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A *SHMT1* variant decreases the risk of nonsyndromic cleft lip with or without cleft palate in Chile

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Abstract

Objective: To assess the association between polymorphic variants from *SHMT1* and *MTHFS* genes, involved in the cytoplasmic futile folate cycle, and the risk of nonsyndromic cleft lip with or without cleft palate (NSCL/P) in the Chilean population.

Subjects and Methods: In a sample of 139 Chilean NSCL/P cases and 278 controls, we obtained the genotypes for nine variants of *SHMT1* and *MTHFS* and the association between them and the phenotype was evaluated using odds ratios (OR) in additive (allele), dominant, and recessive models.

Results: After correction for multiple comparisons, only the variant rs1979277 (G > A; p.Leu474Phe) from *SHMT1* showed a significant and protective effect for additive (OR 0.60; 95% CI 0.42–0.86; p = .0054, q = 0.0488) and dominant models (OR 0.48; 95% CI 0.29–0.75; p = .0009; q = 0.0081). Our bioinformatic prediction plus functional evidence from previous reports demonstrate that the A allele for this missense variant decreases the enzymatic activity.

Conclusions: Owing to the rs1979277 A allele, which reduces the cytoplasmic SHMT activity and has a higher frequency in controls than in NSCL/P cases, we hypothesized that a low enzyme activity may increase the cytoplasmic concentration of folates and, therefore, explain the protective role against OFCs.

KEYWORDS

folates, orofacial clefts, *SHMT1*, single nucleotide polymorphisms

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1 | INTRODUCTION

Folates (folic acid in its synthetic form) are essential nutrients involved in the transfer of one-carbon units (methyl groups) to molecules related to de novo synthesis of DNA nucleotides and also ensure proper levels of S-adenosylmethionine (SAM), the main methyl-donating molecule for DNA, histones, and other methylated proteins (Lucock, 2000). Thus, folates play a role in epigenetic modifications, which thence modulate genetic expression, a pivotal mechanism for normal embryonic development (Desai, Sequeira, & Quadros, 2016). In this context, it has been extensively demonstrated that maternal periconceptional folic acid supplementation is an effective intervention against nonsyndromic cleft lip with or without cleft palate (NSCL/P) (Jahanbin, Shadkam, Miri, Shirazi, & Abtahi, 2018). In addition, several variants of the genes involved in folate transport and metabolism have been associated with NSCL/P in diverse populations (Bhaskar, Murthy, & Venkatesh Babu, 2011; Blanton et al., 2011; Wang et al., 2018). In Chile, this birth defect is one of the most prevalent and its prevalence has not decreased despite the increase of folic acid intake by the mandatory fortification of wheat flour since 2000. (López-Camelo, Castilla, & Orioli, 2010; Nazer & Cifuentes, 2014). Thus, we have hypothesized that the Chilean population possesses genetic variants which may alter folate metabolism and in turn to be associated with NSCL/P. Supporting this hypothesis, we have previously reported the association between the variant c.677C > T of *MTHFR* gene and NSCL/P in Chile (Ramírez-Chau, Blanco, Colombo, Pardo, & Suazo, 2016).

Besides the main folate cycle, a lesser known process has been described called the cytoplasmic folate futile cycle (Misselbeck, Marchetti, Priami, Stover, & Field, 2019). Although this process does not seem to be the primary source of folates for SAM synthesis, this pathway appears to be important for the maintenance of one-carbon metabolism especially in stages of high proliferative rates such as cancer and embryonic development (Girgis, Suh, Jolivet, & Stover, 1997). The futile folate cycle includes the conversion of 5-formyltetrahydrofolate (5-FTHF) into 5,10-methenyltetrahydrofolate (5,10-CH-THF) by 5,10-methenyltetrahidrofolate synthetase (MTHFS) in the presence of ATP. 5,10-CH-THF is in turn converted to 5-FTHF by the cytoplasmic serine hydroxymethyltransferase (SHMT1) (Dayan et al., 1995). In a NADPH-dependent reaction, 5,10-CH-THF is reduced to 5,10-methylenetetrahydrofolate (5,10-CH2-THF), a substrate subsequently reduced to 5-methyltetrahydrofolate (5-MTHF) by MTHFR (Girgis et al., 1997; Skibola et al., 2002). This last folate is necessary for homocysteine remethylation and the subsequent synthesis of SAM (Blanton et al., 2011). Additionally, cytoplasmic SHMT catalyses the reversible conversion of tetrahydrofolate (THF) into 5,10-CH2-THF (5,10-methylene-THF) in a reaction where serine is synthesized from glycine. THF is a cofactor for enzymes involved in the synthesis of purines and in a multistep pathway is converted to 5-MTHF (Skibola et al., 2002).

