THREE-DIMENSIONAL MORPHOMETRIC ANALYSIS OF THE CRANIOFACIAL COMPLEX IN THE UNAFFECTED RELATIVES OF INDIVIDUALS WITH NONSYNDROMIC OROFACIAL CLEFTS

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Numerous studies have described altered patterns of craniofacial form in the unaffected relatives of individuals with nonsyndromic oral clefts. Unfortunately, results from these studies have been highly variable and have failed to provide a reliable method for discriminating at-risk relatives from controls. In the present study, we compared craniofacial shape between a sample of unaffected relatives (33 females; 14 males) from CL/P multiplex families and an equal number of age/sex/ethnicity-matched controls. A total of 16 *x,y,z* facial landmark coordinates derived from 3D photogrammetry were analyzed via Euclidean Distance Matrix Analysis (EDMA), while 14 additional linear distances from direct anthropometry were analyzed via t-tests. Variables identified as significantly different ($p \leq 0.10$ from EDMA; 0.05 from t-tests) were then entered into a two-group discriminant function analysis. All analyses were carried out for each sex separately. Compared to controls, female unaffected relatives demonstrated increased upper facial width, midface reduction and lateral displacement of the alar cartilage. A single discriminant function was derived (canonical correlation = 0.43; $p = 0.01$) which correctly classified 70% of female unaffected relatives and 73% of female controls. Male unaffected relatives demonstrated increased upper facial and cranial base width, increased lower facial height and decreased upper facial height. Again, a single discriminant function was derived (canonical correlation = 0.79; $p < 0.001$) which correctly classified 86% of male unaffected relatives and 93% of male controls. In both males and females, upper facial width contributed most to group discrimination. Based on the discriminant function results, unaffected relatives were classified into risk/liability classes (high risk or low risk) based on the degree of phenotypic divergence from controls. Results suggest that the craniofacial shape differences characterizing unaffected relatives are partly sex-specific and perhaps more pronounced in males. The pattern

of relative-control differences observed in both sexes is in broad agreement with previous findings from both humans and animal models. Although preliminary, these results suggest that a quantitative assessment of the craniofacial phenotype may allow for the identification of at-risk individuals within CL/P multiplex families. Importantly, the identification of such individuals could lead to improvements in recurrence risk estimation and gene mapping.

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PREFACE

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1.0 BACKGROUND AND SIGNIFICANCE

1.1 INTRODUCTION

Collectively, oral clefts (OCs) are one of the most common birth defects that affect human beings. They are the most common *craniofacial* birth defect. It has been estimated based on global birth rates that a child is born with a cleft every 2.5 minutes (Mossey and Little, 2002). Due to their frequency and the extensive treatment often required, OCs represent a major public health problem. It is commonplace for children with clefts to require extensive (and often multiple) surgical repairs, hearing and speech therapy, help with feeding, orthodontic and otolaryngology treatment, and a host of psychosocial counseling services (Berk and Marazita, 2002). OCs are associated with excess infant mortality and significant lifelong morbidity (Czeizel and Sankaranarayanan, 1984; Christensen et al., 2004), particularly in developing countries with inadequate health care systems (Murray et al., 1997; Croen et al., 1998). The costs, both financial and personal, are enormous.

Unfortunately, despite decades of dedicated research and substantial progress, the specific factors responsible for the vast majority of OCs remain unknown. There is abundant evidence that OCs have a substantial genetic basis; nevertheless, except in rare Mendelian forms, no single genetic marker sufficient to cause a cleft has been identified to date. This means that for most individuals with a cleft as well as their unaffected family members, the risk of passing the trait on to their children will be based solely on empirical recurrence risk estimates, which may not apply equally well to all individuals within cleft families (Aylsworth, 1985). Numerous lines of evidence suggest that both cleft cases and their unaffected relatives possess a suite of physical characteristics that differ on average from the general population, including facial features (Weinberg et al., 2006a). Consequently, it may be possible to assign risk status to individuals within cleft families based on measurable aspects of their phenotype – the idea being

that the phenotype can serve as a proxy for the genetic/epigenetic factors underlying the condition. The present study seeks to determine whether a quantitative assessment of facial morphology can provide a means for assigning the unaffected relatives of cleft individuals into risk categories. This research has the potential to not only improve recurrence risk estimates, but also to increase the power of gene mapping and association approaches.

1.2 CLASSIFICATION AND PHENOTYPIC VARIABILITY

Although numerous classification schemes have been proposed (Davis and Ritchie, 1922; Veau, 1931; Kernahan, 1971), OCs are typically divided into three main classes based on descriptive anatomical and embryological considerations (Fogh-Andersen, 1942; Millard, 1976): clefts affecting the primary palate, including the lip and alveolus (CL or CL/A), clefts affecting both the primary and secondary palate (CLP), and clefts affecting only structures posterior to the incisive suture, including the soft palate (CP). Based on epidemiological data (see next section) clefts of the first and second type are almost always considered as a single disease entity, cleft lip with or without cleft palate (CL/P). Traditionally, OCs have also been divided into syndromic and nonsyndromic forms. Currently, there are more than 400 recognized syndromes that included OCs as a feature (Gorlin et al., 2001; Cohen, 2002). The majority of these syndromes are the result of single gene disorders (i.e., they follow a simple Mendelian pattern of inheritance), but many others are caused by chromosomal rearrangements or specific teratogenic exposures during early development. In all likelihood, there are still many more unrecognized syndromes with clefting as part of the phenotype. The vast majority of OCs, however, are not associated with any other major malformations; such clefts are considered nonsyndromic or isolated. Roughly 75 % of all CL/P cases are considered nonsyndromic, while this hold true for only about 50% of CP cases (Murray, 2002). The focus of the present investigation is on nonsyndromic clefting, specifically nonsyndromic CL/P.

Within each class of nonsyndromic cleft there is considerable phenotypic variation (Eppley et al., 2005). In CL/P, expression of the disease ranges from microforms of the external upper lip which manifest only as a subtle displacement of the alar cartilage during normal lip function (cleft nasal deformity) to a total lack of tissue extending posteriorly from the external

lip through the soft palate (complete cleft lip and palate). At the minimal end of the cleft spectrum, recent findings using high resolution ultrasound suggest that the range of variation may even extend beyond the externally visible lip; occult subepithelial clefts manifesting as small breaks in the superior *orbicularis oris* muscle have been shown to be present at elevated rates in the unaffected relatives of individuals with overt CL/P (Martin et al., 2000; Weinberg et al., 2003; Neiswanger et al., in press). The defect in CL/P can either be present unilaterally (80%) or bilaterally (20%), the latter being more severe (Mossey and Little, 2002). In a similar manner, isolated CP can range from a relatively minor anomaly such as a bifid uvula to a full midline defect involving the entire secondary hard palate and soft palate.

Importantly, the proper delineation of cleft cases as syndromic or nonsyndromic can improve clinical management and patient care, recurrence risk estimation, and the outcome of studies designed to assess etiology (because heterogeneity will be limited). In reality, though, the dichotomy between syndromic and nonsyndromic forms of clefting is oversimplistic, and a universally accepted set of criteria for distinguishing these two forms of clefting does not exist (Saal, 2002). Typically, the term "nonsyndromic" is used interchangeably with the term "isolated" suggesting, at a minimum, that no other major anomaly should be present. It is less clear, however, how to deal with so called minor physical anomalies (MPAs). These anomalies are morphological variations that are thought to represent subtle deviations from normal development and may have diagnostic relevance (Jones, 1997). Unlike major anomalies, MPAs are of no functional consequence and require no medical interventions. Nevertheless, they are quite rare, occurring in less than 4% of the general population (Marden, 1964; Spranger, 1982; Leppig, 1987). Jones (1988) has suggested that cleft cases possessing more than two MPAs should not be considered isolated, whereas Tolorová and Cervenka (1998) classified cases with multiple MPAs as isolated, unless a specific and recognizable pattern of anomalies was present.

The line between syndromic and nonsyndromic forms of clefting is becoming ever more blurred. Consider, for example, Van der Woude syndrome (VWS). VWS is a rare autosomal dominant condition characterized by two main features: clefting (CL/P and CP) and bilateral lower lip fistulas (Kondo et al., 2002). However, it is not uncommon for affected individuals to present with an oral cleft in the absence of any other physical defect. Moreover, variants of the gene responsible for VWS, *IRF6*, also show evidence of genetic association in a subset of cases with nonsyndromic CL/P (Zucchero et al., 2004; Ghassibe et al., 2005), indicating a common

etiology for the two conditions. Accordingly, a number of studies are now strategically using these rare Mendelian syndromes that include isolated clefts as part of their phenotype, such as VWS, as a model to understand the genetic basis of nonsyndromic clefting (Schutte and Murray, 1999; Stanier and Moore, 2004; Jurgessur and Murray, 2005).

1.3 EPIDEMIOLOGICAL PATTERNS

Based on large-scale population studies, it has been estimated that CL/P accounts for about 75% of all nonsyndromic cleft cases (Saal, 2002). Within CL/P, defects involving both the lip and palate are typically observed more often than those involving the lip alone (Fogh-Andersen, 1942; Mossey and Little, 2002). Interestingly, in populations with a higher cleft prevalence, the ratio of CLP to CL is higher (Tolarová and Cervenka, 1998; Mossey and Little, 2002). Because CL and CLP often occur in the same family and have similar demographic profiles, they are considered variants of the same condition, CL/P (Fraser, 1970; Harville et al., 2005). CL/P and CP, however, are considered pathogenically distinct diseases, based primarily on embryological and epidemiological evidence; they differ in terms of incidence, sex ratio, distribution among different ethnic groups, frequency of additional anomalies and tendency to be associated with a syndrome (Mossey and Little, 2002). The secondary palate defect in CL/P is thought to occur via a different mechanism from that observed in CP (Trasler and Fraser, 1963; 1977). Fogh-Andersen (1942) was the first to note that CL/P and CP very rarely occur together in the same family. When both cleft types co-occur within a family, it is usually a hallmark of certain rare syndromic forms of clefting such as Van der Woude syndrome or Kallmann syndrome (Lidral and Murray, 2004). Furthermore, in families with a history of CL/P, the recurrence risk of having a child with CP is at the same level as the general population (Saal, 2002).

World-wide, the birth prevalence of CL/P is estimated at approximately 1/700 (Murray, 2002). This estimate varies widely by ancestry, from around 1/300 to 1/3000 (Vanderas, 1987; Croen et al., 1998; Tolarová and Cervenka, 1998). Among Caucasian populations, the prevalence hovers around 1/1000 (Wyszynski et al., 1996). In general, Native Americans are reported to have the highest prevalence, followed by Asian-derived populations, Caucasians and Hispanics, and African-derived populations (Mossey and Little, 2002); of course, within each of

these broad categories there exists considerable variation. Both genetic and/or environmental (e.g., socioeconomic) factors have been proposed to explain the observed geographical variation in CL/P (Leck, 1972; Ching and Chung, 1974; Croen et al., 1998). The prevalence of CP is lower than CL/P, at around 1/1500 (Lidral and Murray, 2004). Further, in contrast to CL/P, the prevalence of CP is less variable across populations, typically ranging between 1/1000 and 1/2000 (Croen et al., 1998; Tolarová and Cervenka, 1998; Mossey and Little, 2002). It should be noted that because of differences in ascertainment methods across studies that report oral cleft prevalence figures, the data can be difficult at times to interpret. For example, some studies combine data from stillbirths as well as live births (Vanderas, 1987), resulting in inflated prevalence estimates because still births have a much greater frequency of OCs (Kraus et al., 1963; Nishimura et al., 1966; Hay, 1971). Moreover, many earlier studies did not differentiate between syndromic and nonsyndromic cases and/or lumped CL/P and CP cases together (Cooper et al., 2006).

Like many birth defects, nonsyndromic OCs exhibit skewed sex ratios (Lubinsky, 1997). It has long been recognized that CL/P occurs more frequently in males than females (Rischbieth, 1910; Fogh-Andersen, 1942). It is universally accepted that roughly twice as many males as females (M:F = 2:1) are affected with CL/P (Wyszynski et al., 1996). This figure seems to be fairly consistent across populations; however, the sex ratio has been shown to co-vary with cleft severity and number of affected family members (Mossey and Little, 2002). The male to female ratio tends to be higher in cases of bilateral CL/P and lower in cases of CL (Fogh-Andersen, 1942; Fraser, 1980; Shapira et al., 1999). Additionally, the male to female ratio is decreased in multiplex versus simplex families (Niswander et al., 1972; Fraser, 1980; 1998). In contrast to CL/P, CP is more common in females than males, although the ratio (3:2) is not as skewed as in CL/P (Saal, 2002). A satisfactory explanation for the sex difference in clefting is lacking, although some have suggested hormonal factors may play a role (James, 2000).

When CL/P manifests as a unilateral defect (~80% of the time), it is characterized by significant laterality (Paulozzi and Lary, 1999; Hallgrímsson et al., 2005). In approximately two-thirds of unilateral CL/P cases, the defect is located on the left side of the face (Fogh-Andersen, 1942; Tolarová, 1987; Shapira et al., 1999). Interestingly, a similar laterality pattern has been described in certain susceptible mouse strains (Trasler and Trasler, 1984). Still, a plausible biological mechanism for the left side predilection in CL/P has not been offered to date.

Interestingly, this laterality pattern (as well as the observed sex difference) is not found in syndromic forms of clefting where the lip is involved (Czeizel and Tusnády, 1971).

Based on the epidemiological patterns outlined above, CL/P and CP are clearly distinct conditions. As a consequence, *the remainder of this report will focus exclusively on nonsyndromic CL/P*.

1.4 EMBRYOLOGY

1.4.1 Normal facial development

Craniofacial development involves an exquisitely complex set of morphogenetic, mechanical and molecular processes occurring in a very precise spatial and temporal sequence (Streeter, 1951; Warbrick, 1960; Hinrishsen, 1985; Diewert and Wang, 1992; Sperber, 2001; 2002; Johnston and Bronsky, 2002; Francis-West et al., 2003; Cox, 2004; Jiang et al., 2006; Morriss-Kay, 2006). In order to understand the aberrant developmental processes leading to CL/P, it is first necessary to understand how the human face develops under normal conditions. Because craniofacial development is an excessively broad topic, the following section will focus on the early morphogenesis of the primary palate. The major events that define the development of the human face occur between the fourth and eighth week post-conception (Hinrishsen, 1985; Yoon et al., 2000; Senders et al., 2003; Jiang et al., 2006). These events are described below and outlined in Table 1.

At approximately four weeks post-conception (Carnegie stage 10), the face consists of little more than a centrally located depression, the stomodeum or future oral cavity, surrounded by three undifferentiated bulges of tissue, i.e., facial prominences. These facial prominences include the relatively large frontonasal mass centrally located above the stomodeum and the paired mandibular prominences inferior to the stomodeum. The mandibular prominences are derived from the first pharyngeal arches. A major tissue component of the facial prominences is neural crest-derived mesenchyme (ectomesenchyme), which migrates into the presumptive facial region early in the fourth week from its origins along the dorsal surface of the developing brain (Hall, 1999; La Douarin and Kalcheim, 1999). This neural crest-derived mesenchyme will

Table 1 Morphogenesis of the upper lip and primary palate from the fourth though the seventh week post-conception

Carnegie Stage	*Age* [a]	*Mouse Stage* [b]	*Major Events* [c]
10	22 days	13 (E8.5)	Mesenchyme of the face is established as neural crest cells migrate into the rostral frontonasal mass overlying the forebrain and the more caudal first pharyngeal arches
11	24 days	14 (E9.0)	Frontonasal and paired mandibular (first arch) prominences are established; the stomodeum is wide and located between these rapidly swelling structures
12	26 days	15 (E9.5)	Paired maxillary prominences emerge at the rostral base of the mandibular arches, bounding the stomodeum bilaterally
13	28 days	16 (E10.0)	Frontonasal prominence expands laterally in response to brain growth; oropharyngeal membrane ruptures; mandibular prominences merge to form continuous lower jaw; surface epithelium thickenings (olfactory placodes) appear at the inferolateral corners of the frontonasal prominence
14	32 days	17 (E10.5)	Mesenchyme surrounding to the olfactory placodes proliferates causing them to invaginate and form nasal pits; the medial and lateral prominences emerge around the nasal pits in an inverted horseshoe pattern with the inferior end open to the stomodeum
15	33 days	18 (E11.0)	Maxillary and medial nasal prominences grow toward one another, transforming the nasal pits into vertically oriented slits; the medial nasal and lateral nasal prominences make contact
16	37 days	19 (E11.5)	The lateral nasal prominences are displaced laterally as the medial nasal and maxillary prominences contact one another; epithelial nasal fin established

Table 1 (continued).

17	41 days	20 (E12.0)	Growth of the maxillary prominences displace nasal structures toward the midline; medial nasal prominences merge at the midline to establish the central portion of the upper lip and nose
18	44 days	21 (E12.5)	Nasal fin begins to disintegrate, allowing nasomaxillary prominences to fuse; cartilaginous nasal septum present; choanal membranes rupture
19	48 days	22 (E13.0)	Continuity of the upper lip complete

[a] Days post-conception

[b] Theiler Stage

[c] Based primarily on descriptions from Sperber (2002) and Jiang et al. (2006)

ultimately give rise to the skeletal and other connective tissues comprising the face (Graham, 2003). Cranial paraxial mesoderm is also contained within the facial prominences, eventually giving rise to the facial musculature (Noden and Francis-West, 2006). The surface of the facial prominences is covered with ectoderm-derived epithelium. Signaling interactions between these different tissues play a key role in governing the coordinated development of the face (Francis-West et al., 1998; Richman and Lee, 2003; Jiang, 2006).

Toward the end of the fourth week post-conception, paired maxillary prominences emerge from the rostral base of the mandibular arches, swelling toward the ventral surface of the face and eventually flanking the stomodeum bilaterally. Soon after, the mandibular prominences begin to merge at the midline forming an intact lower law. By the fifth week, the face consists of a central cavity (the stomodeum) surrounded by four bulging facial prominences (frontonasal, left and right maxillary, and mandibular). Around this time, two bilateral localized thickenings in the surface ectoderm of the frontonasal prominence (the nasal placodes) appear just superior to the stomodeum (Muller and O'Rahilly, 2004; Schlosser, 2006). Rapid cell proliferation in the underlying mesenchyme immediately adjacent to the placodes leads to their invagination, ultimately resulting in the formation of nasal pits – predecessor of the nostrils (Waterman and Meller, 1973). As the swellings that surround the nasal pits continue to grow, they quickly take on the appearance of an inverted horseshoe with an open end at the inferior margin, establishing continuity with the stomodeum (Sperber, 2002). Each nasal swelling is eventually divided into two tissue domains based on their relationship to the embryo's midline, a medial nasal prominence and a lateral nasal prominence. It is the subsequent fusion of the facial prominences in the nasomaxillary region that serves as the basis for the morphological integrity of the primary palate and upper lip (Trasler, 1968; Gaare and Langman, 1977a; Johnston and Millicovsky, 1985; Sun et al., 2000).

In the course of normal development, the facial prominences continue to grow and approach one another until, at the end of the fifth week, the free tips of the medial nasal, lateral nasal and maxillary prominences on each side of the face come into physical contact at the inferior border of the nasal pit (Hinrishsen, 1985; Yoon et al., 2000; Senders et al., 2003). Coordinated outgrowth of the facial prominences is essential for proper morphogenesis and is controlled by molecular signals (e.g., *Bmp4*) passed between the epithelium and the underlying

mesenchyme (Francis-West et al., 1994; Cox, 2004; Jiang et al., 2006). As the prominences make contact, a transient vertically oriented seam of epithelial tissue called the nasal fin is established. The breakdown of this epithelial seam is required for the maxillary and lateral nasal prominences to fuse with the medal nasal prominences (Gaare and Langman, 1977b). It is currently believed that process involves restricted apoptosis and/or epithelial-mesenchymal transformation (see Jiang et al., 2006 for discussion). The initial continuity of the upper lip is completed during the sixth week as the two medial nasal prominences approximate one another and merge in the midline, providing a single body of tissue that will eventually develop into the central third of the upper lip (including the philtrum), the nasal tip and columella, and the alveolar and palatal bone anterior to the incisive suture (Sperber, 2002). By the end of the seventh week, the nasal fin has disintegrated completely, allowing the mesenchyme from the once-separate facial processes to intermingle. Continued anterior and vertical growth of the midface along with lateral expansion of the head and upper face ensures that by the beginning of the eighth week post-conception, the basic form of the human face is established (Diewert and Lozanoff, 1993a; 1993b; 2002; Diewert et al., 1993; Avery, 1994). The transformation of the human face from the sixth to tenth week is depicted in Figure 1.

1.4.2 Developmental basis of CL/P

Embryological explanations for birth defects can be traced back to antiquity (Ballantyne, 1904). According to Warkany (1977), the preeminent physician and scientist William Harvey suggested an empirically-based embryological mechanism for OCs as early as the mid-17th century; Harvey suggested that OCs were the result of a developmental arrest during the early formation of the face (Harvey, 1651). Ostensibly, clefting of the primary palate results from a failure of the medial nasal prominence to either *make contact* or *maintain contact* with the adjacent maxillary and/or lateral nasal prominence (Scott, 1966; Trasler and Fraser, 1977; Vermeij-Keers et al., 1983). In terms of pathogenesis, the failure of adjacent facial prominences to make contact suggests that hypoplasia of one or more facial prominences or spatial-temporal factors may be involved (Trasler and Fraser, 1977). In contrast, failure to maintain contact suggests a localized breakdown involving tissues at the contact site. Thus, different underlying pathogenic mechanisms can result in the same disease phenotype. This fact, coupled with the relatively high

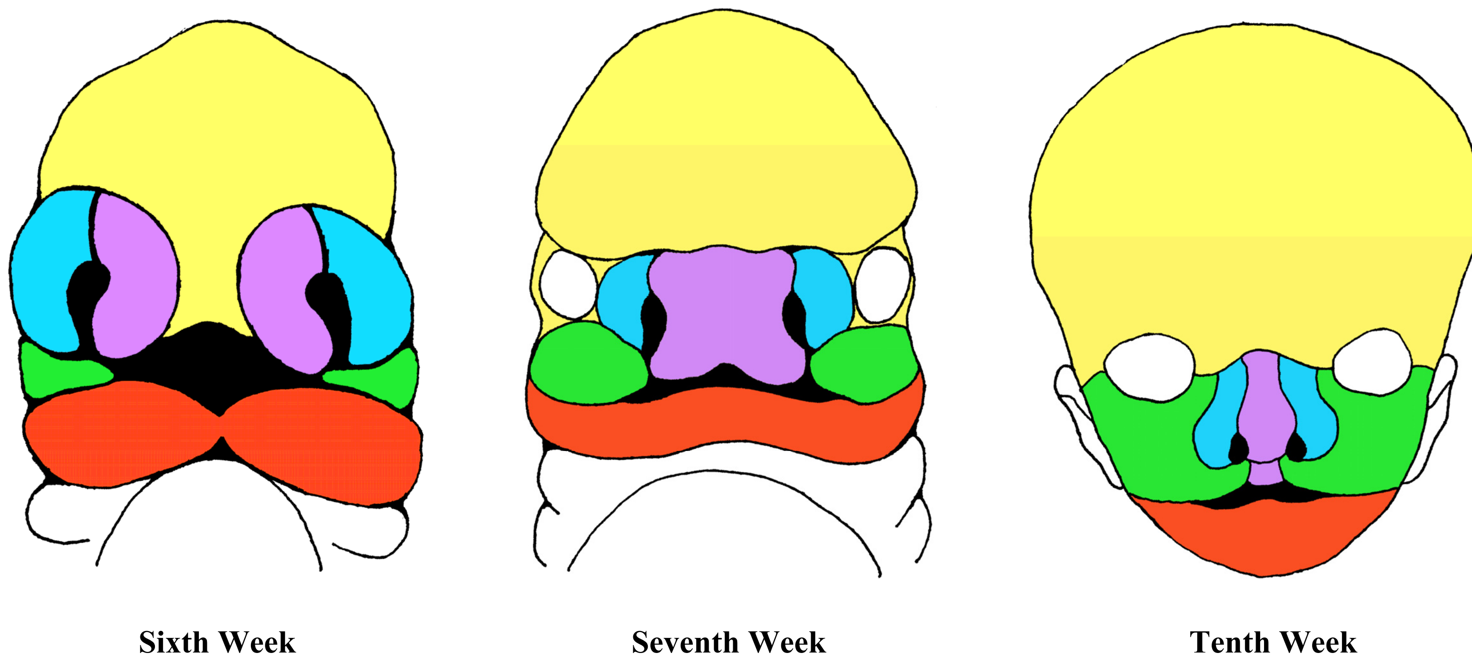

Figure 1 Craniofacial morphogenesis from the 6th to 10th week.

The embryonic facial prominences are color-coded to show their contribution to definitive facial structures. Yellow = Frontonasal prominence, Purple = Medial nasal prominence, Blue = Lateral nasal prominence, Green = Maxillary prominence and Orange = Mandibular prominence.

population incidence of CL/P, argues that the primary palate represents an anatomical region highly susceptible to errors in morphogenesis – a developmental weak link.

Most of our knowledge on the pathogenesis of CL/P stems from experimental studies on staged embryos. A number of morphometric and histological studies have been carried out on the embryos of inbred mouse strains (e.g., A/J, A/WySn, CL/Fr) with high liability to spontaneous or induced CL/P (Diewert and Lozanoff, 2002). One of the most common findings in cleft-susceptible mouse strains is diminished size of one or more facial prominences comprising the future nasomaxillary complex compared with non-susceptible strains (Reed, 1933; Trasler, 1968; Millicovsky et al., 1982; Trasler and Leong, 1982; Trasler and Ohannessian, 1983; Wang et al., 1995). Reduced growth of facial prominences has also been shown to occur in phenytoin- and retinoic acid-treated mice, which have an increased rate of CL/P (Sulik et al., 1979; Helms et al., 1997). Similar findings have been reported for humans (Johnston and Hunter, 1989). Work by Diewert and coworkers have shown that a cascade of precisely coordinated morphogenetic events involving the entire craniofacial complex are required for normal primary palate formation (Diewert and Lozanoff, 1993a; 1993b; Diewert et al., 1993). Significant reduction in the outgrowth of one or more adjacent facial prominences disrupts the highly coordinated morphogenesis of the primary palate by altering the normal spatial and/or temporal relationship of the facial processes as they approach one another (Diewert and Lozanoff, 2002). This increases the probability that fusion will either be delayed or fail to occur altogether.

The lack of facial process outgrowth in susceptible mouse strains is likely the result of mesenchymal deficiency (Wang et al., 1995). Both Diewert and Shiota (1990) and Mooney and coworkers (1991) offered further evidence of mesenchymal deficiency in staged human embryos with CL/P. Such a deficiency could stem from problems with the early migration of neural crest cells into the facial prominences and/or a lack of proliferation of already intact crest-derived mesenchyme (Trasler and Fraser, 1977; Johnston and Bronsky, 2002). Since outgrowth of the facial prominences appears to be controlled by molecules (e.g., *Bmp4*) emanating from the overlying ectoderm (Jiang et al., 2006), the observed mesenchymal deficiency could result from localized defects in epithelial-mesenchymal signaling (Cox, 2004).

Closely related to evidence for a discrepancy in the size or growth of the facial prominences relative to one another, stands a large body of evidence indicating that altered

orientation of the facial processes during early facial morphogenesis is a major factor in the pathogenesis of CL/P (Trasler, 1968; Juriloff and Trasler, 1976). This idea is known as the "face shape hypothesis." Because the "face shape hypothesis" provides much of the conceptual and evidentiary foundation for the present study, it will be discussed at length in a later section (Section 1.6). Suffice to say at this juncture that numerous studies in both mouse and human populations support the notion that face shape plays an important role in the etiology of CL/P.

Fusion of the medal nasal prominences with the lateral nasal and maxillary prominences not only requires physical contact, but a subsequent host of localized cellular events involving the epithelia at the contact site. These events include selective apoptosis and removal of superficial peridermal cells, the formation of cellular adhesions across adjacent basal cell layers, the establishment of filopodial bridges between neighboring facial prominences, and the eventual replacement (transformation) of the remaining epithelia by mesenchyme (Gaare and Langman, 1977b; Millicovsky and Johnston, 1981; Johnston and Bronsky, 2002; Jiang et al., 2006). In principle, at least, a breakdown at any one of the steps could result in failed or incomplete fusion. For example, in two mouse strains with elevated rates of spontaneous CL/P (A/WySn and CL/Fr), epithelial activities specifically associated with fusion (e.g., peridermal breakdown, establishment of filopodial attachments) are greatly reduced (Millicovsky and Johnston, 1981; Millicovsky et al., 1982; Forbes et al., 1989). Importantly, Millicovsky et al. (1982) noted a wide range of variation in epithelial activity at the time of fusion in these highly susceptible mouse strains; they suggested that this variation may correlate with the extent of the defect (i.e., the severity of the cleft).

In a separate series of studies, Wang et al. (1995) described the internal aspects of the primary palate in five mouse strains with varying degrees of cleft susceptibility. Compared to non-susceptible strains (BALB/cByJ and C57BL/6J), susceptible stains (A/J, A/WySn and CL/Fr) demonstrated reduced growth of the maxillary prominences, delayed epithelial contact and formation of the nasal fin, reduced epithelial contact area, delayed infiltration of mesenchyme across the contact site, and a deficient mesenchymal bridge formation between the medial nasal and maxillary prominences (Wang et al., 1995). In their view, the delayed and deficient pattern of development would result in an epithelial contact site too tenuous to hold the adjacent facial prominences together as the midface advanced anteriorly (Diewert and Lozanoff, 2002). Interestingly, the degree of delay and deficiency was positively correlated with the rate of

clefting across the susceptible strains. Diewert (1993) described a similar pattern in staged human fetuses; Japanese fetuses exhibited delayed mesenchymal bridge formation compared to Caucasian fetuses, which could help explain the higher CL/P prevalence in the Japanese population.

1.5 ETIOLOGY

1.5.1 CL/P as a complex disease

CL/P is a complex disease trait. In the context of genetic epidemiology, complexity refers to a situation were multiple factors contribute to the presence of a disease phenotype. Such phenotypes are often referred to as multifactorial (Jorde et al., 2003). Importantly, these factors often included both genetic and non-genetic (environmental) components as well as interactions between these components. One ramification of the multifactorial nature of complex diseases is that the translation from genotype to phenotype is less direct and more convoluted. Another consequence is that, in contrast to single-gene disorders, complex diseases are characterized by inheritance patterns that tend not to fall into any of the simple Mendelian categories like autosomal dominant or recessive. Such complex inheritance patterns make it more difficult to predict the probability a given individual will pass on the trait. Worse yet, many of the most powerful methods for identifying genetic loci, such as linkage analysis, are ill-suited for dealing with complex disease traits.

Of course, the distinction between monogenetic and complex diseases is somewhat heuristic. Geneticists fully recognize the fact that many monogenetic phenotypes are modified, if even to a small extent, by additional genetic and/or environment factors while, at the same time, many complex phenotypes probably have significant single-gene contributions (Beaudet et al., 2001). The difference is more a matter of degree than of kind. Complex diseases have all the difficulties associated with Mendelian conditions (e.g., reduced penetrance, variable expressivity, the presence of phenocopies, and heterogeneity), but in many instances, to a greater extent. Add to this, higher-order complications like additive and/or multiplicative gene-gene and gene-environment interactions and it is clear why the search for solutions to complex diseases is

so daunting. An unfortunate fact of life is that many of the most common diseases that affect humans, including CL/P, are complex in nature. Unraveling the causes of complex and common diseases is likely to be a major focus of genetic epidemiology over the next century.

In the sections that follow, the evidence for both environmental and genetic contributions to CL/P will be presented.

1.5.2 Environmental factors

1.5.2.1 Teratogens A teratogen is defined as any exogenous environmental agent capable of producing malformations in a developing organism (Wilson and Fraser, 1977). The role of environmental factors in the etiology of congenital malformations was appreciated by early scholars (Warkany, 1977). There is an extensive body of literature on humans as well as laboratory animals implicating a wide variety of environmental agents, acting alone or in combination with predisposing genetic factors, as causative in the occurrence of OCs (see Shashi and Hart, 2002 and Hayes, 2002 for extensive reviews). For example, the use of a variety of prescription medications during early pregnancy, including anti-convulsants (Abrishamchian et al., 1994), retinoids (Abbott and Pratt, 1991), glucocorticoids (Czeizel and Rockenbauer, 1997; Carmichael and Shaw, 1999), benzodiazepines (Dolovich et al., 1998) and folate antagonists (Hernandez-Diaz et al., 2000) have been associated with clefting in offspring. Regarding recreational substances, there is abundant evidence that both maternal smoking (Ericson et al., 1979; Kallen, 1997; Wyszynski et al., 1997) and alcohol use (Werler et al., 1991; Munger et al., 1996) contribute to nonsyndromic clefting in offspring, typically in a dose-dependent manner (Shaw and Lammer, 1999; Chung et al., 2000). Conversely, there appears to be no evidence for a teratogenic effect stemming from caffeine use (Rosenberg et al., 1982). Additional teratogenic factors that may be related to CL/P include various toxins, such as organic solvents and pesticides, and certain maternal infections associated with fever (Hayes, 2002; Shashi and Hart, 2002).

On the whole, the results of studies linking teratogenic exposures to CL/P are difficult to interpret. For virtually every environmental agent where experimental or epidemiological evidence for an association with CL/P exists, there is evidence to the contrary. Clearly methodological differences in study design, sample composition and statistical methodology

account for a considerable proportion of these discrepancies. Many epidemiological studies are retrospective, for example, and exposure data may be inaccurate due to recollection bias or affected by confounders. Even in prospective cohort studies that attempt to control for confounders, detailed data on exposures (dose and/or timing) is often lacking. Although using animal models to assess teratogens affords researchers some control over exposures and potential confounders, it remains unclear how to extrapolate data gleaned from such studies to humans.

At a more fundamental level, however, difficulty assessing the etiological role of teratogens in CL/P likely has to do with the complex nature of the disease. The CL/P sample in any given study will be comprised of an etiologically heterogeneous mix of cases, of which relatively few are likely to be caused by the exclusive action of an isolated teratogen. The complex etiology of clefting dictates that a combination of environmental and genetic factors acting in a synergistic manner probably influences cleft liability. This realization has lead to studies specifically designed to investigate the role of gene-environment interactions in CL/P (Murray, 2002; Zeiger and Beaty, 2002). In mice, it has long been recognized that susceptibility to teratogen-induced CL/P varies with strain (Trasler and Ohannessian, 1983). A handful of epidemiological studies exploring the interaction between specific candidate genes (e.g., *Tgf-a*) and teratogenic exposures (i.e., smoking or alcohol) have been carried out in human populations (Hwang et al., 1995; Shaw et al., 1996; Maestri et al., 1997; Romitti et al., 1999). These studies have demonstrated that the odds of a teratogen resulting in CL/P are largely moderated by the presence of particular genetic variants.

1.5.2.2 Maternal factors Factors associated with maternal health and disposition have also been suggested to play a role in the occurrence of CL/P (Munger, 2002; Shashi and Hart, 2002). In studies using a variety of animal models, induced vitamin deficiencies (e.g., folic acid, vitamin A, and various B vitamins) have repeatedly been shown to result in OCs (Munger, 2002). Similar deficiencies have been implicated in humans as well (Faron et al., 2001). Moreover, numerous large-scale population-based studies have been carried out to assess the relationship between maternal vitamin supplementation and clefting in offspring (reviewed in Munger, 2002). The results of these studies have been contradictory, with some reports showing a rather dramatic decrease in CL/P with vitamin supplementation during early pregnancy (Shaw et al., 1995; Tolarová and Harris, 1995; Czeizel et al., 1996), while other studies show little or no

change (Hill et al., 1988; Hayes et al., 1996; Werler et al., 1999). Some studies have also shown evidence of an interaction between maternal nutritional status and the genotype of offspring in determining CL/P risk (Shaw et al., 1998a), which could explain some of the discrepant findings.

In a recent report from Sweden, maternal obesity was identified as a risk factor for CL/P (Cedergren and Kallen, 2005) and at least one earlier report has implicated maternal diabetes (Spilson et al., 2001). The idea that maternal hormone imbalances may be involved in clefting has been around for some time. Much of the early work in this area was done on animal models, particularly after the discovery that corticosteroids could induce cleft palate (Baxter and Fraser, 1950). More recently, James (2000) has offered indirect evidence (sex ratios in unaffected sibs within cleft families) that the mothers of CL/P offspring possess a specific hormonal profile during early pregnancy. In another recent report, Czeizel et al. (2003) were able to demonstrate a protective effect of severe nausea during early pregnancy against having an offspring with CL/P; nausea during early pregnancy has been shown to correlate with the presence of specific hormones. Related to the hormonal hypothesis is the idea that excess psychological stress can induce clefting in offspring, conceivably though activation of the hypothalamic-pituitary-adrenal axis (Peer and Strean, 1956; Barlow et al., 1975a). Support for this hypothesis comes from experiments on mice involving the exposure of pregnant females to a variety of stresses, such as excess movement, physical restraint, crowding and deprivation (Brown et al., 1972; Hamburgh et al., 1974; Barlow, 1975b; Montenegro et al., 1995). Although an early study by Fraser and Warburton (1964) suggested that the association between maternal psychological stress and clefting in humans was lacking, a handful of studies have since provided evidence (albeit typically weak evidence) to the contrary (Drillen et al., 1966; Saxen, 1974; Montenegro et al., 1995; Carmichael and Shaw, 2000; Hansen et al., 2000). Adding to this line of evidence, a recent study by Ács and co-workers (2006) has demonstrated that mothers with untreated panic disorder during pregnancy were over three times more likely to have a child with CL/P than mothers without panic disorder.

1.5.3 Genetic factors

1.5.3.1 Mode of inheritance CL/P is one of the first examples of a birth defect shown to aggregate in families. This is likely due to the conspicuous nature of the defect coupled with the

fact that a large proportion of affected individuals go on to reproduce. As pointed out by Rischbieth (1910) and Fogh-Andersen (1942), the first definitive report of oral cleft transmission within a multigenerational pedigree was reported by Trew (1757). Just over 50 years later, citing several known cases where multiple family members were affected, the preeminent German scientist Johann Friedrich Meckel (1812) emphasized heredity as the primary explanation for the defect. Numerous hereditary case reports of clefting were recorded over the next 50 years (reviewed in Rischbieth, 1910 and Fogh-Andersen, 1942), including one famous case reported by Sproule (1863) of the disease in multiple members within the author's own family – Darwin made reference to this particular case in his widely read work *Variations of Animals and Plants under Domestication* (Darwin, 1875). The first reports describing large series of cleft cases appeared during the late nineteenth century (see Fogh-Andersen, 1942). Typically, these reports indicated that between 10 and 20 percent of cases show a hereditary tendency. Fogh-Andersen (1942), however, argues that such figures are probably underestimates, due to the methodological limitations of these earlier reports.

It wasn't until after the rediscovery of Mendel's work at the turn of the 20th century by Hugo De Vries and Carl Correns, that scientists began to offer explanations as to the particular manner in which clefting was inherited. A number of descriptive works published on moderately sized samples of hereditary cleft cases appeared between the mid-1920s and 1940, the results of which are amply summarized by Fogh-Andersen (1942). These reports often disagreed on the manner of inheritance; most suggested that the disease was recessive, while others found evidence for dominant, sex-linked, or irregular (incomplete) dominant patterns of inheritance. This was the state of affairs when the Danish physician Poul Fogh-Andersen published his seminal work *Inheritance of Harelip and Cleft Palate* in 1942. Based mainly on recurrence data obtained on the families of 703 cleft cases, Fogh-Andersen was the first to appreciate the etiological distinction between CL/P and CP. He concluded that the majority of CL/P cases were genetic in nature and that the manner of inheritance was "that of 'conditioned dominance' with sex-limitation to males."

The major problem with the simple Mendelian models offered by many early investigators was that they were inconsistent with the patterns of disease recurrence observed in a large proportion of families with CL/P. Something of a paradigm shift occurred in the early 1960s, when multifactorial models of inheritance began to be applied to disease phenotypes.

These models – an extension of the polygenic models developed by Ronald Fisher and other population geneticists in the 1930s – implicated a number of genetic and/or environmental factors and were originally developed to explain the inheritance of traits with a continuous distribution. However, largely influenced by the animal experiments of Gruneberg (1952), Carter and others argued that such models could also explain the inheritance of discrete phenotypes (including disease phenotypes), as long as one assumed that the underlying liability for developing the trait followed a continuous distribution (Carter, 1961; 1976; Falconer, 1965). Only those individuals beyond a certain point, or threshold, along the liability continuum would develop the trait/disease. The multifactorial threshold (MFT) model was applied to CL/P almost immediately (Woolf et al., 1963; 1964; Fraser, 1970; 1976; Carter et al., 1982; Hu et al., 1982). It should be emphasized, however, that these studies were entirely descriptive in nature and did not attempt any formal statistical tests of the MFT model.

