Evaluating LINE-1 methylation in cleft lip tissues and its association with early pregnancy exposures

Khan, Mohammad Faisal J.; Little, Julian; Mossey, Peter A.; Steegers-Theunissen, Régine P. M.; Autelitano, Luca; Lombardo, Ilenia

Published in:
Epigenomics

DOI:
10.2217/epi-2017-0081

Publication date:
2018

Document Version
Peer reviewed version

Abstract

Aim: To pilot investigation of methylation of long interspersed nucleotide element-1 (LINE-1) in lip tissues from infants with non-syndromic cleft lip, and its association with maternal periconceptional exposures.

Methods: The lateral and medial sides of the cleft lips of 23 affected infants were analyzed for LINE-1 methylation by bisulfite conversion and pyrosequencing.

Results: The medial side showed 1.8% higher methylation compared to the lateral side; p=0.031, particularly in male infants (2.7% difference; p=0.011) or when the mothers did not take folic acid during periconceptional period (2.4% difference; p=0.011). These results were not statistically significant when Bonferroni adjustment was used.

Conclusion: The observed differences in DNA methylation, although non-significant after correction for multiple comparisons, suggest that differential regulation of the two sides may impact lip fusion and warrant larger-scale replication.

Keywords: LINE-1, DNA methylation, Cleft lip with or without cleft palate.

Running title: LINE-1 methylation in cleft lip tissues

Introduction

Orofacial clefts (OFC) are congenital anomalies affecting the lip, palate or both and categorized in two broad phenotypes, cleft lip with or without cleft palate (CL/P) and cleft palate (CP) [1]. The occurrence of the two phenotypes shows a sex based disparity, with a male predominance in CL/P and a female predominance in CP [2, 3]. About 30% of cases of OFC are syndromic. The non-syndromic cases are thought to be due to multiple genes and environmental factors [4, 5].

Like most other chronic diseases, the heritability of developing non-syndromic OFC is only to a small extent accounted for by the major risk loci so far identified, indicating that the infant's genetic profile alone cannot explain the origin of this malformation. And as the formation of the lip and palate starts early in pregnancy, is vulnerable to perturbation of the maternal nutritional and non-nutritional milieu [6] that can affect its epigenetic programing. Therefore, cleft of the lip and palate can arise as a result of any change that impacts its normal development such as genetic variation [7, 8], and environmental factors including maternal nutrients, smoking and hormones [9, 10, 11, 12], but the role of these factors in etiology is still inconclusive [13, 14].

Numerous studies suggest that the risk for OFC is increased by disturbance of the one-carbon metabolism cycle [15], although the role of specific nutrients such as folate remains controversial [16, 17, 18]. Folate feeds into the one-carbon metabolism cycle that results in the formation of methyl groups [19]. The level of supplementation of methyl donors in pregnancy has been shown to influence the levels of DNA methylation in infants [20], especially in the periconceptional period [21]. Moreover, in a mouse study, an increase in the level of dietary methyl donors has been found to increase genomic DNA methylation levels in the offspring [22].

DNA methylation of the pyrimidine base cytosine in DNA may be one of the mechanisms underlying differential programming of cell lineages in mammalian development, as suggested by the erasing or reshuffling of methylation marks in the early embryo and its reestablishment after implantation [23, 24, 25]. This process establishes basic adult methylation patterns prior to organogenesis. DNA methylation is in a state of flux during gametogenesis and early embryogenesis, which can be modulated by embryonic environmental exposures. Potentially, investigation of the methylation of long interspersed nucleotide element-1 (LINE-1) repetitive elements, generally accepted to be a surrogate measure of global DNA methylation content

[26, 27], could provide insight into the role of the environment in regulating whole genome DNA methylation.