The human gene *MTHFS* (15q25.1) encodes a protein of 203 amino acid, located in cytoplasm (85%) and the mitochondria (15%) (Dayan et al., 1995; Girgis et al., 1997). MTHFS is essential for mice development due to its involvement in the de novo purine synthesis (Field, Anderson, & Stover, 2011). Activity deficiency in humans by missense mutations is associated with hypomyelination of brain tissues, epilepsy, and microcephaly (Rodan et al., 2018). Single nucleotide polymorphisms (SNPs) in human *MHTFS* have been associated with congenital heart defects (Chowdhury et al., 2012; Nembhard et al., 2017). A microdeletion of the chromosome region containing *MTHFS* has been detected in a patient showing cleft lip and palate, among other craniofacial defects (Sing et al., 2011). In addition, a SNP of this gene has been associated with the risk of NSCL/P in a population of Mexican ancestry in United States (Blanton et al., 2011). Meanwhile, the *SHMT1* gene (17p11.2) encodes the cytoplasmic SHMT enzyme, composed of 483 residues (Garrow et al., 1993). Mice *Shmt1-/-* are viable and fertile (MacFarlane et al., 2008). However, a low-folate diet significantly influences the occurrence of neural tube defects among the offspring of *Shmt1-/-* females (Beaudin et al., 2012). To the best of our knowledge, only one study has associated SNPs from *SMHT1* and nonsyndromic cleft palate in a Norwegian population (Boyles et al., 2009).

Considering the evidence gathered here, the aim of the current study was to evaluate the association between common variants of the genes encoding the futile folate cycle enzymes, *MTHFS* and *SMHT1*, and the risk of NSCL/P in a Chilean population using a case–control design. In a population-based association between a biallelic genetic marker and a phenotype, the model of inheritance for the risk allele is unknown (Salanti et al., 2009). Thus, we proposed to evaluate three genetic models: (a) additive or allele model, where the effect of an individual risk allele (*a*) is evaluated and carriers of 2 copies of this allele have twice the risk of heterozygous; (b) a dominant model, which assumes that carriers of 2 copies of *a* have the same risk of heterozygous; (c) a recessive model, where the risk is associated with two copies of *a* (Salanti et al., 2009).

2 | METHODS

2.1 | Subjects

Our sample was composed of 139 unrelated NSCL/P cases. This group contained 35.9% females, and 70% had no family history of orofacial clefts. These cases were recruited during 2017 and 2018 from the following centers (Santiago, Chile): The Craniofacial Malformations Unit, School of Dentistry, Universidad de Chile; The Cleft Lip/Palate Center, Hospital Exequiel Gonzalez Cortes; The Dental Service, Hospital Roberto del Rio; and The Maxillofacial Service, Hospital San Borja-Arriaran. Cases associated with maternal exposure to teratogenic substances during pregnancy, such as phenytoin and warfarin, were excluded. The control group, composed of 278 unrelated individuals (34.8% females), was recruited among blood donors of the Blood Banks of San Jose and San Juan de Dios Hospitals (Santiago, Chile). All control subjects had a negative family

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history of orofacial clefts (OFCs). Cases and controls do not significantly differ for gender proportions (p = .828). Population stratification by ethnicity may generate spurious results of association in case–control studies (Santos, Pérez, Carrasco, & Albala, 2002). Thus, for 120 of the 138 cases we also recruited both biological parents, conforming a population of 120 case–parent trios considering that this design is insensible to population stratification (Santos et al., 2002). The cases of this sample were 33.3% females, and 69% have no familial history of orofacial clefts. Thus, this is a sample from the same population where cases and controls were recruited. Our study was approved by The Institutional Review Board of the Faculty of Dentistry, Universidad de Chile (Protocol #2017/07), and all participants or their legal representatives provided their written informed consent. This study was performed under the guidelines of the Declaration of Helsinki.

