



Gene-gene interaction for nonsyndromic cleft lip with or without cleft palate in Chilean case-parent trios

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ABSTRACT

Objective: Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a birth defect for which several genes susceptibility genes been proposed. Consequently, it has been suggested that many of these genes belong to common inter-related pathways during craniofacial development gene-gene interaction. We evaluated the presence of gene-gene interaction for single nucleotide polymorphisms within interferon regulatory factor 6 (*IRF6*), muscle segment homeobox 1 (*MSX1*), bone morphogenetic protein 4 (*BMP4*) and transforming growth factor 3 (*TGFB3*) genes in NSCL/P risk in Chilean case-parent trios.

Design: From previous studies, we retrieved genotypes for 13 polymorphic variants within these four genes in 152 case-parent trios. Using the *trio* package (R) we evaluate the gene-gene interaction in genetic markers pairs applying a 1°-of-freedom test (1df) and a confirmatory 4°-of-freedom (4df) test for epistasis followed by both a permutation test and a Benjamini-Hochberg test for multiple comparisons adjustment.

Results: We found evidence of gene-gene interaction for rs6446693 (*MSX1*) and rs2268625 (*TGFB3*) (4df $p = 0.024$; permutation $p = 0.015$, Benjamini-Hochberg $p = 0.001$).

Conclusions: A significant gene-gene interaction was detected for rs6446693 (*MSX1*) and rs2268625 (*TGFB3*). This finding is concordant with research in animal models showing that *MSX1* and *TGFB3* are expressed in common molecular pathways acting in an epistatic manner during maxillofacial development.

1. Introduction

Expression and susceptibility to complex traits are characterized by the interaction of genes and environmental factors and by gene-gene (GxG) interaction (also known as epistasis). Thus, any study aiming to elucidate the genetic architecture of a complex trait, should consider GxG interaction analysis (Carlborg & Haley, 2004). Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a complex disorder representing the most common birth defect affecting human craniofacial development (Jugessur & Murray, 2005; Watkins, Meyer, Strauss, & Aylsworth, 2014). Consequently to its complex etiology, NSCL/P potential causal mutations have been detected within regulatory and coding regions if at least 20 candidate genes encoding products involved in different molecular pathways (Dixon, Marazita, Beaty, &

Murray, 2011; Leslie & Marazita, 2013; Vieira, 2008).

In the best of our knowledge, there is no genome-wide association studies for this birth defect analyzing exclusively Chilean subjects. However, our research group has been assessed the association between *loci* interferon regulatory factor 6 (*IRF6*, 1q32.2), muscle segment homeobox 1 (*MSX1*, 4p16.2), bone morphogenetic protein 4 (*BMP4*, 14q22.2) and transforming growth factor 3 (*TGFB3*, 14q24.3) and NSCL/P based on previous reports in other populations (Paradowska-Stolarz, 2015; Tang, Wang, Han, Guo, & Wang, 2013; Watkins et al., 2014; Wattanawong, Rattanasari, McEvoy, Attia, & Thakkinian, 2016). Thus, in single-gene studies we found cleft risk haplotypes composed by polymorphic variants (single nucleotide polymorphisms or SNPs) within these *loci* using a sample of NSCL/P case-parent trios from a Chilean population (Suazo, Santos, Jara, & Blanco, 2008; Suazo,

Abbreviations: BMP4, bone morphogenetic protein 4; GxG interaction, gene-gene interaction; IRF6, interferon regulatory factor 6; MSX1, muscle segment homeobox 1; NSCL/P, nonsyndromic cleft lip with or without cleft palate; SNP, single nucleotide polymorphism; TGFB3, transforming growth factor beta 3

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Santos, Jara, & Blanco, 2010; Suazo, Santos, Jara, & Blanco, 2010; Suazo, Santos, Scapoli, Jara, & Blanco, 2010).