Numerous studies have shown changes in LINE-1 DNA methylation associated with the onset of specific conditions such as gestational diabetes, preeclempsia, congenital heart diseases; CHD and neural tube defects; NTDs [28, 29, 30, 31] and with prognosis of diseases such as several types of cancer [32, 33, 34]. Studies in animals or animal derived tissues have shown the involvement of DNA methylation in the development of OFC, one study involved lip tissue [35], but the majority of investigations are limited to secondary palate tissues with an intact upper lip [36, 37]. Most human OFC cases involve clefting of the upper lip [38, 39, 40]. Moreover, the lip and palate have separate embryological origins and therefore may have different etiologies and DNA methylation status [41]. This is supported by recent data showing distinct methylation profile in different cleft subtypes using blood DNA [42]. In addition Alvizi *et al*. observed that DNA methylation correlated with the penetrance of nonsyndromic cleft lip and palate (nsCL/P) [43]

There is a lack of epigenetic data on the DNA methylation of cleft tissues in humans. To overcome this shortcoming, we undertook a pilot study of LINE-1 methylation in lip tissue taken from humans undergoing surgical repair of cleft lip (CL) to address epigenetic changes. We also investigated the association of LINE-1 methylation with reported maternal periconceptional folic acid supplementation, sex and cleft subtype.

Materials and methods

Cases

Infants with non-syndromic cleft lip with or without palate were identified in the context of the ongoing PENTACLEFT project [44]. The PENTACLEFT project protocol includes the recruitment of non-syndromic CL/P cases, their parents and maternal grandparents, and the collection of genomic DNA from peripheral blood or buccal swab samples. The project was approved by local IRB (prot. N.08-2011), and case enrolment required written parental informed consent. Families of consecutive cases were invited to enrol in the study at the Regional Centre for Orofacial Clefts and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy, at the time of the first surgical intervention on the index child. Infants with recognized syndromic clefts or the Pierre Robin sequence were excluded from the study. Parents of included infants were asked to respond to a specific questionnaire that was administered by personal interview when the affected child was brought to the surgical centre to undergo the

primary surgery. Information on educational status, ethnic group and family history of both parents was collected, along with data on maternal medical and reproductive history, exposure to environmental risk factors, use of drugs, medications and supplements such as folic acid (FA) during the periconceptional period (from three month before to three months after conception). Lip tissue samples were collected from non-syndromic CL/P cases at the time of first surgery.

Tissue samples

The lip tissue samples were collected from Twenty-three non-syndromic CL/P cases, with an average age of 6.5 (95% CI 5.0-7.1) months at the time of surgery: 12 males (7 CL, 5 CLP; 3 cases with preconceptional FA, preFA, and 9 without preconceptional FA, No-preFA); and 11 females (6 CL, 5 CLP; 2 cases with preFA, 7 No-preFA, and 2 with missing preFA data). Samples were collected immediately in lysis buffer (pH7.4) from both lateral and medial side of CL. The samples were then transferred to the laboratory at University of Ferrara where they were processed for epigenetic study.

DNA extraction and sodium bisulfite treatment

The cases' lateral and medial side cleft lip tissues collected in lysis buffer were homogenized separately, with a view to primarily include the connective tissue portion of the upper lip, with minimum contribution from the epidermis. The genomic DNA was extracted from the homogenate 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 bisulfite converted using EZ-DNA Methylation-Lightning™ Kit (Zymo Research, Irvine, CA, USA).

Pyrosequencing

The LINE-1 DNA methylation level was measured for all the study samples with pyrosequencing on PyroMarkQ96 ID using PyroMark Gold reagents (Qiagen). LINE-1 region including 4 CpG sites (position 305 to 331 in accession no. X58075) was amplified by PCR using the following primers: 5'-TTTTGAGTTAGGTGTGGGATATA-3' and 5'-Bio-AAATCAAAAAATTCCCTTTC-3'. LINE-1 PCR products represent a pool of approximately 15 000 genomic loci interspersed across the whole human genome [45, 46]. PCR reactions were performed in duplicate to achieve precision between runs with total volume of 25µl containing 10X PCR buffer, 50mM$MgCl_2$, 2.5mMdNTPs, 10pM of each primer, 5U Taq

polymerase and 2.5µl of bisulfite 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 analyzed on 8% polyacrylamide gel using silver staining. The PCR plate with each well containing 20µl of PCR product, 20µl of RNAse free distilled water, 3µl of sepharose beads containing streptavidin and 37µl of binding buffer; thus a 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).