2.2 | Molecular analyses

A sample of venous peripheral blood was obtained from each participant, and genomic DNA was extracted from this sample according to a standard method (Chomczynski & Sacchi, 1987). Genotypes for *MTHFS* and *SHMT1* SNPs were obtained performing an Infinium Global Screening Array-24 BeadChip (Illumina) at the Human Genomics Facility (HuGe-F) in Erasmus MC, Netherlands, according to the manufacturer's guidelines. All subjects and markers here studied passed the quality control tests which were applied according to previously stated criteria (Anderson et al., 2010). For cases and controls, a genotype call rate ≥95% was used. The SNPs selected for analysis met the following criteria: (a) located between 5 kb upstream from the transcription start site and 5 kb downstream from the stop triplet (according to GRCh37 assembly of the human genome); (b) minor allele frequency (MAF)> 0.10; (c) not in linkage disequilibrium (LD) with other SNPs contained in the array (r^2 > .8); (d) no significant departure from the Hardy–Weinberg equilibrium (HWE) in the control population (p < .01); and (e) located within autosomes. Table 1 describes the list of SNPs analyzed in this study.

2.3 | Functional annotation and prediction

The effect of a SNP on gene expression was evaluated using the Genotype-Tissue Expression (GTEx) database (https://www.gtexportal.org/home/). RegulomeDB score (http://www.regulomedb.org/) (Boyle et al., 2012) was used to predict the effect of a non-coding SNP on regulatory function by means of information regarding epigenetic signatures related to gene expression (enhancer histone marks, promoter histone marks, DNAse hypersensitive sites) and protein-binding sites (Chromatin ImmunoChIP seq). For non-synonymous variants, protein functionality was predicted using the online tool SIFT (https://sift.bii.a-star.edu.sg/), where scores <0.05 are considering damaging for protein function.

2.4 | Statistical analyses

Both genotype set manipulation and all statistical analyses were performed using PLINK 1.9 (http://www.cog-genomics.org/plink/1.9/). An exact test was applied in order to detect HWE departures. A logistic regression analysis was performed to assess association of SNPs under the allele (additive), dominant, and recessive models. Additive model was also adjusted by sex, due to prevalence of this congenital malformation is 1.5–2 times higher in males than females (Watkins, Meyer, Strauss, & Aylsworth, 2014), and we have previously described that genetic association differs when subjects are stratified by sex (Ramírez-Chau et al., 2016; Suazo et al., 2011). For multiple comparisons correction, false discovery rate (FDR) was applied according to the Benjamini–Hochberg method (Benjamini & Hochberg, 1995), considering a threshold for FDR-adjusted p-values (q-value) of .05. Markers with significant association after FDR correction were also analyzed in the sample of case–parent trios. In this sample, the transmission disequilibrium test (TDT; Spielman, Mcginnis, & Ewens, 1993) was applied assessing the distortion of allele transmission from parent to affected progeny. In our study, case–control and case–parent trios partially share the population of cases. Therefore, the results from these samples are correlated (Martin & Kaplan, 2000) being the reason why a combined OR including a homogeneity test was applied for additive model in case–controls and allele transmission in case–parent trios (Kazeem & Farrall, 2005). The statistical power for genetic association based on the allele model (case–controls) and allele transmission (case–parent trios) was computed using Quanto 1.2.4 (http://biostats.usc.edu/Quanto.html).

3 | RESULTS

Table 1 describes the chromosome position, alleles, and its frequencies in both cases of controls, for the 9 SNPs in *MTHFS* and *SHMT1* considered for this study. Allele (additive) association of these markers and NSCL/P in our Chilean sample showed that only the two SNPs within the *SHMT1* gene are associated with the phenotype: rs1979277 (OR 0.60; 95% CI 0.42–0.86; p = .0054) and rs2273027 (OR 1.36; 95% CI 1.01–1.83; p = .0424). However, after multiple comparisons correction by FDR, only the association with rs1979277 remains borderline significant (q = 0.0488) (Table 2). The effect of sexes on the association between rs1979277 and NSCL/P is evident when it was adjusted for by this covariable and after FDR correction (OR 0.62; 95% CI 0.43–0.89; p = .0083; q = 0.0744) (Table 2). When the dominant model was considered for association, only rs1979277 showed a significant association with NSCL/P (OR 0.48; 95% CI 0.29–0.75; p = .0009; q = 0.0081). On the other hand, for the recessive model of association none of the nine SNPs exhibited significant association (Table 3). Using Quanto software and considering the MAF for rs1979277 in controls and its OR for additive model, the statistical power (1-beta) to detect association reaches 89%. As previously mentioned,

