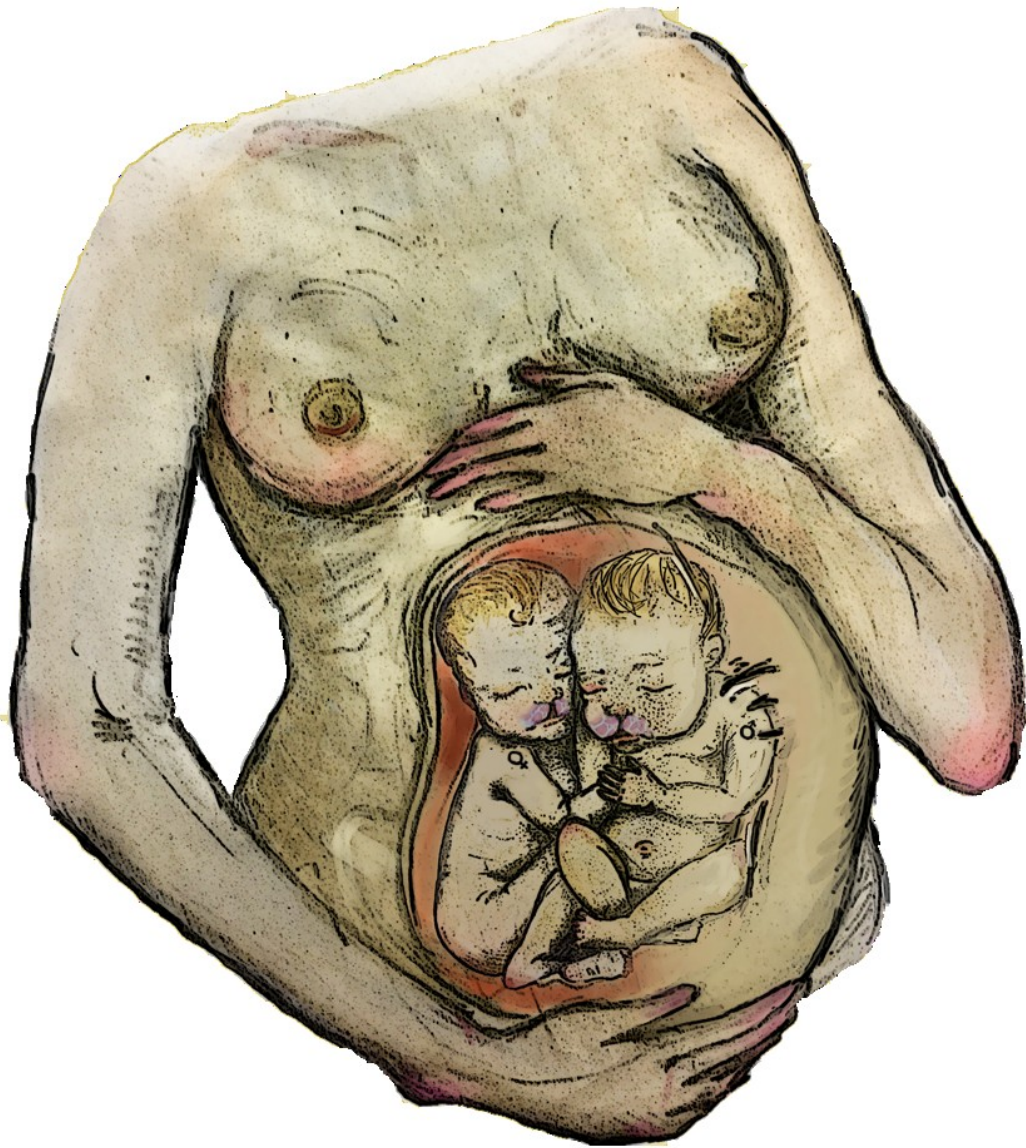
GENETIC, EPIGENETIC AND HISTOLOGICAL STUDY OF OROFACIAL CLEFT

INFLUENCE OF SEX IN DISEASE SUSCEPTIBILITY



MOHAMMAD FAISAL JAMAL KHAN

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Mohammad Faisal Jamal Khan

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MOLECULAR MEDICINE AND PHARMACOLOGY

Thesis, University of Ferrra, Italy

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PART ONE

GENERAL INTRODUCTION

Chapter 1

General introduction, objective and outline of thesis

General outline on Birth Defects or Congenital Anomalies

Birth defects can be defined as structural or functional abnormalities, which are present from birth and may result in either the death of the infant within weeks of birth or long-term disabilities. The term congenital disorder or anomalies is considered to have the same definition; the two terms are used interchangeably (Christianson *et al*., 2006).

The March and Dimes yearly global report on birth defects lists at least 7.9 million people born under birth defects, and out of which at least 3.3 million die before the age of 5 (Christianson *et al*., 2006). In addition to this the report by World Health Organization (World Health Organization, 2016) estimated the death of 276,000 babies within four weeks of birth every year from congenital defects (Ludwig *et al*, 2016).

There are more than 7,000 known types of birth defects, fourth most common is the Orofacial Cleft (OFC), with an incidence rate of 250,000 babies born with cleft worldwide (Merritt, 2005). The OFC not only enormously affect the individuals and their families by emotional and financial strain but also significantly affect the society as well as economical costs for the health care system. In Italy, the health care costs for approximately 800 children born with orofacial clefting per year has been estimated at around 80 million Euros (Bianchi *et al*., 2000).

The Orofacial Cleft

Overview of OFC history and current status

The OFC is a result of incomplete closure of the lip, palate, or both, caused by both genetic and environmental factors (Wheby *et al*., 2010) and may involve the lip, the hard palate (the roof of the mouth) and/or the soft palate (back of the mouth). The OFC can more rarely affect other part around the oral cavity which can further extend to other facial structures resulting in oral, facial and craniofacial deformity (Mossey *et al*., 2009).

For most of history, a cleft has guaranteed a distressing existence or indeed no existence at all, since one common way of dealing with clefts has been to murder those born with them. In ancient Rome and Sparta, babies were drowned or left out in the wilderness (Converse *et al*., 1930). In parts of Europe in the middle ages, clefts were seen as offering proof not just of the child’s satanic nature but of the mother’s too. (Accordingly, both mother

and baby were often abandoned). This misogynistic association with evil is reflected in the confusing origins of the term “hare lip”. Although first coined because of the supposed resemblance between an untreated cleft lip and a hare’s mouth, the name became bound up, in the 16th and 17th centuries, with the superstition that witches often took the shape of hares. If a pregnant woman was startled by a hare, the theory ran, her offspring would bear a “mark” in the form of a facial abnormality (Bhattacharya *et al*., 2009).

Even when children with clefts made it through infancy, their lives, in the past, can’t have been good. Although cleft lip repairs were known before 20th century but the earliest recorded was in China around 390BC (Morse., 1934; Wu., 1936), which were inevitably crude with severe disfigurement. Palates, meanwhile, generally went untreated until the mid-20th century, guaranteeing, among other things, lifelong problems with speech. One effect of such shortcomings was that there was little check on prejudice. Ignorant assumptions about those with clefts, that they were subnormal, malevolent, a curse, received apparent confirmation in physical setups.

The sane sense of thought on cleft appeared in the 16th century when Fabricius ab Aquapendente suggested the embryological basis of cleft, reflecting the fusion of the upper lip with the mid line at a very late stage (Aquapendente, 1600). Although the most convincing explanation was given by Blandin in the early 19th century, suggesting it as a resultant failure of fusion of premaxilla and maxillary segment (Blandin, 1836; 1838).

Today, while the situation in wealthy countries is much improved, in many poorer regions things remain dire. Ignorance and superstition are still widespread. Although at present OFC being one of the most common birth defect, with an incidence of roughly 1.77 in 1000 in the Europe (EuroCleftNet, 2015), the pathology, particularly in developed countries is seldom discussed and largely invisible. In most developing nations, there is no state provision for repairing clefts, which leaves responsibility for tackling the problem at the doors of charities.

Whereas, recent initiatives by European Cleft Network (EuroCleftNet) in conjugation with European Cleft Organization (ECO) helped address treatment (quality of care) and prevention (genetics and environment) in Europe. Meanwhile, the present tragedy is that, government in Europe question the wisdom of devoting resources to clefts. After all, they

argue, prioritizing the fund to other pressing needs such as cancer treatment, raising a question ‘Where does the Cleft treatment stand in priority list’?

Epidemiology

Cleft lip and cleft palate (CL and CP) are the most common and immediately recognizable craniofacial anomalies. Some are diagnosed prenatally on ultrasound; more often, they are first seen after the mother delivers. The infant if born with cleft may have a unilateral, bilateral, complete, or incomplete cleft and it may involve the lip only, the palate only, or both (Figure 1.1A-1E). Cleft lip and/or cleft palate (CL/P) often has an isolated non-syndromic occurrence; however, when associated with other abnormal physical findings, a recognizable syndrome may be present. Around 25% of CL/P and 50% of the CP cases are found to occur in combination with other malformation or as part of syndrome and hence considered as syndromic cleft (Jones, 1998; Leslie *et al*, 2016). Although, the study in the present thesis is focused on addressing the nonsyndromic CL/P (nsCL/P) and CP (nsCP).

In general, the incidence of nsCL/P is estimated to be approximately 1 in 700 live birth (Dixon *et al*., 2011). Recent review conducted by Panamonta *et al*., 2015; on cleft birth prevalence (95% confidence interval) per 10,000 live in different continent, is listed in Table 1.1. Whereas the OFC prevalence in Europe as listed by the European cleft network (EuroCleftNet, 2015) accounts to 1.77 per 1000 live birth.

Table 1. 1 Prevalence of Cleft birth according to continent

Continental Region	Prevalence per 10,000 live birth	95% confidence interval
Asia	1.57	1.54-1.60
North America	1.56	1.53-1.59
Europe	1.55	1.52-1.58
Oceania	1.33	1.30-1.36
South America	0.99	0.96-1.02
Africa	0.57	0.54-0.60

Adapted from: Panamonta *et al*., 2015

The males predominate in the occurrence of CL/P (60-80% of cases), whereas the females constitute the majority within CP group. (Fogh-Andersen., 1961; Mossey and Little., 2002). Cleft lip and palate deformity is strongly associated with bilateral cleft lips (BCLs,

86% of cases); the association decreases to 68% with unilateral cleft lip (UCL). The left side is most commonly involved in unilateral cleft lip cases. However, the predominance of syndromic forms, defined by the presence of other abnormalities in addition to CL/P is lower in males (Mossey and Castilla, 2001). Curiously, the frequency in females is higher when the father is greater than 40 years (Rittler *et al*., 2004).

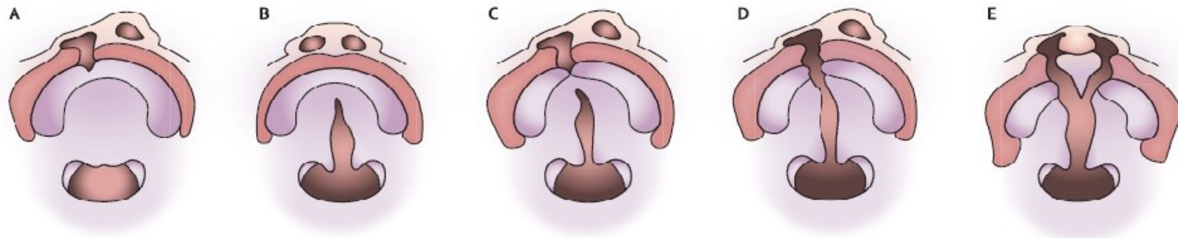

Figure 1. 1 Schematic representation of nonsyndromic orofacial clefts

(A) Cleft lip and alveolus. (B) Cleft palate. (C) Incomplete unilateral cleft lip and palate. (D) Complete unilateral cleft lip and palate. (E) Complete bilateral cleft lip and palate.

Adapted from: Shaw WC. Orthodontics and occlusal management. Oxford: Butterworth-Heinemann, 1993.

Development of Cleft lip and Palate

The formation of the lip and palate involves complex series of events which requires close co-ordination between series of programs of cell migration, growth, differentiation and apoptosis (Mossey *et al*., 2009). The development of the face begins in the 4th week, where the neural crest cells migrate to form the five facial primordia: the frontonasal prominence, the paired mandibular processes, and the paired maxillary processes (MxP) (Figure 1.2a). These structures are composed of ectodermally derived epithelium and neural crest derived mesenchyme. By the end of the 4th and beginning of the 5th week of gestation the facial prominences are formed, followed by invagination of the nasal placodes to form the medial (MNP) and lateral (LNP) nasal processes (Figure 1.2b). During the 6th and 7th weeks of gestation, the nasal processes grow and merge, forming the upper lip and primary palate (Jiang *et al*., 2006; Dixon *et al*., 2011). The nostrils are formed from the fusion of the MNP, LNP, and the MxP (Figure 1.2c). Failure in growth or fusion of these processes results in orofacial clefting involving the upper lip, alveolus, and/or primary palate (Leslie, 2012).

The development of the secondary palate starts by the 7th week of gestation as outgrowth from the MxP (Figure 1.2d). The palatal shelves grow vertically on either side of

the tongue and subsequently, elevate to the horizontal position above the tongue and move along the medial direction, once in contact, they commence fusion forming the medial epithelial seam (MES) (Figure 1.2e).. The disappearance of the epithelial seams by apoptosis and epithelial-mesenchymal transformation marks the complete fusion (Jiang *et al*, 2006), which ultimately results in the separation of oral space into nasal and oral cavities (Figure 1.2f). The partial or total absence of the merging or fusion of these prominences or disruption of distinct morphogenetic processes at different stage of embryological development may cause clefting of the lip and may extend to jaw and palate (Shkoukani *et al*., 2013).

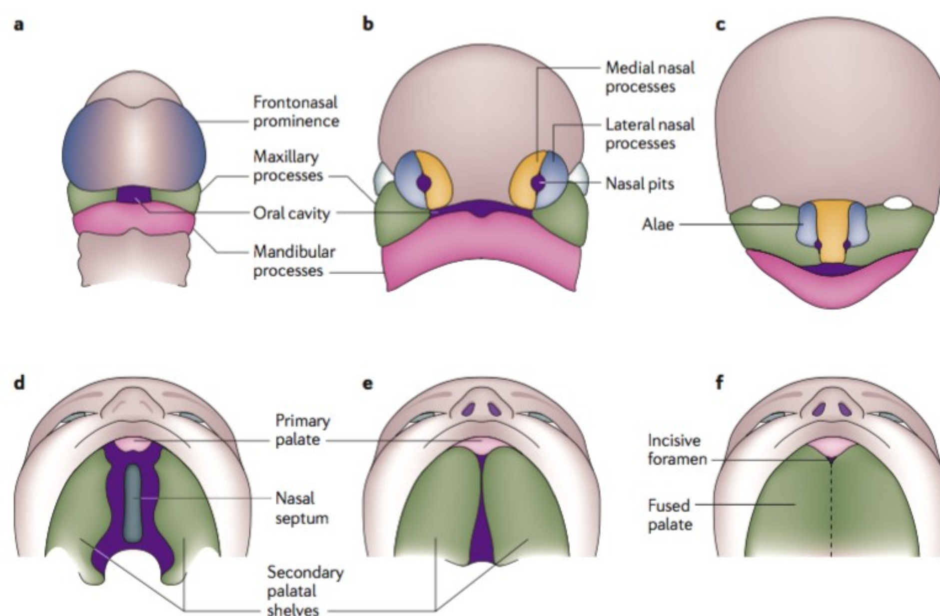

Figure 1. 2 Development of the lip and palate

a-c, Development of the lip; d-f, Development of the palate.
Adapted from: Dixon *et al*., 2011

Orofacial Cleft Phenotype

The complete formation of the lip occurs during the sixth week of gestation and the palate during the last week of first trimester. Therefore, it is possible to have cleft lip alone (CL) or along with cleft palate (CLP), and those affecting late in development may have cleft palate only (CP). Moreover, this broad sub-division of anatomical defects is consistent with the distinct developmental origins of the lip/primary palate versus the secondary palate. Although Cleft is classified in three broad categories as listed above but still resides among heterogeneous disorder, hence its classification system is based on the severity of the pathology (Marazita, 2012). Ideally, the development of the primary and the secondary palate

have distinct developmental origin, but the severity of the clefting in the lip can lead to the cleft of the secondary palate, converging CL and CLP to the common term cleft lip with or without palate CL/P (Fogh-Andersen., 1942; Fraser., 1955). Yet, varying with the approach and interpretation of the surgeon, pathologist and geneticists for the single term (Wang *et al*., 2014; Watkin *et al*, 2014). Although few studies have distinguished CL from CLP, with CLP being common and generally excess in males (Mossey *et al*., 2002; Jensen *et al*., 1988; Shapira *et al*., 1999).

In addition, OFC are further classified as “syndromic OFC” and ‘nonsyndromic OFC”. In syndromic, the cleft is associated with other malformation. Usually, it is due to a single gene (monogenic or Mendalian) disorder. The syndromic cleft may also occur due to other chromosomal abnormalities due to involvement of multiple genes (Hennekam *et al*., 2013). The term “syndromic” may, but does not necessarily, suggest the presence of a recognizable syndrome. Conversely, the term “nonsyndromic OFC” describes an OFC that occurs in a patient who may or may not have other structural or functional anomalies, but where none of the other morbidity (if present) is known or thought to be causally related to the OFC of interest. Approximately, 70-80% of CL/P and 50% of CP are considered nonsyndromic (Jones, 1998; Leslie *et al*, 2016).

The sub-clinical Phenotype

The cleft phenotype appears to be much more complex and characterised by a number of subclinical features (Weinberg *et al*., 2006). These can be associated with skeletal, dental, and soft tissue abnormalities, all of which may represent an incomplete manifestation (i.e., forme fruste) of the clefting process. Examples of these markers include micro (subclinical)-defects of the orbicularis oris muscle (Marazita, 2007), the presence of dental anomalies (Menezes *et al*., 2008), and variations in face shape Mossey *et al*., 2010). The subclinical phenotype of the cleft palate includes submucous cleft palate, characterized by bifid uvula and bony notch in the posterior edge of the hard palate (Chen *et al*., 1994; Reiter *et al*., 2012). Figure 1.3.

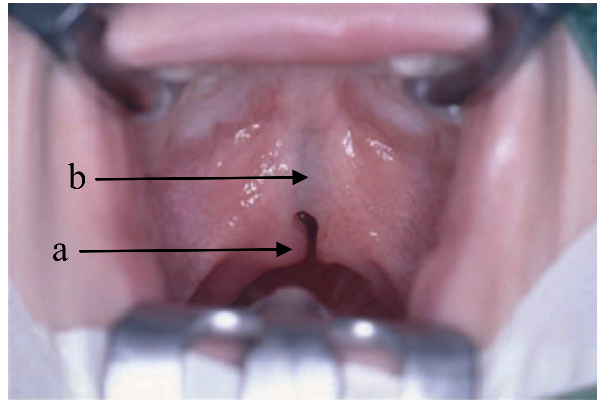

Figure 1. 3 Submucous cleft palate with the two main morphological symptoms

(a) bifid uvula and (b) a transluscent zone lacking a posterior nasal spine, leading to a bony notch in the posterior end of the hard palate.
Adapted from: Reiter *et al*., 2012

Recent studies focused on discontinuities of the orbicularis oris (OO) muscles in clinically unaffected relatives of individuals with OFC (Neiswanger *et al*., 2007) and in monozygotic twins (Leslie *et al*., 2016) suggest that the determination of subepithelial OO defects may eventually reflect important in a clinical setting, as a means of providing more accurate recurrence risk estimates to relatives in CL/P families and in offspring's of monozygotic twins respectively.

Etiology of Cleft lip and Cleft Palate

CL/P is etiologically heterogeneous with both genetics and environmental contributions. Over an extended population, the genetic contribution to this heterogeneous trait varies depending on penetrance frequency of the causative allele and more commonly, with allele of low penetrance interacting with the environmental factors (Watkin *et al*., 2014). With the advent of the genomics era and advances in both quantitative and molecular analysis techniques, there have been great improvements in the identification of causative genetic mutations and associations underlying syndromic forms of CL/P, Table 1.2. On the other hand, there is currently little progress in identifying and understanding of the genetic etiology of isolated (nonsyndromic) CL/P cases (6-9), the genes associated with nsCL/P is listed in Table 1.3.

Table 1. 2 Genes associated with syndromic orofacial clefting

Syndrome	Gene	Loci	Reference
Cleft lip ± cleft palate (CL/P)			
Van der Woude, popliteal pterygium	*IRF6*	1q32-q41	Kondo *et al*., 2002
Holoprosencephaly, type 3	*SHH*	7q36	Roessler *et al*., 1996
Zollinger syndrome-3	*PXMP3*	8q21.1	Raynold *et al.,* 1995
Waardenburg syndrome, type I	*PAX3*	2q35	Giacoia *et al.,* 1969
Hartsfield, Kallmann	*FGFR1*	8p11	Dode *et al*., 2003; Simonis *et al.,* 2013
Tooth agenesis with or without cleft	*MSX1*	4p16	van den Boogaard *et al.,* 2000
Cleft Palate Only			
Van der Woude	*GRHL3*	1p36.11	Mangold *et al.,* 2016; Leslie *et al.,* 2016
Stickler syndrome, type II	*CLO11A2*	6p21.3	Snead and Yates., 1999
DiGeorge	*TBX1, COMT*	22q11.21	Packham and Brook., 2003
Pierre Robin Sequence	*SOX9*	17.q24.3-q24.1	Benko *et al.,* 2009; Foster *et al*., 1994
Popliteal pterygium	*IRF6*	1q32-q41	Dixon, 2011; kondo et., 2002
Teacher Collins	*TCOF1*	5q32-q33	Group TCC, 1996
Apert, Crouzon	*FGFR2*	10q26	Reardon *et al*., 1994; Wilkie *et al.,* 1995
Midline cleft lip			
Opitz G/BBB	*MID1*		Quaderi *et al.,* 1997
Orofacialdigital type I	*OFD1*		Ferrante *et al*., 2001

Adapted and modified from Allam *et al*., 2014; Lakhanpal *et al*., 2013 and Burg *et al*., 2016

Table 1. 3 Genes associated with nonsyndromic orofacial clefting

Gene	Loci	Reference
IRF6	1q32.2	Blanton *et al.*, 2005; Birnbaum *et al.*, 2009; Beaty *et al.*, 2010;
SPRY2	13q31.2	Ludwig *et al.,* 2012
TPM1	15q22	Ludwig *et al.,* 2012; Leslie *et al.*, 2015
TBK1	12q14	Beaty *et al.,* 2011
PAX7	1p36.13	Ludwig *et al.*, 2012
PAX9, TGFB3,BMP4	14q21–24	Marazita *et al.*, 2009
COL8A1/FILIPIL	3q12	Beaty *et al.,* 2013
TP63	3q27–28	Marazita *et al.,* 2009
VAX1	10q25.3	Mangold *et al.,* 2010; Beaty *et al.,* 2010; Butali *et al.,* 2013
FOXE1	9q22.33	Marazita *et al.,* 2009; Ludwig *et al.,* 2014
GREM1	15q13.3	Mangold *et al.,* 2010
ABCA4	1p22.1	Beaty *et al.,* 2010
TGFA	2p13	Ardinger *et al.*, 1989; Marazita *et al.,* 2009
SUMO1	3q27-28	Alkuraya *et al.,* 2006; Gupta *et al.,* 2006
MSX1	4q16	Machado *et al.,* 2016; Suziki *et al.*, 2004
NOG	17q22	Mangold *et al.,* 2010; Leslie *et al.,* 2015
PRR2	19q13	Martinelli *et al.,* 1998; Warrington *et al.,* 2006
GRHL3	1p36.11	Mangold *et al.,* 2016; Leslie *et al.,* 2016

Adapted and modified from Carinci *et al.,* 2007 and S. T. Sonis (ed.), Genomics, Personalized Medicine and Oral Disease, 2015.