The pyrosequencing runs were performed to obtain a pyrogram from each PCR reaction, using software for analysis in AQ (allele quantification) mode, in a total volume of 40µl per well, including 38.4µl of annealing buffer and 1.6µl of 10pM sequencing primer with suspended beads containing the sample DNA. The assays was created according to the manufacturer's recommendations and the output of the two pyrosequencing runs was averaged. The nucleotide dispensation order was: ACTCAGTGTGTCAGTCAGTTAGTCTG. LINE-1 DNA methylation values were detected at positions +306, +318, +321 and +328 in Genebank sequence X58075. The CpG site at position +328) was not considered for subsequent analyses, as precision of methylation values was insufficient, probably due to the adjacent CT dinucleotide. Using the combined average data, the overall LINE-1 DNA methylation values was calculated as the mean of the proportions of C (%) at the 3 CpG sites analyzed, (positions +306, +318 and +321) and this indicated the level of methylation of LINE-1 elements [47].

Statistical analysis

All the statistical analysis was performed using the IBM SPSS Statistics 21. All p-values were 2-sided, with a threshold for declaring statistical significance of $p<0.05$. The distributions of LINE-1 methylation levels were checked for normality using the Shapiro-Wilk test that is appropriate for small samples; none departed from normality. For within case comparison between lateral and medial cleft side, a paired student's t-test was used. For comparison of 2-level categories of periconceptional use of supplements containing folic acid, sex and cleft subtype, the unpaired student's t-test was performed. In view of possible concerns about multiple comparisons, we also applied the Bonferroni correction to comparisons within and between cases. This was a secondary analysis because of the known limitations of the Bonferroni correction and inapplicability of other forms of adjustment to this study [48, 49]. We adopted the most conservative approach of adjusting for all 13 comparisons reported.

Results

Our results are based on samples that showed a normal distribution of LINE-1 methylation for both medial (p=0.124) and lateral (p=0.773) sides. Initial analysis using the nominal $p<0.05$ threshold showed that the DNA in tissue taken from the medial side of the cleft lip was found to have 1.8% more methylation compared to DNA in tissue taken from the lateral side (p=0.031; Table 1). In analysis stratified on sex, no significant difference in methylation between the sexes for either the lateral (males 71.5±3.1% verses females 72.0±1.2%; p=0.748) or medial (males 74.2±3.0% verses females 73.0±2.4%; p=0.293) sides was observed (Figure 1). However, the methylation levels between lateral and medial sides in males was observed to be significantly different (lateral 71.5±3.10% verses medial 74.2±3.0%; p=0.011), (Table 1). When methylation levels within and between CL and cleft lip and palate (CLP) subtype were analyzed, no significant differences were observed (Table 1). To evaluate the role of periconceptional folic acid as an environmental factor affecting the level of global DNA methylation, we calculated the average methylation level on lateral and medial cleft sides in infants born to mothers with and without periconceptional folic acid supplementation. Comparison between these two groups showed no significant difference. However, methylation on the medial side was 2.7% higher than that on the lateral side in the cases whose mothers did not take periconceptional folic acid supplements, (p=0.011; Table 1). In secondary analysis using a Bonferroni corrected threshold (p=0.004) , this finding was not statistically significant.

Discussion

In this first pilot study using human lip tissue obtained from infants with non-syndromic CL/P, we observed differences in LINE-1 DNA methylation between tissues on the lateral and medial side of the cleft. These differences were apparent in boys but not in girls, and in infants whose mothers did not take supplements containing folic acid in the periconceptional period but not in the offspring of women who took supplements. There were no differences in methylation by sex or cleft subgroup.

