REVISITING THE RECURRENCE RISK OF NONSYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE

by

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PURPOSE: Cleft lip with or without cleft palate (CLP) is a common birth defect, with phenotypes ranging from overt clefts to minimal microforms. Occult defects of the superior orbicularis oris (OO) muscle appear to be a part of this phenotypic spectrum. Analysis of the OO phenotype as a clinical tool is hypothesized to improve recurrence risk estimates in families.

METHODS: Upper lip ultrasound images were collected as a component of the Oral-Facial Cleft (OFC) study. Breaks in the continuity of the OO muscle visualized on ultrasound were scored as OO defects. Occurrences of CLP were compared between families with ≥ 1 family member with an OO defect and families without OO defects. Recurrence risks of CLP and of OO muscle defects among siblings and first degree relatives (FDRs) of probands with CLP were calculated using empiric proportions. Similar methods were used to calculate the recurrence risks of CLP and of OO defects among siblings and FDRs of probands with isolated OO defects.

RESULTS: The occurrences of CLP in families with and without a history of OO defects are 0.1863 and 0.1165, respectively ($p < 0.01$, OR = 1.735). The sibling recurrence risk of CLP in this cohort is 9.1%; the FDR risk is 15.7%, which are both significantly different from published CLP recurrence risk data. The likelihoods of one or more siblings or FDRs of a proband with CLP to have an OO defect are 14.7% and 11.4%, respectively. The sibling recurrence of isolated OO muscle defects in this cohort is 17.2%; the FDR recurrence is 16.4%. The chances for one or more siblings or FDRs of a proband with an OO defect to have a CLP are 3.3% and 7.3%,

respectively, which are similar to the published recurrence risk estimates of nonsyndromic (NS) CLP.

CONCLUSIONS: This study supports OO muscle defects as being part of the CLP spectrum and suggests an improvement in the accuracy of recurrence risk estimates of CLP. Carefully defining the CLP phenotype has considerable public health relevance, as it is a critical component to the enhancement of genetic studies investigating the etiology of CLP.

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1.0 INTRODUCTION

This research was conducted through the Center for Craniofacial and Dental Genetics at the University of Pittsburgh. The primary goal of the center is to identify genes that contribute to complex human phenotypes, primarily those involved in craniofacial and dental disorders. In order to reach this goal, the team collects phenotypic and genetic data worldwide, using statistical and molecular methods for gene mapping and identification. Investigations into phenotype descriptions as well as behavioral and epidemiological factors contributing to these disorders are also taking place.

It has long been appreciated that there is significant familiality of nonsyndromic cleft lip with or without cleft palate (CLP); although, for the most part, inheritance patterns are not clearly Mendelian. Fogh-Andersen's 1942 doctoral thesis was the first population-based study to suggest that there was a significant amount of heritability involved in the etiology of CLP. He proposed that CLP was inherited in a "conditional dominant" fashion (dominant with reduced penetrance). He observed a higher incidence of CLP in males than females and found that CLP and isolated cleft palate (CP) seemed to act as separate entities with regard to their inheritance (Fogh-Andersen 1942).

Since that time, the mode of inheritance of CLP has been a much debated topic. For over thirty years, CLP was considered to follow a multifactorial threshold (MFT) model of inheritance, where the accumulation of a number of small genetic and environmental effects is

tolerated by a developing fetus until a threshold is reached, beyond which is a risk for CLP (Marazita 2002). Theoretically, this model seemed to explain the complex inheritance pattern of CLP and early studies were published in support of the MFT model (Bear 1976; Woolf and others 1964). However, once investigators began to perform critical statistical tests on the predictions and goodness-of-fit of the MFT model, it became clear that the MFT model was frequently rejected in favor of a mixed model (major locus plus multifactorial background) (Chung and others 1986; Marazita and others 1984) or a major locus alone, with variable penetrance (Hecht and others 1991; Marazita and others 1992; Nemana and others 1992). Since this clarification has come about, investigators have focused on the quest to identify major genes implicated in the CLP phenotype (Marazita 2002).

The identification of genes that predispose to CLP will assist in the recognition of individuals at risk, enhance understanding of the development of orofacial clefting and facilitate the study of gene-environment interactions, with the hope of leading toward prevention strategies for such birth defects (Yazdy and others 2007). In addition, there have been reports that individuals born with clefts have shorter lifespans, increased risk of hospitalizations for psychiatric illnesses as adults, abnormal brain development and increased risk for certain types of cancers. Clearly, there is plenty of opportunity to improve the counseling for families with an increased risk for clefts as well as to gain insight into other areas of research including psychiatric genetics and cancer (Vieira 2008).

The first step in any gene identification or gene mapping process is to know what is to be mapped; in other words, to define the precise phenotype (Haines and Pericak-Vance 1998). The phenotypic range of visible CLP is very broad, ranging from minimal scars on the upper lip to overt clefts of the lip and palate (Eppley and others 2005). There is evidence to suggest that this

spectrum should be expanded to include occult defects or discontinuities of the superior orbicularis oris (OO) muscle. This suggestion is supported by the significant increase in frequency of OO muscle defects in unaffected relatives of CLP probands when compared to controls with no family history of clefting (Martin and others 2000; Neiswanger and others 2007). CLP recurrence risk estimates that consider the OO phenotype have not yet been investigated. If the phenotype of CLP is redefined to include OO muscle defects, a clearer segregation of the clefting phenotype may be observed within affected families, allowing genetic studies to further delineate the genetic factors implicated in the development of cleft lip with or without cleft palate.

The current study examines the recurrence risks of CLP and of OO muscle defects, with careful consideration of the OO muscle status of unaffected relatives. The following is a literature review to provide a rationale for this research and includes information about the epidemiology and development of cleft lip with or without cleft palate, genes that may be implicated in the clefting process, phenotypes within the orofacial clefting spectrum and the clinical importance of increasing our knowledge toward expanding this phenotypic spectrum.

1.1 THE MORBIDITY OF CLEFT LIP AND PALATE

1.1.1 Epidemiology

The broad diagnosis of "cleft lip and palate" can be divided into two distinct categories: cleft lip with or without cleft palate and cleft palate only. These two groups are thought to be etiologically distinct, as suggested by analyses of familial segregation of these traits and their

embryological origins (Carinci and others 2007; Ferguson 1988; Fraser 1955). Further, CLP can be classified into cases that are associated with known clinical syndromes and those that are isolated or nonsyndromic (NS). The majority (70%) of cases of CLP are nonsyndromic (Jones 1988). Of those, males are more frequently affected with CLP than females, unilateral defects are more common than bilateral defects and of unilateral defects, CLP on the left side is more common than on the right. Although there are no clear explanations for these preferences, a MFT model with an underlying continuous liability for CLP with two thresholds that vary, depending on sex, has been proposed as a reasonable framework to discuss the sex differences observed in NS CLP (Neiswanger and others 2007). In addition, certain genetic variants, discussed later in this document, may be involved in sex-dependent susceptibility to clefting (Blanco and others 2001).

Syndromes that include CLP as part of their phenotype include over 300 malformation syndromes (Trisomy 13, holoprosencephaly), Mendelian disorders (Van der Woude syndrome, Waardenburg syndrome) and teratogens (phenytoin, retinoic acid) (Jugessur and Murray 2005). Because of the clustering of NS CLP in some families and the increased risk of recurrence for siblings of affected individuals, nonsyndromic forms of clefting must involve some genetic contribution. Monozygotic twins show a 40 to 60 percent concordance rate for NS CLP (Murray 2002), suggesting that although genetics do influence the development of orofacial clefts, genetics does not appear to be the only etiological factor.

Various environmental factors have been known to play a role in the development of CLP. Maternal smoking during pregnancy provides a moderate increase in risk of CLP (Little and others 2004). Maternal folic acid deficiency during pregnancy has also been linked to an increased risk of clefts (Munger 2002), leading to the suggestion of a beneficial effect of

increased folic acid intake to reduce risk (Botto and others 2004). Epidemiologic studies support a role for environmental factors in clefting, especially in regions of low socioeconomic status (SES) (Cembrano and others 1995; Lasa and Manalo 1989). Incidences of CLP are lower in regions of high SES, and when SES does not change but geographic location does, no change in frequency of CLP is noted (Christensen and others 1995). Nutritional or toxic environmental exposures of some kind may contribute to as much as one-third of cleft cases, and etiologies may be most identifiable in indigent populations (Murray 2002).

NS cleft lip with or without cleft palate is a significant public health concern, being one of the most common birth defects worldwide. The surgical, nutritional, dental, speech, medical and behavioral interventions required to treat affected individuals impose a substantial economic burden (Strauss 1999). The lifetime health care costs for a single individual with cleft lip and/or palate is estimated to be over $100,000, not including out of pocket expenses incurred by families (CDC 1995). In the United States alone, approximately 6,800 births each year are affected by oral facial clefts (Canfield and others 2006).

The prevalence of CLP shows ethnic variation; in general, a relatively high prevalence of 1.19 to 2.0 per 1000 live births has been reported in Asians (Cooper and others 2006) and Native Americans (Wyszynski 2002), followed by an average of 1.0 per 1000 live births in Caucasians and less than 0.5 per 1000 live births in those of African descent (Gundlach and Maus 2006). These differences are most often attributed to racial variations in the timing and coordination of cellular morphologic patterns, particularly the development of the median nasal process (Eppley and others 2005). There are exceptions to these summaries; however, with some particular geographic areas having high frequencies thought to be related to founder effects and/or environmental triggers (Murray 2002).

1.1.2 Development of the upper lip, the secondary palate and of CLP

A cleft lip is a unilateral or bilateral opening in the upper lip, which forms during the fourth through seventh week of embryonic development. Cleft palate, on the other hand, is an opening in the hard or soft palate, which forms from the fifth through twelfth weeks of development. Developmental differences in the embryology of CL and CP have considerable significance toward their etiological distinctions (Spritz 2001). An appreciation of normal facial development is critical to the understanding of how this process may be interrupted to result in a CLP phenotype.

Development of the human face begins in the fourth week of embryogenesis, with migrating neural crest cells (future facial skeleton) combining with the core mesoderm (future facial muscles) and the epithelial cover to establish the facial primordia. At this stage, the facial primordia consists of five separate prominences surrounding the stomodeum, also called the primitive mouth (Figure 1). The frontonasal prominence (also referred to as the globular prominence or the medial nasal prominence, at different times in development) is located at the rostral side of the primitive mouth; two maxillary prominences are bound bilaterally to the stomodeum; and paired mandibular processes are bound caudally. The frontonasal prominence then widens as the forebrain gives rise to primitive cerebral hemispheres and the medial ends of the mandibular processes gradually merge to form the mandible (lower lip and jaw). A thickening of surface ectoderm occurs bilaterally on the ventrolateral surface of the frontonasal prominence, giving rise to the nasal placodes and eventually nasal pits and nasal processes (Jiang and others 2006).

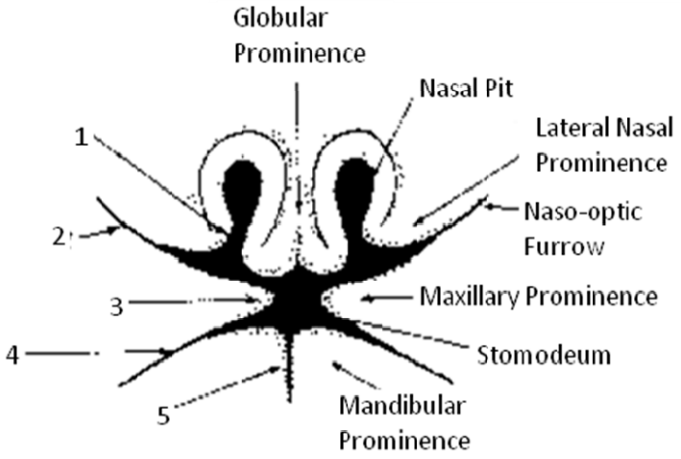

Figure 1. Embryonic lines of fusion, from (Sperber 2002b).