Like any model, the MFT model makes certain testable predictions: recurrence risks should increase with the number of family members affected, the severity of the disease phenotype, and the sex of the proband (particularly when the proband is of the less commonly affected sex). One also expects a rapid non-linear decease in recurrence risk as the degree of relationship with the proband becomes more distant. When these predictions were examined in CL/P populations, the results were ambiguous; some predictions were borne out, others were not (Bear, 1976; Melnick et al., 1980; Nemana et al., 1992). A number of researchers also utilized goodness-of-fit approaches and analyzed recurrence risk patterns in samples of CL/P families in order to evaluate the merits of the MFT as well as alternative models (Melnick et al., 1980; Mendell et al., 1980; Marazita et al., 1986; Farrall and Holder, 1992; Mitchell and Risch, 1992). Unfortunately, most of these studies did not find overwhelming evidence for either the MFT or single locus models. Segregation analyses, a model-fitting approach designed to formally test alternative models of inheritance on pedigrees, produced equally ambiguous results (Marazita, 2002; Mitchell, 2002a). Some segregation analyses have found evidence in favor of a major locus (recessive and dominant) model, while others indicated that multifactorial or mixed models provided a better overall fit (reviewed in Marazita, 2002). In some instances it was not possible to discriminate between alternative models at all. Such conflicting results may be due to the obfuscating effects of etiological heterogeneity and/or the low discriminatory power of segregation analysis techniques (Mitchell, 2002a).

Another pillar of evidence supporting the genetic basis for CL/P comes from twin studies. Although the occurrence of twins with CL/P is quite rare (Christensen and Fogh-Andersen, 1993), these cases provide an excellent opportunity to understand the relative genetic contribution to the disease. One potential problem, however, is that the twinning process itself could result in elevated rates of clefting. There is some evidence that twins, in particular monozygotic twins, are generally at increased risk for congenital malformations (Hay and Wehrung, 1970; Myrianthopoulos, 1976; Layde et al., 1980). With regard to clefting, the majority of the data suggests a slight increase in CL/P in multiple births over singletons (Layde et al., 1980; Little and Nevin, 1989; Mastroiacovo et al., 1999). However, these differences typically failed to reach statistical significance due to inadequate sample sizes. It does not appear to be the case that CL/P risk differs between monozygotic and dizygotic twins or between same-sex and opposite-sex pairs (Christensen and Fogh-Andersen, 1993; Mitchell, 2002b). Probandwise concordance rates for CL/P in monozygotic twins range from 28-67% (48% average), while concordance rates for dizygotic twins range from 3-5%, similar to the recurrence risk observed for non-twin full-sibs (Mitchell, 2002b). Thus, the probandwise concordance rate for monozygotic twins is far less than 100% (indicating a substantial role for environmental factors) yet is as much as 20 times higher than that for dizygotic twins. Based on the observed concordance rates, heritability estimates for CL/P are generally stated to be in the .60 to .70 range (Christensen and Fogh-Andersen, 1993).

Patterns of familial recurrence can also illuminate the genetic basis of CL/P (Farrall and Holder, 1992; Mitchell and Risch, 1992; Mitchell and Christensen, 1996; Schliekelman and Slatkin, 2002). Once a child with CL/P is born, the risk to future sibs increases dramatically compared to the general population (Wyszynski et al., 1996). For example, the recurrence risk for first degree relatives is around 4%, 40 times the general population prevalence of ~.001% (for Caucasians). The risk of recurrence increases as more individuals in a family are affected and when the defect is more severe (Mitchell and Risch, 1993). Analysis of recurrence risk patterns in familial data can also provide estimates of the number of genes that underlie a trait, the magnitude of genetic effects, and the mode of inheritance (Risch, 1990). In general, studies of recurrence risk point to an oligogenetic model involving a handful of loci (most likely 3-6, but perhaps as many as 14) operating in a multiplicative manner. It is estimated that the maximum effect of any single locus would be about a six-fold increase in risk (Mitchell, 2002a).

There is overwhelming evidence that genetic factors play a major role in CL/P susceptibility. Namely, CL/P tends to aggregate in families (20-25% of cases are familial), monozygotic twins demonstrate excess concordance, and recurrence risk are elevated well above the population prevalence. The precise mode of inheritance, however, is still a matter of debate. The most likely picture is one where a handful of genes with major (i.e., detectable) effects are superimposed onto a multifactorial background. The consensus view is that anywhere from 3-14 genes are involved (Lidral and Murray, 2004; Marazita and Mooney, 2004). The identification of these major genes has been hampered by the fact that these loci exhibit reduced penetrance and complex interaction effects. In addition, genetic heterogeneity both within and between CL/P samples has been a major impediment.

1.5.3.2 Identifying candidate genes/regions The number of genes involved in facial morphogenesis is vast. Large-scale expression profiles of embryonic murine orofacial tissue have detected as many as 7000 genes during the critical stages of primary palate development (Mukhopadhyay et al., 2004). Similar studies are now being carried out on human craniofacial tissues (e.g., COGENE project); results thus far have been comparable (Cai et al., 2005). From among this complex network of transcription factors, growth factors and signaling molecules, certain genes have garnered disproportionate attention from researchers. This is because independent lines of data suggest that such genes are likely to play a key role in the processes underlying primary palate morphogenesis. Sometimes, general knowledge about a gene's function is enough warrant the interest of researchers (e.g., genes involved in cell adhesion). More often, however, candidate genes for CL/P have been identified by observing expression patterns in relevant tissues, performing experimental studies on animal models, employing linkage mapping approaches including whole-genome scanning for regions of interest, and by applying knowledge from syndromic forms of CL/P where the putative genes have been identified. Other recently developed methods such as genome-wide association have yet to be applied to CL/P samples.

Candidate genes identified through expression must be present in the right place at the right time. The number of genes expressed in the developing face is far too great for a comprehensive treatment of the subject; in fact little is known about the expression pattern or function for the vast majority of these genes. Many potential candidate genes for clefting

involve developmental processes that take place at very early stages; for example, the multitude of genes that regulate neural crest formation, migration and patterning (for review see Cobourne, 2000; Francis-West et al, 2003; Richman and Lee, 2003; Cox, 2004; Helms et al., 2005; Morris-Kay, 2006). It is probable, however, that most of these genes are not good candidates for *non-syndromic* CL/P, because disruptions at this level of development are likely to result in far more dramatic effects on the craniofacial complex. Therefore, this brief discussion will be limited to a handful of genes expressed slightly later in development, in the immediate tissues of the naso-maxillary region (i.e., orofacial prominences) that give rise to the primary palate.

Several bone morphogenetic protein (Bmp) isoforms are expressed in the developing orofacial prominences. In chicks, *Bmp2* and *Bmp4* are heavily expressed in the distal epithelial tips (the pre-fusion contact sites) of the medial nasal, lateral nasal and maxillary prominences (Francis-West et al., 1994). These Bmp signals are required for mesenchymal proliferation, which results in outgrowth of the facial prominences (Ashique et al., 2002). This is accomplished through activation of downstream targets *Msx1* and *Msx2* (Barlow and Francis-West, 1997), which are expressed in the mesenchyme immediately adjacent to the overlying sites of ectodermal Bmp expression. Virtually identical patterns of expression and function for these molecules have been described in mice (Gong, 2001; Gong and Guo, 2003). Sonic hedgehog (Shh) is a powerful morphogenetic protein expressed in the epithelia of the orofacial prominences (Hu and Helms, 1999; Jeong et al., 2004). *Shh* is thought to play a key role in both the early survival and later proliferation (outgrowth) of the facial prominences (Jeong et al., 2004). Hu and Helms (1999), for example, have shown that localized application of exogenous *Shh* results in horizontal expansion of the frontonasal prominence in mice. Just prior to primary palate fusion, *Shh* is downregulated at the epithelial contact site of the maxillary prominence, which may facilitate apoptosis and cell adhesion required to establish the epithelial seam (Cox, 2004). A number of fibroblast growth factor (Fgf) isoforms are expressed in the orofacial prominences, although the role of *Fgf8* has been emphasized. Like *Bmp4* and *Shh*, *Fgf8* is expressed in the epithelia of the facial prominences and is suspected to play a role in their outgrowth. Marked Fgf8 expression in the regions of the olfactory placodes suggests a key role in the formation of the medial and lateral nasal prominences (and the nasal pits) by controlling differential growth of the frontonasal mesenchyme (Bachler and Neubuser, 2001). A recent study by Lan et al. (2006) has shown differential *Wnt3* and *Wnt9b* expression in the epithelia of

the facial prominences of mice prior to lip fusion. During lip fusion, only *Wnt9b* was expressed at the contact site between the medial and lateral nasal processes. Furthermore, canonical Wnt signaling (TOPGAL) was detected in the epithelial seam as well as the adjacent mesenchyme. For a more in-depth account of the expression profile, signaling pathways and interactions involving these and other potential CL/P candidate genes see Francis-West et al. (1998), Chong et al. (2002), Depew et al. (2002), Cox (2004) and Jiang et al. (2006). The following website is also useful: http://www.informatics.jax.org/menus/expression_menu.shtml.

Transgenic mouse models and mouse strains with spontaneous CL/P have provided a wealth of information about potential candidate genes. Mice with targeted inactivation of one or more genes (knockout mice) most often present with clefts of the secondary palate or median clefts, however, some also possess isolated primary palate clefts (analogous to the human condition) as part of their phenotype (see Francis –West et al., 2003). For example, mice lacking both *Msx1* and *Msx2* present with bilateral CL/P (Cox, 2004). Similarly, mice with null mutations for *Bmp4* and *Bmpr1a* (Liu et al., 2005), *Wnt9b* (Carroll et al., 2005), *Ap2α* (Nottoli et al., 1998), *Gli3* (Juriloff, 2002) and *Egfr* (Miettinen et al., 1999) exhibit CL/P in varying degrees of frequency and severity. Certain strains of inbred mice also demonstrate naturally occurring CL/P (Juriloff, 2002). For example, in A-strain mice (A/J, A/HeJ, A/WySn) anywhere from 5-30% of newborns display CL/P, depending on the particular strain (Kalter, 1979; Juriloff, 1982). Other strains of mice such as CL/Fr (20-25%) and L-line (~10%) exhibit similar rates of spontaneous CL/P. Over the past 25 years Diana Juriloff and co-workers have attempted to uncover the genetic basis of CL/P in these mouse strains. Based on a large set of breeding experiments and linkage studies (reviewed in Juriloff, 2002), a model involving genes at two epistatically interacting loci, *clf1* and *clf2*, was offered (Juriloff et al., 2001; 2004); *clf1* has been mapped to a 2cM region on mouse chromosome 11 (human homologue 17q), while *clf2* has been mapped to a 4cM region on mouse chromosome 13 (human homologue 5q/9q). Numerous genes are present within these candidate regions, some of which have clear expression patterns in the developing mammalian face (Juriloff et al., 2004). In a recent report, Juriloff et al. (2006) identified *Wnt9b* as the putative gene for *clf1*. Although the gene for *clf2* has yet to be identified, it is currently believed to be a modifier with three alleles, the combination of which influences CL/P penetrance in the presence of the *Wnt9b* mutation. Syndromic mice with spontaneous mutations such as *Dancer* (*Dc*) and *Twirler* (*Tw*) also have CL/P as part of their phenotype in the

homozygous lethal state. The *Dancer* phenotype apparently results from a mutation in *Tbx10* that causes misexpression of the gene in the craniofacial region (Bush et al., 2004). The gene responsible for the *Twirler* phenotype is still unidentified, but the mutation has been mapped to mouse chromosome 18 (human 18q).

Early linkage mapping studies were severely limited by a paucity of genetic markers. The first study to evaluate linkage in a series of CL/P families utilized a set of 42 polymorphic markers and was able to demonstrate significant linkage to a gene (*F13A*) on the short arm of chromosome 6 (Eiberg et al., 1987). With the advent of high throughput sequencing technology and the strides achieved through the human genome project, new approaches to linkage mapping became possible (Gelineau-van Waes and Finnell, 2002). Applied primarily to human samples, genome-wide scanning/screening is an approach designed to survey the entire genome simultaneously in order to identify chromosomal regions that may harbor putative genes (Haines, 1998; Altmuller et al., 2001). This is accomplished by estimating linkage between a disease phenotype (and consequently the gene or genes responsible for that phenotype) and a large number of random polymorphic genetics markers (typically microsatellites or SNPs) spaced at regular intervals across the genome. Importantly, genome-wide scanning is a hypothesis generating approach; it is not designed to test *a priori* hypotheses regarding linkage of specific candidate genes/regions. From 2000 to 2005, a total of eight genome-wide scans have been carried out in samples of CL/P families (Prescott et al., 2000; Marazita et al., 2002a; Wyszynski et al., 2003; Zeiger et al., 2003; Marazita et al., 2004a; 2004b; Field et al., 2004; Lidral and Moreno, 2005). Results have generally been modest, with maximum LOD scores typically falling well-below the threshold for provisional linkage (LOD of 3.0). Suggestive LOD scores (> 1.0, < 3.0) for the following chromosomal regions have been replicated: 1p31-36 (*SKI1, LHX8, MTHFR*), 2p12-13 (*TGFA*), 2q35-37 (*SATB2*), 3p21-24 (*AP2, END1*), 3q24-28 (*P63*), 4q32-33, 9q21-22 (*PTCH, FOXE1*), 12q24, 14q11-12 (*TGFB3, BMP4*), 17q11, 18q11-12 (Lidral and Moreno, 2005). Using a slightly different approach, Marazita et al. (2004b) combined genotype data from seven independent samples (388 CL/P families; 2551 individuals) and performed a genome scan. Due most likely to the increased power derived from using such a large sample, significant parametric multipoint HLOD scores were observed for multiple regions: 1p12-13 (3.58), 6p23 (3.74), 6q23-25 (3.55), 9q21 (6.62), 14q21-24 (3.92) and 15q15 (3.65). Importantly, the large hit on 9q (*FOXE1*) represents the most significant linkage result

observed to date for CL/P. Furthermore, a meta-analysis combining the results from 13 independent genome scans (574 CL/P families; 3584 individuals) was performed. Twelve significant regions were identified, including several novel regions: 1q32 (*IRF6*), 2q32-35, 3p25, 6q23-25, 7p12 (*EGFR, GLI3*), 8p21, 8q23, 12p11, 14q21-24, 17q21 (*RARA*, *WNT9B*), 18q21 and 20q13 (*BMP7*). Also, a recent genome scan on Indian CL/P families revealed strong evidence in favor of linkage to a marker at 13q33.1-34, a novel region (Radhakrishna et al., 2006).

For many Mendelian syndromes associated with CL/P, the causative genes have already been identified. A number of these syndromes, specifically those with more mild phenotypes, have served as a source of candidate genes for non-syndromic CL/P (reviewed in Spritz, 2001; Cobourne, 2004; Cox, 2004; Stanier and Moore, 2004; Jugessur and Murray, 2005). Van der Woude syndrome (VWS; OMIM 199,300), for example, is an autosomal dominant disease typically characterized by CL/P or CP, lower lip fistulas and occasional hypodontia, although the disease exhibits variable expressivity and affected individuals have been documented to present with oral clefts as the only phenotype (Rizos and Spyropoulos, 2004). It is the most common Mendelian form of CL/P, accounting for about 1% of syndromic cases (Cobourne, 2004). VWS is caused by mutations in the *IRF6* gene located at 1q32 (Kondo et al., 2002). Critically, a strong relationship between specific alleles at the *IRF6* locus and non-syndromic CL/P has been demonstrated in more than one population (Zucchero et al., 2004) and this result has been independently replicated (Blanton et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005). A number of additional Mendelian syndromes with CL/P as part of their phenotype are currently considered models for non-syndromic CL/P, including CL/P with dental agenesis (*MSX1*; van den Boogaard et al., 2000), CL/P with ectodermal dysplasia 1 or CLPED1 (*PVRL1*; Suzuki et al., 2000), Gorlin's syndrome or nevoid basal cell carcinoma (*PTCH1*; Hahn et al., 1996), ectrodactyly ectodermal dysplasia clefting (EEC) syndrome and ankyloblepharon ectodermal dysplasia clefting (AEC) syndrome (*P63*; Celli et al., 1999), Kallmann syndrome (*FGFR1*; Dode et al., 2003) and X-linked Opitz syndrome (*MID1*; Cox et al., 2000). Active research projects are currently underway to examine the role of all of these genes in non-syndromic CL/P.

In addition to the methods outlined above, information about potential candidate genes/regions can be gleaned from cases where CL/P is due to naturally occurring chromosomal anomalies, including translocations, duplications, insertions, inversions and deletions (Brewer et al., 1998; 1999). Such chromosomal rearrangements may arise *de novo* or be transmitted within

families in some cases (e.g., 22q deletion syndrome). An approach called breakpoint mapping can be used to localize the position on a chromosome where the putative gene resides (in theory the gene should be very near or at the breakpoint). For example, a balanced translocation of the *SATB2* gene at 2q32 has been shown to result in isolated CP (FitzPatrick et al., 2003).

1.5.3.3 Testing candidate genes/regions Based on the various approaches outlined in Section 1.5.3.2, a number of candidate genes/regions have been identified. Ideally, for a gene to be a good candidate one would expect confirmation of a positive result from several independent research groups and/or overlapping evidence from alternative experimental modalities (e.g., expression analysis and/or genome-wide screening). Unfortunately, for CL/P, this has been the exception rather than the rule. A large number of studies have used positional cloning approaches such as linkage or association mapping or, more rarely, direct DNA sequencing/screening for mutations to test specific candidate genes. Because a comprehensive review of these studies is not feasible here, the current review will be limited to handful of the most important findings. For a more detailed account of candidate gene findings in CL/P see Wyszynski et al., 1996; Spritz, 2001; Vieira and Orioli, 2001; Carinci et al., 2003 and Cobourne, 2004.

Linkage analysis statistically assesses the extent to which two traits (a disease phenotype and a genetic marker of known location) are inherited together (i.e., co-segregate) more often than expected in families (Pericak-Vance, 1998; Teare and Barrett, 2005). In contrast with the genome-wide screening approach, a "focused" linkage analysis is carried out with the express purpose of testing particular candidate genes/regions chosen *a priori*. To date, the candidate linkage approach has been used to evaluate the 1q21-32 region (Pierpont et al., 1995; Wong et al., 2000; Martinelli et al., 2001a), the 2p13 region (Hecht et al., 1991; Vintiner et al., 1992; Field et al., 1994; Wyszynski et al., 1997b; Pezzetti et al., 1998; Scapoli et al., 1999; Wong et al., 2000; Schultz et al., 2004), the 4q25-31 region (Beiraghi et al., 1994; Pierpont et al., 1995; Blanton et al., 1996; Wong et al., 2000; Schultz et al., 2004), the 6p23-25 region (Hecht et al., 1993; Vintiner et al., 1993; Carinci et al., 1995; Blanton et al., 1996; Scapoli et al., 1997; 1999; Pezzetti et al., 1998; 2000; Wong et al., 2000), the 17q12-21 region (Shaw et al., 1993; Vintiner et al., 1993, Stein et al., 1995), the 19q13 region (Stein et al., 1995; Wyszynski et al., 1997c; Martinelli et al., 1998; Wong et al., 2000; Fujita et al., 2004) and the 22q11 region (Pierpont et

al., 1995). From all of these studies, statistically significant linkage results (LOD ≥ 3) are sporadic at best (Wyszynski, 2002). In fact, only the 6p23-25 region has showed a significant linkage finding in more than one study; it must be noted, however, that all of these analyses were performed on the same Italian population (Carinci et al., 1995; Scapoli et al., 1997; Pezzetti et al., 1998).

In genetic association analysis, allele frequencies of markers in or near candidate genes are compared statistically between individuals with a disease and unaffected controls (Hodge, 1993; Cordell and Clayton, 2005). Controls can come from either the general population (case-control) or from within families (family-based association) (Hodge, 1994). The goal in association analysis is to demonstrate a population-level correlation between the presence of a particular allele and a disease state. In general, association analysis is considered a more powerful approach than traditional linkage analysis for mapping genes in complex diseases like CL/P, where a number of genes with weak effects are likely involved (Risch and Merikangas, 1996). Thus, a large number of association studies have been carried out on CL/P (see Marazita and Neiswanger, 2002). In one of the first candidate gene association studies for CL/P, Ardinger et al. (1989) demonstrated a significant association between the disease and alleles at two polymorphic markers (C2 allele at Taq1 and A2 allele at BamHI) within the *TGFA* gene. Following this report, a number of attempts were made to replicate the *TGFA* finding in various CL/P populations, some finding support for an association (Chenevix-Trench et al., 1991; 1992; Holder et al., 1992; Sassani et al., 1993; Feng et al., 1994; Jara et al., 1995; Tanabe et al., 2000) and other failing to find support (Stoll et al., 1992; Field et al., 1994; Beaty et al., 1997; Lidral et al., 1997; Marazita et al., 2002b; Jugessur et al., 2003; Moreno et al., 2004; Suzuki et al., 2004). A meta–analysis of earlier case-control studies provided evidence in favor of an association (pooled OR = 1.42; CI = 1.16 – 1.73), but also found considerable heterogeneity (Mitchell, 1997). The current consensus view is that *TGFA* is probably a modifier gene, neither necessary nor sufficient to cause CL/P (Lidral and Moreno, 2005; Vieira, 2006).

In addition to *TGFA*, association approaches have also been carried out to investigate the following candidate genes/regions: *MSX1*(Lidral et al., 1997; 1998; Beaty et al., 2001; 2002; Blanco et al., 2001; 2004; 2005; Marazita et al., 2002b; Mitchell et al., 2001; Jugessur et al., 2003; Vieira et al., 2003; Moreno et al., 2004; Suzuki et al., 2004; Modesto et al., 2006), *4q31* (Mitchell et al., 1995; Paredes et al., 1999; Marazita et al., 2002b), *TGFB3* (Lidral et al., 1997;

1998; Maestri et al., 1997; Tanabe et al., 2000; Beaty et al., 2001; 2002; Mitchell et al., 2001; Sato et al., 2001; Jugessur et al., 2003; Vieira et al., 2003; Moreno et al., 2004; Suzuki et al., 2004; Ichikawa et al., 2006), *TGFB2* (Lidral et al., 1997; Tanabe et al., 2000), *RARA* (Chenevix-Trench et al., 1992; Shaw et al., 1993; Vintiner et al., 1993; Mitchell et al., 2003; Maestri et al., 1997; Kanno et al., 2002; Moreno et al., 2004), *BCL3* (Stein et al., 1995; Amos et al., 1996; Maestri et al., 1997; Wyszynski et al., 1997c; Lidral et al., 1998; Martinelli et al., 1998; Beaty et al., 2001; Marazita et al., 2002b; Gaspar et al., 2002; Blanco et al., 2004), *MTHFR* (Shaw et al., 1998b; Mills et al., 1999; Gaspar et al., 1999; Wyszynski and Diehl, 2000; Blanton et al., 2000; 2002; Martinelli et al., 2001b; 2006; Prescott et al., 2002; Shotelersuk et al., 2003; Jugessur et al., 2003; Pezzetti et al., 2004; Mostowska et al., 2006; Zhu et al., 2006), *PVRL1* (Sözen et al., 2001; Scapoli et al., 2004; Ichikawa et al., 2006; Neiswanger et al., 2006), *GABRB3* (Tanabe et al., 2000; Scapoli et al., 2002) and *IRF6* (Zucchero et al., 2004; Blanton et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005; Ghassibe et al., 2005). With the possible exception of *IRF6*, results have been contradictory for the majority of these candidate genes (Marazita and Neiswanger, 2002; Murray 2002). As discussed earlier, mutations in the *IRF6* gene have been shown to cause Van der Woude syndrome. Recently, in a multi-population study, Zucchero and co-workers (2004) demonstrated a strong association between a particular *IRF6* variants (V-allele) and non-syndromic CL/P. At the population level, the putative *IRF6* variant was found to be responsible for 12% of the genetic contribution to CL/P and tripled the recurrence risk in families with one affected member. This result has now been validated in a number of additional CL/P populations (Blanton et al., 2005; Scapoli et al., 2005; Srichomthong; et al., 2005; Ghassibe et al., 2005).

In a small number of studies, direct DNA sequencing has been carried out in order to identify possible functional mutations in candidate genes. Jezewski et al. (2003) performed a mutation screen on various coding and non-coding regions of the *MSX1* gene in a large sample of CL/P cases. Probable etiologic mutations were observed in 2% of affected individuals and no controls. This result has been replicated subsequently by a number of independent researchers (Suzuki et al., 2004, Vieira et al., 2004; Lace et al., 2006; Tongkobpetch et al., 2006). Potentially putative mutations in the *PVRL1* gene have also been identified in cases of non-syndromic CL/P (Sözen et al., 2001; Scapoli et al., 2004; 2006; Turhani et al., 2005), but see Tseng et al. (2006). In a recent study, Vieira et al. (2005) performed a mutation screen on 20

candidate genes in two CL/P populations (Iowa and Philippines). They identified a total of 16 missense mutations in nine genes, including point mutations in *FOXE1*, *GLI2*, *MSX2*, *SKI*, *SATB2,* and *SPRY2*. Taken together, Vieira and colleagues suggest that these mutations account for as much as 6% of severe non-syndromic CL/P cases.

Despite the best effort of researchers, the candidate gene approach has provided a vast amount of contradictory evidence when applied to complex diseases (Hattersley and McCarthy, 2005; Munafo, 2006). For almost every CL/P candidate gene subjected to linkage or association analysis, the results have been marred with inconsistencies. There are a number of plausible reasons for this. Traditional parametric linkage analysis was originally designed to detect genes of large effect that segregate in a Mendelian fashion. This analytical approach only began to be applied to CL/P after a number of segregation analyses failed to provide support for the prevailing MFT model (e.g., Marazita et al., 1984). Many of the requirements for parametric linkage analysis to operate effectively, namely that the mode of inheritance be specified correctly and that the relative contribution of the disease-causing gene be of sufficient magnitude, simply may not be tenable given the complex etiological nature of CL/P. Moreover, to detect a gene of minor effect (e.g., a modifier gene) using parametric linkage would require an extremely large and/or informative sample of families. One approach to deal with these problems has been the use of less model-based statistics (e.g., nonparametric linkage approaches). Another has been to employ data pooling and meta-analytic methods to acquire very large samples.

A more general problem, however, is that CL/P is a heterogeneous condition. Genetic heterogeneity can take two basic forms: locus heterogeneity, where a disease is due to mutations in more than one gene, and allelic heterogeneity, where a disease is due to different mutations in the same gene. CL/P is probably characterized by both types. There can also be other kinds of etiological heterogeneity; for example, some CL/P cases may be due to maternal effects or teratogens (i.e., sporadic cases). Careful screening of cases may be sufficient to deal with this problem. In addition, heterogeneity could be due to the racial/ethnic background of a sample. Finally, excess heterogeneity could result from inadequate specification of the disease phenotype (see next section). The presence of non-penetrant individuals as well as the combining of etiologically distinct conditions can obscure results. For example, at least one recent study has suggested that CL and CL/P may be etiologically distinct conditions and should not be combined prior to genetic analysis (Harville et al., 2005). Of course, every time a disease phenotype is

broken-down further, the sample size is reduced which could effectively offset any gains in resolution. In principle, all of these forms of heterogeneity can not only occur between samples (resulting in contradictory findings), but also within a given sample (resulting in a loss of power because heterogeneous families may nullify one another). For these reasons heterogeneity, whatever its form, poses a major problem for both linkage and association mapping approaches and is likely a major culprit in the demise of the candidate gene approach to mapping complex diseases.

1.5.4 Refining the cleft phenotype

Definition of a disease phenotype is the first step in mapping disease genes (Aylsworth, 1998; Haines and Pericak-Vance, 1998; Rice et al., 2001). This all important step, however, is perceived too often as an afterthought by geneticists. Accurate phenotype definition is particularly crucial when dealing with complex diseases, where the translation from genotype to phenotype is more sinuous (Funalot et al., 2004). Nevertheless, in many instances, complex disease phenotypes are based solely on convenient diagnostic conventions having little or nothing to do with basic biology (Aylsworth, 1998). Importantly, inadequate phenotyping can seriously compromise the statistical power in gene mapping approaches (Rice et al., 2001). For example, the affection status of individuals could be specified incorrectly, particularly when the disease in question is characterized by reduced penetrance and variable expressivity. In this scenario, family members with very subtle disease manifestations run the risk of being incorrectly diagnosed as unaffected. It is not difficult to comprehend how misdiagnosis can translate into a loss of statistical power; in the context of linkage analysis, it will distort the co-segregation of traits within families. Problems can also arise when diseases are characterized by large amounts of etiological heterogeneity. With such diseases, lumping heterogeneous cases or families into a single class will inevitably result in a loss of resolution. Typically, an attempt is made to mitigate etiological heterogeneity by screening study participants for family history, environmental exposures, etc. prior to genetic analysis. While this approach may eliminate the most obvious problems, a good deal of residual heterogeneity is still likely to persist. Rigorous phenotyping could provide an additional solution; if it can be shown that etiological variation has

observable phenotypic correlates, it may be possible to generate more homogenous subsets based on these phenotypic descriptors.

Diagnostic uncertainty has been a major issue in the field of psychiatry for many years and numerous attempts have been made to devise a more rigorous approach to phenotype definition (Lenzenweger, 1999; Gottesman and Gould, 2003; Braff and Light, 2005). Conversely, relatively little effort has been made to refine the phenotype associated with CL/P. This likely stems, at least in part, from the fact that the condition is a visible birth defect. Most often, genetic studies of CL/P simply divide subjects on the basis of a presence/absence criterion; individuals with a plainly observable defect are classified as affected. Occasionally, attempts have been made to further subdivide case samples. For example, some investigators have placed affected individuals along a graded scale of severity, while others have taken into account the laterality of the defect. While these are steps in the right direction, such schemes are still rooted in a system basically defined by superficial anatomical description, a kind of typology that has existed primarily to serve the surgical community. Due to this fact, such phenotypic classifications may not serve the needs of geneticists seeking to uncover the etiological factors underlying the disease. On this issue, Melnick writes, "In our search for solutions…we have failed primarily because we have attempted to elucidate etiological correlates to the wrong phenotype(s). CL±P is a distant consequence of more relevant and proximate 'inborn errors of morphogenesis.' As with any other genetic disorder, if the phenotype is vaguely or incorrectly defined, the chance of understanding its etiology is minimal." (Melnick, 1992; p. 10).

Since the 1960s it has become increasingly clear that both CL/P cases and often their "unaffected" relatives are characterized by a statistical overrepresentation of a number of rather subtle morphological deviations (reviewed in Weinberg et al., 2006a). These morphological deviations include excess asymmetry (Adams and Niswander, 1967, Woolf and Gianas, 1976; Werner and Harris, 1989, Neiswanger et al., 2002), increased non-right-handedness (Fraser and Rex, 1985; Wentzlaff et al., 1997; Jeffery and Boorman, 2000), aberrant dermatoglyphic patterns (Vormittag et al., 1979; Balgir, 1993; Kobyliansky et al., 1999; Scott et al., 2005a), occult defects of the orbicularis oris muscle (Martin et al., 2000; Weinberg et al., 2003; Neiswanger et al., 2006), a variety of dental anomalies (Böhn, 1963; Ranta, 1986; Harris, 2002), altered somatic growth trajectories (Becker et al., 1998; Wyszynski and Wu, 2002), increased frequency of minor physical anomalies/variants (Shprintzen et al., 1985; Pelz and Amling, 1996; Scott et al.,

2005b), structural brain differences (Nopoulos et al., 2000; 2001; 2002), various vertebral anomalies (Ross and Lindsay, 1965; Ugar and Semb, 2001; Rajion et al., 2006) and altered face shape (covered extensively in Section 1.6). In light of this evidence, it has become clear that the traditional phenotypes used to study the etiological basis of CL/P have been far too narrowly defined. Of particular interest to the genetics community are those phenotypic traits capable of differentiating the unaffected relatives of CL/P individuals from the general population. The potential value of these "subclinical" phenotypes to the genetics of clefting has not been overlooked by researchers. In an editorial to the American Journal of Medical Genetics, David Bixler expressed concern over the way CL/P phenotypes were being defined in genetic studies; he writes, "…if we cannot better define this cleft phenotype, such studies will yield distorted and incorrect information since 'gene carriers' (those non-cleft persons with increased genetic liability) will be missed." (Bixler, 1991; p. 271).

It is a given that in the majority of multiplex families, some fraction of unaffected individuals will possess cleft susceptibility loci. The problem is that based on current phenotypic definitions (overt cleft = affected), all unaffected family members are treated as if their genetic risk is equivalent. In principle, some or all of the aforementioned subclinical phenotypes may represent the outward manifestation of latent genetic liability for CL/P. Therefore, any method allowing for the detection of clinically unaffected, but genetically "at risk", individuals within cleft families has the potential to boost the informativeness of study samples and subsequently the power of genetic analyses based on those samples. This approach was utilized in a series of recent pilot studies of multiplex CL/P families, where the identification (via ultrasound) of occult defects in the upper lip musculature of a subset of unaffected relatives resulted in a reassessment of their affection status. Both genetic linkage and association analyses based on this phenotypically-enriched sample demonstrated an increase in statistical power over analyses using traditional diagnostic criteria (Weinberg et al., 2003; Neiswanger et al., 2006). One logical conclusion is that those superficially "unaffected" family members with muscular defects are harboring the putative risk alleles.

1.6 THE 'FACE SHAPE HYPOTHESIS'

1.6.1 Definition and origin

In its most elemental form, the ‘face shape hypothesis’ states that heritable aspects of facial morphology are a predisposing factor in the pathogenesis of isolated orofacial clefting. The idea that certain facial features represent a kind of *forme fruste* for orofacial clefting probably dates to the mid to late 19th century. The eminent Edinburgh surgeon Sir William Fergusson is cited as the first to point out the fact that parents of affected children often possessed certain distinctive facial features (Dixon, 1966). In a well-known (at the time) lecture on cleft lip and palate, Fergusson states, “…I am of the opinion that the defect [clefting] arises from the breed, and that it occurs where there is a predisposition in the parents. I fancy that I can detect this in the features of the father or mother, or of both” (Fergusson, 1867; p. 63). Elaborating on this point in *A System of Practical Surgery*, Fergusson writes “…I fancy I can in general detect the parent of a child thus born [that is, born with a cleft] by the appearance of the face, and often while listening to a mother’s story about some conjectural cause for her infant’s deformity, I have thought that a glance at her own features in a looking-glass might have given her a more plausible reason for the condition of her offspring” (Fergusson, 1870; p. 496). Perhaps influenced by Fergusson’s observations as well as accumulating evidence for the hereditary basis of orofacial clefting, other surgeons integrated an examination of parents’ facial features as part of their clinical routine. For example, Rose writes, “An examination of the parents’ mouths should always be made when possible, and very commonly it will be found that one or both possess a short upper lip, and a high arched narrow palate” (Rose, 1891; p. 23). These statements, unfortunately, fail to provide adequate detail regarding the precise nature of the facial differences observed in the parents of children with clefts. The notion, however, that the faces of parents differ in ways detectable to a trained observer was established.

A rebirth of the “face shape hypothesis” occurred during in 1960s as the MFT model was applied to orofacial clefting. Critically, the MFT model provided a set of rules for understanding and predicting the distribution of genetic liability within families when the trait in question did not follow a simple Mendelian inheritance pattern. The idea that variation in facial form should have some relationship with genetic liability to CL/P is not surprising, particularly given the fact

that virtually all candidate genes for CL/P have been shown to play a key role in normal facial development. As stated earlier, genetic liability to CL/P is usually modeled as a continuous trait, with a threshold located near the upper end of the distribution, beyond which the disease is expressed. The MFT model predicts that those individuals located toward the disease end of the distribution will have higher genetic susceptibility. Presumably, those individuals located beyond the threshold (i.e., those with an overt manifestation of the disease) posses some critical number or combination of cleft susceptibility loci. Just below the threshold, however, the presence of etiologic genes may simply result in a particular conformation of facial features associated with increased risk for developing a cleft or, in the case of parents, having a child with a cleft.

Consequently, to test the 'face shape hypothesis,' studies since the 1960s have focused on comparing facial morphology between various at-risk samples and low-risk samples. In animal models, this test involves a comparison of facial morphology between mouse strains with elevated rates of spontaneous or teratogen-induced isolated clefting and one or more normal or control strains. In humans, the comparison is typically made between a sample of unaffected relatives (e.g., parents and/or sibs) of individuals with isolated clefts and population controls with no family history of the disease. The evidence from these studies will be considered in the sections that follow.

1.6.2 Evidence from mouse models

1.6.2.1 Qualitative descriptions of facial morphology Unlike many other lines of research in science, the study of face shape as a predisposing factor in mouse strains susceptible to CL/P has an unequivocal beginning. In 1968, Daphne Trasler published a study comparing the developing faces of embryos from two inbred mouse strains: the A/J strain, which is susceptible to spontaneous CL and the C57BL/6 strain, which is not (Trasler, 1968). It was noted that by day 10, CL-susceptible mice were characterized by less distance between the nasal pits as well as an anterior and medial reorientation of the medial nasal prominences. This reorientation had the effect of decreasing the angle of divergence between contralateral medial nasal prominences, while at the same time, making them appear conspicuously prominent. Regarding the implications of these morphological differences for the pathogenesis of CL/P, Trasler speculates,

“This lack of divergence may result in a decrease or failure of the epithelial fusion between the medial and lateral nasal processes, and consequently a lack of consolidation of the isthmus” (Trasler, 1968; p. 37). Trasler interpreted these morphological findings within the context of the MFT model; indices of “face shape” were continuously distributed traits capable of influencing the probability that adjacent oronasal prominences would make contact and fuse.

Millicovski et al. (1982) compared facial development between cleft-susceptible CL/Fr mice (36% spontaneous CL) and normal C57BL/6 mice (0% spontaneous CL). Compared to the C57 strain, CL/Fr mice demonstrated altered geometry of the medial nasal prominences, such that their growth was directed in a more medial and anterior direction. These results were virtually identical to those obtained by Trasler (1968) on A/J mice. CL/Fr mice, however, also, displayed variable hypoplasia of the lateral nasal prominences and reduced surface epithelial activity, which the authors suggest is the reason for the exaggerated rate of CL in this strain. That same year, Trasler and Leong (1982) performed a histomorphological comparison between embryonic C57BL/6 mice (both normal and exposed to 6-aminonicotinamide or 6-AN in utero), mice with the *dancer* mutation (20% CL), and CL/Fr mice. Dancer mice displayed retarded nasal placode development at embryonic day 10 and, compared to stage-matched C57BL/6 embryos, had reduced medial and lateral nasal prominences. In contrast with Millicovski et al. (1982), CL/Fr mice were not found to have hypoplasia of the lateral nasal prominences. Also, unlike Millicovski and co-workers, Trasler and Ohannessian (1983) found no evidence of reduced surface epithelial activity at the contact site in either the A/J or CL/Fr strains compared to control C57BL/6 mice. Forbes et al. (1989), in a direct comparison of CL/Fr and A/J embryos, observed reduced epithelial surface activity and medial nasal prominence hypoplasia, but no difference in medial nasal prominence divergence in the more susceptible CL/Fr strain. Thus, the mechanisms underlying the exaggerated rate of CL in the CL/Fr strain are still unclear.

1.6.2.2 Quantitative assessment of facial morphology In an attempt to expand upon initial descriptive reports, Juriloff and Trasler (1976) attempted a quantitative assessment of embryonic facial morphology in three separate lines of mice: L-line mice that present with spontaneous CL at a rate of 3-9%, M-line mice that present with high rates of midline clefting when exposed to 6-AN in utero, and C-line control mice. A series of six measurements (and one index of measurements) were obtained from standardized photographs of day 10 embryos from each

mouse line; these measurements were designed to capture relevant aspects of frontonasal width, medial nasal prominence divergence and projection, and overall face size. Results indicated that L-line mice possess significantly less distance between the nasal pits compared to either the M- or C-lines, in both absolute and relative terms. A non-significant trend toward less divergent medial nasal prominences was also observed in the L-line. Frontonasal process width, overall face size and medial nasal prominence projection did not differ across lines. In general, these quantitative results support the original descriptions by Trasler (1968).