There is evidence of interaction among the products of *IRF6*, *MSX1*, *BMP4* and *TGFB3* during maxillofacial development in animal models. Thus, the expression of *Msx1* and *Bmp4* seems to be reciprocally regulated in the mesenchymal tissues of murine palatal shelves where the expression of the *Bmp4* protein reverts *Msx1* deficiency and rescues the cleft palate phenotype (Smith, Lozanoff, Yyyanar, & Nazarali, 2013; Zhang et al., 2002). On the other hand, a mice knock-out line for *Tgfb3* exhibits an expression reduction of *Msx1* and a proliferation deficiency of the palatal shelves which possibly explain the cleft palate observed in these animals (Del Río et al., 2011). Based on these evidences and in the fact that, as we previously mentioned, we found NSCL/P-risk haplotype in our triads population, instead single-marker association, we decided to evaluate GxG interaction between haplotypes composed by SNPs from these four genes. Unfortunately, in the best of our knowledge, there are no developed methods for haplotype interaction for case-parent trio samples. Alternatively, in a Chilean case-control sample we recently assessed haplotype-based interaction of these same *loci* for NSCL/P expression and found GxG interaction between haplotypes composed by SNPs of *BMP4* and *IRF6* (Blanco, Colombo, Pardo, & Suazo, 2017). However, population-based design (such as case-control) may generate spurious associations due to population stratification by ethnicity, a phenomenon detected in the contemporary urban Chilean population (Palomino, Palomino, Cauvi, Barton, & Chacaborty, 1997; Santos, Perez, Carrasco, & Albala, 2002; Valenzuela, 1988).

The case-parent trio design is insensitive to population stratification due to the construction of genetic matched pseudo-controls based on the non-transmitted alleles from parents to affected progeny (Santos et al., 2002). Using case-parent trio design, for the current study we propose to assess the single-marker GxG interaction between 13 SNPs within *IRF6*, *MSX1*, *BMP4* and *TGFB3* genes in the risk of NSCL/P in a sample of 152 Chilean case-parent trios. In order to achieve our aim, we propose to apply a step-wise method based in two statistical tests. First, we have used a Wald test of 1°-of-freedom (1df) in order to identify pairs of interacting SNPs. These interactions must be confirmed due to 1 df test may detect significant results not only due to epistasis but also due to of independence between *loci* (Cordell, 2002; Xiao et al., 2016). Thus, a 4°-of-freedom (4df) test was used as a confirmatory test of these SNP pairs (Cordell, 2002). To detect GxG interaction based on several genes and/or markers is always complex due to it is necessary to assess multiple hypotheses with the need of multiple comparisons affecting the statistical power of the tests, even for large sample sizes studies (Xiao et al., 2016). This is the reason why we decide to confirm our 4df test using two alternative methods for multiple comparisons correction.

2. Materials and methods

2.1. Subjects

The sample was composed of 152 unrelated NSCL/P Chilean case-parent trios. Cases were 38% females and 69% of them had no family history of orofacial clefts. According to the clinical classification, 92% were cataloged as cleft lip and palate whereas 8% exhibited only a cleft lip. These cases and their parents were obtained between the years 2008 and 2011 after in depth interviews of at least three family members in order to reconstruct a family history. Interviews considered maternal exposure to teratogenic substances (phenytoin, warfarin and ethanol) during pregnancy. These nuclear families were recruited at the following centers: Craniofacial Malformations Unit, School of Dentistry, Universidad de Chile; Cleft Lip/Palate Center, Hospital Exequiel Gonzalez Cortes; Dental Service, Hospital Roberto del Rio; Maxillofacial Service, Hospital San Borja-Arriaran; and Maxillofacial Service, Hospital Sótero del Rio (all of them located in the city of Santiago, Chile). Our study was approved by School of Medicine (Universidad de Chile) Institutional Review Board (Protocols #2005-11 and #018-

Table 1
IRF6 *MSX1*, *BMP4* and *TGFB3* SNPs considered for the current study.