From our results it appears that the medial side of clefts have higher global methylation levels, especially in male infants. This pattern is also apparent in infants from pregnancies in which supplements containing folic acid were not taken during the periconceptional period, but we acknowledge that numbers are very small. We also recognize that the inability to obtain normal lip tissues with which to compare our lip tissue samples is a shortcoming that prevents

the direct determination of whether this epigenetic difference between of the two sides is a pattern present in the general population, rather than being specific to clefting, i.e. a real cause or consequence of clefting. However, we suggest that this difference may reflect the fact that these tissues develop during separate embryonic stages and therefore possibly experience different environmental exposures. The lateral aspects of the upper lip originate from the maxillary process (MxP) during the 4th week of embryonic development, while the medial aspects of the upper lip originates from the medial nasal process (MNP), beginning in the 5th week [4]. It is possible that the two separate windows of origin may have been exposed to different environmental milieus that resulted in differential methylation of the two sides of the cleft, in turn influencing the closure of the two processes and the occurrence of a cleft lip.

Another possible explanation for the observed differences in methylation of the two cleft sides could lie in the developmental field concept, and act in a spatial and temporal manner [50]. For example, in normal circumstances, the lateral and medial aspects of the upper lip originate from two different embryonic units that constitute a morphogenetic reactive unit. It is possible that this part of the embryo's reactive unit in the two aspects of the upper lip may have experienced different spatial and temporal forces of organization and differentiation (epimorphic field) leading to dysmorphogenesis of the two sides, reflected as a difference in methylation as observed in our study. Some empirical support is provided by a recent animal study that shows temporal regulation of Sonic Hedgehog (SHH), resulting in down-regulation of *Foxf2* expression and reduced proliferation of medial nasal process mesenchymal cells that are required for upper lip closure [51]. We postulate that differential expression of a single gene on the two sides of the developing lip could be regulated by different epistatic factors, and hence we plan to investigate expression of specific genes implicated in human clefting in future work.

An influence of in-utero environment on epigenetic modulation is compatible with previous reports showing associations between DNA methylation changes and neural tube defects [52] and congenital heart defects [53, 54]. Neural tube defects are clearly linked with low folate status [55], and there is some evidence that this is also the case for several types of congenital heart defect [56]. There appears to be no previous reports on the association between folic acid intake during the periconceptional period or pregnancy and global LINE-1 DNA methylation in humans in general, or specific to the development of CL/P [57]. In infants born to mothers who did not use periconceptional folic acid supplementation we found a suggestion of a trend of increased methylation on the medial side.

In the cleft subgroup analysis, we found no significant difference in methylation between the two sides, a result that is compatible with the similar DNA methylation profile of CL and CLP reported by Sharp *et al*. [42]. Similarly, sex subgroup analysis showed no significant difference in methylation between the two sides. However, comparison within males showed a significantly higher methylation for the medial side that may be an outcome of differential developmental programming in males, who have an increased susceptibility to CL/P [2, 3]. This may reflect a role of sex in cleft etiology. We acknowledge that our results are based on small numbers, because collecting tissues from the cleft cases is of great challenge [58]. Statistical power is low, and we note that all nominally significant results in this study were non-significant in the secondary analysis applying the Bonferroni correction. Therefore, we urge that until replication of our results with in a larger sample size, the clear answer to this primarily evidence should be taken with caution.

According to a recent study, infants with cleft lip and cleft lip and palate subtypes may have similar rates of development, suggesting that epigenetic changes associated with development may not be a confounding factor in epigenetic studies of cleft lip, and cleft lip and palate [42]. Children with CL and CLP underwent surgery and thus had samples taken at approximately similar ages. There are reports of the absence of an age effect on LINE-1 methylation [59, 60, 61], and from a study on lip tissues collected from 4-month-old CL and CLP cases that shows an independent expression of genes associated with ageing [60]. Therefore, we consider that our observation is little influenced by ageing. Moreover, the advantage of using tissues derived from same individuals with relatively similar age in our study overcomes the influence of DNA sequence on DNA methylation and possibly the influence of age on DNA methylation, if any. Although the targeting of lip tissues in this study potentially would give the most direct insight into epigenetic changes associated with the occurrence of cleft lip, we are aware that heterogeneity could arise in these tissues from neural-crest derived connective tissue and muscles, and in-situ derived epidermis. However, we sought to overcome this limitation by collecting tissues in a lysis buffer to minimize the contribution from the epidermis and primarily include the connective tissue portion of lip tissues. Moreover, the observation of similar correlations between blood and tissue methylation in nsCL/P epigenetic study of Sharp *et al*. [42] and Alvizi *et al*. [43] suggests that the two tissues can be considered to be exchangeable in nsCL/P methylation studies at least. An aim of our future work in newly recruited cases is to collect blood and investigate correlation between methylation in blood with that in tissue from the lateral and medial sides of clefts.