A rotation and advancement of the nasal placode permits the bilateral nasal prominences to sweep over the maxillary process to join with the medial nasal process and collectively form the basis of the upper lip-nasal unit, including the primary (hard) palate (Figure 2). Virtually all cases of overt cleft lip are attributed to the failure of the medial nasal process to either contact or maintain contact with the lateral nasal and maxillary processes, which typically occurs around 7 weeks postconception (Johnston 1990). It is thought that microform cleft lip may result from either partial failure in this fusion mechanism, or otherwise from spontaneous late fetal repair of an overt cleft lip (Pace and others 2006).

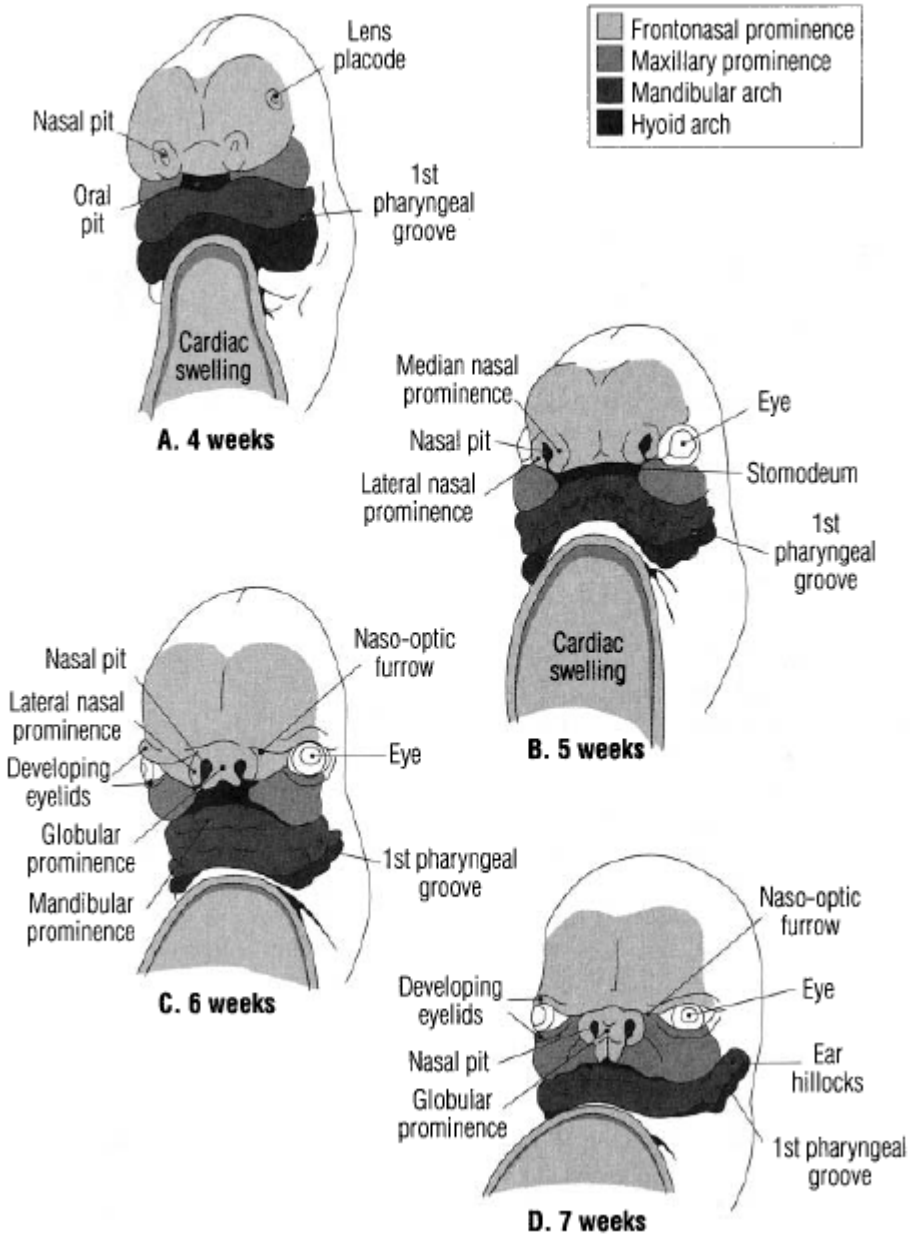

Figure 2. Development of the face at 4 weeks (A), 5 weeks (B), 6 weeks (C) and 7 weeks (D) (Sperber 2001).

The formation of the primary palate and the projection of the two lateral palatal processes into the stomodeum from the maxillary prominences are both required for the formation of the secondary (soft) definitive palate. The creation of bilateral, vertical palatal shelves occurs during

week 7 of human development, on the maxillary processes, lateral to the developing tongue. These vertical palatal processes flow quickly into the horizontal plane, enabling them to establish contact with each other in the midline, with the primary palate anteriorly and with the lower edge of the nasal septum (Figure 3). Fusion of the palatal shelves is dependent on epithelial-mesenchymal transformation (Sperber 2002a). The requirement of proper primary palate formation prior to the formation of the secondary palate is clearly important, as this likely contributes to the etiology of a combined cleft lip and cleft palate phenotype.

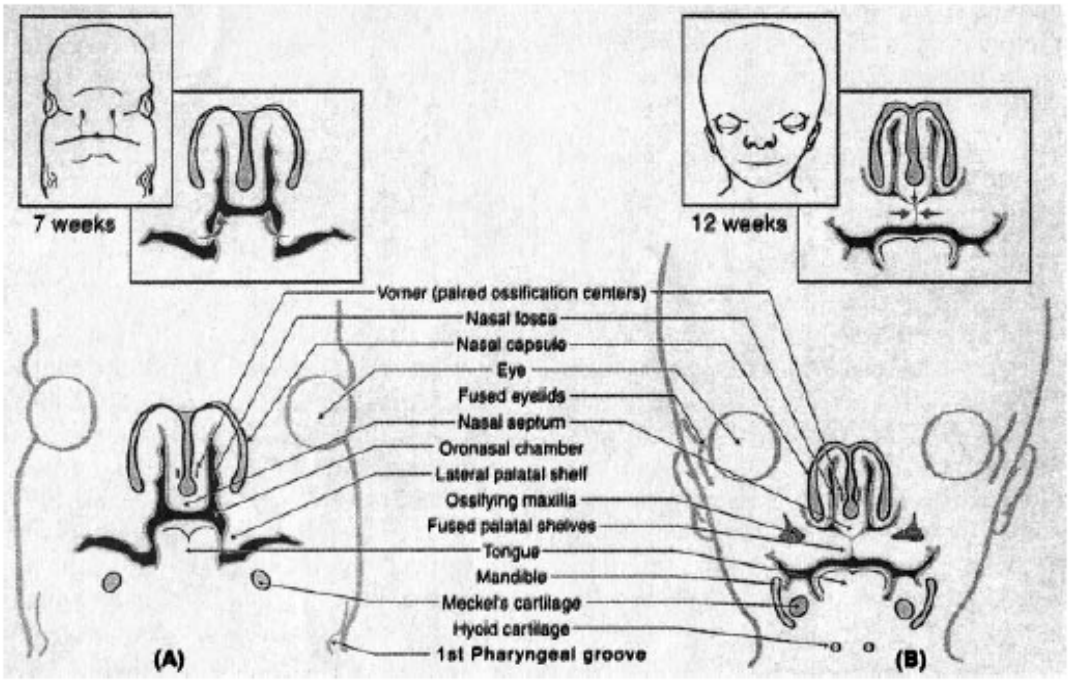

Figure 3. Development of the secondary palate; coronal sections of the human fetal head at 7 weeks (A) and 12 weeks (B) (Sperber 2002a).

1.2 THE GENETICS OF OROFACIAL CLEFTING

Studies have identified a number of genes and loci potentially implicated in orofacial clefting based on evidence from animal models, expression analyses, and human linkage and association studies. Some of the genes that appear to be associated with NS CLP have also been identified as being linked to syndromic forms of the phenotype. The molecular events involved in the proper formation of orofacial structures are under the strict control of a variety of genes, including fibroblast growth factors, sonic hedgehog, bone morphogenic proteins, members of the transforming growth factor β superfamily and a number of transcription factors (Jugessur and Murray 2005). Therefore, genes involved in the clefting process typically have functions assisting in cell migration, adhesion, growth, differentiation and apoptosis, related to the careful embryologic regulation required in order to perform the complex process of facial development.

A summary of genes and loci linked to NS CLP can be found in Table 1. Many of these genetic factors have been confirmed by subsequent analyses and refuted by others. Conflicting results seen in the literature are partially caused by differences in both study design and populations (Vieira 2006). Below is a short review of some of the associated genes and loci. Comprehensive reviews of the human genetic factors associated with NS CLP have been published and serve as excellent references for this material (Carinci and others 2007; Murray 2002; Vieira 2006).

Table 1. Genetic Links to NS CLP

Gene/Locus	Function
TGFα; 2p13	Mammalian growth factor
BCL3; 19q13	Proto-oncogene
4q25-4q31.3	Various genes, including *SCD5* (key regulator of energy metabolism)
MSX1; 4q16	Growth promoter, inhibits differentiation
IRF6; 1q32-q41	Interferon regulatory factor
PVRL1; 11q23	Cell-cell adhesion
TP63; 3q27	Transcription factor, tumor suppressor
13q33.1-q34	Multiple genes, including: *DP1* (transcription factor), *ING1* (tumor suppressor) and *COL4A1* (α-1 chain of collagen IV)
SUMO1; 2q32.2-q33	Modification of proliferating cell nuclear antigen; regulator of *MSX1*
MTHFR; 1q36	Folic acid metabolism
TGFβ3; 14q24	Growth factor; palate seam fusion
RARα; 17q21.1	Retinoic acid receptor
6p24	Multiple genes, including *EDN1* (endothelin 1; vasoconstrictor)
GABRB3; 15q11	Subunit of the GABA receptor, necessary for palate formation in the mouse
BMP4; 14q22-q23	Bone morphogenetic protein, functions in mesoderm induction, tooth development, limb formation, bone induction and fracture repair; activates *MSX1*

Transforming Growth Factor Alpha (TGFα) is a well-characterized mammalian growth factor. Its role in clefting appears small but significant, as suggested by various case-control and linkage studies. *TGFα* most likely acts as a genetic modifier of clefting, in agreement with the oligogenic model suggested for NS orofacial clefts (Vieira 2006).

BCL3 is a transcription factor involved in cell-lineage determination and in cell-cycle regulation. Linkage data has implicated this gene to be involved with sporadic and familial NS CLP (Stein and others 1995; Wyszynski and others 1997). *BCL3* has been proposed to be either a modifier or an additive gene for CLP etiology (Martinelli and others 1998).

Linkage studies have suggested chromosome 4q as a cleft susceptibility locus (Marazita and others 2002; Mitchell and others 1995). In addition, individuals with balanced translocations interrupting the SCD5 gene at 4q21 have been identified as having a cleft lip phenotype (Beiraghi and others 2003).

MSX1 may be involved in syndromic as well as NS clefting, within the context of both CLP and isolated CP (Carinci and others 2007). Mutations in *MSX1* are associated with autosomal dominant Cleft lip and palate-oligodontia syndrome; a specific nonsense mutation was found to segregate with a CLP plus tooth agenesis phenotype in a large family (van den Boogaard and others 2000). It has been proposed that missense mutations in conserved regions of the MSX1 gene alone could contribute to as many as 2% of total NS cleft lip and palate cases (Jezewski and others 2003). It also appears that interactions between specific *MSX1* variants and maternal smoking and alcohol consumption increase the risk of CLP (Romitti and others 1999). Further, Blacno et al. found a marginally significant “sex-dependent” association between *MSX1* and CLP in male Chilean patients, but not in females, suggesting a role for *MSX1* in the increased frequency of CLP in males (Blanco and others 2001).

