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Genetic Susceptibility to Non-Syndromic Cleft Lip and Palate and Enamel Hypomineralization: Evidence from Variants and Gene-Gene Interactions in Calcium Homeostasis Genes

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Abstract

Objective

To investigate associations between single nucleotide polymorphisms (SNPs) in calcium homeostasis-related genes and combined phenotypes defined by non-syndromic cleft lip with or without palate involvement (NSCL ± P) and enamel hypomineralization (MIH and/or HSPM), including SNP-SNP interactions.

Materials and Methods

In this cross-sectional study with a comparison group, 230 children aged 6–14 years (125 with NSCL ± P; 105 controls) were clinically assessed for MIH/HSPM by calibrated examiners. Variants in *VDR, CYP24A1, PTH*, and *CALCR* were genotyped by real-time PCR. Participants were classified into four combined phenotype groups according to NSCL ± P and hypomineralization status. Genotype distributions were evaluated under codominant, dominant, and recessive models, followed by multinomial logistic regression. SNP-SNP interactions were analyzed using model-based multifactor dimensionality reduction.

Results

MIH/HSPM was more frequent in participants with NSCL ± P than in controls (44.8% vs. 37.1%), although not significantly (p = 0.240). *PTH* rs694 was the only SNP associated with the combined phenotype groups. Under the recessive model, the CC genotype was associated with lower odds of NSCL ± P with MIH/HSPM compared with controls without MIH/HSPM (OR = 0.29; 95% CI: 0.10–0.84; p = 0.023). A significant interaction between *PTH* rs6256 and *CALCR* rs1801197 was also identified for the concomitant presence of NSCL ± P and MIH/HSPM (p_perm = 0.007).

Conclusion

Calcium homeostasis-related genetic variation may contribute to the concomitant occurrence of NSCL ± P and MIH/HSPM, particularly through *PTH*-related pathways.

Clinical relevance:

Identifying genetic factors shared by NSCL ± P and MIH/HSPM may improve understanding of susceptibility to enamel defects in children with clefts and support more individualized preventive dental care.

1 Introduction

Molar-incisor hypomineralization (MIH) was defined in 2001 as a qualitative enamel defect affecting one to four permanent first molars and is frequently associated with affected incisors [1]. Clinically, MIH is characterized by changes in enamel translucency and increased enamel porosity, which may result in dental hypersensitivity, post-eruptive enamel breakdown, and restorative difficulties [2]. These alterations significantly impair patients' quality of life and pose a considerable clinical challenge, increasing the need for dental treatment and the risk of

treatment failures [3]. Similar defects have also been observed in second primary molars and are referred to as hypomineralized second primary molars (HSPM). The presence of HSPM has been considered a predictor of MIH [3, 4]. Indicating that HSPM and MIH present similar clinical manifestations of a common developmental disturbance affecting enamel mineralization, potentially sharing similar etiological factors and biological pathways.

Studies have reported a higher prevalence of developmental defects of enamel (DDE), including MIH/HSPM, among individuals with craniofacial anomalies, such as non-syndromic cleft lip with or without palate involvement (NSCL ± P) [5–7]. Orofacial clefts are among the most common congenital anomalies affecting the orofacial region [8] and arise from failure of fusion of the facial processes during embryogenesis and have a multifactorial etiology. In addition to the morphofunctional alterations associated with clefts, individuals with NSCL ± P frequently present dental anomalies, including tooth agenesis, supernumerary teeth, taurodontism, and DDE, including MIH and HSPM [9]. The concomitant occurrence of NSCL ± P and dental anomalies raise the possibility that shared biological mechanisms may contribute to their co-occurrence, supporting the investigation of genetic factors in the context of combined phenotypic presentations.