Another potential limitation of our study is that the tissue DNA methylation measurement in infancy may have been indirectly influenced by the presence of a cleft lip and so may differ from that at the time of lip fusion in embryonic development. But for ethical reasons, this is the only accessible, and the closest, tissue associated with OFC that can be studied in humans. It is obviously difficult to collect lip tissue specimens from normal babies and this limits making direct causal inference.

Of note, our study is based on small sample size (with possibility of both Type I and Type II errors) and being aware of this limitation, splitting our samples based on factors (sex, cleft subtype and pre-FA) thought to affect methylation, was an attempt to provide preliminary data. Our primary analysis did not include Bonferroni correction because of known limitations including Type II error [48] and the inapplicability of other forms of adjustment [49] in this exploratory study, in view of being cautious of not missing a possible effect worthy of future investigation. Since the recruitment of cleft cases is still ongoing in the PENTACLEFT project, we hope to replicate and better justify our preliminary finding using larger number of cases and to investigate epistatic regulation of genes implicated in OFC.

In conclusion, the observed difference in methylation between tissue taken from the lateral and medial sides of a cleft lip may reflect the fact that these tissues develop during separate embryonic stages and therefore possibly experience different environmental exposures that can regulate DNA methylation patterns differently. The finding of a difference in DNA methylation in male but not female infants should be further investigated. Our findings suggest that epigenetic mechanisms may be important in the etiology of OFC, warranting replication in a larger study.

Summary points

- The etiology of non-syndromic orofacial cleft (OFC) is only in part explained by genetic variants. We hypothesized the possible role of early pregnancy epigenetic programming in the pathogenesis of OFC.
- There is lack in epigenetic data on the DNA methylation of cleft tissues in humans. Therefore, to overcome this shortcoming, this pilot study is the first comparative assessment of long interspersed nucleotide element-1 (LINE-1) methylation between tissues taken from the two sides of infants with cleft lip, and investigate possible association with reported maternal periconceptional environmental exposures.

- We show that LINE-1 methylation of tissues from medial side of the lip is higher compared to the lateral side, and that is particularly apparent for male infants. In addition, we show that the medial side methylation is higher for infants whose mothers did not take supplements containing folic acid during periconceptional period.
- The observed differences in methylation between tissue taken from lateral and medial sides of cleft lip may reflect the fact that these tissues develop during separate embryonic stages and therefore possibly experience different environmental exposures that can modulate DNA methylation patterns differently.
- The differences in methylation between males and females may reflect a play of chance.
- This study suggests differential methylation of two cleft side that may impact lip fusion, warranting replication in a larger study.

Financial & competing interests disclosure

The Authors have no conflicts of interest to declare

Ethics approval and consent to participate

Research ethical approval was granted by the local IRB (prot. N.08-2011) and required written parental informed consent was collected for enrolled case.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Marazita ML. The evolution of human genetic studies of cleft lip and cleft palate. Annu Rev Genomics Hum Genet 13 263-283 (2012).
2. Fogh-Andersen P. Incidence of cleft lip and palate: constant or increasing? Acta Chir Scand 122 106-111 (1961).
3. Mossey P. A., Little J. Epidemiology of oral clefts: an international perspective. In: Cleft lip and palate: from origin to treatment. Wyszynski DF (Ed.), Oxford University Press, New York, USA, 127–58 (2002).
4. Dixon MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet 12(3), 167-178 (2011).
   • **Describes the embryonic development of lip and palate with potential role of epigenetics in the etiology of OFC.**