Beside the genetic factors, environmental factors also play a significant role in the eitiology of CL/P, with different magnitude of risk in CL/P and CP (Watkin *et al*., 2014). The maternal cigarette smoking (Zhang *et al*., 2010; Källén, 1997; Chung *et al*., 2000; Dietrich *et al*., 2003; Lammer *et al*., 2004; Little *et al*., 2004a, b; Meyer *et al*., 2004; Honein *et al*., 2007; Shi *et al*., 2008) and alcohol consumption (Romitti *et al*., 2007) during pregnancy, has been identified as posing risk factors for OFC, with increased risk for CL/P than for CP only. However, the use of folic acid supplementation during pregnancy has been inversely related to development of OFC (Murray, 2002; Jia *et al*., 2011; Badovinac *et al*., 2007). Although some reports do not claim a benefit from maternal folate supplementation (Little *et al*., 2008), a recent meta-analysis indicated a significant reduction in risk of CLP with maternal folic acid use (Butali *et al*., 2013). Furthermore, using improved methods for estimating nutrient intake, Wallenstein *et al*., 2013 similarly observed a decreased risk for CLP with maternal folate intake as well as for other micronutrients such as niacin, riboflavin and vitamin B12. Interestingly, studies report a significant folate-risk relationship for CLP only (not CPO), which may be a hallmark distinguishing these phenotypes. In addition, ambiguous results have been found for maternal disease and stress during pregnancy, chemical exposure and corticosteroids (Hayes *et al*., 2002).

Role of Genetics in Cleft lip and Palate

Several linkage analysis and candidate gene approach carried to check association for CL/P, has different confirmation status. The genes with confirmed evidence includes *IRF6* and *Vax1* and the genes with likely evidence are *ABCA4, BAMP4, FGFR2, FOXE1, MAFB, MSX1, MYH9* (Dixon, *et al* 2011). Moreover, there are some genes with varying consensus, and includes *TGFA, MTHFR, GSTT1, SUMO1*. In addition, genome-wide association studies have identified 15 (Cura *et al*., 2016) additional risk loci (Birnbaum *et al*., 2009; Grant *et al*., 2009; Beaty *et al*., 2010; Mangold *et al*., 2010, Ludwig *et al*., 2012 & 2016), with some of these replicating in subsequent studies (e.g., Jia *et al*., 2015), Figure 1.4 . For most of these loci, the causative variant/gene has yet to be identified, however, large-scale sequencing efforts surrounding these loci may yield compelling candidates for further interrogation (Leslie *et al*., 2015).

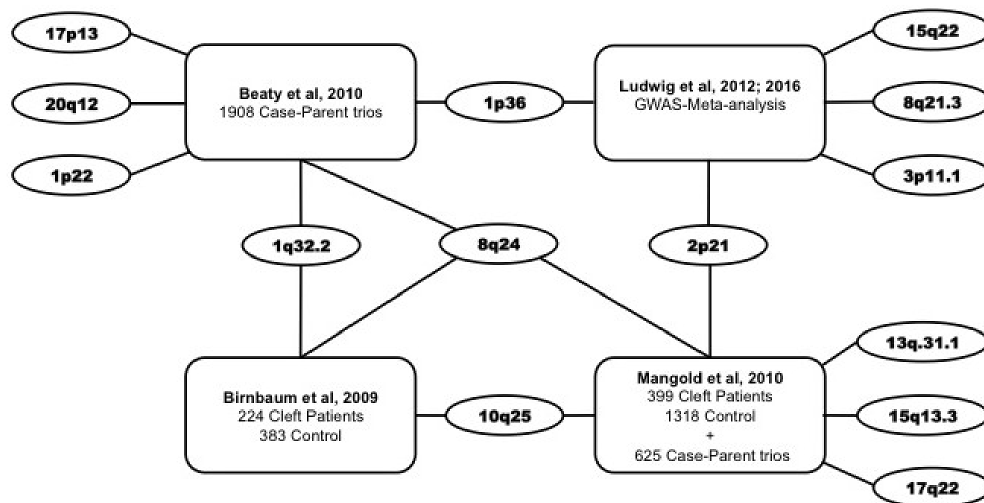

Figure 1. 4 Schematic representation of GWAS risk loci for nonsyndromic CL/P

Adapted from: Birnbaum *et al*., 2009; Beaty *et al*., 2010; Mangold *et al*., 2010; Ludwig *et al*., 2012 and 2016.

The Genome wide association study (GWAS) and linkage study are more inclined to CL/P, with little focus on CP, in order to identify common gene variants. Few studies comparing the nsCL/P and nsCP have found no association between CP and candidate genes for CL/P, further corroborating that these two phenotypes have separate genetic etiology (Bohmer *et al*., 2013). Although the only risk loci for which there is evidence of an overlapping effect for both phenotype is a region on 9q21, which has been initially identified in a linkage study of nonsyndromic clefting families (Marazita *et al*., 2004; 2009). Subsequent, fine mapping identified two SNPs around *FOXE1* that accounted for linkage to both nsCL/P and nsCP (Moreno *et al*., 2009).

At present, only one linkage study (Koillinen *et al*., 2005) and three GWAS (Koillinen *et al*., 2005; Beaty *et al*., 2011 and Leslie *et al*., 2016) study have been done solely on nsCP. The linkage study focused exclusively on nsCP was performed in 24 Finnish family along with GWAS scan in 9 families with extended pedigree (Koillinen *et al*., 2005). Although the outcome of the study was not significant for any gene, but showed suggestive linkage for loci 1p34, 2p24,-p25 and 12q21. Recently, target sequencing of coding region of Grainyhead – like 3 gene (*GRHL3*) showed evidence of association between nsCP and SNP (rs41268753),

(Mangold *et al*., 2016). Moreover, this returned significant by an independent GWAS performed in nsCP/control cohort of European ancestry (Leslie *et al*., 2016).

Role of Environment in Cleft lip and Palate

Given the complexity of the fetal growth, each step becomes crucial and is prone to developmental mischief, it is not surprising that more than 7000 birth defects are known to occur, the common of them is OFC. The cause of OFC is largely a mystery, although some genetic and few environmental perpetrators have been identified.

The basic environment to which a developing fetus is exposed, directly or indirectly banks on any exposure that occurs by the way of mother. However, pinning down the role of environmental agents in OFC remains a daunting challenge, as it is an outcome of an interplay between the gene and environment. Epidemiological data supports the role of environmental risk factors in developing OFC. Lack of sufficient folic acid in the diet for example, is one of the risk factors for OFC, among other maternal environmental exposures implicated for OFC includes other nutrient imbalances (zinc deficiency), maternal smoking and alcohol use.

Maternal exposure to tobacco smoking has been consistently associated with increased risk with clefting and a meta-analysis has suggested a modest relative risk for CL/P; 1.34 (95% CI: 1.25 to 1.44) and for CP; 1.22 (95% CI: 1.10 to 1.35), but a more recent study found an increased relative risk for both CL/P; 1.62 (95% CI: 1.35-1.95) and CP; 1.38 (95% CI: 1.04-1.83), with both studies comparing active smoking against none at all (Little *et al.,* 2004; Butali *et al.,* 2013). With an extension to the role of smoking in OFC, a meta-analysis on passive smoking is reported recently, with relative risk for CL/P; 2.05 (95% CI: 1.27-3.3) and CP; 2.11 (95% CI: 1.23-3.62), with an overall increase risk of 1.5 fold for nsOFC (Sabbagh *et al.,* 2015).

Although, many studies have been conducted to evaluate the role of folate in the prevention of OFC, the data on folate use and its risk in humans remain inconclusive. It has also been shown that folic acid supplementation during the periconceptional period significantly reduces the risk of having a child with OFC (Tolarova, 1982; Czeizel, 1993; Tolarova and Harris, 1995; Wilcox *et al.*, 2007; Wehby and Murray, 2010; Figueiredo *et al.,* 2014). Whereas, several studies on maternal use of folate and risk for OFC have shown no

significant association between the two (Johnson and Little, 2008; Little *et al.,* 2008; Li *et al*., 2012; Butali *et al.,* 2013). However, there has been a reported decrease in OFC in North America since the mandatory fortification of grains with folic acid in the late 1990's (Parker *et al.,* 2010; Saad *et al.,* 2014) this decline is seen only in CL/P, while CPO rates have remained fairly constant (Johnson and Little, 2008).

Maternal alcohol use during pregnancy is a known cause of birth defects associated with the fetal alcohol syndrome, but its role in OFC is not well understood (Bille *et al.,* 2007; Romitti *et al.,* 2007), even though it has been shown in animal models to have a disruptive effect on neural crest cells, which contribute to the development of both lip and palate (Bell *et al.,* 2014). However, several discrepancies between studies on role of alcohol and risk of developing OFC could be an outcome of confounding by other risk factors such as maternal nutritional status, smoking and stress. More through studies are still need to be done to establish the role of maternal alcohol use and nutritional intake as risk for OFC, but obtaining accurate data is challenging and complicated due to multiple environmental risk factors.

Role of Sex or Sex differences in diseases and birth defects

The importance of considering sex differences matters in human physiology and in determining the disease phenotypes, to help shape more effective therapies. A recent review emphasized the differences in the sexes relying on classical hormonal differences and increasing evidence of genetic factors contributing to this difference (Ober *et al.*, 2008). In this context, sex can be considered an 'environmental' variable that could differentially regulate the developmental pathways in the two sexes. Therefore, sex might interact with the genotype in a manner similar to other environmental factors i.e. gene-sex interaction. However, unlike most other environmental factors, sex is easily observable and usually distinct. Such sex-specific genetic architecture suggests new models of susceptibility for common diseases and sheds light on potential mechanisms of sexual dimorphism in human phenotypes (Ober *et al.,* 2008). In humans, sexual dimorphism is observed in the prevalence, course and severity of many common diseases, including cardiovascular diseases (Barrett-Connor, 2007), autoimmune diseases (Whitacre *et al.,* 1999) and asthma (Loisel *et al.,* 2011). Of note, these sex based differences in the prevalence of the disease can be an outcome of hormonal profile or due differences in the genetic architecture of the two sexes.

As evident from previous studies on regulation of the genes in the two sexes being conserved over evolution (Ober *et al*., 2008), a subset of gene expression could be differentially regulated in the sexes giving an expression of important phenotypic differences, including developmental, physiological and/or behavioral in the two sexes. This regulatory differences between males and females, can result in beneficial outcome to each sex or can also contribute to different gene-environment interactions in the two sexes. In turn, such differences might result in sex-specific susceptibility to a disease.

The programming of the intrauterine growth environment is the foundation for the health of neonate. In general, the intrauterine environment is not only governed by pregnant mothers but is also thought to be regulated by the sex of their developing fetuses, where sex of developing fetuses has influenced the development of pathologies such as preterm birth (PTB), pregnancy induced hypertensive disorders (PIHD) and gestational diabetes mellitus (GDM), (Verburg *et al.*, 2016). This reflects that as much as males and females can do the same thing, their biology isn't the same, the severity and prevalence of many diseases may differ between the sexes, alongside the environment they regulate. Hence, we need to be cognizant of these variables and their interaction with the developing environment i.e. sex-environment interactions, while we are analyzing a research data or come up with medical treatments.

Sex differences in a variety of specific birth defects have been observed for over 40 years Gittelsohn and Milham, 1964; Fernando *et al.,* 1978; Lubinsky, 1997) and its prevalence in birth defects in a large population has been reported (Lary and Paulozzi., 2001). Beside this, endogenous sex differences have been found with occurrence of OFC (CL/P and CP) phenotypes, but the cause is mainly unknown. In course, a study on Taiwanese has shown higher prevalence of CL/P with multiple pregnancies, gestational age ≤37 weeks, and lower birth weight (1.5kg), (Lei *et al.,* 2013). His finding showed, male dominance in the CL/P and female in the CP phenotype respectively. This is in accordance with epidemiological data across different populations (Nagase *et al*., 2010; Reddy *et al.,* 2010) for the prevalence of cleft between the two sexes, reflecting a sex-environment regulated neonatal outcome (Weng *et al.,* 2015), for OFC.

The studies on animal model suggest that genotype–sex interactions are widespread (Cunningham, 1900) and that many important genes will be missed if such interactions are

ignored. In context, the present thesis test for genotype–sex interactions in the three association studies, particularly for sexually dimorphic phenotypes such as CL/P and CP. In addition the present thesis also targets the role of sex dependent interaction of infant genotype with mothers environmental exposures, such as periconceptional folic acid consumption and smoking during pregnancy, sex-genotype-environment interaction. Moreover, sex based epigenetic differences on the two sides of cleft tissue specimens is also factored in the present thesis.

Role of Histology in Cleft lip and Palate

Histology is the microscopic study of animal and plant cell and tissues through sectioning, staining and examining under a microscope (Alturkistani *et al.*, 2015). Histology has been demonstrated to provide enhance knowledge in clinical reasoning, disease diagnosis and treatment (Shaw *et al.,* 2012). Histology has helped elucidate many morphological features of different tissues including lip and palate.

Histology and histopathology are correlated, for histopathological interpretation, understanding the normal histology is essential. At first the histological slides are prepared to examine for cells and tissues being healthy or diseased, if there appears variation in the normal tissue morphology the histological slides are categorized as histopathological. In process, it is plausible to state that histology and histopathology is interdependent.

In many instances, histopathological analysis is restricted to anticipated target tissues based on the work priority that may include identification of generalized gross abnormalities; hence majority of tissues considered irrelevant are not analyzed. This non-comprehensive, ascertainment-biased approach indisputably carries the risk of missing important pathological findings and additional phenotype identification. Although, the histological examination of the lip and palate in mouse models has bespoken pivotal role in providing important insights into the morphological consequences and mechanisms of gene functions (Schofield *et al.*, 2011).

Ideally, in humans born with cleft, histology has been an integral part in the screening of different cleft phenotypes. Some studies associated histology with identification of sub-clinical or microform cleft phenotypes in the course of severity and degree of penetrance (Lehman, 1976; Heckler *et al.,* 1979; Martin *et al.,* 1993). In CLP settings, histology has also

been used as confirmatory measure of the true defects in the OO muscles over artifacts observed on images drawn using ultrasound (Rogers *et al.*, 2008). In process, the present thesis makes use of the histological sections from the cleft lip specimens in term to identify changes in the lip muscle making use of an image processing tool to complement better restoration of the cleft lip.

Objective of the Thesis

The above background reflects that even after many years of investigation, the mode of inheritance of isolated CL/P and CP remains controversial and challenging, probably due to the samples and model employed. Progressively, many approaches has been employed for identification of genes contributing to isolated CL/P and CP, ranging from linkage studies to genome wide association studies, but had modest success. The limited success of these studies could be due to restricted genetic approach without employment of environmental factors, which ideally is linked and influence the development of isolated CL/P and CP. Secondly, it can be reasoned to ignorance of sexual dimorphism, while identifying the genes contributing to these etiologies.

Ideally, the CL/P and CP have different developmental origin and considered to be etiologically different varying with environment and sex of an individual. In order to gain insights in the genes associated with the two cleft phenotype with different etiological origin, the present study aims to elucidate the following objectives:

- Part 2. Family based association studies: Identification of genetic and environmental risk factors for isolated CL/P and CP.
- Part 3. Epigenetic regulation: Evaluating global LINE-1 DNA methylation in CL/P tissues in response to mothers environmental factors.
- Part 4. Evaluation of Orbicularis oris muscle: Explore differences in OO muscle arrangement using histological images.

Outline of the Thesis

The workflow of the thesis starts from Part 1 which includes three family based association studies. Chapter 2 deals with association of *TGFA* in nsCL/P and other two studies Chapter 3 and Chapter 4 presents the association of *LOXL3* and *GRHL3* gene in developing nsCPO. A new direction to better identification of nsCL/P etiological factor, by

evaluation of global LINE-1 DNA methylation in human cleft lip tissue in response to mothers environmental factor is reported in Part 3; Chapter 5. This study is the first to include human lip tissue specimen to investigate DNA methylation. The final work of this thesis, Part 4; Chapter 6 is involves the use of cleft lip tissues to evaluate muscle fibre diameter using an inherent and easy to use imaging tool from image analysis software, ImageJ. The last and final part of the thesis Part 5; Chapter 7 is a concise discussion on outcome of each study presented in this thesis and its implications.

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PART TWO

FAMILY BASED ASSOCIATION STUDIES

A. Identification of risk factors for isolated CL/P

Chapter 2

Opposite effect in males and females of a deletion polymorphism in *TGFA* gene in determining the risk of nonsyndromic orofacial cleft

Abstract

Introduction: Orofacial clefts (OFCs), including cleft lip with/without cleft palate (CL/P) and cleft palate (CP) is the most common craniofacial congenital anomalies and can occur in context of syndromic, or as multifactorial nonsyndromic cases (nsOFCs). The *TGFA* gene regulates proliferation and differentiation of palatal epithelial cells during palate closing. The association of 4nt deletion polymorphisms of *TGFA* gene (TaqI variant) to clefting was first documented in 1989. However, after 26 years, it still remains controversial, and a recent GWAS analyses had not included the gene among the major nsCL/P loci.

Objestive: To assess association of newly identified 11nt deletion polymorphism in intron 4 of *TGFA* (11nt-del variant) and TaqI variant with risk of developing cleft, and test for eventual gene-environment interactions.

Methods: We genotyped nsOFC case-parents trios (from EUROCRAN studies) recruited in nine European countries in the period 2001-2014, for *TGFA*/TaqI and *TGFA*/11nt insertion/deletion variant. Genetic association of the two variant was evaluated using transmission disequilibrium test (TDT) and log-linear regression analysis of relative risks (RR) using STATA package. Family-based TDT analysis exhibited no statistical significance for the two *TGFA* variants, but a relatively meaningful association was shown on sex stratification, with a significant under-transmission among males (4nt-del: p=0.007; 11nt-del: p=0.047) and a significant protective effect (RR 0.61 (95%CI 0.42-0.88), p=0.009) of TaqI variant. Instead, females presented a trend towards over transmission of TaqI variant and an increased risk of nsOFC (RR=1.55 (95%CI 0.98-2.44), p=0.06). As regards to the RR, the effect of sex turned out highly significant (p=0.0018). Furthermore, we found no association of the two *TGFA* variants with environmental factors (smoking and folic acid).

Conclusion: Our results provide that the TaqI deletion has opposite effect in the two sexs, suggesting a possible complex interaction between *TGFA* and components of sex-determining factors expressed during embryogenesis.

Introduction

The development of human face is a complex morphogenetic process involving hundreds of genes controlling the coordinated pattern, proliferation and differentiation of tissues having multiple embryological origins (Stephen *et al.*, 2015). The congenital abnormal growth and/or development of the head and facial bones may lead to craniofacial birth defects. The most common of which is the abnormal opening of the lip and/or the roof of the mouth (palate), the Orofacial cleft (OFC).

In the development of lip and palate, the complete formation of the lip occurs during the sixth week of gestation and the palate during the last week of first trimester. Therefore, it is possible to have cleft lip alone (CL) as a single entity or along with cleft palate (CLP). The two thirds ($2/3^{rd}$) of all cases of clefting involves the lip with or without involvement of the palate, whereas one third ($1/3^{rd}$) of all cases occur as an isolated deformity of the palate. In general, the incidence of non syndromic cleft lip with or without palate (nsCL/P) is estimated to be approximately 1 in 700 live birth (Dixon *et al*., 2011) but in Europe the prevalence is about 1/1000 (Lidral and Moreno, 2005). The males predominates in the occurrence of CL/P (60-80% of cases), whereas the females constitute the majority within CP group. (Fogh-Andersen, 1961; Mossey and Little, 2002). Cleft lip and palate deformity is strongly associated with bilateral cleft lips (BCLs, 86% of cases); the association decreases to 68% with unilateral cleft lip (UCL). The left side is most commonly involved in unilateral cleft lip cases. However, the predominance of syndromic forms, defined by the presence of other abnormalities in addition to CL/P is lower in males born with CL/P (Mossey and Castilla, 2001). Curiously, the frequency in females is higher when the father is greater than 40 years (Rittler *et al*., 2004).

The etiology of CL/P is still unclear, where the studies carried in various countries and regions mark it as an outcome of genetic and environmental conditions or a combination of both. In siblings, if the first-born child has got CL/P the chances of the second born having CL/P is 3-4%. If two children within a family have it, the probability of the third child being born with CL/P is 9% (Review-Scotland, 2006). Previous studies have been consistent in finding a positive association of alcohol consumption, cigarette smoking, and vitamin intake as an environmental factors implicated in the aetiology of CL/P (Maestri *et al*., 1997; Werler *et al*., 1999; Itikala *et al*., 2001). CL/P is therefore considered a complex multifactorial disorder (Mangold *et al*., 2011).