The etiology of MIH/HSPM has not yet been fully elucidated and is considered multifactorial, involving environmental, systemic, and genetic factors [10, 11]. Adequate mineral homeostasis, particularly calcium and phosphate (P) balance, is essential for enamel formation and mineralization. These minerals account for more than 90% of enamel's inorganic content and are therefore largely responsible for its high hardness. Calcium homeostasis is regulated through a complex network of molecular interactions involving vitamin D, parathyroid hormone, and calcitonin [12], whose effects are mediated by several genes, including Vitamin D Receptor (*VDR*), Cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*), Parathyroid Hormone (*PTH)*, and Calcitonin Receptor (*CALCR*) [13–17]. Therefore, genetic alterations such as single nucleotide polymorphisms (SNPs) may influence dental mineralization and potentially contribute to susceptibility to developmental dental defects.

Although previous studies have investigated associations between these genetic variants and dental or craniofacial conditions, including dental caries [18], MIH [19, 20], delayed dental maturation [21] mandibular retrognathism [22] and isolated NSCL ± P [23], evidence regarding their potential involvement in phenotypes characterized by the concomitant occurrence of NSCL ± P and MIH/HSPM remains lacking.

Both NSCL ± P and MIH/HSPM are complex traits with multifactorial etiologies, in which genetic susceptibility is unlikely to be explained by the effect of a single variant. In addition to individual genetic effects, interactions among variants may contribute to phenotypic expression, a phenomenon known as epistasis, in which the effect of one gene or genetic variant is modulated by the presence of another [24]. In the context of mineral homeostasis and dental development, investigating SNP-SNP interactions may provide a more comprehensive understanding of the underlying genetic mechanisms, particularly when multiple physiological pathways, such as those regulated by *VDR*, *CYP24A1*, *CALCR* and *PTH*, converge on related biological processes.

Therefore, the primary aim of this cross-sectional study with a comparison group was to investigate the association between SNPs in genes involved in calcium homeostasis (*VDR*, *CYP24A1*, *CALCR* and *PTH*) and combined phenotypic presentations defined by the presence or absence of NSCL ± P and MIH/HSPM. Additionally, SNP-SNP interactions among these genes were investigated within the NSCL ± P group according to the presence or absence of MIH/HSPM.

2 Methods

2.1 Ethical aspects

This cross-sectional study with a comparison group was conducted in accordance with the Strengthening the Reporting of Genetic Association Studies (STREGA) checklist guidelines [25]. The study protocol was approved by the Research Ethics Committee of the Paraná State Health Department (protocol no. 5,100,185) and by the Research Ethics Committee for Human Studies in the Health Sciences Sector of the Federal University of Paraná (protocol no. 3,752,172), both located in Curitiba, Paraná, Brazil. Participation in the study was authorized through written informed consent signed by the participants' legal guardians and assent obtained from the children.

2.2 Sample selection

The study sample consisted of children of both sexes, aged between 6 and 14 years, allocated into two groups: a group composed of children with NSCL ± P (NSCL ± P group) and a comparison group consisting of children without clefts (control group). Participants in the NSCL ± P group were recruited from the Center for Comprehensive Care for Patients with Cleft Lip and Palate (CAIF), located in Curitiba, Paraná, Brazil. The control group was recruited from the Pediatric Dentistry Clinic of the Federal University of Paraná (UFPR), in the same city. The absence of NSCL ± P was confirmed by clinical examination. The presence of syndromes or other craniofacial malformations not involving clefts was considered an exclusion criterion.

The NSCL ± P group included different clinical manifestations of clefts: isolated cleft lip (CL) and cleft lip and palate (CLP). Cases of isolated cleft palate (CP) were not included because this condition has a distinct embryological origin compared with the other cleft types [26]. Cleft classification was established through clinical examination and confirmed by medical records. Data collection was performed between January 2022 and August 2023.

Sample size calculation was based on previous data regarding the frequency of dental anomalies in individuals with clefts, with an estimated prevalence of 38.8% [9]. A 95% confidence level, 80% statistical power, and an estimated odds ratio of 3.14 were adopted. Considering the possibility of sample losses, an additional 20% was added to the initial estimate. Therefore, the ideal sample size was defined as 100 to 120 participants per group.