5. Mangold E, Ludwig KU, Nöthen MM. Breakthroughs in the genetics of orofacial clefting. Trends Mol Med 17(12), 725-733 (2011).
6. Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. Int J Obes (Lond) 39(4), 633-641 (2015).
• A review that describes the role of nutrients in early gestational programming that can affect the epigenome.
7. Dubois L, Ohm Kyvik K, Girard M et al. Genetic and environmental contributions to weight, height, and BMI from birth to 19 years of age: an international study of over 12,000 twin pairs. PLoS One 7(2), e30153 (2012).
8. Godfrey KM, Barker DJ. Fetal programming and adult health. Public Health Nutr 4(2B), 611-624 (2001).
9. Grieger JA, Clifton VL. A review of the impact of dietary intakes in human pregnancy on infant birthweight. Nutrients 7(1), 153-178 (2014).
10. Walsh JM, Mcauliffe FM. Impact of maternal nutrition on pregnancy outcome--does it matter what pregnant women eat? Best Pract Res Clin Obstet Gynaecol 29(1), 63-78 (2015).
11. Martelli DR, Coletta RD, Oliveira EA et al. Association between maternal smoking, gender, and cleft lip and palate. Braz J Otorhinolaryngol 81(5), 514-519 (2015).
12. Gresham E, Byles JE, Bisquera A, Hure AJ. Effects of dietary interventions on neonatal and infant outcomes: a systematic review and meta-analysis. Am J Clin Nutr 100(5), 1298-1321 (2014).
13. Murray JC. Gene/environment causes of cleft lip and/or palate. Clin Genet 61(4), 248-256 (2002).
14. Yuan Q, Blanton SH, Hecht JT. Genetic causes of nonsyndromic cleft lip with or without cleft palate. Adv Otorhinolaryngol 70 107-113 (2011).
15. Ebadifar A, Ameli N, Khorramkhorshid HR, Salehi Zeinabadi M, Kamali K, Khoshbakht T. Incidence Assessment of MTHFR C677T and A1298C Polymorphisms in Iranian Non-syndromic Cleft Lip and/or Palate Patients. J Dent Res Dent Clin Dent Prospects 9(2), 101-104 (2015).
16. Johnson CY, Little J. Folate intake, markers of folate status and oral clefts: is the evidence converging? Int J Epidemiol 37(5), 1041-1058 (2008).
17. Butali A, Little J, Chevrier C et al. Folic acid supplementation use and the MTHFR C677T polymorphism in orofacial clefts etiology: An individual participant data pooled-analysis. Birth Defects Res A Clin Mol Teratol 97(8), 509-514 (2013).

18. Rozendaal AM, Van Essen AJ, Te Meerman GJ et al. Periconceptional folic acid associated with an increased risk of oral clefts relative to non-folate related malformations in the Northern Netherlands: a population based case-control study. Eur J Epidemiol 28(11), 875-887 (2013).
19. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. Vitamins and hormones 79 1-44 (2008).
20. Sinclair KD, Allegrucci C, Singh R et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proceedings of the National Academy of Sciences of the United States of America 104(49), 19351-19356 (2007).
21. Pauwels S, Ghosh M, Duca RC et al. Maternal intake of methyl-group donors affects DNA methylation of metabolic genes in infants. Clinical epigenetics 9 16 (2017).
• The study shows the role of periconceptional methyl donors in modulating DNA methylation.
22. Hollingsworth JW, Maruoka S, Boon K et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. The Journal of clinical investigation 118(10), 3462-3469 (2008).
23. Miyoshi N, Stel JM, Shioda K et al. Erasure of DNA methylation, genomic imprints, and epimutations in a primordial germ-cell model derived from mouse pluripotent stem cells. Proceedings of the National Academy of Sciences of the United States of America 113(34), 9545-9550 (2016).
24. Kafri T, Ariel M, Brandeis M et al. Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev 6(5), 705-714 (1992).
25. Reik W, Santos F, Mitsuya K, Morgan H, Dean W. Epigenetic asymmetry in the mammalian zygote and early embryo: relationship to lineage commitment? Philos Trans R Soc Lond B Biol Sci 358(1436), 1403-1409; discussion 1409 (2003).
26. Fryer AA, Nafee TM, Ismail KM, Carroll WD, Emes RD, Farrell WE. LINE-1 DNA methylation is inversely correlated with cord plasma homocysteine in man: a preliminary study. Epigenetics 4(6), 394-398 (2009).
27. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3), e38 (2004).