Van der Woude syndrome is a Mendelian disorder that closely resembles NS CLP; pits in the lower lip are the only additional remarkable characteristic. The syndrome is caused by mutations in the gene for interferon regulatory factor 6 (*IRF6*) (Kondo and others 2002). Lip pits are found in approximately 85% of cases of the syndrome; therefore, 15% of cases may be clinically indistinguishable from NS CLP. This phenotypic feature encouraged researchers to investigate the potential role of variations in *IRF6* related to NS CLP. A study of 1968 families with isolated CLP showed highly significant transmission disequilibrium for the V274I variant in the IRF6 gene. The group found that *IRF6* has an attributable risk of about 12 percent of NS CLP, suggesting that *IRF6* plays a substantial role in the causation of NS CLP. They modified the risk of recurrence of CLP to be approximately 9% among siblings in families with a history of CLP whose parents are at risk for having a child with the homozygous risk allele (Zucchero and others 2004).

Cleft lip/palate-ectodermal dysplasia syndrome (CLPED1) is characterized by CLP, hidrotic ectodermal dysplasia, syndactyly and, in some cases, mental retardation. The causative gene for this syndrome is *PVRL1*, an immunoglobulin-related transmembrane cell-cell adhesion molecule (Suzuki and others 2000). A highly significant association between heterozygosity for a specific nonsense mutation (W185X) and NS CLP has been reported (Sözen and others 2001). Conclusions state that *PVRL1* variants contribute to nonsyndromic CLP in multiple populations, making a minor contribution to the sporadic forms of orofacial clefting (Avila and others 2006)

TP63 mutations are implicated in at least four human malformation syndromes, including Ankyloblepharon-ectodermal dysplasia-clefting syndrome (AEC), Ectrodactyly ectodermal dysplasia and facial clefts (EEC), Rapp-Hodgkin syndrome (RHS) and Limb-mammary syndrome (LMS). Clear syndrome-specific mutation patterns explain the spectrum of

features associated with *TP63* mutations (van Bokhoven and Brunner 2002). Since mutations in the TP63 gene underlie several monogenic malformation syndromes manifesting CLP, mutation analysis has been performed in patients with NS CLP, identifying a novel mutation that seems to segregate with a NS CLP phenotype. TP63 gene mutations are suggested to play a role in a small number of cases with NS CLP (Leoyklang and others 2006).

Trisomy 13 is a known malformation syndrome which often includes CLP as part of its phenotype. Interestingly, two Indian pedigrees, with NS CLP segregating as an autosomal dominant trait, demonstrated linkage to 13q33.1-q34, providing evidence to support the involvement of a region on chromosome 13 in some cases of NS CLP (Radhakrishna, 2006).

Animal models have confirmed the role of the Small Ubiquitin-like Modifier 1 (SUMO1) gene in upper lip and palate formation. *SUMO1* is known to modify numerous cellular proteins, thereby affecting their metabolism and function. Ubiquitin and SUMO proteins compete for modification of proliferating cell nuclear antigen, which is an essential processivity factor for DNA replication and repair. Of note, *SUMO1* is able to regulate MSX1 gene expression by sumoylation (Gupta and Bei 2006), implicating the possible role of both *SUMO1* and *MSX1* in NS orofacial clefting. A case report describes an individual born with isolated CLP, identified as carrying a balanced chromosome translocation in which the SUMO1 gene was disrupted on chromosome 2 (Alkuraya and others 2006). In addition, *SUMO1* polymorphisms have been found to be associated with NS CLP (Song and others 2008).

Reduced maternal folic acid intake during pregnancy has been reported to be a risk factor for NS CLP and MTHFR, a key enzyme in folic acid metabolism, has been implicated in the etiology of NS CLP. The C677T *MTHFR* polymorphism results in an enzyme with reduced activity, leading to elevated plasma homocysteine levels and reduced plasma folate (Frosst and

others 1995). A significantly higher *MTHFR* mutation frequency in mothers of CLP patients versus controls has been reported. This is thought to be related to maternal hyperhomocysteinemia being a risk factor for having CLP offspring (Martinelli and others 2001). Homozygosity for the common C677T polymorphism is significantly more frequent in patients with CLP, particularly females, lending support to the importance of folate metabolism in affected individuals (Mills and others 1999).

"Treatment" of mice with TGFβ protein isoforms accelerates palatal fusion, suggesting a role of TGFβ in the regulation of palate formation (Brunet and others 1993). Linkage disequilibrium was found for *TGFβ3* in CLP patients (Maestri and others 1997) and *TGFβ3* has been found to be associated with CLP and/or isolated CP phenotypes, varying by the study (Ichikawa and others 2006; Jugessur and others 2003; Vieira and others 2003). An interaction between *MSX1* and *TGFβ3* has also been suggested as contributing to CLP (Vieira and others 2003).

Certain alleles of the retinoic acid receptor alpha (RARα) gene are reported to be significantly different between NS CLP cases and unrelated controls (Chenevix-Trench and others 1992). Linkage analysis has shown that particular genetic variants of this gene are involved in the formation of CLP. *RARα* has also been suggested to be a modifier of CLP severity (Shaw and others 1993).

Some linkage studies have suggested 6p23-6p24 as an important locus implicated in the CLP phenotype (Carinci and others 1995; Scapoli and others 1997). Published data implies the presence of a CLP locus in this region; however, given the complexity of the condition and the limited number of families studied, additional verification is required. Notably, some individuals

with CLP have been characterized as having 6p chromosome abnormalities, including translocations and deletions (Davies and others 1995).

GABRB3 encodes one of thirteen subunits that combine to form a receptor for gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter. The functional gene also appears to be required for normal palate development in the mouse. An analysis of the *GABRB3* locus showed evidence of linkage disequilibrium with CLP, suggesting an etiological role in human clefting (Scapoli and others 2002).

Mouse models have shown that conditional knockouts of *BMP4* have an unusual "healed" CL phenotype (Liu and others 2005), motivating researchers to look at *BMP4* as a CLP candidate gene. The frequency of *BMP4* mutations in those with microform CLP and OO defects is significantly higher than in controls. This data suggests that *BMP4* alterations may result in delayed lip closure (Suzuki and others 2009).

1.3 THE PHENOTYPIC SPECTRUM OF CLEFT LIP AND PALATE

Congenital orofacial clefts show a wide variety of anatomical disruptions extending outward from the oral cavity with varying degrees and frequencies. The spectrum of orofacial clefting continues far beyond the phenotypes of CLP and CP alone, although CLP and CP are certainly the most common of all orofacial clefts (Tolarová and Cervenka 1998). Even within the context of CLP, there is a wide spectrum of phenotypes. We typically use descriptions to distinguish a particular cleft type; the most common include: unilateral, bilateral, complete, incomplete, vermillion notch and microform. A microform or minimal cleft lip may appear as a minor defect in the mucocutaneous border, a nostril deformity (Heckler and others 1979) or a faint band of

fibrous tissue running from the edge of the red lip to the nostril floor; appearing as a “scar” on the upper lip (Thaller and Lee 1995). A vermillion notch can be variable in size; this term is used to describe a small cleft, or notch, of the upper lip that typically does not extend far beyond the vermillion border (border between the upper lip and philtrum). An incomplete cleft lip may or may not involve the entire lip area, a complete cleft lip continues up through the nasal cavity and a complete CLP further continues into the palate (Figure 4).

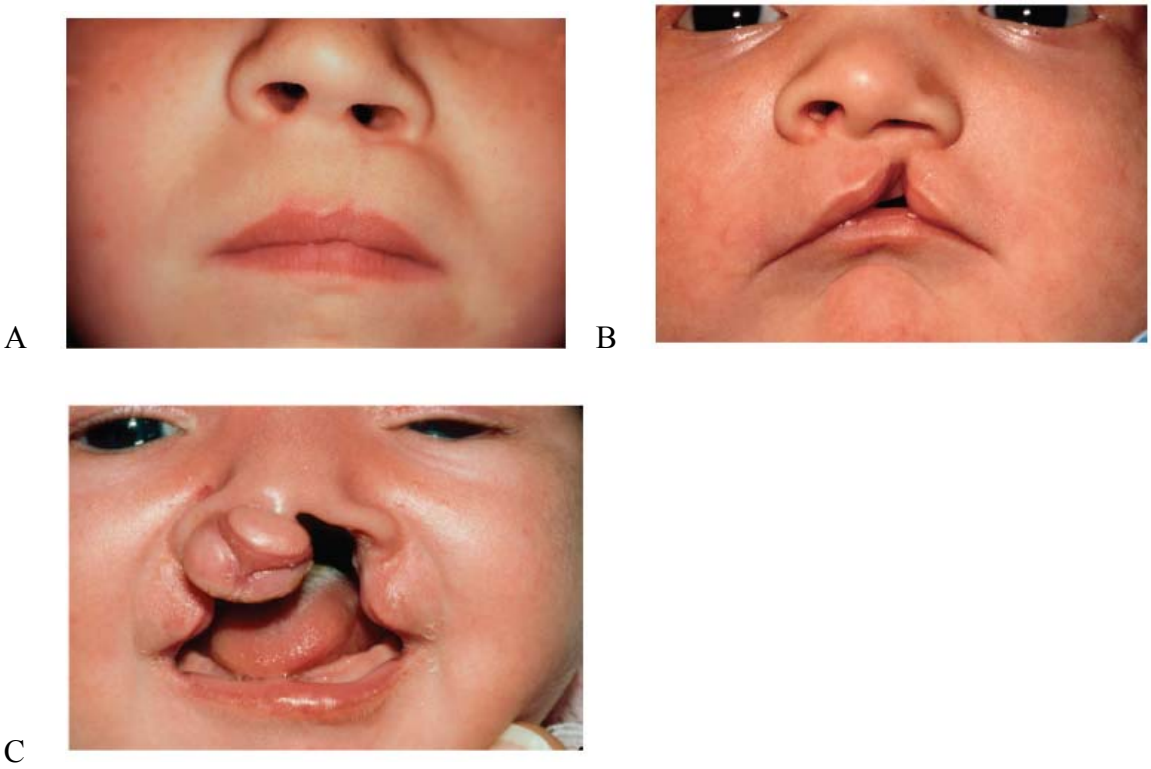

Figure 4. The variability of CLP: (A) microform CL; (B) incomplete unilateral CL; (C) complete bilateral CL and CP. Adapted from (Eppley and others 2005).

1.3.1 Other phenotypes associated with the NS CLP spectrum

In an effort to develop a thorough understanding of the etiology of NS CLP there has been a significant focus on finely characterizing and describing associated phenotypic features observed

in individuals with CLP and/or family members of those affected with CLP. Some of these include dental anomalies, structural brain anomalies, non-right handedness, differences in dermatoglyphic patterns, craniofacial morphology and velopharyngeal incompetence. These phenotypes have all been reported in the general population, but appear to be more frequently associated with families and/or individuals affected with CLP (Weinberg and others 2006).

A number of dental anomalies have been reported in individuals affected with NS CLP. Associated dental anomalies can range from a single malformed tooth in the vicinity of the cleft to dentition-wide reductions in tooth size or multiple congenitally missing teeth. The most commonly reported anomaly is hypodontia (dental agenesis) with other reported dental anomalies including supernumerary teeth, increased oral asymmetry, enamel formation defects and delayed dental age. These various dental anomalies are suggested to represent either microforms of orofacial clefting or generalized developmental disturbances (Harris 2002). Of note, in the case of dental agenesis, it has been shown that as clefting increases in severity, a greater number of teeth are missing. Following this finding, it has been proposed that dental anomalies could serve as markers for the definition of cleft sub-phenotypes (Menezes and Vieira 2008).

In an attempt to gather more information about the expanded CLP phenotype in the context of dental anomalies, it would be useful to know whether isolated tooth defects are more common in unaffected family members of CLP probands than in the general population. Studies of the dentition in unaffected parents and siblings of CLP patients have been inconsistent to date. Some have shown no significant differences in dental anomalies between unaffected CLP relatives and controls; others have reported higher frequencies of such anomalies in unaffected sibling groups (Weinberg and others 2006).