2.3 Clinical data collection

The presence of MIH/HSPM was assessed through oral clinical examination using the criteria proposed by Ghanim et al. (2015) for epidemiological studies on enamel hypomineralization. Participants were examined in a dental setting under artificial light using a dental mirror. Prior to data collection, two examiners (GFS and VST) were calibrated according to the protocol proposed by Ghanim et al. (2015). This classification system categorizes MIH and HSPM lesions according to their severity, including white/cream opacities, yellow/brown opacities, post-eruptive enamel breakdown, atypical restorations, atypical carious lesions, and atypical extractions. In addition to MIH and HSPM, the criteria also encompass other DDE, such as diffuse opacities, enamel hypoplasia, amelogenesis imperfecta, and demarcated opacities [27].

Calibration was conducted in two stages. Initially, the examiners participated in a theoretical training session during which 20 intraoral photographs depicting different types of DDE were evaluated. Subsequently, each examiner independently assessed a distinct set of 30 clinical photographs showing different presentations of

DDE. After a seven-day interval, the same set of photographs was re-evaluated individually. The examiners' responses were compared with those of a gold-standard examiner. Intra- and inter-examiner reliability were assessed using Cohen's kappa coefficient, and agreement values exceeded 0.80, indicating excellent reproducibility.

2.4 Genetic analysis

Cell samples were collected through a mouth rinse with 5 mL of sterile saline solution, from which genomic DNA was extracted according to the protocol described by Küchler et al. (2012) .

SNPs in genes associated with calcium homeostasis were selected, including rs1544410, rs7975232, and rs2228570 in *VDR*; rs927650 in *CYP24A1*; and rs1801197 in *CALCR;* rs6256, rs307247, and rs694 in *PTH*. SNP selection was based on minor allele frequency (MAF > 10%) and evidence of their potential influence on the amelogenesis [13–17]. It was hypothesized that these genes may be involved in dental enamel development and that common genetic variants could contribute to the occurrence of MIH/HSPM. Detailed information regarding the investigated genes and their respective SNPs is presented in Table 1.

Table 1
Characteristics of the selected genes and SNPs.

Gene	Gene influence in amelogenesis	SNP	Position	Allele	Funcion	MAF
Vitamin D Receptor (*VDR*)	• It plays crucial roles during the final stage of tooth maturation [13]. • It exerts a direct and local influence on the regulation of enamel mineralization. Its absence results in premature maturation and hypermineralization of the tissue [14].	rs1544410	chr12:47846052	T/C	Intron	T = 0.381
		rs7975232	chr12:47845054	A/C	Intron	C = 0.464
		rs2228570	chr12:47879112	A/G	Initiator Codon Variant	A = 0.377
Cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*)	• Regulates vitamin D homeostasis through the degradation of calcitriol, contributing to processes related to the mineralization of hard tissues [29].	rs927650	chr20:54156202	C/T	Intron	T = 0.451
Parathyroid hormone (*PTH*)	• It participates in the regulation of odontogenesis together with *CALCR*, acting specifically on dental papilla cells to regulate enamel secretion [16].	rs6256	chr11:13492506	G/T	Stop gained	T = 0.127
		rs307247	chr11:13491931	A/G	500B Downstream Variant	A = 0.393
		rs694	chr10:77790869	C/T	Intron	C = 0.429
Calcitonin receptor (*CALCR*)	• It participates in the regulation of odontogenesis together with *CALCR*, acting specifically on dental papilla cells to regulate enamel secretion [16].	rs1801197	chr7:93426441	A/G	Missense	G = 0.306

Abbreviation: MAF - minor allele frequency.

Genotyping was performed using real-time polymerase chain reaction (PCR) with allelic discrimination assays on the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The success rate of the genetic assays ranged from 96.9% to 99.1% (Supplementary Table 1).

2.5 Statistical analysis

Descriptive analyses were performed to characterize the study sample. Categorical variables were summarized as absolute and relative frequencies, whereas age was expressed as mean and standard deviation. The comparability of the NSCL ± P and control groups was assessed by comparing sex distribution using the chi-square test and mean age using the independent-samples t-test.

Hardy–Weinberg equilibrium (HWE) was assessed for each SNP using the chi-square test through the online tool available at https://wpcalc.com/en/equilibrium-hardy-weinberg. Deviations from HWE were considered when the chi-square value exceeded 3.84 (Supplementary Table 1).