The identification of genes involved in the orofacial cleft development can supplement towards a better understanding of the prevention and diagnosis of this developmental anomaly. Therefore, the genetic risk factors of nsOFC has been a subject of intensive research and numerous candidate genes have been identified, one of which is *TGFA* (Ardinger *et al*., 1989, Don and Ma., 2015).

The transforming growth factor alpha (*TGFA*) gene occurs in wide spectrum of normal tissue from pre-implantation period in mouse embryo to adult life (Coffey *et al*., 1987; Rappolee *et al*., 1988; Mead *et al*., 1989; Mydlo *et al*., 1989). The *TGFA* encoded protein binds to the epidermal growth factor receptor (EGFR) situated at the palatal epithelium during palate closing (Jugessur *et al*., 2003; Passos-Bueno *et al*., 2004) which may function as a normal embryonic version of EGF-related growth factor (Lee *et al*, 1985). EGF/*TGFA* and glucocorticoids are believed to regulate the proliferation and differentiation of palatal epithelial cells both *in vitro* and *in vivo* (Feng *et al*, 2014). Moreover, the continued presence of EGF inhibits the fusion process; *TGFA* is likely to have similar effects. These biological studies suggest that polymorphism in the *TGFA* gene might contribute to the development of CL/P. A transmittance of *TGFA* rare allele from heterozygous parents to an offspring in the European descent is well documented (Mitchell, 2000) which further explains the correlation between cleft development and *TGFA* polymorphism. (Miettinen *et al*., 1999).

The *TGFA* gene located on short arm of chromosome 2 (2p13) includes 80 kilobases (kb) of genomic DNA and comprises six exons, coding a polypeptide of 50 amino acids (Vieira and Orioli, 2001). When treated with Taq1 restriction enzyme the *TGFA* gene shows a restriction fragment length polymorphism (RFLP) with mutant allele (C2, 2.7kb fragment) represented by a four-base (TAAT) deletion. *TGFA*/Taq I polymorphism is located at intron 5, 1602bp in the 5' direction of the acceptor site of exon 6 (Vieira, 2006).

Although the association of TGF/Taq1 polymorphism is well characterized and found to be associated with the development of CL/P, there exists an inconsistency in number of studies (Vieira, 2006; Lijia *et al*., 2015) which could be an outcome of both differences in the design of study and population. In weigh, the present study examines the pool of European trios (from EUROCRAN studies) for frequency of transmission of a newly identified *TGFA*/11nt insertion/deletion (rs3830586, distant 960bp of Taq1 deletion located at intron 4, 100bp upstream of Exon 5) along with the common *TGFA*/TaqI insertion/deletion variant to risk of developing nsCL/P. Additionally, the test of gene-environment interactions between

the two *TGFA* insertion/deletion variants and the two common maternal exposures (smoking and folic acid supplementation) during pregnancy are factored in this study.

Materials and methods

Samples

The study recruited 632 nsOFC case-parents trios (from the EUROCRAN studies) from nine European countries in the period 2001-2014, Table 2.1. A written informed consent was taken for all patients from their respective parents. Each trio was associated with detailed clinical phenotype, and a questionnaire was circulated to garner data on environmental factors (mother's, periconceptional supplementation with folic acid and/or exposure to tobacco smoking during the first trimester of pregnancy).

Genotyping

The DNA from nsOFC trios were isolated from peripheral blood and buccal swab using Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH) and QIAamp DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manufacturer's instruction and quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA).

The genotype of *TGFA*/Taq1 and *TGFA*/11nt insertion deletion polymorphism was carried out using 5ng of genomic DNA amplified in 10µL of polymerase chain reaction (PCR) reaction mixture, with final concentrations: dNTP 2.5mM, $MgCl_2$ 50mM, Taq DNA polymerase 5.0U and 20 pmol/µl of each primer. The respective primers selected is quoted in Table 2.2

The PCR conditions followed were: For Taq1 insertion/deletion-Initial cycle of 94°C, 64°C and 72°C followed with 10 cycles of 94°C for 15s, 63°C for 30s and 72°C for 30s with a final 25 cycles of 94°C for 15s, 62°C for 50s, 72°C for 30s and 72°C for 120s. For 11nt insertion/deletion (rs3830586) - Initial cycle of 94°C, 64°C and 72°C followed with 10 cycles of 94°C for 15s, 63°C for 30s and 72°C for 30s with a final 24 cycles of 94°C for 15s, 62°C for 50s and 72°C for 30s, using Thermal cycler 2700 (PerkinElmer, Waltham MA). The amplified PCR product was genotyped on 10% polyacrylamide gel: *TGFA*/TaqI-amplicon size 117bp for insertion and 113bp for deletion and *TGFA*/11nt-amplicon size 189bp for insertion and 178bp for deletion, and visualized using silver staining.

Statistical analysis

The patients with isolated OFC (absence of other non-cleft defects) was used for analysis and graded in accordance to type of cleft. The genetic association of the two insertion/deletion polymorphisms (Taq1 and 11nt) in *TGFA* gene was evaluated using transmission disequilibrium test (TDT) (Speilman *et al*., 1993). The relative risks associated with interaction between genotypes and environmental factors (periconceptional folic acid supplementation or exposure to tobacco smoking during early pregnancy) was evaluated with log-linear model using STATA software. For all analysis, homozygous and heterozygous variant genotype was compared with homozygous wildtype genotype as reference. Moreover, the parent-of-origin effect was evaluated for each variant.

Results

The TDT analysis showed no notable evidence of asymmetric segregation of deletion alleles from parents of cleft cases, and log-linear regression analyses resulted in no significant relative risk (Figure 2.1) for nsOFC associated with *TGFA* genotypes. Table 2.3. However, stratification by case sex revealed a significant under-transmission of deletion alleles among males (TaqI: p=0.007; 11nt-del: p=0.047, Figure 2.2) and a significant protective effect of TaqI deletion (RR 0.61 (95%CI 0.42-0.88), p=0.009). Instead, females presented a trend towards over-transmission of TaqI deletion and an increased risk of nsOFC (RR=1.55 (95%CI 0.98-2.44), p=0.06). As regards to the RR, the effect of sex turned out highly significant (p=0.0018); Figure 2.2, Table 2.3.

Furthermore, stratification on cleft types reveled a significant trend of transmission for *TGFA*/TaqI in CL/P and CPO, with an under-transmission in males (CL/P, p=0.046; CPO, p=0.033) and over-transmission in females. But the calculated relative risk in male carriers of *TGFA*/TaqI display a significant protective effect for CPO (RR=0.24 (95%CI (0.08-0.74), p=0.013); Figure 2.2.

The two *TGFA* deletion variant is not significantly affected by maternal periconceptional folic acid supplementation during early pregnancy, Figure 2.3. While the cleft subtypes reveals, a significant under-transmission of *TGFA*/11nt in cases with CL/P and CLP born to mothers unexposed to folic acid (CL/P, p=0.039; CLP, p=0.033); Table 2.4. For mothers who reported smoking, there appeared no evidence of nsCL/P risk with infant genotype, Figure 2.4.

Discussion

The susceptibility of an individual gene could play an important role in the development of most diseases. Therefore, studies of genetic susceptibility are helpful for disease prevention and diagnosis. In milieu, numerous candidate genes have been identified as genetic risk factor of nsCL/P one of which is *TGFA* reported long back by Ardinger *et al*., 1989 in Caucasian population. From then on, growing number of studies have resulted statistically significant for association of *TGFA*/TaqI polymorphism and risk of nsCL/P such as Desai *et al*., 2014; in an Indian population, Holder *et al*., 1992; in a British population, Jara *et al*., 1995; in a South American population, Tanable *et al*., 2000; in a Japanese population, Lu *et al*., 2013; in a Chinese population and Letra *et al*., 2012; in a Brazilian population. However the present study is in contrary, but likewise in accordance with study done by Lindral *et al*., 1997; in the Phlipines and Hecht *et al*., 1991; in Rochester.

Ardinger *et al*. (1989) suggested the association of *TGFA* gene and the DNA sequences in an adjacent region in cleft pathogenesis. Since, many variants in *TGFA* gene has been identified contributing to cleft, no studies have been conducted to mark the association of *TGFA*/11nt insertion/deletion variant. Therefore, in addition to the common TaqI variant the present study included a new insertion/deletion variant (11nt) of *TGFA* gene for their association to risk of nsCL/P in European population. In addition, the maternal environmental exposure (smoking and folic acid supplementation) is factored in the study.

At the overall analysis, the genetic association between the two *TGFA* variants and nsOFC seems primarily inclined towards 4nt-del (TaqI) in intron 5 rather than to the 11nt-del in intron 4 of the gene. On the analysis by sex, we noted an opposite effect in males and females, with an under-transmission in males and an over-transmission in females for *TGFA*/TaqI variant. Moreover, after stratifying on cleft types, significant trend was found in CL/P and CPO subgroup for *TGFA*/Taq1, with both showing significant under transmission in males and over transmission in females. Whereas the relative risk was more significant in CPO for *TGFA*/TaqI polymorphism. Among the mothers reported for smoking and/or peri-conceptional folic acid supplementation, we observed no evidence of nsCL/P risk with infant genotype. However, the cleft subtypes showed significant under transmission of *TGFA*/11nt in cases born with CL/P and CLP to non folic acid supplemented mothers.

The exclusion of significant genetic association of *TGFA*/TaqI may have gone unnoticed in the present study, until stratified by sex for nsCL/P and nsCPO in EUROCRAN population. This infers, a possible complex interaction between *TGFA* and components of sex-determining factors expressed during embryogenesis, leading to study sex difference as a factor influencing and modifying genetic risk. This could provide clues that will aid the development of targeted prevention and intervention efforts contributing to the etiology of Cleft.

It may seem implausible, but there are examples of genetic association in opposite direction in each sex. (Ober *et al.*, 2008). In humans, sexual dimorphism is observed in the prevalence, course and severity of many common diseases, including cardiovascular diseases, autoimmune diseases and asthma. One of the most remarkable sex specific association concern is *IFN*-gamma in asthma, with a heterozygous genotype, but with opposite direction in males and females (Loisel *et al*., 2011). Based on these studies and our result it appears reasonable to suggest differential gene regulation mechanism in males and females that can influences human phenotypes, including reproductive, physiological and disease traits. The influence of sexual dimorphism in gene association studies of nsOFC is generally ignored. Hence, this study provides a new directional approach of considering sexual dimorphism as one of the factors influencing a gene, and should be taken into consideration while conducting new or re-analysis of conventional GWAS.

In conclusion, our results provide evidence that the TaqI deletion has opposite effect in the two sexs. While the endogenous causes of the sex differences observed in nsOFC are largely unknown, the situation in other diseases is not much different. On the other hand, the present study values the influence of sex differences in nsOFC disease susceptibility, and provide information to research's to re-analyze the GWAS complete datasets to uncover sex-specific associations, which may have been totally missed if males and females had associations in opposite directions as presented in this study.

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Table 2. 1 Sex and Cleft type of cases included in the study

	OFC		CL/P		CL				CLP		CPO	
TGFA							**UCL/P**		**BCL/P**			
Taq1	632		476		178		224		75		156	
	male	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*	*female*
	359	258	305	163	108	69	146	77	51	20	53	95
11nt del	368		284		105		145		33		84	
	male	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*	*female*
	198	156	179	103	65	40	91	53	24	8	18	63

CL/P, cleft lip with or without palate; CL, cleft lip; CLP, cleft lip and palate; UCL/P, unilateral cleft lip and palate; BCL/P, bilateral cleft lip and palate; CPO, cleft palate only

Table 2. 2 Oligonucleotide sequence used for Genotyping

TGFA Gene	Sequences	Amplicon Size
TaqI	5’-GAATTTGGCCTATGAAAGGTCT-3’	
	5’-ACATCCTGATGTCTTCATGGAT3’	117bp
11nt del	5’-AGCAGCTGCAAAGACACAGA-3’	
	5’-AAACCACTGCCCTACAATGC-3’	189bp

Table 2. 3 A sex based Transmission disequilibrium test (TDT) for the transmitted and non-transmitted *TGFA*/Taq1 and *TGFA*/11nt deletion variants in children with different cleft subgroups

TGFA	**Taq1**		**11nt del**	
	T/NT	**p-value**	**T/NT**	**p-value**
OFC	107/120	0.388	44/57	0.196
male	51/82	**0.007**	21/36	0.047
female	50/33	0.062	21/21	1.000
CL/P	82/88	0.645	31/43	0.163
male	45/66	**0.046**	20/43	0.157
female	33/19	0.052	10/12	0.670
CL	35//37	0.814	13/10	0.532
male	18/26	0.228	6/7	0.782
female	16/10	0.239	7/3	0.206
CLP	47/51	0.686	18/33	**0.036**
male	27/40	0.112	14/23	0.139
female	17/9	0.117	3/9	0.083
UCL/P	35/39	0.642	18/21	0.631
male	21/32	0.131	10/13	0.532
female	13/7	0.180	7/7	1.000
BCL/P	14/13	0.847	5/9	0.285
male	6/10	0.317	5/6	0.763
female	6/3	0.317	0/3	0.083
CPO	25/32	0.354	13/14	0.847
male	6/16	**0.033**	1/5	0.102
female	17/14	0.590	11/9	0.655

Bold form indicates $p < 0.05$

Table 2. 4 Transmission disequilibrium test (TDT) for the transmitted and non-transmitted *TGFA*/Taq1 and *TGFA*/11nt deletion variants in children of different cleft subgroup based on maternal environmental exposures.

TGFA	**Taq1**		**11nt del**	
	T/NT	**p-value**	**T/NT**	**p-value**
OFC				
folic acid (yes)	81/86	0.699	35/39	0.642
folic acid (no)	26/35	0.249	9/19	0.059
smoking (yes)	26/31	0.508	11/16	0.336
smoking (no)	79/90	0.397	31/41	0.239
CL/P				
folic acid (yes)	62/62	1.000	26/29	0.686
folic acid (no)	20/26	0.376	5/14	**0.039**
smoking (yes)	21/24	0.655	8/13	0.275
smoking (no)	60/64	0.719	22/29	0.327
CL				
folic acid (yes)	29/29	1.000	11/7	0.346
folic acid (no)	6/8	0.593	2/3	0.655
smoking (yes)	5/11	0.134	2/3	0.655
smoking (no)	30/26	0.593	11/7	0.346
CLP				
folic acid (yes)	33/33	1.000	15/22	0.250
folic acid (no)	14/18	0.480	3/11	**0.033**
smoking (yes)	16/13	0.577	6/10	0.317
smoking (no)	30/38	0.332	11/22	0.056
UCL/P				
folic acid (yes)	26/28	0.785	14/12	0.695
folic acid (no)	9/11	0.655	4/9	0.166
smoking (yes)	6/8	0.593	3/4	0.705
smoking (no)	28/31	0.696	14/16	0.715
BCL/P				
folic acid (yes)	10/9	0.819	4/7	0.366
folic acid (no)	4/4	1.000	1/2	0.564
smoking (yes)	3/3	1.000	3/2	0.655
smoking (no)	11/10	0.827	2/7	0.096
CPO				
folic acid (yes)	19/24	0.446	9/9	1.000
folic acid (no)	6/8	0.593	4/5	0.739
smoking (yes)	5/7	0.564	3/3	1.000
smoking (no)	19/25	0.366	9/11	0.655

Bold form indicates $p < 0.05$

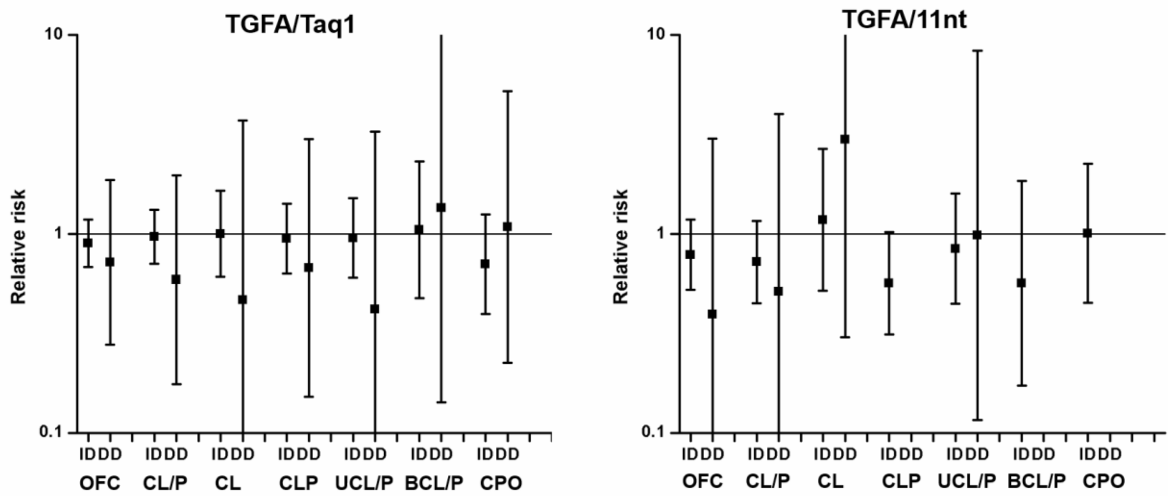

Figure 2. 1 Relative risk of *TGFA* Taq1 and *TGFA* 11nt-del in cleft sub-types

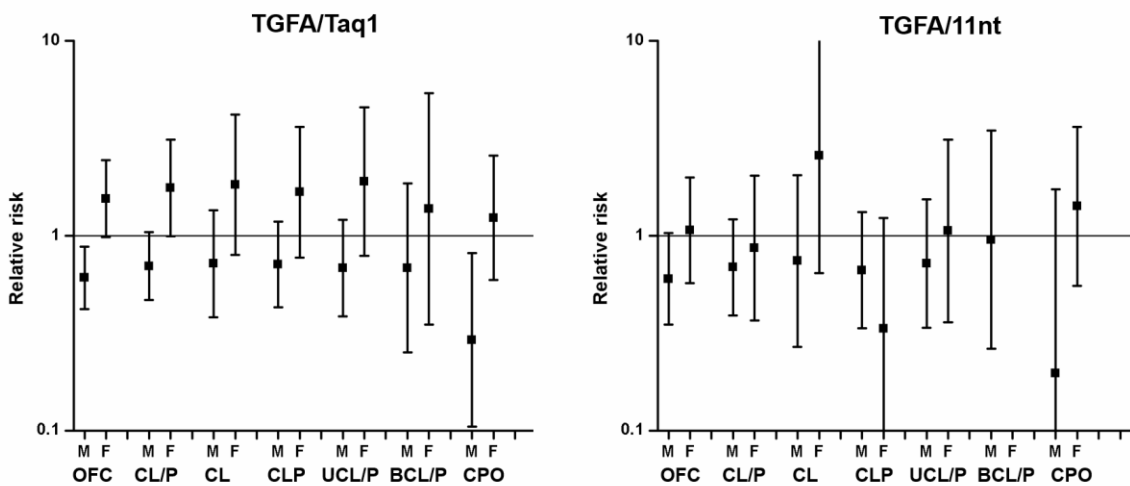

Figure 2. 2 Relative risk of *TGFA* Taq1 and *TGFA* 11nt-del in males and females

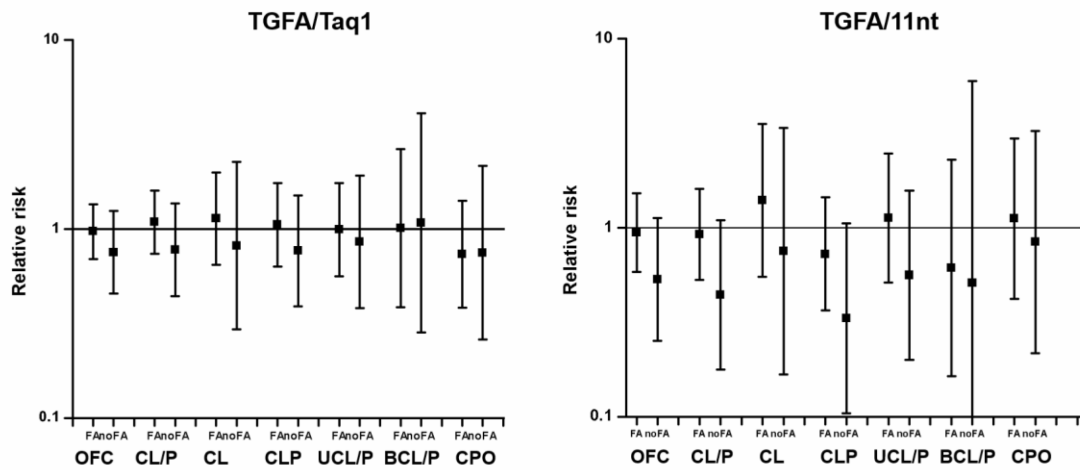

Figure 2. 3 Relative risk of *TGFA* Taq1 and *TGFA* 11nt-del with folic acid supplemented mothers

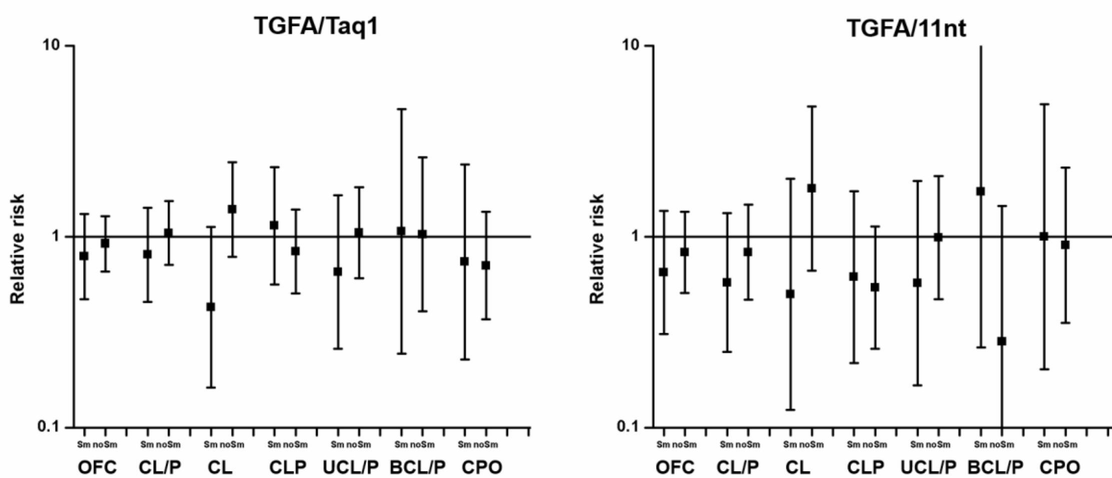

Figure 2. 4 Relative risk of *TGFA* Taq1 and *TGFA* 11nt-del with smoking mothers

B. Identification of risk factors for isolated CP

Chapter 3

Lysyl Oxidase like 3 (*LOXL3*) in susceptibility of developing nonsyndromic cleft palate

Abstract

Introduction: The lysyl-oxidase like-3 (*LOXL3*) is a copper dependent amine oxidase found to play an important role in collagen cross linking and maintaining its stability. Previous research has established that inhibition or deletion of this gene can lead to perinatal lethality and development of cleft palate in mice. Additionally, a missense variant in human *LOXL3* has been identified in a family with autosomal recessive Stickler syndrome, characterized with cleft palate. This accounts for possible link between *LOXL3* and developing isolated cleft palate.