Trasler and Machado (1979) extended these initial findings by exploring the hypothesis that the embryonic face shape changes that characterize CL-susceptible mice persist into later stages of development. Both skeletal and soft tissue measurements were obtained on newborn and adult mice from five lines/strains with varying degrees of cleft susceptibility: CL/Fr (23% spontaneous CL), A/J (~12% spontaneous CL), L-line (3-9% spontaneous CL), M-line (0% spontaneous CL, but 17% midline cleft with 6-AN exposure) and C57BL/6 (0% spontaneous CL). While univariate comparisons showed significant inter-strain differences for all measurements, post-hoc tests revealed that many of these differences were among CL-susceptible strains (CL/Fr, A/J, L-line). For skeletal measurements, the only variable that consistency distinguished all CL-susceptible mice (newborn and adult) from all non-susceptible mice was a significant reduction in premaxillary length. Nasal bone length, premaxillary width and interorbital distance varied considerably among CL-susceptible strains. That being said, a discriminant function model based on these four skeletal predictor variables was capable of discriminating between CL-susceptible (A/J and L-line) and non-susceptible (M-line and C57BL/6) adult mice, with the CL/Fr strain remaining largely separate. Homologous skeletal measurements did not discriminate as well in newborns. For soft tissue measurements, CL-susceptible mice consistently demonstrated reduced snout and premaxilla length in a susceptibility-specific manner; as projective length of the anterior oronasal complex increased, CL-susceptibility decreased.

A morphometric analysis of CL-susceptible dancer mice was carried out by Jacobson and Trasler (1992). Mice heterozygous for the dancer mutation ($Dc^{+/-}$) and normal dancer mice ($Dc^{+/+}$) were mated to R stock mice, and the offspring of these crosses were compared. Relative to $Dc^{+/+}$ embryos at day 10/hour 21 (crescent stage), $Dc^{+/-}$ embryos were characterized by significantly reduced total facial prominence surface area and volume, reduced medial nasal

prominence surface area and volume, reduced lateral nasal prominence surface area and volume, and smaller maximum head width. Embryos did not differ, however, in anterior projection of the medial nasal prominences or crown rump length; thus, the observed reductions in *Dc*$^{-/+}$ crossed embryos were not due to a general somatic growth deficit. Wang and Diewert (1992) performed an extensive morphometric analysis of craniofacial growth patterns in mouse embryos from the A/WySn (28% spontaneous CL) and C57BL/6J control strains. During the early phase of primary palate closure (35-39 somite stage), A/WySn mice showed deficient anterior growth of the maxillary prominences, a rapid decrease in nasal pit width and delayed lateral and anterior-posterior forebrain growth. At this same developmental stage, control embryos demonstrated normal maxillary growth, relatively little change in nasal pit width and rapid forebrain growth. These results indicate that the coordination between medial nasal prominence growth and brain growth is altered in A/WySn mice, i.e., there is a loss of normal morphological integration or covariation between structures during development. Based on these findings, Wang and Diewert suggested that altered brain growth may be a primary factor in the development of CL in these mice; an interesting observation given the apparent structural brain differences in humans with CL/P (Nopoulos et al., 2000; 2001; 2002).

In a recent paper, Hallgrímsson et al. (2004) utilized a variety of landmark-based morphometric approaches to examine differences in craniofacial shape, developmental stability and morphological integration between adult A/WySnJ and C57BL/6J mouse skulls. To briefly summarize the main morphological findings, A/WySnJ mice exhibited significant shape differences (most notably, a relative shortening of the neurocranium and widening of the face), excess directional (but not fluctuating) asymmetry, and reduced morphological integration (particularly in the face and palate). The loss of integration and coordination between developing craniofacial structures observed in CL-susceptible mice echoes the findings of Wang and Diewert (1992). It is not clear, however, how to reconcile Wang and Diewert's description of reduced forebrain width and increased midface narrowing during early primary palate formation with the excess face width reported by Hallgrímsson and colleagues. One factor may relate to differences in the developmental stage of the samples; the former authors performed their analysis on embryonic mice whereas the latter study utilized adult skeletal tissue.

1.6.3 Evidence from humans

There is an extensive literature describing the effects of oral clefts on the growth and final form of the craniofacial complex. The vast majority of this literature is focused on outcome assessment by way of morphological comparison between surgically repaired cases and unaffected controls (for overview see Ross, 2002; Ward et al., 2002). Due to the confounding effects of early surgery on craniofacial growth, however, such studies are of relatively little use for understanding the relationship between isolated clefting and facial form. A number of research designs have been employed to circumvent the problem of surgical intervention. For example, comparative morphological studies have been carried out on fetal specimens affected with clefts (Siegel et al., 1987; 1991; Kimes et al., 1988; Mooney et al., 1988; 1992; Diewert and Shiota, 1990; Smith et al., 1997; Sherwood et al., 2001; Hansen et al., 2005), affected children prior to surgery (Krogman et al., 1975; Šmahel et al., 1985; Šmahel and Müllerová, 1986; Farkas et al., 1993; Kreiborg and Hermann, 2002; Zemann et al., 2002), and adults that never received surgical treatment (Ortiz-Monasterio et al., 1959; Bishara et al., 1976; 1985; 1986; Mars and Houston, 1990; Yoshida et al., 1992; Capelozza et al., 1993; Silva Filho et al., 1998; Lambrecht et al., 2000; Shetye and Evans, 2006). Numerous differences between such cases and controls have been reported for virtually every region of the craniofacial complex, the extent of the dysmorphology a function of the severity of the defect (Šmahel et al., 1985). Such differences are not surprising given the loss of structural and functional integrity that results from the presence of an oral cleft. The problem with these study designs, however, is that they preclude the possibility of distinguishing dysmorphic features which confer risk for developing a cleft (i.e., risk markers) from those which are secondary to the presence of a cleft once it has formed. Morphological features belonging to the former class are most suitable for predicting CL/P risk status. Consequently, the most common strategy to investigate craniofacial risk markers has been to examine the unaffected relatives of individuals with overt CL/P.

1.6.3.1 Early qualitative studies on unaffected relatives Many early family studies of clefting reported outwardly visible minor manifestations of the disease or "microforms" including notching or scarring of the upper lip and asymmetry of the nostrils in the otherwise normal relatives of cleft individuals (Fogh-Andersen, 1942). Beginning in the early 1960s, a

small number of studies began to use radiographs in order to examine even more surreptitious aspects of facial morphology in superficially unaffected relatives, with the expressed goal of identifying gene carriers. These studies tended to focus on the identification of discrete nasal and/or palatal defects. For example, Fukuhara and Saito (1962) examined frontal radiographs taken on a random sample of 21 Japanese families with at least one affected child; some form of occult dysplasia in the nasal floor and/or palate was evident in at least one unaffected relative (parent and/or sib) in 11 families. In a follow-up case report, Fukuhara and Saito (1963) observed comparable defects in an additional 12 families. Similarly, Rusconi and Brusati (1966) obtained radiographs of the nasal cavities of unaffected first-degree relatives in six Italian families; in all but one family, at least one relative had some form of observable nasopalatal defect. In a much larger and more systematic study, Niswander (1968) examined frontal radiographs for a variety of minor nasopalatal defects in 87 families with at least one affected member and 83 control families. Case-control comparison of defect frequencies revealed a slight albeit non-significant increase in unaffected case relatives; the rate of observable defects in CL/P family members (sporadic and familial cases) was slightly greater than 11%, compared to 9% in control family members. In a separate study on the same sample, Mills et al. (1968) investigated the morphological configuration of the palate from dental casts. Although the authors noted a slight tendency for unaffected first-degree relatives to have more high arched and narrow palates, these differences failed to reach statistical significance. It should be noted that, with the exception of Niswander (1968), the above studies included a combination of CL/P, CP and/or mixed cleft families. Furthermore, it is unclear whether any syndromic cases were included.

1.6.3.2 Quantitative studies comparing unaffected relatives to population controls Early attempts to quantitatively assess facial morphology in the unaffected relatives of cleft children tended to focus on one particular feature of anatomical region. Dixon (1966) examined the facial profile by assessing relative jaw relationships (ANB angle) from radiographs in a sample of 82 non-cleft parents of affected children. Overall, a very high proportion (30%) of the parental sample met the clinical criteria for a Class III relationship, indicating a substantial degree of relative midface retrusion. This finding held even after the parental sample was broken down by their child's cleft type; 29% Class III in parents of CL/P cases and 28% Class III in parents of CP cases. A few years later, Mills et al. (1968) compared measurements of maxillary arch width and

length derived from palatal casts between unaffected relatives (parents and sibs) and matched controls; no differences were observed. Coccia et al. (1969) compared ocular and orbital measurements from 100 unaffected relatives in 11 multiplex CL/P and CP families to 66 controls. In contrast to reports showing excess distance between orbits in cleft affected individuals (e.g., Moss, 1965), results indicated that unaffected relatives were significantly more hypoteloric than controls.

The first comprehensive attempt to describe quantitative patterns of craniofacial variation associated with increased CL/P risk was made by Fraser and Pashayan (1970). Using a combination of direct anthropometry, photogrammetry and physioprints, a series of 15 vertical, horizontal and projective measurements were compared between 50 unaffected parents of children with CL/P and 50 controls. To summarize, the parental group was found to have significantly increased upper face width, increased total face height and excess flattening of the midface. A gestalt assessment of face shape was also carried out by multiple blind raters from frontal photographs, with faces categorized as either round, ovoid, rectangular, trapezoidal or square. The parental group was characterized by a significantly higher frequency of rectangular and trapezoidal face shapes than the control group, indicating a relatively broader upper third of the face.

Following Fraser and Pashayan's landmark study, a cephalometric analysis of 40 unaffected parents and 40 controls was carried out by Coccaro and colleagues (1972). A total of 27 linear and angular measurements covering the facial skeleton and cranial base were obtained from lateral radiographs; significant relative-control differences were identified for 12 variables. The parental group possessed a significantly more acute cranial base angle, reduced upper face height, decreased palate length, reduced nasal bridge length, increased mandibular corpus length and less pronounced facial convexity (i.e., a flatter facial profile). Using a combination of photogrammetry and measurements derived from palatal casts, Erickson (1974) found that the unaffected sibs of CL/P cases tended to have a flatter facial profile, a more tapering maxillary arch and displayed some evidence of increased palatal arching.

In a comprehensive analysis, Kurisu et al. (1974) collected a set of 20 dimensions from lateral and frontal radiographs on a sample of 131 unaffected mothers and 92 unaffected fathers of CL/P probands. Compared to male controls, fathers were shown to exhibit significantly increased lateral orbital width, decreased total and upper face height, decreased facial convexity

and decreased (more acute) gonial angles. Mothers for the most part showed the same pattern of altered morphology when compare to their female control counterparts (i.e., excess facial width, reduced upper face height and increased facial flattening), however, the differences tended to be more subtle. These results were confirmed by factor analysis. Also in this same study, data from a second independent control group was included and it was found that the differences between the two control groups were often more substantial than those between unaffected parents and their initial sex-matched controls. This finding underscores the importance of selecting an appropriate control group for comparison. Furthermore, using a multivariate approach to measure biological distance (Q-mode correlation analysis), Kurisu and coworkers were able to demonstrate a negligible impact of the severity of the child's cleft on the parents' craniofacial morphology.

During the same year, Figalová and Šmahel (1974) published a study utilizing direct soft-tissue anthropometry to investigate craniofacial morphology in a variety of unaffected relatives (fathers and mothers, grandfathers and grandmothers, and uncles and aunts) from families with at least one cleft affected member. All groups of relatives were compared separately to a sample of 50 female and 50 male controls derived from the same region (Czech Republic). Focusing on the unaffected parents, both mothers and fathers were characterized by significant reductions in head length, head width and cranial base width. In contrast to previous reports, however, both sets of parents showed a significant increase in upper face height. In addition, fathers specifically demonstrated reduced upper face width and increased nose length (both absolute and relative) compared to male controls, while mothers demonstrated reduced mandibular width and increased intercanthal distance compared to female controls. Interestingly, the vast majority of the above findings were consistent across most additional relative groups. However, considering the excessive number of individual univariate comparisons (~200), many of the observed statistical differences may simply be false positives. Finally, in contrast with Kurisu et al. (1974), these authors report a tendency of more pronounced dysmorphology in families where the affected individual is more severe.

Based on their analysis of lateral radiographs, Shibasaki et al. (1978) observed that unaffected fathers of CL/P cases possessed significantly increased cranial base flexure, reduced palate length, reduced upper face height, decreased soft-tissue thickness of the upper lip, increased lower face height and a mandible with a shorter body, more obtuse gonial angle and

more open rotation compared to male controls. In contrast to earlier studies, unaffected fathers were also found to have a more convex facial soft-tissue profile. The morphological tendencies in unaffected mothers were generally similar to unaffected fathers, yet the relative-control differences were typically less pronounced; statistically significant deviations were noted only for cranial base flexure and soft-tissue thickness of the upper lip. Nakasima and Ichinose (1983) compared midparent values on 53 cephalometric variables to those from a sample of 110 male-female control pairs. The parental sample was divided by the type of cleft affecting their child: CL/P (180 parental pairs), CL (45 parental pairs) and CP (26 parental pairs). Compared to controls, the CL/P parent group demonstrated significantly reduced head length and width, maxillary depth and upper face height as well as significantly increased lower face height and various craniofacial width measures (upper face, orbital, nasal and mandibular). The experimental group was further characterized by an open mandibular rotation. A similar phenotype was observed for the CL only parents. Following the initial univariate analysis, a subset of the 53 variables was subjected to multiple discriminant function analysis. On the basis of these variables, all parental groups were easily distinguished from controls; measures of relative face width made the greatest contribution to group discrimination. In a separate study, Nakasima and Ichinose (1984) devised a method to acquire overall area measures of the neurocranium, upper face and lower face from frontal and lateral radiographs. These area measurements were then compared between parents of children with CL/P and parents of control children. The authors found that the parents of CL/P children showed a significant reduction in neurocranial area, particularly around the parietal region. No differences were observed for upper face or lower face area measurements. In another Japanese cephalometric study, Sato (1989) found that the unaffected fathers and mothers of children with CL/P had significantly increased interorbital and nasal cavity width, upper lip height and lower face height. Both parent groups also showed a tendency for reduced upper face height. Conversely, unaffected mothers and fathers had distinct facial profiles; the face of fathers was found to be more convex while the face of mothers was found to be more flattened compared to sex-specific controls. This difference was significant only for fathers. Based on these variables, discriminant function analysis was able to correctly classify over 90% of parents and controls to their respective groups.

Ward and colleagues applied an entirely different approach to the problem (Ward et al., 1989). They criticized earlier studies for assuming (based on a decidedly orthodox interpretation of the MFT model and its predictions) that genetic liability to CL/P would be equally distributed across parents. Ward and colleagues state that such studies "…make no allowance for the possibility that one parent may contribute more to the susceptibility for oral clefting than does the other" (p. 318). They go on to say, "…if one assumes etiologic heterogeneity in the production of facial clefts, parental contribution should be minimal in some cases, heavily weighted to one parent in others, and approximately equal only in those instances where by chance each parent happens to possess the same degree of predisposing factors" (p. 319). Thus, instead of simply describing how a group of unaffected relatives differs *en mass* from controls for a given set of measures, Ward and co-workers utilized hierarchical cluster analysis to classify individual relatives into relatively homogeneous, naturally occurring subsets based on their composite phenotype. With this design, the major goal is to identify that subset of relatives whose craniofacial phenotype is distinct from the general population.

Seventeen dimensions were collected from lateral radiographs on 82 unaffected parents of CL/P children. All data were standardized against published sex-, age- and population-specific normative cephalometric data to generate Z scores. Following cluster analysis, three main subsets/clusters of relatives were identified. One parental cluster (N = 39) was characterized by craniofacial features that did not deviate substantially from the published normative data. In contrast, the other two clusters of relatives clearly differed from controls in a variety of ways. For example, both deviant clusters tended to have increased cranial base length, increased palate length, greater total face height and greater lower face height. In other ways, however, these two "at-risk" clusters were unique with respect to their morphological tendencies. One cluster, for example, tended to have a shorter mandibular body, an increased gonial angle and a flatter facial profile compared with published norms. On the other hand, the remaining cluster tended to have more cranial base flexure, larger mandibular measurements, a decreased gonial angle and excess posterior face height. These three parental groups were also compared against an independent sample of CL/P affected individuals. Not surprisingly, the cluster whose craniofacial measurements did not deviate far from normative values was least similar to the CL/P affected group. Conversely, the pattern of craniofacial morphology in the other two parental clusters was much closer to the CL/P group, arguing that these clusters represent a

subset of individuals at increased risk for harboring CL/P susceptibility factors. Interestingly, when both parents of a given affected child were included in the analysis, it was observed that in almost half the instances one parent was assigned to an "at risk" cluster while the other was assigned to the normative cluster.

Blanco et al. (1992) compared eight variables from frontal radiographs between unaffected parents and sibs of CL/P probands and sex- and age-matched controls. Both younger (age 6-12) and older (age 13 and greater) male siblings did not differ in any appreciable way from male controls. Unaffected fathers, however, did show significant increases in neurocranial width. Female sibs in the younger age group demonstrated a significant increase in total face height and a tendency toward increased upper face height over female controls. The older female sib group showed a different pattern of changes; they showed a significant increase in upper face width and interorbital distance. Unaffected mothers did not differ from female controls on any measure. The paucity of statistically significant findings in this study was most likely due to the small sample sizes. Raghavan et al. (1994) compared mean cephalometric measurements (midparent values) from 38 pairs of parents with CL/P children to 24 control parental pairs. Unaffected parents showed a number of differences; major findings included significant decreases in cranial base flexure, upper face height and almost all measures of upper and lower face width. Significant increases were noted for palate length, gonial angle, midface prominence and nasal cavity width, which was the only horizontal facial dimension larger in the experimental group.

Mossey and co-workers (1998a) examined lateral radiographs on a sample of 83 unaffected parents of CL/P children born in Scotland. Compared to sex- and ethnicity-matched controls, unaffected fathers and mothers demonstrated rather unique morphological differences. Fathers, for example, showed evidence of reduced mandibular and maxillary area, reduced palate length and decreased cranial base angle. Mothers, on the other hand, were characterized by increased mandible length, increased total face height and increased cranial base length. Both mothers and fathers possessed reduced overall calvarial area. Discriminant function analysis was able to correctly classify over 80% of male parents and controls and over 90% of female parents and controls. A similar approach was taken by AlEmran and co-workers (1999); however, they utilized data derived from fontal radiographs. They found that unaffected fathers had significantly increased nasal cavity width yet decreased maxillary alveolar width. Unaffected

mothers also had significantly reduced maxillary alveolar width in addition to reduced head width and upper face width. Stepwise logistic regression was able to correctly classify 74% of the male relatives and controls (based on nasal cavity and alveolar width) and 77% of the female relatives and controls (based on head width). Suzuki et al. (1999) included frontal as well as lateral radiographs and limited their experimental sample to unaffected parents from multiplex cleft families. Initial univariate tests were carried out on 55 linear distance, angle and ratio variables. Compared to a large control sample, parents demonstrated significantly increased interorbital, intercondylar and nasal cavity width (both in absolute and relative terms) as well as increased cranial base length. Parents did not differ from controls on any angular variables. Furthermore, no interaction effects between sex (father or mother) and parental status (unaffected relative or control) was noted for any variable. A subset of highly significant variables was then entered into a discriminant function analysis; about 68% of parents and controls were classified correctly. An independent sample of unaffected parents from simplex CL/P families and controls was then used to validate the initial discriminant function model; only 62% of the follow-up sample was classified correctly using the initial model parameters. These classification results are in stark contrast with those of Mossey et al. (1998a).

McIntyre and Mossey (2003a) compared over 400 individual measurements from frontal radiographs between an etiologically mixed sample of 92 unaffected parents (i.e., their children had either CL/P or CP) and an ethnically-matched control sample. A total of 133 linear distances, ratios, angles and cross-sectional areas covering virtually every region of the craniofacial complex differed significantly between parents and controls. Most notably, parents were characterized by an expansion of the superolateral portions of the face (e.g., lateral orbital and zygomatic regions) and a concomitant reduction of central and midface nasomaxillary components. A subset of 36 highly significant variables was entered into a discriminant function analysis, which correctly classified over 90% of parents and controls. McIntyre and Mossey (2004) further characterized craniofacial morphology in this sample through a variety of geometric morphometric approaches designed to separate shape from size (principle components analysis of shape, Euclidean distance matrix analysis and thin plate spline transformation). Overall face shape was determined to be statistically different between unaffected parents and controls and between parents of CL/P and CP children. No shape differences were observed between unaffected mothers and fathers (which may itself indicate a deviation from the normal

pattern of human sexual dimorphism). Parent-control shape differences were largely localized to a few anatomical regions; parents displayed lateral and inferior displacement of the superolateral portions of the face, decreased upper face height resulting from superior displacement of the nasal floor, superior displacement of the mandible and unilateral expansion of the nasal cavity. Interestingly, the prior finding of nasomaxillary restriction (McIntyre and Mossey, 2003a) was not confirmed; in fact, the opposite pattern was observed. Given that the same parent and control samples were used for both studies, this difference must reflect statistical methodology.

Perkiomaki et al. (2003) analyzed lateral radiographs from a sample of 28 Costa Rican families with a history of CL/P. Unaffected relatives (mothers, fathers and sibs) showed evidence of reduced anterior cranial base length and palate length compared with sex- and age-matched normative data; these changes were most prominent in unaffected male sibs. Mothers also showed some evidence of reduced head length. One major design flaw in this study is that only variables found to be significantly different between male and female CL/P cases and controls were subjected to testing in unaffected relatives; many of these differences could arise from surgery or simply as a secondary consequence of the cleft itself. Using an identical study design, Yoon et al. (2004) analyzed frontal radiographs on these same families. In general, unaffected family members were found to have significantly decreased head width, mandible width, total facial height and lower facial height. Furthermore, unaffected family members displayed significantly wider facial dimensions, including measures of the interorbital region, nasal cavity, upper face and maxilla. Upper face height tended to be increased, specifically in unaffected fathers and male sibs. This pattern of facial height differences (i.e., a decreased lower face and an increased upper face) is in direct contradiction to the bulk of prior evidence and may be idiosyncratic to this particular population. Interestingly, the pattern of craniofacial differences observed in parents largely mimicked those in their affected children.

1.6.3.3 Discordant twin designs A relatively small number of morphological studies on twins discordant for CL/P have been carried out, likely due to the rarity of the phenomenon. Most studies focus on comparisons between affected and unaffected twins (Ross and Coupe, 1965; Cronin and Hunter, 1980; Trotman et al., 1993; Laatikainen, 1999). An alternative study design with relevance to the face shape hypothesis is to compare the craniofacial morphology of noncleft twins to an unaffected control sample. Only three studies to date have carried out such

an analysis. Johnston and Hunter (1989) compared the noncleft twins from 25 monozygotic pairs to matched normative cephalometric data derived from the Burlington Growth Center database. The major finding of this study was the presence of reduced nasal cavity width in the noncleft twin sample. It was also noted, however, that the values for this trait showed a bimodal distribution, with reduced widths in roughly two-thirds and increased widths in the other third of the noncleft twin sample. Interestingly, a similar distribution of nasal cavity size was observed in CL/P cases (Liu et al., 1992). Johnston and Hunter hypothesize that these morphological findings stem ultimately from discrepancies in the size of the embryonic nasal prominences and may indicate the presence etiologically distinct subgroups.

Laatikainen et al. (1996) examined a sample of noncleft twins from 33 monozygotic and dizygotic pairs where the affected twin had either CL/P or CP. The only major difference noted was a more open mandibular rotation in the noncleft twins. More recently, Chatzistavrou et al. (2004) performed a cephalometric analysis comparing the noncleft twins from 25 monozygotic pairs (CL and CL/P) to sex-, age- and ethnicity-matched controls. Although differences were generally subtle, the noncleft twins did demonstrate significantly reduced interorbital, maxillary and nasal cavity width, reduced upper face height and increased cranial base length. There was also some evidence, in contrast to Laatikainen et al. (1996), that noncleft twins exhibited a more closed mandibular rotation pattern. Furthermore, the finding of reduced facial width in their noncleft twin sample is at odds with many of the findings in studies on unaffected parents. A major advantage to the discordant twin design is the obvious fact that the noncleft twin is genetically indistinguishable from the affected co-twin, at least when the sample is comprised of monozygotic twins. Thus the same gene(s) responsible for the cleft must be present, but not fully expressed, in the noncleft twin. Nevertheless, it is also possible that multiple births could result in altered craniofacial morphology due to intrauterine factors; this fact could effectively bias the sample.

1.6.3.4 Studies relating population variation in craniofacial form to cleft prevalence

Another indirect line of evidence supporting the face shape hypothesis is the relationship between facial features and CL/P incidence across populations. As reviewed earlier, epidemiological studies show that the prevalence of CL/P varies widely by population, with certain Asian-derived groups having the highest rates, followed by European-derived and lastly

African-derived populations. It has been suggested that populations with the highest incidence of CL/P should possess facial features similar to those observed in other “at-risk” groups, such as the noncleft relatives of cleft individuals (Fraser and Pashayan, 1970; Burdi et al., 1972; Siegel and Mooney, 1986). For instance, a number of anthropological studies have shown that, compared to other populations, some East-Asian groups are characterized on average by a broader upper face and palate coupled with less facial convexity (Enlow, 1990; Hajniš et al., 1994; Gill, 1998). To date, however, very few studies have formally tested this hypothesis.

Fraser and Pashayan (1970) qualitatively assessed overall face shape from frontal photographs and physioprints in a sample of 20 normal Japanese subjects, as well as 50 unaffected parents of Caucasian CL/P probands and 50 Caucasian controls. Compared Caucasian controls, rectangular and trapezoidal face shapes were greatly over-represented in the Asian sample, as was the tendency for a flat facial profile. Critically, these findings match those observed in the sample of unaffected relatives also included in the study. Chung and Kau (1985) compared cephalometric data across six ethnic groups from Hawaii with varying rates of CL/P. The higher risk groups (Japanese, Chinese and Filipinos) were observed to have reduced cranial base length, upper face height, palate length and mandible length compared to the lower risk groups (Caucasians and native Hawaiians). Additionally, relative upper face width was found to correlate positively ($r = 0.872$; $p = 0.027$) with CL/P incidence by ethnic group. That is, groups with the higher rates of CL/P had relatively wider upper faces. Incidentally, this latter finding is supported by experimental studies on animal models demonstrating a relationship between patterns of excessive growth in maxillary width and rates of CL/P (Smiley et al., 1971; Siegel and Mooney, 1986).

1.6.3.5 Attempts to synthesize prior studies Even a cursory consideration of the available evidence for face shape change in unaffected relatives is sufficient to paint a complex and confusing picture. The data are peppered with inconsistent and contradictory findings. As a consequence, reaching a consensus on the precise facial characteristics that best define this group of "at risk" individuals has been difficult. In spite of these difficulties, it is worth mentioning that *not a single study has failed to find some evidence of craniofacial alteration in the clinically unaffected relatives of cleft cases*. Thus, we know their faces are "different," though of course the devil is in the details. In recent years, a few attempts have been made to synthesize the

available evidence. Ward et al. (2002) provided a comprehensive narrative review of the morphometric literature on CL/P cases as well as their unaffected family members. They conclude that the most promising morphological markers for CL/P risk include excess interorbital and midfacial width, greater lower face height and an open (posterior) rotation of the mandible. In support of this claim, they point to the fact that these same features are present in affected individuals, both prior to and following surgery.

McIntyre and Mossey (2002) offered a slightly more systematic review of the cephalometric literature. A formal literature search was carried out to identify relevant studies, followed by a process of data abstraction. After comparing data across 15 studies comparing unaffected parents to controls, the following conclusion was reached: the craniofacial phenotype of unaffected parents does indeed differ from the general population, although it is not possible to identify a consistent pattern of distinguishing features. The authors attribute this inability to a variety of methodological inconsistencies and ethic variability in the populations studied. Maulina and co-workers (2006) reached the same conclusions as McIntyre and Mossey in their updated review of the literature.

A systematic review was also carried out by Weinberg et al. (2006b); however these authors used a more formal meta-analytic approach to quantitatively synthesize the available cephalometric evidence. Following a literature search, a total of nine unaffected parent-control studies were identified that met the study's inclusion criteria. Analyses were carried out with both maternal and paternal data combined (28 independent variables) and within each parent group separately (18 independent variables). To summarize the main findings, unaffected parents of children with CL/P possessed significantly wider interorbital, nasal cavity and upper facial dimensions, narrower cranial vaults, longer cranial bases, longer and more protrusive mandibles, shorter upper faces and longer lower faces compared with controls. Some sex-specific patterns were also noted. Expressly, unaffected mothers (but not fathers) were characterized by significantly reduced head width, increased cranial base length and increased SNB angles. Alternatively, unaffected fathers (but not mothers) were characterized by significantly reduced upper face height. This study also revealed that for roughly half of the included variables, significant across-study heterogeneity was present. Moreover, the magnitude of the effect sizes for over 80% of the variables was in the small (0.20 - 0.49) to very small (< 0.20) range, with only a handful of variables falling within the moderate range. Taken together,

these facts indicate that the morphological changes present in the parental sample are quite subtle and, in many cases, highly variable. The most robust finding in unaffected parents was an increased nasal cavity width; this trait was significant in the maternal, paternal and combined analyses, displayed minimal across-study heterogeneity and had the largest effect size.

1.6.3.6 Combining genetics and morphometrics Combining morphometric and genetic information in the analysis of CL/P would seem a logical step in the search for relevant liability factors. Unfortunately, very few studies have attempted to combine morphometric and genetic data. Ward et al. (1994) reported on a single large multiplex family demonstrating five generations of CL/P. Based on their relationships within the pedigree and clinical status (and assuming a major gene model), family members were divided into presumed "obligate carriers", "obligate normals", affected individuals and "unknown" cases. Groups were compared on a set of seven cephalometric variables, chosen for their suspected relevance to CL/P liability. Univariate comparisons revealed that "obligate carriers" had significantly a wider nasal cavity, increased interorbital distance and reduced lower face height compared to the "obligate normal" group. Stepwise discriminant function analysis identified a combination of four variables (nasal cavity width, facial profile, palate length and lower face height) capable of classifying 88.9% of family members into their correct group. The biological distance in multivariate space between the carrier and normal group centers (centroids) was statistically significant (p = 0.0035). Discrimination between the at-risk carrier and normal relative groups was influenced by a combination of increased facial width dimensions (interorbital, upper facial and nasal cavity), decreased lower face height and facial profile flattening. It was suggested by Ward et al. (1994), that these results could indicate the presence of a major gene effect operating within this family. It was subsequently noted by Ward and colleagues (2002) that when this particular pedigree was subjected to linkage analysis a separate study, all of the morphologically deviant "obligate carriers" possessed genetic markers linked to the short arm of chromosome 4.

Mossey et al. (1998b) performed an association analysis on three polymorphic markers (BamHI, RsaI, TaqI) within the *TGFA* gene. It was observed that the distribution of alleles at the BamHI marker differed significantly between the unaffected parents of CL/P cases and CP cases. Using a logistic regression model, this genetic marker data was able to correctly classify 68.3% of parents according to the affection status of their offspring. Interestingly, by incorporating a

set of three cephalometric variables observed to differ between these parental groups (cranial area, mandibular length and total mandibular area) into the regression model, the classification accuracy increased to 85%. Thus, by informing their genetic analysis with morphometric information, the ability to predict parental group membership improved dramatically over either type of data considered in isolation.

1.6.3.7 Problems and limitations of previous research As McIntyre and Mossey (2002), Weinberg et al. (2006b) and other have pointed out, methodological inconsistencies among studies have led to confusion as to the precise nature of the craniofacial differences between unaffected relatives and controls. Prior studies typically suffer from one or more of the following problems: 1) a reliance on cephalometry, which can only provide the most rudimentary information on craniofacial form and precludes the analysis of many potentially important soft tissue structures; 2) lack of standardization regarding variables used to summarize craniofacial form; 3) the use of simple univariate statistical methods that are incapable of separating size differences from shape differences; 4) a failure to account for sex differences; 5) the inclusion of a large number of simplex families, where the etiology is more likely to be sporadic (i.e., non-genetic) in origin; and 6) a failure to utilize methods capable of assigning risk to individual relatives on a case by case basis.

1.7 THE POTENTIAL SIGNIFICANCE OF THIS RESEARCH

Based on both human and mouse studies, it has been hypothesized that certain heritable aspects of craniofacial form (e.g., facial widths) represent either a predisposing factor to CL/P or a subtle manifestation of the disease. As outlined above, numerous studies have attempted to identify differences in craniofacial form in the unaffected relatives of children with clefts compared to population controls. Unfortunately, due to methodological inconsistencies and inadequate study designs, the precise nature of these differences remains elusive. The purpose of this study is to improve upon previous attempts to characterize the craniofacial complex of unaffected relatives by employing state of the art three-dimensional surface imaging technology in conjunction with the application of multivariate statistical methods that allow for a more rigorous assessment of

craniofacial form/shape, as well as the differential assignment of liability to individuals. As a consequence, the proposed study has the potential to overcome many of the limitations that have plagued previous research. Ultimately, this approach should allow for a more detailed understanding of the differences that characterize the facial phenotype in unaffected relatives. More broadly, though, exploration of craniofacial morphology in non-cleft family members is directly relevant to our understanding of the underlying causes of isolated CL/P. If indeed the differences in facial morphology between unaffected CL/P relatives and population controls relate to an underlying genetic susceptibility, then the identification and subsequent inclusion of superficially unaffected, but genetically informative, individuals into genetic analyses should improve gene mapping results. Further, if a set of craniofacial variables predictive of latent genetic risk can be established, this has direct implications for genetic counseling and the generation of recurrence risk estimates.

1.8 THE PRESENT STUDY: HYPOTHESES AND GOALS

Our general working hypothesis is that the unaffected relatives from CL/P multiplex families will display quantitative differences in craniofacial soft tissue morphology when compared to demographically-matched controls. A secondary related hypothesis is that the pattern of craniofacial shape change present in unaffected relatives will be sex-specific. That being said, the primary objective of the present study is to utilize 3D surface imaging and statistical shape analysis to describe the precise nature of these differences. Furthermore, this study aims to develop a predictive model capable of classifying unaffected relatives into liability categories based on their facial phenotype.

2.0 MATERIALS AND METHODS

2.1 SAMPLE RECRUITMENT

Two main groups comprised the current study sample: unaffected relatives of individuals with CL/P and unaffected controls with no prior history of the disease in their family.

2.1.1 Unaffected relatives

The unaffected relatives of individuals with CL/P were identified through index cases (affected probands) treated by the Cleft Craniofacial Center at Children's Hospital of Pittsburgh. The Cleft Craniofacial Center provides comprehensive care (surgical, speech, hearing) for craniofacial anomalies, serving families that reside predominantly in Western Pennsylvania. The initial identification of suitable families was performed by chart review following HIPPA approval. To be eligible to participate in the study a family had to be multiplex (have at least one additional affected individual other than the proband) and of Caucasian ancestry. It was not necessary that the second affected individual be a first degree relative of the proband or that they have the same type of cleft as the proband. A small number of "mixed" families (CL/P and CP) and CP only families (N = 2) were also included. Families with known syndromic forms of clefting were excluded; all families were screened for syndromes following their initial identification by board certified medical geneticists. Eligible families were then recruited via phone or letter. Typically contact was initiated with a member of the proband's nuclear family, but both first degree and extended family members were invited to participate. Once a family decided to participate, they were mailed a copy of the informed consent documents to review. Upon arrival at the research facility, all family members were explained the study in detail and signed consent was obtained.

2.1.2 Unaffected controls

Unaffected controls with no history of orofacial clefting (syndromic or non-syndromic) were ascertained through a variety of methods. Regardless of their mode of ascertainment, controls with a personal or family history of orofacial clefting or any other disease associated with craniofacial dysmorphology or with personal history of facial plastic or reconstructive surgery were not eligible to participate. Further, recruitment of controls was restricted to individuals of Caucasian (European) ancestry. Methods of control recruitment included referral (using a market research firm to provide contact information on individuals willing to participate in research studies) and solicitation (posting flyers in and around the university to advertise the study). In addition, members of the university staff and faculty as well as their friends and family members participated as controls. Potential control subjects were contacted by study recruiters and screened to ensure they met the inclusion criteria. Upon arrival at our research facility, informed consent was obtained. The vast majority of controls ascertained through the above methods permanently reside in western Pennsylvania. Additional controls were obtained in eastern Ohio during the 2005 Twins Days Festival, as part of a related research project. Over the course of a two day period, self-referred individuals were screened and consented on-site. In general, control participants were ascertained as unrelated individuals; however, occasionally related controls (nuclear families or sibs) were recruited.

All participants (cleft families and controls) were reimbursed for their participation. All aspects of subject recruitment and the data collection protocols outlined below have been approved by the Internal Review Board of the University of Pittsburgh.

2.2 DATA COLLECTION PROCEDURE

2.2.1 Craniofacial data

Quantitative data on the soft tissues of the head and face were obtained by a combination of two methods: 3D photogrammetry-based indirect anthropometry (3D landmark coordinate locations and linear distances) and caliper-based direct anthropometry (linear distances).

2.2.1.1 Indirect 3D surface anthropometry A FaceCam 250 imaging system was used to obtain 3D surface captures of the face. The FaceCam 250 is a digital 3D photogrammetric system developed by Genex Technologies, Inc. (Bethesda, MD). The system is completely self-contained, comprised of an external metal housing within which sit three cameras (center, right and left) arranged at fixed angles to provide overlapping fields of view (Figure 2). Also within the main housing is a centrally positioned projector that briefly shines a grid pattern of structured (striped) white light onto the target. The pattern of deformation as the light hits the surface of the target is recorded by the cameras during capture and provides the raw data for reconstructing 3D surface geometry. The entire capture phase takes 400 milliseconds. The data are then sent to a local PC where proprietary software is utilized to generate a single 3D composite. In its most basic form, this composite consists of a very dense 3D polygonal mesh skeleton (~300,000 individual data points connected by vertices). This polygonal mesh is essentially a pure geometrical representation of the captured surface (i.e., it contains the surface geometry). The precise location in 3D space of every data point comprising the mesh is defined by a set of unique *x,y,z* coordinates. Both color and texture data (acquired at the time of capture) are superimposed on top of the mesh in order to improve interpretability. The final product is a geometrically accurate representation of the surface of the face with realistic surface rendering. The Genex system has been extensively validated in terms of measurement precision and accuracy (Weinberg et al., 2004; 2006c).

For the present study, the data of interest are the *x,y,z* coordinate locations of specific facial soft tissue landmarks. In an effort to obtain the broadest anatomical coverage with the minimum number of landmarks, an initial set of 22 standard soft tissue landmarks (Farkas, 1994; Kolar and Salter, 1997) was chosen. A list of these landmarks and their definitions is presented

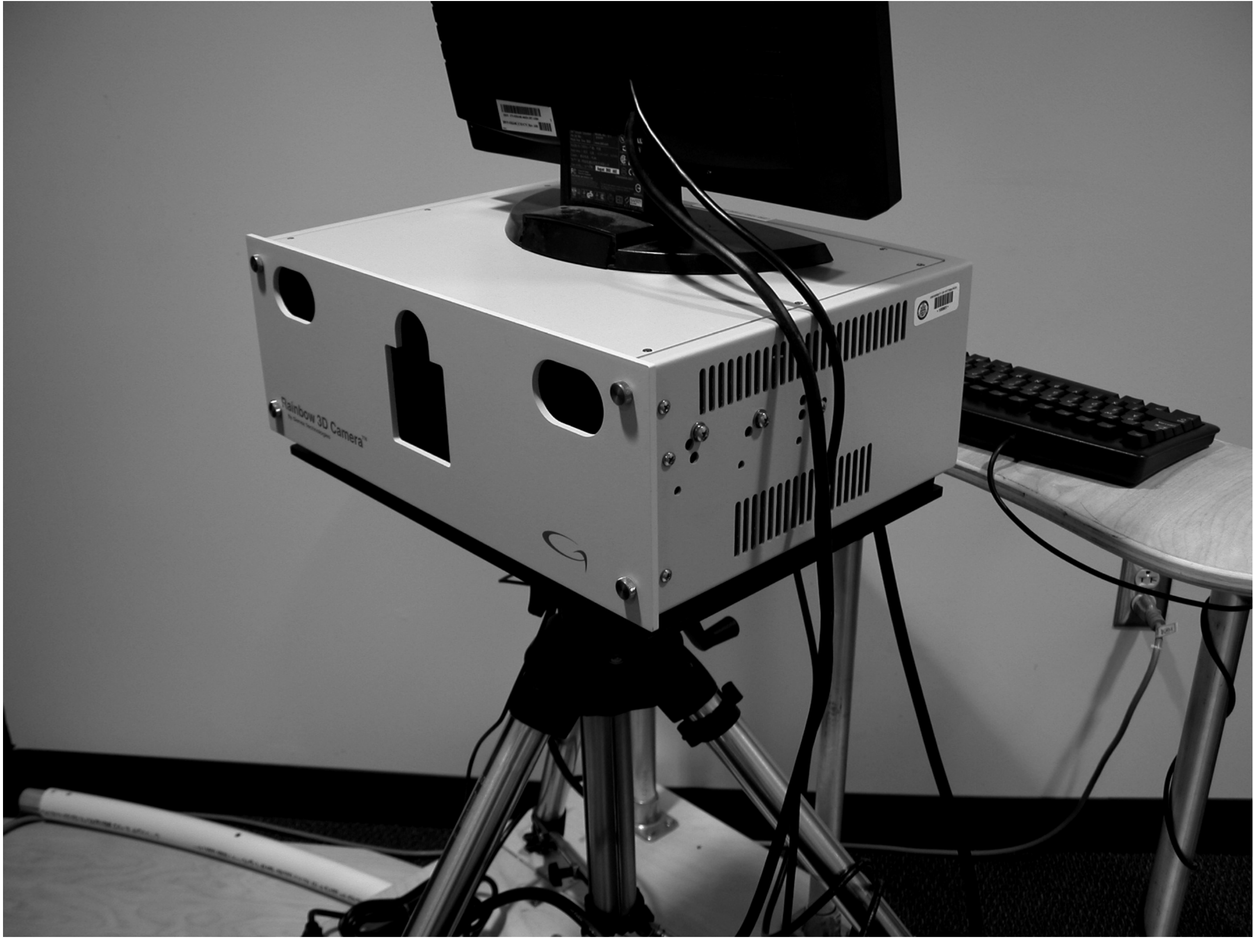

Figure 2 Genex 3D FaceCam 250 imaging system.