With regard to structural brain anomalies, neuroimaging work has shown that, when compared with a matched sample of healthy controls, the brains of men with NS CLP and CP have regional differences in cerebral and cerebellar tissue volume, a reduction in cerebrospinal fluid and an enlargement of certain midline brain structures. Deviations from the normal pattern of asymmetry have been observed for temporal lobe gray matter, occipital lobe white matter and cerebellar gray matter (Nopoulos and others). These structural anomalies have not yet been investigated in unaffected relatives of individuals with CLP, and the etiological factors responsible for these anomalies remain unclear. It is plausible that certain structural brain anomalies may share a common underlying etiology with CLP. Development of the brain and the craniofacial complex are intimately related due to their common tissue origins, overlapping gene expression patterns and functional growth dependencies (Weinberg and others 2006).

Human handedness is often used as an indicator of brain lateralization. The frequency of atypical cerebral lateralization is much higher in left-handed individuals than right-handed (Warrington and Pratt 1973). Similar to the reason for investigating structural brain differences in individuals with CLP, non-right handedness has been suspected to be a feature of individuals with NS CLP because of the biological relationship between asymmetrical fusion of the embryological facial prominences and abnormal brain lateralization. A higher than normal incidence of non-right handedness in NS CLP populations has consistently been reported; however, results have been inconsistent when considering the relationship between the side of unilateral clefts and handedness. Importantly, unaffected first degree relatives of those with CLP are also more likely to be non-right handed than controls, supporting the hypothesis of familial effects on handedness and CLP (Weinberg and others 2006).

Dermatoglyphic pattern types are suspected to reveal information about the nature of prenatal development in general. A preponderance of arches is considered by some researchers to be indicative of a developmental delay or disturbance coinciding with the formation of the finger print. For example, there is an increased frequency of arch patterns on the finger prints of subjects exposed prenatally to teratogens and affected with various disorders involving developmental delay (Babler 1991). Studies show that compared with controls, individuals with CLP seem to have an increased frequency of arches and ulnar loops and a decreased frequency of whorl patterns (Deshmukh and others 1979). One study also found that unaffected relatives had significantly more loops and fewer whorls compared to average, suggesting a pattern of adverse developmental events within CLP families (Scott and others 2005).

Dysmorphology of the craniofacial structure has been noted in some cases with CLP. Unrepaired complete bilateral CLP has been associated with numerous major structural deviations of the neurocranium and viscerocranium in adults (da Silva Filho and others 1998). Facial widths in CLP populations have been reported to be greater than average (Smahel and others 1985). Greater interorbital and nasal cavity width are suggested to be the two most consistent craniofacial abnormalities associated with CLP; these features may also be observed within unaffected relatives, hence the idea that heritable facial morphology may be a predisposing factor in the genesis of oral clefts (Ward and others 2002).

The production of normal speech requires the coordination of several muscle groups of the soft palate and the nasopharynx (McWilliams and others 1984). Defects in any aspect of the nasopharyngeal anatomy and/or physiology may lead to velopharyngeal incompetence (VPI). VPI is characterized by hypernasality, nasal air emission and compensatory articulation disorders (Boorman and others 2001). Cleft palate (both syndromic and nonsyndromic forms) is the most

common cause of VPI; however VPI has been reported in the absence of CP. In such cases, VPI may be due to various submucosal muscular defects, a disproportion between the size of the nasopharynx and the length of the palate, or a mechanical disruption due to scarring or contracture (Riski 2002). The presence of any one of these features may elevate the risk for producing offspring with CLP, particularly if there is a family history of CLP. Some level of VPI is present in nearly one quarter of unaffected relatives of those with CLP, supporting the notion that occult soft palate defects may be a subclinical marker for clefting (Weinberg and others 2006).

1.3.2 The superior orbicularis oris muscle

The superior orbicularis oris muscle is the upper portion of the sphincter muscle that surrounds the mouth. As described earlier, a critical sequence of steps is necessary for the fusion of the future upper lip elements. Consideration of the OO muscle is important throughout this developmental process. After the fusion of the maxillary prominences with the medial nasal prominence, transformation of the epithelial cells into mesenchymal tissue occurs, completing the process of lip fusion. At this point, mesoderm migrates across the fused prominences; by 8 weeks post-conception, a dense, continuous band of mesenchyme (the future OO muscle) can be seen and the complete OO muscle architecture is visible by 16 weeks gestation. It is certainly possible that a delay in fusion of the maxillary and medial nasal processes could alter the migration of mesoderm into the medial upper lip, resulting in a subepithelial OO defect that can only be visualized by ultrasound (Marazita 2007).

In cleft lip patients, the OO muscle fibers diverge from their typical horizontal organization and orient parallel to the potential cleft line; although, the involvement of the OO

muscle in a cleft lip may vary (Figure 5). Histological studies show that microform cleft lip defects may also extend to the muscle fibers of the OO muscle (Heckler and others 1979). In the context of plastic surgery, it has long been emphasized that the re-orienting of OO muscles during cleft lip repair process is critically important to a successful surgery (Randall and others 1974).

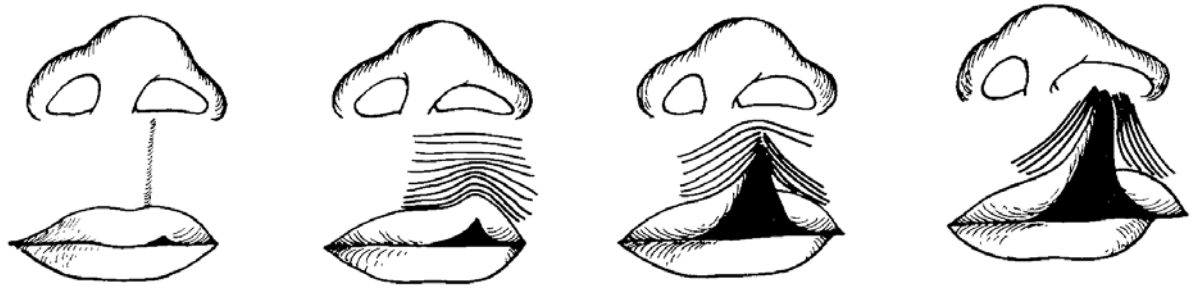

Figure 5. Diagrams of OO muscle fiber orientation in cleft lips of varying severity; from far left: minimal/microform CL, two incomplete CLs and complete CL. Taken from (Heckler and others 1979).

In 2000, Martin et al. revealed that discontinuities of the OO muscle are more frequently observed in unaffected family members of those with CLP as compared to controls. This outcome had been hypothesized, as OO muscle discontinuities reported among individuals with CLP have frequently been observed, and it is well known that there are clear developmental implications to the failure of upper lip closure. For this study, ultrasound was used as a method to evaluate the OO muscle in seemingly unaffected relatives of those with CLP. They examined the OO muscles of unaffected first-degree relatives of 21 children seen in a craniofacial clinic with CLP and 52 controls. Three raters from the group scored each image as having an OO defect or not; the OO muscle for each subject was defined as abnormal if any two of the three reviewers scored a scan as positive. When visualized by high-resolution ultrasound, the OO muscle typically appears as a single, continuous, smooth strand of dense muscle that is located

just above the alveolar ridge. A typical defect in the OO muscle was either an obvious focal echogenic area or a considerable thinning of the muscle. A statistically significant increase between the presence of OO defects in first-degree relatives versus controls (40% vs. 13%, respectively) was noted ($p < 0.002$). The researchers concluded that defects of the OO muscle visualized by ultrasound are more frequently noted in families with a history of CLP. They propose that there may be an embryological attempt at repairing the underlying OO defect with a deposition of collagen at the edges of the OO muscle, as in a scar that represents the ultrasonic defect detected in this study (Martin and others 2000).

In 2007, Neiswanger et al. provided a new set of data with a larger sample size to support Martin's hypothesis and findings. The study used high-resolution ultrasound to compare the frequency of discontinuities in the OO muscle in 525 unaffected relatives of individuals with NS CLP versus 257 unaffected controls. OO muscle discontinuities were observed in 10.3% of the non-cleft relatives, compared to 5.8% of the controls ($p = 0.04$). It is noteworthy that this new study only considered an OO defect to be present if it contained an obvious focal echogenic area (Figure 6). Thinning of the muscle, as long as the muscle was continuous, was not measured as a defect. In addition, this study looked at the presence of OO defects in all relatives that were in their research database, not only first degree relatives as Martin et. al. had done. These data confirmed the hypothesis that sub-epithelial OO muscle defects are a mild manifestation of the CL phenotype (Neiswanger and others 2007).

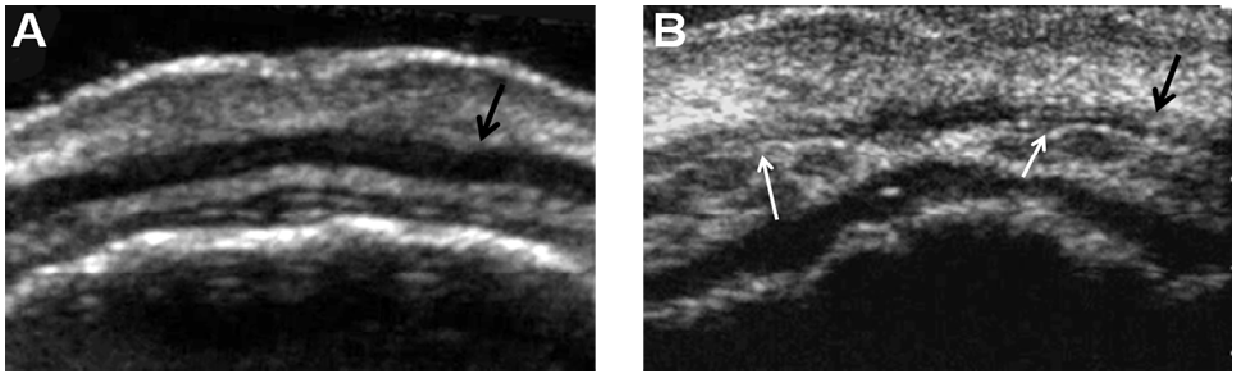

Figure 6. A typical OO muscle (A) and an OO muscle with a bilateral defect (B).

In both studies looking at OO muscle defects in families with a history of CLP, families with CP only were excluded from analysis, as a discontinuity in the OO muscle is not thought to be a microform of the cleft palate only phenotype. A recent study investigated the presence of OO defects in families with a history of CLP versus those with a history of isolated CP, and found that there is an increase in OO defects observed in unaffected relatives of probands with cleft lip only versus cleft palate only. Although the increase is not statistically significant, these data support the claim that cleft lip and cleft palate are etiologically distinct and provide further evidence that discontinuities in the superior OO muscle are a microform of the CLP phenotype only (Klotz and others 2008).

Although isolated CP and CLP are etiologically distinct, the possibility of an occult CL phenotype accompanying an overt CP phenotype should be taken into consideration. It is possible that some individuals with seemingly isolated CP may also have a subepithelial OO muscle defect, which may increase the risk for future pregnancies to have a CLP phenotype. A recent study used high-resolution ultrasound to identify occult discontinuities within the OO muscle in a subset of individuals with isolated CP. These findings raised question about the accuracy of the isolated CP designation and brought up the possibility that a portion of existing

families with seemingly isolated CP may actually fit into a CLP family grouping. It is important to specify the type of cleft defect, as genetic counseling and recurrence risk estimates are different for CP and CLP (Weinberg and others 2008).

Histological studies have shown support for the OO phenotype as being a microform of CLP. One study identified defects in the OO muscles of two 18-week fetuses with no obvious visible clefts, suggesting that the CLP phenotypic spectrum might also include occult subepithelial clefts (Martin and others 1993). Another study examined the histology of the OO muscle in 32 cadavers unaffected with overt CLP in order to characterize OO muscle defects visualized on ultrasound, as there is no way to directly inspect and verify the presence of an OO defect visualized by ultrasound in a live individual. The upper lip muscles of the cadavers were visualized by ultrasound and rated as being normal or abnormal before being dissected for histological sectioning. The family history of all subjects was unknown. One of the 32 subjects was identified to have an OO defect by ultrasound; the muscle fibers in the OO sections from this individual appeared more disorganized than those from the OO "normal" individuals, with a dense band of connective tissue being observed at the site identified as echogenic by ultrasound. They concluded that in general, the histological findings of the OO muscle correlated well to the ultrasonographic findings (Rogers and others 2008).