Objective: We explore a missense variant (rs17010021) in *LOXL3* gene as susceptibility marker for developing isolated cleft palate and test for gene-environment interaction.

Methods: We genotyped nsCPO case-parents trios (from EUROCRAN studies) collected from nine European countries in the period 2001-2014, for *LOXL3* missense (rs17010021) variant. The variant selection was done using UCSC and Ensembl genome browser based on MAF≥2 with predictable deleteriousness on PolyPhen2. Genetic association of the variant was evaluated using transmission disequilibrium test (TDT) and log-linear regression analysis for relative risks (RR) using STATA package.

Results: The TDT result showed no significant segregation of minor allele from parent to off-springs. There was no risk with mothers genotype in developing nsCPO. A child carrying two minor alleles (AA) had an increased risk (RR=4.80 (95%CI 1.21-19.09), p=0.026) for developing CP compared to child carrying one minor allele (TA), (RR=0.54 (95%CI 0.27-1.09), p=0.086). The calculated parental asymmetry showed increased transmittance of minor allele (A) from father (p=0.052). Furthermore, on classifying based on sex the trend was more in females (RR=9.78 (95%CI 2.50-38.26), p=0.001).

Conclusion: The conclusion drawn from our present study is nominal for the transmittance of minor allele from the father and increased risk with infant genotype. Although the study entails the sex based segregation of minor allele for the present variant, it may seem plausible to identify more functional variant in *LOXL3* gene. The observed results should be considered with austerity and should be replicated using large set of case-parent trios to get substantial validation for its association in developing CP.

Introduction

Before the end of sixth week the medial nasal process merge with the maxillary process to form upper lip and the primary palate. While the secondary palate develops from bilateral, medially directed outgrowths of the maxillary processes, first grow vertically on either side of the tongue. Subsequently, the palatal shelves elevate to a horizontal position above the tongue characterized by robust cell proliferation and synthesis of extracellular matrix (ECM) proteins such as hyaluronic acid (HA), which has evident role in palatal shelf remodelling (Wilk *et al.* 1978; Brinkley and Vickerman 1982). This remodelling process undergoes a series of morphogenetic movements that brings the medial edge epithelium to contact one another and commence fusion. Disruption of any steps up to and including fusion can lead to a cleft palate.

Isolate cleft palate (CPO), the rarest of the congenital orofacial malformation varies essentially with geography and ethnicity affecting 1 to 25 per 10,000 live birth, with the highest in non-Hispanic Whites and lowest in Africans (Burg *et al.,* 2016). In addition, the frequency of CPO differs by sex with 1:2 male to female ratio. Approximately, 50% of CPO is inclined to its unique isolated occurrence as nonsyndromic cleft palate and the remaining half is often found associated with other genetic syndrome or malformation (Mai *et al*, 2014; Watkin *et al.,* 2014). Recent research links both syndromic and nonsyndromic CPO as part of single spectrum, as some genes responsible for syndromic CPO may also be candidate genes for nonsyndromic CPO (Stanier and Moore, 2004), due which the etiology of the CPO is much more complex, linked both to genetic and environmental factors. Whereas, the influence of genetics in CPO is high with increased recurrence rate among first-degree relatives of affected individuals, as evident from studies in Norway and Denmark (Siversten *et al.,* 2008; Grosen *et al.,* 2010).

Although, the mechanism driving the palatal shelf elevation is unknown but it has been suggested that palatal epithelium, mesenchymal cells and extracellular matrix (ECM) is important for this process, where the palate ECM infrastructure mainly includes collagen, fibronectin and hyaluronic acid (Morris-Wiman and Brinkley, 1993). Nevertheless, these ECM components and in particular the type and amount of collagen remodelling, is essential for determining the mechanical properties of the oral soft connective tissue (Cornelissen *et al.*, 2000).

Several genetic factors have been implicated in nonsyndromic oral clefts, mainly focused on cleft lip and/or palate (CL/P) and little on CPO. However, a single nucleotide polymorphism (SNP) near *FAF1* gene has been shown to be associated with CPO in European trios (Ghasibe-Sabbagh *et al.,* 2011). Whereas, this *FAF1* locus is partially associated with heritability of CPO and hence we hypothesize that other genetic risk factor may exist.

In order, we explored a gene from lysyl oxidase (LOX) family, the Lysyl oxidase like-3 (*LOXL3*). The *LOXL3* is more similar to *LOXL2* and *LOXL4* in structure, located on chromosome 2. It is a copper dependent amine oxidase, which catalyzes the cross-linking of various tropocollagen molecules to form collagen fibrils (CFs). Collagen cross-linking can augment CFs stability (Shoulders *et al.,* 2009). Previous study have reported development of cleft palate on inhibition of LOXs by a natural inhibitor β-aminopropionitrile (BAPN), (Pratt *et al*, 1972). This indicate a need to understand the significant role of LOXs based collagen cross-linking in the etiology of developing cleft palate.

Recent evidence suggest perinatal lethality and impaired development of the palatal shelves with deletion of *LOXL3* gene in a mice model (Zhang *et al.,* 2015). In addition *LOXL3* and its variant *LOXL3*-sv1 is suggested to have a possible functional role in bone development or maintenance (Lee *et al.,* 2006). Furthermore, a missense variant in human *LOXL3* has been identified in a family with autosomal recessive Stickler syndrome (Alzahrani *et al.,* 2015). Although, the Stickler syndrome is commonly caused by mutations in different collagen genes and exhibit cleft palate, the recent association of *LOXL3* in Sticker syndrome indicate a possible link between *LOXL3* based collagen remodeling and developing cleft palate. Despite the importance of *LOXL3* in developing cleft palate in mice, there remains a paucity of evidence in humans, marking *LOXL3* as candidate gene implicated in CP development. Therefore, we speculated *LOXL3* as possible candidate gene in developing nsCPO. Based on this hypothesis, we inspected as many as 25 missense variant in the *LOXL3* gene and selected a missense variant with the highest MAF (MAF ≥ 13%, T>A) located on exon 10, with an assigned rs17010021, which convert isoleucine to phenylalanine. We evaluated case-parent trios from European ancestry for its association with nsCPO. Additionally, we factored the influence of parental imprinting.

Materials and Methods

Samples

The study recruited 390 nsCPO case-parent trios of European descent under the aegis of European collaboration on craniofacial anomalies (EUROCRAN and ITALCLEFT) network. The written and informed consent was taken from all the patients and their respective parents.

Selection of functional Variant

The selection of *LOXL3* functional variant was done using UCSC Genome browser assembly hg/19 (https://genome-euro.ucsc.edu/) and Ensembl genome browser 86 (http://www.ensembl.org/) and subsequently filtered missense variant with minor allele frequency, MAF ≥ 2. On screening we selected one missense variant on exon 10, with MAF ≥ 13 having an assigned dbSNP rs17010021 (dbSNP146), as a predictable functional marker associated with nsCPO. In addition we garnered the prediction of functional effects of rs17010021 using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) and variant table option of Ensembl genome browser 86.

Genotyping

The DNA of trios were isolated from peripheral blood and buccal swab using Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH) and QIAamp DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manufacturer's instructions and quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA).

TaqMan allelic discrimination assays were performed for a missense variant (rs10017021) located on exon 10 of *LOXL3* gene as per standard protocols from the manufacturer (Applied BioSystems, Foster City, CA).

Statistical analysis

The patients with isolated CPO were used for analysis and genetic association of *LOXL3* variant was evaluated using transmission disequilibrium test (TDT) (Speilman *et al.,* 1993). Using a log linear model we screened for possible parent-of-origin effect which access on risk increment (I_M) in the offspring and entails on the allele transmitted from the mother compared to the father (Weinberg *et al.*, 1998; Wilcox *et al.,* 1998). In addition, the relative risks (RR) associated with the infants genotype was also calculated using a Log-Linear model.

Results

The screening the *LOXL3* gene for possible missense variant with MAF≥2 resulted in three polymorphism (MAF≥2.5, rs73949682 and rs73949683 and MAF≥13, rs17010021). Among the three we selected one with highest MAF and high predicted deleteriousness (PolyPhen2, http://genetics.bwh.harvard.edu/pph2/). The genetic association was evaluated using Transmission Disequilibrium Test (TDT) which revealed no significant segregation of minor allele from the parent to the off-spring. Table 3.1. The log linear analysis showed no risk with mother's genotype in developing nsCPO. Figure 3.1. Whereas, the child carrying two minor allele (AA) was at increased risk (RR=4.80 (95%CI 1.21-19.09), p=0.026) compared to the child carrying one minor allele (RR=0.54 (95%CI 0.27-1.09), p=0.086), Figure 3.2. In addition, we calculated the parental asymmetry, a test to evaluate the contribution of each parent in transmitting the minor allele and our result showed a significant transmittance of the minor allele from the father (p=0.052), Table 3.2. Furthermore, on stratifying the case sex, the trend of developing nsCPO was more inclined to females (RR=9.78 (95%CI 2.50-38.26), p=0.001). Figure 3.3.

Discussion

The candidate gene studies have been the core of cleft research and its identification has traditionally relied on gene expression and developmental analysis in model organisms, particularly the mouse. More recently, extrapolation from the study of syndromic forms of CL/P or CPO has proven to be a useful supplement to this approach (Dixon *et al.,* 2011).

Abnormal *LOXL3* function has been reported for multiple types of defects in humans as well as in animals. A recent study identified a homozygous missense mutation (c.2027G>A, p.C676Y) in exon 12 of the *LOXL3* gene as the cause of autosomal recessive Stickler syndrome in a consanguineous family, with cleft palate phenotype (Alzahrani *et al.,* 2015). In animal studies, deletion of lox3b in zebrafish led to craniofacial abnormalities (van Boxtel *et al.,* 2011), and *Loxl3*$^{-/-}$ mice demonstrated development of cleft palate. Therefore, to our knowledge, the present study is the first to evaluate *LOXL3* missense variant as functional marker in etiology of isolated cleft palate. However, deleterious mutation in *LOXL3* are rare and less likely prevalent among large populations.

To identify the genetic marker within the *LOXL3* for developing isolated cleft palate, the screening of missense variant obtained using UCSC Genome Browser (https://genome-euro.ucsc.edu/) and filtered based on minor allele frequency (MAF>2%) resulted in selection of three missense variant (rs73949682, rs73949683 and rs17010021). However, out of the three we ruled out the two variants with lower MAF (MAF≥2.5, rs73949682 and rs73949683) and preferred one missense variant with the highest MAF (MAF≥13%, rs17010021) located on exon 10 of the gene. Subsequent, analysis of rs17010021 via the variant effect predictor generated strong evidence of deleteriousness (Polyphen-2, http://genetics.bwh.harvard.edu/pph2/).

Our result demonstrate no significant association of the *LOXL3* missense variant (rs17010021) with nsCPO. In addition, we observed no risk with mothers genotype but we observed a higher transmittance of minor allele (A) from the father to the affected child. Furthermore, we observed a sex based difference with an inclination toward females. Overall, a child with AA genotype of rs17010021 conferred an increased risk, whereas a child with TA genotype were protective for developing nsCPO. Moreover, all infants carrying AA genotype were females, this could be by chance or may be the case of sex dependent transmission of minor allele.

It is known that common variants usually contribute to complex diseases such as nsCPO with modest effect (Manolio *et al.,* 2009). The genetic effect of an individual rare variant such as rs17010021 might not be statistically significant with sample paucity. As such, statistical significance of rs17010021 can be validated using large case-parent trios. Although, our present study, focused on discovering rs17010021as a potential functional variant in risk of developing nsCPO. Alternatively, it may seem plausible identifying more functional variants of *LOXL3*.

In conclusion, we selected the missense variant in *LOXL3* and performed the first association study with nsCPO case-parent European trios. Our result provides nominal evidence on transmittance of minor allele from the father and increase risk with infant genotype. This study also entails the influence of sex based segregation of minor alleles. Currently, the sex dependent and parental based preferential transmission of *LOXL3* missense variant should be considered with prudence for its association with nsCPO and should be replicated using large set of case-parent trios to get substantial validation.

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Table 3. 1 Transmission disequilibrium test (TDT) for the transmitted and non-transmitted *LOXL3* missense variants in children with CPO alongside maternal environmental exposures

LOXL3							
rs17010021	**CPO**	**Sex**		**Folic acid**		**Smoking**	
T>A		**M**	**F**	**Y**	**N**	**Y**	**N**
T	17	5	12	10	6	4	13
NT	22	8	14	15	7	4	18
TDT	0.64	0.69	0.15	1	0.08	0	0.81
p-value	0.423	0.405	0.695	0.317	0.782	1	0.369
*Significance at p < 0.05							

Table 3. 2 Parental asymmetry test

LOXL3	
rs17010021	**CPO**
T>A	
Mother	3
Father	10
PAT	3.77
p-value	**0.052**

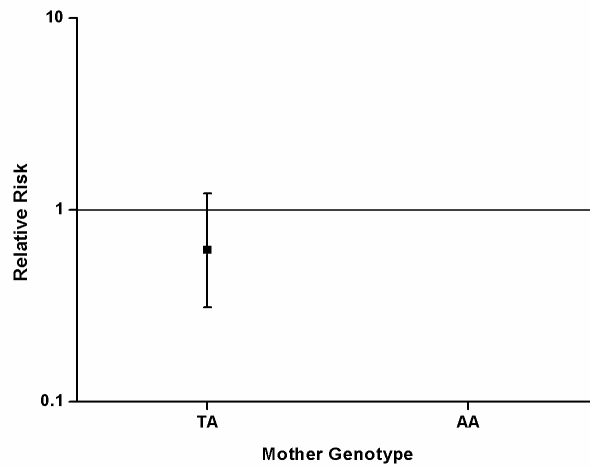

Figure 3. 1 Relative risk of *LOXL3* variant with mothers genotype

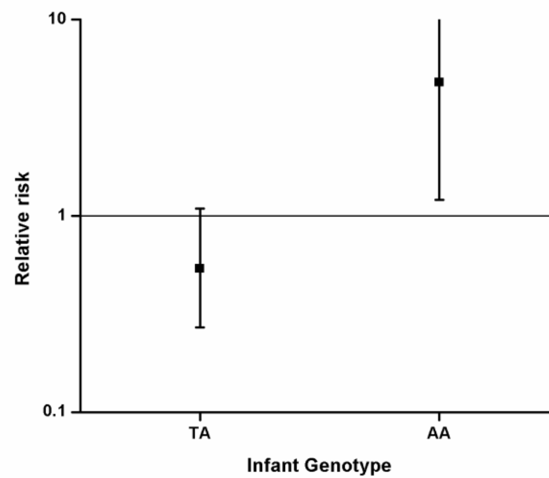

Figure 3. 2 Relative risk of *LOXL3* variant with infant genotype

Infant with one minor allele is at reduced risk and infant homozygous for minor allele at increased risk

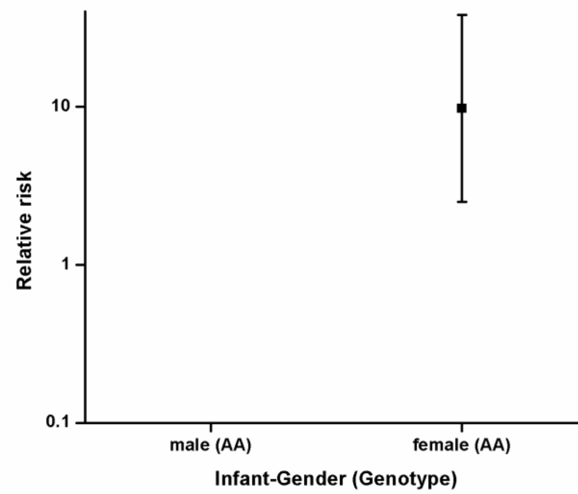

Figure 3. 3 Relative risk of *LOXL3* variant with infant sex

The figure shows a complete biased risk towards female

Chapter 4

Replication of Grainyhead like 3 (*GRHL3*) in susceptibility of developing nonsyndromic cleft palate

Abstract

Background: The Grainyhead-like 3 (GRHL3) encodes a transcriptional factor in the grainyhead family with multiple conserved role in the developmental processes including neural tube closure, epidermal barrier formation, wound healing and craniofacial development. This gene and its missense variant (rs41268753) has been recently conferred risk for developing nonsyndromic cleft palate only (nsCPO).

Objective: To replicate and test the effect of this missense variant for their association in developing nsCPO and test for gene-environment interaction.

Methods: We genotyped nsCPO case-parents trios (from EUROCRAN and ITALCLEFT studies) collected from nine European countries in the period 2001-2014. Genetic association of the variant was evaluated using transmission disequilibrium test (TDT) and log-linear regression analysis for relative risks (RR) using STATA package.

Results: The TDT result showed significant segregation of minor allele from parent to off-springs ($\chi2$= 7.68; p=0.006). Moreover, there appeared a significantly higher transmission of minor allele in female infants (($\chi2$= 6.76; p=0.009). There appeared no risk with mothers genotype in developing nsCPO. While the risk of gene-variant was influenced by infant genotype, where an infant carrying two minor alleles (TT) had 3-fold increased risk RR=14.93 (95%CI 3.21-69.43); p=0.001) for developing nsCP compared to infant carrying one (CT) minor allele, (RR=2.05 (95%CI 1.07-3.94); p=0.031). Moreover, when classified based on sex, the gene-variant showed biased trend towards females (RR=15.04 (95%CI 3.58-63.09); p<0.001). Furthermore, for GRHL3 variant, infants born to mothers without periconceptional folic acid supplementation and non-smoking mothers were at significant risk (RR=25.54 (95%CI 3.11-n/c); p=0.003) and smoking (RR=15.91 (95%CI 3.00-84.21); p=0.001).

Conclusion: Our study provides additional evidence that missense variant (rs41268753) in GRHL3 contribute to the risk of developing nsCPO.

Introduction

Collectively, the cleft lip with or without palate (CL/P) and cleft palate only (CPO) is one of the most common and recognizable birth defect, with an incidence of 1 in 500 (Mossey *et al*., 2009) and 1 in 2500 live birth, respectively (Burg *et al*., 2016). These two phenotypes are considered syndromic or nonsyndromic based on their occurrence with or without other malformations. Around 25% of CL/P and 50% of the CPO cases are found to occur in combination with other malformation or as part of syndrome and hence considered as syndromic cleft (Jones., 1998; Leslie *et al*, 2016). Whereas, the remaining CL/P and CPO often has a nonsyndromic occurrence with a heterogeneous etiology including both genetic and environmental contributions (Mangold *et al*., 2011).

The few genetic studies comparing the nsCL/P and nsCPO have found no association between CPO and candidate genes for CL/P, demonstrating that these two phenotypes have separate genetic etiology (Bohmer *et al*., 2013). The only risk loci for which there was evidence of an overlapping effect for both phenotype is a region on 9q21, which has been initially identified in a linkage study of nonsyndromic clefting families (Marazita *et al*., 2004; 2009). Subsequent, fine mapping identified two SNPs around FOXE1 that accounted for linkage to both nsCL/P and nsCP (Moreno *et al*., 2009).

The CL/P and CP can occur in context of complex malformation syndrome. One of the most common of these is Van der Woude syndrome (VWS, [MIM 119300]), caused by mutation in IRF6 gene (Kondo *et al*., 2002). Further mutation screening in VWS families without IRF6 mutation demonstrated GRHL3 as second gene reported with mutations resulting in VWS characterized by orofacial cleft (OFC) and lower lip pit (Peyrard-Janvid *et al*., 2014).

GRHL3 has four known transcripts and is considered indispensable in the courses of neural tube closure (Ting *et al*., 2003a; Ting *et al*., 2003b; Wilanowski *et al*., 2002), skin barrier formation (Ting *et al*., 2005), wound healing (Caddy *et al*., 2010) and craniofacial development (Peyrard-Janvid *et al*., 2014; Dworkin *et al*., 2014). Functional analyses has shown Grhl3 as key downstream target of Irf6 in the process of periderm differentiation, but no genetic interaction between IRF6 and GRHL3 has been observed in mouse (de la Garza *et al*., 2013). Whereas, phenotypic analyses of murine embryos with double heterozygous knockout (Irf6+/-; Grhl3+/-) indicated that both genes play pivotal roles in the development of the functional periderm, and disturbing this process leads to VWS (Peyrard-Janvid *et al*.,

2014). Taken together, previous studies demonstrate mutations in both IRF6 and GRHL3 cause almost the same clefting phenotypes.

Though, analysis of GRHL3 mutant has not been reported but loss of GRHL3 in mouse results in oral adhesions and aberrant elevation of the palatal shelves, leading to cleft palate (ECLAMC). Moreover, the recent sequencing approaches has indicated a missense variant (rs41268753) in the coding region of GRHL3 gene as predictive marker of nsCPO (Mangold *et al*., 2016). This was independently confirmed in GWAS study on nsCPO case-control cohort from European ancestry (Leslie *et al*., 2016). In trail, the present study is a replication of the current GWAS outcome to confirm missense variant (rs41268753) in GRHL3 as susceptibility marker in the etiology of developing nsCPO. Furthermore, the test of gene-environment interaction between the GRHL3 missense variant and the two common maternal exposures (smoking and folic acid supplementation) during pregnancy are factored in the current study.