The three digital cameras and the striped light projector are housed within the unit.

in Table 2. These landmarks generally correspond to the anatomical boundaries of facial soft tissue structures (e.g., *alare*) or to the underlying boney morphology (e.g., *nasion*). Landmark positions were marked directly on the subject's face with an Onyx liquid eyeliner pencil. Landmark labeling prior to image capture not only allows for more precise landmark localization from the captures, but also dramatically increased the speed of data collection (Weinberg et al., 2004). In order to reduce the invasiveness of the landmark labeling procedure, five landmarks were left unlabeled: left and right *endocanthion*, left and right *chelion*, and *stomion*. This did not present a major problem, since identification of these landmarks is relatively straightforward on the 3D capture. For the same reason, the right and left *exocanthion* could not be labeled directly. In this case, however, a small mark was made just below the outer edge of each eye (1-2cm below) with the subject gazing upwards. These marks were used as a guide to localize *exocanthion* during landmark extraction.

After the facial landmarks were labeled, 3D image capture could proceed. The imaging parameters of the FaceCam 250 specify a capture field with a depth of 35cm (14in). The anterior boundary of this capture field is 155cm (45in) from the front plate of the FaceCam 250 while the posterior boundary is 150cm (59in) from the front plate (Figure 3). The simplest set up is to position the camera so that the front plate is parallel to and exactly 59in from an interior wall. This set-up ensures that any object within the space extending from the wall to 14in out will be within the designated capture field. The capture process begins with the subject seated on an adjustable stool placed against a wall with neutral-tone paint (e.g., beige). Positioned directly in front of the FaceCam system, the subject is asked to rest the back of their head against the wall in order to minimize involuntary movement. The operator adjusts the tilt of the subject's head to approximately 10 degrees above Frankfort horizontal to ensure that the subnasal/submental regions are within the camera's visual range. Once the subject's head is in the correct orientation, the height of the FaceCam unit is adjusted until the subject's head is centered vertically within the camera's field of view (this is facilitated by a preview feature of the capture software that allows the operator to view the subject from the camera's perspective in real-time video). Any hair obscuring the subject's face or ears is tucked away.

At this point, the subject is asked to make no facial expression, relax their face and breathe calmly through their nose. The center (frontal) capture is then taken. Immediately following the capture, a preview image is produced, which can be used to check for motion

Table 2 Craniofacial landmarks used in 3D photogrammetry

Region	*Landmark*	*Symbol*	*Location*	*3D Capture* [a]	*Definition* [b]
Head	Tragion	t	Bilateral	L, R	The point at the superior margin on the tragus where the cartilage and skin meet
Face	Gnathion	gn	Midline	C, L, R	The most inferior point on the inferior surface of the mandibular symphysis [c]
	Nasion	n	Midline	C, L, R	The point corresponding the location of the frontonasal suture in the midline [c]
	Sublabiale	sl	Midline	C	The point at the midline of the labiomental sulcus
	Subnasale	sn	Midline	C, L, R	The point at the intersection of the columella and the skin of the upper lip
	Stomion	sto	Midline	C	The midpoint along the labial fissure [d]
Orbits	Endocanthion	en	Bilateral	C	The point marking the medial angle (inner corner) where the upper and lower eyelids meet [d]
	Exocanthion	ex	Bilateral	C	The point marking the lateral angle (outer corner) where the upper and lower eyelids meet
Nose	Alar curvature	ac	Bilateral	L, R	The most posterolateral point along the nasal ala where the cartilage meets the skin of the cheek
	Alare	al	Bilateral	C	The most lateral point on the alar cartilage of the nose
	Pronasale	prn	Midline	C, L, R	The most anterior point on the tip of the nose along the midline
	Subalare	sbal	Bilateral	C	The point at the posteroinferior margin of the nose where the alar cartilage meets the skin of the upper lip

Table 2 (continued).

Mouth	Chelion	ch	Bilateral	C	The point marking the angle where the upper and lower lips meet [d]
	Crista Philtri	cph	Bilateral	C	The point where the philtral ridge meets the vermillion portion of the upper lip

[a] C = Center capture, L = Left capture, R = Right capture

[b] Based on Kolar and Salter (1997)

[c] Requires palpation to locate

[d] Landmark not pre-labeled on subject

artifacts and data coverage. Based on this preview the user has the option of discarding and repeating the image capture or saving the data for later processing. This is the first of three captures. With the subject remaining in the same position, the camera is then repositioned to obtain 45 degree left and 45 degree right captures. This is accomplished by sliding the camera along a custom made track (Figure 3); due to its arc-shaped design, the track system ensures that the distance between the FaceCam unit and the subject remains constant at every point along the track. Assuming none of the images need to be retaken, all three captures can be obtained in under 30 seconds. Finally, the subject is instructed to remove the landmark labels from their face.

The need for multiple captures on the same subject stems from the FaceCam 250's inherently limited horizontal field of view; a limitation that translates into an inability to acquire surface information from ear to ear in a given frontal capture. Thus, any landmarks in and around the ears (e.g., *tragion*) are not visible in a center capture. While, in principle, multiple captures can be "stitched" together into a single composite during post-processing, in practice, a number of potential problems could arise during this process. For example, because captures with the FaceCam system are not obtained synchronously (but sequentially) any change in facial expression between captures will add considerable noise to the data (Weinberg and Kolar, 2005). Currently, the process of combining multiple captures in a sequential capture system like the FaceCam 250 lacks empirical validation.

At this point all of the data necessary for the generation of a 3D image exist, albeit not in a visually interpretable form. The data embedded within each capture must be reconstructed to produce a 3D polygonal mesh with surface rendering. With the Genex FaceCam capture software this is an automated process that takes about 40 seconds and can be done at any time following the initial image capture. After processing, the 3D capture is available immediately and can be visually inspected and modified (if necessary) through the use of a variety of orientation, rendering and editing tools. For each 3D capture, any extraneous information (i.e., anything other than the subject’s face) contained within the capture field was selectively deleted to reduce the size of the capture. Further, the 3D polygonal mesh was inspected for obvious defects, such as holes or spikes. If present, such defects were corrected using a range of available data filling and smoothing tools. Finally, the 3D capture was saved locally and backed-up on a server.

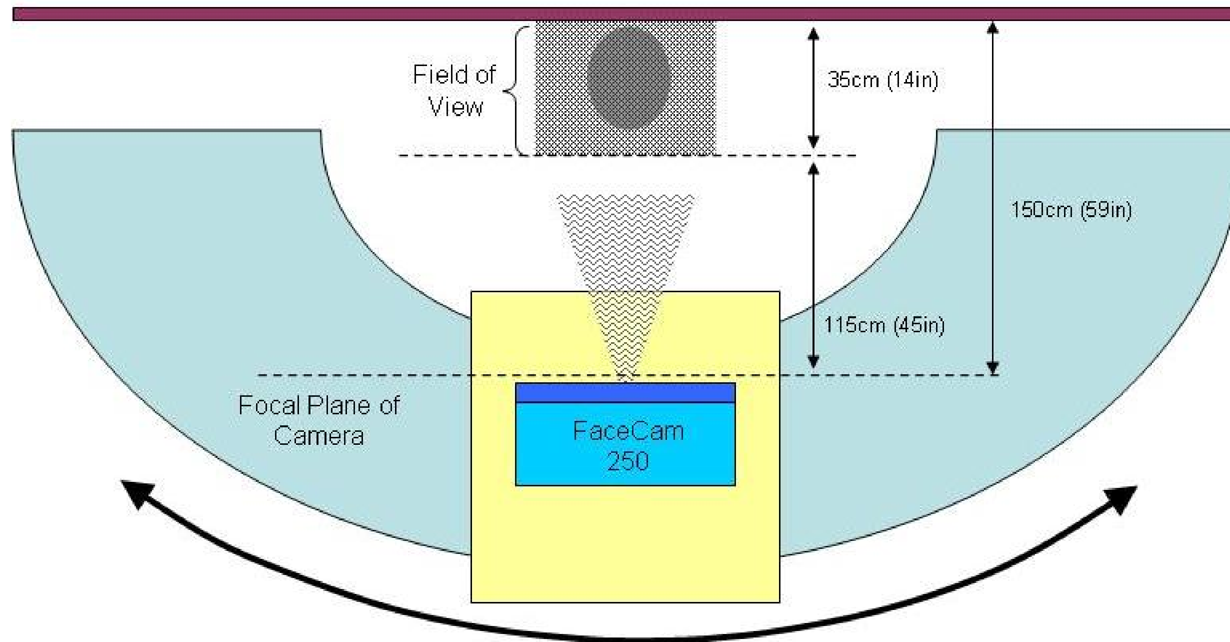

Figure 3 Set-up for 3D imaging system.

The camera is moved by the user in a stable arc (indicated by arrow) in order to obtain 45 degree right and left 3D captures. This design allows for a constant distance to be maintained between the focal plane of the camera and the stationary subject when the camera is moved to any position along the arc.

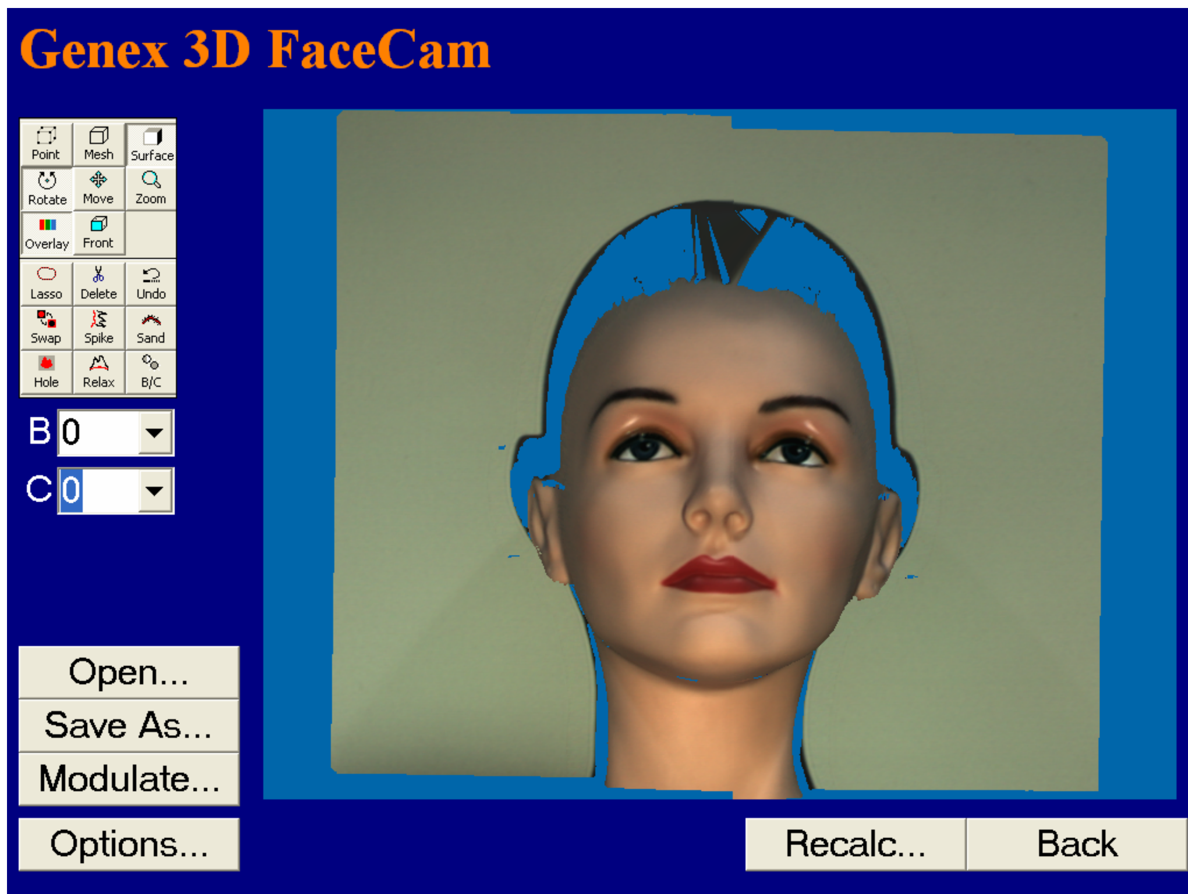

Figure 4 Genex 3D capture software interface.

The interactive GUI allows the user to alter the orientation, size and surface rendering of the 3D capture with the menu of tools located at the right side of the frame. Furthermore, the user is able to edit the 3D capture by removing extraneous data (e.g., background data) and altering surface properties (e.g., spike removal and hole-filling).

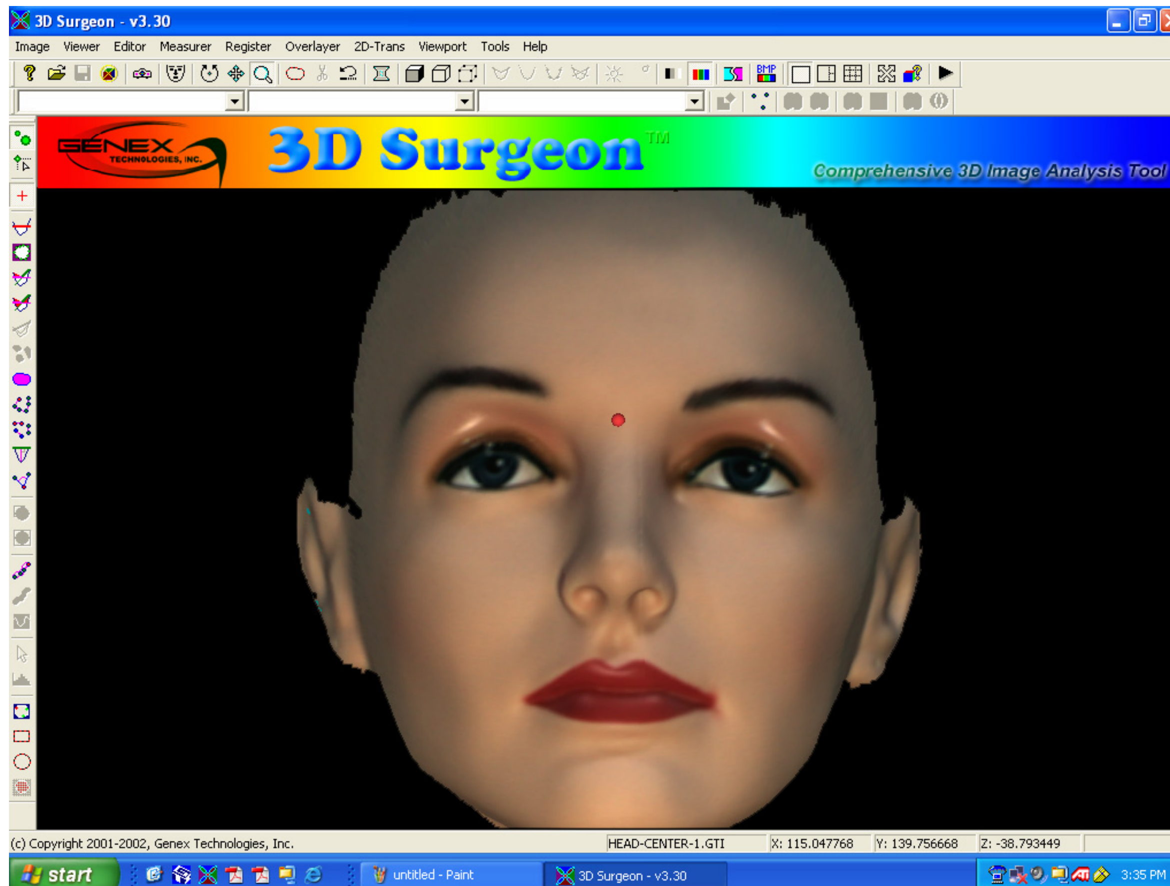

Figure 5 Genex 3D Surgeon software interface.

The interactive GUI allows the user to alter the orientation, size and surface rendering of the 3D capture. In addition, this program allows 3D coordinate and linear distance data to be obtained from 3D facial captures, using the menu of measurement tools located along the right side of the frame. Note the single landmark (red point) positioned at *nasion*. The *x,y,z* coordinates associated with is landmark are displayed along the bottom of the frame.

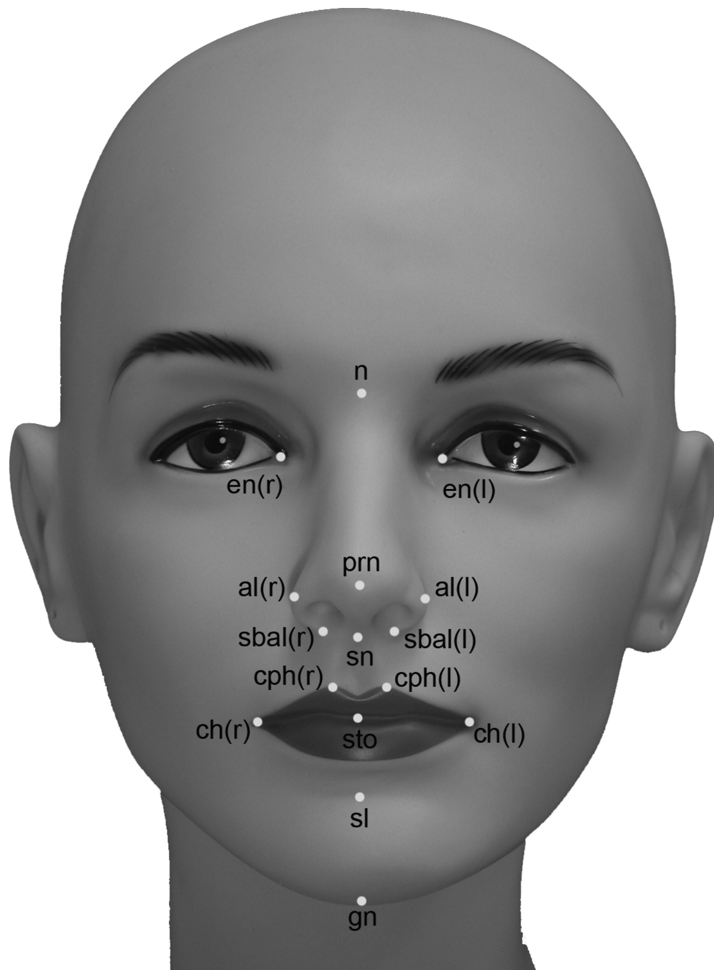

Figure 6 The sixteen facial surface landmarks used in the present analysis.

The coordinate locations of these landmarks were subjected to Euclidean distance matrix analysis.

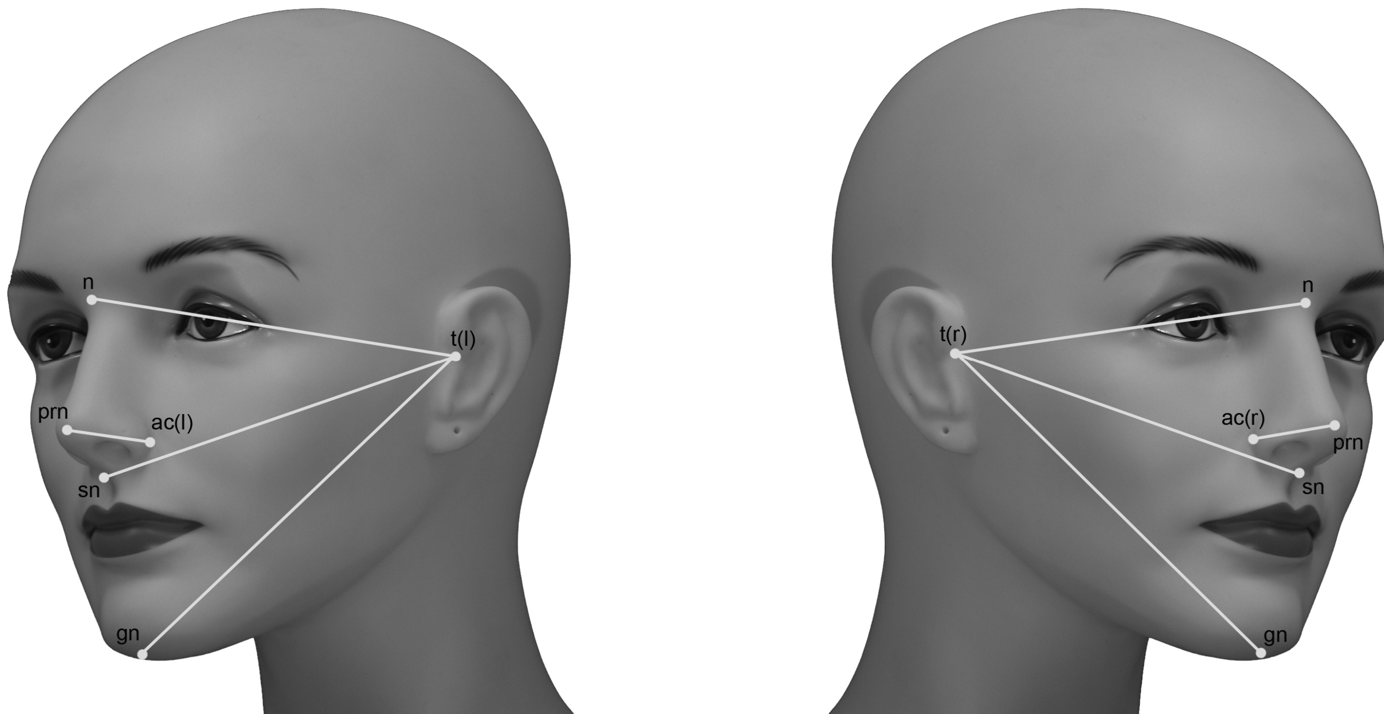

Figure 7 Additional linear distances from 3D photogrammetry.

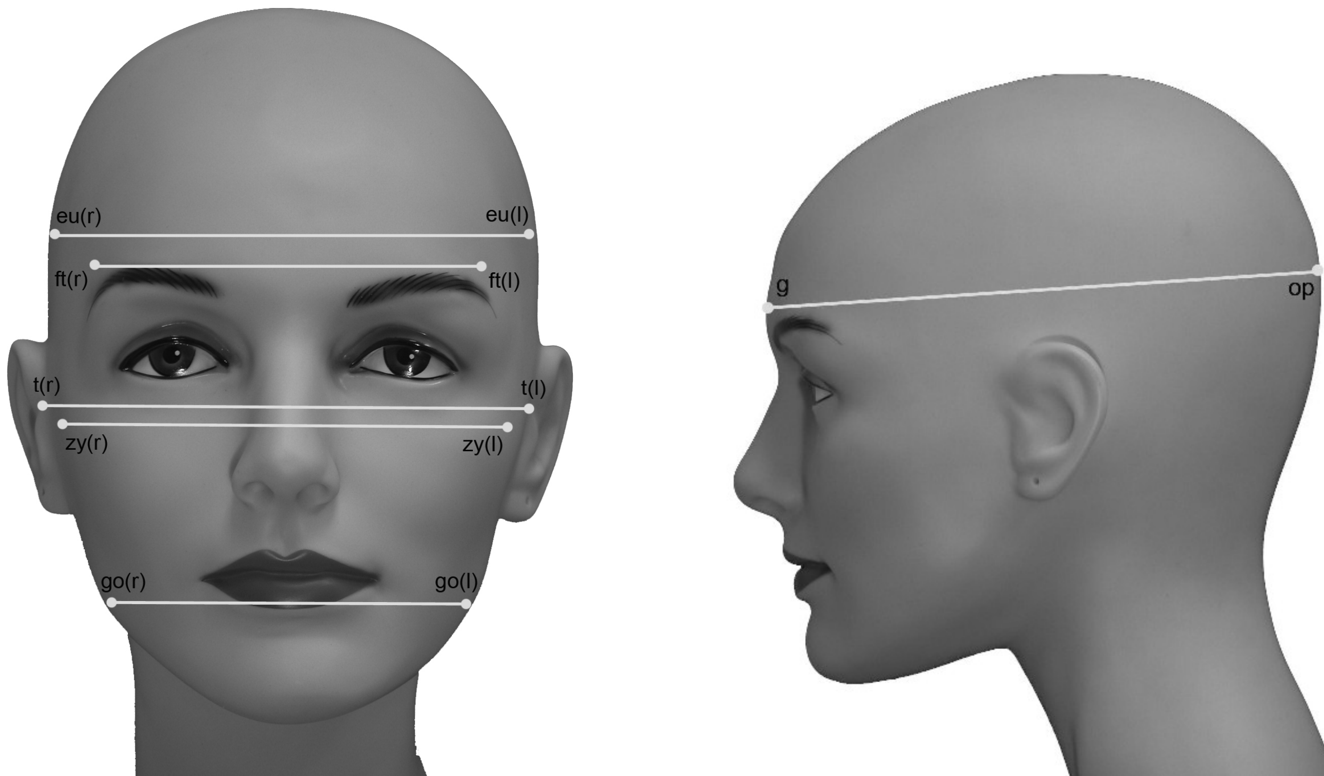

Figure 8 Additional linear distances from direct anthropometry.

Data in the form of *x,y,z* coordinates were then extracted from the 3D captures. Each capture was loaded into *3D Surgeon*, the proprietary measurement software program developed by Genex. This program offers users a broad set of measurement tools for extracting quantitative information from 3D captures, including user-specified 3D coordinates. The GUI allows the user to interact with the 3D capture, altering its size, orientation and surface rendering to provide optimal visualization (Figure 4). A given landmark's 3D coordinates are obtained by positioning the curser over the landmark's location and right-clicking. Once selected, the point is permanently marked on the capture with a colored label (Figure 5). This process is repeated, one by one, until each landmark of interest has been selected. The *x,y,z* coordinate locations are then saved and written to a text file, compiled in the order the landmarks were selected. From the center 3D capture, an initial set of coordinates were obtained corresponding to the location of 18 soft tissue landmarks. However, the number of landmarks was ultimately reduced to 16 because the left and right *exocanthion* could not be located in a large number of individuals. Figure 6 shows the 16 landmarks derived from the center capture. From the left and right 3D captures, coordinates corresponding to the location of six landmarks (four overlapping with the center capture) were obtained. Unfortunately, because the coordinate system for each 3D capture is unique the landmark data derived from each capture cannot simply be combined post-hoc. As a result, the data obtained from each capture must be treated separately during statistical analysis.

For the center capture, the raw coordinates were used for analysis. Alternatively, the coordinates derived from the left and right captures were used to calculate a set of four paired (left and right) linear distances (Table 3 and Figure 7), which were then subjected to statistical analysis. All linear distance calculations were carried out in Excel using the standard formula for deriving the Euclidean distance between two points (landmarks) defined by Cartesian coordinates in 3D space.

2.2.1.2 Direct anthropometry Spreading calipers (GPM, Switzerland) were used to acquire six additional soft tissue craniofacial linear distances directly on subjects. All of these additional measurements involve landmarks that cannot be located using indirect (non-contact) methods and/or are so large that they span an area outside of the field of view of a single 3D capture (Weinberg and Kolar, 2005). These additional landmarks are listed in Table 4. Table 3

Table 3 Additional linear distances used in the present study

Linear Distance	*Symbol*	*Method*
Maximum head width	eu-eu	Direct anthropometry (spreading calipers)
Forehead Width	ft-ft	Direct anthropometry (spreading calipers)
Maximum upper face width	zy-zy	Direct anthropometry (spreading calipers)
Cranial base width	t-t	Direct anthropometry (spreading calipers)
Mandible width	go-go	Direct anthropometry (spreading calipers)
Maximum head length	g-op	Direct anthropometry (spreading calipers)
Upper face depth (L)	t(l)-n	3D photogrammetry (left capture)
Upper face depth (R)	t(r)-n	3D photogrammetry (right capture)
Midface depth (L)	t(l)-sn	3D photogrammetry (left capture)
Midface depth (R)	t(r)-sn	3D photogrammetry (right capture)
Lower face depth (L)	t(l)-gn	3D photogrammetry (left capture)
Lower face depth (R)	t(r)-gn	3D photogrammetry (right capture)
Alar cartilage length (L)	ac(l)-prn	3D photogrammetry (left capture)
Alar cartilage length (R)	ac(r)-prn	3D photogrammetry (right capture)

Table 4 Craniofacial landmarks used in direct anthropometry

Region	*Landmark*	*Symbol*	*Location*	*Definition* [a]
Head	Euryon	eu	Bilateral	The most lateral point on the cranial vault [b]
	Frontotemporale	ft	Bilateral	The most medial point on the temporal crest
	Glabella	g	Midline	The most anterior point in the midline between the supraorbital ridges
	Opisthocranion	op	Midline	The most posterior point on the occipital region of the cranial vault [b]
	Tragion	t	Bilateral	The point at the superior margin on the tragus where the cartilage and skin meet
Face	Gonion	go	Bilateral	The most lateral point at the mandibular angle [b]
	Zygion	zy	Bilateral	The most lateral point on the zygomatic arch [b]

[a] Based on Kolar and Salter (1997)

[b] Identified during course of measurement

contains a description of these anthropometric measurements and they are represented visually in Figure 8. All are standard anthropometric measurements (Kolar and Salter, 1997).

2.2.2 Demographic information

Basic demographic information such as age, sex, ethnicity, height and weight were obtained from each subject at the time of assessment. Further, as part of a broader medical history examination, the specific type of cleft present in the proband and additional family members was verified. The LAHSHAL code was used to facilitate cleft classification (Kriens, 1989); this system is designed provide a quick descriptive summary of both the laterality (unilateral or bilateral) and the anatomical location(s) of the defect.

2.3 STATISTICAL ANALYSIS

2.3.1 Overall study design

2.3.1.1 Statistical design Data analysis was divided into two phases. In the first phase, the goal was to identify a subset of variables that best distinguishes unaffected relatives from matched controls. To accomplish this goal, two different statistical approaches were utilized based on the type of data available. The raw *x,y,z* coordinates derived from the center 3D captures were subjected to Euclidean Distance Matrix Analysis (EDMA), a morphometric approach designed to handle landmark-based coordinate data directly (described in Section 2.3.3.1). Alternatively, separate linear distances derived from the left/right 3D captures and direct anthropometry were analyzed using standard parametric statistics (described in Section 2.3.3.2). In both types of analysis, a straightforward two-group comparison (unaffected relatives versus matched controls) was carried out. For this comparison, males and females were treated separately; that is, male unaffected relatives were compared to male controls and female unaffected relatives were compared to female controls. The decision to carry out the analysis in a sex-specific manner was based on both the findings of previous studies and some preliminary

results showing that relative-control morphological differences were influenced by sex (Kurisu et al., 1974; Weinberg et al., 2006b). Importantly, during the first phase of the analysis only an initial subset of unaffected relatives was included for statistical comparison (based on criteria outlined in Section 2.3.1.2). All additional relatives with valid data not included in the first phase were held for the second phase.

The goal of the second phase of the analysis was to develop a multivariate model to facilitate the classification of unaffected relatives into risk categories. To this end, a two-group discriminant function analysis (DFA) was carried out on the same sex-specific relative-control sample as in phase one (see Section 2.3.4.2). The choice of variables to enter into the DFA was informed by the results of phase one; only variables found to differ significantly in phase one were included in phase two. This approach both ensures a parsimonious model and substantially increases the subject to variable ratio, which, if too low, can seriously undermine the validity of multivariate analyses (Hair et al., 1998). Once the discriminant model was generated, the parameters from that analysis (i.e., discriminant weights) were applied to the raw data from the additional unaffected relatives excluded from phase one. The purpose of this was to predict the group membership of these additional unaffected relatives. In this manner, all unaffected relatives were either correctly classified as relatives or misclassified as controls.

2.3.1.2 Unaffected relative selection To date, craniofacial data has been collected on 124 unaffected relatives of cleft individuals in 41 multiplex families (33 CL/P, 6 mixed CL/P and CP, 2 CP). Of these 124 individuals, 54 were male and 70 were female. Since most conventional statistical methods assume that entities within a given sample are independent of one another, including multiple individuals from the same family in a case-control design presents an obvious quandary. The simplest way around this problem is to select a subset of unrelated individuals from the available families for analysis. A decision has to be made, therefore, regarding who to include and who to exclude. The first step in this selection process was to examine the pedigrees and family history notes of all 41 families to determine on which side of the family the disease is being transmitted. Armed solely with this information, family members in each pedigree were classified naively as either potential carriers or non-carriers (Figure 9). From the subset of identified potential carriers, an initial sample of relatives was chosen based on the following criteria: 1) subjects had to be a first degree relative of an affected individual; 2) subjects had to

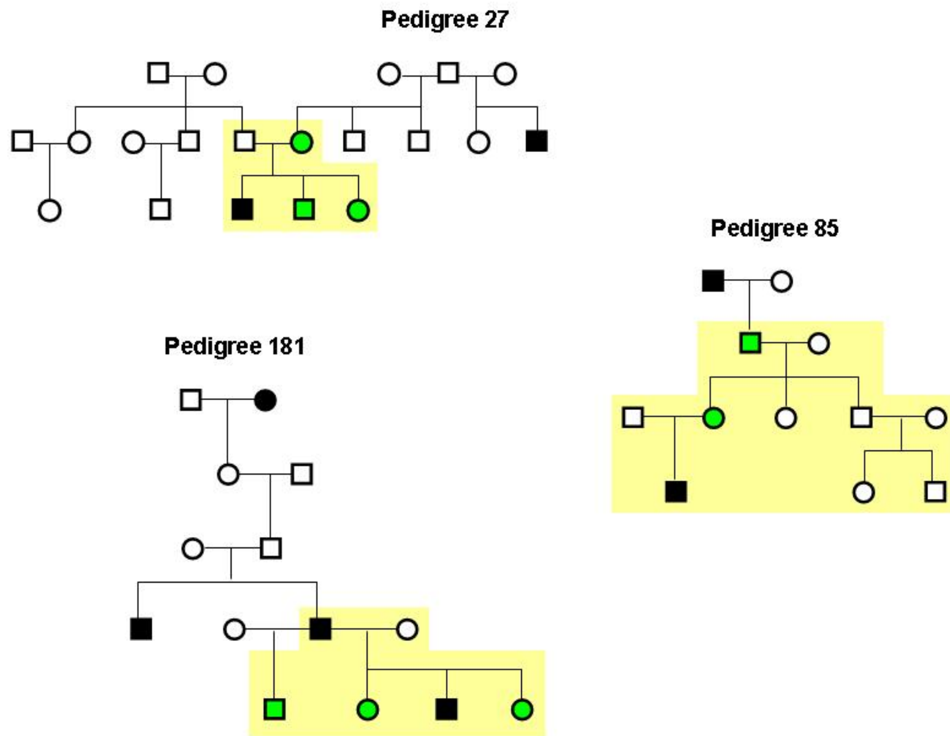

Figure 9 Example of multiplex CL/P pedigrees.

Complete pedigrees are presented. The segment of the pedigree highlighted in yellow represents the portion of the family available for study. Affected individuals are shown in black, probable gene carriers are shown in green and other unaffected relatives are shown in white. Selection of unaffected relatives for the initial sample was made from among the probable gene carriers.

have coordinate data for all 16 facial landmarks derived from the center 3D capture; 3) subjects had to be unrelated to one another. In cases where more than one individual within a family satisfied the first and second criteria (e.g., two full sibs of a proband), older individuals were selected over younger individuals. This decision was based on the fact that the 3D data in older individuals (in particular children) tend to have less motion artifacts.

Because male and female relatives were analyzed separately, it was possible for one member of each sex from the same family to be included in the initial case-control phase of the analysis. Despite the fact that these individuals were related to one another, this would have no bearing on the analysis because each sex was treated as a separate sample. Regardless of sex, each relative had to meet all of the criteria outlined above to be included in the first phase of the analysis.

Of the 124 available unaffected relatives, a sample of 47 individuals (14 males and 33 females) from 36 families was selected for the first phase of the analysis. Twenty two of these 47 individuals were opposite sex pairs from the same family; thus, 11 families had both a male and female member included in the initial sample. A total of 46 additional unaffected relatives (22 males and 24 females) with valid data were excluded from the first phase of the analysis for one or more of the following reasons: they were designated as a non-carrier, they were related to an individual already in the initial sample and/or they were not a first degree relative of an affected individual. Data for these additional relatives were set aside for the second phase of the analysis. Thirty one unaffected relatives had unusable data and were excluded from all parts of the analysis.

2.3.1.3 Control matching criteria A sex-, age- and ethnicity-matched sample of 47 unrelated healthy control individuals (14 males and 33 females) was selected out of a possible 227 controls (143 females and 84 males) for comparison with the 47 unaffected relatives. Relatives and controls were matched for age within one year. As with unaffected relatives, controls with missing coordinate data were excluded. If multiple controls were available for a given relative, the control individual most similar in height was chosen so as to minimize any size discrepancy between samples. Age and height statistics for the relative and control groups are reported in Table 5.

Table 5 Descriptive statistics for relative-control matching criteria

	N	*Age*	*Height*
Females			
Relatives	33	27.8 (±14.9)	63.96 (±3.82)
Controls	33	27.8 (±15.1)	64.23 (±3.25)
Males			
Relatives	14	26.9 (±18.2)	65.85 (±6.59)
Controls	14	27.1 (±18.1)	63.45 (±6.80)

2.3.2 Assessment of measurement error

The Genex imaging system has been extensively validated for precision and accuracy (Weinberg et al., 2004; 2006c). In Weinberg et al. (2004), a set of 19 linear distances were obtained repeatedly on 20 normal healthy subjects by two separate observers with both calipers and the Genex 3D imaging system. In addition, subjects were measured both with and without landmarks pre-labeled. Thus each subject was measured a total of 12 times. This multifactorial design allowed intra- as well as inter-observer precision to be assessed and compared across techniques. Four different precision estimates were calculated, the mean absolute difference (MAD), the relative error magnitude (REM), the technical error of measurement (TEM) and the intraclass correlation coefficient of reliability (R). Further, accuracy was assessed by comparing caliper-derived measurements directly with Genex-derived measurements via paired t-tests. Regardless of the precision estimate, results indicated that the Genex system was capable of extremely high precision, particularly when subjects were pre-labeled (Table 6). Importantly, Genex-derived measurements were observed to have significantly higher precision than caliper-derived measurements. This was true for both intra- as well as inter-observer precision, although intra-observer precision was expectedly higher. Regarding accuracy, seven measurements were significantly different between calipers and the Genex system, but the magnitude of the difference was generally small (within 2mm) suggesting fairly good congruence overall. In almost all cases (14 out of 19 variables), the mean Genex measurements were smaller than the mean caliper measurements, suggesting that the Genex system may have a slight tendency toward underestimation.

In a recent study, measurements from the Genex system were also compared to those from a comparable imaging system (Weinberg et al., 2006c). Twelve linear distances were measured on a sample of 18 mannequin heads via three methods: the Genex imaging system, a 3dMD imaging system, and calipers. All measurements were performed twice with each method so that observer precision could be assessed. A two-factor repeated measures ANOVA was carried out to test simultaneously for mean differences across methods and for differences in precision across methods. While significant method differences were observed for nine linear distances, the magnitude of these differences was quite small (at the sub-millimeter level). Thus,

Table 6 Intraobserver precision statistics for the Genex 3D imaging system

Variable	*Precision index*			
	Mean absolute difference (mm)	*Relative error magnitude*	*Technical error of measurement (mm)*	*Intraclass correlation coefficient*
Facial height	0.21	0.17	0.25	0.91
Upper facial height	0.21	0.28	0.41	0.92
Lower facial height	0.26	0.37	0.26	0.91
Glabella-Subnasion height	0.27	0.41	0.48	0.93
Upper facial depth (Left)	0.24	0.20	0.20	0.92
Midfacial depth (Left)	0.23	0.18	0.24	0.92
Lower facial depth (Left)	0.38	0.26	0.33	0.92
Intercanthal width	0.60	1.95	0.58	0.91
Biocular width	0.19	0.22	1.25	0.92
Auricular length (Left)	0.23	0.38	0.94	0.92
Nasal width	0.11	0.33	0.12	0.92
Nasal height	0.17	0.31	0.24	0.93
Nasal projection	0.13	0.61	0.13	0.93
Alar length (Left)	0.24	0.74	0.30	0.92
Philtrum width	0.16	1.29	0.22	0.92
Labial fissure width	0.38	0.76	0.42	0.92
Upper lip height	0.29	1.37	0.34	0.92

Table 6 (continued).