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TABLE 1 Single nucleotide polymorphisms (SNPs) in the futile folate cycle considered for association with NSCL/P in a Chilean population

rsID	Gene	Chromosome: position[a]	Alleles[b]	Frequency in cases (*N* = 139)[c]	Frequency in controls (*N* = 278)[c]
rs8032039	*MTHFS*	15:80126770	A/G	0.229	0.280
rs60843106	*MTHFS*	15:80132626	A/G	0.142	0.161
rs16971460	*MTHFS*	15:80157583	T/C	0.129	0.119
rs6495449	*MTHFS*	15:80158159	G/A	0.116	0.094
rs17285375	*MTHFS*	15:80160011	C/A	0.094	0.109
rs2562744	*MTHFS*	15:80174388	A/C	0.455	0.474
rs2115540	*MTHFS*	15:80190308	A/G	0.481	0.511
rs1979277	*SHMT1*	17:18232096	G/A	0.192	0.282
rs2273027	*SHMT1*	17:18250527	C/T	0.436	0.362

[a]Based on Human GRCh37.p13 assembly.

[b]Major allele/Minor allele.

[c]Minor allele frequency.

TABLE 2 Allele (additive) association between SNPs in genes involved in the futile folate cycle and NSCL/P in a Chilean population

			Unadjusted			Adjusted by sex		
rsID	Gene	Minor allele	OR (95% CI)	*p*-value	FDR *q*-value	OR (95% CI)	*p*-value	FDR *q*-value
rs8032039	*MTHFS*	G	0.76 (0.54–1.07)	.1235	0.3705	0.74 (0.51–1.07)	.1073	0.3219
rs60843106	*MTHFS*	G	0.86 (0.57–1.31)	.4913	0.6744	0.86 (0.57–1.31)	.4813	0.6739
rs16971460	*MTHFS*	C	1.09 (0.70–1.72)	.6760	0.6760	1.09 (0.69–1.73)	.6832	0.6832
rs6495449	*MTHFS*	A	1.26 (0.78–2.01)	.3391	0.6744	1.34 (0.79–1.98)	.3482	0.6739
rs17285375	*MTHFS*	A	0.85 (0.52–1.40)	.5246	0.6744	0.86 (0.54–1.39)	.5463	0.6739
rs2562744	*MTHFS*	A	0.92 (0.69–1.24)	.6037	0.6760	0.92 (0.69–1.24)	.5990	0.6739
rs2115540	*MTHFS*	G	0.88 (0.66–1.19)	.4277	0.6744	0.88 (0.65–1.19)	.4163	0.6739
rs1979277	*SHMT1*	A	0.60 (0.42–0.86)	.0054	0.0488	0.62 (0.43–0.89)	.0083	0.0744
rs2273027	*SHMT1*	T	1.36 (1.01–1.83)	.0424	0.1906	1.36 (1.01–1.84)	.0438	0.1969

Note: FDR *q*-value: *p*-value corrected by false discovery rate.

population stratification by ethnicity may generate false-positive results of genetic association in population-based studies (Santos et al., 2002). Therefore, we confirm our findings for rs1979277 in a case–parent trio sample. TDT analysis reveals that the A allele was undertransmitted from parents to affected progeny (OR 0.60; 95% CI 0.38–0.96; $p = .0304$; data not shown), supporting the protective role for this allele (1-beta of 76%). In addition, combined OR showed an absence of heterogeneity between the results of both case–control and case–parent trio samples ($p = .9573$) and reinforces their individual findings (OR 0.60; 95% CI 0.45–0.79; $p = .0003$; data not shown).

According to dbSNP (https://www.ncbi.nlm.nih.gov/snp/), the variant rs1979277 is located in exon 12 of the human gene *SHMT1* and the substitution G > A translates as a missense variant (p.Leu474Phe, accession number NP_004160.3). Considering the sequence homology, conservation, and the properties of amino acids, the SIFT tool predicts that the Leu474Phe is potentially damaging for protein function, with a score of 0.02 (data not shown).