The association between NSCL ± P and MIH/HSPM was initially assessed using the chi-square test. A combined phenotype variable was then created based on the concomitant presence or absence of NSCL ± P and MIH/HSPM, resulting in four groups: (1) NSCL ± P with MIH/HSPM, (2) NSCL ± P without MIH/HSPM, (3) controls with MIH/HSPM, and (4) controls without MIH/HSPM. Associations between the combined phenotype groups and genotype distributions were assessed using the chi-square test under codominant, dominant, and recessive inheritance models. For genotype distributions showing a statistically significant association with the combined phenotype groups in the chi-square analysis, multinomial logistic regression was subsequently performed to identify the phenotype group comparisons underlying the overall association and to estimate its magnitude and direction. Controls without MIH/HSPM were used as the reference phenotype category. Odds ratios (ORs) and their respective 95% confidence intervals (95% CIs) were calculated, with the genetic reference category defined according to the inheritance model evaluated. Statistical significance was set at 5% ($p < 0.05$). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 20.0 (SPSS Inc., Chicago, IL, USA).

Interactions among SNPs in the *VDR* (rs1544410, rs7975232, and rs2228570), *CYP24A1* (rs927650), *CALCR* (rs1801197), and *PTH* (rs6256, rs307247, and rs694) genes were evaluated using the model-based multifactor dimensionality reduction (MB-MDR) approach, implemented through the mbmdr package in R software. Analyses were adjusted for sex, ethnicity, and NSCL ± P status, with cross-validation and 1,000 permutations applied to minimize false-positive interactions. STRING, a protein–protein interaction network analysis tool, was used to investigate functional interactions among the analyzed genes (https://string-db.org/) [28].

3 Results

A total of 292 children underwent recruitment and screening. Sixty-two were excluded due to the presence of syndromes (n = 13), craniofacial anomalies other than oral clefts (n = 5), cleft palate (n = 20), incomplete clinical examination data (n = 13), or the absence of an available DNA sample (n = 11). The final study sample therefore included 230 children, comprising 125 participants with NSCL ± P and 105 controls. Males predominated in the NSCL ± P group (58.4%), whereas females predominated in the control group (52.3%), with 125 boys and 109 girls overall. Among participants with NSCL ± P, 42 had CL and 83 had CLP. Mean age was comparable between the groups: 8.85 years (SD = 2.12) in the NSCL ± P group and 8.60 years (SD = 2.01) among controls, with an overall mean age of 8.74 years (SD = 2.07). No statistically significant differences were observed between the groups regarding sex distribution or age ($p > 0.050$) (Supplementary Table 2).

MIH/HSPM was more frequent among participants with NSCL ± P than among controls (44.8% vs. 37.1%). However, this difference was not statistically significant ($p = 0.240$) (Supplementary Table 3).

Genotype distributions across the combined phenotype groups were similar for all SNPs under the codominant, dominant, and recessive inheritance models, except for rs694 in *PTH*. Under the recessive model, the distribution of the CC and combined CT + TT genotypes differed significantly among the four phenotype groups ($p = 0.049$) (Table 2). Multinomial logistic regression was subsequently performed to identify the phenotype group comparison underlying this association and to estimate its magnitude and direction. Using controls without

MIH/HSPM as the reference phenotype category and the combined CT + TT genotypes as the genetic reference, the CC genotype was associated with lower odds of belonging to the NSCL ± P with MIH/HSPM group (OR = 0.29; 95% CI: 0.10–0.84; p = 0.023), suggesting a potential protective effect against the combined phenotype. No significant associations were observed for controls with MIH/HSPM (OR = 1.28; 95% CI: 0.53–3.08; p = 0.579) or for participants with NSCL ± P without MIH/HSPM (OR = 0.73; 95% CI: 0.33–1.64; p = 0.451), compared with controls without MIH/HSPM (Table 3).

Table 2
Genotype distributions among combined NSCL ± P and MIH/HSPM phenotype groups according to codominant, dominant, and recessive inheritance models.