Lateral upper lip height (Left)	0.21	1.33	0.56	0.93
Upper lip length (Left)	0.37	0.99	0.42	0.92

Data adapted from Weinberg et al. (2004), see text for more detail on precision indices

All values based on data from 3D capture with landmarks pre-labeled, except for intercanthal width

at a practical level, all three methods produced congruent data. No differences in precision were observed across methods.

Observer related measurement error (precision) for the six additional caliper-derived measurements was calculated as well. All six measurements were collected twice on 90 subjects by the same observer. Intraclass correlation coefficients (ICC) were then calculated in SPSS (two-way mixed-effects model with absolute agreement). The average ICC across the six variables was very high (0.984), with ICC values ranging from 0.965 to 0.992.

2.3.3 Phase I. Identifying an informative variable set

2.3.3.1 Analysis of 3D coordinates with EDMA Euclidean Distance Matrix Analysis (EDMA) is a morphometric approach based on the principle of form invariance; meaning that "the form of an object is that characteristic that remains invariant under any translation, rotation, or reflection of the object" (Richtsmeier et al., 2002; p. 72). Thus EDMA, unlike superimposition-based approaches (i.e., geometric morphometrics), does not require fitting landmark data into a common coordinate system prior to the comparison of forms. This is critical because any approach based on superimposition of landmark data must adopt a particular superimposition scheme (e.g., procrustes superimposition; see Dryden and Mardia, 1998; O'Higgins and Jones, 1998). The problem is that the choice of superimposition scheme is arbitrary and can seriously affect the estimation of parameters of interest (e.g., mean form, variance, and covariance structure; see Siegel and Benson, 1982; Rohlf and Slice, 1990). A full account of this problem is provided in Lele (1991; 1993) and Lele and McCulloch (2002). EDMA avoids the nuisance parameters of translation, rotation and reflection by focusing on a definition of form unaffected by such factors.

The form of an object in EDMA is defined by the complete set of linear distances between all possible landmark pairs. For a form comprised of K landmarks, there will be $K(K - 1)/2$ linear distances. The set of linear distances representing a given object's form can be written as a square symmetric matrix with K rows and K columns; in EDMA this is termed a form matrix (FM). Alternatively, a FM can be written as a vector of the off-diagonal upper (or lower) elements of the matrix. Just as a single FM can represent a summary of form for a given object, average form matrices can be constructed to represent a sample of objects. A comparison

of forms in EDMA constitutes a comparison of two mean form matrices. Thus, for two hypothetical samples, denoted X and Y, FM(X) would be compared directly to FM(Y). The result of this comparison is summarized as a form difference matrix, denoted as FDM(X,Y). The FDM can be calculated as either a matrix of ratios of homologous linear distances, written as FM(X)/FM(Y), or as a matrix of arithmetic differences between homologous linear distances, written as FM(X) – FM(Y). The FM contains information about both the size and shape of an object. It is also possible however to limit the analysis to only shape by scaling the FM by some measure of size. Scaling the FM (an option within EDMA) results in a shape matrix (SM) and the comparison between two shape matrices results in a shape difference matrix or SDM (Lele and Cole, 1996).

Statistical testing with EDMA is divided into two components. The first involves a null hypothesis test of overall form (or shape) difference between samples. With this test one is simply seeking to determine whether two forms (or shapes) are statistically equivalent (the null hypothesis is that they are equivalent). To perform this test, a test statistic is calculated based on data contained within the FDM (or SDM). An empirical distribution of this test statistic is then constructed through either non-parametric or parametric (Monte Carlo) bootstrap re-sampling. The location of the observed test statistic within the empirical distribution (given a designated alpha level) determines its statistical significance. The second component involves the localization of specific form (or shape) differences through the construction of confidence intervals around each element (linear distance) in the form or shape difference matrix (Lele and Richtsmeier, 1995). Again, either non-parametric or parametric bootstrapping is used; this time to generate an empirical distribution of values for each linear distance. If the upper and lower bounds of the distribution for a given linear distance (defined by the designated alpha level) exclude the null value (the value indicating no difference between groups), then that linear distance can be said to significantly differ across the two groups compared. Outlined below is a more detailed account of the specific methods used in the present study. A full treatment of statistical testing in EDMA is given in Lele and Richtsmeier (2001).

For the present analysis, EDMA was performed on the 16 facial landmark coordinates derived from the center 3D capture. Following an initial screen for outliers, mean form matrices consisting of 120 linear distances were generated for both unaffected relatives and controls. This was done for each sex separately, resulting in the creation of four distinct form matrices: 1)

unaffected male relatives, 2) male controls, 3) unaffected female relatives, and 4) female controls. In order to remove size from the data, each mean FM was scaled in order to generate mean shape matrices. In each case, the geometric mean of the linear distances contained within a given FM was used as the scaling factor. The geometric mean is simply the n-th root of the product of n numbers (linear distances in this case). Since it takes into account all available values, it can be considered a global measure of a size. Use of the geometric mean in morphometric studies is well established (Darroch and Mosimann, 1985; Jungers et al., 1995). For each sex, a SDM was generated by comparing the unaffected relative SM to the control SM, followed by a null hypothesis test of the equivalence of shape and the construction of confidence intervals. Based on the recommendations set forth by Lele and Richtsmeier (1995), an alpha level of .10 instead of the conventional alpha level of .05 was used for all statistical tests. On this point Lele and Richtsmeier (1995) state, "In general, for bootstrap confidence intervals, it is better to aim for a 90% confidence interval instead of the usual 95% confidence interval. Since the tails of the distribution of the estimator are estimated less precisely than the middle portion, a 95% confidence interval tends to be more unstable than a 90% or an 80% confidence interval." All calculations and tests were performed using the SHAPE MODULE within the EDMA statistical package for Windows, WinEDMA 1.0.1 (Cole, 2003). What follows are the specific step-by step procedures EDMA uses to perform a null hypothesis test for the equivalency of shape between two samples (adapted from Lele and Cole, 1996 and Lele and Richtsmeier, 2001).

STEP 1. Calculate the mean FM for each of the two samples of interest, X and Y

STEP 2. Calculate a scaling factor for each sample, $C(X)$ and $C(Y)$, and divide each entry in the FM by its respective scaling factor to produce two shape matrices, SM(X) and SM(Y).

STEP 3. Calculate the SDM(X,Y) consisting of the arithmetic differences between homologous linear distances in SM(X) and SM(Y).

STEP 4. Determine the test statistic Z by locating the element within the SDM(X,Y) that differs the most (in either direction) from zero.

STEP 5. Re-sample with replacement to form two new samples, X^* and Y^*, using a parametric (Monte Carlo) bootstrapping algorithm. Then, re-calculate the SDM(X^*,Y^*) and determine Z^* for the bootstrapped samples.

STEP 6. Repeat STEP 5 10,000 times to obtain a bootstrap distribution of Z^* (Z^*_1, Z^*_2, . . ., Z^*_{100}, . . ., $Z^*_{10,000}$).

STEP 7. Sort the values within bootstrap Z^* distribution in ascending order. The 90% confidence interval for this sorted distribution is delimited by Z^*_{500} and Z^*_{9500}. If the interval does not contain zero reject the null that the two mean shapes are equivalent.

As part of the SHAPE procedure in EDMA, the same steps are carried out separately for the scaling factor *C*, to determine if the two samples differ in size.

A non-parametric bootstrap procedure was used to calculate 90% confidence intervals for each of the 120 scaled linear distances represented in the SDM. This procedure involves the following steps (adapted from Lele and Richtsmeier, 1995; 2001):

STEP 1. For each of two samples, *X* and *Y*, obtain a random sample with replacement equal to the size of the original sample. These new samples are termed X^* and Y^*.

STEP 2. Calculate the mean SDM(X^*,Y^*) based on the samples obtained in STEP 1.

STEP 3. Repeat STEP 1 and STEP 2 10,000 times, producing 10,000 shape difference matrices.

STEP 4. Write the SDM for each of the 10,000 bootstrapped samples as a vector containing 120 elements (each element is an arithmetic difference between homologous linear distances).

STEP 5. Re-arrange the data into a matrix with 10,000 columns (one column per bootstrap iteration) and 120 rows (one row per SDM element).

STEP 6. Sort each row in the matrix independently in ascending order.

STEP 7. The 90% confidence interval for each linear distance is delimited by removing the first and last 500 values of each sorted row (upper and lower 5% of distribution). The minimum and maximum values remaining in each row constitute respectively the lower and upper bounds of the confidence interval. If a given interval does not contain zero, then there is at least a 90% probability that the linear distance associated with that interval differs between the two groups.

As Lele and Richtsmeier (1995) point out, the purpose these confidence intervals is to facilitate the discovery of regions where shape differences are most apparent between groups, not to provide a means for determining whether two forms are statistically significantly different from one another overall. Because each bootstrap confidence interval is calculated in an element-wise manner (one per row), their distributions can be considered independent of one another. Alternately stated, each confidence interval consists of a slightly different set of data. Thus, there is no common distribution (like a t-distribution) among the 120 confidence intervals. This is one of the reasons why Bonferroni-type corrections for multiple testing are not required in EDMA (see Richtsmeier et al., 2006). The other reason is that the shape localization component of EDMA is an exploratory endeavor; that is, no *a priori* hypotheses are offered as the nature of the shape difference between samples.

2.3.3.2 Analysis of selected linear distances The additional 14 linear distances derived from the left/right 3D captures and calipers were also compared between unaffected relatives and controls by sex. Once the 3D capture and caliper data were compiled in Excel, any missing values were imputed with group-specific means. The geometric mean was then calculated from the 14 linear distances and used to scale the original data. The scaled data were then imported into SPSS v11.5 (Chicago, IL), checked for normality and screened for outliers. Fourteen separate two-sample t-tests were performed. An alpha level of at least 0.05 was required to attain statistical significance.

2.3.4 Phase II. Constructing a multivariate predictive model

2.3.4.1 Discriminant function analysis The criteria for which predictor variables to include in discriminant function analysis (DFA) is a contentious area within statistics. Based on the recommendations of Duarte Silva and Stam (1995) and the methods used in previous discriminant analyses (Suzuki et al., 1999; McIntyre and Mossey, 2003), variables found to differ significantly between unaffected relatives and controls from EDMA ($p < 0.90$) and t-tests ($p < 0.05$) were entered into a DFA. The primary goal in DFA is classification (Klecka, 1980; Meyers et al., 2006). Superficially, DFA is similar to logistic regression, except that the predictor variables in DFA must be continuous. DFA uses a weighted linear combination of

continuous predictor variables to determine the probability of membership in a discrete number of groups. This linear combination of variables (termed a discriminant function) is weighted so as to maximize between-group variance and minimize within-group variance, resulting in the maximal separation between groups. The number of discriminant functions estimated is one less than the number of groups; thus, in a two group example only one discriminant function is produced. A given individual's membership in a group is probability-based and can be represented as either a discriminant score or a multivariate distance measure, termed the Mahalanobis distance or D^2. A discriminant score is calculated for each individual in the analysis by plugging their raw trait values into the discriminant function equation. The group means for these scores are termed centroids. In a two group situation, the score that falls exactly between the group centroids is called the cut score (because discriminant scores are standardized, the cut score will be zero). Each individual is assigned membership in a group based on whether their discriminant score falls above or below the cut score. The Mahalanobis distance is the distance between a centroid and an individual case. Since there are two centroids (one per group), each case will have two Mahalanobis distances. The smaller of the two distances determines to which group a given case will be assigned. The overall classification performance of the discriminant function can be assessed through the prediction hit rate – the percentage of cases assigned to the correct group. A formal test of the classification accuracy of the discriminant function (Press's Q Statistic; see Hair et al., 1998) is obtained by comparing the observed hit rate to that expected by chance (50% in a two group situation).

Aside from prediction and classification, DFA offers the researcher a set of descriptive tools for revealing the nature of the difference between groups. For example, the substantive importance of each predictor variable to the overall discriminant function (i.e., the variables contributing most to group separation) can be determined. Similar to variable loadings in factor analysis, the correlation between each predictor variable in the model and the discriminant function is calculated. Both the effect size and direction of the correlations give the researcher a sense of the relative importance and substantive meaning of the predictors. The significance of the overall discriminant function model is determined by testing whether the means for each group on the discriminant function (i.e., the group centroids) are equivalent. The strength of the relationship between the discriminant function and the outcome variable (i.e., the grouping

variable) is estimated as a canonical correlation coefficient; squaring this coefficient provides a measure of the proportion of variance accounted for by the discriminant function.

In the present study, separate discriminant function analyses were carried out on males and females, each utilizing a set of sex-specific variables identified as statistically significant in phase I. In each analysis, all predictor variables were entered simultaneously as a single block. Because only two groups were involved, relatives and controls, a single discriminant function for each sex was estimated. Each individual was classified as either a relative or control based on the methods outlined above. The analysis was carried out in SPSS v11.5 (Chicago, IL).

Prior to performing each DFA, the variables to be included were tested for multivariate assumptions. First, the shape of the distribution of each variable was visually inspected for normality. Next, the degree of multicollinearity was assessed in a two-stage process. Multicollinearity occurs in multivariate designs when multiple predictor variables are very highly correlated with one another. The problems associated with excess multicollinearity are well known; for example, multicollinearity can cause difficulties in the interpretation of a discriminant function model by distorting discriminant coefficients (Hair et al., 1998; Meyers et al., 2006). The first step in the assessment of multicollinearity was to construct a simple correlation matrix consisting of the bivariate Pearson product-moment correlations between each pair of variables. If correlations greater than 0.90 were found between any two variables, one variable was dropped. The decision as to which variable to retain was based on an estimate of informativeness; the variable with the greatest relative-control difference observed in either EDMA or the univariate t-tests was retained. The second step in assessing multicollinearity involved obtaining a set of formal multicollinearity statistics on all remaining variables. This was accomplished using the Collinearity Diagnostics procedure within SPSS. This procedure provides a number of indices (e.g., the variance inflation factor or VIF) useful for gauging the degree of multicollinearity in a given data set (Field, 2000). Any further collinearity was dealt with in the manner described previously. Eliminating variables due to multicollinearity has the added benefit of increasing the subject to variable ratio. Finally, with the final set of variables intact, the equality of the variance-covariance matrices between groups was evaluated (Box's M test) and the data were screened for multivariate outliers (Mahalanobis D^2 method).

2.3.4.2 Classification of additional family members Recall from Section 2.3.1.2 that only a subset of unaffected relatives meeting very specific criteria was used to estimate the discriminant function models; a total of 46 unaffected relatives with valid data were excluded from this portion of the analysis. The reason for this was to ensure unbiased results, while attempting to maximize the likelihood of uncovering relative-control differences. The parameter estimates from the sex-specific discriminant functions were used to predict the group membership of these 46 additional unaffected relatives. To accomplish this, discriminant scores and Mahalanobis distance statistics were obtained for each additional relative. Discriminant scores can be calculated manually by simply weighting the raw trait scores of the new individuals by their unstandardized coefficients derived from the appropriate discriminant function. In SPSS, the classification of the additional relatives was accomplished simply by entering their trait values in the original analysis, while leaving their group status indeterminate. SPSS does not use the data from these "unknown" cases to build its discriminant model, but will classify them nevertheless based on the model's parameters. In this way, every unaffected relative in the sample with valid data was assigned to either the relative or control group.

2.3.4.3 Detection of at-risk relatives The ultimate goal of the DFA is to facilitate the identification of those unaffected relatives at elevated risk for carrying orofacial cleft susceptibility factors through quantitative evaluation of their craniofacial morphology. The DFA forces each unaffected relative into one of two groups: relatives or controls. It is hypothesized that the subset of unaffected relatives classified with controls share a distinct pattern of facial characteristics more common in those at reduced risk for orofacial clefting. Alternatively, those unaffected relatives classified as relatives represent a distinct cluster possessing facial features dissimilar from both low risk relatives and controls, features hypothesized to be indicative of elevated cleft risk. For the present study, an unaffected relative had to meet two criteria to be considered "at-risk": 1) they had to be classified as a relative via DFA, and 2) they had to have a Mahalanobis distance greater than 1.96 from the control group's centroid. This latter criterion is based on the fact that Mahalanobis distances are measured in standard deviation units; therefore, a Mahalanobis distance greater than 1.96 from a given centroid would indicate a less than 5% ($p < 0.05$) chance of an individual belonging to that group.

2.3.4.4 Comparison of risk classes Following each relative's assignment to a particular risk class, basic descriptive characteristics were compared statistically between the "high risk" and "low risk" relative subgroups, including sex ratio, age and type of cleft in the family. The relationship between risk status in relatives and cleft severity was assessed in two different ways. Unaffected relatives were first classified into severity categories based on the type of cleft affecting the proband in their family. Severity categories were based on both the laterality and the extent of the defect present in probands. These categories were, in order of severity, unilateral clefts of the primary palate, bilateral clefts of the primary palate, unilateral clefts of the primary and secondary palate, and bilateral clefts of the primary palate. The proportion of "low risk" versus "high risk" relatives across each severity category was then compared via chi-squared analysis. In addition, a parametric analysis was carried out, were the mean biological distance (D^2) of relatives from the controls was compared across severity categories via ANOVA. The distribution of high risk relatives within cleft families as well as their degree of relationship to affected individuals was also described.

3.0 RESULTS

3.1 PHASE I. IDENTIFYING AN INFORMATIVE VARIABLE SET

3.1.1 EDMA results

3.1.1.1 Female sample Craniofacial landmark data obtained from a sample of 33 female unrelated, unaffected relatives from 33 multiplex nonsyndromic cleft families, along with an equal number of female age-matched controls, were subjected to EDMA. Mean age (years) for the relative sample was 27.79 (±14.94) versus 27.76 (±15.13) for the control sample (t = 0.008, df = 64, p = 0.993; see Table 5). The mean form matrices containing all 120 unscaled linear distances for the female relative and control samples are shown in Table 7 and Table 8, respectively. Overall, the relative and control samples did not differ statistically in terms of size, estimated by the geometric mean (relatives = 35.95, controls = 35.93). As stated in Section 2.3.3.1, EDMA tests for differences in size between two samples by estimating a confidence interval based on an empirical distribution of bootstrapped size differences (10,000 re-samples). Since the 90% confidence interval of the bootstrapped size difference distribution (-0.71 – 0.66) contains zero, the null hypothesis of size equivalence between groups can not be rejected. The geometric means for each group were used to scale their respective mean form matrices, resulting in mean shape matrices (form – size = shape). The mean shape matrices for each group are provided in Table 9 and Table 10.

Results of the null hypothesis test of shape difference indicate that the shape of the face differs, on the whole, between female unaffected relatives and female controls. Interestingly, the 90% confidence interval of the bootstrapped Z distribution (-0.08 – 0.08) does contain zero, which would seem to suggest that face shape does not differ statistically between groups. A major caveat when testing the null hypothesis of shape equivalency in EDMA is that the ability

Table 7 Mean form matrix for female relative sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	44.38	0														
sn	52.13	19.26	0													
sto	70.67	36.06	19.10	0												
sl	87.78	53.39	36.80	17.74	0											
gn	115.21	81.91	65.24	46.28	28.59	0										
en(r)	23.25	45.90	46.60	62.52	78.10	103.81	0									
en(l)	23.80	45.33	46.07	62.04	77.49	103.16	30.94	0								
al(r)	46.31	26.80	19.64	32.09	47.64	74.32	33.88	46.33	0							
al(l)	46.94	26.15	19.02	31.56	46.90	73.59	47.04	34.01	33.22	0						
sbal(r)	50.70	23.67	10.24	22.58	39.11	66.72	41.02	46.89	10.24	26.26	0					
sbal(l)	51.18	23.20	9.55	22.07	38.53	66.20	47.63	41.12	26.38	10.30	17.68	0				
cph(r)	63.81	29.05	13.06	9.85	26.29	54.48	55.24	58.05	23.28	30.20	14.26	19.91	0			
cph(l)	64.13	29.19	13.28	9.81	25.96	54.14	58.80	54.95	31.02	22.80	20.80	13.89	12.24	0		
ch(r)	77.95	51.13	35.25	25.83	27.78	46.87	62.58	72.57	32.70	50.54	29.75	40.92	24.62	34.07	0	
ch(l)	78.08	50.87	35.14	25.92	27.36	46.40	72.97	62.04	50.92	32.30	41.32	29.70	34.03	24.40	45.69	0

Table 8 Mean form matrix for female control sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	44.48	0														
sn	52.05	19.16	0													
sto	70.71	36.40	19.38	0												
sl	87.35	53.39	36.69	17.35	0											
gn	114.10	81.85	64.86	45.70	28.46	0										
en(r)	22.62	46.50	47.28	63.18	78.28	103.15	0									
en(l)	23.58	46.08	46.78	62.79	77.77	102.51	30.70	0								
al(r)	45.93	26.30	19.17	31.84	47.11	73.48	34.65	46.48	0							
al(l)	46.75	26.28	18.69	31.45	46.47	72.64	46.99	34.59	32.11	0						
sbal(r)	50.40	23.77	10.56	22.89	39.00	66.23	41.46	47.36	9.56	25.83	0					
sbal(l)	50.98	23.47	9.95	22.53	38.55	65.74	48.08	41.54	26.03	9.59	18.09	0				
cph(r)	64.14	29.58	13.57	9.68	25.67	53.70	56.23	59.06	23.27	30.20	14.84	20.62	0			
cph(l)	64.47	29.66	13.68	9.77	25.47	53.41	59.72	55.98	30.83	22.96	21.30	14.53	12.37	0		
ch(r)	77.18	51.09	35.27	25.82	27.85	46.54	62.53	72.49	32.44	49.79	29.51	41.03	24.37	34.04	0	
ch(l)	77.45	50.63	34.87	25.78	27.48	45.98	72.90	62.09	50.14	31.76	41.15	29.21	33.74	23.94	45.68	0

Table 9 Mean shape matrix for female relative sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	1.235	0														
sn	1.450	0.536	0													
sto	1.966	1.003	0.531	0												
sl	2.442	1.485	1.024	0.494	0											
gn	3.205	2.279	1.815	1.287	0.795	0										
en(r)	0.647	1.277	1.296	1.739	2.173	2.888	0									
en(l)	0.662	1.261	1.282	1.726	2.156	2.870	0.861	0								
al(r)	1.288	0.746	0.546	0.893	1.325	2.068	0.943	1.289	0							
al(l)	1.306	0.727	0.529	0.878	1.305	2.047	1.309	0.946	0.924	0						
sbal(r)	1.411	0.659	0.285	0.628	1.088	1.856	1.141	1.305	0.285	0.731	0					
sbal(l)	1.424	0.645	0.266	0.614	1.072	1.841	1.325	1.144	0.734	0.286	0.492	0				
cph(r)	1.775	0.808	0.363	0.274	0.731	1.516	1.537	1.615	0.647	0.840	0.397	0.554	0			
cph(l)	1.784	0.812	0.370	0.273	0.722	1.506	1.636	1.528	0.863	0.634	0.579	0.386	0.340	0		
ch(r)	2.168	1.422	0.981	0.718	0.773	1.304	1.741	2.019	0.910	1.406	0.828	1.138	0.685	0.948	0	
ch(l)	2.172	1.415	0.978	0.721	0.761	1.291	2.030	1.726	1.417	0.899	1.149	0.826	0.947	0.679	1.271	0

Table 10 Mean shape matrix for female control sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	1.238	0														
sn	1.449	0.533	0													
sto	1.968	1.013	0.539	0												
sl	2.431	1.486	1.021	0.483	0											
gn	3.176	2.278	1.805	1.272	0.792	0										
en(r)	0.630	1.294	1.316	1.758	2.179	2.871	0									
en(l)	0.656	1.283	1.302	1.748	2.165	2.853	0.854	0								
al(r)	1.278	0.732	0.534	0.886	1.311	2.045	0.964	1.294	0							
al(l)	1.301	0.731	0.520	0.875	1.293	2.022	1.308	0.963	0.894	0						
sbal(r)	1.403	0.662	0.294	0.637	1.086	1.843	1.154	1.318	0.266	0.719	0					
sbal(l)	1.419	0.653	0.277	0.627	1.073	1.830	1.338	1.156	0.724	0.267	0.504	0				
cph(r)	1.785	0.823	0.378	0.269	0.715	1.495	1.565	1.644	0.648	0.841	0.413	0.574	0			
cph(l)	1.794	0.826	0.381	0.272	0.709	1.487	1.662	1.558	0.858	0.639	0.593	0.404	0.344	0		
ch(r)	2.148	1.422	0.982	0.719	0.775	1.295	1.740	2.018	0.903	1.386	0.821	1.142	0.678	0.948	0	
ch(l)	2.156	1.409	0.971	0.718	0.765	1.280	2.029	1.728	1.396	0.884	1.145	0.813	0.939	0.666	1.271	0

Table 11 Sorted shape difference matrix for female sample

No.	*Distance*	*LB*	*Estimate*	*UB*	*No.*	*Distance*	*LB*	*Estimate*	*UB*	*No.*	*Distance*	*LB*	*Estimate*	*UB*
1	al(r)-al(l)	-0.058	**-0.030**	-0.002	41	sto-al(r)	-0.029	**-0.007**	0.017	81	cph(r)-cph(l)	-0.007	**0.004**	0.015
2	n-gn	-0.073	**-0.029**	0.015	42	cph(r)-ch(r)	-0.036	**-0.006**	0.023	82	prn-al(l)	-0.027	**0.004**	0.035
3	gn-al(l)	-0.067	**-0.025**	0.016	43	sbal(r)-ch(r)	-0.031	**-0.006**	0.018	83	en(l)-al(r)	-0.026	**0.005**	0.035
4	gn-al(r)	-0.063	**-0.022**	0.020	44	en(r)-en(l)	-0.030	**-0.006**	0.018	84	al(l)-cph(l)	-0.020	**0.005**	0.029
5	al(r)-ch(l)	-0.061	**-0.021**	0.020	45	prn-ch(l)	-0.052	**-0.006**	0.041	85	sl-en(r)	-0.028	**0.006**	0.041
6	gn-cph(r)	-0.061	**-0.021**	0.020	46	n-en(l)	-0.025	**-0.006**	0.014	86	prn-sbal(l)	-0.024	**0.008**	0.039
7	n-ch(r)	-0.056	**-0.020**	0.016	47	n-sbal(l)	-0.034	**-0.005**	0.024	87	sn-sto	-0.011	**0.008**	0.027
8	al(l)-ch(r)	-0.058	**-0.020**	0.017	48	al(r)-cph(l)	-0.033	**-0.005**	0.023	88	sto-sbal(r)	-0.010	**0.009**	0.029
9	al(l)-sbal(l)	-0.034	**-0.020**	-0.006	49	sto-cph(r)	-0.020	**-0.005**	0.010	89	sn-sbal(r)	-0.009	**0.009**	0.027
10	gn-cph(l)	-0.060	**-0.019**	0.023	50	n-al(l)	-0.033	**-0.005**	0.023	90	sl-en(l)	-0.026	**0.009**	0.045
11	al(r)-sbal(r)	-0.034	**-0.019**	-0.004	51	sbal(r)-ch(l)	-0.040	**-0.004**	0.032	91	prn-sto	-0.019	**0.010**	0.040
12	n-en(r)	-0.038	**-0.017**	0.004	52	sto-ch(l)	-0.036	**-0.003**	0.028	92	n-cph(r)	-0.024	**0.010**	0.045
13	sl-cph(r)	-0.044	**-0.017**	0.011	53	sl-gn	-0.032	**-0.003**	0.026	93	n-cph(l)	-0.025	**0.010**	0.045
14	gn-en(r)	-0.060	**-0.017**	0.028	54	sn-sl	-0.032	**-0.003**	0.028	94	sn-cph(l)	-0.010	**0.011**	0.033
15	gn-en(l)	-0.058	**-0.016**	0.026	55	prn-sn	-0.026	**-0.002**	0.021	95	sn-sbal(l)	-0.008	**0.011**	0.030
16	n-ch(l)	-0.052	**-0.016**	0.019	56	sto-al(l)	-0.026	**-0.002**	0.021	96	sbal(r)-sbal(l)	-0.011	**0.012**	0.034
17	sto-gn	-0.051	**-0.015**	0.021	57	sl-sbal(r)	-0.030	**-0.002**	0.026	97	en(l)-sbal(l)	-0.014	**0.012**	0.039
18	al(l)-ch(l)	-0.042	**-0.015**	0.013	58	n-sn	-0.032	**-0.001**	0.029	98	en(r)-sbal(r)	-0.013	**0.013**	0.038
19	sl-al(r)	-0.044	**-0.014**	0.017	59	en(l)-ch(r)	-0.037	**-0.001**	0.034	99	sto-sbal(l)	-0.008	**0.013**	0.034
20	prn-al(r)	-0.044	**-0.013**	0.018	60	sto-cph(l)	-0.016	**-0.001**	0.015	100	en(r)-sbal(l)	-0.017	**0.013**	0.044
21	sl-cph(l)	-0.041	**-0.013**	0.015	61	en(r)-ch(l)	-0.038	**-0.001**	0.037	101	prn-cph(l)	-0.017	**0.014**	0.045
22	sbal(l)-ch(l)	-0.040	**-0.013**	0.013	62	en(r)-al(l)	-0.032	**-0.001**	0.031	102	en(l)-sbal(r)	-0.016	**0.014**	0.043

Table 11 (continued).

23	sn-al(r)	-0.034	**-0.013**	0.009	63	prn-gn	-0.050	**-0.001**	0.049	103	sbal(r)-cph(l)	-0.010	**0.014**	0.038
24	gn-sbal(r)	-0.052	**-0.013**	0.027	64	en(r)-ch(r)	-0.031	**0.000**	0.030	104	sn-cph(r)	-0.007	**0.014**	0.035
25	cph(l)-ch(l)	-0.045	**-0.012**	0.020	65	prn-ch(r)	-0.044	**0.000**	0.043	105	prn-cph(r)	-0.015	**0.015**	0.045
26	gn-sbal(l)	-0.051	**-0.012**	0.028	66	cph(l)-ch(r)	-0.033	**0.000**	0.032	106	sbal(r)-cph(r)	-0.005	**0.016**	0.037
27	al(l)-sbal(r)	-0.037	**-0.011**	0.014	67	al(r)-cph(r)	-0.024	**0.000**	0.025	107	en(l)-al(l)	-0.009	**0.017**	0.043
28	sl-al(l)	-0.043	**-0.011**	0.020	68	sto-ch(r)	-0.028	**0.000**	0.029	108	prn-en(r)	-0.023	**0.018**	0.059
29	gn-ch(l)	-0.053	**-0.011**	0.031	69	ch(r)-ch(l)	-0.039	**0.000**	0.041	109	sbal(l)-cph(l)	-0.005	**0.018**	0.040
30	n-sl	-0.048	**-0.011**	0.027	70	al(l)-cph(r)	-0.026	**0.001**	0.027	110	sto-en(r)	-0.011	**0.019**	0.050
31	sto-sl	-0.033	**-0.011**	0.011	71	prn-sl	-0.039	**0.001**	0.041	111	sn-en(r)	-0.011	**0.020**	0.050
32	n-al(r)	-0.038	**-0.010**	0.018	72	sn-ch(r)	-0.031	**0.001**	0.033	112	sbal(l)-cph(r)	-0.005	**0.020**	0.045
33	al(r)-sbal(l)	-0.037	**-0.009**	0.017	73	sl-sbal(l)	-0.029	**0.001**	0.031	113	sn-en(l)	-0.011	**0.020**	0.051
34	sn-gn	-0.049	**-0.009**	0.031	74	n-sto	-0.029	**0.002**	0.034	114	prn-en(l)	-0.022	**0.022**	0.064
35	sn-al(l)	-0.030	**-0.009**	0.012	75	sl-ch(r)	-0.030	**0.002**	0.036	115	sto-en(l)	-0.010	**0.022**	0.054
36	gn-ch(r)	-0.050	**-0.008**	0.034	76	en(l)-ch(l)	-0.028	**0.003**	0.033	116	en(r)-al(r)	-0.003	**0.022**	0.046
37	cph(r)-ch(l)	-0.043	**-0.008**	0.027	77	prn-sbal(r)	-0.026	**0.003**	0.033	117	en(r)-cph(l)	-0.009	**0.026**	0.061
38	n-sbal(r)	-0.036	**-0.008**	0.021	78	n-prn	-0.035	**0.004**	0.042	118	en(r)-cph(r)	-0.005	**0.028**	0.061
39	sn-ch(l)	-0.041	**-0.007**	0.027	79	sbal(l)-ch(r)	-0.031	**0.004**	0.037	119	en(l)-cph(r)	-0.006	**0.029**	0.064
40	al(r)-ch(r)	-0.033	**-0.007**	0.020	80	sl-ch(l)	-0.032	**0.004**	0.039	120	en(l)-cph(l)	-0.006	**0.030**	0.065

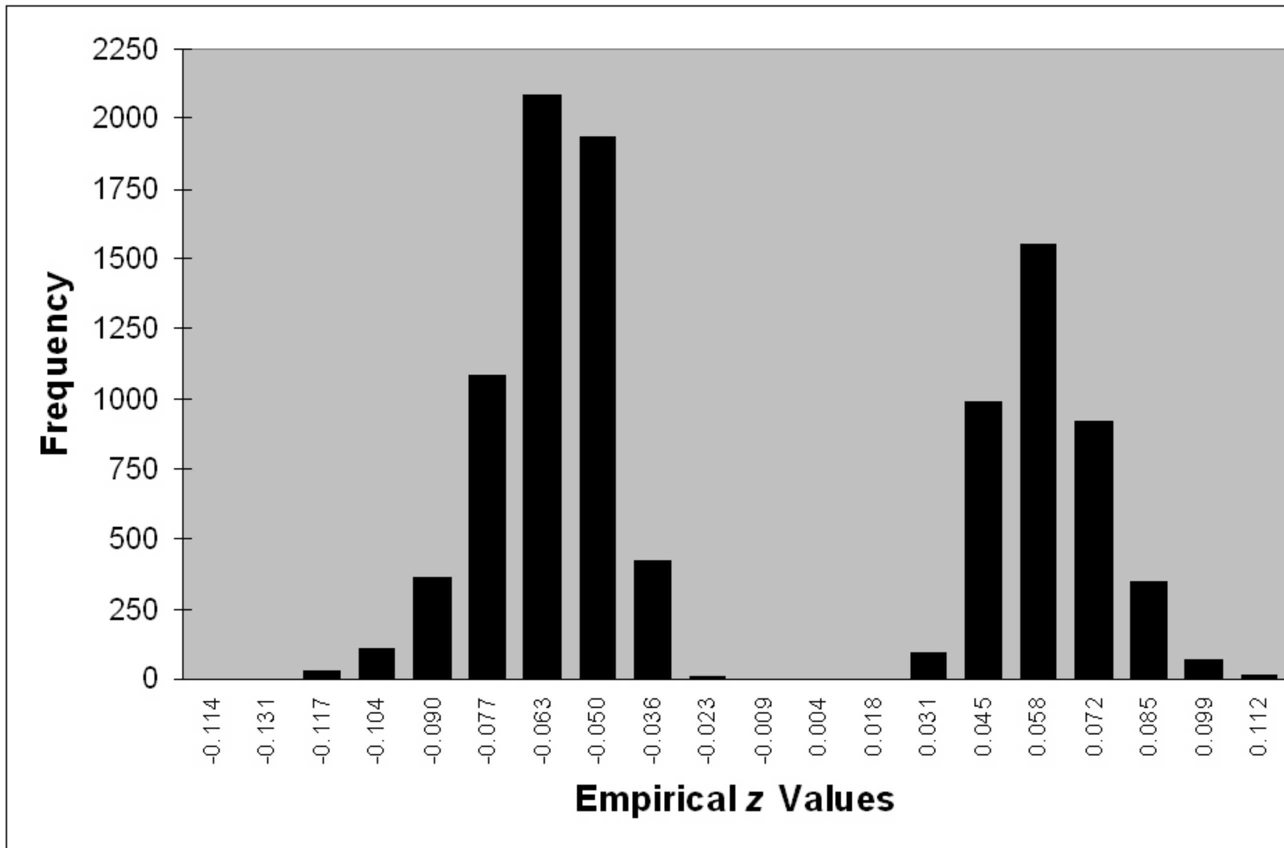

Figure 10 Omnibus test of shape difference between female relatives and controls.

This chart represents the frequency distribution of Z values generated from the bootstrapping routine of EDMA. Z is a maximal estimate of the difference in face shape between relatives and controls. The distribution is based on 10,000 random re-samples with replacement.

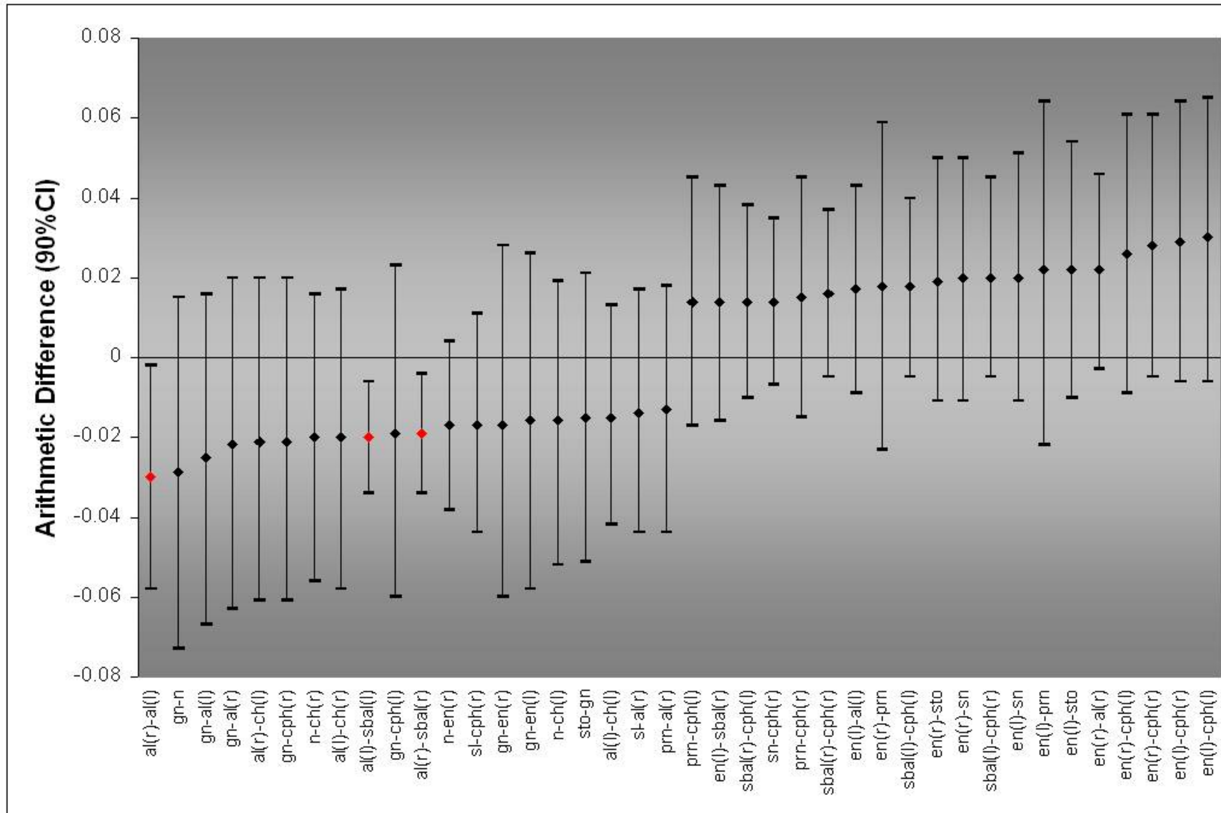

Figure 11 Tail ends of the sorted shape difference matrix for females.