Gene	SNP	Physical Location ^a	MAF ^b	HW P value ^c
<i>IRF6</i>	rs2235371 (C > T)	Chr1: 209790735	0.197	0.870
	rs2235375 (C > G)	Chr1: 209792242	0.515	0.148
	rs2236909 (G > A)	Chr1: 209798310	0.498	0.272
	rs764093 (T > C)	Chr1: 209809986	0.289	0.014
<i>MSX1</i>	rs6446693 (T > C)	Chr4: 4853353	0.473	0.528
	rs3775261 (A > C)	Chr4: 4862018	0.403	0.853
	rs12532 (G > A)	Chr4: 4863419	0.490	0.090
<i>BMP4</i>	rs2855532 (T > C)	Chr14: 53953247	0.318	0.018
	rs762642 (T > G)	Chr14: 53956335	0.455	0.079
	rs1957860 (T > C)	Chr14: 53962637	0.413	0.667
<i>TGFB3</i>	rs3917201 (A > G)	Chr14: 75963212	0.286	0.853
	rs2268625 (A > G)	Chr14: 75973291	0.239	0.957
	rs2268626 (A > G)	Chr14: 75978424	0.415	0.383

^a Chromosome location according to GRCh38 genomic assembly at dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>).

^b MAF: minor allele frequency estimated in an all parental genotypes.

^c HW P value: significance for deviations from Hardy-Weinberg equilibrium for genotype frequency distribution in the parental sample.

2009) and all participants or its legal representative gave their informed consent.

2.2. Genotype data extraction

For GxG interaction estimation, genotype data for 13 SNPs (Table 1) were extracted from previous studies including the same Chilean case-parent trios population here described (Suazo et al., 2008; Suazo et al., 2010a, 2010b; Suazo, Santos and Scapoli et al., 2010). All SNP genotypes were obtained by a polymerase chain reaction followed by a restriction fragment length polymorphism analysis as was described in our previous reports (Suazo et al., 2008; Suazo et al., 2010a, 2010b; Suazo, Santos and Scapoli et al., 2010). As quality control, amplicons for a subset of samples were directly sequenced confirmed the genotypes obtained by polymerase chain reaction followed by a restriction fragment length polymorphism analysis and then used as internal controls (data not shown). Polymorphisms considered in the current report, their alleles frequencies and genomic positions are listed in Table 1.

2.3. Statistical analyses

We calculated allele frequencies based on simple proportions using parental genotypes. Departures from the Hardy-Weinberg equilibrium in genotype distributions in parents were evaluated through a chi-square goodness-of-fit test implemented in STATA 12 statistical package. In order to select candidate SNPs pairs for GxG interaction, we performed a conditional logistic regression (1df Wald test). This conditional model is based in the assumption of an additive mode of inheritance for a pair of SNPs randomly selecting one among three pseudo-controls and, therefore, containing one interaction parameter (Li et al., 2010), providing a rapid analysis of pairwise GxG interactions (Xiao et al., 2016). As we previously mentioned, 1df Wald test may be considered as evidence of epistasis but also may reflect a violation of interaction model (Cordell, 2002; Xiao et al., 2016). Thus, these results were confirmed applying the 4df likelihood ratio test which considers the genotypes for the case and three possible genotypes (pseudo-controls) based on both parental genotypes (Cordell, 2002; Schaid, 1999; Xiao et al., 2016). Due to the multiple hypotheses evaluated in this study, it is necessary to adjust the significant results by a multiple comparison correction. The most popular method, named Bonferroni correction, can increase the false-positive rate when SNPs pairs are not independent (i.e. they are in linkage disequilibrium) reducing the statistical power (Nyholt, 2004). Therefore, we have opted for a

permutation test following the criteria of Xiao et al. (2016). There are 45 pairs of SNPs for interaction, hence the adjusted p-value must be less than $0.05/45 = 0.0011$, being the reason why we considered 1000 permutations as adequate for our aim. To confirm the interaction signal, a random 1000 permutation data sets was originated for each significant pair for 4df test and the permutation p-value was calculated by the proportion of permuted 4df statistic exceeding the observed among the 1000 permuted data (Xiao et al., 2016). All of these tests (1df, 4df and permutations) were included in the *trio* package (Schwender et al., 2014) and performed in RStudio software version 1.0.136. Due to small sample sizes may affect the statistical power of permutations tests (Gadbury, Page, Heo, Mountz, & Allison, 2003), we decide to apply the Benjamini-Hochberg test (Benjamini & Hochberg, 1995) as a complementary multiple comparison correction test. This test is based on the control of false discovery rate to avoid Type-I errors. A rank from the more significant p-value to the less significant one is constructed. Then a corrected value is assigned for each individual p-value computed by means of the quotient of its rank position and the total number of tests and multiplied by the recommended false discovery rate of 0.05. Thus, if a corrected p-value is < 0.05 , it is considered as significant after multiple comparisons correction (Benjamini & Hochberg, 1995)