Materials and Methods

Samples

The study recruited 390 nsCPO case-parent trios of European descent under the aegis of European collaboration on craniofacial anomalies (EUROCRAN and ITALCLEFT) network. The written and informed consent was taken from parents for their respective children.

Genotyping

The DNA of trios were isolated from peripheral blood and buccal swab using Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH) and QIAamp DNA Blood Mini Kit (QIAGEN, Hilden DE) according to the manufacturer's instructions and quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA). TaqMan allelic discrimination assays were performed for a missense variant (rs41268753) located in exon 11 of *GRHL*3 gene as per standard protocols from the manufacturer (Applied BioSystems, Foster City, CA).

Statistical analysis

The cases with nsCPO were used for analysis and genetic association of *GRHL*3 missense variant (rs41268753) was evaluated using transmission disequilibrium test (TDT) (Speilman *et al.,* 1993). In addition, the relative risks (RR) associated with interaction of

infacts genotype and mothers environmental factors (periconceptional folic acid supplementation or exposure to tobacco smoking during early pregnancy) was calculated using a Log-Linear model with STATA package.

Results

The genetic association was evaluated using transmission disequilibrium test (TDT) which showed a significant segregation of minor allele from the parent to the off-spring (χ^2= 7.68; p=0.006). Moreover, there appeared a significantly higher transmission of minor allele in female infants ((χ^2= 6.76; p=0.009), Table 4.1.

The log linear analysis showed no risk with mothers genotype in developing nsCPO. Whereas, under co-dominant model the risk of rs41268753 gene-variant was influenced by infant genotype with a significantly three fold increased risk for infants carrying two minor alleles (RR=14.93 (95%CI 3.21-69.43); p=0.001) compared to one minor allele (RR=2.05 (95%CI 1.07-3.94); p=0.031). Figure 4.1. Assuming a recessive model, the risk still returned significant for infants homozygous for minor allele (RR=6.66 (95%CI 1.84-24.01); p=0.004).

Moreover, when classified based on sex, the risk was completely biased towards female infants homozygous for the minor allele (RR=15.04 (95%CI 3.58-63.09), p<0.001), Figure 4.2A, whereas female infants with one minor allele showed a non-significant trend for risk (Female: RR=2.03 (95%CI 0.87-6.11); p=0.095). Figure 4.2B.

Furthermore, for *GRHL*3 variant, infants born to mothers without folic acid supplementation showed significant increased risk (RR=25.54 (95%CI 3.11-n/c); p=0.003), Figure 4.3; and smoking (RR=15.91 (95%CI 3.00-84.21); p=0.001), Figure 4.3.

Discussion

Recently a missense variant (rs41268753) in *GRHL3* has been conferred as risk for developing nsCPO (Mangold *et al*., 2016; Leslie *et al*., 2016) and in order to confirm, the present replication study was carried using nsCPO case-parent trios from European population. Beside this we investigated for gene-environment interactions previously reported for their association in the etiology of nsCPO. Our result confirms and provides additional evidence that mutations in *GRHL3* contribute to the risk of nsCPO in European population.

We found a significant transmission of minor allele from parent to the offspring in our EUROCRAN and ITALCLEFT case-parent trio samples, warranting a confounding free

confirmatory evidence of association between *GRHL3* variant (rs41268753) and nsCPO in European population, which was not apparent in the recent GWAS of Leslie *et al*., 2016. This difference could be an outcome of low MAF of this variant in European population and the differences in the sample size included in the two study. Moreover, our result demonstrate a higher transmission of the variant allele in female infants, a limitation imposed in the recent case-control GWAS (Leslie *et al*., 2016) and sequencing studies (Mangold *et al*., 2016). We observed no risk with mothers genotype but instead a dose dependent risk with infants genotype. Of note, a threefold increased risk was observed for infants homozygous for the variant allele RR= 14.93; p=0.001, compared to infants heterozygous for the variant allele RR=2.05; p=0.031, Figure 1. This allelic RR of 2.05, matches with that observed by Mangold *et al*., 2016 for the central European population (allelic RR=2.15), validating the consensus of result observed in the two studies for European population.

Besides the confirmation of GRHL3 as susceptibility marker for developing nsCPO, the present study is advantaged with sex based subgroup analysis which shows a female biased risk for the variant allele. The homozygous female infants; RR=15.04; p<0.001 (Figure 2A) showed higher risk compared to heterozygous female infants; RR=2.03; p=0.095 (Figure 2B). In addition, the present study tested the effect of mothers folic acid supplementation and smoking as risk factors for developing nsCPO, we observed significant increased risk in infants born to mothers without folic acid supplementation ((RR=25.54, p=0.003); Figure 3) and smoking ((RR=15.91, p=0.001); Figure 4).

Overall, our findings are in line with recent GWAS studies on *GRHL3* with more robust confirmation, making use of large sample size recruited from nine European countries with less vulnerable case-parent trio design (Mossey *et al*., 2016). This further validates the association of *GRHL3* missense variant (rs41268753) as the first successful marker and *GRHL3* as first gene directly implicated in developing nsCPO in European population. In addition, our result demonstrate a female biased risk for this variant, justifying the need of well-designed sex based GWAS to unravel sex specific associations, if any. Furthermore, the present study demonstrates the protective effect of periconceptional folic acid supplementation.

In summary, this GWAS replication study was important to further understand the roles of chance, bias and/or population heterogeneity and the role of environment in regulating the genetic susceptibility of *GRHL3* in nsCPO. Our study confirms *GRHL*3 missense variant

(rs41268753) as susceptibility maker for nsCPO risk in European population. In addition, our result showed increased risk with infant genotype and a biased risk towards female carrying two minor allele. Moreover, the study entails the sex based segregation of minor allele for the gene-variants studied and shows the protective effect of periconceptional folic acid supplementation, but it may seem plausible to identify more functional variant in the genes and test for gene-gene and gene-environment interactions.

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Table 4. 1 Transmission disequilibrium test (TDT) for the transmitted and non-transmitted *GRHL3* missense variants in children with CPO alongside maternal environmental exposures.

GRHL3							
rs41268753	**CPO**	**Sex**		**Folic acid**		**Smoking**	
C>T		**M**	**F**	**Y**	**N**	**Y**	**N**
T	33	12	19	15	16	5	28
NT	14	6	6	8	6	2	10
TDT	7.68	2.00	6.76	2.13	4.55	1.29	8.53
p-value	**0.006**	0.157	**0.009**	0.144	**0.033**	0.257	**0.004**
*Significance at p < 0.05							

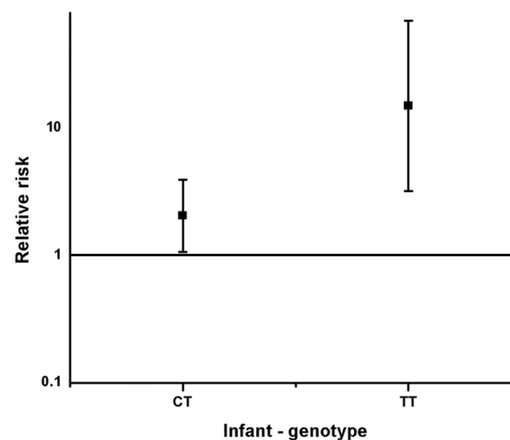

Figure 4. 1 Relative risk of *GRHL3* variant with infant genotype

Infant with one minor allele is at reduced risk and infant homozygous for minor allele at increased risk

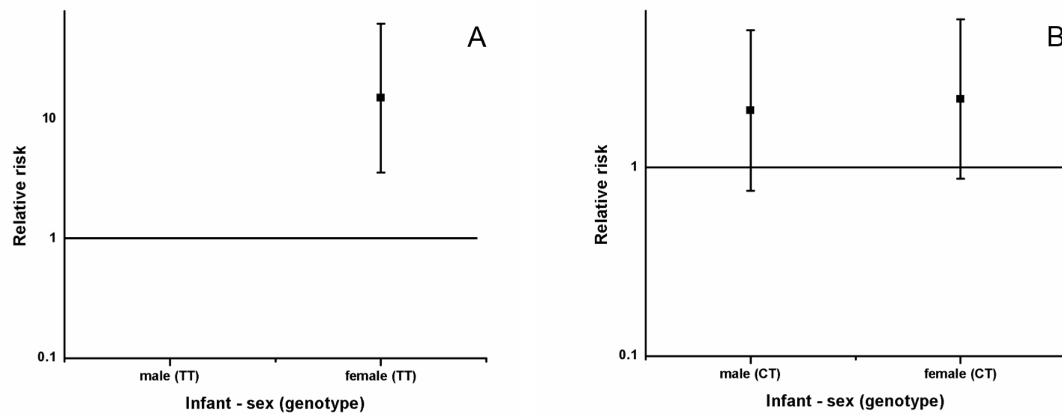

Figure 4. 2 Relative risk of *GRHL3* variant with infant sex

(A) Complete biased risk towards female with two minor allele. **(B)** The male and female with one minor allele shows trend for risk.

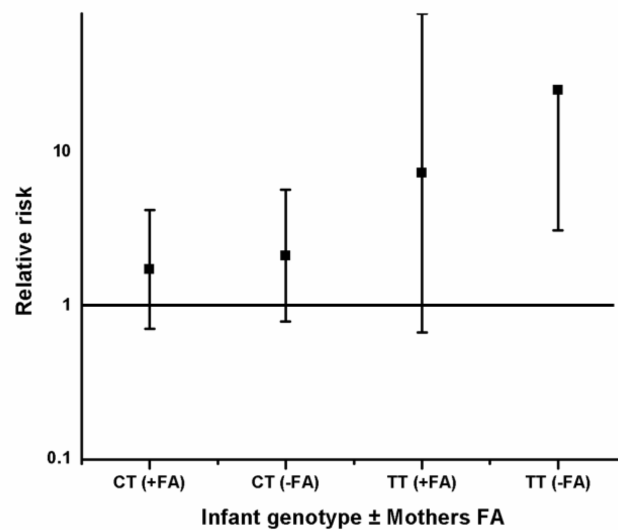

Figure 4. 3 Relative risk of *GRHL3* variant with Folic acid supplemented mothers

The graph shows mothers who reported folic acid (FA) supplementation, there was no evidence of nsCP risk with infant genotype. Whereas the infants homozygous for the minor allele and born to mothers without FA supplementation were at increased risk.

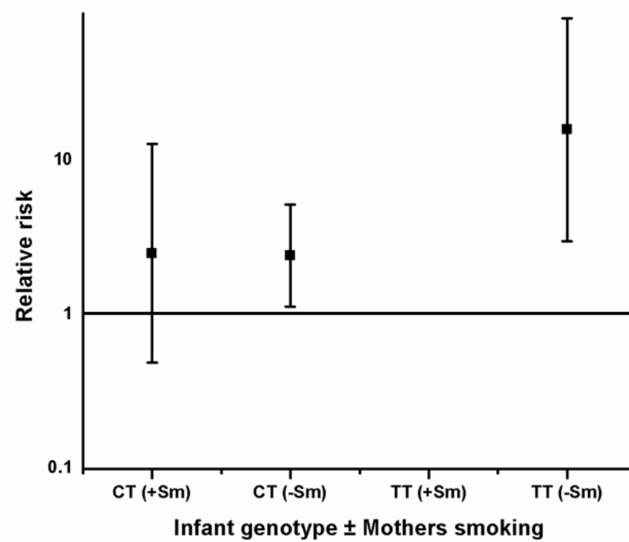

Figure 4. 4 Relative risk of *GRHL3* variant with smoking mothers

The graph shows mothers who reported smoking, there was no evidence of nsCP risk with infant genotype. Whereas the infants homozygous for the minor allele and born to mothers without smoking were at increased risk.

PART THREE

EPIGENETIC REGULATION

Chapter 5

Evaluating global DNA methylation in CL/P tissues in response to mothers environmental factors

Abstract

Introduction: DNA methylation is a key epigenetic modification which in mammals, occurs mainly at CpG dinucleotides. Most of the CpG methylation in the genome is found in repetitive regions, rich in dormant transposons and endogenous retroviruses.

Objective: In the present work we determined whether DNA methylation of the repetitive element (LINE-1) is altered on the lateral and medial side of the cleft cases using lip tissues.

Methods: The homogenized tissue DNA from lateral and medial cleft sides were bisulfite converted and amplified using conventional PCR. The LINE-1 global DNA methylation was measured with pyrosequencing using AQ mode. For comparison of mean methylation value between lateral and medial side a paired student's t-test was performed. While all other comparison involving two categories, the unpaired t-test was used.

Results: Our result demonstrates a significantly higher methylation level on the medial side compared to the lateral side; p=0.040. The comparison within and between the sexes and cleft phenotype did not show significant score. While the comparision of the two sides in males showed significantly higher methylation for the medial side; p=0.020. The level of medial side methylation was comparatively higher for infants of non-supplemented folic acid pregnancies.

Conclusion: A higher methylation of the medial side restricted to folate depleted pregnancies. This could be an outcome of different time of developmental origin of the two sides, experiencing two different environmental windows and hence could be regulated with different methylation patterns leading to differences in the two sides. The outcome of the study gives a new directional window to cleft research and raises concern of epigenetic mechanism in the etiology of cleft, warranting replication of the study using larger cases.

Introduction

Orofacial cleft is a congenital deformity affecting the lip, palate or both. It is categorized in two broad phenotype, cleft lip with or without cleft palate (CL/P) and cleft palate (CP). The frequency of the two phenotype varies with sex, CL/P predominate the males and CP among females (Fogh-Andersen, 1961; Mossey and Little., 2002). The OFC can be a part of syndrome or can occur as an isolated cleft; termed nonsyndromic cleft, the etiology of which is multifactorial, contributed by both diverse genetic and environmental factors (Mangold *et al.*, 2011).

This congenital oral deformity arises due to failed fusion of the lateral nasal process (LNP) with the medial nasal process (MxP) during 6th and 7th week of gestation (Dixon *et al.,* 2011), indicating the critical period of plasticity during pregnancy whereby the development of the lip may be influenced by environmental factors, such as maternal nutrients, smoking and hormones (Waterland *et al.,* 2010; Grieger *et al.,* 2014; Walsh *et al.,* 2015; Martelli *et al.,* 2015; Gresham *et al.,* 2014), as well as inherent genetic profiles (Dubois *et al.,* 2012; Godfrey *et al.,* 2001), but is still elusive (Murray, 2002; Yuan *et al.,* 2011). Numerous studies reveal that cleft risk is increased by disturbed one-carbon metabolism cycle (Ebadifar *et al.,* 2015), and particular nutrients such as folate are known to impact this risk with an inverse association of periconceptional folic acid supplementation (Johnson and Little, 2008; Butali *et al.,* 2013).

The one-carbon metabolism cycle results in the formation of methyl group that are required for methylation of DNA. Folate feeds into this cycle and has been shown to alter the levels of DNA methylation in women of childbearing age (Crider at al., 2011). A decrease in the level of dietary folate has been found to decrease genomic DNA methylation levels (Grieger *et al.,* 2014) and is an independent risk factor for neural tube defects; NTDs, congenital heart diseases; CHD (Czeizel, 2013) and cleft (Wheby and Murray, 2011).

DNA methylation of the pyrimidine base cytosine in DNA may be one of the mechanisms underlying differential programming of cell lineages in mammalian development, which is evident from erased methylation mark in early embryo and its de-novo re-modification after implantation (Kafri *et al.,* 1992; Reik *et al.,* 2003). This serves to re-establish the basic adult methylation patterns prior to organogenesis and show that DNA methylation are in a state of flux during gametogenesis and early embryogenesis, which can

be modulated by embryonic environmental exposures. Considering this hypothesis, exploring the global DNA methylation using surrogate LINE-1 DNA methylation (Fryer *et al.*, 2009; Yang *et al.,* 2004) can act as model representing the interplay of environment in regulating the whole genome DNA methylation. Numerous studies have shown changes in LINE-1 methylation with the development of disease with hypomethylation in NTDs (Wang *et al.,* 2010) and poor cancer prognosis (Ogino *et al.,* 2008). The studies on animals or animal derived tissues has evidenced the involvement of DNA methylation in the development of oral clefts (Mukhopadhyay *et al.,* 2015; Juriloff *et al.,* 2014; Seelan *et al.,* 2012), but the studies are limited to palatal tissues.

The palate are embryologically separate and originate at different time in development origin compared to lip (Pansky, 1982). Hence, the etiology of cleft lip and cleft palate is different and the methylation status of these tissues may prove to be different. In order, to overcome this shortcoming of cleft lip tissue methylation, the present study is the first to use human tissue specimens to gain insight into human cleft tissue epigenetic profile. Where LINE-1 global DNA methylation is explored, using tissues from lateral and medial sides of the cleft, and marks the influence of sex and cleft phenotype on global DNA methylation status in relation to periconceptional folic acid supplementation.

Material and methods

Cases

Cases were identified at the San Paolo Hospital, Milan, Italy, and recruited in the context of the ongoing Italian PENTACLEFT study. The study protocol was approved by local IRB (prot. N.08-2011), and case enrollment was dependent upon parent's signature of informed consent. Overall, 24 cases with an average age of 6.5 months were included in the study: 13 males (8 CL and 5 CLP cases) and 11 females (5 CL and 6 CLP cases) along with details of maternal periconceptional folic acid supplementation.

Tissue samples

The lip tissues from the lateral and medial cleft side of infants undergoing first surgical interventions were collected in lysis buffer (pH 7.4) and were transported from Milan to Ferrara, kept at room temperature until processed.

DNA extraction and sodium bisulfite treatment

For the cases the tissue were homogenized and genomic DNA extracted using Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH) according to the manufacturer's instructions and quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA). The DNA with concentration >10ng on Qubit® instrument was selected and bi-sulfite converted using EZ DNA Methylation-Lightning™ Kit (Zymo Research, Irvine, CA, USA).

Pyrosequencing

Line-1 methylation level was measured for all the study samples with pyrosequencing on PyroMarkQ96 ID using PyroMark Gold reagents (Qiagen). Figure 5.1 shows the primers for LINE-1 that amplifies a region (position 305 to 331 in accession no. X58075), which includes 4 CpG cites. PCR reactions were performed in duplicates with total volume of 50µl containing 10X PCR buffer, 50mM$MgCl_2$, 2.5mMdNTPs, 10pM of each primer, 5U Taq polymerase and 5µl of bisulfte modified DNA with the following cycling profile: 27 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 2 min. The amplicon of 147bp was analysed on 8% polyacrylamide gel using silver staining. The residual PCR product was transferred in two PCR plates with each well containing 20µl of PCR product, 20µl of RNAse free distil water, 3µl of sepharose beads containing streptavidin and 37µl of binding buffer; i.e. total volume of 80µl of the mixture was placed on the thermo-mixture. Following this, the PCR product was made single-stranded to act as a template in a pyrosequencing reaction by washing with ethanol 70% and denaturation buffer using a Pyrosequencing Vacuum Prep Tool (Bio-Stage). Two pyrosequencing runs were performed from each PCR reaction, using AQ mode in a total volume of 40µl per well, including 38µl of annealing buffer and 1.6µl of 10pM sequencing primer with suspended beads containing the sample DNA. The assays was created according to manufacturer's instruction and the output of the two pyrosequencing run was combined. The nucleotide dispensation order was: ACT CAG TGT GTC AGT CAG TTA GTC TG, Figure 5.1. Using the combined data achieved through pyrosequencing, the average LINE-1 methylation level was calculated as the mean of the proportions of C (%) at the 3 CpG sites analysed, which were located at positions +319, +322 and +329 (positions of the corresponding Guanine in the forward DNA strand, in relation to the first nucleotide base of the consensus promoter sequence) and this indicated the level of methylation of LINE-1 elements.

Statistics

All the statistical analysis was performed using the IBM SPSS Statistics 21. All p-value were 2-sided. The mean value of LINE-1 methylation was compared between lateral and medial cleft side by paired student's t-test and for all other test for variables with two categories the unpaired student's t-test was used.

Result

The LINE-1 methylation level was determined using tissues from lateral and medial cleft sides in a total number of 24 CL/P specimens. The characteristic LINE-1 methylation data for the specimens are listed in Table 5.1.

The average scores in the level of global DNA methylation between the lateral and medial side of cleft tissue was compared using paired sample t-test. On average, medial side were shown to have 1.7% more methylation compared to lateral side, with a significant score of p=0.040; Figure 5.2. We then classified our samples based on two different sexes and evaluated if there are any methylation differences within and between the sexes for the lateral and medial sides compared. There appeared no significant difference in methylation between the sexes for lateral (males 70.9±0.01% verses females 72.0±0.02%; p=0.522) and medial (males 73.3±0.00% verses females 73.0±0.00%; p=0.840) sides. Interestingly, the methylation on the medial and lateral sides in males were observed to be significantly different (lateral 73.3±0.00% verses medial 70.9±0.01%; p=0.020). However, when methylation level within and between CL and CLP phenotype were analyzed, it showed no significant difference in mean score. To further dissect the role of periconceptional folic acid as an environmental factor affecting the level of global DNA methylation, we calculated the average methylation level of lateral and medial cleft sides in infants born to mothers with and without periconceptional folic acid supplementation. Comparison between the two periconceptional pools showed no significant difference. Whereas, a statistical significant difference in scores were observed on equating the lateral and medial side within the pool of non-periconceptional folic acid supplementation, p=0.011; with result showing 2.5% more methylation level on the medial side compared to lateral side. Figure 5.3 (a-b) represents the average methylation scores on medial and lateral side for each comparison.

Discussion

In the early embryogenesis, there occurs great shuffling of methylation events with multiple levels of epigenetic regulation, evident from prior study carried by Kafri *et al.*, 1992. He showed that DNA methylation patterns are erased in primordial germ cells and in the interval immediately following fertilization. This shows the establishment of new methylation pattern in the developing embryo prior to organogenesis, which could be thought to have differential modulation or methylation design depending on the provided in-utero

environment, such as periconceptional folic acid supplemented environment. Moreover, the final methylation profile observed in somatic tissues act to stabilize and reinforce the prior events that regulate the activity of tissue specific genes (Monk *et al.*, 1987).