This chart shows a subset of 40 scaled linear distances with the greatest mean arithmetic difference between relatives and controls. The 20 point estimates to the left of the central baseline represent variables larger in relatives. Likewise, the 20 point estimates to the right of the central baseline represent variables smaller in relatives. Each mean difference estimate is accompanied by an empirical 90% confidence interval generated via nonparametric bootstrapping. Variables where the confidence interval does not include zero are said to be significantly different between relatives and controls. Significantly different variables are indicated by red diamonds.

of the confidence interval to be informative depends on the nature of the Z distribution (see Lele and Richtsmeier, 2001; p. 185). Because Z is an arithmetic difference, it is often the case that the bootstrapped Z distribution will be bimodal. This occurs when the majority of the arithmetic differences deviate (both positively and negatively) from zero, a Z value of zero indicating no difference between groups. Unfortunately, in this situation the estimated confidence interval fails to provide the information necessary for meaningful interpretation of shape difference. According to Lele and Richtsmeier (2001), it is appropriate to reject the null hypothesis that two shapes are equivalent when presented with a bimodal Z distribution with a large dip around zero. Figure 10 shows the bimodal bootstrapped Z distribution based on 10,000 re-samples.

In addition to evidence for an overall shape difference between female relatives and female controls, there was evidence indicating that the shape differences were localized to particular facial regions. Table 11 shows the shape difference matrix (SDM) written as a vector sorted in ascending order. Recall from Section 2.3.3.1, that each element in the SDM is accompanied by a 90% confidence interval allowing for the identification of specific shape differences between groups. Figure 11 shows the upper and lower 10% of the 120 arithmetic differences comprising the sorted SDM along with their respective 90% confidence intervals. In sum, only three linear distances were identified as significantly ($p < 0.10$) larger in female unaffected relatives compared with controls: 1) the distance between the right and left *alare* points, what is traditionally called soft tissue nose width; 2) the distance between the left *alare* and the left *subalare*; and 3) the distance between the right *alare* and the right *subalare*. The magnitude of the relative-control difference for these three linear distances was quite small: 3mm, 2mm and 2mm, respectively. All three dimensions involve the landmark *alare* and, in combination, suggest that lateral displacement of the alar cartilage (relative to controls) is a characteristic facial feature of the female unaffected relatives of CL/P individuals.

3.1.1.2 Male sample Using the same procedure described above, EDMA was performed on a sample of 14 male unrelated, unaffected relatives from 14 multiplex nonsyndromic cleft families and an equal number of matched controls. The mean age for the male relative and control samples did not differ significantly (relatives 26.93 [±18.18], controls 27.14 [±18.06], t = -0.031, df = 26, p = 0.975; see Table 5). The mean form matrices containing all 120 unscaled linear distances for the male relative and control samples are shown in Table 12 and Table 13,

Table 12 Mean form matrix for male relative sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	44.85	0														
sn	52.46	18.65	0													
sto	72.69	37.30	20.92	0												
sl	90.24	55.81	39.42	18.65	0											
gn	120.40	87.01	70.63	49.94	31.29	0										
en(r)	24.50	48.01	48.72	65.66	81.24	109.49	0									
en(l)	24.81	47.79	48.27	65.58	81.01	109.14	32.94	0								
al(r)	46.24	27.28	20.71	34.10	50.15	79.63	34.62	47.96	0							
al(l)	47.35	26.94	19.61	33.53	49.28	78.61	49.07	35.57	34.08	0						
sbal(r)	51.06	23.84	11.18	24.26	41.35	71.76	42.48	49.02	10.55	27.21	0					
sbal(l)	51.74	23.45	10.40	24.08	41.01	71.37	50.11	43.02	27.89	10.14	19.14	0				
cph(r)	66.62	30.85	15.64	9.55	26.65	57.55	59.11	62.44	25.99	32.77	16.71	22.64	0			
cph(l)	67.10	31.02	15.69	9.68	26.35	57.15	63.11	59.41	33.77	25.50	23.23	16.55	13.03	0		
ch(r)	80.15	53.13	37.88	27.18	28.66	50.16	65.07	76.02	34.91	52.90	31.67	43.78	25.54	35.71	0	
ch(l)	80.41	52.58	37.12	27.15	28.21	49.59	76.57	65.25	53.29	34.07	43.48	31.21	35.32	25.00	47.83	0

Table 13 Mean form matrix for male controls

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	45.50	0														
sn	53.83	19.97	0													
sto	74.18	38.83	21.06	0												
sl	92.13	56.91	39.56	18.52	0											
gn	119.37	85.37	67.87	46.93	28.49	0										
en(r)	24.15	49.57	50.40	67.61	83.90	109.20	0									
en(l)	24.84	49.03	50.04	67.43	83.64	108.93	32.01	0								
al(r)	47.67	28.25	20.78	34.38	50.72	77.21	36.95	49.65	0							
al(l)	48.49	27.96	20.59	34.35	50.41	76.82	50.25	37.02	35.20	0						
sbal(r)	52.51	25.28	11.50	24.61	41.85	69.22	44.40	50.89	10.37	28.58	0					
sbal(l)	52.76	24.81	11.27	24.78	41.87	69.22	51.29	44.21	28.60	10.24	20.21	0				
cph(r)	68.08	32.32	15.66	9.71	26.54	54.59	61.16	64.26	26.11	33.71	16.88	23.47	0			
cph(l)	68.49	32.33	15.83	9.86	26.32	54.34	64.85	61.21	34.08	26.14	23.72	17.13	13.25	0		
ch(r)	81.45	54.28	37.72	27.19	29.51	48.20	67.24	77.75	34.78	53.89	31.36	44.42	25.35	35.87	0	
ch(l)	81.38	53.18	36.85	26.77	28.97	48.01	77.63	66.83	53.37	33.93	43.64	30.78	35.07	24.47	48.18	0

Table 14 Mean shape matrix for male relative sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	1.184	0														
sn	1.385	0.492	0													
sto	1.919	0.985	0.552	0												
sl	2.382	1.473	1.040	0.492	0											
gn	3.178	2.297	1.864	1.318	0.826	0										
en(r)	0.647	1.267	1.286	1.733	2.144	2.890	0									
en(l)	0.655	1.261	1.274	1.731	2.138	2.881	0.869	0								
al(r)	1.220	0.720	0.547	0.900	1.324	2.102	0.914	1.266	0							
al(l)	1.250	0.711	0.517	0.885	1.301	2.075	1.295	0.939	0.900	0						
sbal(r)	1.348	0.629	0.295	0.640	1.091	1.894	1.121	1.294	0.279	0.718	0					
sbal(l)	1.366	0.619	0.274	0.636	1.082	1.884	1.323	1.135	0.736	0.268	0.505	0				
cph(r)	1.758	0.814	0.413	0.252	0.703	1.519	1.560	1.648	0.686	0.865	0.441	0.597	0			
cph(l)	1.771	0.819	0.414	0.256	0.695	1.508	1.666	1.568	0.891	0.673	0.613	0.437	0.344	0		
ch(r)	2.116	1.402	1.000	0.717	0.756	1.324	1.717	2.007	0.921	1.396	0.836	1.156	0.674	0.943	0	
ch(l)	2.122	1.388	0.980	0.716	0.745	1.309	2.021	1.722	1.407	0.899	1.148	0.824	0.932	0.660	1.262	0

Table 15 Mean shape matrix for male control sample

	n	*prn*	*sn*	*sto*	*sl*	*gn*	*en(r)*	*en(l)*	*al(r)*	*al(l)*	*sbal(r)*	*sbal(l)*	*cph(r)*	*cph(l)*	*ch(r)*	*ch(l)*
n	0															
prn	1.185	0														
sn	1.402	0.520	0													
sto	1.932	1.012	0.549	0												
sl	2.400	1.483	1.030	0.482	0											
gn	3.110	2.224	1.768	1.223	0.742	0										
en(r)	0.629	1.291	1.313	1.761	2.186	2.845	0									
en(l)	0.647	1.277	1.304	1.757	2.179	2.837	0.834	0								
al(r)	1.242	0.736	0.541	0.896	1.321	2.011	0.963	1.293	0							
al(l)	1.263	0.728	0.536	0.895	1.313	2.001	1.309	0.964	0.917	0						
sbal(r)	1.368	0.658	0.300	0.641	1.090	1.803	1.157	1.326	0.270	0.745	0					
sbal(l)	1.374	0.646	0.294	0.646	1.091	1.803	1.336	1.152	0.745	0.267	0.526	0				
cph(r)	1.773	0.842	0.408	0.253	0.691	1.422	1.593	1.674	0.680	0.878	0.440	0.611	0			
cph(l)	1.784	0.842	0.412	0.257	0.685	1.415	1.689	1.594	0.888	0.681	0.618	0.446	0.345	0		
ch(r)	2.122	1.414	0.983	0.708	0.769	1.256	1.751	2.025	0.906	1.404	0.817	1.157	0.660	0.934	0	
ch(l)	2.120	1.385	0.960	0.697	0.755	1.251	2.022	1.741	1.390	0.884	1.137	0.802	0.914	0.637	1.255	0

Table 16 Sorted shape difference matrix for male sample

No.	Distance	LB	Estimate	UB	No.	Distance	LB	Estimate	UB	No.	Distance	LB	Estimate	UB
1	gn-cph(r)	-0.172	**-0.097**	-0.023	41	sn-cph(r)	-0.042	**-0.005**	0.032	81	sbal(l)-cph(r)	-0.031	**0.014**	0.060
2	sn-gn	-0.178	**-0.096**	-0.015	42	sto-al(r)	-0.050	**-0.005**	0.041	82	n-cph(r)	-0.056	**0.015**	0.085
3	sto-gn	-0.169	**-0.096**	-0.021	43	sn-sto	-0.033	**-0.004**	0.025	83	prn-al(r)	-0.049	**0.016**	0.082
4	gn-cph(l)	-0.168	**-0.093**	-0.018	44	al(r)-cph(l)	-0.060	**-0.004**	0.054	84	prn-en(l)	-0.077	**0.016**	0.111
5	gn-sbal(r)	-0.174	**-0.091**	-0.010	45	sl-al(r)	-0.055	**-0.003**	0.049	85	en(l)-sbal(l)	-0.031	**0.016**	0.063
6	gn-al(r)	-0.175	**-0.090**	-0.008	46	n-ch(l)	-0.096	**-0.003**	0.091	86	prn-al(l)	-0.052	**0.017**	0.087
7	sl-gn	-0.138	**-0.084**	-0.029	47	prn-ch(l)	-0.120	**-0.002**	0.115	87	al(r)-al(l)	-0.030	**0.017**	0.066
8	gn-sbal(l)	-0.162	**-0.080**	0.001	48	sn-cph(l)	-0.040	**-0.002**	0.036	88	n-sn	-0.037	**0.018**	0.072
9	gn-al(l)	-0.158	**-0.074**	0.009	49	sbal(r)-cph(r)	-0.043	**-0.001**	0.039	89	n-sl	-0.053	**0.018**	0.088
10	prn-gn	-0.169	**-0.073**	0.025	50	sl-sbal(r)	-0.047	**-0.001**	0.044	90	en(l)-ch(l)	-0.057	**0.019**	0.092
11	n-gn	-0.163	**-0.068**	0.023	51	al(l)-sbal(l)	-0.023	**-0.001**	0.020	91	en(l)-ch(r)	-0.070	**0.019**	0.106
12	gn-ch(r)	-0.158	**-0.068**	0.020	52	sto-sbal(r)	-0.036	**0.001**	0.037	92	sn-al(l)	-0.018	**0.019**	0.056
13	gn-ch(l)	-0.155	**-0.058**	0.040	53	sto-cph(r)	-0.025	**0.001**	0.028	93	sn-sbal(l)	-0.013	**0.019**	0.050
14	gn-en(r)	-0.143	**-0.045**	0.050	54	cph(r)-cph(l)	-0.023	**0.001**	0.026	94	n-sbal(r)	-0.029	**0.020**	0.069
15	gn-en(l)	-0.139	**-0.043**	0.049	55	sto-cph(l)	-0.025	**0.001**	0.029	95	sbal(r)-sbal(l)	-0.016	**0.021**	0.060
16	en(r)-en(l)	-0.082	**-0.036**	0.011	56	en(r)-ch(l)	-0.096	**0.001**	0.096	96	n-al(r)	-0.027	**0.021**	0.071
17	cph(l)-ch(l)	-0.101	**-0.022**	0.057	57	n-prn	-0.069	**0.001**	0.073	97	prn-cph(l)	-0.038	**0.023**	0.085
18	sbal(l)-ch(l)	-0.090	**-0.022**	0.046	58	sbal(l)-ch(r)	-0.079	**0.001**	0.083	98	en(r)-cph(l)	-0.058	**0.024**	0.103
19	sn-ch(l)	-0.106	**-0.020**	0.067	59	sn-sbal(r)	-0.026	**0.004**	0.036	99	prn-en(r)	-0.068	**0.024**	0.117
20	sto-ch(l)	-0.100	**-0.019**	0.066	60	sbal(r)-cph(l)	-0.043	**0.005**	0.053	100	en(l)-al(l)	-0.017	**0.025**	0.067
21	sbal(r)-ch(r)	-0.077	**-0.019**	0.041	61	n-ch(r)	-0.084	**0.006**	0.097	101	sto-en(l)	-0.041	**0.026**	0.092
22	cph(r)-ch(l)	-0.104	**-0.019**	0.069	62	al(l)-ch(r)	-0.076	**0.007**	0.092	102	en(l)-cph(r)	-0.049	**0.026**	0.100
23	n-en(r)	-0.061	**-0.018**	0.026	63	al(l)-cph(l)	-0.036	**0.008**	0.053	103	en(l)-cph(l)	-0.045	**0.026**	0.096
24	sn-ch(r)	-0.093	**-0.017**	0.061	64	sl-sbal(l)	-0.042	**0.008**	0.058	104	al(l)-sbal(r)	-0.015	**0.026**	0.068

Table 16 (continued).

25	al(r)-ch(l)	-0.105	**-0.016**	0.074	65	n-sbal(l)	-0.040	**0.009**	0.057	105	prn-sto	-0.026	**0.027**	0.079
26	al(r)-ch(r)	-0.074	**-0.015**	0.045	66	al(r)-sbal(l)	-0.035	**0.009**	0.053	106	sn-en(r)	-0.037	**0.027**	0.092
27	al(l)-ch(l)	-0.082	**-0.015**	0.052	67	sbal(l)-cph(l)	-0.031	**0.009**	0.049	107	en(l)-al(r)	-0.033	**0.027**	0.086
28	cph(r)-ch(r)	-0.081	**-0.014**	0.056	68	prn-sl	-0.057	**0.010**	0.075	108	prn-sbal(l)	-0.036	**0.027**	0.091
29	sl-cph(r)	-0.050	**-0.012**	0.026	69	sto-al(l)	-0.032	**0.010**	0.052	109	prn-cph(r)	-0.032	**0.028**	0.087
30	sbal(r)-ch(l)	-0.096	**-0.011**	0.076	70	sto-sbal(l)	-0.026	**0.010**	0.046	110	prn-sn	-0.015	**0.028**	0.072
31	sl-cph(l)	-0.051	**-0.010**	0.031	71	sl-ch(l)	-0.075	**0.010**	0.096	111	sto-en(r)	-0.042	**0.028**	0.098
32	sn-sl	-0.056	**-0.010**	0.035	72	prn-ch(r)	-0.091	**0.012**	0.117	112	prn-sbal(r)	-0.033	**0.029**	0.093
33	sto-sl	-0.045	**-0.010**	0.026	73	sl-ch(r)	-0.057	**0.012**	0.084	113	sn-en(l)	-0.033	**0.030**	0.092
34	sto-ch(r)	-0.081	**-0.009**	0.069	74	sl-al(l)	-0.042	**0.012**	0.066	114	en(l)-sbal(r)	-0.024	**0.032**	0.086
35	al(r)-sbal(r)	-0.032	**-0.008**	0.016	75	n-cph(l)	-0.059	**0.013**	0.084	115	en(r)-cph(r)	-0.043	**0.033**	0.108
36	cph(l)-ch(r)	-0.086	**-0.008**	0.074	76	al(l)-cph(r)	-0.038	**0.013**	0.065	116	en(r)-ch(r)	-0.040	**0.034**	0.108
37	n-en(l)	-0.052	**-0.008**	0.036	77	n-al(l)	-0.035	**0.013**	0.062	117	en(r)-sbal(r)	-0.013	**0.036**	0.083
38	ch(r)-ch(l)	-0.109	**-0.007**	0.096	78	en(r)-sbal(l)	-0.048	**0.013**	0.074	118	sl-en(l)	-0.032	**0.041**	0.113
39	al(r)-cph(r)	-0.053	**-0.006**	0.043	79	en(r)-al(l)	-0.047	**0.014**	0.074	119	sl-en(r)	-0.032	**0.041**	0.115
40	sn-al(r)	-0.041	**-0.005**	0.032	80	n-sto	-0.048	**0.014**	0.075	120	en(r)-al(r)	0.002	**0.049**	0.096

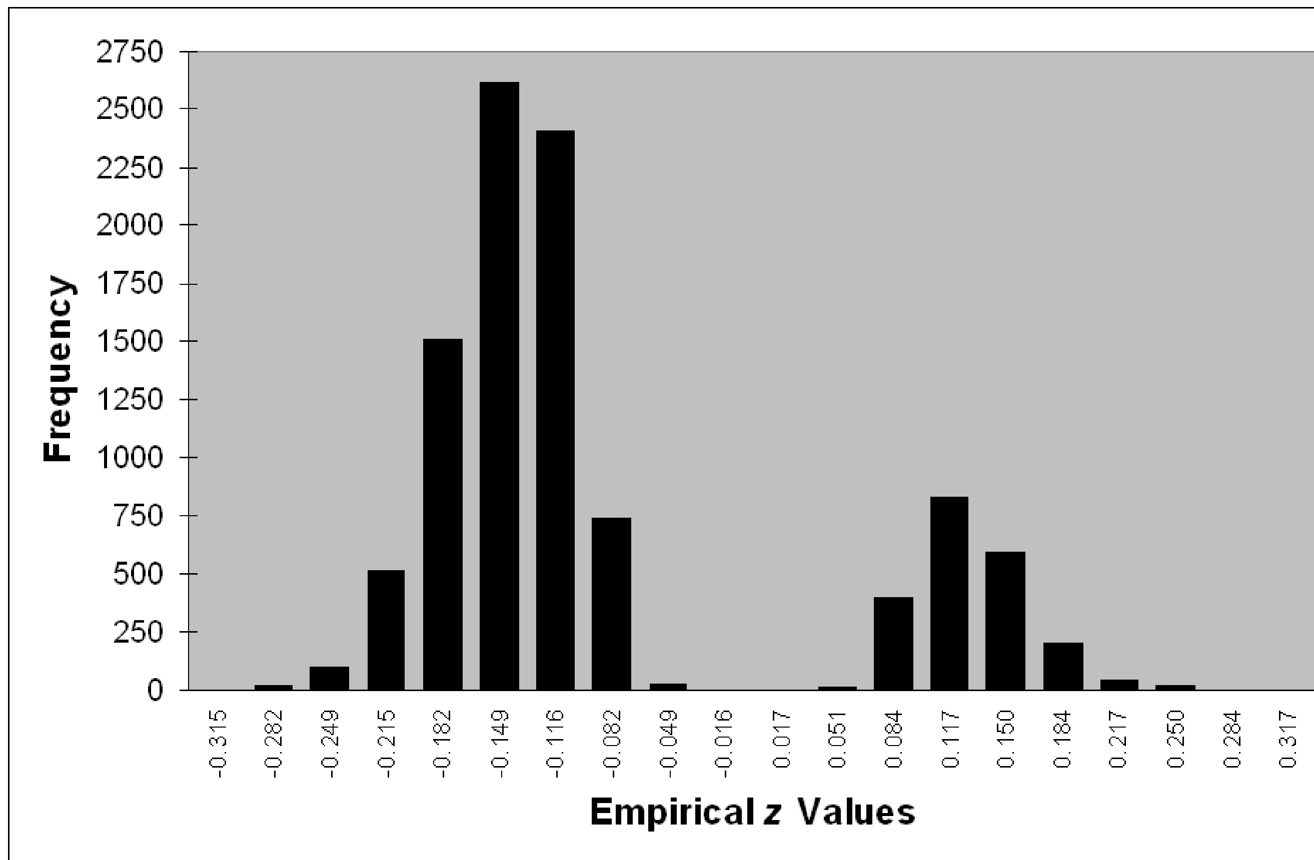

Figure 12 Omnibus test of shape difference between male relatives and controls.

This chart represents the frequency distribution of *Z* values generated from the bootstrapping routine of EDMA. *Z* is a maximal estimate of the difference in face shape between relatives and controls. The distribution is based on 10,000 random re-samples with replacement.

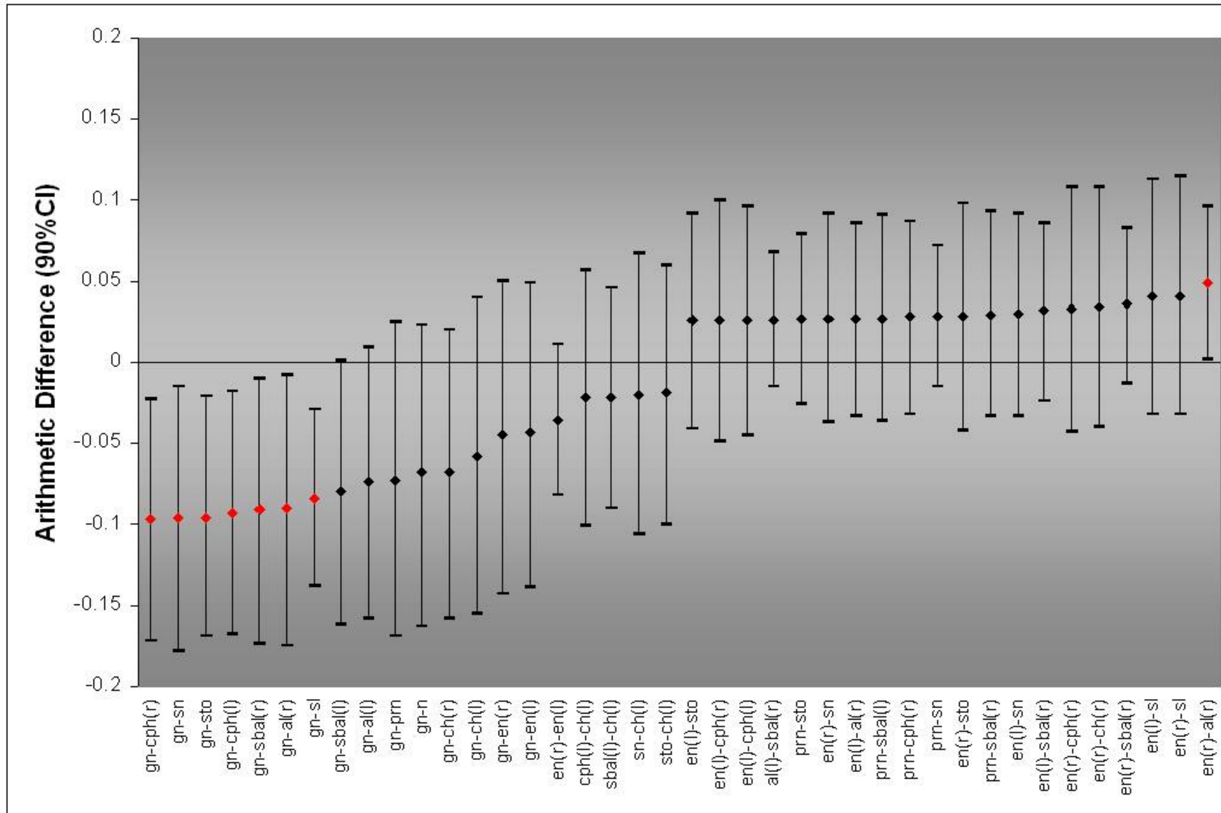

Figure 13 Tail ends of the sorted shape difference matrix for males.

This chart shows a subset of 40 scaled linear distances with the greatest mean arithmetic difference between relatives and controls. The 20 point estimates to the left of the central baseline represent variables larger in relatives. Likewise, the 20 point estimates to the right of the central baseline represent variables smaller in relatives. Each mean difference estimate is accompanied by an empirical 90% confidence interval generated via nonparametric bootstrapping. Variables where the confidence interval does not include zero are said to be significantly different between relatives and controls. Significantly different variables are indicated by red diamonds.

respectively. Similar to the female samples, the male relatives and controls did not differ statistically in terms of overall size; the difference between the geometric mean for relatives (37.89) and controls (38.39) was only 0.50. The 90% confidence interval of the bootstrapped size difference distribution clearly contained zero (-1.08 – 2.09). The mean shape matrices for each group are provided in Table 14 and Table 15.

As with females, results of EDMA's null hypothesis test of omnibus shape difference indicate that the shape of the face differs between male unaffected relatives and male controls (Figure 12). However, in contrast to females, these shape differences were localized to different facial regions. Table 16 shows the data comprising the shape difference matrix (SDM) for males written as a vector sorted in ascending order. Figure 13 shows the upper and lower 10% of these 120 arithmetic differences along with their respective 90% confidence intervals. In the male sample, eight linear distances were identified as significantly ($p < 0.10$) different in unaffected relatives versus controls. Seven of these dimensions were larger in unaffected relatives. All of these measurements represent aspects of lower facial height and involve the landmark *gnathion*. The magnitude of the relative-control difference for these seven distances ranged from 8mm to 10mm, greater than the differences observed in females. A single linear distance was found to be significantly smaller (5mm) in male relatives, the distance between the right *endocanthion* and the right *alare*. Although not statistically significant, the next seven linear distances at the extreme end of the SDM all involve *endocanthion* (mostly right) and vertically span the upper/midface region (Figure 13). This indicates a strong trend toward reduction in upper/mid facial height, possibly with a more marked expression on the right side of the face. Taken together, these results suggest that a combination of morphological features including increased vertical length of the lower face and decreased vertical length of the upper face characterize male unaffected relatives of CL/P individuals.

3.1.2 Univariate results

3.1.2.1 Female sample Regarding the 14 additional scaled linear distances, two were found to differ significantly ($p < 0.05$) between female unaffected relatives and controls (Table 17). Specifically, female unaffected relatives possessed significantly greater upper facial width ($p = .02$) and significantly reduced left midfacial depth ($p = 0.04$). The effect sizes (Hedges

Table 17 Effect sizes and t-test results for females on 14 additional linear distances

Distance	*Relative mean* [a] *(sd)* *n = 33*	*Control mean* [a] *(sd)* *n = 33*	*Effect size (g)* [b]	*t statistic*	*Significance*
Maximum head width	1.400 (0.046)	1.401 (0.045)	-0.026	-0.108	0.914
Forehead Width	0.997 (0.043)	0.988 (0.031)	0.240	0.985	0.328
Maximum upper face width	1.277 (0.035)	1.258 (0.033)	0.571	2.346	0.022
Cranial base width	1.250 (0.037)	1.249 (0.035)	0.033	0.137	0.892
Mandible width	0.981 (0.041)	0.963 (0.038)	0.447	1.835	0.071
Maximum head length	1.774 (0.063)	1.802 (0.075)	-0.414	-1.699	0.094
Upper face depth (L)	1.128 (0.025)	1.136 (0.025)	-0.302	-1.239	0.220
Upper face depth (R)	1.129 (0.026)	1.130 (0.022)	-0.049	-0.203	0.840
Midface depth (L)	1.159 (0.031)	1.173 (0.023)	-0.509	-2.098	0.040
Midface depth (R)	1.165 (0.032)	1.177 (0.023)	-0.430	-1.756	0.084
Lower face depth (L)	1.294 (0.036)	1.300 (0.047)	-0.144	-0.590	0.557
Lower face depth (R)	1.309 (0.040)	1.308 (0.044)	0.042	0.176	0.861
Alar cartilage length (L)	0.297 (0.020)	0.299 (0.019)	-0.077	-0.318	0.751
Alar cartilage length (R)	0.302 (0.020)	0.299 (0.018)	0.126	0.523	0.603

[a] Data reflect raw values scaled by group-specific geometric mean (relative = 104.62, control = 103.80)

[b] Hedges *g*, positive value indicates relative group larger than control group

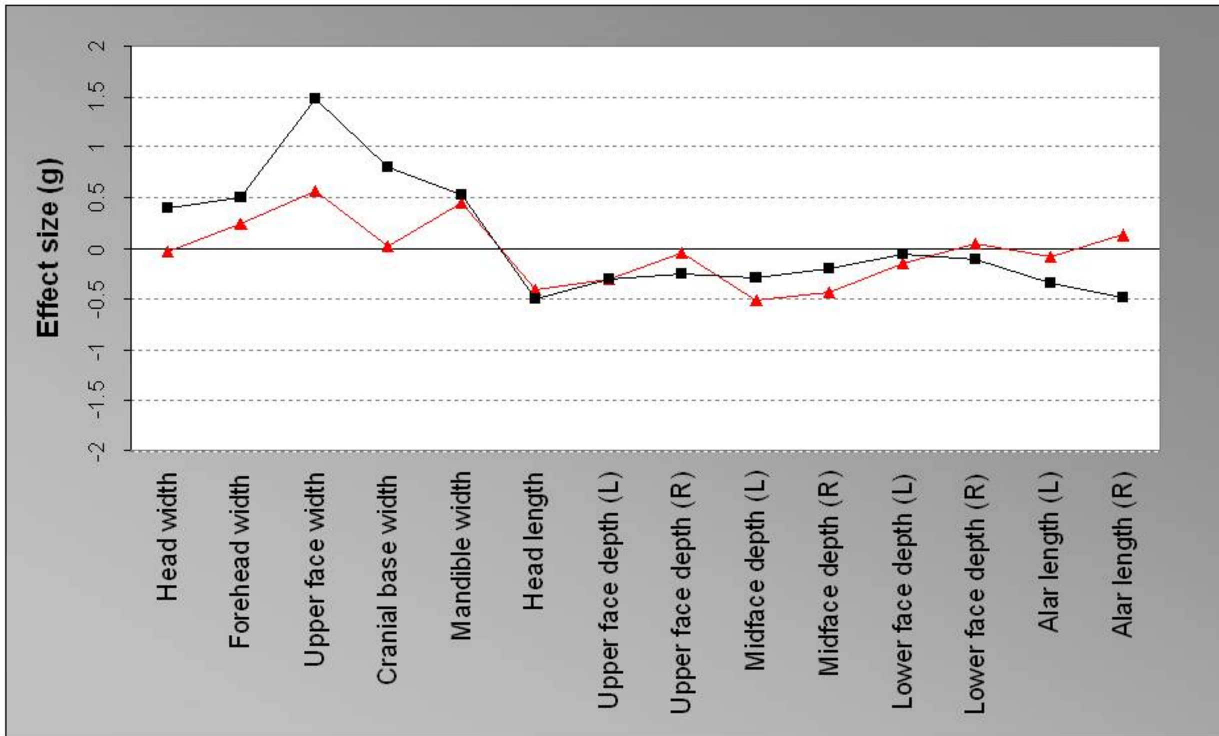

Figure 14 Effect sizes for the 14 additional linear distances.

Effect sizes represent the magnitude of the mean difference between relatives and controls. Values to the left of the baseline indicate smaller dimensions in relatives, while values to the right of the baseline indicate larger dimensions in relatives. The male sample is represented by solid black squares. The female sample is represented by red triangles.

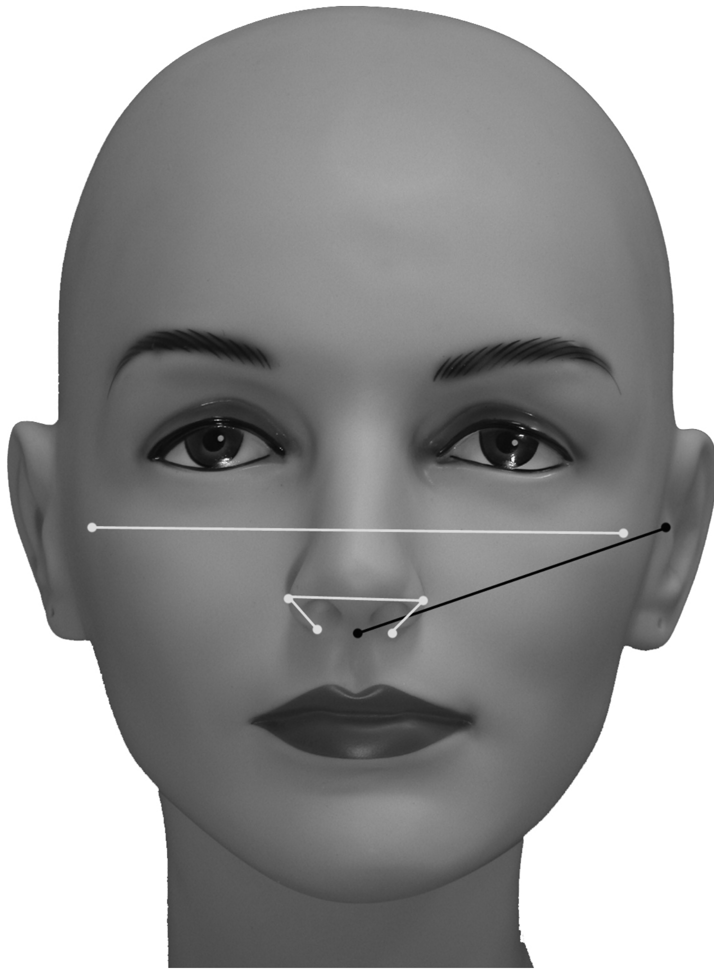

Figure 15 Significantly different dimensions in female sample.

Dimensions found to be significantly larger in relatives versus controls are indicated by white lines. Dimensions found to be significantly smaller in relatives are indicated by black lines.

g) for these two dimensions were 0.57 and -0.51, respectively. The magnitude of these effect sizes are within the moderate range (Cohen, 1988). Effect sizes for the remaining 12 variables were in the small (0.20 – 0.49) to very small (0 - 0.20) range (Figure 14). Non-significant trends ($0.05 > p > 0.10$) toward increased mandibular width, reduced head length and reduced right midfacial depth were also observed in the unaffected relative group. All facial dimensions observed to differ significantly are depicted in Figure 15.

3.1.2.2 Male sample Similar to the female relative group, male unaffected relatives were characterized by significantly wider upper faces ($p < .001$) compared to male controls. Male relatives also possessed significantly wider cranial bases ($p = .04$), however, unlike female relatives, there were no observed differences in midfacial depth. The effect sizes for these two variables were quite large: 1.47 for upper face width and 0.79 for cranial base width. In general, effect size magnitudes were greater for males than females, indicating a greater degree of divergence between male unaffected relatives and male controls. These results are detailed in Table 18 and depicted in Figure 14 and Figure 16.

3.1.3 Summary of phase I results

Through EDMA and a series of univariate t-tests, a subset of informative variables capable of differentiating the unaffected relatives of CL/P individuals from a sample of matched controls was identified. The relative-control differences were largely sex-specific. Female unaffected relatives differed from female controls on five size-adjusted variables; craniofacial differences included an increase in upper facial width, midface reduction and lateral displacement of the left and right alar cartilage in relatives. In contrast, male unaffected relatives demonstrated a distinct pattern of craniofacial differences; a total of eight variables were observed to differentiate male relatives from male controls. The major differences included increased lower facial height, some evidence for decreased upper/midfacial height (particularly on the right side of the face) and increased upper facial width in unaffected male relatives. Interestingly, the only consistent facial feature distinguishing unaffected relatives from controls in both sexes was an increase in facial width. These informative variables were utilized in phase II of the analysis (see below).

Table 18 Effect sizes and t-test results for males on 14 additional linear distances

Distance	*Relative mean [a] (sd)* *n = 14*	*Control mean [a] (sd)* *n = 14*	*Effect size (g) [b]*	*t statistic*	*Significance*
Maximum head width	1.392 (0.055)	1.369 (0.063)	0.385	1.053	0.302
Forehead Width	0.974 (0.033)	0.956 (0.040)	0.496	1.349	0.189
Maximum upper face width	1.279 (0.038)	1.229 (0.029)	1.472	4.013	< 0.001
Cranial base width	1.263 (0.032)	1.238 (0.030)	0.787	2.145	0.041
Mandible width	0.986 (0.020)	0.969 (0.039)	0.527	1.456	0.157
Maximum head length	1.754 (0.062)	1.793 (0.089)	-0.497	-1.354	0.187
Upper face depth (L)	1.125 (0.033)	1.135 (0.029)	-0.312	-0.852	0.402
Upper face depth (R)	1.127 (0.020)	1.134 (0.033)	-0.254	-0.688	0.498
Midface depth (L)	1.170 (0.038)	1.179 (0.019)	-0.280	-0.760	0.454
Midface depth (R)	1.179 (0.026)	1.184 (0.022)	-0.199	-0.542	0.592
Lower face depth (L)	1.331 (0.064)	1.334 (0.039)	-0.053	-0.142	0.888
Lower face depth (R)	1.323 (0.042)	1.329 (0.058)	-0.109	-0.299	0.768
Alar cartilage length (L)	0.291 (0.024)	0.300 (0.029)	-0.340	-0.926	0.363
Alar cartilage length (R)	0.296 (0.022)	0.308 (0.027)	-0.481	-1.304	0.204

[a] Data reflect raw values scaled by group-specific geometric mean (relative = 110.15, control = 109.59)

[b] Hedges *g*, positive value indicates relative group larger than control group

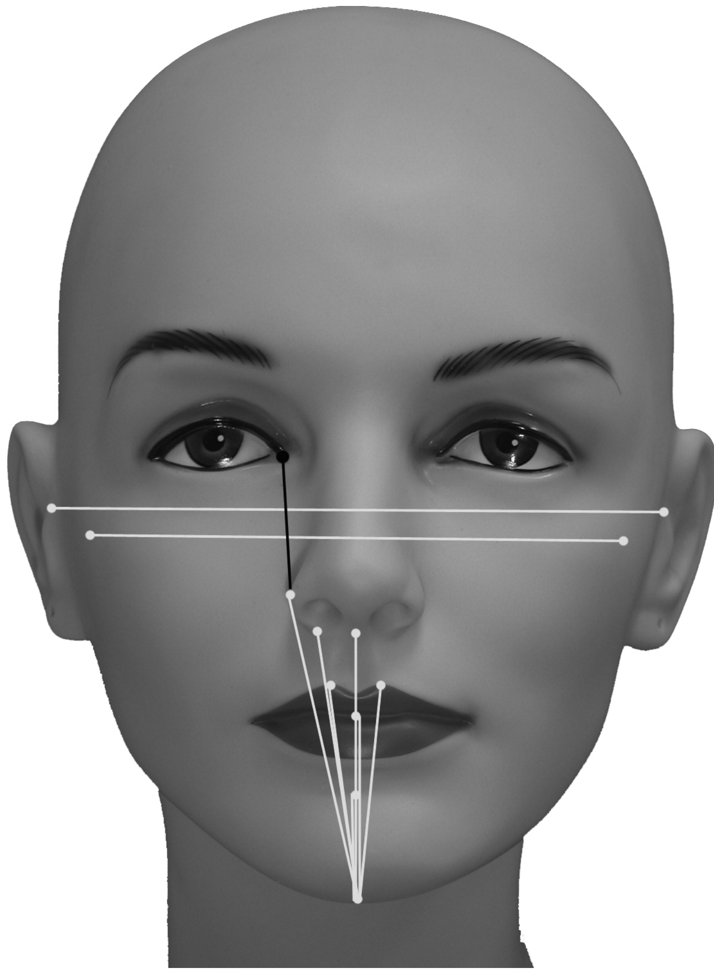

Figure 16 Significantly different dimensions in male sample.

Dimensions found to be significantly larger in relatives versus controls are indicated by white lines. Dimensions found to be significantly smaller in relatives are indicated by black lines.

3.2 PHASE II. CONSTRUCTING A MULTIVARIATE PREDICTIVE MODEL

3.2.1 Multivariate assumptions

3.2.1.1 Female sample The five informative variables identified from phase I were first assessed for multicollinearity. Based on a simple Pearson correlation matrix, no pair of variables was observed to correlate at a level greater than 0.90 (Table 19). However, formal tests for multicollinearity revealed a potential problem with two variables: *al(r)-al(l)* and *al(l)-sbal(l)*. These results are presented in Table 20. Consequently, the variable *al(l)-sbal(l)* was dropped from further analysis because, of the two variables in question, it was the weaker discriminator between unaffected relatives and controls. Each of the remaining four variables was observed to be distributed normally. Moreover, the assumption of homogeneity of the variance-covariance matrices between the unaffected relative and control groups was met (Box's M test; $p = 0.583$) and no multivariate outliers were detected ($D^2 < 18.47$ for all subjects).

3.2.1.2 Male sample For the male sample, five out of 10 of the initially informative variables were dropped due to excessive multicollinearity. This is perhaps not surprising given the obvious overlap in linear distances indicating increased lower facial height in the unaffected relatives (Figure 16). Six variables – *al(r)-gn*, *sbal(r)-gn*, *cph(r)-gn*, *cph(l)-gn*, *sto-gn*, and *sn-gn* – were observed to have bivariate correlations greater than or equal to 0.90 (Table 21). Due to these excessive correlations, only a single variable *sn-gn* was retained. Using the five remaining variables, formal tests for multicollinearity were performed – no additional problems were found (Table 22). All variables were observed to be distributed normally, homogeneity of the variance-covariance structure across groups was verified (Box's M test; $p = 0.632$), and no multivariate outliers were detected ($D^2 < 20.52$ for all subjects).