3. Results

Table 1 shows the list of SNPs used in this report, their genomic position and allele frequencies in the parental sample. Two of them, rs764093 (*IRF6*) and rs2855532 (*BMP4*) were excluded for interaction analysis due to their deviation of Hardy-Weinberg equilibrium (Table 1). Therefore, GxG interaction assessment was performed using 11 SNPs given 45 possible pairs of markers. Table 2 resumes the significant results for GxG interaction for NSCL/P risk in this stepwise study. Thus, for the screening step using 1df Wald statistic, we found four SNPs pairs with evidence of interaction. This is the case of rs2236909 (*IRF6*) and rs1957860 (*BMP4*) ($p = 0.004$), rs2235375 (*IRF6*) and rs1957860 (*BMP4*) ($p = 0.019$), rs6446693 (*MSX1*) and rs2268625 (*TGFB3*) ($p = 0.027$), rs3775261 (*MSX1*) and rs2268625 (*TGFB3*) ($p = 0.038$), and rs1957860 (*BMP4*) and rs3917201 (*TGFB3*) ($p = 0.034$) (Table 2). After the confirmatory step (4df Cordell statistic) only one pair remains significant, rs6446693-rs2268625 ($P = 0.024$), markers within *MSX1* and *TGFB3* genes, respectively (Table 1). In addition, these SNPs pair showed a significant *P* value extracted for 1000 permutations ($p = 0.015$) and after Benjamini-Hochberg correction ($p = 0.001$), confirming the evidence of GxG interaction for these markers in NSCL/P risk (Table 2).

4. Discussion

It has been well documented that family-based designs, such as case-parent trios, show advantages over the population-based ones, especially regarding the insensibility to population stratification and the possibility of parent-of-origin assessment (Santos et al., 2002; Weinberg, 1999). Despite the advantages of family-based designs, the

majority of methods for GxG interaction detection has been developed for case-control and cohort designs (Li et al., 2010). Based on a stepwise method, we found evidence of interaction between a SNPs pair located within *MSX1* (rs6446693) and *TGFB3* (rs2268625) genes in the increase of NSCL/P risk (Table 2). Although these SNPs have no individual effect on the association with this malformation, they are part of the haplotypes which showed transmission distortions in this same sample of trios (Suazo et al., 2010a; Suazo, Santos and Scapoli et al., 2010). These markers are not coding or regulatory variants and therefore, the interaction herein detected may reflect the presence of interacting cleft causal mutations in linkage disequilibrium with these SNPs, which must be detected in future studies. A report of Vieira et al. (2003), described interaction between polymorphic variants (a different set of markers to those considered in the current study) from *MSX1* and *TGFB3* in nonsyndromic orofacial cleft risk using trios from eight Latin-American countries including Chilean nuclear families. Although this last report apply a different statistical method, can be considered supporting our results in Chile.

There is evidence that the products of *MSX1* and *TGFB3* belong to common pathways in murine maxillofacial development. Independent studies have demonstrated that knock-out mice for *Msx1* and *Tgfb3* exhibit cleft palate among other craniofacial abnormalities (Satokata & Maas, 1994; Kaartinen et al., 1995). In addition, *Tgfb3*^{-/-} mouse showed a significant decrease in the proliferation of the palatal mesenchymal which might explain the absence of contact and fusion of these structures during palatal development (Del Río et al., 2011). Associated to the absent of *Tgfb3* in these mice, there is a lack of the normal antero-posterior and medial-lateral gradient of *Msx1* expression in the palatal shelves mesenchymal but no alteration of other genes pattern, such as *Bmp2*, *Bmp4* or *Shh* (Del Río et al., 2011). In accordance with these last results, when epidermal growth factor pathway is blocked *Tgfb3*^{-/-} in palate culture, the mesenchymal pattern of *Msx1* expression is restored and palate fusion is rescued (Barrio et al., 2014). Bioinformatic analyses have demonstrated that *TGFB3* and *MSX1* products belong to the same molecular network modulating cell proliferation not only in craniofacial territories but also in other developmental process (Dai, Yu, Si, Fang, & Shen, 2015). Taking together, the results of these reports demonstrated an epistatic interaction among *Tgfb3* and *Msx1* in craniofacial development in animal models.