1.4 GENETIC COUNSELING AND PUBLISHED NS CLP RECURRENCE RISKS

When assessing a family's risk to have a child with a CLP, the genetic counselor considers many factors, such as: the number of individuals in the family affected with CLP, the relationship of those individuals to the proband/pregnancy, the presence of other birth defects associated with

clefting in that particular family, and the possibility of a syndrome that explains the occurrence of the CLP. Recurrence risk estimation for NS CLP is derived from empiric data. In general, the genetic counselor typically discusses a broad "multifactorial" inheritance pattern with the family, whereby both genetic and environmental factors are involved in the etiology of such defects, explaining why we might see more than one CLP in a given family.

Estimates of the relative risk of CLP for first degree relatives of those affected with CLP compared with the population prevalence range from 24-fold to 82-fold (Mitchell and Christensen 1996; Sivertsen and others 2008; Skjaerven and others 1999). The term recurrence risk is most often used to describe the chance that the same congenital anomaly will occur in a subsequent pregnancy born to the same parents; this is purely a sibling recurrence risk. Some literature broadens the recurrence risk definition to include other relatives, including first-degree (parents, siblings, children), second-degree (grandparents, aunts/uncles) and beyond. In terms of percentages, the increases in sibling risk of CLP translate from roughly 0.1% in the general population to 3-5% for families with one affected child (Chakravarti 2004).

The severity of the CLP does not seem to be a variable that is important to the calculation of familial recurrence risks. Based on a number of small studies, it was once thought that the increased severity of a CLP was related to an increased recurrence risk. Under the MFT model of inheritance, individuals with severe CLP phenotypes presumably had a higher number of genetic and environmental factors involved in the etiology of their CLP. Given that the MFT model has been rejected as an appropriate model of inheritance for CLP and current studies have increased statistical power with increased sample sizes, evidence has shown that mild or severe clefting in one child does not decrease or increase the risk of a subsequent child being affected (Sivertsen and others 2008). This is an important consideration with respect to OO muscle defects. If OO

muscle defects are considered a mild or microform cleft lip, an unborn sibling's risk for an overt cleft may be similar to that what it would have been, had the same proband had a CLP.

The recurrence risk for OO defects alone has not yet been reported. Given the current knowledge of the recurrence risk of CLP as well as the strong suggestion of OO defects fitting well into the phenotypic spectrum of CLP, recurrence risk estimation of OO defects as well as for CLP in the context of OO defects should be investigated.

2.0 SPECIFIC AIMS OF THIS STUDY

The specific aims that will be addressed in this study are threefold: 1. To investigate the occurrences of CLP in families with ≥ 1 family member with an OO defect versus those families without OO defects; 2. To estimate the recurrence risks of overt CLP and of OO defects in relatives of probands with CLP; and 3. To estimate the recurrence risks of overt CLP and of OO defects in relatives of probands with OO defects. Recurrence risk is defined as the likelihood that a trait or disorder present in one family member will occur again in other family members in the same or subsequent generations (NLM 2009) and, for the purposes of this project, will be calculated for both siblings and first degree relatives of probands.

3.0 METHODS

This study is funded by various grants awarded to Mary L. Marazita by the National Institutes of Health (Appendix A). The OFC study originally received approval by the University of Pittsburgh's Institutional Review Board (IRB) in 1998, with annual renewals and modifications as needed (Appendix B).

3.1 ORAL-FACIAL CLEFT (OFC) STUDY

Current challenges to clarifying the complexities of CLP include identifying contributory genes, investigating gene by gene and gene by environment interactions, and exploring the expression and function of etiological genes. A major difficulty in the discovery of genetic risk factors to the development of CLP is a poor definition of the phenotype (Weinberg and others 2006). The search for the genetic basis of any disease should begin with a concrete definition and assessment of the phenotype. In the case of a complex disease such as CLP, phenotype definition is especially important, as variability in expression can confound a seemingly straightforward trait. Numerous research groups across the world are investigating the genetic contributions to CLP. When large data sets and multiple research sites are used to search for genetic components to disease, it is critical that each involved research center is using the same precise diagnostic

criteria. Even a small margin of error in the phenotypic diagnosis can translate into false representation of a data set (Haines and Pericak-Vance 1998).

The Oral-Facial Cleft (OFC) study was designed to identify and evaluate associated phenotypic features in affected CLP families, with the hope of expanding and clarifying the CLP phenotype to aid genetic studies. Data collection sites include Pittsburgh, St. Louis, Texas, Ohio, Hungary, Guatemala, China, Argentina, and Spain. Notification of IRB approval was obtained from each of the foreign sites. When required, the informed consent and questionnaires of the OFC protocol are translated to the local language for each site. The complete protocol typically requires a one-time visit of 3-6 hours. This time requirement is adjusted by site and situation. For example, data from Guatemala is collected during medical and surgical missions with Children of the Americas (COTA). Typically, OFC research subjects in Guatemala are seen by the research team for less than one hour, as subjects have clinic appointments and surgical times to attend to. All subsets of the research procedures are listed in the original IRB document.

Families with a history of nonsyndromic cleft lip and/or palate are recruited through a local cleft craniofacial center, a registry, or word of mouth, depending on the site. Multiplex as well as simplex families are recruited into the OFC study; however, until approximately one year ago, the majority of families recruited were multiplex, with more than one individual in the family being affected with a CLP. As information is gathered about each family, case families are further stratified, depending on the type of cleft(s) that are observed. Individuals with CLP are examined and questioned regarding associated birth defects and/or syndromic features in order to ensure each participant has a NS form of CLP. Control families are recruited from the general population at each research site, typically via advertisements or word of mouth. It is certainly a goal of the OFC project for the controls to be case-matched based on age, gender,

race and ethnicity. Controls should not have a personal nor family history of cleft lip or cleft palate, genetic syndromes, or severe birth defects. For the purposes of this current set of analyses, it is important to note that families are not ascertained based on the status of their OO muscle phenotype.

Upon obtaining informed consent, each subject's personal information is de-identified with a family identification number (given to all members of the same family), an individual identification number (indicating the degree of relationship to the proband), as well as a study identification number (with no indication of family number nor degree of relatedness). Parents or legal guardians are required to give consent for subjects who are less than 18 years of age. Subjects typically complete at least a core protocol, including medical history and pedigree information, collection of DNA samples via saliva or blood, and basic demographic information. When appropriate, psychosocial and behavioral traits as well as pregnancy histories are assessed with questionnaires. Physical assessments vary by the location of data collection, as some research sites do not allow the time for the entire protocol to be completed with each subject. Assessments may include a videotaped speech sample, 3-D image craniofacial measurements, a high-resolution ultrasound of the upper lip, dermatoglyphics, minor physical variants, dental phenotype and lip prints.

Data collected in all sites is returned to Pittsburgh. All teleforms from the questionnaires, including information regarding family history, cleft types, etc., are scanned, verified and entered into a Progeny database. DNA is extracted from saliva and blood samples and stored for use in genetic studies investigating genes and polymorphisms that contribute to the cleft phenotype. With regard to physical assessments, only the protocol for obtaining and rating the upper lip ultrasound will be further discussed in this document.

3.1.1 Ultrasounds of the superior orbicularis oris muscle

High resolution ultrasound of the upper lip is performed on OFC study subjects in an effort to identify subclinical muscular variations that may be informative for orofacial clefting. Technicians and research assistants who perform such ultrasounds have been trained to perform the standard ultrasound procedure (outlined below) and to readily identify the OO muscle. The ultrasound machinery varies, depending on the data collection site. In general, each participant's study identification number is recorded along with the ultrasound video. The technician may make notes, referring to the subject's study ID number. Notes may only include comments that affect the quality of the ultrasound image, such as the presence of a mustache, a screaming child, etc. No notes are recorded regarding the cleft affection status of each participant or their family members.

Each ultrasound is performed while the subject is in the supine position, with the lips and mouth relaxed. Gel is applied to the transducer, such that there is a gel pad between the probe and the skin of the upper lip. The transducer is placed perpendicular to each patient's upper lip; location is central on the upper lip and minimal pressure is applied. Once the technician has successfully located the OO muscle, the transducer is moved slowly across both sides of the upper lip, in order to obtain a complete picture of the OO muscle. Images are generally one minute long. The images are stored internally on the ultrasound machine and subsequently transferred onto a USB drive until they are assessed by our research team.

3.1.2 Rating and assessment of ultrasound images

Continuous video ultrasound images are rated independently by three raters who have been trained to recognize discontinuities in the superior OO muscle. All raters are blinded to the CLP affection status of all participants and their family members. Rating scores are: 1 = no discontinuity of the OO muscle identified; 4 = clear discontinuity in the OO muscle identified; or 9 = unratable image. All ratings of 4 are further assessed in order to record the precise location of the OO defect on the upper lip; for example, a unilateral defect on the right or left, or a bilateral defect. Ratings of 9 are avoided when possible, but those images that are given a final score of 9 are typically very poor and unclear. No individual with a rating of 9 was used in the analysis of this study.

After each image is rated independently, the same three raters discuss their ratings until a single consensus rating is reached for each image. The consensus rating is recorded and entered into the same Progeny database where the remainder of the OFC data is pooled.

3.2 DATASET

Data used for these analyses are extrapolated from the complete OFC dataset. The variables that were required for the analysis of this particular study were: “famid” (the family identification number), “indid” (the individual identification numbers), “folder” (the site at which the data was collected), “aff” (the affection status of each participant), “cleftpix” (the type of cleft that each affected individual has), and “finalrating” (the final OO rating for each participant).

Since this study is looking specifically at recurrence, multiple new family variables were created and utilized (rather than using variables to describe each individual study subject). Each new family variable that was created is described below, within the context of that particular study objective.

3.3 DATA MANAGEMENT AND STATISTICAL METHODS FOR SPECIFIC AIM 1

The goal of specific aim 1 is to investigate the occurrences of CLP in families with ≥ 1 family member with an OO defect versus those families without OO defects. Given that sub-epithelial OO muscle defects appear to be a mild manifestation of the CLP phenotype (Neiswanger and others 2007), the hypothesis for specific aim 1 was that the occurrence of CLP would be higher in families with OO defects than those without. For this aim, both CLP case and control families were included, as long as our database included OO data on at least one member of the family who was unaffected with a CLP.

From the original OFC data set, an "OOMFamStatus" variable was created. This variable is binary and scores either 0 or 1, depending on the OO muscle status of the family as a whole. An OOMFamStatus score of 0 was given to families in which all members who are unaffected with a CLP have an OO rating of 1 (no defect). A score of 1 was given to families in which at least one member who is unaffected with a CLP has an OO rating of 4 (defect noted). An additional family variable called "clpstatus" was created. Similarly, this variable is binary (0, 1) and a family's score depends on whether there is a family history of CLP (1), or the family is a control family (0).

Using the statistical program R (http://www.r-project.org/), a 2 x 2 table was created using the OOMFamStatus variable versus the clpstatus family variable. From the 2 x 2 table, the occurrences of CLP in the two family groups (all OOM = 1 and at least one OOM = 4) were calculated, based on proportions. The sensitivity, specificity, positive and negative predictive values were calculated using the values from the 2 x 2 table (Haines and others 2006). A logistic regression was performed in order to compare the proportions of CLP in OOM+ vs. OOM- families. Logistic regression was chosen over the option of a chi square test in order to allow for adjustments to be made with any confounding variables (although no adjustments were subsequently made). Using R, the results of a logistic regression analysis provide a 2-sided p value, indicating whether or not the proportions compared are statistically different from each other, and a β coefficient, which provides an indication of the strength of the association. e^{β} = OR is the equation used to calculate an odds ratio.