28. Reichetzeder C, Dwi Putra SE, Pfab T et al. Increased global placental DNA methylation levels are associated with gestational diabetes. Clin Epigenetics 8 82 (2016).
29. Choudhury M, Friedman JE. Epigenetics and microRNAs in preeclampsia. Clin Exp Hypertens 34(5), 334-341 (2012).
30. Stolk L, Bouwland-Both MI, Van Mil NH et al. Epigenetic profiles in children with a neural tube defect; a case-control study in two populations. PLoS One 8(11), e78462 (2013).
31. Wang L, Wang F, Guan J et al. Relation between hypomethylation of long interspersed nucleotide elements and risk of neural tube defects. Am J Clin Nutr 91(5), 1359-1367 (2010).
32. Furlan C, Polesel J, Barzan L et al. Prognostic significance of LINE-1 hypomethylation in oropharyngeal squamous cell carcinoma. Clin Epigenetics 9 58 (2017).
33. Ye D, Jiang D, Li Y, Jin M, Chen K. The role of LINE-1 methylation in predicting survival among colorectal cancer patients: a meta-analysis. Int J Clin Oncol doi:10.1007/s10147-017-1106-1 (2017).
34. Ogino S, Nosho K, Kirkner GJ et al. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. J Natl Cancer Inst 100(23), 1734-1738 (2008).
35. Juriloff DM, Harris MJ, Mager DL, Gagnier L. Epigenetic mechanism causes Wnt9b deficiency and nonsyndromic cleft lip and palate in the A/WySn mouse strain. Birth Defects Res A Clin Mol Teratol 100(10), 772-788 (2014).
36. Mukhopadhyay P, Greene RM, Pisano MM. Cigarette smoke induces proteasomal-mediated degradation of DNA methyltransferases and methyl CpG-/CpG domain-binding proteins in embryonic orofacial cells. Reprod Toxicol 58 140-148 (2015).
    • **Describes the potential role of environment in modulating DNA methylation of early embryonic tissues that contribute to the formation of lip.**
37. Seelan RS, Mukhopadhyay P, Warner DR, Webb CL, Pisano M, Greene RM. Epigenetic regulation of Sox4 during palate development. Epigenomics 5(2), 131-146 (2013).Watkins SE, Meyer RE, Strauss RP, Aylsworth AS. Classification, epidemiology, and genetics of orofacial clefts. Clin Plast Surg 41(2), 149-163 (2014).
38. Gritli-Linde A. The etiopathogenesis of cleft lip and cleft palate: usefulness and caveats of mouse models. Curr Top Dev Biol 84 37-138 (2008).