Using the same SNPs set considered for the current report, our group reported evidence of interaction between haplotypes composed by markers within *IRF6* and *BMP4* genes in the risk of NSCL/P in the Chilean population (Blanco et al., 2017). This latter report was based on a case-control design where the cases are the same subjects who have been herein analyzed together with their progenitors. Due to ancestral origin, the contemporary urban Chilean population shows a gradient among Amerindian admixture and socioeconomic generating a population stratification (Palomino et al., 1997; Valenzuela, 1988) which may generate spurious results of association (Santos et al., 2002). This is the reason why it has been suggested that case-control results may be ideally confirmed by a case-parent trio design study (Kazem & Farrall, 2005). This previous study (Blanco et al., 2017) was performed as a haplotype-based interaction method which is more powerful than

Table 2
Significant GxG interaction between SNP pairs in the step-wise analysis in the NSCL/P Chilean case-parent trios.

SNP 1 (gene)	SNP2 (gene)	1df Wald statistic	<i>P</i> value	4df Cordell statistic	<i>P</i> value	Perm <i>P</i> value [#]	BH <i>P</i> value [*]
rs2236909 (<i>IRF6</i>)	rs1957860 (<i>BMP4</i>)	8.36	0.004	3.96	0.410	–	
rs2235375 (<i>IRF6</i>)	rs1957860 (<i>BMP4</i>)	5.48	0.019	2.38	0.660	–	
rs6446693 (<i>MSX1</i>)	rs2268625 (<i>TGFB3</i>)	4.89	0.027	11.23	0.024	0.015	0.001
rs3775261 (<i>MSX1</i>)	rs2268625 (<i>TGFB3</i>)	4.27	0.038	4.78	0.310	–	
rs1957860 (<i>BMP4</i>)	rs3917201 (<i>TGFB3</i>)	4.48	0.034	N.C.	–	–	

N.C. Not calculated possibly because the model considers that the SNPs are in linkage disequilibrium.

[#] *P* value estimated for 1000 permutations (see text).

^{*} *P* value after Benjamini-Hochberg multiple comparison correction.

single-marker methods due to its capability to capture the variability of a wide genomic region (Liu, Zhang, & Zhao, 2008). In the best of our knowledge there are no haplotype-based interaction methods for case-parents trio design. In conclusion, we have considered that both studies of our group have valid results and cannot be considered as complementary each other.

We have found three previous studies applying the step-wise method here performed for GxG interaction assessment in the risk of NSCL/P. The first one described interaction of SNPs belonging to wingless-type mmtv integration gene family and other left genes in samples of Asian and European trios (Li et al., 2015). Later, Xiao et al. (2016) evaluated the possible interaction between 13 NSCL/P susceptibility loci in European and Asian trios. These authors reported several pairs of SNPs from these loci showing significant evidence of interaction (Xiao et al., 2016). Finally, a third report described evidence of interaction for SNPs from genes within 16p13.3 region in Chinese trios (Liu et al., 2017). The authors of all just mentioned articles have highlighted the advantages of the statistical model for GxG interaction assessment in triads. Thus, in comparison to other methods, this one perform an effective screening approach in order to detect possible interacting pairs of SNPs which is later confirmed by the 4df Cordell likelihood ratio test model (Cordell, 2002; Li et al., 2015, Xiao et al., 2017). The 4df likelihood ratio test method has the advantage that can detect evidence of epistasis even for markers or genes with low effects on a phenotype associated to permutations for multiple comparisons, a method more effective than Bonferroni correction in presence of linkage disequilibrium (Xiao et al., 2016). In addition, in our study, all included SNPs have a minor allele frequency ≥ 0.2 which increase the power to detect interaction (Xiao et al., 2016). In this context, Emily, Mailund, Hein, Schauser, & Schierup (2009) have estimated that for loci with allelic frequency lower than 0.1, sample sizes greater than 3000 individuals may be necessary to detect two-locus interaction. Unfortunately, the fact that this method considers all possible parental genotypes does not allow identifying interacting risk allele. Thus, additional functional/molecular methods may be useful for detecting a combined allele effect from the significant SNPs pair. This further studies can complemented our results in order to get closer to translational research.