4 | DISCUSSION

It has been well documented that variants within genes involved in the folate cycle are associated with NSCL/P in several populations (Bhaskar et al., 2011; Blanton et al., 2011; Wang et al., 2018). This fact was previously evaluated in the Chilean population where the *MTHFR* SNP rs1801133 (c.677C > T; p.Ala222Val) is a risk factor for this birth defect (Ramírez-Chau et al., 2016). The current report focused on a folate cycle, called the futile folate cycle, which seems to maintain a normal one-carbon metabolism during fast proliferative stages such as development and neoplasia (Girgis et al., 1997). We have analyzed the association between SNPs of genes encoding the two enzymes of this futile cycle (*SHMT1* and *MTHFS*) in a case–control study. After applying filters by MAF, Hardy–Weinberg, and linkage disequilibrium, a total of nine markers were included in this association study (Table 1). Among these markers, after multiple comparisons correction, only the SNP rs1979277 of *SHMT1* gene was significantly associated with NSCL/P for our sample (Table 2)

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TABLE 3 Dominant and recessive association between SNPs genes involved in the futile folate cycle and NSCL/P in a Chilean population

		Dominant model (aa + Aa vs. AA)[a]			Recessive model (aa vs. Aa + AA)[a]		
rsID	Gene	OR (95% CI)[b]	*p*-value[b]	FDR *q*-value[b]	OR (95% CI)	*p*-value	FDR *q*-value[b]
rs8032039	*MTHFS*	0.70 (0.45–1.09)	.0947	0.4262	0.76 (0.24–2.12)	.5848	0.8772
rs60843106	*MTHFS*	0.75 (0.44–1.23)	.2332	0.5439	2.12 (0.47–9.37)	.2416	0.7155
rs16971460	*MTHFS*	1.10 (0.65–1.83)	.7126	0.9162	0.82 (0.08–5.10)	.7324	0.9416
rs6495449	*MTHFS*	1.31 (0.75–2.26)	.3022	0.5439	1.03 (0.09–7.29)	.9727	0.9727
rs17285375	*MTHFS*	0.87 (0.48–1.56)	.6430	0.9162	0.60 (0.06–3.21)	.5290	0.8772
rs2562744	*MTHFS*	1.02 (0.63–1.67)	.9181	0.9256	0.73 (0.42–1.26)	.3180	0.7155
rs2115540	*MTHFS*	1.02 (0.61–1.73)	.9256	0.9256	0.72 (0.41–1.23)	.1590	0.7155
rs1979277	*SHMT1*	0.48 (0.29–0.75)	.0009	0.0081	0.93 (0.38–2.13)	.8498	0.9560
rs2273027	*SHMT1*	1.27 (0.81–2.02)	.2691	0.5439	1.96 (1.08–3.54)	.01681	0.1513

Note: FDR Q-value: *p*-value corrected by false discovery rate.

[a]A: major allele; a: minor allele.

[b]Unadjusted.

where the minor allele (A) presented a higher frequency in controls than in cases and, therefore, can be considered a protective factor. In order to evaluate the effect of population stratification, the additive model result was compared with the allele transmission distortion in case–parent trio sample. Thus, the protective effect in case–controls was supported by these last design findings as well as by the combined OR. In addition, this significance also appears when a */dominant model was evaluated. Thus, genotypes AA and GA are more represented in unaffected subjects than in affected ones (Table 3). When the results of the allele association were adjusted by sex, the difference between cases and controls was not significant after FDR correction (Table 2). Sex is a covariable with an important effect on association between genetic markers and NSCL/P. Thus, the prevalence of this congenital malformation is 1.5–2 times higher in males than females (Watkins et al., 2014). In addition, we have described in Chile that genetic association between this phenotype and variants for *BMP4* and *IRF6* genes differs when subjects are stratified by sex (Ramírez-Chau et al., 2016; Suazo et al., 2011). Despite these evidences, we did not include an analysis stratified by sex in order to avoid the effect on the statistical power related to construct subsets of the sample.

As previously mentioned, *SHMT1* encodes the cytoplasmic enzyme SHMT which transfers a one-carbon unit to 5,10-CH2-THF from THF coupled with the conversion of glycine into serine (Skibola et al., 2002). This cytoplasmic enzyme may be modified by SUMOylation which leads to its translocation to the nucleus to ensure the availability of folates for thymidylate biosynthesis (Palmer, Kamynina, Field, & Stover, 2017). According to the *Bgee* expression database for animals (https://bgee.org), this gene is expressed in the frontonasal and maxillary processes during mouse craniofacial development. To the best of our knowledge, there is only one previous report concerning variants for *SHMT1* gene and its role in nonsyndromic OFCs. Thus, rs2168781 was considered as a risk factor for cleft palate in a Norwegian population (Boyles et al., 2009). Therefore, the present study is the first reporting association between *SHMT1* SNPs and NSCL/P.