In the present study we observed medial side of the cleft to be globally hypermethylated. While sex based and cleft type subgroups showed no significant difference in methylation for the two sides. However, within males, the medial side appeared more methylated compared to the lateral side. Analysis including mothers environmental factors, showed more methylation scores for the medial side in infants born to mothers not supplemented with periconceptional folic acid.

Apparently, it appears from our result that in clefted individuals the medial side is more methylated compared to the lateral side and seem more restricted to pregnancies started without folic acid supplementation. This distinct methylation of the two cleft sides may be reflective of different time in developmental origin and their respective environment. For example, the lateral side originate from maxillary processes (MxP) that develop at the 4th week of gestation while the medial side from medial nasal process (MNP) develop by the end of the 4th week or beginning 5th week (Dixon *et al.*, 2011). This shift in the window of development coupled with environmental disruption during this period of pregnancy might be related to the differential methylation of the two cleft sides observed in our study. The observation fits in well with the developmental field concept where the medial side of the lip possibly acts in a temporal and spatially coordinated manner. It seems plausible that the late medial development is epistatically regulated compared to the development of early lateral side. On account of observed methylation differences and thought on epistatic regulation of the genes on the two cleft sides, it becomes wise to explore expression of a single gene implicated in cleft eg. IRF6 on the two sides, to gain sight of the progressive epistatic regulation of the gene on the two sides, if any.

Although the influence of in-utero environment in epigenetic modulations have been convincible with previous report showing association of DNA methylation changes with other birth defects such as neural tube defects; NTDs and congenital heart diseases CHDs (Wang *et al.,* 2015; Serra-Juhe *et al.,* 2015; Sheng *et al.,* 2014). Of note, no research in humans, to date have found association of folic acid intake during pregnancy and global DNA methylation (LINE-1 methylation), (Geraghty *et al.*, 2016) especially in context to development of the CL/P. Progressively, the present study was an attempt to gain insight into

periconceptional folic acid and global LINE-1 DNA methylation, which showed increased methylation of the medial sides in infants born to mothers without periconceptional folic acid supplementation. Based on our result it can be hypothesized that supplementation of periconceptional folic acid could counter balance the excess methylation and help maintain proper methylation between the intermaxillary and mediolateral prominences during upper lip development.

Additionally, our attempt of sex and cleft phenotype specific analysis showed no-significance. While within sample comparison of males, lateral and medial side showed more methylation on the medial side, and surprisingly these males dominated the pool of non-folic acid supplemented pregnancies. If the process in our study occurs similar to as demonstrated by an animal based study of Maloney *et al.*, 2011. Where he showed how methyl donor nutrients in early pregnancy changed glucose metabolism in offspring, with effect being sex dependent, with male biased alterations. Giving a possibility of differential methylation trend towards our male cases as a response to compensate with environment of folic acid replete mothers and the programming of which can start early in pregnancy leading to the observed changes in our male specimens.

Even though GWAS (Genome-Wide Association Study) of major loci involved in non-syndromic CL/P (nsCL/P) has not completely reflected its heritability, indicating that the infants genetic profile alone cannot explain the origin of this malformation. Meanwhile epidemiologic studies show an association of low socioeconomic status to nsCL/P, pointing out an important environmental contribution. In an attempt to opens this new window of cleft research, the present study is the first to report the global DNA methylation using cleft tissue specimens.

The advantage of using tissue specific epigenetic profiling over peripheral blood lymphocytes (PBL) makes this study well accepted and free from confounding cellular heterogeneity of PBL. Of note, our study is limited to small sample size (making the statistical power of the experimental implant insufficient) and being aware of this limitation, splitting our samples based on factors (sex, cleft phenotype and pre-FA) thought to affect methylation, was an attempt to provide only descriptive data to access possible trends. Therefore, to better justify the outcome of this preliminary finding, the study should be replicated using larger number of cases. Presuming the outcome of this study, it is plausible to suggest that in animal models epigenetic analysis should mainly focus on search of

epimutations in the intermaxillary prominences, and determine if folic acid supplementation can restore normal methylation level at this site and protect from clefting.

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Table 5. 1 Global LINE-1 DNA methylation level (%) of medial and lateral cleft sides.

Mean ± standard deviation (SD) values of total CL/P cases or cases categorized by sex, cleft phenotype and pre-folic supplementation (pre-FA) are shown, along with mean difference ; 95% confidence interval (C.I) and p-value of t-test.

	Medial side ± SD	**Lateral side** ± SD	Mean difference (95% C.I.) p-value
Total cases	**73.20** ± 3.60	**71.50** ± 4.14	1.72 (0.08 to 3.36) *p*=0.040
Males	**73.35** ± 4.42	**70.96** ± 4.40	2.40 (0.44 to 4.32) *p*=0.020
Females	**73.04** ± 2.40	**72.10** ± 1.20	0.94 (-2.13 to 4.01) *p*=0.510
Mean difference (95% C.I.) p-value	0.30 (-2.80 to 3.40) *p*=0.840	-1.13 (-4.60 to 2.43) *p*=0.520	
CL	**74.01** ± 2.56	**72.13** ± 3.36	1.87 (-0.38 to 4.13) *p*=0.095
CLP	**72.30** ± 4.42	**70.71** ± 4.97	1.54 (-1.30 to 4.37) *p*=0.253
Mean difference (95% C.I.) p-value	1.75 (1.44 to 4.74) *p*=0.286	1.42 (-2.12 to 4.96) *p*=0.415	
No-pre-FA	**73.80** ± 2.91	**71.32** ± 4.03	2.46 (0.64 to 4.27) *p*=0.011
pre-FA	**71.01** ± 4.94	**70.98** ± 4.17	0.037 (-4.91 to 4.98) *p*=0.986
Mean difference (95% C.I.) p-value	2.77 (-0.76 to 6.30) *p*=0.320	0.35 (-3.71 to 4.40) *p*=0.902	

Abbreviations: SD, standard deviation; CI, confidence interval; CL, cleft lip; CLP, cleft lip and palate; No-pre-FA, no periconceptional folic acid; pre-FA, periconceptional folic acid

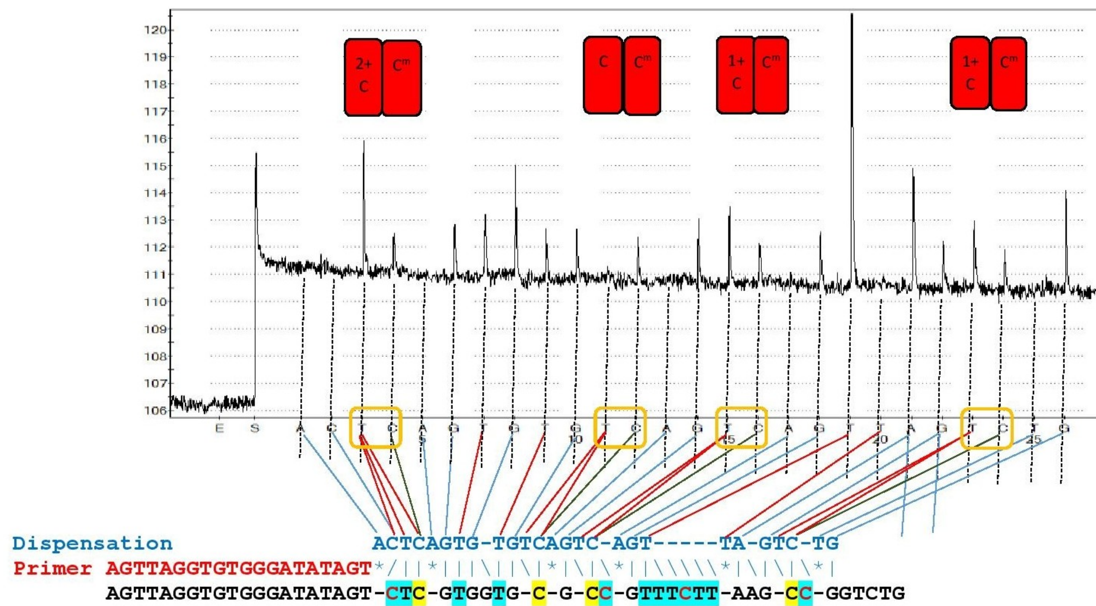

Figure 5. 1 Pyrogram showing nucleotide dispensation order

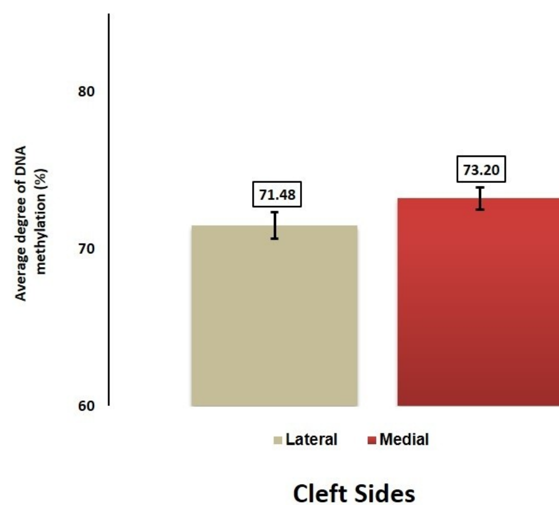

Figure 5. 2 Shows average degree of LINE-1 methylation on the two cleft sides

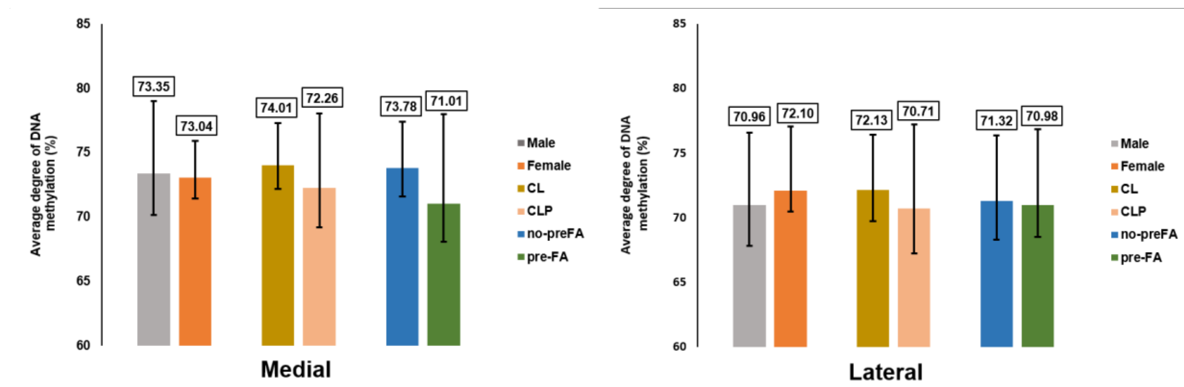

Figure 5. 3 Shows average degree of LINE-1 methylation based on sample stratification

(a) Medial side (b) Lateral side.

Chapter 6

Muscle fibre diameter on lateral and medial side of cleft lip using an image processing tool.

Abstract

Introduction: The existing histological muscle fiber quantification requires fresh tissue specimens and an alternative method is not well estabilished.

Objective: To introduce a new image processing tool for ascertaining differences in muscle fiber diameter between lateral and medial sides of cleft lip (CL) in histological images.

Methods: CL tissues with or without palate were collected from 18 children at the time of lip surgery from lateral and medial side of CL. The tissues were fixed in buffered formalin, sectioned and stained with haematoxylin and eosin. The histological images were processed using ImageJ user-enabled plugin. Student's t-test was performed to compare differences in muscle fiber diameter between lateral and medial side of CL, extending to check differences between sexes and two cleft phenotype. A p value of 0.05 was considered significant for all measured parameters.

Results: In general, there was no significant difference between lateral and medial muscle diameter. On classifying based on two sexes, we found significant difference on the medial side at p=0.032. Meanwhile, classifying on cleft types, CL and CLP showed no significant difference between lateral and medial muscle fibre diameter.

Conclusion: The observed change in the muscle diameter on the medial side of cleft lip between two sexes values the role of sex in the etiology of cleft. This study should be replicated using large sample for conclusive outcome, which can be supplemented with our present image processing method for faster and easy evaluation, which can impact better restoration of the anatomical and functional component of the upper lip.

Introduction

The Cleft lip with or without palate (CL/P) is the most common craniofacial congenital anomaly worldwide. Labial architecture is dramatically altered in CL/P cases, with slight difference among sexes and cleft sub-phenotypes (Carroll *et al.,* 2012). Of the structures affected in Cleft lip, the orbicularis oris (OO) muscle is obviously of particular significance.

The OO muscle is one of the muscles of facial expression, forming the sphincter of the oral cavity, is intimately associated in maintaining the upper lip form and function. The OO muscle is composed of different types of muscle fibres. (Schiaffino and Reggiani, 2011) which are of two different types: (a) type I (slow twitch), characterized by resistance to fatigue, slow contraction and small fibre diameter and (b) type II (fast twitch) muscle fibre with fast contraction rate, and low resistance to fatigue, and also has larger muscle diameter. Hence, the functional distribution of these muscle fibre types help in contraction of OO muscle fibres leading to change in shape of the lips and is integral part of actions used in feeding (Tamura *et al.,* 1998; Jacinto-Gonçalves *et al.,* 2004) and in human speeching (Rastatter & DeJarnette, 1984; Rastatter *et al.,* 1987; Standring, 2004; Regalo *et al.,* 2005; Raphael *et al.,* 2007).

The predominant hypothesis regarding normal development of the embryonic upper lip is that the medial-nasal processes bilaterally fuse with the inter-maxillary process during the 6th week of gestation (Dixon *et al.,* 2011), with the latter resulting in the philtrum of the upper lip. The disappearance of the epithelial seams by apoptosis and epithelial-mesenchymal transformation marks the complete lip fusion (Jiang *et al.,* 2006). The migration of the mesenchymal cells across the fused prominences give rise to a dense and continuous band of mesenchymal cells corresponding to the future OO muscle. It seems plausible that the delayed or failed union of lip primordial prominences to the formation of continuous OO muscle may prevent the correct orientation of the OO muscles. This lack of merging results in varying degree of damage and reduction of OO muscle function in cleft patients (Lazzeri *et al.,* 2008).

Patients with cleft lip (CL) only or cleft lip and palate (CLP) require a number of treatments for complete habitation, one such procedure is the surgical reconstruction of the OO muscles (Cohen *et al.,* 2004). During primary surgical repair of the lip, it is not possible to reconstruct the muscular sling anatomically and physiologically due to the creation of scar

tissue, and contraction of this on healing results in unsightly bulging of the muscle on either side of the lip scar, depressions, and asymmetries that are further noticeable during animation, and give the lip an unnatural look (Cohen *et al.,* 2004).

Although many histological studies have contributed to understanding of the development of cleft lip, few studies have been done on the histological changes in the OO muscle at the edges of the cleft lip (Schendel *et al.,* 1989; Raposio *et al.,* 1998; Wijayaweera *et al.,* 2000) and between lateral and medial edge of cleft lip (Wijayaweera *et al.,* 2000). Neiswanger *et al.,* 2007and Mittal *et al.,* 2012 contributed to this understanding with a new directional approach, by using ultrasound technique to speculate OO muscle in individuals with cleft lip. Both studies showed differences in OO muscles in first degree relatives, which was in agreement with the finding of Martin *et al.,* 2000. In another study, Weinberg *et al.,* 2006 showed significant score of OO defects in extended unaffected relatives of CL/P proband. These support the hypothesis of OO discontinuities as a subclinical phenotype in CL cases. Yet, there is little or no information in the literature characterising the variation in OO muscle fibre diameter on either sides of the cleft lip. Such information could provide a potential for different muscle type on either side of the cleft, which could affect the approach towards reconstruction of the cleft lip.

The evaluation of the muscle fibre diameter in addition to the generalized histological and ultrasound approach could provide an additional support towards better reconstruction of the cleft lip. In order to access the muscle fibre diameter on lateral and medial side of the cleft, the present study introduces an image processing tool and underscores its importance to evaluate a sex based differences in the OO muscle diameter, with a view to ascertain differences in in cleft phenotypes using histological images.

Material and methods

Cases

Cases were identified at the San Paolo Hospital, Milan, Italy, and recruited in the context of the ongoing Italian PENTACLEFT study. The study protocol was approved by local IRB (prot. N.08-2011), and case enrollment was dependent upon parent's signature of informed consent. Overall, 18 cases were included in the study: 9 males (5 CL and 4 CLP cases) and 9 females (4 CL and 5 CLP cases).

Tissue Samples

Lip tissue samples were collected from 18 CL±P children at time of lip surgery. Samples were obtained from both medial and lateral side of cleft lip and immediately fixed in buffered formalin solution. Samples were then transferred to laboratory at University of Ferrara where they were processed for paraffin embedding. Histological sections (6µm) were cut and stained with hemotoxylin and eosin (H&E) using routine protocol.

Image acquisition and processing

OO muscle fibres were observed under 40X objective lens and images were captured using Nikon Eclipse E600 microscope coupled with DS-5 M video camera, Lucia G 4.81, Laboratory Imaging Ltd., Praha, Hostiva. Images were taken at a resolution of 1280 X 960 pixel and saved in tiff format. At least three 40X magnification fields were acquired with at least 65 muscle fibre diameter measurements for each specimen.

The H&E stained images were processed using ImageJ (Ver. ImageJ 1.49h, Dresden, Germany), an open source image processing program. The original H&E image was first converted to greyscale image by “8bit” ImageJ command, an intermediate operation before creating a binary image. Using standard commands of ImageJ and subsequently assigned commands were followed for the input image in order to obtain the desired area of each OO muscle bundle. The sequence of events is illustrated in Figure 6.1 (a-e).

Since the internal calculations were performed in pixel units, it was necessary to convert this into physical units through calibration of the input image. Using the line tool of ImageJ, the number of pixels representing a known distance in the image was measured. These values, which change with images, were calculated with each input image to complete the calibration.

The inherent analyse particle command takes the pre-processed binary image (Fig. 6.1c) and measures area in physical units (μm^2). Color-coding was prepared based on criteria that depended on standard output or calculated parameters (e.g., area). The color (Ferreira, 2014) overlaid each muscle fibre (Fig.6.1e), helped in the visualization of different muscle bundle area. The results were made available in textual and spreadsheet for further analyses.

Statistical Analysis

All the statistical analysis was performed using the IBM SPSS Statistics 21. Muscle fibre diameter comparisons between lateral and medial sides of cleft were carried out using paired student's t-test. The effect of sex or cleft type was assayed using unpaired student's t-test. Considering a number of 18 cases included in this study, at least 51 muscle fibre measurements per each tissue sample would be required in order to detect a 30% difference between cleft sides assuming an α-error (bilateral) of 0.05 and a β-error of 0.20. According to these parameters, the number of muscle fibre measurements were also enough to detect a 40% difference between sex groups or between cleft type groups.

Results

The mean and standard deviation of lateral and medial side of cleft tissue muscle diameter, grouped according to sex and two cleft phenotype is shown in Table 1. There was no significant difference of scores between lateral (9.12 ± 1.82 μm) and medial (9.09 ± 1.21 μm) side, p=0.095, Table 6.1.

The samples were subsequently classified into male and female, and compared for lateral and medial muscle fibre diameter. There was no significant difference between lateral and medial side within each sex. While comparing between sexs showed a significant difference in score for the medial side, p=0.032, Table 6.1.

Further analysis was concerned to evaluate lateral and medial muscle fibre diameter in two cleft phenotypes, CL and CLP. The statistical comparison of lateral and medial muscle fibre diameter within CL and CLP showed no significance at levels, CL (p=0.378) and CLP (p=0.427). Likewise, analysis between CL and CLP showed no significance for lateral side (p=0.379) and medial side (p=0.378), Table 6.1.

Discussion

Mounting evidence have indicated that the structure of the upper lip is intimately associated with the OO muscle (Schiaffino and Reggiani, 2011) and demonstrated varying distribution of OO muscle fibre types along the upper lip (Dong *et al.,* 2015). Moreover, Fara (1975); Gundluck (1979) showed increased disorganization of muscle fibre with the degree of clefting. In addition, previous histological studies evaluating the OO muscle on lateral and medial edges of cleft lip observed paucity and less disorganized muscles on the medial side

with a greater disorganization on the lateral side (Wijayaweera *et al.,* 2000). Currently, the ultrasound technology is used to visualize and assess the OO muscles (Roger *et al.,* 2008). Interestingly, a normal ultrasound shows a continuous hypoechogenic (black on ultrasound) band of uniform thickness with no obvious breaks in the black band. A discontinuity typically looks like an echogenic break which is a sign of a missing segment of muscle or an abrupt thinning (Marazita, 2007). Of note, Rogers study demonstrated varying degree of hyper and hypo-echoic area with depth of the upper lip.

On account of differences documented in the above studies for lateral and medial muscle fibre arrangement in a cleft individual, the present study was designed to undertake histological image processing method to measure muscle fibre diameter taking advantage of lateral and medial cleft tissue specimen, with an objective to explore differences if any, with respect to sex and two different cleft phenotypes, in order to supplement better restoration of the anatomical and functional component of the upper lip.

We observed no significant change in the muscle fibre diameter on lateral and medial side of the cleft, p=0.957. While comparing medial side of male against female appeared significant; p=0.036, with male (8.49 ± 1.07) having smaller muscle diameter compared to female (9.69 ± 1.09). Moreover, there was no significant difference within and between the two cleft phenotypes, for lateral and medial sides compared. Furthermore, the histographic analysis; Figure 6.2, represent majority of fibre diameter frequency within range of 7-11 µm, for both cleft side compared, demonstrate values previously reported in isolated cleft lip specimens, with type I fibres of 10.2 µm and Type II fibres of 7.4 µm (Schendel *et al.,* 1993).

Apparently, the sex based dissimilarity on the medial muscle fibre diameter could be a resultant outcome of inherent sex related developmental differences (Natsume *et al.,* 1988), or differential response to a hormone. For example, till the 11th week, the progesterone production is governed by the maternal corpus luteum, after which its production is taken over by the fetally guided placenta (Larsen *et al.,* 2001). This shift from maternal to fetal hormone production occurs around the same time that the OO muscle is forming (Neiswanger *et al.,* 2007). To reason if the process occurs slight differently depending on the sex of the fetus, or has a slightly different effects on the formation of the OO muscle in male compared to female fetuses, it could possibly be related to sex based differences in muscle fibre diameter observed in our present study.