Although the advantages of the method here used and above mentioned, we need to consider our findings with caution due to the main limitation related to our modest sample size and the large number of test performed for all possible SNPs pairs, which can contribute decreasing the statistical power. Thus, we applied a second method for multiple comparisons correction (Benjamini & Hochberg, 1995) more appropriated for small or modest sample sizes in comparison to Bonferroni method (Streiner, 2015). Another limitation is the number of genes selected, which may limit the probability to detect interaction. As we previously mentioned, there is evidence that the products of these genes belong to common molecular networks in the maxillofacial development in animal models. However, it is possible that many other unknown proteins are acting in these networks in a manner not detected in the current study. Thus, it is necessary to amply the number of genes analyzing large scale groups as those derived from genome-wide studies, for example. As we previously mentioned, there is no genome-wide association studies for orofacial clefts in Chile, which could be considered a disadvantage for the current report. However, a genome-wide association studies combined analyzed samples from Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Uruguay, and Venezuela was reported (Leslie et al., 2016) which did not show evidence of association for the 4 loci reported by our group. These differences may be explained by genetic heterogeneity. In this context, the contemporary Central and South American populations share a common Amerindian-Caucasian-African admixture, but each country show certain particularities in its genetic background. At Spanish arrival, Amerindian populations were sparsely distributed in Argentina and Brazil in comparison to Andean countries. In addition, African descendants were

mainly concentrated in Central America and in the northern region of South America (Vieira, Karras, Orioli, Castilla, & Murray, 2002). Therefore, our contemporary population is the product of the admixture of Amerindian and Caucasian genes with a slight contribution of African alleles (Eyheramendy, Martinez, Manevy, Vial, & Repetto, 2015).

In summary, we found evidence based on a step-wise method and confirmed by two independent methods for multiple comparisons, of gene-gene interaction for polymorphic variants belonging to *TGFB3* and *MSX1* genes in the expression of nonsyndromic cleft lip with or without cleft palate in the Chilean population. Although our results may be considered with caution due to the modest sample size of our trio population, they may be supported by a previous report including Chilean samples and by findings in animal models showing that these are expressed in common molecular pathways in an epistatic manner during maxillofacial development. Further studies with larger sample size and/or in independent samples may confirm these results. In addition, molecular studies in both animal and human cell models may help us to elucidate the direct interaction (epistasis) among these two genes or the presence of other intermediary proteins and to identify specific risk allele combination, getting closer to a translational approach.

Conflict of interest

All authors declare no conflicts of interest.