406 39. Gritli-Linde A. The mouse as a developmental model for cleft lip and palate research.
407 Front Oral Biol 16 32-51 (2012).
408 40. Pansky B. Review of medical embryology, Embryome Sciences, Inc 1301 Harbor Bay
409 Parkway, Alameda, CA, 94502 (1982).
410 41. Sharp GC, Ho K, Davies A et al. Distinct DNA methylation profiles in subtypes of
411 orofacial cleft. Clin Epigenetics 9 63 (2017).
412 **•• Benchmarking study showing methylation in human cleft subtypes.**
413 42. Alvizi L, Ke X, Brito LA et al. Differential methylation is associated with non-
414 syndromic cleft lip and palate and contributes to penetrance effects. Scientific reports
415 7(1), 2441 (2017).
416 **•• Benchmarking study showing contribution of methylation in penetrance effects**
417 **in human nonsyndromic cleft lip and palate.**
418 43. University of Ferrara, projects: { HYPERLINK "http://www.unife.it/progetto/pentacleft" }.
419 44. Knothe C, Shiratori H, Resch E et al. Disagreement between two common biomarkers
420 of global DNA methylation. Clinical epigenetics 8 60 (2016).
421 45. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for
422 estimating global DNA methylation using bisulfite PCR of repetitive DNA elements.
423 Nucleic acids research 32(3), e38 (2004).
424 46. Tournier B, Chapusot C, Courcet E et al. Why do results conflict regarding the
425 prognostic value of the methylation status in colon cancers? The role of the preservation
426 method. BMC cancer 12 12 (2012).
427 47. Opitz JM. The developmental field concept in clinical genetics. J Pediatr 101(5), 805-
428 809 (1982).
429 **• Describes the concept of epimorphic field in an embryo that reacts as a spatially**
430 **and temporally coordinated manner to the localized forces of organization and**
431 **differentiation.**
432 48. Perneger TV. What's wrong with Bonferroni adjustments. Bmj 316(7139), 1236-1238
433 (1998).
434 49. Armstrong RA. When to use the Bonferroni correction. Ophthalmic & Physiological
435 Optics: the journal of the British College of Ophthalmic Opticians 34(5), 502-508
436 (2014).
437 50. Everson JL, Fink DM, Yoon JW et al. Sonic Hedgehog regulation of Foxf2 promotes
438 cranial neural crest mesenchyme proliferation and is disrupted in cleft lip
439 morphogenesis. Development doi:10.1242/dev.149930 (2017).

•• **Benchmarking study showing temporal regulation of gene in the medial aspect of a cleft lip.**

51. Wang X, Guan Z, Chen Y et al. Genomic DNA hypomethylation is associated with neural tube defects induced by methotrexate inhibition of folate metabolism. PLoS One 10(3), e0121869 (2015).
52. Serra-Juhe C, Cusco I, Homs A, Flores R, Toran N, Perez-Jurado LA. DNA methylation abnormalities in congenital heart disease. Epigenetics 10(2), 167-177 (2015).
53. Sheng W, Qian Y, Zhang P et al. Association of promoter methylation statuses of congenital heart defect candidate genes with Tetralogy of Fallot. J Transl Med 12 31 (2014).
54. Arth A, Kancherla V, Pachon H, Zimmerman S, Johnson Q, Oakley GP, Jr. A 2015 global update on folic acid-preventable spina bifida and anencephaly. Birth Defects Res A Clin Mol Teratol 106(7), 520-529 (2016).
55. Liu S, Joseph KS, Luo W et al. Effect of Folic Acid Food Fortification in Canada on Congenital Heart Disease Subtypes. Circulation 134(9), 647-655 (2016).
56. Geraghty AA, Lindsay KL, Alberdi G, Mcauliffe FM, Gibney ER. Nutrition During Pregnancy Impacts Offspring's Epigenetic Status-Evidence from Human and Animal Studies. Nutr Metab Insights 8(Suppl 1), 41-47 (2015).
57. Stock NM, Humphries K, Pourcain BS et al. Opportunities and Challenges in Establishing a Cohort Study: An Example From Cleft Lip/Palate Research in the United Kingdom. Cleft Palate Craniofac J 53(3), 317-325 (2016).
58. El-Maarri O, Walier M, Behne F et al. Methylation at global LINE-1 repeats in human blood are affected by gender but not by age or natural hormone cycles. PloS one 6(1), e16252 (2011).
59. Zhang FF, Cardarelli R, Carroll J et al. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics 6(5), 623-629 (2011).
60. Chalitchagorn K, Shuangshoti S, Hourpai N et al. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene 23(54), 8841-8846 (2004).
61. Jakobsen LP, Borup R, Vestergaard J et al. Expression analyses of human cleft palate tissue suggest a role for osteopontin and immune related factors in palatal development. Experimental & molecular medicine 41(2), 77-85 (2009).