3.4 DATA MANAGEMENT AND STATISTICAL METHODS FOR SPECIFIC AIM 2

The goal of specific aim 2 was to calculate the risk of recurrence of CLP and of OO defects among probands with CLP. For each recurrence (CLP or OO defect), the risk was calculated for siblings of the proband as well as first degree relatives. Published data quote a 3-5% risk of CLP recurrence for siblings and first degree relatives (FDR) (Chakravarti 2004; Sivertsen and others 2008); therefore, our hypothesis for the CLP recurrence risk was that our cohort would have similar recurrence risks as previously published data. The frequency of OO muscle defects specifically among siblings and FDRs of probands with CLP has not previously been reported,

although Neiswanger's study found that 10.3% of unaffected relatives of CLP probands had an OO defect. Note that a first degree relative is defined as a parent, sibling or child of the proband.

No control families were included in this particular analysis. Families included in specific aim 2 were those in which the proband is affected with a CLP. To analyze the recurrence among siblings of those probands, families were included if at least one sibling of the proband was in our database. To analyze the recurrence among first degree relatives (FDRs) of probands, families were included if at least one FDR of the proband was in our database. Depending on whether the recurrence of CLP or OO defects was being investigated, the family was only included if we had the relevant data for the appropriate family member(s) (CLP and/or OO status).

A "CSiblings" binary variable was created; this was coded as 0 if none of the proband's siblings had a CLP and 1 if there was a recurrence of CLP in at least one of the proband's siblings. Similarly, "CRelatives" was created and coded as 0 if none of the proband's FDRs had a CLP and 1 if there was at least one recurrence of CLP in the proband's FDRs. An "OOSiblings" variable was coded as 0 if none of the CLP proband's siblings who were unaffected with a CLP had an OO muscle defect, and as 1 if at least one sibling unaffected with a CLP had an OO muscle defect. An "OORelatives" variable was coded as 0 if none of the CLP proband's FDRs who were unaffected with CLP had an OO muscle defect, and as 1 if at least one FDR unaffected with a CLP did have an OO muscle defect. The new family variables created for specific aim 2 are summarized below in Table 2.

Table 2. Key of binary family variables created for specific aim 2 – all probands have CLP

Variable name	Code = 0	Code = 1
CSiblings	Proband has no siblings with CLP	Proband has at least one sibling with CLP
CRelatives	Proband has no FDRs with CLP	Proband has at least one FDR with CLP
OOSiblings	Proband has no siblings with OO defects	Proband has at least one sibling with an OO defect
OORelatives	Proband has no FDRs with OO defects	Proband has at least one FDR with an OO defect

Separate tables were created in R to estimate a) the proportion of siblings of CLP probands who had a recurrence of CLP; b) the proportion of FDRs of CLP probands with a recurrence of CLP; c) the proportion OO muscle defects among unaffected siblings of probands with CLP; and d) the proportion of OO muscle defects among unaffected FDRs of probands with CLP. These proportions were used as empiric estimates of the recurrence risks in our cohort.

3.5 DATA MANAGEMENT AND STATISTICAL METHODS FOR SPECIFIC AIM 3

The goal of specific aim 3 was to calculate the rate of recurrence of CLP and of OO defects among probands with OO muscle defects. Both CLP case and control families were included in this particular analysis. For each recurrence (CLP or OO defect), the risk was calculated for siblings of the proband as well as first degree relatives. At this time, there has been no published

data reporting CLP or OO muscle defect recurrence risks for probands who have OO muscle defects.

To analyze the recurrence among siblings of probands with OO muscle defects, families were included if at least one sibling of the proband was in our database. To analyze the recurrence among first degree relatives (FDRs) of probands, families were included if at least one FDR of the proband was in our database. Depending on whether the recurrence of CLP or OO defects was being investigated, the family was only included if we had the relevant data for the appropriate family member(s) (CLP and/or OO status). Note that all probands included in the analysis of specific aim 3 were not affected with CLP, but had an isolated OO defect. This is important to keep in mind, since it is well known that OO defects are usually present in individuals with overt CLP (Heckler and others 1979).

A "SiblingsOO" binary variable was created; this was coded as 0 if none of the proband's unaffected siblings had an OO defect and 1 if there was at least one recurrence of OO defects in the proband's unaffected siblings. Similarly, "RelativesOO" was created and coded as 0 if none of the proband's unaffected FDRs had an OO defect and 1 if there was at least one recurrence of OO defects in the unaffected FDRs. A "SiblingsCleft" variable was coded as 0 if none of the OO proband's siblings had an overt a CLP, and as 1 if at least one sibling was affected with CLP. A "RelativesCleft" variable was coded as 0 if none of the OO proband's FDRs were affected with CLP and as 1 if at least one FDR was affected with a CLP. The new family variables created for specific aim 3 are summarized below in Table 3.

Table 3. Key of binary family variables created for specific aim 3 – all probands have OO defects

Variable name	Code = 0	Code = 1
SiblingsOO	Proband has no siblings with OO defects	Proband has at least one sibling with an OO defect
RelativesOO	Proband has no FDRs with OO defects	Proband has at least one FDR with an OO defect
SiblingsCleft	Proband has no siblings with CLP	Proband has at least one sibling with CLP
RelativesCleft	Proband has no FDRs with CLP	Proband has at least one FDR with CLP

Separate tables were created in R to estimate a) the proportion of isolated OO defects in siblings of probands with OO defects; b) the proportion of isolated OO defects in FDRs of probands with OO defects; c) the proportion of siblings affected with CLP of probands with OO defects; and d) the proportion of FDRs affected with CLP of probands with OO defects. These proportions were used as an empiric estimate of the recurrence risks in our cohort.

3.6 FURTHER COMPARISONS

Some of the results obtained in specific aims 1 through 3 were compared to those from published literature and/or to each other. Proportions were compared using chi square and Fisher's exact tests (using R); the specific test used in each case depends on the sample size of that particular proportion. Any value in a 2 x 2 table less than 5 required the use of a Fisher's exact test.

4.0 RESULTS

The following results are separated by specific aim under which they were investigated. In general, a total of 2616 individuals had OO muscle ultrasounds performed, rated and used for these analyses, with 2033 (77.71%) of them having a rating of 1, 438 (16.75%) having a rating of 4 and 145 (5.54%) with a rating of 9.

4.1 SPECIFIC AIM 1

The goal of specific aim 1 was to investigate the frequency of CLP in families with OO muscle defects versus families without OO muscle defects. A total of 718 families were used in this analysis.

Table 4. Occurrences of CLP in families with OO muscle defects (OOM+) versus families without OO muscle defects (OOM-)

	Yes family history of CLP	No family history of CLP	Total
OOM+ families	65 (18.6%)*	284 (81.4%)	349
OOM- families	43 (11.7%)*	326 (88.3%)	369
Total	108	610	718

* $p < 0.01$, OR = 1.74

The occurrence (proportion) of CLP in OOM+ families is 0.1863, corresponding to the positive predictive value of using the OO muscle defect as a screen for CLP in the family. The occurrence (proportion) of CLP in OOM- families is 0.1165. The negative predictive value of using the OO muscle defect as a predictor for CLP in the family is 0.8835. The sensitivity of using the OO muscle defect as a predictor for CLP in the family is 60.19%; the specificity is 53.44%.

The comparison between the proportions of CLP in OOM+ versus OOM- families is significant, with a p value < 0.01. The odds of having an individual affected with CLP in the family are increased by 1.74-fold if a relative is identified as having an OO muscle defect.

4.2 SPECIFIC AIM 2

Specific aim 2 investigated the recurrence risks of CLP and of OO muscle defects among siblings and FDRs of probands with CLP. The following results are summarized below in Table 5. A total of 176 families were included in the analysis of sibling recurrence of CLP (Appendix C, Table 8, Calculation A). 16 of the 176 families (9.1%) had a recurrence of CLP among one or more siblings of the proband. 382 families were included in the analysis of FDR recurrence of CLP (Appendix C, Table 8, Calculation B). 60 of the 382 families (15.7%) had a recurrence of CLP among one or more FDRs of the proband. 129 families were included to calculate the proportion of isolated OO muscle defects among siblings of probands with CLP (Appendix C, Table 8, Calculation C). 19 of the 129 CLP probands (14.7%) had at least one sibling with an OO defect. 379 families were included to calculate the proportion of isolated OO muscle defects

among FDRs of probands with CLP (Appendix C, Table 8, Calculation D). 43 of the 379 CLP probands (11.4%) had at least one FDR with an OO defect.

Table 5. Summary of results from specific aim 2

A) Sibling recurrence of CLP		
Yes, sibling recurrence of CLP	No sibling recurrence of CLP	Total families
16 (9.1%)	160 (90.9%)	176
B) FDR recurrence of CLP		
Yes, FDR recurrence of CLP	No FDR recurrence of CLP	Total families
60 (15.7%)	322 (84.3%)	382
C) Proportion of families with OO muscle defects among siblings of probands with CLP		
Yes, sibling(s) with OO defects	No sibling(s) with OO defects	Total families
19 (14.7%)	110 (85.3%)	129
D) Proportion of families with OO muscle defects among FDRs of probands with CLP		
Yes, FDR(s) with OO defects	No FDR(s) with OO defects	Total families
43 (11.4%)	336 (88.6%)	379

4.3 SPECIFIC AIM 3

Specific aim 3 investigated the recurrence risks of CLP and of OO muscle defects among siblings and FDRs of probands with OO muscle defects. The following results are summarized in Table 6. A total of 29 families were included in the analysis of sibling recurrence of OO defects (Appendix C, Table 9, Calculation A). 5 of the 29 families (17.2%) had a recurrence of OO

muscle defects among one or more siblings of the proband. 67 families were included in the analysis of FDR recurrence of OO defects (Appendix C, Table 9, Calculation B). 11 of the 67 families (16.4%) had a recurrence of OO muscle defects among one or more FDRs of the proband. 30 families were included to calculate the proportion of CLP among siblings of probands with OO muscle defects (Appendix C, Table 9, Calculation C). 1 of the 30 probands (3.3%) with OO muscle defects had at least one sibling with an overt CLP. 82 families were included in calculating the proportion of CLP among FDRs of probands with OO muscle defects (Appendix C, Table 9, Calculation D). 6 of the 82 probands (7.3%) with OO muscle defects had at least one FDR with an overt CLP.

Table 6. Summary of results from specific aim 3

A) Sibling recurrence of OO defects		
Yes, sibling recurrence of OO defect	No sibling recurrence of OO defect	Total families
5 (17.2%)	24 (82.8%)	29
B) FDR recurrence of OO defects		
Yes, FDR recurrence of OO defect	No FDR recurrence of OO defect	Total families
11 (16.4%)	56 (83.6%)	67
C) Proportion of families with CLP among siblings of probands with OO defects		
Yes, sibling(s) with CLP	No sibling(s) with CLP	Total families
1 (3.3%)	29 (96.7%)	30
D) Proportion of families with CLP among FDRs of probands with OO defects		
Yes, FDR(s) with CLP	No FDR(s) with CLP	Total families
6 (7.3%)	76 (92.7%)	82

5.0 DISCUSSION

These data provide a step toward improving recurrence risk estimates for families affected by CLP. This study is the first of its kind to calculate recurrence risks of superior orbicularis oris muscle defects, and is also unique by including the OO defect status within the context of familial CLP recurrence risk estimates. The data used for these analyses are an outcome of multiple years of international data collection performed by the Oral-Facial Cleft (OFC) study, based out of Pittsburgh, PA. The main goal of this particular project was to support and encourage the utility of the OO phenotype with regard to future recurrence risk estimation and genetic counseling of CLP. These data suggest that sub-epithelial OO defects are associated with an increased risk of CLP among family members of probands with OO defects and attempt to delineate a quantitative value for that increase.

5.1 CLP AMONG FAMILIES WITH AND WITHOUT OO DEFECTS

It has previously been noted that the occurrence of OO defects among relatives of probands with CLP is significantly higher than control families without a history of CLP (Neiswanger and others 2007). The current study has found that the occurrence of CLP is significantly higher among families with unaffected relatives that have isolated OO defects than families without OO defects ($p < 0.01$). This result certainly strengthens the finding of occult OO defects being within

the spectrum of CLP, as the two phenotypes appear to segregate together, whether families are ascertained for analysis based on their prior CLP status or their prior OO defect status.