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References

- Barrio, M. C., Del Río, A., Murillo, J., Maldonado, E., López-Gordillo, Y., Paradas-Lara, I., et al. (2014). Epidermal growth factor impairs palatal shelf adhesion and fusion in the Tgf- β 3 null mutant. *Cells Tissues Organs*, *199*, 201–211.
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*, 289–300.
- Blanco, R., Colombo, A., Pardo, R., & Suazo, J. (2017). Haplotype-based gene-gene interaction of bone morphogenetic protein 4 and interferon regulatory factor 6 in the etiology of non-syndromic cleft lip with or without cleft palate in a Chilean population. *European Journal of Oral Sciences*, *125*, 102–109.
- Carlborg, O., & Haley, C. S. (2004). Epistasis: Too often neglected in complex trait studies? *Nature Review Genetics*, *5*, 618–625.
- Cordell, H. J. (2002). Epistasis: What it means, what it doesn't mean: And statistical methods to detect it in humans. *Human Molecular Genetics*, *11*, 2463–2468.
- Dai, J., Yu, H., Si, J., Fang, B., & Shen, S. G. (2015). Irf6-Related gene regulatory network involved in palate and lip development. *Journal of Craniofacial Surgery*, *26*, 1600–1605.
- Del Río, A., Barrio, M. C., Murillo, J., Maldonado, E., López-Gordillo, Y., Martínez-Sanz, E., et al. (2011). Analysis of the presence of cell proliferation-related molecules in the Tgf- β 3 null mutant mouse palate reveals misexpression of EGF and Msx-1. *Cells Tissues Organs*, *193*, 135–150.
- Dixon, M. J., Marazita, M. L., Beaty, T. H., & Murray, J. C. (2011). Cleft lip and palate: Understanding genetic and environmental influences. *Nature Review Genetics*, *12*, 167–178.
- Emily, M., Mailund, T., Hein, J., Schauser, L., & Schierup, M. H. (2009). Using biological networks to search for interacting loci in genome-wide association studies. *European Journal of Human Genetics*, *17*, 1231–1240.
- Eyheramendy, S., Martínez, F. I., Manevy, F., Vial, C., & Repetto, G. M. (2015). Genetic structure characterization of Chileans reflects historical immigration patterns. *Nature Communications*, *6*, 6472.
- Gadbury, G. L., Page, G. P., Heo, M., Mountz, J. D., & Allison, D. B. (2003). Randomization tests for small samples: An application for genetic expression data. *Journal of the Royal Statistical Society. Series C (Applied Statistics)*, *52*, 365–376.
- Jugessur, A., & Murray, J. C. (2005). Orofacial clefting: Recent insights into a complex trait. *Current Opinion in Genetics & Development*, *15*, 270–278.
- Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., et al. (1995). Abnormal lung development and cleft palate in mice lacking TGF- β 3