SNP	Allele	Group				Total	P-value[a]
		Controls without MIH/HSPM	Controls with MIH/HSPM	NSCL ± P without MIH/HSPM	NSCL ± P with MIH/HSPM		
rs1544410 (*VDR*)							
Codominant model	TT	9 (33.3)	4 (14.8)	12 (44.4)	2 (7.4)	27	0.160
	CT	27 (25.7)	16 (15.2)	35 (33.3)	27 (25.7)	105	
	CC	30 (31.6)	19 (20.0)	21 (22.1)	25 (26.3)	95	
Dominant model	CC	30 (31.6)	19 (20.0)	21 (22.1)	25 (26.3)	95	0.179
	CT + TT	36 (27.3)	20 (15.2)	47 (35.6)	29 (22.0)	132	
Recessive model	TT	9 (33.3)	4 (14.8)	12 (44.4)	2 (7.4)	27	0.117
	CT + CC	57 (28.5)	35 (17.5)	56 (28.0)	52 (26.0)	200	
rs7975232 (*VDR*)							
Codominant model	AA	26 (31.7)	12 (14.6)	31 (37.8)	13 (15.9)	82	0.063
	AC	21 (21.9)	20 (20.8)	27 (28.1)	28 (29.2)	96	
	CC	18 (40.0)	6 (13.3)	9 (20.0)	12 (26.7)	45	
Dominant model	AA	26 (31.7)	12 (14.6)	31 (37.8)	13 (15.9)	82	0.080
	AC + CC	39 (27.7)	26 (18.4)	36 (25.5)	40 (28.4)	141	
Recessive model	CC	18 (40.0)	6 (13.3)	9 (20.0)	12 (26.7)	45	0.185
	AC + AA	47 (26.4)	32 (18.0)	58 (32.6)	41 (23.0)	178	
rs2228570 (*VDR*)							
Codominant model	AA	11 (32.4)	3 (8.8)	11 (32.4)	9 (26.5)	34	0.532
	AG	27 (24.3)	24 (21.6)	32 (28.8)	28 (25.2)	111	
	GG	28 (33.7)	12 (14.5)	25 (30.1)	18 (21.7)	83	
Dominant model	GG	28 (33.7)	12 (14.5)	25 (30.1)	18 (21.7)	83	0.595
	AG + AA	38 (26.2)	27 (18.6)	43 (29.7)	37 (25.5)	145	

SNP	Allele	Group				Total	P-value[a]
		Controls without MIH/HSPM	Controls with MIH/HSPM	NSCL ± P without MIH/HSPM	NSCL ± P with MIH/HSPM		
Recessive model	AA	11 (32.4)	3 (8.8)	11 (32.4)	9 (26.5)	34	0.585
	AG + GG	55 (28.4)	36 (18.6)	57 (29.4)	46 (23.7)	194	
rs927650 (*CYP24A1*)							
Codominant model	CC	19 (28.8)	9 (13.6)	21 (31.8)	17 (25.8)	66	0.693
	CT	31 (29.0)	23 (21.5)	29 (27.1)	24 (22.4)	107	
	TT	16 (29.6)	6 (11.1)	19 (35.2)	13 (24.1)	54	
Dominant model	CC	19 (28.8)	9 (13.6)	21 (31.8)	17 (25.8)	66	0.861
	CT + TT	47 (29.2)	29 (18.0)	48 (29.8)	37 (23.0)	161	
Recessive model	TT	16 (29.6)	6 (11.1)	19 (35.2)	13 (24.1)	54	0.596
	CT + CC	50 (28.9)	32 (18.5)	50 (28.9)	41 (23.7)	173	
rs1801197 (*CALCR*)							
Codominant model	AA	24 (27.9)	19 (22.1)	26 (30.2)	17 (19.8)	86	0.277
	AG	28 (27.2)	12 (11.7)	30 (29.1)	33 (32.0)	103	
	GG	13 (33.3)	7 (17.9)	13 (33.3)	6 (15.4)	39	
Dominant model	AA	24 (27.9)	19 (22.1)	26 (30.2)	17 (19.8)	86	0.280
	AG + GG	41 (28.9)	19 (13.4)	43 (30.3)	39 (27.5)	142	
Recessive model	GG	13 (33.3)	7 (17.9)	13 (33.3)	6 (15.4)	39	0.415
	AG + AA	52 (27.5)	31 (16.4)	56 (29.6)	50 (26.5)	189	
rs6256 (*PTH*)							
Codominant model	GG	48 (28.2)	26 (15.3)	54 (31.8)	42 (24.7)	170	0.721
	GT	14 (28.6)	10 (20.4)	14 (28.6)	11 (22.4)	49	
	TT	4 (57.1)	1 (14.3)	1 (14.3)	1 (14.3)	7	
Dominant model	GG	48 (28.2)	26 (15.3)	54 (31.8)	42 (24.7)	170	0.744
	GT + TT	18 (32.1)	11 (19.6)	15 (26.8)	12 (21.4)	56	