Table 19 Simple correlation matrix of informative variables for female sample

	zy-zy	*t(l)-n*	*al-al*	*al(r)-sbal(r)*	*al(l)-sbal(l)*
zy-zy	1.000	-	-	-	-
t(l)-n	-.410	1.000	-	-	-
al-al	.169	.036	1.000	-	-
al(r)-sbal(r)	-.019	-.047	.377	1.000	-
al(l)-sbal(l)	.107	.175	.838	-.030	1.000

Bold values indicate correlations ≥ 0.90

Table 20 Multicollinearity statistics on informative variable subset for female sample

Eigenvalue	*Condition index*	*zy-zy*	*t(l)-sn*	*al-al*	*al(r)-sbal(r)*	*al(l)-sbal(l)*
5.979	1.000	.00	.00	.00	.00	.00
.015	20.022	.00	.00	.00	.41	.00
.005	34.436	.02	.01	.03	.00	.04
.001	77.828	.32	.18	.02	.01	.02
.000	140.203	.07	.04	**.94**	.58	**.92**
.000	220.724	.60	.77	.01	.00	.02
	Tolerance	.785	.753	.122	.422	.142
	Variance inflation factor (VIF)	1.274	1.327	8.230	2.370	7.061

Bold values indicate excess collinearity

Table 21 Simple correlation matrix of informative variables for male sample

	zy-zy	*t-t*	*al(r)-gn*	*sbal(r)-gn*	*cph(r)-gn*	*cph(l)-gn*	*sn-gn*	*sto-gn*	*sl-gn*	*al(r)-en(r)*
zy-zy	1.000	-	-	-	-	-	-	-	-	-
t-t	.677	1.000	-	-	-	-	-	-	-	-
al(r)-gn	.011	-.186	1.000	-	-	-	-	-	-	-
sbal(r)-gn	.001	-.215	**.976**	1.000	-	-	-	-	-	-
cph(r)-gn	.117	-.132	.874	.882	1.000	-	-	-	-	-
cph(l)-gn	.096	-.152	.882	.893	**.996**	1.000	-	-	-	-
sn-gn	-.033	-.220	**.963**	**.959**	**.895**	**.908**	1.000	-	-	-
sto-gn	.069	-.115	**.929**	**.952**	**.941**	**.943**	**.939**	1.000	-	-
sl-gn	.046	-.112	.840	.849	.807	.804	.828	.875	1.000	-
a(r)l-en(r)	-.087	-.051	-.210	-.107	.113	.118	-.065	.025	-.121	1.000

Bold values indicate correlations ≥ 0.90

Table 22 Multicollinearity statistics on informative variable subset for male sample

Eigenvalue	*Condition index*	*zy-zy*	*t-t*	*sn-gn*	*sl-gn*	*al(r)-en(r)*
5.980	1.000	.00	.00	.00	.00	.00
.015	20.140	.00	.00	.01	.18	.04
.004	40.892	.03	.01	.00	.01	.83
.001	66.409	.02	.01	.80	.76	.04
.000	119.208	.72	.05	.08	.04	.08
.000	175.535	.23	.93	.11	.01	.02
	Tolerance	.584	.538	.292	.302	.982
	Variance inflation factor (VIF)	1.712	1.857	3.425	3.306	1.019

Bold values indicate excess collinearity

3.2.2 Discriminant analysis results

3.2.2.1 Female sample For the female sample, four variables were entered simultaneously into a two-group DFA: maximum face width (*zy-zy*), left midface depth (left *t-sn*), nose width (*al-al*), and right *alare-subalare* distance (right *al-sbal*). These variables are shown in Figure 17. A single statistically significant discriminant function was derived ($\Lambda = 0.81$; $\chi^{2\,(4df)} = 12.76$; $p = 0.01$), indicating that the combination of these four predictor variables was capable of differentiating between female unaffected relatives and female controls (see Table 23 for discriminant model descriptive statistics). A canonical correlation of .43 was observed, indicating that the discriminant function was able to account for 18.5% (squared canonical correlation) of the variance in the outcome variable. Overall, 71% of relatives and controls were classified correctly. Broken down by group, 70% of relatives and 73% of controls were classified correctly (Table 24). Empirical cross-validation (Jackknife routine) of the classification procedure resulted in a slight reduction in the overall classification accuracy to 67%. The ability of the discriminant function to classify cases into their correct group was significantly better than expected by chance (Press's Q = 29.33; $p < 0.001$). The average biological distance (Mahalanobis D^2) of these 33 relatives from the centroid of the control group was 1.83 standard deviations (Figure 18). Evaluation of the structure matrix coefficients (Table 25) revealed that maximum face width had the highest loading on the discriminant function ($r = 0.61$), followed in decreasing order by right *alare-subalare* length ($r = 0.57$), left midface depth ($r = -0.55$) and nose width ($r = 0.53$). The narrow range of values for these coefficients suggested that all four predictor variables approximated one another in terms of their substantive importance to the discriminant function.

3.2.2.2 Male sample For the male sample, five variables (see Figure 19) were entered simultaneously into a two-group DFA: maximum face width (*zy-zy*), cranial base width (*t-t*), lower face height (*sn-gn*), *sublabiale-gnathion* length (*sl-gn*) and right *alare-endocanthion* length (right *al-en*). Based on these five predictor variables, a single statistically significant discriminant function was derived ($\Lambda = 0.38$; $\chi^{2\,(5df)} = 23.05$; $p < 0.001$). Table 26 contains descriptive statistics for the discriminant model. A large canonical correlation of .79 was

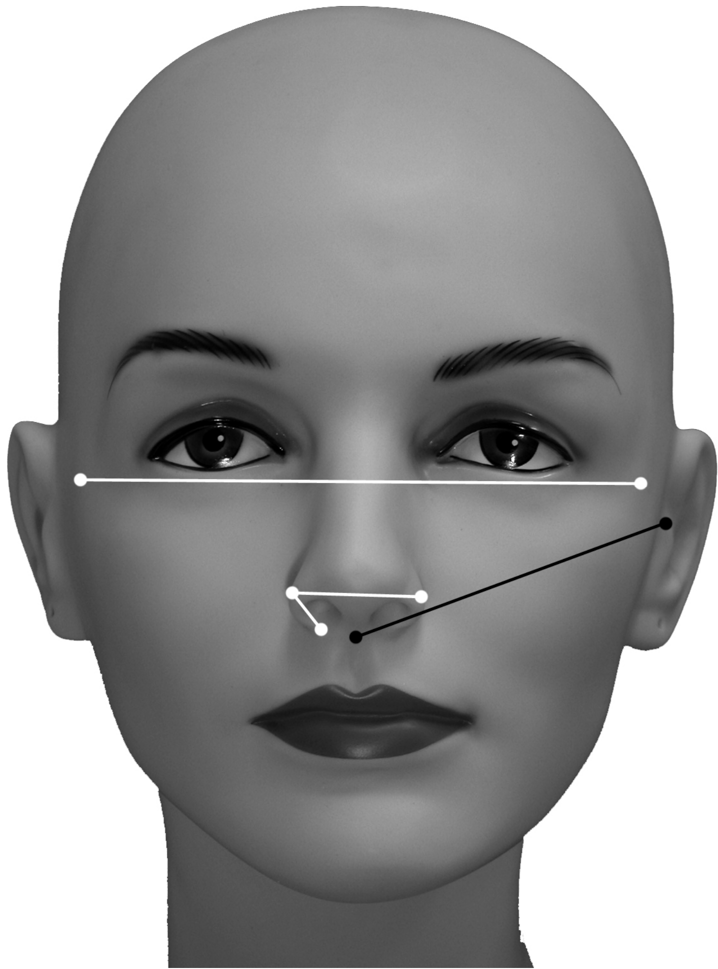

Figure 17 Variable subset entered into DFA for female sample.

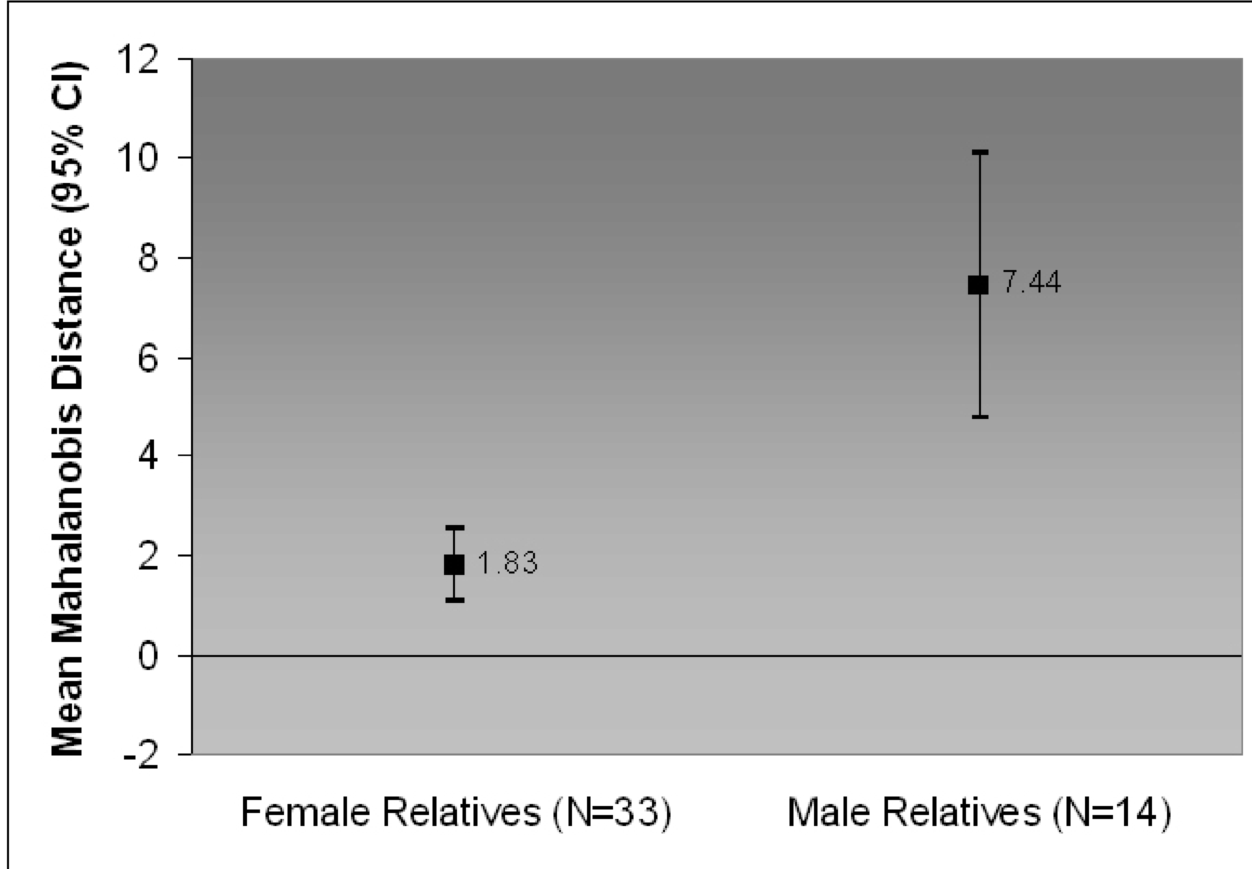

Figure 18 Mean Mahalanobis distance of relatives from control centroid.

Distance is measured in standard deviation units. Control centroid represented by zero baseline.

Table 23 Discriminant function model descriptive statistics for female sample

Function	*Eigenvalue*	*Canonical corr. (r)*	*Sq. canonical corr. (r^2)*	*Wilks' lambda*	*Chi-square*	*df*	*Significance*
1	.229	.431	.186	.814	12.761	4	.013

Four variables included in model

Table 24 Classification accuracy for female sample

		Predicted group	
		Relative	*Control*
Actual group	*Relative*	23 (69.7%)	10 (30.3%)
	Control	9 (27.3%)	24 (72.7%)

Overall correct classification rate = 71.2%

Press's Q = 29.33; $p < 0.001$

Table 25 Discriminant model coefficients for female sample

Predictor variables	*Standardized coefficients*	*Unstandardized coefficients*	*Structure matrix coefficients*
zy-zy	.472	13.848	.613
t(l)-sn	-.424	-15.704	-.549
al-al	.351	5.713	.528
al(r)-sbal(r)	.511	13.961	.572
Constant		-8.337	

observed, indicating that the discriminant function was able to account for as much as 62.6% of the variance in the outcome variable. The classification accuracy was very high; 86% of male relatives and 93% of male controls were classified correctly, with an overall correct classification rate of 89% (Table 27). The Jackknife cross-validation classification procedure resulted in an accuracy of 86%. As with the female sample, the ability of the discriminant function to assign male cases to their correct group was significantly better than expected by chance (Press's Q = 17.29; $p < 0.001$). The average biological distance of these 14 relatives from the centroid of the control group was 7.44 standard deviations (Figure 18). Evaluation of the structure matrix coefficients (Table 28) revealed that maximum face width had by far the highest discriminant function loading ($r = 0.61$), followed in decreasing order by *sublabiale-gnathion* length ($r = 0.34$), cranial base width ($r = 0.33$), right *alare-endocanthion* length ($r = -0.32$) and lower face height ($r = 0.30$). The similarity in coefficient values for the four latter variables suggests that their substantive importance to group discrimination was more or less equivalent.

3.2.3 Identification of at-risk relatives

For each unaffected relative included in the discriminant function analyses, a discriminant score and Mahalanobis distance measure were calculated allowing for a probability-based classification. As stated in Section 2.3.4.3, unaffected relatives were considered "high risk" if, following the DFA, they were assigned to the relative group (not the control group) and had a Mahalanobis distance of at least 1.96 from the control centroid. Based on these criteria, a total of 11 female (33%) and 12 male (86%) unaffected relatives were classified as "high risk" individuals (see Figure 20 and Figure 21, respectively).

Using the parameters from the DFA, 46 additional unaffected relatives not included in the creation of the initial discriminant function models were assigned to a risk class. A total of eight out of 24 additional unaffected female relatives (33%) and nine out of 22 additional unaffected male relatives (41%) met the "high risk" criteria. Thus, in total, 40 unaffected relatives could be classified as "high risk" and 53 as "low risk." Six unaffected relatives could not be assigned to a risk class because they had missing data (Figure 22).

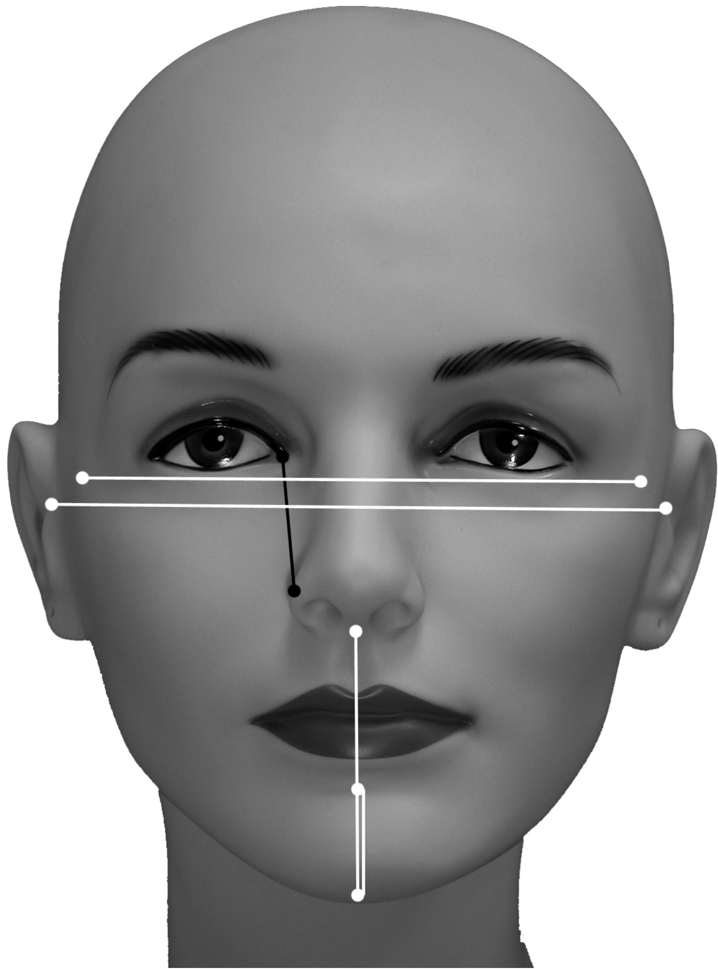

Figure 19 Variable subset entered into DFA for male sample.

Table 26 Discriminant function model descriptive statistics for male sample

Function	*Eigenvalue*	*Canonical corr. (r)*	*Sq. canonical corr. (r^2)*	*Wilks' lambda*	*Chi-square*	*df*	*Significance*
1	1.666	.791	.626	.375	23.045	5	< .001

Five variables included in model

Table 27 Classification accuracy for male sample

		Predicted group	
		Relative	*Control*
Actual group	*Relative*	12 (85.7%)	2 (14.3%)
	Control	1 (7.1%)	13 (92.9%)

Overall correct classification rate = 89.3%

Press's Q = 17.29; $p < 0.001$

Table 28 Discriminant model coefficients for male sample

Predictor variables	*Standardized coefficients*	*Unstandardized coefficients*	*Structure matrix coefficients*
zy-zy	.881	26.356	.610
t-t	.152	4.935	.326
sn-gn	.547	4.339	.298
sl-gn	.211	2.179	.336
al(r)-en(r)	-.565	-9.750	-.317
Constant		-39.690	

3.2.4 Characterization of risk classes

The sex distribution in the "high risk" and "low risk" relative groups differed significantly (χ^2 = 5.626; p = 0.02). The percentage of males and females in the "high risk" group was almost equal at 52.5% and 47.5%, respectively; this resulted in a male to female ratio of 1.1 (Table 29). In contrast, among "low risk" relatives the sex distribution was skewed heavily toward females (71.7% vs. 28.3%), giving a male to female sex ratio of 0.4. The mean age of the "high risk" group was 30.7 years (±18.1), compared to 31.0 years (±14.1) for the "low risk" group; this difference was not statistically significant (p = 0.929). There was no statistical relationship between cleft severity and risk status in unaffected relatives. The distribution of "high risk" and "low risk" relatives did not differ by cleft severity (χ^2 = 0.680; p = 0.712). Additionally, the mean Mahalanobis distance of relatives from controls did not differ across severity categories (F = 0.538; p = 0.586).

The distribution of risk status in unaffected relatives differed by the type of cleft family (Table 30). The ratio of high risk to low risk relatives was highest in the small number of CP families included (1.5), followed by CL/P families (0.76) and mixed CL/P-CP families (0.56). Due to the small number of mixed (N = 6) and CP (N = 2) families, it is likely that these figures are dubious. Furthermore, the number of high risk relatives was strongly correlated with the degree of relatedness to the proband; that is, the vast majority of high risk relatives (72.5%) were first degree relatives of cleft probands. Only 9 of high risk relatives (22.5%) were second degree relatives. These proportions, however, are likely biased by the fact that many extended family members had either missing data or did not participate. A total of 29 families studied (41.4%) had no high risk relatives of any kind, while 14 (34.1%) families had just a single high risk relative (Figure 23). One family had a total of five high risk relatives, which was the highest number observed in the present sample.

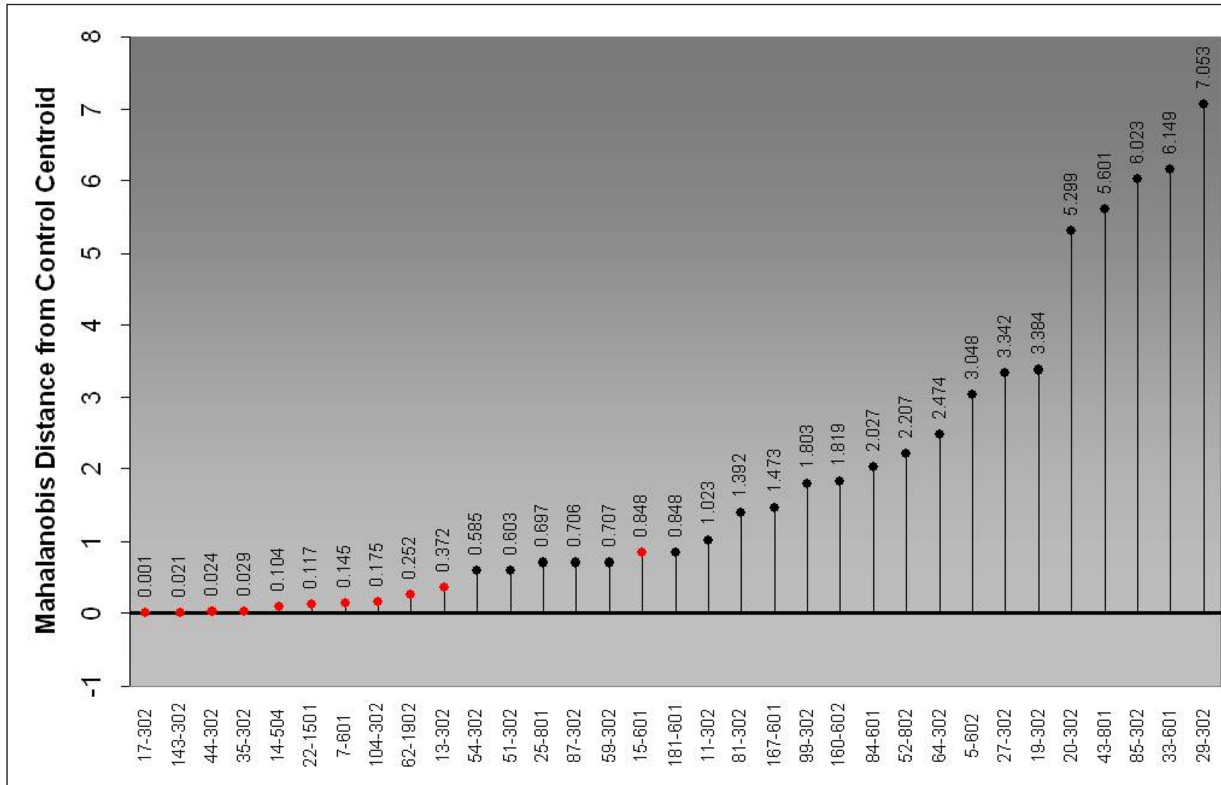

Figure 20 Mahalanobis distance of each female relative from control centroid.

Distance is measured in standard deviation units. Female control centroid indicated by zero baseline. Stippled line indicates the 1.96 distance threshold. Red circles represent unaffected relatives misclassified as controls from DFA. Black circles represent unaffected relatives correctly classified as relatives from DFA.

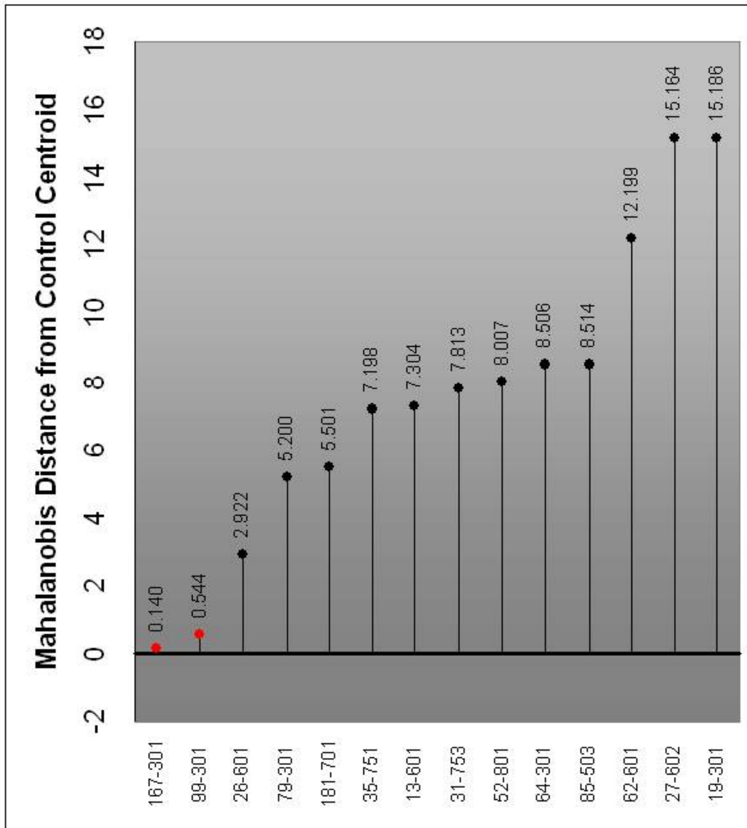

Figure 21 Mahalanobis distance of each male relative from control centroid.
Distance is measured in standard deviation units. Male control centroid indicated by zero baseline. Stippled line indicates the 1.96 distance threshold. Red circles represent unaffected relatives misclassified as controls from DFA. Black circles represent unaffected relatives correctly classified as relatives from DFA.

Figure 22 Mahalanobis distance of all relatives from control centroid.

This figure includes data from the 46 additional relatives excluded from the initial model building phase of the DFA. Distance is measured in standard deviation units. Control centroid indicated by zero baseline. Stippled line indicates the 1.96 distance threshold. Red circles represent unaffected relatives misclassified as controls from DFA. Black circles represent unaffected relatives correctly classified as relatives from DFA.

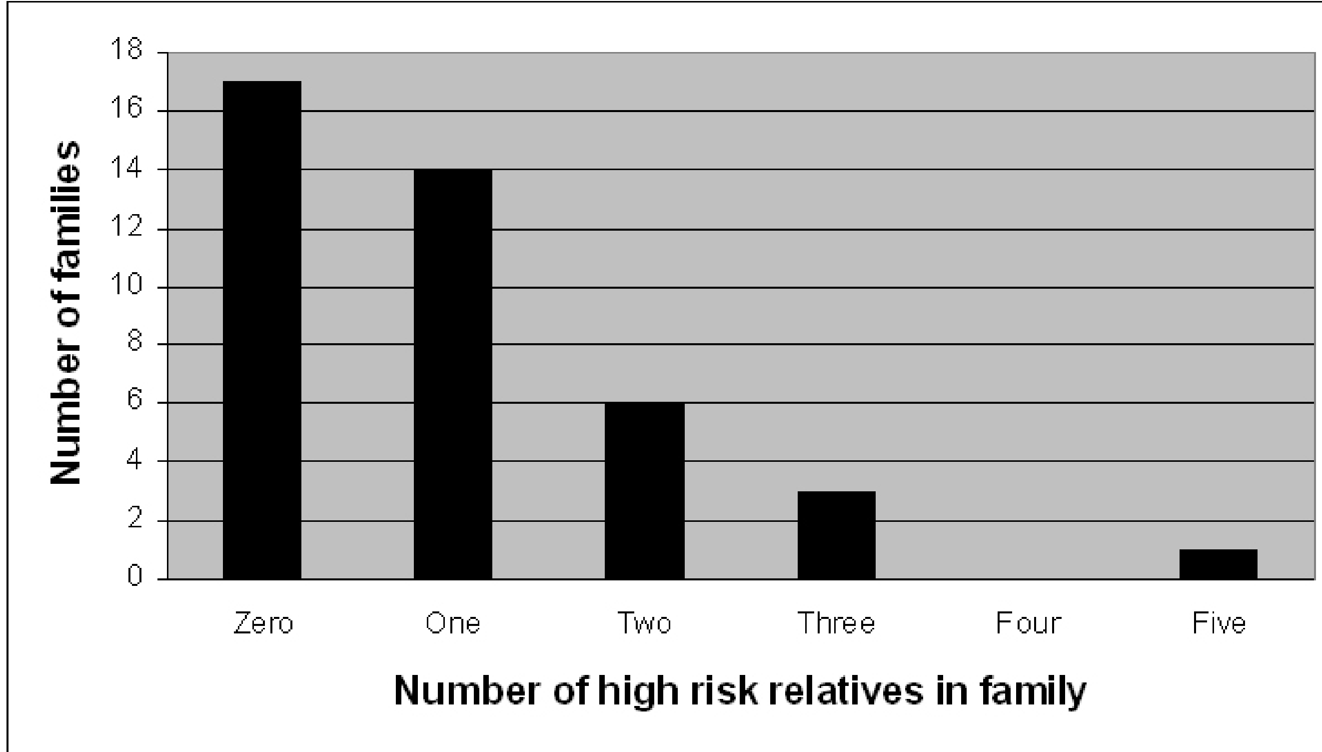

Figure 23 Number of families by number of "high risk" relatives.

Table 29 Sex ratio of risk status among unaffected relatives

	N	*Male*	*Female*	*M:F*	χ^2	*p*
High risk relatives	40	21 (52.5%)	19 (47.5%)	1.1	5.626	0.02
Low risk relatives	53	15 (28.3%)	38 (71.7%)	0.4		

Table 30 Distribution of risk status by type of cleft family

Family type	*Number of families*	*High risk relatives*	*Low risk relatives*	*Unknown risk relatives*	*High risk: Low risk*
CL/P	33	32	42	93	.76
Mixed (CL/P + CP)	6	5	9	9	.56
CP	2	3	2	5	1.50
Totals	41	40	53	107	

4.0 DISCUSSION

4.1 SUMMARY OF THE MAJOR MORPHOLOGICAL RESULTS

Using a combination of 3D photogrammetry and direct anthropometry, the present study compared aspects of craniofacial shape between a genetically loaded sample of unaffected first-degree relatives (parents and sibs) from multiplex nonsyndromic cleft families and a demographically-matched sample of unaffected controls. Through the implementation of Euclidean Distance Matrix Analysis (EDMA), a form-invariant approach to statistical shape analysis based on 3D landmark coordinate data, it was observed that both unaffected male and female relatives differed in overall craniofacial shape (but not size) compared to their respective, sex-specific control groups. Furthermore, these shape changes were localized to specific regions of the face in a partially sex-specific manner. For the female relative to control comparison, three out of a possible 120 shape variables (i.e., scaled inter-landmark distances) were observed to be significantly different ($p < 0.10$). All three linear distances involved the landmark *alare*, located on the most lateral aspect of the nasal alar cartilages, and all indicate that unaffected female relatives are characterized by a lateral displacement of the left and right nasal alar cartilage; that is, an increased soft tissue nose width. Certain morphological trends were also observed in female relatives; in the context of EDMA, such trends refer to shape variables that are not necessarily statistically different across groups, but are relatively more divergent than other variables. Such variables are located at the tail ends of the sorted EDMA shape difference distribution (e.g., see Figure 11). Based on the variables contained within the upper and lower 5% of the distribution, female relatives demonstrated non-significant trends toward increased lower face height and decreased upper face height over controls. It is tempting to suggest that with a larger sample, at least some of these differences might become significant. Separate, yet complimentary, univariate analyses were carried out on a set of 14 additional soft tissue

dimensions covering more expansive portions of the craniofacial complex. It was observed that unaffected female relatives had, in addition to the differences noted above, significantly ($p < 0.05$) broader upper faces and excess midface retrusion.

Regarding the male unaffected relative sample, EDMA results highlighted variables contributing to vertical aspects of facial morphology. Significant differences were noted for 10 variables, almost all involving landmarks located in the mid to lower portion of the central face, particularly *gnathion* – the most inferior midline point on the mandible. Specifically, nine of these 10 variables indicated that male relatives had significantly longer lower faces, while the remaining variable signified a reduction in upper face height compared to male controls. Interestingly, the single observed difference in the upper face was localized to the right side of the face, suggesting a possible pattern of asymmetry. Considering those variables showing a non-significant morphological trend away from controls, all suggested evidence for reduced upper face height and were also largely biased toward the right side of the face. For the additional 14 anthropometric variables, the results of univariate t-tests further demonstrated significant increases in the width of the upper face and cranial base.

To reiterate the major morphological differences, unaffected female relatives were found to possess significantly wider upper faces, less maxillary protrusion and a wider inter-alar distance (i.e., soft tissue nose width). Females also showed a slight trend toward increased lower and decreased upper face height. Male unaffected relatives were also observed to have significantly wider upper faces, but were further characterized by significantly increased width of the cranial base, increased height of the lower face and reduced height of the upper face.

Following the initial descriptive phase of the analysis, a subset of the above informative variables was entered into a multivariate discriminant function analysis (DFA). This subset was selected based on discriminatory potential and degree of inter-correlation with other variables. As stated in the methods section, DFA provides the tools necessary to assign individuals to a discrete number of groups based on an optimally weighted combination of predictor variables. In the present analysis, DFA was used to generate a predictive model capable to assigning unaffected relatives and controls to their respective groups. Furthermore, the discriminant weighting scheme derived from the above model was applied to a set of additional relatives excluded from the initial portion of the analysis for a variety of reasons (see Section 2.3.1.2). In this manner, all unaffected relatives with valid data were eventually assigned to a group.

For the female sample, a total of four predictor variables were entered simultaneously into the DFA (upper face width, nose width, left midface depth and right *alare* to *subalare* distance). The resultant discriminant model was statistically significant ($p = 0.01$), indicating a high degree of separation between the unaffected female relative group and the female control group. Overall, just over 70% of cases were assigned to their correct group. Upper face width had the largest impact on group discrimination, although all of the included variables approximated one another in terms of their substantive importance. For males, a total of five predictor variables were entered in the analysis (upper face width, lower face height, right alare to endocanthion distance [taken as a measure of upper face height], *sublabiale* to *gnathion* distance [chin height] and cranial base width). The discriminant model was highly significant ($p < 0.001$), indicating substantial divergence in craniofacial morphology between male unaffected relatives and male controls. This fact was reflecting in the classification statistics; an overall correct classification rate of 89% was achieved. As in the female sample, upper face width had the greatest effect on group discrimination. Interestingly, in contrast to the female sample, the remaining four variables had substantially lower loadings. This finding highlights the relative importance of upper face width in differentiating male relatives and controls.

Thus, for both females and males, a small subset of highly informative craniofacial dimensions was capable of successfully discriminating between unaffected relatives and controls. Overall classification accuracy was above average in females and excellent in males. The pattern of variable contribution to the discriminant function was similar between females and males in some respects, but different in others. In both sexes, upper face width was the most important to group discrimination, suggesting that it is the single most consistent predictor of group status.

4.2 COMPARISON OF RESULTS TO PREVIOUS STUDIES

On the whole, the results observed in the present study agree with the findings of previous reports that have compared the unaffected relatives of CL/P individuals to controls (see Table 31). This is not to say that there are no discrepancies. Indeed, the heterogeneous nature of the available evidence virtually ensures some disagreement (see Section 1.6.3.2). The finding in the

present investigation of excess upper face width in unaffected relatives has been replicated by several prior studies (Fraser and Pashayan, 1970; Nakasima and Ichinose, 1983; Blanco et al., 1992; Yoon et al., 2004), although in some cases the findings have been sex specific. Moreover, some additional studies have observed non-significant trends toward increased face width in relatives (Suzuki et al., 1999; McIntyre and Mossey, 2004). Nevertheless, other reports have found just the opposite – that upper face width is significantly *reduced* in relatives (Figalová and Šmahel, 1974; Raghavan et al., 1994; AlEmran, 1994). It could be argued that additional measures of the upper facial region, such as inter-orbital distance, might provide supplementary evidence for a general increase in upper face width. Although we did not identify any significant differences in inter-orbital distance between our unaffected relative and control groups, many other studies have reported excess interorbital dimensions in relatives using cephalometry (Kurisu et al., 1974; Figalová and Šmahel, 1974; Nakasima and Ichinose, 1983; Sato, 1989; Blanco et al., 1992; Suzuki et al., 1999; Yoon et al., 2004; Weinberg et al., 2006b). The other major craniofacial width measure, cranial base width, was found to be significantly increased in male relatives. To date, only a single additional study has considered this characteristic (Figalová and Šmahel, 1974); in contrast to the present study, they found that cranial base width was significantly reduced in their sample of unaffected parents from the Czech Republic. Consequently, due to a paucity of evidence it is not possible to put these findings within a broader context.

In the current analysis, female relatives displayed lateral alar cartilage displacement resulting in a widening of the soft tissue component of the nose. Only two studies have examined the soft tissue dimensions of the nose in unaffected relatives; Fraser and Pashayan (1970) documented a non-significant trend toward increased nose width in unaffected parents, while Figalová and Šmahel (1974) found significant increases in extended family members, in particular the unaffected grandparents of probands. In the latter study, the authors suggest that this finding is likely due to the considerable age difference between the grandparental sample and the control sample, since cartilage continues to grow with age. In the present study, relatives and controls were age-matched to within one year; therefore, it is highly improbable that age influenced any of the present results. Since the overwhelming majority of prior studies are based on cephalometry, an exactly equivalent measure of soft tissue nose width was not available. Nevertheless, many studies have documented changes in the horizontal dimensions of the nasal

Table 31 Comparison of major results from the present analysis with those of previous studies

Studies	*Upper face width*	*Cranial base width*	*Lower face height*	*Upper face height* [b]	*Nasal width* [c]	*Midface depth (convexity)* [d]
Present analysis	↑	↑♂	↑♂	↓♂	↑♀	↓♀
Dixon, 1966						↓
Fraser and Pashayan, 1970 [e]	↑				ns	↓
Coccaro et al., 1972				↓		↓
Erickson, 1974						ns
Kurisu et al., 1974				↓♂		↓♂
Figalová and Šmahel, 1974 [f]	↓♀	↓		↑	ns	ns
Shibasaki et al., 1978			↑♂	↓♂		↑♂
Nakasima and Ichinose, 1983	↑ [g]		↑	↓	↑	
Sato, 1989	ns		↑	ns	↑	↑♂
Ward et al., 1989 [h]			↑	ns		ns
Blanco et al., 1992 [i]	↑♀		ns	ns		
Raghavan et al., 1994	↓		ns	↓	↑ [g]	ns
Mossey et al., 1998a			ns	ns		ns
AlEmran et al., 1999	↓♀				↑♂	
Suzuki et al., 1999	ns		ns	ns	↑	ns
McIntyre and Mossey, 2003a	ns			↓	ns	
Perkiomaki et al., 2003			ns	↓		ns
McIntyre and Mossey, 2004 [j]	ns			ns	ns	

Table 31 (continued).

Yoon et al., 2004	↑ [g]		↓	↑♂	↑	
Chatzistavrou et al., 2004 [k]	ns		ns	↓	↓	ns
Weinberg et al., 2006b [l]	↑ [g]		↑	↓♂	↑	ns

↑ = significantly larger in relatives, ↓ = significantly smaller in relatives, ns = non-significant difference

Mills et al. (1968), Coccia et al. (1969) and Nakasima and Ichinose (1984) were not included because they did not consider any of the above variables. Ward et al. (1994) not included because they studied only a single pedigree.

[a] Only CL/P results included from above studies

[b] Defined as *n-sto* from anthropometry and typically as *n-ans* from cephalometry

[c] Defined as *al-al* from anthropometry and *nc-nc* from cephalometry

[d] In the present study measured as t-sn, but in cephalometry usually measured as an angle between soft or hard tissue landmarks

[e] Used a combination of direct anthropometry, photogrammetry and physioprints

[f] Used direct anthropometry

[g] Relative measurement (ratio)

[h] Comparison of atypical clusters to normative data

[i] Only data on unaffected parents considered

[j] Only EDMA results considered

[k] Compared noncleft twins from discordant CL/P monozygotic pairs to controls

[l] Meta-analysis of cephalometric data on unaffected parents

cavity from frontal radiographs. A total of six studies have shown significant increases in nasal cavity width in unaffected relatives (Nakasima and Ichinose, 1983; Sato, 1989; Raghavan et al., 1994; AlEmran et al., 1999; Suzuki et al., 1999; Yoon et al., 2004) while only one study reported the opposite (Chatzistavrou et al., 2004). This latter study was based on a comparison between a sample of noncleft twins from monozygotic pairs discordant for CL/P and controls, whereas in the other studies unaffected parents comprised the experimental sample. Interestingly, Johnston and Hunter (1989) observed that nasal cavity width was distributed in a bimodal manner in their sample of noncleft co-twins, with two-thirds of twins displaying reduced nasal cavity width and the other third displaying increased nasal cavity width. It is not clear if the same pattern holds true for the twin sample examined by Chatzistavrou et al. (2004), because the authors do not provide data on trait distributions. It is certainly conceivable that distinct patterns of midface development could be present in discordant CL/P twins.