The associated variant rs1979277 (G > A) is located in the last exon of *SHMT1* encoding a missense variation in protein sequence (p.Leu474Phe) with a potential effect on protein function according to the bioinformatic prediction by SIFT. In addition, the literature regarding this missense variant showed that it has been associated with an increase in the risk of some neoplasia such as prostate and colorectal cancer (Collin et al., 2009). Nevertheless, a meta-analysis detected a protective effect for solid tumors in Asian populations (Zhao et al., 2015). Regarding the relation between this variant and birth defects, the rs1979277 G > A substitution was considered as a risk factor for neural tube defects (NTD) in an Indian population (Rebekah, Tella, Buragadda, Tiruvatturu, & Akka, 2017). However, another report in Britains describes this variant as a maternal protective factor against NTD (Relton et al., 2004).

The protective effect rs1979277 A allele reported in the current study may be explained by its biological role previously described. Compared with the wild type enzyme, the p.Leu474Phe polymorphism decreases the affinity of human SHMT1 for its substrates (Fu, Hunt, Schirch, Safo, & Chen, 2005). Mothers carrying the AA genotype have lower levels of plasma homocysteine and higher counts for both red blood cells and plasma folates than those mothers with GG genotype (Heil et al., 2001). Thus, the decrease in the activity of this enzyme may slow down the conversion of THF to 5,10-CH2-THF, increasing the availability of THF as a cofactor for purine synthesis and its conversion to 5-MTHF, providing one-carbon units for SAM synthesis (Skibola et al., 2002). This metabolic block hypothesis is also supported by others authors who described a protective effect for NTD for this variant (Fu et al., 2005). In addition, p.Leu474Phe variant distorted the SUMOylation of the cytoplasmic SHMT and potentially alters its translocation to the nucleus (Woeller, Anderson, Szebenyi, &

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Stover, 2007). However, the decrease in the nuclear activity of SHMT may be superseded by the mitochondrial enzyme isoform which, in addition to the cytosolic enzyme, is translocated to the nucleus.

The strengths of the current study are based on, first, the statistical power of the association detected (89%) in case–control. Although the association for this marker in case–parent trios is lower (76%), the combined OR significantly increases the statistical power of each single sample (Glaser & Holmans, 2009). Second, the evidence of a biological role of this variant described in the preceding paragraphs. On the other hand, the main weakness of our work is the lack of evidence for association of this variant with nonsyndromic OFCs in the genome-wide association analyses (GWAS) over several populations. However, this absence of previous evidence may support our general hypothesis about variants for folate metabolism genes belonging to the Chilean population which can explain the null effect of the national policy of folic acid fortification on the prevalence of orofacial clefts (López-Camelo et al., 2010; Nazer & Cifuentes, 2014). In previous paragraphs, we discussed the evidence for *SHMT1* gene expression in developing craniofacial structures in animals. These findings and the absence of studies evaluating the role of p.Leu474Phe variant related to normal or abnormal maxillofacial development warrant further investigation.

In conclusion, we found evidence for association of the variant rs1979277 (G > A) and NSCL/P in a Chilean population. The allele A showed a protective effect against this birth defect. Considering the significance of the dominant model, we can infer that an embryo carrying at least one copy of the A allele has a lower risk of developing an orofacial cleft in comparison with a non-carrier. Bioinformatic prediction plus literature evidence demonstrate that this variant introduces a missense mutation (p.Leu474Phe) in SHMT enzyme sequence, which decreases its activity. Our results suggest that low enzyme activity may increase the cytoplasmic concentration of folates and, therefore, explain its protective role against OFCs. To the best of our knowledge, this is the first study reporting association between this variant and OFCs.

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AUTHOR CONTRIBUTIONS

C. Salamanca, J. Suazo and P. Gonzalez-Hormazabal have designed the study and applied the molecular and statistical analyses. Recabarren AS, Recabarren P.A., Pantoja R., Pardo R.A and Leiva N contributed to recruit the samples. All authors participate in drafting the manuscript and approved the final version.

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