SNP	Allele	Group				Total	P-value[a]
		Controls without MIH/HSPM	Controls with MIH/HSPM	NSCL ± P without MIH/HSPM	NSCL ± P with MIH/HSPM		
Recessive model	TT	4 (57.1)	1 (14.3)	1 (14.3)	1 (14.3)	7	0.415
	GT + GG	62 (28.3)	36 (16.4)	68 (31.1)	53 (24.2)	219	
rs307247 (*PTH*)							
Codominant model	AA	13 (32.5)	9 (22.5)	14 (35.0)	4 (10.0)	40	0.232
	AG	33 (32.7)	16 (15.8)	27 (26.7)	25 (24.8)	101	
	GG	20 (23.0)	13 (14.9)	28 (32.2)	26 (29.9)	87	
Dominant model	GG	20 (23.0)	13 (14.9)	28 (32.2)	26 (29.9)	87	0.252
	AG + AA	46 (32.6)	25 (17.7)	41 (29.1)	29 (20.6)	141	
Recessive model	AA	13 (32.5)	9 (22.5)	14 (35.0)	4 (10.0)	40	0.134
	AG + GG	53 (28.2)	29 (15.4)	55 (29.3)	51 (27.1)	188	
rs694 (*PTH*)							
Codominant model	CC	17 (35.4)	12 (25.0)	14 (29.2)	5 (10.4)	48	0.131
	CT	35 (30.4)	17 (14.8)	32 (27.8)	31 (27.0)	115	
	TT	14 (21.2)	10 (15.2)	23 (34.8)	19 (28.8)	66	
Dominant model	TT	14 (21.2)	10 (15.2)	23 (34.8)	19 (28.8)	66	0.306
	CT + CC	52 (31.9)	29 (17.8)	46 (28.2)	36 (22.1)	163	
Recessive model	CC	17 (35.4)	12 (25.0)	14 (29.2)	5 (10.4)	48	0.049*
	CT + TT	49 (27.1)	27 (14.9)	55 (30.4)	50 (27.6)	181	

Abbreviations: NSCL ± P, non-syndromic cleft lip with or without cleft palate; MIH, Molar Incisor Hypomineralization; HSPM, Hypomineralized Second Primary Molars.

[a]Chi-square test

*Statically significant result ($p < 0.05$).

Table 3

Genotype distribution and multinomial logistic regression analysis of the association between the PTH rs694 variant and combined NSCL ± P and MIH/HSPM phenotype groups under the recessive model.

Phenotype group	CC, n (%)	CT + TT, n (%)	Crude OR (95% CI)	p-value
Controls without MIH/HSPM	17 (25.8)	49 (74.2)	Reference	–
Controls with MIH/HSPM	12 (30.8)	27 (69.2)	1.28 (0.53–3.08)	0.579
NSCL ± P without MIH/HSPM	14 (20.3)	55 (79.7)	0.73 (0.33–1.64)	0.451
NSCL ± P with MIH/HSPM	5 (9.1)	50 (90.9)	0.29 (0.10–0.84)	0.023*

Abbreviations: NSCL ± P, non-syndromic cleft lip with or without cleft palate; MIH, molar incisor hypomineralization; HSPM, hypomineralized second primary molars; OR odds ratio; CI, confidence interval.