5.2 RECURRENCE RISK CALCULATIONS

5.2.1 Recurrence risks of CLP among siblings and FDRs in our cohort

The recurrence risks for CLP among siblings and FDRs in this data set were calculated to be 9.09% and 15.71%, respectively. These are important calculations for multiple reasons. First, it is important to investigate whether the data collected and the families included within the broad OFC cohort are consistent with published recurrence risk values. Next, these CLP recurrence risk values could ideally be used as "baseline" quantitative figures to be compared with the other risks and proportions calculated throughout the current study.

The 9.09% sibling recurrence risk of CLP was compared to a published sibling recurrence risk value of 4.55% using a chi square test, resulting in a significant p value of < 0.01. Likewise, the 15.71% FDR recurrence risk of CLP was compared to a published risk value of 4.17%, with a p value of $<< 0.001$. The significance of these results is an important detail for our research group to be aware of. There is an ascertainment bias in the OFC data with regard to multiplex families. Until approximately one year ago, data for the OFC study was primarily collected from multiplex families. Since that time, simplex families have been included; however, it is now clear that the data collection is still skewed toward multiplex families. That being said, the OFC dataset should perhaps not be used at this time for CLP recurrence risk estimates, since we are not considered to have a broad representation of families with CLP.

Another interpretation of the increased CLP recurrence risks could be that simply the recurrence risk from our dataset is higher than that which has been previously calculated in other cohorts. This dataset includes families from multiple countries, and since an effort was made to maximize our sample size, the dataset was not stratified by location. Therefore, it is possible that a higher incidence of CLP and potentially higher CLP recurrence risks in various populations (Guatemala and Argentina, for example) may be contributing to the CLP recurrence risk results we obtained.

5.2.2 OO defects among siblings and FDRs of probands with CLP in our cohort

If OO muscle defects and CLP segregate together in affected families, we would anticipate a higher than expected number of OO defects among siblings and FDRs of probands with CLP. Earlier studies using OFC data investigating OO muscle defects in association with clefting have reported values of a 10.3% prevalence of OO defects among unaffected relatives of probands with CLP and a 5.8% prevalence of OO defects among relatives of probands without a personal or family history of CLP (Neiswanger and others 2007).

The current study is the first to stratify the OFC data by relation (siblings, FDRs). The proportions of OO defects in siblings and FDRs of those with CLP (14.73% and 11.35%, respectively) were compared to 10.3%. Both p values ($p = 0.2727$ and $p = 0.5584$) were not significant. The majority of families included in the OFC cohort are nuclear families; therefore, the 10.3% originally reported with this cohort primarily reflected the proportion of OO defects observed in close family members of probands with CLP. The results reported in specific aim 2 stratify the occurrence of OO muscle defects to siblings and FDRs of probands with CLP, and it is not surprising that these values are not significantly different from 10.3%. Still, with

regard to recurrence risk estimates, there may be an importance of the specific degree of relation among those with OO defects and/or CLP and hopefully an increase in sample size will help clarify this matter.

We are unable to compare the OO recurrence risk estimates to a "general population" prevalence of OO defects, as this information has not been reported. To our knowledge, OO data has only been collected in the context of CLP analysis, whereby families have already been stratified into those with or without a history of CLP.

5.2.3 Recurrence risks of OO muscle defects among siblings and FDRs in our cohort

The recurrence risk of isolated OO defects has not previously been reported. It is important to note that the individuals considered in this data set have isolated OO defects and individuals with overt CLP were not included in these analyses. In addition, the ascertainment criteria for the entire OFC study is based on whether or not the family being recruited has a history of CLP, rather than their OO muscle status. In this study, we report an OO defect recurrence of 17.24% among siblings and 16.42% among FDRs. Of note, it was difficult to capture families with an individual who had an OO defect, where we also had data collected on his or her siblings and/or FDRs, explaining why the sample sizes were lower for these specific analyses.

If OO muscle defects are on the spectrum of the CLP phenotype and if CLP is inherited in an autosomal dominant or autosomal recessive fashion, we would expect recurrence risk estimates of OO defects to approach 50% or 25%, respectively. The calculated recurrences of OO defects alone are certainly higher than the 3-5% recurrence risk for CLP reported in the literature. An OO defect recurrence of 16-17% approaches values that are in accordance with autosomal dominant or recessive forms of inheritance, with reduced penetrance. These results

suggest a heritable component to the OO defect as a phenotype on its own, transcending more than one generation, and also perhaps give some insight with regard to the inheritance pattern of the CLP phenotype.

5.2.4 CLP among siblings and FDRs of probands with OO defects in our cohort

Examining the incidence of CLP among siblings and FDRs of probands with OO defects was a very important component of this study. These data provide us with estimates of the chance to have a sibling or FDR with a CLP if a proband is identified by ultrasound as having an OO defect. These results offer a first step toward suggesting quantitative values that may be used to create an additive recurrence risk estimate clinically, whereby CLP statuses as well as OO statuses of family members are included in the risk estimate.

In our data set, the chances for a sibling or FDR to have a CLP if a proband is found to have an OO defect are 3.33% and 7.3%, respectively. These numbers are not significantly different from published recurrence risk estimates of CLP among siblings and FDRs when the probands have an overt cleft ($p = 1$ and 0.25, respectively), suggesting that the OO muscle defect imposes a CLP risk that is very similar to the risk imposed by a prior CLP in the family. These results are consistent with the hypothesis that OO defects are on the spectrum of CLP.

The results from this specific analysis were also compared to the average population incidence of CLP (1/1000, or 0.1%). A 3.33% CLP frequency among siblings of those with OO defects is significantly different from 0.1% ($p = 0.03$). Similarly, a 7.3% CLP frequency among FDRs of those with OO defects is also significantly different from 0.1% ($p << 0.001$). We recognize that our dataset does not represent a typical population-based sample, as we have ascertained our families based on whether or not they fit a certain phenotype description (CLP).

Still, the risks of CLP for both siblings and FDRs of probands with OO defects are significantly greater than for the general population, further suggesting important counseling implications of being aware of one or more OO defect(s) in the family.

We also compared the CLP frequencies among siblings and FDRs of probands with OO muscle defects to the CLP recurrence risks that were calculated in our sample (from specific aim 2). A 3.33% CLP frequency among siblings of those with OO defects is not significantly different from our calculated sibling CLP recurrence risk of 9.09% ($p = 0.518$). This lack of significance is surprising, given that our calculated sibling CLP recurrence risk is over twice the CLP frequency among siblings of those with OO defects. It is important to note that the method to compare these two values used a Fisher's exact test looking specifically at the proportion of sibling CLP (1/30). The test obviously accounted for the 1/30; recognizing that a bold conclusion cannot be made with such a small sample size. The results of these calculations could perhaps be better interpreted if the sample sizes were greater. A similar comparison was made between the 7.3% CLP frequency among FDRs of probands with OO defects and the 15.71% calculated FDR CLP recurrence risk ($p = 0.05$). The difference between these two values is significant and this interpretation is likely to be more accurate, given that the sample size used for the FDR CLP frequency was higher (6/82).

5.3 LIMITATIONS OF THE STUDY

Careful consideration has gone into the processing and analysis of this data. Still, there are a number of limitations to this study.

First, the dataset that was used for these analyses only includes family members that have been enrolled in the OFC study. There are certainly instances where a family reports additional relatives with CLP; however, we do not have data on those reported individuals unless they have been recruited and consented into the study. The Center for Craniofacial and Dental Genetics makes an effort to recruit and include data from as many family members as possible; however, this is not always feasible.

Our methods of obtaining ultrasound images of the superior OO muscle and the rating of such images are still imperfect. For roughly 5% of individuals who participate in the OFC study and who have their OO muscle visualized by ultrasound, the research team is not able to come to a consensus regarding whether or not that individual has an OO defect. These ratings of "unratable, or 9" were excluded from analysis for the purpose of the current study; however, if we are able to rate them appropriately as being either "1" or "4", perhaps this would strengthen any OO family analyses.

The variable for specific aim 1 was created by identifying families in the OFC dataset (both case and control families) where one or more relatives who is not affected with a CLP has an isolated OO defect. In some cases, this variable has identified a family as OOM+ if a second- or third-degree relative of the proband was the only individual with an OO defect. We might expect the probands of these families to be at a lesser risk for CLP than if the relative with the OO defect was a first-degree relative.

These analyses have not yet been stratified into male and female family members with CLP or OO muscle defects. Sex differences with regard to OO defects have been reported; there is a significant increase of OO defects in unaffected male relatives of individuals with CLP over male controls (Neiswanger and others 2007). Of note, in general, there is also an increased

incidence of NS CLP in males over females worldwide, and the discovery of sex differences of OO defects seems to fit the CLP phenotypic observation.

Due to an attempt to maximize the number of families included in this study, the analyses were not stratified by geographical location. Families included were from all over the world (Appendix C), and it is possible that the OO muscle phenotype, the prevalence of CLP and environmental factors vary between sites.

The recurrence risks calculated for "first degree relatives" were done so in order to include CLP and OO muscle data from parents and children of probands, in addition to their siblings. It would be appropriate to stratify this FDR variable in order to have more accurate estimates, depending on the precise relation of the affected relative(s). The recurrence risk estimates also did not take into consideration the number of siblings or FDRs affected with either OO defects or CLP, they simply accounted for the fact that there was at least one sibling or FDR affected with the appropriate phenotype. It may be required that more precise recurrence risk analyses are performed, so that such risks could be additive. Ideally, precise recurrence of CLP should be given, depending on if one or both parents has a CLP combined with the information relating to if one or both parents has an OO defect, combined with the number of siblings affected with CLP and the number of siblings affected with OO defects. The current study gives more general data regarding recurrence risks and does not delve into particular phenotypes in specific relatives. Performing these stratifications for the current project would have substantially reduced our sample size, decreasing the power of this study.

In order for the upper lip ultrasound to have clinical utility, it must be clear that our interpretation of an OO defect visualized by ultrasound is the correct interpretation. Histological studies already mentioned in this document (Heckler and others 1979; Martin and others 1993;

Rogers and others 2008) have been the first step at confirming OO ultrasound interpretations. These types of studies are in need of a reliable method for the analyzing and rating of OO muscle ultrasound images. Our method of using three independent, blinded raters seems adequate in the research setting, but there is no way of verifying our final rating in live study subjects. Therefore, at this point, we cannot be fully confident in the clinical utility of our current rating system. Improvements in higher resolution imaging may help clarify all images, particularly those in which are labeled as "unratable".

Although this study includes a large number of families, certainly the inclusion of more families will assist in the interpretation of recurrence risk results. In particular, it would be highly beneficial to confirm these results with more families in which data is available on OO muscle defects for first degree relatives. Within our data collection, it will also be useful to continue to attempt to collect both multiplex and simplex families as well as controls – as we have seen with specific aim 2, in order to calculate accurate recurrence risks, the data set must not be biased toward one type of family over another.

5.4 FUTURE DIRECTIONS

Many of the limitations described above may be addressed in future studies to further strengthen the utility of the OO muscle phenotype being used as a clinical tool. The ultimate goal of this research is to provide additional evidence that the determination of subepithelial OO defects may eventually become important in a clinical setting, as a means of providing more accurate recurrence risk estimates to relatives of CLP families. The upper lip ultrasound is readily

available, non-invasive, simple to learn and not time-consuming or expensive, lending it to be a reasonable means of assessment.

In the future, once more families are recruited and more OO data is obtained and rated, the analyses from the current study should be stratified into sex, geographic location, specific relation of relatives affected with CLP and/or OO defects, as well as the number of relatives with such defects. In accordance with this, family-specific recurrence risks for CLP may be calculated, depending on the OO status and CLP status of each relative ascertained in the assessment.

Genome wide analyses are underway in order to identify genomic locations associated with the OO muscle defect phenotype. The hope is that these genetic analyses give additional insight into genes that are specifically associated with the OO muscle phenotype and/or the CLP phenotype. Similar studies could be performed with other associated phenotypes discussed earlier (non-right handedness, dermatoglyphics, etc.) to provide more clues toward the complex etiology of CLP.