Observed differences in the vertical dimensions of the face are also largely supported by earlier reports. Regarding the excess lower face height observed in the present sample of male relatives, at least four studies have corroborated this finding (Shibasaki et al., 1978; Nakasima and Ichinose, 1983; Sato, 1989; Ward et al., 1989). In three of these studies, the same pattern was noted for female relatives as well. Recall, however, that a trend toward increased lower face height was observed in the current unaffected female sample. Only one study to date has reported relatives to have a significant reduction in lower face height compared with controls (Yoon et al., 2004), while many others have found no significant difference for this dimension (e.g., Raghavan et al., 1994; Suzuki et al., 1999). A number of studies have also confirmed the present finding of reduced upper face height in unaffected relatives (Coccaro et al., 1972; Kurisu et al., 1974; Shibasaki et al., 1978; Nakasima and Ichinose, 1983; Raghavan et al., 1994; McIntyre and Mossey, 2003a; Perkiomaki et al., 2003; Chatzistavrou et al., 2004). In at least two studies (Kurisu et al., 1974; Shibasaki et al., 1978), this difference was significant only in males, which echo the current findings. Nonetheless, both Figalová and Šmahel (1974) and Yoon et al. (2004) found the opposite – unaffected relatives had significantly *increased* upper face height.

A significant reduction in midface depth (distance from *tragion* to *subnasale*) was observed in unaffected female relatives. Since a concomitant decrease in lower face depth (an indirect measure of mandibular size or projection) was not obvious, this suggests a pattern of

selective midface retrusion resulting in a flattening of the facial profile. A similar approach to the measurement of midface depth with direct anthropometry was attempted by Figalová and Šmahel (1974); although they found that unaffected relatives did possess more retrusive midfaces, this characteristic was not significantly different from controls. Fraser and Pashayan (1970) used a different approach based on facial contour mapping from photographs of the face in profile; in agreement with the present study, a significant midface reduction was noted in unaffected parents. Notwithstanding the aforementioned studies, the typical approach to characterize the facial profile or relative jaw position is to construct angles from either soft or hard tissue landmarks on a lateral radiograph (e.g., ANB angle). Using this approach, at least three studies have documented significantly reduced facial convexity (Dixon, 1966; Coccaro et al., 1972; Kurisu et al., 1974), two studies reported significantly *increased* facial convexity in unaffected male relatives exclusively (Shibasaki et al., 1978; Sato, 1989) and numerous others report no major alterations in facial profile (see Table 31).

It is also useful to consider the present results in light of the evidence from Weinberg and co-worker's (2006b) recent meta-analysis, since meta-analyses are generally thought to provide a more accurate estimate of a given experimental effect than any single study (Kline, 2004). Critically, when compared with the current study, a virtually identical pattern of increased upper face width, nasal cavity width and lower face height along with decreased upper face height was seen in unaffected parents. On the other hand, no detectable difference in facial profile convexity was noted. On the whole, then, it is reasonable to conclude that the majority of previous reports are in agreement with the main morphological findings of this study. Some minor discrepancies exist and these may be due to either methodological differences or true biological differences between study samples (see Section 4.3. for further discussion).

The results of discriminant function analysis were also in line with most previous reports in terms of the proportion of individuals classified correctly. A total of 71% of female relatives and controls were assigned to their correct group; this number improved to 89% in males. Combined, this works out to an 80% overall correct classification rate. Nakasima and Ichinose (1983) were able to correctly classify roughly 84% of their sample of Japanese unaffected relatives (CL/P families) and controls. Interestingly, as in the present study, measures of relative facial width were observed to contribute most to group discrimination. Sato (1989) reported a correct classification rate of 92.6% in their sample, which is extremely high for a non-clinical

population. Similar rates were observed by Mossey et al. (1998a), with 82.9% of male relatives and controls and 95.4% of female relatives and controls classified correctly. In a related study, MacIntyre and Mossey (2003a) reported an overall accuracy rate just under 91%. The lowest rate was reported by Suzuki et al. (1999) at 67.9%, with relative nasal cavity width contributing most to group discrimination. Interestingly, their sample (like the one used here) was comprised of relatives from multiplex families. Finally, AlEmran et al. (1999) used a related procedure (stepwise logistic regression) and were able to assign 74.4% of their male sample and 76.9% of their female sample correctly. Once again, increased nasal cavity width was identified as an important factor in the model.

Sex-specific differences in craniofacial morphology between relatives and controls were apparent. Both male and female relatives demonstrated excess upper face width compared with controls. Male relatives possessed a significantly wider cranial base and displayed significant differences in upper and lower face height, although female relatives showed a similar trend for these last two dimensions as well. In contrast to males, female relatives exhibited significant changes in soft tissue nose breadth and midface depth. Importantly, sex-based discrepancies in the specific pattern of relative-control craniofacial differences have been observed in virtually every study that has examined males and females separately, and this phenomenon has subsequently been confirmed by meta-analysis. Moreover, in the current analysis, examination of the magnitude of the relative-control differences revealed that male relatives had a more deviant facial phenotype than female relatives. This is evident both in the results from EDMA (Figure 11 vs. Figure 13) and the univariate analysis (Figure 14). Comparison of the mean biological distance (D^2) between the unaffected relatives of each sex to their respective control group clearly indicates the increased deviation present in males (Figure 18). This pattern is reflected in the classification accuracy rate as well. The degree of difference present in male relatives is a particularly interesting finding considering the fact that the current male sample is less than half the size of the female sample. In a similar manner, a more extreme facial phenotype was observed in male relatives by Kurisu et al. (1974) and Shibasaki et al. (1978). However, Mossey et al. (1998a) found the opposite pattern, in that their sample of female relatives was in fact *more* deviant. Furthermore, several others have found the magnitude of the relative-control disparities to be more or less equivalent between the sexes (AlEmran et al., 1999; Suzuki et al., 1999; McIntyre and Mossey, 2004; Yoon et al., 2004; Weinberg et al., 2006b).

Thus, most studies have failed to corroborate this finding. It is difficult make sense of these discordant findings, and the current results may reflect properties largely unique to our sample of relatives.

4.3 METHODOLOGICAL EXPLANATIONS FOR DISCREPANCIES BETWEEN STUDIES

It is likely that at least some of the apparent discrepancies between the results observed here and those of prior studies are due to differences in study design and/or methodology. As stated earlier, almost all previous attempts to characterize the craniofacial morphology of unaffected relatives from CL/P families have relied on standard cephalometry. Since the current analysis is based entirely on measurements between soft tissue surface landmarks and not bone, it should be immediately apparent that many potential variables will not be comparable. This is less of a problem, perhaps, when considering soft tissue measurements based on landmarks that relate closely to the underlying bone (e.g., *nasion* or *gnathion*). Studies directly comparing such measurements from cephalometry to direct surface anthropometry generally find moderate to high correlations, although the raw values still differ due to the intervening soft tissue (Farkas et al., 1999; Landes et al., 2002; Budai et al., 2003). However, the relationship between a variable like soft tissue nose width and hard tissue nasal cavity width is probably much more tenuous, despite the fact that they are both measures of midface width. Measures of inter-orbital distance provide yet another example; many cephalometric studies have documented excess inter-orbital width in unaffected relatives. Yet no such difference was observed in our sample and it is possible that this discrepancy could be due to the quite different methods of determining inter-orbital dimensions in cephalometry and surface-based anthropometry. More alarming still is that even two cephalometric studies examining the same sample of CL/P family members, using the same normative control data, and ostensibly measuring the same dimension can find contradictory results. Both Perkiomaki et al. (2003) and Yoon et al. (2004) examined upper face height in the same Chilean sample, but came to opposite conclusions; the former reported that upper face height was significantly reduced in unaffected relatives, while the latter found it to be significantly increased. The difference between these two studies was a very slight alteration in

the landmarks utilized to define upper face height and the type of radiograph utilized (lateral versus frontal, respectively). This example illustrates how even relatively subtle changes in methodology can affect outcome.

It has been recognized for decades that cephalometry is a suboptimal method for describing the complex three-dimensional anatomy of the craniofacial complex (Moyers and Bookstein, 1979). The surface-based techniques used in the present study (direct anthropometry and indirect 3D photogrammetry) provide a more realistic assessment of craniofacial form than traditional cephalometry, because the 3D morphology of the head is not forced into a flat two-dimensional plane. It is certainly reasonable to suspect that some discrepancies between cephalometric and surface-based studies could arise as a result of this flattening process, particularly with measurements involving landmarks that are not in the same plane because the distortion will be the greater. Only two previous studies have employed surface-based methods comparable to those used in the present analysis, although the number of variables considered was limited. Both Fraser and Pashayan (1970) and Figalová and Šmahel (1974) collected measurements using direct surface anthropometry (calipers). Nevertheless, for upper face width, cranial base width and upper face height, our results are in direct contradiction to those of Figalová and Šmahel (1974). In the present study, both upper face width and cranial base width were derived from direct anthropometry, while upper face height was derived from 3D photogrammetry. Only a small portion of the variables used in the present study were derived from direct anthropometry, as the majority were obtained through indirect 3D photogrammetry. In general, studies comparing linear measurements derived from 3D photogrammetric technology with those derived from traditional direct anthropometry demonstrate an acceptable level of congruence (Weinberg et al., 2004; 2006c). Thus, there likely are additional factors underlying these discrepancies.

As McIntyre and Mossey (2003b) and others have pointed out, the conventional cephalometric method of calculating a set of simple linear distances without adjusting for size is inadequate for a rigorous description or comparison of craniofacial shape. Most cephalometric studies simply compare raw linear distances, although some include ratios which can serve as a crude approximation of shape. In contrast, all of the 134 linear distances included in the current analysis were scaled by a global measure of craniofacial size (the geometric mean), resulting in shape variables – that is, variables that define the form of an object after size has been removed.

Thus, all 134 measures were expressed in relative terms. Only one previous study has actually examined face *shape* (as opposed to some combination of shape and size) in unaffected relatives via modern morphometric methods (McIntyre and Mossey, 2004); unfortunately, this study was still based on 2D cephalometric data. It is unclear if lack of scaling is a factor in any of the observed discrepancies between studies. Interestingly, however, one of the major findings in the meta-analysis by Weinberg et al. (2006b) was that upper face width, which was by far the single best discriminator between unaffected relatives and controls in the present study, was only significantly different between unaffected parents and controls when the variable was scaled by some other dimension, such as maximum head width.

Another important factor could be the specific population from which the experimental and control samples were derived. To date, studies comparing unaffected relative to controls have been carried out in the U.S., Canada, Costa Rica, Chile, Japan, the United Kingdom, India, Saudi Arabia and the Czech Republic. Undoubtedly, there is wide variability in craniofacial form (shape) across geographically distinct populations (Hennessy and Stringer, 2002). It is entirely plausible that population-specific patterns of relative-control differences exist as well. For example, with regard to facial profile convexity, it may be noteworthy that the only two studies that report increased convexity in relatives are based on a Japanese sample (Shibasaki et al., 1978; Sato, 1989). Such population-specific patterns could relate to underlying etiological heterogeneity; that is, in certain populations CL/P susceptibility may be due to one set of etiologic factors and in others, another set may be involved. Ultimately, these different sets of etiologic factors could have distinct manifestations in the facial phenotype of family members. In their meta-analysis, Weinberg et al. (2006b) attempted to evaluate the role of population as a moderator of among-study variability in relative-control facial differences. Although no significant relationship was noted, this may very well have been due to the small number of studies included, hence a result of insufficient power.

Finally, one of the major differences between the present study and previous reports is the nature and composition of the CL/P family sample. For one thing, the current study ascertained multiplex families exclusively. This was a deliberate decision, the goal being to bias the sample toward increased genetic informativeness and relative homogeneity. Only one previous study included exclusively multiplex relatives (Suzuki et al., 1999); the rest include a mix of familial and sporadic (simplex) cases. Importantly, some fraction of sporadic cases is

likely to be non-genetic in origin – that is, they did not receive genetic susceptibility factors from either of their unaffected parents. Therefore, including unaffected relatives from simplex families together with relatives from multiplex families has the potential to increase the etiological heterogeneity in the unaffected relative sample. If this heterogeneity has phenotypic correlates (particularly, in the face), the effect of mixing in a large portion of sporadic cases could be to obscure certain differences in craniofacial morphology when comparing unaffected relatives to controls. The distribution of different types of cleft families in the relative sample could also be an important factor. While the present study sample was comprised overwhelmingly of CL/P families, a small number of mixed and CP families were included. This was not the case in many previous studies. All of these factors should be kept in mind when comparing and contrasting results across studies.

It is worth noting that even given all of the methodological caveats outlined above, the results of this study are remarkably consistent with those of earlier reports. If nothing else, this speaks to the fact that the phenotypic consequences of increased genetic liability to orofacial clefting can be reliably detected in the unaffected relatives of affected individuals through a quantitative assessment of craniofacial form. This overall concordance further argues that the observed morphological changes between unaffected relatives and controls are not simply artifacts of biased sampling or some other methodological flaw. These results give credence to the idea that at some point it may be possible to utilize similar phenotypic parameters in the clinical assessment of genetic risk within cleft families.

4.4 DEVELOPMENTAL BASIS FOR CRANIOFACIAL PHENOTYPE IN RELATIVES

The developmental basis for the differences in craniofacial morphology observed in unaffected relatives is unclear. This is undoubtedly due to the fact that the morphogenetic factors underlying variation in human craniofacial shape are complex and still poorly understood. Certainly, many of the features present in unaffected relatives are biologically plausible as risk markers for orofacial clefting. It is not terribly difficult to imaging how excess width of the face during embryogenesis, for example, could lead to a scenario whereby adjacent facial

prominences would be less likely to contact one another. However, the root developmental mechanisms that underlie variation in facial width are not clear. One possible factor could be bilateral expansion of the forebrain, which, due to its position relative to the developing face, plays a central role in establishing the basic plan of the anterior head (Wang and Diewert, 1992; Diewert et al., 1993; Kjaer, 1995; Maracucio et al., 2005). In this scenario, an increase in the width of the forebrain during early ontogeny could result in excess horizontal displacement of morphogenetic components that give rise to the upper face. This would include derivatives of the maxillary prominences like the malar eminences as well as the developing nasal placodes, the latter essential for establishing the continuity of the upper lip. Interestingly, Hu and Helms (1999) have shown in mice that excess lateral expansion of the frontonasal prominence (which houses the forebrain) due to exogenous application of *Shh* often results in clefting. Nevertheless, many reports describing embryonic facial morphology in cleft susceptible mouse strains actually find evidence of *reduced* facial and frontonasal width (Trasler, 1968; Millicovski et al., 1982; Jacobson and Trasler, 1992; Wang and Diewert, 1992). To make matters even more confusing, studies of face shape in *adults* from some of these same mouse strains confirm the presence of excess face width, similar to that seen in studies of unaffected relatives in CL/P families (Hallgrímsson et al., 2004). In addition, structural neuroimaging studies on the brains of human adults with CL/P have shown a relative increase in the size of the forebrain (Nopoulos et al., 2000; 2002). Consequently, the presence of excess lateral growth of the embryonic forebrain may also explain the increased nasal cavity and inter-orbital distances often noted in unaffected relatives. Of course, it must be remembered that mouse brains and human brains are quite distinct, particularly in the post-embryonic period. The very assumption that morphological patterns present during embryogenesis will extend into latter periods of development may be untenable. Unfortunately, demonstrating the ontological continuity of complex morphological traits is difficult for obvious reasons. A study by Trasler and Machado (1979), however, was able to show greater discrimination between susceptible and non-susceptible adult mice compared with newborn mice based on a variety of facial measurements. This suggests the intriguing possibility that at least some of the craniofacial manifestations of cleft liability may have a more pronounced expression later in development.

Documented changes in the growth, shape and/or position of other facial regions in cleft susceptible mice may offer some additional clues. Wang and Diewert (1992) and others, for

example, have reported reduced outgrowth of the maxillary prominences in A/WySn mice. It is hypothesized that this reduction leads to a scenario whereby the presumptive maxillary and nasal components will be more divergent from one another during the critical period of primary palate formation, ultimately decreasing the likelihood of contact and fusion (Diewert and Lozanoff, 2002). Interestingly, Trasler and Machado (1979) reported a decrease in the anterior growth of the maxilla in both *newborn* and *adult* mice susceptible to CL, suggesting that midface reduction may persist as a risk marker. These results are consistent with the hypothesis that intrinsic mesenchymal deficiency is a primary factor in the etiopathogenesis of CL/P. It is tempting to speculate that an analogous reduction in the maxillary prominences takes place in humans susceptible to CL/P; this could help explain some of the observed phenotypic patterns in unaffected relatives such as reduced midface advancement and/or reduced upper face height. Initial outgrowth of the facial prominences is controlled by a cascade of molecules with overlapping expression patterns (Cox, 2004; Jiang et al., 2006). These include signaling proteins in the *Bmp*, *Msx*, *Hedgehog* and *Fgf* families. Variation in the expression patterns of any or all of these morphogenes could be involved in modulating the facial features outlined above.

Regarding midface width, the more medial orientation and position of the medial nasal prominences often reported in cleft susceptible mice (Trasler, 1968; Juriloff and Trasler, 1976; Millicovski et al., 1982; Wang and Diewert, 1992) would seem at the outset to be difficult to reconcile with the common finding of increased nasal cavity width in adult unaffected relatives. However, nasal cavity width may relate more to the relative position of the maxillary and lateral nasal prominences than the position of medial nasal prominences, as the medial nasal prominences contribute mostly to midline structures (e.g., nasal tip, nasal septum, etc.). Indeed, increased horizontal growth of the palate (a derivative of the maxillary prominence) has been implicated as a risk factor for clefting in animal models (Smiley et al., 1971; Siegel and Mooney, 1986). It is highly probable that the increased soft tissue nose width observed in present sample of female relatives relates in some way to lateral nasal prominence morphogenesis, since it is from this tissue that the nasal alar cartilage is derived. This is supported by a handful of studies that have found differences in lateral nasal prominence growth in cleft susceptible embryonic mice (Sulik et al., 1979; Millicovski et al., 1982; Trasler and Leong, 1982).

The increased lower face height observed in the unaffected relative sample may be more difficult to explain from a developmental point of view. Ward et al. (2002) suggested that lower

facial height may be a byproduct of changes in mandibular rotation. Although we were not able to measure this characteristic in our sample, a handful of previous studies have reported that unaffected relatives tend to have a more open mandibular rotation compared with the general population (Shibasaki et al., 1978; Nakasima and Ichinose, 1983; Laatikainen et al. 1996). The developmental basis for changes in mandibular orientation is unclear, although to some degree it could be reflection of an increased gonial angle. Alternatively, this morphological trend may be related to concomitant changes occurring within the cranial base. The cranial base, much like the brain, is considered a prime mover in craniofacial development and is thought to play a major role in guiding and establishing the growth patterns of adjacent anatomical regions, in particular the intimately related viscerocranium (Enlow and Hunter, 1968; Chinappi; 1971; Anderson and Popovich, 1983; Kerr and Adams, 1988; Kasai et al., 1995; Singh et al., 1997; Hilloowala et al., 1998; Siebert and Swindler, 2002). The cranial base has these properties due to its cartilaginous origin; cartilage, unlike bone or soft tissue, has a largely intrinsic growth potential (Baume, 1961; van Limborgh, 1970; Koski, 1975). The present study was limited to a single measure of the cranial base (maximum width) because only soft tissue assessments were possible, and this dimension was found to be larger in unaffected male relatives. It is worth noting, though, that similar changes have been described in cleft affected individuals (Mølsted et al., 1995; Smith et al. 2002). Previous cephalometric studies have characterized cranial base morphology in unaffected relatives more thoroughly (Coccaro et al., 1972; Kurisu et al., 1974; Ward et al., 1989; Mossey et al., 1998a). In general, relatives seem to have longer cranial bases but decreased cranial base angles. Due to their close anatomical relationship, an increase in cranial base flexure could alter the rotation pattern and position of the mandible relative to the rest of the face. Such a change could result in an inferior displacement of anterior mandible and, hence, an increase in lower face height.

Increased cranial base flexure also tends to be associated with a reduction in midface depth, which was observed in female unaffected relatives. The developmental and evolutionary relationship between facial protrusion and cranial base flexure is well established (Mooney et al., 2002). However, if previous reports are correct that unaffected relatives are characterized by increased cranial base length, this could offset the effects of increased kyphosis on midface projection. This is because during early development, the nasal septum (the most anterior portion of the cranial base) transmits anterior displacement forces emanating from the growing

cartilaginous cranial base to the midface (Scott, 1953; Ford, 1956; Burdi, 1965; 1969; Siegel, 1976; Dixon, 1997). These forces effectively draw the maxilla forward and serve as the driving force behind midface advancement. Unfortunately, these discrepancies are not able to be reconciled at this time.

4.5 AN ASSESSMENT OF RISK STATUS IN UNAFFECTED RELATIVES

Clearly, some portion of unaffected relatives in our sample of CL/P multiplex families possess excess genetic risk factors for the disease and are therefore at elevated risk for passing them on to their offspring. Given the complex genetics underlying nonsyndromic oral clefts, however, it is not possible (as it might with a simple Mendelian disorder) to assign genetic risk status to family members based solely on their position within a pedigree. Nonsyndromic clefting is probably still best understood as a multifactorial disease, although not in the strict mathematical sense of many genes each with a small additive effect. As Fraser (1989a; 1989b) and others have pointed out, the multifactorial model allows for loci to make differential contributions to disease susceptibility – some will have larger effects, others smaller. Furthermore, it does not preclude the possibility that genetic susceptibility within families will be unequally distributed across members. Thus, genetic risk will vary within families, but in patterns that are difficult to predict. There are at least two good reasons for wanting to be able to predict an individual's genetic risk for a disease like CL/P. From a practical standpoint, the ability to forecast disease recurrence is based on an assessment of risk (Young, 1999); traditionally, such an assessment is based on some combination of empirical population parameters (e.g., incidence) coupled with specific information from the family in question (e.g., how many members affected, disease severity, etc.). Still, however, the estimates are very crude and can be terribly wrong. The other reason relates to the search for causes. Gene identification approaches benefit from increased power when each individual's affection status is an accurate reflection of their genetic risk. As it stands now, a large proportion of genetically informative individuals are misclassified as unaffected, which inevitably disrupts the statistical relationship between phenotype and genotype.

One way to improve our ability to forecast genetic risk is to develop reliable phenotypic risk markers. Such phenotypic markers can be thought of as proxies for underlying genetic risk.

A well established method for identifying risk markers is to compare the relatives of affected individuals on some measurable aspect of their phenotype to a control group with no history of the disease. With CL/P this approach has generally focused on comparisons of facial morphology; however, other phenotypes have also been explored (see Weinberg et al., 2006a). Therefore, the initial aim of this study was to identify a suite of quantitative facial features capable of distinguishing the unaffected relatives of individuals with overt nonsyndromic clefts from demographically-matched controls. An additional goal, however, was to use the information derived from this comparison to develop a predictive model for assigning all unaffected family members an index of risk. Based on this index, risk or liability classes could be constructed with each unaffected relative assigned to a class.

The designation of unaffected relatives to risk classes was based on the results of the discriminant function analysis. Recall that separate discriminant function models were constructed for male and female samples. However, there were also a number of additional unaffected relatives (N = 46) not included in the construction of the initial discriminant models. The initial exclusion of these individuals was based on a variety of factors (see Section 2.3.1.2), the most important being that they were related to another subject already in the sample. Importantly, the presence of non-independent cases (e.g., individuals from the same family) violates a basic assumption of most parametric statistics. Not including these related individuals at the outset helped ensure that the discriminant models would be unbiased. One of the main advantages of DFA is that it provides the tools to classify new unknown cases, using parameters estimated from the original model. Accordingly, the parameters (i.e., unstandardized discriminant function coefficients) from the female and male discriminant models (Table 25 and Table 28) were used to predict the group membership each of the additional relatives initially excluded.

For the present study, only two risk classes were considered: high and low. Each unaffected relative was assigned to either the "high risk" class or the "low risk" class based on two criteria. These were, 1) whether they were classified as a relative or control from the DFA and 2) the degree of phenotypic divergence from the control sample. For the former criterion, to be considered "high risk", unaffected relatives had to be classified correctly as relatives and not misclassified as controls. For the latter criterion, a multivariate measure of biological distance (Mahalanobis D^2) was used. This useful statistic provides a quantitative estimate of how

different each unaffected relative is from the control group mean (or centroid), based on a combination of informative facial features. Because distance is measured in standardized deviation units, a threshold for phenotypic deviation from the control centroid can be established on statistical grounds. Risk status is then determined by an individual's location relative to this threshold. For the present analysis, the threshold was set at 1.96 (basically two standard deviation units), although the cutoff line is flexible and can be made more or less stringent. Thus, unaffected relatives with facial phenotypes placing them greater than two standard deviations from the norm were candidates for "high risk" status. This approach allows for the identification of a subset of unaffected relatives who, by virtue of their facial morphology, are most different from controls. On the flipside, it also allows for the identification of those relatives that are at reduced risk (or possess a different set of risk factors) because they do not deviate far from the control group – their faces are essentially indistinguishable from controls. An important feature of this method of risk allocation is that each relative is able to be assigned to a class on a case by case basis.

It was only after meeting *both* of the above criteria that a final decision regarding risk status of relatives was made. Ultimately, out of 93 unaffected relatives subjected to the aforementioned classification scheme, a total of 40 relatives (43%) met the criteria for assignment to the "high risk" category; that is, they were both classified from DFA as a relative (not a control) and had a D^2 value greater than 1.96 from the control centroid. In contrast, 53 relatives (67%) met the criteria for the "low risk" designation. All unaffected relatives along with their risk classification statistics are depicted in Figure 22.

Comparing "high risk" to "low risk" relatives revealed some interesting differences. Not surprisingly, the pattern of craniofacial phenotype differences was identical to those observed between unaffected relatives and controls. Moreover, it was observed that risk status was not distributed across the sexes evenly. The ratio of males to females in the "high risk" group was almost equal (1.1:1). In the "low risk" group, conversely, there was an overwhelming majority of females (71.7% vs. 28.3%), resulting in a male to female ratio of 0.4:1. This is perhaps also not surprising, given that relative-control facial differences tended to be more extreme in the male sample. Furthermore, from epidemiological studies it is well-established that more males are affected with CL/P, at about a 2:1 ratio of males to females. Thus, a potential relationship between cleft liability (as determined from facial phenotype) and sex ratio seems to exist. The

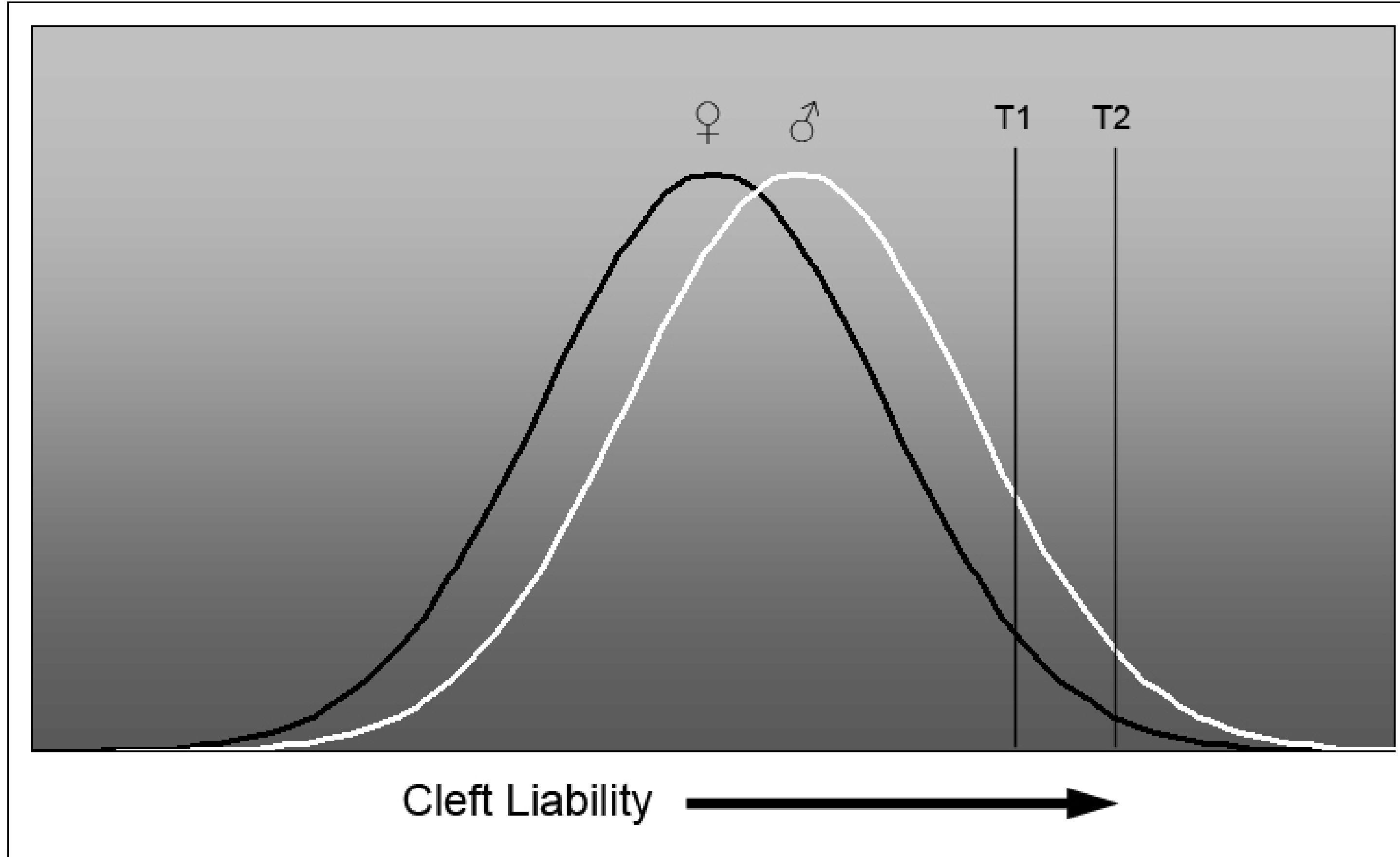

Figure 24 Distribution of cleft liability with separate liability curves for females (black) and males (white).
The hypothesized first threshold (T1) represents the point beyond which an individual manifests a subclinical cleft phenotype. The second threshold (T2) represents the point beyond which an individual is clinically affected. In this scheme, “high risk” relatives tend to be located between T1 and T2. Figure based on Fraser (1998).

proportion of males drops as one moves down the liability continuum from those affected with CL/P (2:1) to "high risk" unaffected relatives (1.1:1) to "low risk" unaffected relatives (0.4:1).

According to Fraser (1998) this pattern is an extended prediction of the multifactorial threshold model of liability, assuming sex-specific distributions (see Figure 24). For a continuous liability distribution, assume the existence of two distinct yet overlapping liability curves; a male curve and a female curve. The liability curve for males will be shifted slightly to the right, signifying the increased genetic susceptibility present in males compared to females. On this liability distribution there is a threshold beyond which an individual develops a cleft; this is the *disease threshold*. Now, imagine a second threshold only slightly lower (to the left) on the liability scale; this threshold represents a *subclinical threshold* and it can be conceptualized as the line dividing "high risk" from "low risk" relatives. That is, beyond the subclinical threshold a relative is considered high risk. Moving left from the cleft threshold toward the subclinical threshold, note that the ratio of the area under the curve for the male distribution shifts relative to female distribution. This shift results in an alteration in the male to female ratio, such that as liability decreases, the male to female ratio also decreases. This is the exact pattern observed in the present sample.

It is important to keep in mind that the designations "high risk" and "low risk" are based entirely on an assessment of phenotypic features. Because the relationship between genotype and facial phenotype is extremely complex, it is uncertain exactly how these categories relate to genetic susceptibility for CL/P. Indeed, the phenotypic differences observed in "high risk" unaffected relatives *may* indeed be indicative of a latent genetic risk for CL/P. The fact that the current relative sample was derived entirely from multiplex families weights the evidence in favor of this view. However, this is a separate hypothesis that was not directly tested in the present analysis. It is only by incorporating this phenotypic information into formal genetic analyses that we can even begin to evaluate such claims. That being said, it is interesting to consider some of the possible etiological implications of these findings.

Etiological heterogeneity is the bane of every researcher who studies the genetics of nonsyndromic clefting. This is because its presence reduces the statistical power to detect putative loci. It also poses a problem for genetic councilors and possibly even for craniofacial practitioners who treat such conditions. This is because clefts due to one set of etiological factors may have a different recurrence risk pattern or respond differently to treatment than clefts

due to a different set of factors. It is imperative, therefore, that patterns of heterogeneity be sorted out among CL/P cases and families. Given this need, it would be of great interest if in fact "high risk" relatives represented a distinct etiological group within CL/P. It was stated early in the introduction that clefting is a common end point resulting from a variety of alterations in the developmental program of the face. In a very general sense, experimental evidence from animal models suggests that clefts of the primary palate arise from either a failure to *make contact* or *maintain contact* between adjacent embryonic facial prominences. In the former scenario, the facial prominences never meet one another, suggesting that either something in their growth or configuration is altered (Reed, 1933; Trasler, 1968; Millicovsky et al., 1982; Trasler and Leong, 1982; Trasler and Ohannessian, 1983; Wang et al., 1995). In the latter scenario, the facial prominences do meet, but there is a localized breakdown at the contact site, suggesting perhaps that an epithelial defect is present (Gaare and Langman, 1977b; Millicovsky and Johnston, 1981; Millicovsky et al., 1982; Forbes et al., 1989). Clearly, these scenarios appear to signify two rather distinct etiopathogenic processes leading to clefting of the primary palate. This does not obviate the possibility there may be considerable heterogeneity within these two main groups as well. It should be fairly evident, nevertheless, that the former scenario points to face shape. This then begs the question of whether the "high risk" subset of relatives represents a distinct etiological risk group based on altered face shape. Unfortunately, due to incomplete data, this question cannot be answered at the present time. Without a doubt, though, the implications of such a finding would be far reaching, particularly if this etiological subgroup can be shown to share a common set of genetic factors.

The assignment of unaffected relatives to risk classes was based on the ability of a linear combination of variables to discriminate between subjects who seem to share a common facial phenotype with controls (low risk) and those who are more deviant (high risk). Admittedly, the distinction between these two "classes" is arbitrary since it is based largely on the dichotomization of a continuously distributed variable (D^2). An alternative method was used by Ward and co-workers (1989) to accomplish a similar goal. These authors used hierarchical cluster analysis to identify naturally occurring subsets of unaffected relatives based on a limited set of cephalometric variables (the results of this study are reviewed in Section 1.6.3.2). This is an extremely well designed original study and offers a useful and valid approach to the problem. The reason a similar approach could not be used here relates to a number of methodological

issues unique to the present study. For one thing, because it is an approach applied to the entire sample, hierarchical cluster analysis is highly sensitive to any form of sample stratification. That means that if one is interested in identifying clusters that will distinguish "high risk" from "low risk" relatives, the sample must be invariant to all other factors that could introduce structure into the data. Examples could include age, sex, ethnicity, body size, etc. Fortunately for Ward et al., they based their analysis on cephalometric variables and thus were able to rely on a large body of pre-existing normative data to generate invariant Z scores. Unfortunately, similar soft tissue normative data sets that cover the span of ages present in the current study do not exist. Furthermore, because EDMA produces a set of linear distances between all possible landmark pairs, many of the resulting dimensions are non-standard and, therefore, are not included in any current anthropometric datasets. Thus, generating invariant Z scores was not possible. A further problem relates to the sample size to variable ratio, which should be very high for hierarchical cluster analysis. Our sample was simply too small to implement this approach and obtain a valid cluster solution.

4.6 STRENGTHS AND LIMITATIONS OF THE CURRENT STUDY

The present study has a number of strengths. In particular, the use of 3D surface imaging is a major advance over other imaging methods used in the past, most of all because it allows for 3D landmark coordinates to be obtained. Importantly, this approach results in data that more accurately captures the complex 3D nature of the craniofacial complex. Furthermore, such landmarks allow for the implementation of a variety of rigorous statistical shape analyses, capable of disentangling size from shape. This includes novel whole-surface-based approaches that are currently being developed (Hammond et al., 2004). Additionally, this is an imaging technique that is fast and non-invasive, with no exposure of subjects to radiation or lasers. This makes it an ideal technology for children as well as control subjects, where the cost-benefit ratio of radiation exposure is typically negative since they receive no direct benefit from research participation. The inclusion of only multiplex families represents another major advantage, since families with sporadic cases tend to increase sample heterogeneity. Excessive heterogeneity can have the negative effect of obscuring group differences during statistical analysis, particularly

when these differences are subtle. Yet another advantage of having multiplex families is that incorporation of phenotypic data into a variety of genetic analyses (a future goal) will be facilitated greatly. Finally, the present study utilized statistical methods that allow for a rigorous assessment of face shape (EDMA) and the development of a predictive model capable of assigning risk to relatives on a case by case basis (DFA and Mahalanobis D^2).

That being said, the limitations of this study are many. First and foremost is the issue of sample size. The small size of the sample placed major limitations on the type of statistical methods that could be utilized. Moreover, this factor likely resulted in a significant loss of power to detect more subtle craniofacial differences, some of which may have had important implications. There were a number of factors that impacted sample size: the relative paucity of multiplex nonsyndromic cleft families, the inability to recruit extended family members, the inability to obtain landmark data on subjects with facial hair and technical issues related to our imaging system (e.g., unusable data, poor data quality, technical glitches). This last factor, in particular, resulted in a loss of data for a good portion of participating family members, effectively cutting our potential sample size in half. The fact that in so many families only one parent could be included made it virtually impossible to distinguish between likely modes of inheritance. Thus, patterns of aggregation and transmission of the observed "high risk" phenotype within families could not be established. Ward et al. (1989) was able to demonstrate based on the distribution of liability within parental pairs that some families seemed to fit a multifactorial model while other did not. Only with a larger, more complete sample would such distinctions be possible.

Related to the sample size issue was our inability to independently validate the discriminant function models. Traditionally, this is accomplished by splitting the total sample into an initial model building group and a holdout validation group. This was clearly not possible given our sample size restrictions. One way around this problem is to use an empirical validation technique; we used the Jackknife re-sampling method offered in SPSS. While this is better than no validation at all, it suboptimal compared with the split sample technique. Another potential limitation with the statistical methodology was the fact that the choice of traits to enter into the DFA was based on prior statistical testing in the same samples. This in no way violates the integrity of the discriminant models, *per se*, but can affect the generalizability of the results.

Regrettably, independent sample validation is also the best one way to test whether or not discriminant model parameters are over-fitted to the data.

Even though the use of 3D surface imaging technology is a major advance for studies of this kind, the fact that we are the first to use such technology inherently limits the parallels that can be drawn with the findings of previous studies. Our particular imaging system (FaceCam 250) also has some design features that limit the kind of data that can be collected. For instance, the limited field of view coupled with the fact the system uses structured light means that full ear-to-ear shots of the face cannot be obtained in a single 3D capture. This results in an inability to obtain certain central face and ear landmarks (e.g., *tragion*) from the same image, without performing a post-capture image registration. Unfortunately, this procedure is capable of introducing considerable noise into the data and is largely unvalidated. Since these anatomical regions must be captured separately, the 3D data from each capture will have a different coordinate system. This fact necessitates analyzing the data from each capture separately. Of course, another obvious limitation is that hard tissue measurements were not possible, which means that a great deal of potentially relevant anatomy was left unexplored. Unfortunately, multi-modal imaging technologies are still not in general use, so obtaining hard and soft tissue data on the same subject is presently difficult.

4.7 FUTURE DIRECTIONS

The validation and replication of these results in an independent sample of unaffected relatives from CL/P multiplex families is a first and necessary step. In addition, extension of these methods to simplex families has exiting potential. Simplex families are typically thought to be too etiologically heterogeneous to be useful for genetic analysis. Presumably, CL/P has a genetic basis in some unknown fraction of simplex families. If genetically susceptible yet unaffected parents or sibs can be identified in a subset of simplex families through an assessment of craniofacial shape, then these families could be included in genetic analyses along with multiplex families. Since there are so many simplex families relative to multiplex families, this would greatly increase the number of families available for analysis.

One of the major objectives for the future will be to explore ways of incorporating these morphometric findings into formal genetic analyses, such as association and linkage approaches. One uncomplicated way to do this would be to renegotiate the affection status of noncleft family members based on their phenotypic risk classification. In this scheme, the disease status of "high risk" relatives is simply changed from unaffected to affected. If the "high risk" category is truly capturing some relevant aspect of genetic susceptibility then this change should result in an increase in the statistical power to detect genetic effects. More sophisticated approaches are also possible. Linkage analysis is specifically designed to handle different liability classes, and these are often used when the trait of interest has a strong sex- or age-specific incidence pattern (Terwilliger and Ott, 1994; Ott, 1999). This is accomplished by altering the linkage parameters (e.g., penetrance) for each individual in the analysis based an index of their liability. This index can take the form of a dichotomy (male / female), ordinal categories (low risk / high risk / affected) or can be fully continuous (Mahalanobis D^2). Importantly, empirical tests have shown that this approach results in a relative increase in power (Shete et al., 2002). These and other analytical options are currently being explored.

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