In order to foster new method over the existing muscle fibre quantification using histological image, the present study introduces a new methodological approach. Where the original H&E stained image once converted to a grey scale image can easily be segmented by threshold command, using image comprising light object on dark background or dark object on light background. Based on that, the lower and upper limits greyscale intensity were adjusted so that the particle of interest are wisely covered. We segmented and detected the muscle fibre in black and white channel by thresholding the image using ImageJ, and in general, threshold values of 0 and 130–150 for white backgrounds, for lower and higher limits respectively, were found suitable for binary image. (Figure 6.1c).

Moreover, for the color-coding overlaid on each muscle bundle with different measured area, an inherent ROI color coder plugin in ImageJ was used. This technique can be exploited to measure different muscle fibre types using a formalin fixed tissue overcoming the adversity of procuring fresh tissue specimens, and using pH sensitive histochemical stain to identify muscle fibre types (de Chalain *et al,* 2001), if the standard threshold diameter of the muscle fibre type is known. For example: As apparent from Figure 6.2, for different frequencies of muscle fibre size on the lateral and medial side, there appears a coherent range between 7-11 µm. Considering the reported diameter of Type I (7.4µm) and Type II (10.2µm) muscle fibres (Schindel *et al,* 1993), it is evident that our coherent range included both, Type I and Type II fibre types. In process, we presumed and categorized range into two groups, A and B, with range limit (>7 - <9) and (>9 - <11) respectively, and evaluated the percentage of fibres in each group. Table 6.2. We observed differences between the groups on the lateral and medial sides, from which it can be ascertained to find differences in muscle fibre-type and diameter, along the two cleft sides.

Remarkably, this observed OO muscle differences can be correlated in order to characterize the defects visualized on ultrasound. Notably, an effort by Marazita's group indicated more disorganized muscles on histological sections from the individual with OO defects on ultrasound. The echoic breaks in ultrasound, which is a sign of a missing segment of muscle or an abrupt thinning (Marazita, 2007) could be an associated outcome of different muscle fibre-types and diameter. This can be reasoned to generalize non-destructive testing of roughness using ultrasound images (Niu *et al.,* 2013). As the roughness varies significantly, the relative proportions of specular reflection and scattering vary correspondingly. So, for the higher roughness, thought to change with varying degree of

muscle fibre-type and diameter, can result in higher proportion of scattered ultrasound and vice-versa. Presuming this hypothesis, the future work in this area may investigate (1) Illustration of the different muscle fibre-types and diameter by comparing the histological slices with corresponding hyper and hypo-echoic areas of an ultrasound imaging, with a view to be included as a subphenotyping measure (2) Quantitative assessment of distribution of Type I and Type II muscle fibres on two cleft sides. (2) Better demonstrate the power of ROI color coder.

Nevertheless, this technique can demonstrate compatibility with the regular pH sensitive histochemical staining (de Chalain *et al.,* 2001), as the reaction at pH 9.4, type 1 (slow-twitch) fibres stain lighter than the type 2 (fast-twitch) fibres. This light and dark stained fibre type can be taken advantage by the ROI color coder, resulting in an output image representing the two fibre type and intermediate fibre subtypes with different color codes, making easier to quantitate and examine the different muscle fibre type (Figure 6.1e) on the histological section from cleft specimens.

Furthermore, the documented method for evaluating muscle fibre diameter is highly economical as it utilizes a less-expensive camera enabled light microscope for image acquisition, and only uses a free open source ImageJ software. The method can automate the measurement of different muscle fibre type and overcome the difficulties of manual procedures. In addition, the method can be readily employed within and as an alternative method to routine laboratory assessment of muscle fibre and diameter measurement. Our method has an advantages of convenience, accuracy, large sample size handling (difficult for histological and histo-chemical measurement), repeatability, with an output delivery time of few seconds, with no special skills or training requirement, and is highly economical.

In conclusion, we observed a sex based differences on the medial muscle fibre diameter and provide a new direction to evaluate any muscle type differences on the two cleft sides, no conclusions can be reached on the basis of the present data alone. The confirmation of phenomena in future studies would suggest the possibility of new knowledge of muscle type differences between the two cleft sides and developmental differences in OO muscle between two sexes. Based on the new methodological approach using ImageJ pre-processing of the histological image and inherent color coded plugin the muscle fibre diameter and type can be quantitated with ease within approximately few seconds.

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Table 6. 1 Orbicularis Oris (OO) muscle fibre diameter (µm) of medial and lateral cleft sides.

Mean ± standard deviation (SD) values of total CL/P cases or cases categorized by sex or cleft phenotype are shown, along with mean difference, 95% confidence interval (C.I.) and p-value.

	Medial side ± SD	**Lateral side** ± SD	Mean difference (95% C.I.) p-value
Total cases	**9.09** ± 1.21	**9.12** ± 1.82	-0.02 (-1.16 to 1.10) *p*=0.95
Males	**8.49** ± 1.07	**8.98** ± 2.30	-0.49 (-2.63 to 1.65) *p*=0.61
Females	**9.69** ± 1.09	**9.25** ± 1.33	0.43 (-0.83 to 1.70) *p*=0.45
Mean difference (95% C.I.) p-value	-1.19 (-2.28 to -0.11) ***p*=0.03**	-0.26 (-2.14 to 1.61) *p*=0.76	
CL	**9.35** ± 1.38	**8.72** ± 1.84	0.62 (-0.91 to 2.16) *p*=0.37
CLP	**8.82** ± 1.03	**9.51** ± 1.83	-0.68 (-2.56 to 1.19) *p*=0.42
Mean difference (95% C.I.) p-value	0.52 (-0.70 to 1.74) *p*=0.37	-0.78 (-2.62 to 1.05) *p*=0.37	

Abbreviations: SD, standard deviation; CI, confidence interval; CL, cleft lip; CLP, cleft lip and palate

Table 6. 2 Fibre Group Analysis

Sample	**Fibre Group (%)**		**Ratio**
	Group A	**Group B**	
Lateral	35.14	29.86	1.17
Medial	44.03	17.98	2.44
Group A: >7 & <9, Group B: >9 & <11			

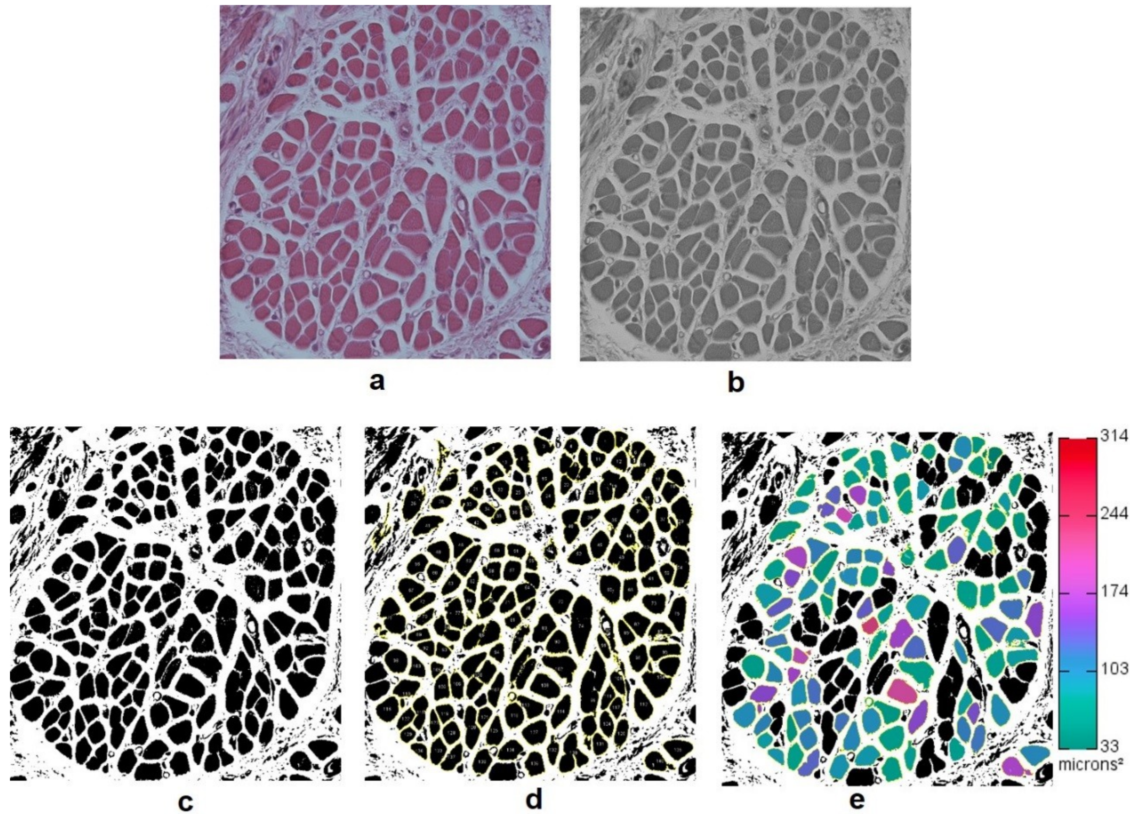

Figure 6. 1 Preprocessing of micrographs using ImageJ standard commands to obtain digital color coded image

(a) original H&E image; **(b)** 8-bit grey-scale image **(c)** thresholding image with "moments" scheme and "no dark background" converts image to binary; **(d)** mask prepared by analyzing the particles with "no exclude edge" options. Output labelled muscle bundles; and **(e)** area based color-coded muscle bundles for better visualization of sizes.

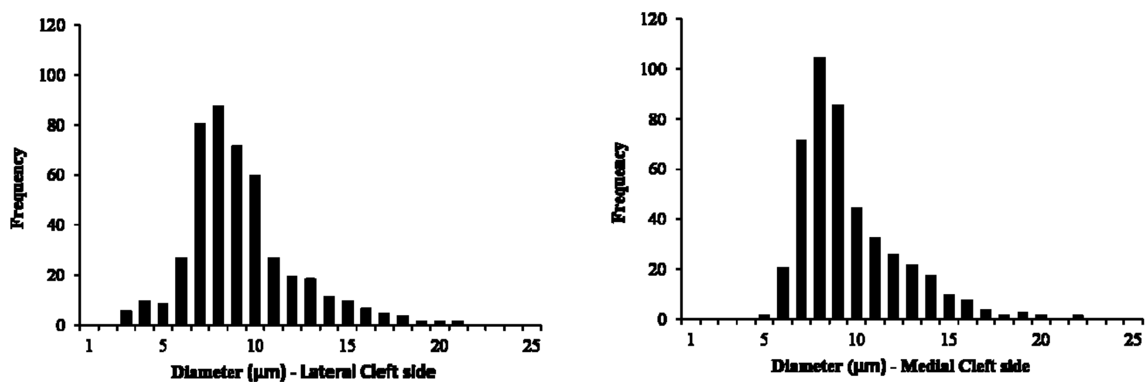

Figure 6. 2 Frequency of muscle fibre diameter for lateral and medial cleft sides

PART FIVE

GENERAL DISCUSSION

Chapter 7

Discussion, conclusion and future direction

Discussion

The studies described in the present thesis was performed with an aim to gain new insight into OFC pathology using multidisciplinary approach. Starting with a more common genetic association studies (Part 2) combined with gene-environment interaction as risk in the two distinct cleft phenotypes; CL/P and CP. This was accomplished using case-parent triads samples from EUROCRAN; the largest study of oral cleft. The use of samples from EUROCRAN in the present thesis makes it unique as it includes samples from countries in southern Europe (Italy and Spain), countries in eastern Europe (Bulgaria, Estonia, Hungary, Slovakia and Slovenia), and countries in northwest Europe (Denmark, the Netherlands and the United Kingdom). This increases the diversity of cleft prevalence and variability in environmental exposures such as; dietary patterns, periconceptional use of supplements and tobacco smoking alongside pregnancy (Mossey *et al.*, 2016; MacKenbach and Mckee, 2013) in the present thesis. Moreover, the use of case-parent trios makes the studies in the present thesis less vulnerable to population stratification warranting the outcome of the studies in present thesis as robust, and free of confounding with greater statistical power.

Keeping in mind the critical period of plasticity in pregnancy, whereby the cleft lip and palate development may be significantly influenced by environmental factors, such as maternal nutrients and smoking during pregnancy, still illusive. The present thesis (Part 3) acknowledges the use of the closest human tissues in the pathology of cleft to emphasize the importance of epigenetic profile, using LINE-1 global methylation in relation to periconceptional folic acid intake. To the best of my knowledge the use of tissue samples to explore epigenetic profile in the etiology of cleft is the first study of its kind. This is advantageous over the use of blood cells, which have cell specific profile based on developmental and environmental changes in the makeup of blood cells. Hence, the use of tissues is free of confounding epigenetic results, which could be discouraging using blood cells.

Taking advantage of the cleft tissue specimens, the final part of the thesis (Part 4) includes the evaluation of the muscle fibre diameter using an imaging tool to underscore its importance in two sexes, with of aim of providing better reconstruction of lip in the two sexes.

Part 2. **Family based association studies**: Identification of genetic and environmental risk factors for isolated CL/P and CP.

Chapter 2. Under the family based association studies, the first part (chapter 2) investigates the susceptibility of two *TGFA* variant (*TGFA*/11nt insertion/deletion and *TGFA*/TaqI insertion/deletion) to risk of developing nsCL/P. Although the association of TGF/Taq1 polymorphism is well characterized and found to be associated with the development of CL/P, there exists an inconsistency in number of studies (Vieira, 2006; Lijia *et al.*, 2015). Whereas the other *TGFA* variant was newly identified and we took advantage to identify its susceptibility in developing nsCL/P. In addition, the test of gene-environment interactions between the two *TGFA* insertion/deletion variants and the two common maternal exposures (smoking and folic acid supplementation) during pregnancy were factored in the study. In the present study there appeared no association of *TGFA*/Taq1 in developing nsCL/P until stratified by sex. Sex analysis, showed an opposite effect in males and females, with an under-transmission in males and an over-transmission in females for *TGFA*/TaqI variant. After stratifying based on the cleft types, the significant association was found in CPO risk for *TGFA*/TaqI polymorphism and among the mothers who reported smoking and/or folic acid supplementation, there was no evidence of nsCL/P risk with infant genotype. The following study reflects the sex difference as a factor influencing and modifying genetic risk, and could provide clues that will aid the development of targeted prevention and intervention efforts contributing to the etiology of Cleft.

The difference in sexes is observed in the prevalence, course and severity of many common diseases, including cardiovascular diseases, autoimmune diseases and asthma. One of the most remarkable sex specific association concern is interferon-gamma (*IFN*-γ) in asthma, with a heterozygous genotype, but with opposite direction in males and females (Loisel *et al.*, 2011). Based on these studies and our result it appears reasonable to suggest the existence of differential gene regulation mechanism in males and females that can influence human phenotypes, including reproductive, physiological and disease traits. The influence of sexual dimorphism in gene association studies of nsOFC is generally ignored. Hence, this study provides a new directional approach and show that sexual dimorphism influences a gene, and this should be considered in the re-analysis or while conducting new conventional GWAS.

Chapter 3 and 4. The recent animal studies showed involvement of *LOXL3* deletion in perinatal lethality and impaired development of the palatal shelves (Zhang *et al.,* 2015), and functional role it's variant in bone development. In addition, *LOXL3* variant has been identified in family with autosomal recessive stickler syndrome, which exhibit CPO. This recent association of *LOXL3* in Sticker syndrome indicate a possible link between *LOXL3* based collagen remodeling and developing cleft palate. Based on this hypothesis, we checked *LOXL3* variant (rs17010021) in the susceptibility of developing nsCPO and additionally gained insight of effect of parental imprinting. To our knowledge, the present study is the first to evaluate *LOXL3* missense variant as functional markers in etiology of isolated cleft palate (nsCPO).

The first step was screening of missense variant using UCSC Genome Browser (https://genome-euro.ucsc.edu/) and filtered based on minor allele frequency (MAF>2%).This resultant output was three missense variant (rs73949682, rs73949683 and rs17010021). However, out of the three we ruled out the two variants with lower MAF (MAF≥2.5, rs73949682 and rs73949683) and preferred one missense variant with the highest MAF (MAF≥13%, rs17010021) located on exon 10 of the gene. Subsequent, the analysis of this (rs17010021) variant using effect predictor generated strong evidence of deleteriousness (Polyphen-2, http://genetics.bwh.harvard.edu/pph2/) and hence this was the selected variant tested for the susceptibility analysis in our EUROCRAN and ITALCLEFT CPO samples.

The result of this study showed no significant segregation of the minor allele, although the transmittance was more from the father. In addition, there was no risk with mothers genotype. Whereas, the risk was influenced by infant genotype, with infant carrying one minor allele were at reduced risk and the infants carrying two minor allele were at increased risk. When classified based on sex there appeared a complete biased trend towards female. Furthermore, for *LOXL3* variant, the mothers who reported folic acid supplementation and smoking showed no evidence of nsCPO risk with infant genotype.

Progressively, we took advantage of the recent GWAS study (Mangold *et al.*, 2016; Leslie *et al.*, 2016), indicating a missense variant (rs41268753) in the coding region of *GRHL*3 gene and occurrence of nsCPO. Our study (chapter 4) was a replication study to test the effect of missense variant (rs41268753) in *GRHL*3 gene, for association in developing nsCPO using our case-parent triads from EUROCRAN and ITALCLEFT. Furthermore, based on same platform as *LOXL3* the test of gene-environment interaction is accounted in the

present study. The overall result of the present study showed significant association of this variant and nsCPO for our case-parent trio samples warranting a confounding free confirmatory evidence of association between GRHL3 variant and nsCPO. Moreover, when classified based on sex, our result demonstrate a higher transmission of the variant allele in female infants, a limitation imposed in the recent GWAS (Leslie *et al.*, 2016) and sequencing studies (Mangold *et al.,* 2016). There was no risk with mothers genotype but instead a threefold risk was observed with infant homozygous for the minor allele in relation to infant with one minor allele. In addition, the present study tested the effect of mothers folic acid supplementation and smoking as risk factors for developing nsCPO and observed a significant increased risk in infants born to mothers without folic acid supplementation and smoking during pregnancy.

Overall, the result of our *GRHL3* study is in line with recent GWAS on nsCPO with more robust confirmation, by making use of large sample size and less vulnerable case-parent trio design. This validates theassociation of *GRHL3* and its variant (rs41268753) as the first gene and first successful marker directly implicated in developing nsCPO in European population. In addition, our result demonstrate a female biased risk for this variant, justifying the need of well-designed sex based GWAS to unravel sex specific associations. Furthermore, the study also shows the protective effect of periconceptional folic acid supplementation.

Our family based association study based on cleft palate, for *LOXL3* gene shows nominal transmittance of minor allele and increased risk with infant genotypes. Whereas for *GRHL3* gene shows a significant transmission of minor allele and a threefold increased risk with infant genotype. As the studies entail the sex based segregation of minor allele for both gene-variants, it may seem plausible to say that sex of the infant is interacting with the genotype of both gene variants in a manner similar to other environmental factors i.e. there appears a gene-sex interaction for both gene variants. Such sex-specific genetic architecture suggests new models of susceptibility in the etiology of nsCPO as well as nsOFC. The present study warrants its use to better understand its mechanisms in complex human phenotypes such as nsOFC.

Part 3. **Epigenetic regulation**: Using lip tissue in conjugation with mothers environmental factors.

Chapter 5. Epigenetics is an additional layer of instructions that controls how our DNA is interpreted, leading a way the genes are controlled and expressed. The mechanism of which changes the way genes are packaged in the cell nucleus, and involve changes in chemical groups that can attach to DNA, or changes in the way RNA molecules interact with our DNA. Some of this epigenetic mechanism can go wrong early in development resulting in diseases such as NTDs (Wang *et al.*, 2010), CHDs (Bahado-Singh *et al.,* 2016). Diet and other environmental factors help regulate the potential child bearing in-utero environment influencing the epigenetic processes (Nilsson *et al.,* 2016) and some of this epigenetic marks can potentially be passed down through generations (Hanson *et al.,* 2016).

The epigenetic work in the present thesis is an attempt to open window of epigenetic regulation and its impact on the development of OFC. This is the first study targeting the cleft etiology with epigenetic profiling of global LINE-1 methylation in tissues from cleft cases in context to sexes and cleft phenotype paralleled with or without periconceptional folic acid supplemented pregnancies.

The result of this work in the thesis is surprising and awakening, showing differences in the average score of global LINE-1 methylation on the two cleft side of cleft cases. This change was more restricted to pregnancies depleted with periconceptional folic acid supplementation. Our hypothesis for the observed change is the difference in developmental timing of the two sides (Dixon *et al.*, 2011), coupled with environmental disruption during this period of pregnancy can together influence differential methylation of the two cleft sides.

Progressively, the present study was an attempt to gain insight into periconceptional folic acid and global LINE-1 methylation, which showed increased methylation of the medial sides in infants born to mothers without periconceptional folic acid supplementation. This reasons that perconceptional folic acid could counter balance the excess methylation and help maintain proper methylation between the intermaxillary and mediolateral prominences during upper lip development.

Moreover, our attempt of sex and cleft phenotype specific analysis showed no-significance. While comparison within male lateral and medial side was significant, and surprisingly the majority of this cases were born to folate deplete mothers. Considering Maloney *et al.*, 2011 study the present result can be reasoned, to different response of males to compensate with environment of mothers devoid of folic acid.

The present study warrants the use of tissues to gain insight on global methylation, especially in the cases such as birth defects to gain true methylation scores which is difficult to achieve using PBL due to its confounding cellular heterogeneity (Skinner *et al*., 2016).

Part 4. **Evaluation of Orbicularis oris (OO) muscle**: Explore differences in OO muscle arrangement using histological images.