Additional studies to consider could be centered on the utility of the ultrasound as a clinical tool and patients' perceptions of that tool as well as of CLP risk assessment. For example, it might be helpful to ask families in the CLP clinic what they think of using an ultrasound to modify their risk and if they would be likely to participate in such an opportunity. Family input regarding their views on recurrence risk modification would be extremely valuable. It has been suggested that a change in risk for CLP from 5% to 9% might not provide any difference in risk perception (Chakravarti 2004); however, families themselves have never been asked this question. The perception of risk involves numerous factors and may be very different for different families. It would be interesting to survey families and invite them to voice their

thoughts on modifying recurrence risk estimates and what effect will be made, if any, by including information about OO muscle defects in a genetic counseling risk assessment.

6.0 CONCLUSIONS

Discontinuities of the superior orbicularis oris muscle appear to be a part of the CLP spectrum. In order to use this information clinically, it is necessary to calculate quantitative values that suggest how we might integrate OO data into CLP recurrence risk calculation and genetic counseling for families.

In conclusion, the prevalence of CLP is significantly higher in families who have a history of OO defects when compared to families who do not have OO defects, supporting the notion that OO muscle defects are on the mild end of the CLP spectrum. The sibling and FDR recurrence risks of CLP in the cohort studied are increased above what has previously been reported in the literature, suggesting either an ascertainment bias, or a higher recurrence risk in our specific cohort. For the first time, we report the sibling and FDR recurrence risks of isolated OO defects, with both being roughly 16-17%. We also reported the risk for OO defects in siblings and FDRs if a proband has a CLP, as well as the risk for a CLP in siblings and FDRs if a proband has an OO muscle defect. The quantitative values reported for the risk of CLP in siblings and FDRs if a proband has an OO muscle defect are especially important, as they perhaps have more clinical efficacy. Although the sample sizes used for these studies are smaller than ideal, we have data to show that risk estimates for CLP in a sibling or FDR of a proband may be very similar if the proband has either an OO defect or an overt CLP. This data is

consistent with studies suggesting that recurrence risk for CLP is not altered with severity of the phenotype (Sivertsen and others 2008).

We anticipate that further directions will include recruiting more families into the OFC studies and stratifying the OO data into specific relatedness, countries and genders with regard to CLP risk. In addition, it must be made a priority to continue to study the utility of the upper lip ultrasound as a predictor of CLP recurrence such that we can move forward to using the ultrasound as a clinical tool.

APPENDIX A

OFC STUDY GRANT FUNDING RELEVANT TO THE CURRENT STUDY, PROVIDED BY THE NIH

Table 7. NIH grant funding

Award #	Title
2 R37 DE008559	Molecular Genetic Epidemiology of Cleft Lip and Palate
5 P50 DE016215	Genetics of Orofacial Cleft Families – Project 2
5 P50 DE016215	Genetics of Orofacial Cleft Families – Biostatistical Core
2 R01 DE014667	Cleft Lip Genetics: A Multicenter International Consortium
1 R21 DE016930	Planning International Orofacial Cleft Genetic Studies
3 P50 DE016215	Genetics of Orofacial Cleft Families – Iowa Supplement
1 R01 DE16148	Extending the Phenotype of Nonsyndromic Orofacial Clefts

APPENDIX B

INSTITUTIONAL REVIEW BOARD APPROVAL PROCESS

This research is conducted primarily under IRB0405013, titled "University of Pittsburgh: Coordinating Center for Oral-Facial Cleft Families: Phenotype and Genetics". Because this particular thesis project is using data that already existed under IRB0405013 and did not involve collecting new data, a new IRB and/or an amendment to the previous IRB was not required. As per Teri Reiche, IRB Program Manager at the University of Pittsburgh, graduate students are considered part of the research team and special IRB approval for them to work with the data under the direction of the PI is not necessary unless they are functioning at a co-investigator level.

Administrative coordination for the following research sites is covered under Pitt IRB0405013: Pittsburgh, PA; St. Louis, MO; West Virginia, Guatemala, Hungary, Madrid, Texas, Denmark, China and Argentina. All sites have their own local IRB approval at their respective institutions. Local IRB approval for Pittsburgh, PA is covered under Pitt IRB0607057, titled "Oral-Facial Cleft Families: Phenotype and Genetics (Pittsburgh Site)". IRB0607057 also covers the local IRB approval for Guatemala, as the organization we collaborate with in order to collect our data (Children of the Americas) does not have an IRB; thus, our institution absorbed

the Guatemala IRB requirements. In addition, the de-identified data from the Philippines, Texas and Argentina is covered under Pitt exempt IRB0402037, titled “Statistical Genetic Analysis of Data on Orofacial Anomalies”. All IRBs are renewed on an annual basis, with the exclusion of the exempt studies, which no longer expire. Modifications are made as needed in between yearly approval times.

B.1 MOST RECENT APPROVAL LETTER FOR IRB0405013

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: DR. MARY MARAZITA

From: SUE BEERS PhD , Vice Chair

Date: 7/16/2008

IRB#: IRB0405013

Subject: University of Pittsburgh: Coordinating Center for Oral-Facial Cleft Families: Phenotype and Genetics

Your research study has received expedited review and approval from the University of Pittsburgh Institutional Review Board under:
45 CFR 46 110.(7)

Please note that the advertisements that were submitted for review have been approved as written.
Please note the following information:

Approval Date: 7/16/2008
Expiration Date: 7/15/2009

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

B.2 MOST RECENT APPROVAL LETTER FOR IRB0607057

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: MARY MARAZITA PHD FACMG

From: SUE BEERS PHD, Vice Chair

Date: 8/13/2008

IRB#: IRB0607057

Subject: Oral-Facial Cleft Families: Phenotype and Genetics: (Pittsburgh Site)

Your research study has received expedited review and approval from the University of Pittsburgh Institutional Review Board under:
45 CFR 46.110.(9)

Please note the following information:

Approval Date: 8/13/2008
Expiration Date: 8/15/2009

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

B.3 MOST RECENT APPROVAL LETTER FOR IRB0402037

University of Pittsburgh
Institutional Review Board

Exempt and Expedited Reviews
Christopher M. Ryan, Ph.D., Vice Chair

Multiple Project Assurance: M-1259

3500 Fifth Avenue
Suite 105
Pittsburgh, PA 15213
Phone: 412.383.1480
Fax: 412.383.1146
e-mail: irbexempt@msx.upmc.edu

TO: Mary Marazita, Ph.D.

FROM: Christopher M. Ryan, Ph.D., Vice Chair

DATE: February 13, 2004

PROTOCOL: Statistical Genetic Analysis of Data on Orofacial Anomalies

IRB Number: 0402037

The above-referenced protocol has been reviewed by the University of Pittsburgh Institutional Review Board. Based on the information provided in the IRB protocol, this project meets all the necessary criteria for an exemption, and is hereby designated as "exempt" under section 45 CFR 46.101(b)(4).

The regulations of the University of Pittsburgh IRB require that exempt protocols be re-reviewed every three years. If you wish to continue the research after that time, a new application must be submitted.

- If any modifications are made to this project, please submit an 'exempt modification' form to the IRB.
- Please advise the IRB when your project has been completed so that it may be officially terminated in the IRB database.
- This research study may be audited by the University of Pittsburgh Research Conduct and Compliance Office.

Approval Date: 02/12/2004
Renewal Date: 02/12/2007

CR:ky

University of Pittsburgh

A Message from the University of Pittsburgh Institutional Review Board

Exempt Studies No Longer Expire

The policy regarding exempt research was revised to eliminate the requirement for renewal after three years. As of April 7, 2006, all exempt studies are being approved without an expiration date. When an exempt study is completed, it is important for investigators to submit to the IRB a termination notification, as well as a summary of the outcomes of their study.

Any previously approved exempt studies that contained an expiration date in the approval letter do not need to be resubmitted. These studies will not expire. It is recommended that you print a copy of this list-bot message to place with that exempt study as documentation that you do not need to submit these exempt studies again for IRB review.

"*IRB mailing for April 11, 2006*

APPENDIX C

ASCERTAINMENT LOCATIONS OF STUDY PARTICIPANTS

Table 8. Locations of ascertainment of families included in calculations for specific aim 2

Location	Number of families from location			
	Calculation A	Calculation B	Calculation C	Calculation D
Pittsburgh	56 (31.8%)	97 (25.4%)	40 (31.0%)	95 (25.1%)
St. Louis	14 (8.0%)	23 (6.0%)	6 (4.6%)	22 (5.8%)
Texas	0 (0.0%)	1 (0.3%)	1 (0.8%)	1 (0.3%)
Hungary	22 (12.5%)	33 (8.6%)	13 (10.1%)	33 (8.7%)
Beijing	0 (0.0%)	60 (15.7%)	0 (0.0%)	60 (15.8%)
Guatemala	53 (30.1%)	92 (24.1%)	41 (31.8%)	92 (24.3%)
Spain	18 (10.2%)	35 (9.2%)	15 (11.6%)	35 (9.2%)
Argentina	13 (7.4%)	41 (10.7%)	13 (10.1%)	41 (10.8%)
Total	**176**	**382**	**129**	**379**

Of the 16 families that had a sibling recurrence of CLP (Table 5; Calculation A), 3 (18.8%) were from Pittsburgh, 4 (25.0%) from St. Louis, 4 (25.0%) from Hungary and 5 (31.2%) from Guatemala. Of the 60 families that had a FDR recurrence of CLP (Calculation B), 17 (28.3%) were from Pittsburgh, 9 (15.0%) from St. Louis, 16 (26.7%) from Hungary, 11 (18.3%) from

Guatemala, 6 (10.0%) from Spain and 1 (1.7%) from Texas. Of the 19 families with OO muscle defects among siblings of probands with CLP (Calculation C), 4 (21.1%) were from Pittsburgh, 3 (15.8%) from Hungary, 10 (52.6%) from Guatemala, 1 (5.3%) from Texas and 1 (5.3%) from Argentina. Of the 43 families with OO muscle defects among FDRs of probands with CLP (Calculation D), 15 (34.9%) were from Pittsburgh, 1 (2.3%) from St. Louis, 3 (7.0%) from Hungary, 17 (39.5%) from Guatemala, 5 (11.6%) from Spain, 1 (2.3%) from Texas and 1 (2.3%) from Argentina.

Table 9. Locations of ascertainment of families included in calculations for specific aim 3

Location	Number of families from location			
	Calculation A	Calculation B	Calculation C	Calculation D
Pittsburgh	10 (34.5%)	21 (31.3%)	10 (33.3%)	27 (32.9%)
St. Louis	1 (3.4%)	2 (3.0%)	1 (3.3%)	3 (3.7%)
Texas	1 (3.5%)	1 (1.5%)	1 (3.3%)	1 (1.2%)
Ohio	2 (6.9%)	6 (8.9%)	2 (6.7%)	6 (7.3%)
Hungary	1 (3.5%)	4 (6.0%)	0 (0.0%)	6 (7.3%)
Guatemala	13 (44.8%)	27 (40.3%)	14 (46.7%)	33 (40.2%)
Spain	0 (0.0%)	4 (6.0%)	0 (0.0%)	4 (4.9%)
Argentina	1 (3.4%)	2 (3.0%)	2 (6.7%)	2 (2.5%)
Total	**29**	**67**	**30**	**82**

Of the 5 families that had a sibling recurrence of an OO muscle defect (Table 6; Calculation A), 3 (60%) were from Pittsburgh and 2 (40%) from Guatemala. Of the 11 families that had a FDR recurrence of an OO muscle defect (Calculation B), 7 (63.6%) were from

Pittsburgh and 4 (36.3%) from Guatemala. The single family with CLP among siblings of the proband with an OO defect (Calculation C) was ascertained from Texas. Of the 6 families with CLP among FDRs of probands with OO defects (Calculation D), 3 (50%) were from Pittsburgh, 2 (33.3%) from Hungary and 1 (16.7%) from Texas.

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