- indicates defects of epithelial-mesenchymal interaction. *Nature Genetics*, *11*, 415–421.
- Leslie, E. J., & Marazita, M. L. (2013). Genetics of cleft lip and cleft palate. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, *163C*, 246–258.
- Leslie, E. J., Carlson, J. C., Shaffer, J. R., Feingold, E., Wehby, G., Laurie, C. A., et al. (2016). A multi-ethnic genome-wide association study identifies novel loci for non-syndromic cleft lip with or without cleft palate on 2p24.2: 17q23 and 19q13. *Human Molecular Genetics*, *25*, 2862–2872.
- Li, Q., Fallin, M. D., Louis, T. A., Lasseter, V. K., McGrath, J. A., Avramopoulos, D., et al. (2010). Detection of SNP–SNP interactions in trios of parents with schizophrenic children. *Genetic Epidemiology*, *34*, 396–406.
- Li, Q., Kim, Y., Sukitipat, B., Hetmanski, J. B., Marazita, M. L., Duggal, P., et al. (2015). Gene–Gene interaction among WNT genes for oral cleft in trios. *Genetic Epidemiology*, *39*, 385–394.
- Liu, N., Zhang, K., & Zhao, H. (2008). Haplotype-association analysis. *Advances in Genetics*, *60*, 335–405.
- Liu, D., Wang, H., Schwender, H., Marazita, M. L., Wang, Z., Yuan, Y., et al. (2017). Gene-gene interaction of single nucleotide polymorphisms in 16p13.3 may contribute to the risk of non-syndromic cleft lip with or without cleft palate in Chinese case-parent trios. *American Journal of Medical Genetics Part A*, *173*, 1489–1494.
- Nyholt, D. R. (2004). A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *American Journal of Human Genetics*, *74*, 765–769.
- Palomino, H. M., Palomino, H., Cauvi, D., Barton, S. A., & Chackraborty, R. (1997). Facial clefting and Amerindian admixture in populations of Santiago: Chile. *American Journal of Human Biology*, *9*, 225–232.
- Paradowska-Stolarz, A. (2015). MSX1 gene in the etiology orofacial deformities. *Postępy Higieny i Medycyny Doswiadczalnej (Online)*, *69*, 1499–1504.
- Santos, J. L., Pérez, F., Carrasco, E., & Albala, C. (2002). Use of case-parents trio for epidemiological studies of association between genetic polymorphisms and complex diseases. *Revista Medica De Chile*, *130*, 1307–1315.
- Satokata, I., & Maas, R. (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nature Genetics*, *6*, 348–356.
- Schaid, D. J. (1999). Case-parents design for gene–environment interaction. *Genetic Epidemiology*, *16*, 261–273.
- Schwender, H., Li, Q., Neumann, C., Taub, M. A., Younkin, S. G., Berger, P., et al. (2014). Detecting disease variants in case-parent trio studies using the bioconductor software package trio. *Genetic Epidemiology*, *38*, 516–522.
- Smith, T. M., Lozanoff, S., Iyyanar, P. P., & Nazarali, A. J. (2013). Molecular signaling along the anterior-posterior axis of early palate development. *Frontiers in Physiology*, *3*, 488.
- Streiner, D. L. (2015). Best (but oft-forgotten) practices: The multiple problems of multiplicity—whether and how to correct for many statistical tests. *American Journal of Clinical Nutrition*, *102*, 721–728.
- Suazo, J., Santos, J. L., Jara, L., & Blanco, R. (2008). Linkage disequilibrium between IRF6 variants and nonsyndromic cleft lip/palate in the Chilean population. *American Journal of Medical Genetics Part A*, *146A*, 2706–2708.
- Suazo, J., Santos, J. L., Jara, L., & Blanco, R. (2010a). Parent-of-origin effects for MSX1 in a Chilean population with nonsyndromic cleft lip/palate. *American Journal of Medical Genetics Part A*, *152A*, 2011–2016.
- Suazo, J., Santos, J. L., Jara, L., & Blanco, R. (2010b). Association between bone morphogenetic protein 4 gene polymorphisms with nonsyndromic cleft lip with or without cleft palate in a Chilean population. *DNA and Cell Biology*, *29*, 59–64.
- Suazo, J., Santos, J. L., Scapoli, L., Jara, L., & Blanco, R. (2010). Association between TGFB3 and nonsyndromic cleft lip with or without cleft palate in a Chilean population. *Cleft Palate Craniofacial Journal*, *47*, 513–517.
- Tang, M., Wang, Y., Han, S., Guo, S., & Wang, D. (2013). Transforming growth factor-beta3 gene polymorphisms and nonsyndromic cleft lip and palate risk: A meta-analysis. *Genetic Testing and Molecular Biomarkers*, *17*, 881–889.
- Valenzuela, C. Y. (1988). On sociogeneticclines. *Ethology and Sociobiology*, *9*, 259–268.
- Vieira, A. R., Karras, J. C., Orioli, I. M., Castilla, E. E., & Murray, J. C. (2002). Genetic origins in a South American clefting population. *Clinical Genetics*, *62*, 458–463.
- Vieira, A. R., Orioli, I. M., Castilla, E. E., Cooper, M. E., Marazita, M. L., & Murray, J. C. (2003). MSX1 and TGFB3 contribute to clefting in South America. *Journal of Dental Research*, *82*, 289–292.
- Vieira, A. R. (2008). Unraveling human cleft lip and palate research. *Journal of Dental Research*, *87*, 119–125.
- Watkins, S. E., Meyer, R. E., Strauss, R. P., & Aylsworth, A. S. (2014). Classification, epidemiology: And genetics of orofacial clefts. *Clinics in Plastic Surgery*, *41*, 149–163.
- Wattanawong, K., Rattanasiri, S., Mcevoy, M., Attia, J., & Thakkestian, A. (2016). Association between IRF6 and 8q24 polymorphisms and nonsyndromic cleft lip with or without cleft palate: Systematic review and meta-analysis. *Birth Defects Research A Clinical and Molecular Teratology*, *106*, 773–788.
- Weinberg, C. R. (1999). Methods for detection of parent-of-origin effects in genetic studies of case-parents triads. *American Journal of Human Genetics*, *65*, 229–235.
- Xiao, Y., Taub, M. A., Ruczinski, I., Begum, F., Hetmanski, J. B., Schwender, H., et al. (2016). Evidence for SNP–SNP interaction identified through targeted sequencing of cleft case-parent trios. *Genetic Epidemiology*, *41*, 244–250.
- Zhang, Z., Song, Y., Zhao, X., Zhang, X., Fermin, C., & Chen, Y. (2002). Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development*, *129*, 4135–4146.