Numerous histological studies have been done to understanding of the development of cleft lip, but only few targeted the OO muscle at the edges of the cleft lip (Schendel *et al.,* 1989; Raposio *et al.,* 1998; Wijayaweera *et al.,* 2000) and between lateral and medial edge of cleft lip (Wijayaweera *et al.,* 2000). Neiswanger *et al.*, 2007 and Mittal *et al.,* 2012 contributed to this understanding with a new directional approach, by using ultrasound technique to speculate OO muscle in individuals with cleft lip. Yet, there seemed a gap in information characterizing the OO muscle with respect to variation in OO muscle fibre diameter on either sides of the cleft lip. Information such as this could provide a potential distribution and role of different muscle type on either side of the cleft, which could lead a way towards reconstruction of the cleft lip. Taking advantage of the tissue samples collected under PENTACLEFT project, the present study made use of an image processing tool to augment understanding of the muscle fibre diameter on the lateral and medial cleft sides in a view to ascertain sex and phenotype based differences in the OO muscle fibre diameter. Our result showed significant change in the muscle fibre diameter on lateral and medial side of the cleft, but on comparing male against female appeared significant for the medial side, with male having smaller muscle diameter compared to female. Moreover, there was no significant difference within and between the two cleft phenotypes, for lateral and medial sides compared. The sex based dissimilarity on the medial muscle fibre diameter observed in the present study could be an outcome of inherent sex related developmental differences (Natsume *et al.,* 1988), or differential response to a hormone. Studies (Neiswanger *et al.,* 2007) shows that the OO muscle is forming around same time when the control of hormones shifts from mother to the fetus, and if the process occurs slightly differently depending on the sex of fetus, it could possibly result in the observed difference in the OO muscle between male and female.

In addition to the histological outcomes of the muscle fibre measurement the present study also facilitates the use of inherent ROI color coded plugin (Ferreira, 2014) of ImageJ as

a mean to evaluate the muscle fibre diameter. This techniques is an added advantage to explore muscle types and diameter in fixed tissue specimens in relation to fresh tissue specimen, once the standard threshold diameter is known. Alongside, it can prove compatible with the regular pH sensitive histochemical staining (de Chalain *et al.,* 2001), to identify lighter stained type 1 (slow-twitch) fibres and dark stained the type 2 (fast-twitch) fibres.

Conclusion

The *TGFA* gene is extensively studied but has resulted in divergent result in different population and additionally it has not been identified as potential risk in conventional GWAS. This evidence the uniqueness of *TGFA* gene in association of developing OFC. Our present study was lucky to capture the mode of its association in etiology of cleft, which could be modulated or influenced by the sex of the fetus or sex determining factors expressed during embryogenesis. This is reflected by opposite effect in males and females for *TGFA*/Taq1 variant in developing nsCL/P, marking sex as an environmental factor regulating the genetic risk and highlights the relevance of gene-sex interaction in nsOFC etiology.

Both the genes (*LOXL3* and *GRHL3*) studied in present thesis to check susceptibility of developing nsCPO are rare variants, and it is difficult to interpret the role of rare variants in complex diseases such as nsCL/P or nsCPO. As reported, the majority of rare variants in individuals with nsCL/P do not segregate in families, as evident in our present study for *LOXL3* gene-variants showing no significant segregation of minor allele. Although most rare variants are functionally deleterious such as our *GRHL3* variant, variants found in complex diseases are less penetrant than their Mendelian counterparts. Because of this, variants could still be relevant genetic risk factors for nsCL/P but require the combined action of additional genetic variants and/or environmental factors to manifest an effect. As per results of the current study this environmental factor could be the sex of the infant, reflecting a sex dependent transmission of minor allele for both gene-variant. Although, there appeared no influence of other environmental exposures in risk of *LOXL3* but *GRHL3* was influenced by periconceptional folic acid supplementation. The genetic effect of an individual rare variant such as rs17010021; *LOXL3* might not be statistically significant with small sample size and hence, the statistical significance can be validated using large case-parent trios. Although, *GRHL3* variant appeared as significant risk for developing nsCPO with the same sample size, as a consequence of their functional deleteriousness.

The outcome of epigenetic study done in the present thesis relies on the tissue specific global methylation profiling, making it wise and free of confounding factors cellular heterogeneity using PBL (Skinner *et al.*, 2016). Moreover, this is the first attempt to gain insight epigenetic regulation of nsOFC etiology using the closest possible tissue which is directly involved in the etiology of cleft, the evaluation of lateral and medial sides adds value to the study as these two sides possibly fuse to form lip and any epigenetic modification in any of the two side could possibly respond differently avoiding proper fusion, leading to cleft. Of note, this is a preliminary study and should be replicated with large number of cases in order to gain more significant result.

The evaluation of the orbicularis oris muscle fibre diameter on either side of the cleft specimen is a unique study which can be correlated with the echoic breaks in ultrasound, thought to be an outcome of different muscle fibre type and diameter. The study values the role of sex in the etiology of cleft with an observed differences in muscle fibre diameter on the medial side of cleft. This study also advents the use of image processing using ROI coder for faster and easy evaluation of muscle fibre diameter to supplement better restoration of the anatomical and functional component of the upper lip.

Future Direction

Considering modulation of collagen rich cervical ECM and the role of lysyl-oxidase family over different time point thoughout pregnancy, regulating the collagen cross-linking leading to change in its elasticity and strength, especially at the gestational day 12 in mice, could affect the normal orofacial palate developmental procedures. Along the process, it is reported a decline in LOX activity in the mouse cervix at early pregnancy, and our study of sex dependent influence in the susceptibility of developing cleft palate, with additional reports on deletion of LOX gene in mouse resulting in peri-natal lethality with impaired development of palatal shelves, gives a direction of possible link between divergent collagen remodeling of cervix and the palate shelves. Moreover, the divergent ECM remodeling of the two phenotypes (cervical, less crosslinked collagen and palatal elevation, more crosslinked collagen) could be an outcome of commonly shared gestational stage and shifts from materal to fetal guided harmone production. Furthermore, as per previous reports, aberrant remodelling of the cervical ECM leads to pre-term birth and this course of pre-term birth is documented to increase risk for childrens to be born with anomalies, such as cleft lip and palate. This shared genetic pool (LOX family) can be taken advantage to unravel the role of

pre-term birth in context to CP development. In addition other functional variants in the *LOXL3* genes can be identified and tested for gene-gene and gene-environment interactions.

GWAS-Identification of major loci involved in nsCL/P has not completely reflected the heritability of nsCL/P, indicating that the child's genetic profile alone cannot explain the origin of this malformation. The coupling of child's genetic profile with the exposure of mothers to specific environment during early pregnancy may be relevant. In order to gain insights the natural child bearing placental environment can be explored in terms of epigenetic profile. The potential benefit is in using a human placenta to achieve optimal understanding of the gene-sex interactions at the placental level. As the two broad cleft phenotypes (CL/P and CPO) are considered to have different etiology and their prevalence differs with sexes, it may seem reasonable to suggest the existence of differential gene regulation in placenta's nourishing the male and female child with different cleft phenotypes and have a different epigenetic profile.

Presuming the outcome of the present epigenetic study, it is plausible to suggest that in animal models epigenetic analysis should mainly focus on search of epimutations in the intermaxillary prominences, and determine if folic acid supplementation can restore normal methylation level at this site and protect from clefting.

The hypothesis that the echoic breaks in ultrasound, which is a sign of a missing segment of muscle or an abrupt thinning is an associated outcome of different muscle fibre-types and diameter. The future work in this area may investigate (1) Illustration of the different muscle fibre-types and diameter by comparing the histological slices with corresponding hyper and hypo-echoic areas of an ultrasound imaging. (2) Quantitative assessment of distribution of Type I and Type II muscle fibres on two cleft sides. (2) Better demonstrate the power of ROI color coder.

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SUMMARY

The Orofacial cleft is the fourth most common birth defect affecting the lip and the palate. It is classified in three broad categories: cleft lip (CL), cleft lip and palate (CLP), consolidated into cleft lip with or without cleft palate (CL/P) and cleft palate only (CP). Further classified as syndromic and nonsyndromic depending on its association with or without any other malformations, respectively. The nonsyndromic CL/P and CP (nsCL/P & nsCP) have separate genetic etiology and are influenced by both genetic and environmental factors with different magnitude of risk for both, hence considered multifactorial. In addition, the prevalence of two cleft phenotypes varies with infant sex, CL/P is more common in males and CP on the contrary are more common in females. In the present thesis we took advantage of sex differences, alongside common phenomenon's affecting the cleft etiology and help value the sex dependent endogenous differences in nonsyndromic orofacial cleft (nsOFC).

Our first approach banked on the family based association analysis, to explore genes-sex influence on the susceptibility of nonsyndromic cleft lip and palate (nsCL/P) and nonsyndromic cleft palate (nsCP). We genotyped nsCL/P and nsCPO case-parent trios (from EUROCRAN and ITALCLEFT studies) collected from nine European countries in the period 2001-2014, for selected Transforming growth factor A (*TGFA*), *TGFA*/Taq1/4nt and *TGFA*/11nt insertion deletion. Where we found a sex dependent association of *TGFA*/taq1/4nt variant in developing nsCL/P, with an opposite effects in male and female infants.

The work was further extended to nsCPO group, where we checked lysyl oxidase like 3 (*LOXL3*) and Grainyhead like 3 (*GRHL3*) in the susceptibility of developing nsCPO and tested for gene-sex and gene-environment interaction. For this, we genotyped nsCPO case-parents trios (from EUROCRAN and ITALCLEFT studies) collected from nine European countries in the period 2001-2014, for selected variants in the two genes. We observed no significant segregation of minor allele for *LOXL3*. Whereas the *GRHL3* showed a significant segregation of minor allele. Moreover, there appeared a female biased risk for both *LOXL3* and *GRHL3* variant. For both tested genes there was no risk with mothers genotype for developing nsCPO. Whereas, the risk of both gene-variant was influenced by infants genotype, with a threefold increased risk for *GRHL3* variant in infants homozygous for the minor allele. Furthermore, for *LOXL3* variant, the mothers who reported folic acid

supplementation and smoking showed no evidence of nsCP risk with infant genotype, while *GRHL3* variant showed significant risk to infants born to mothers without folic acid supplementation and smoking.

The interaction of the fetal genes with the mothers intra-uterine environment is a critical window for proper development of the fetus, where any disruptions or modifications can influence fetal development as well can lead to birth related anomalies such as NTDs, CHDs and OFC. In the present work we checked modification in global LINE-1 DNA methylation on the closest possible tissue (lip) involved in the etiology of cleft. We observed significantly increased methylation on the medial side compared to the lateral side. In addition we also observed significant scores inclined to males born to non-folic acid supplemented mothers. The outcome of the present study entails the importance of epigenetic modification and opens a new directional window in the etiology of cleft. This study is the first to explore methylation status in cleft and warrants replications with a larger set of samples for consolidating the outcome of this preliminary study.

The OO muscle is one of the skeletal muscle of the upper lip intimately associated in maintaining the upper lip structure and is composed of different types of muscle fibres. Having an advantage of CL/P lip tissue bank, directed us to different level from genes to tissues, where we explored sex dependent differences in the orbicularis oris (OO) muscle diameter on the lateral and medial sides of the cleft lip, in order to provide an advantage towards better reconstruction of the cleft lip based on sexes. There was no significant change in the muscle fibre diameter on lateral and medial side of the cleft. Instead, comparing medial side of male against female appeared significant, with males having smaller muscle diameter compared to females.

Both the family based association study on nsCL/P and nsCPO, entails the sex based segregation of minor allele, consolidating our emphasis of sexual dimorphism in gene association studies of nsOFC, which is generally ignored. Moreover the epigenetic study throws light on sex based modification of genes, reflecting that the two sexes can respond differently to the mothers in-utero environment. In addition our histological evaluation on OO muscle diameter in the two sexes further consolidates the endogenous sex based differences observed in the nsOFC and gives a new direction for sex dependent reconstruction of the upper lip. Based on our result it appears reasonable to suggest

differential gene regulation mechanism in males and females that can influences human phenotypes, including reproductive, physiological and disease traits, such as OFC.

RIASSUNTO

Le Schisi Orofacciali sono malformazioni congenite che interessano il labbro ed il palato e rappresentano il quarto difetto congenito più comune. Sono classificate in tre grandi categorie: labioschisi (CL), labiopalatoschisi (CLP) e palatoschisi (CP). CL e CLP vengono generalmente raggruppate in labioschisi con o senza palatoschisi (CL/P). CL/P e CP sono inoltre classificate come sindromiche e non-sindromiche, a seconda della presenza o assenza di altre malformazioni. Le forme non sindromiche di CL/P e CP (nsCL/P e nsCP) hanno un'eziologia genetica multifattoriale, che comprende fattori eziologici sia genetici che ambientali. Inoltre, la prevalenza delle diverse tipologie di schisi varia con il sesso dei bambini: le CL/P sono più comuni tra i maschi, mentre le CP sono più comuni tra le femmine. Nel presente studio si è voluto investigare il ruolo delle differenze di sesso nella eziologia delle schisi orofacciale non sindromiche (nsOFC), in relazione agli aspetti sia genetici che epigenetici.

Il nostro primo approccio si è concentrato sull'analisi di associazione nelle famiglie nucleari (triadi genitori-figlio affetto), per esplorare la relazione tra varianti genetiche e suscettibilità a sviluppare nsCL/P e nsCP. Abbiamo genotipizzato due varianti comuni del gene TGFA codificante il Transforming growth factor α (TGFA/Taq1/4nt del e TGFA/11nt ins/del) in triadi nsCL/P o nsCPO provenienti dagli studi EUROCRAN e ITALCLEFT raccolte in nove Paesi Europei nel periodo 2001-2014. È stata trovata una associazione sesso-dipendente relativa alla variante TGFA /Taq1/4nt del che, relativamente al rischio di sviluppare nsCL/P, manifesta nei maschi un effetto opposto a quello che presenta nelle femmine.

Lo studio genetico è stato esteso, inoltre, a indagini specifiche per le forme di nsCPO. E' stata analizzata l'associazione tra varianti funzionali nei geni LOXL3 (Lysyl oxidase like 3) e GRHL3 (Grainyhead like 3) ed il rischio di sviluppare nsCPO, nonché investigate le interazioni gene-sesso e gene-ambiente. Inizialmente abbiamo analizzato triadi genitori-figlio di casi di nsCPO (dagli studi EUROCRAN e ITALCLEFT) raccolte da nove Paesi Europei nel periodo 2001-2014, studiando varianti candidate nei due geni. I risultati non hanno evidenziato alcuna significativa segregazione asimmetrica degli alleli minori delle varianti di LOXL3 e GRHL3. Per ambedue i geni non è emersa alcuna associazione tra nsCPO e genotipo materno, mentre il rischio di sviluppare nsCPO è risultato influenzato dal genotipo

del caso indice, con un rischio relativo di nsCPO aumentato di tre volte negli omozigoti per l'allele minore di GRHL3. Mentre nel caso della variante di LOXL3 della madre la supplementazione in gravidanza con acido folico o l'esposizione al fumo di tabacco è risultata non influenzare il rischio di nsCPO nel nascituro, la variante GRHL3 è risultata significativamente associata alla mancata supplementazione con acido folico e alla esposizione al fumo di tabacco.

L'interazione dei geni fetali con l'ambiente intrauterino materno costituisce un elemento critico che condiziona il corretto sviluppo del feto, ed alterate combinazioni genotipo-ambiente possono portare ad anomalie di sviluppo, quali i difetti di chiusura del tubo neurale, patologie cardiache congenite (CHDs) e nsOFC.

La disponibilità di una biobanca di campioni labiali di pazienti con nsCL/P ha permesso di eseguire indagini epigenetiche ed istologiche.

Nel presente lavoro abbiamo eseguito per la prima volta indagini epigenetiche nelle nsCL/P, analizzando i livelli di metilazione genomica globale (promotore di LINE-1) in campioni di DNA estratti dai tessuti più prossimi alla porzione labiali coinvolte nelle labioschisi (nsCL). I risultati hanno mostrato un significativo aumento di metilazione sul lato mediale del labbro superiore rispetto al lato laterale. La differenza è risultata particolarmente evidente nei maschi nati da madri che durante la gravidanza non hanno praticato la supplementazione con acido folico.

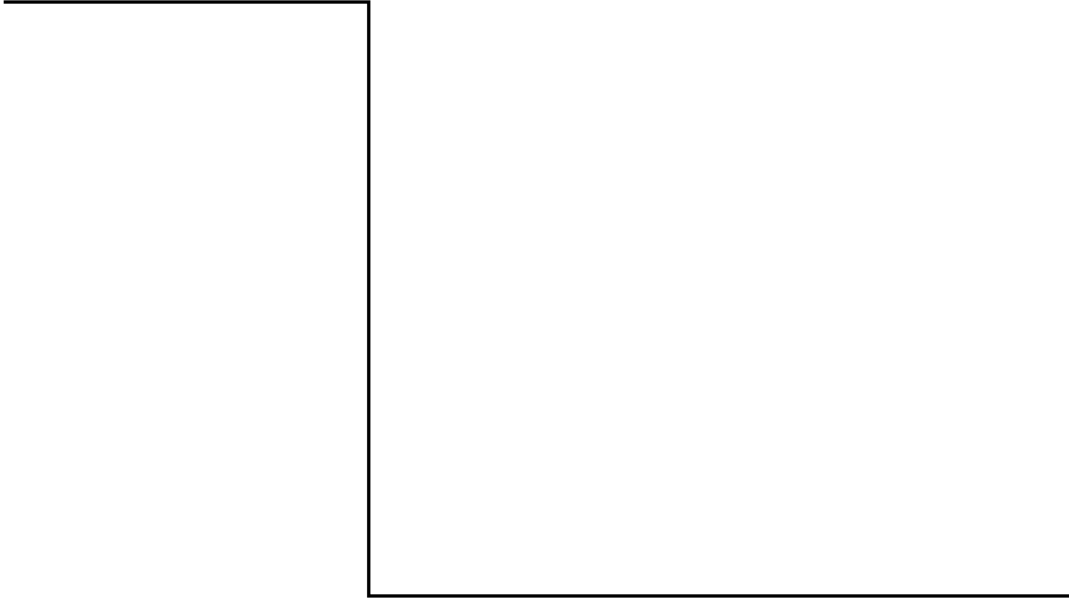
I risultati di questo studio, seppur preliminari, evidenzia l'importanza dell'epigenetica nell'eziologia delle nsCL/P ed aprono la strada a nuove e più estese indagini finalizzate a valutare come l'esposizione a fattori di rischio ambientale possano determinare alterazioni epigenetiche che contribuiscono alla patogenesi delle nsCL/P.

Il muscolo OO è uno dei muscoli scheletrici del labbro superiore che serve a mantenere la struttura labbro superiore ed è composto da diversi tipi di fibre muscolari. Avendo a disposizione una banca dei tessuti labiali di casi con nsCL/P si sono potuti effettuare indagini finalizzate a misurare le dimensioni delle fibre muscolari delle sezioni del muscolo orbicularis oris (OO) ai due lati della schisi labiale. L'indagine non ha rilevato alcuna significativa differenza di diametro tra le fibre muscolari del lato mediale e quelle del lato laterale della schisi. Tuttavia, confrontando il valori rilevati nei due sessi, è risultato che nei maschi la sezione delle fibre muscolari nel lato mediale è significativamente più piccola rispetto a quella osservata nelle femmine.

In conclusione, entrambi gli studi di associazione genetica condotti in triadi nsCL/P e nsCPO hanno prodotto risultati che sono specifici per sesso, dando enfasi al ruolo del dimorfismo sessuale negli studi di associazione genetica di nsOFC, cosa che finora è stata essenzialmente ignorata negli studi pubblicati in letteratura scientifica. Lo studio epigenetico condotto getta luce sulla modificazione dei geni in base del sesso, e tale situazione si riflette sul fatto che i due sessi possono rispondere in modo diverso all'interno dell'utero materno. In aggiunta, la nostra valutazione istologica sul diametro del muscolo OO nei due sessi consolida ulteriormente le differenze endogene di sesso osservate nei casi con nsOFC e può fornire nuove consocenze, potenzialmente utili per migliorare le tecniche di ricostruzione del labbro superiore.

I risultati degli studi condotti suggeriscono l'esistenza differenze nello sviluppo del labbro nei due sessi, le quali possono influenzare l'impatto del genotipo e della esposizione ambientale nel generare condizioni di rischio per lo sviluppo di nsOFC. Queste differenze sesso-specifiche possono potenzialmente essere estese anche ad altri distretti anatomici, ed avere un ruolo anche in altre anomalie congenite.

APPENDICES



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LIST OF GENE NAMES

ABCA4 ATP-binding cassette, sub-family A, member 4

BMP4 Bone morphogenetic protein 4

FAF1 Fas Associated Factor 1

FGFR2 Fibroblast growth factor receptor 2

FOXE1 Forkhead box E1

GRHL3 Grainyhead-like 3

GSTT1 Glutathione S-transferase (GST) theta 1

IRF6 Interferon Regulatory Factor 6

LOXL2 Lysyl oxidase like 2

LOXL3 Lysyl oxidase like 3

LOXL4 Lysyl oxidase like 4

MAFB v-maf musculoaponeurotic fibrosarcoma oncogene homolog B

MSX1 Msh homeobox 1

MTHFR Methylenetetrahydrofolate reductase

MYH9 Myosin Heavy Chain 9

SUMO1 SMT3 suppressor of mif two 3 homolog 1

TGFA Transforming growth factor alpha

VAX1 Ventral anterior homeobox 1

LIST OF ABBREVIATIONS

BAPN = β-aminopropionitrile

BCL = Bilateral cleft lip

CL = Cleft lip

CLP = Cleft lip plus cleft palate

CL/P = Cleft lip with or without cleft palate

CP = Cleft palate

CF = Collagen fibril

DNA = Deoxy-ribonucleic acid

ECM = Extracellular matrix

EGF = Epidermal growth factor

EUROCRAN = European collaboration for craniofacial anomalies

GWAS = Genome-wide association study

H&E = Hematoxylin and Eosin

HA = Hyaluronic acid

ITALCLEFT = Italian cleft network

LINE = Long interspersed nuclear element

LNP = Lateral nasal process

MAF = Minor allele frequency

MES = Medial epithelial seam

MxP = Maxillary process

MNP = Medial nasal process

nsOFC = Nonsyndromic Orofacial cleft

nsCL/P = Nonsyndromic Cleft lip with or without cleft palate

nsCP = Nonsyndromic Cleft palate

OFC = Orofacial cleft

OO = Orbicularis oris muscle

RFLP = Restriction fragment length polymorphism

ROI = Region of Interest

RR = Relative risk

SNP = Single nucleotide polymorphism

TDT = Transmission disequilibrium test

UCL = Unilateral cleft lip

VWS = Van der Woude syndrome