The reference category for the multinomial logistic regression was controls without MIH/HSPM. Genotypes were analyzed according to the recessive genetic model (CC vs. CT + TT).

*Statistically significant result ($p < 0.05$).

Pairwise interactions among SNPs in the evaluated genes were investigated to determine whether combinations of variants were associated with the concomitant presence of NSCL ± P and MIH/HSPM. Considering the simultaneous presence of NSCL ± P and MIH/HSPM, a significant interaction was identified between rs6256 in PTH and rs1801197 in CALCR, which remained significant after permutation testing (p_perm = 0.007) (Table 4).

Table 4
SNP–SNP interactions among VDR, CYP24A1, CALCR, and PTH evaluated using model-based multifactor dimensionality reduction (MB-MDR).

NSCL ± P without MIH/HSPM	SNP1	SNP2	NH[a]	betaH[b]	NL[c]	betaL[d]	p-value[e]	Permutation p-value [f]
	rs1801197 (*CALCR*)	**rs927650 (*CYP24A1*)**	**2**	**1.2132**	**0**	**NA**	**0.008**	**0.05**
rs107247 (*PTH*)	**rs1544410 (*VDR*)**	**0**	**NA**	**1**	**-1.0457**	**0.02**	**0.13**	
rs1801197 (*CALCR*)	**rs7975232 (*VDR*)**	**1**	**2.0941**	**1**	**-2.1854**	**0.04**	**0.19**	
rs927650 (*CYP24A1*)	**rs7975232 (*VDR*)**	**1**	**1.2030**	**0**	**NA**	**0.04**	**0.28**	
NSCL ± P with MIH/HSPM	rs6256 (*PTH*)	rs1801197 (*CALCR*)	1	1.523	2	-1.8328	0.002	0.007*
	rs1801197 (*CALCR*)	rs1544410 (*VDR*)	1	1.415	0	NA	0.02	0.07
	rs927650 (*CYP24A1*)	rs2228570 (*VDR*)	0	NA	1	-1.0367	0.03	0.13
	rs1801197 (*CALCR*)	rs927650 (*CYP24A1*)	1	1.596	1	-1.0809	0.04	0.14
	rs927650 (*CYP24A1*)	rs1544410 (*VDR*)	1	2.168	0	NA	0.04	0.17

Abbreviations: NSCL ± P, non-syndromic cleft lip with or without cleft palate; MIH, Molar Incisor Hypomineralization; HSPM, Hypomineralized Second Primary Molars.

*Statically significant result ($p < 0.05$).

[a] Number of significant high-risk genotype combinations in the interaction. [b] Regression coefficient in step 2 for high-risk exposure. [c] Number of significant low-risk genotype combinations in the interaction. [d] Regression coefficient in step 2 for low-risk exposure. [e] P-value for the interaction model adjusted for covariates. [f] P-value for the permutation-based interaction model.

4 Discussion

Patients with NSCL ± P have a higher frequency of dental anomalies than individuals without clefts [9], including DDE such as MIH/HSPM. In the present study, although MIH/HSPM was more frequent in the NSCL ± P group than in the control group (44.8% vs. 37.1%), this difference was not statistically significant. In a previous study conducted by the same research group using a larger, partially overlapping sample of children with NSCL ± P and a population-based control group, MIH was more frequent among children with NSCL ± P than among controls (29.3% vs. 18.3%) [7]. Although the partial overlap between the cleft samples precludes interpretation of these findings as an independent replication, the previous results support the high frequency of enamel defects among individuals with NSCL ± P.

Other studies have also reported a high prevalence of MIH/HSPM among individuals with oral clefts, ranging from 52.1% to 67.7% [5, 6]; however, neither study included a control group. The absence of a significant association in the present study may be related to the characteristics of the control group, which was recruited from a dental school clinic that serves as a referral center for the diagnosis and treatment of DDEs. This recruitment strategy may have overestimated the frequency of MIH/HSPM among children without NSCL ± P, particularly considering that a prevalence of approximately 12% was previously reported in a population-based sample of schoolchildren without clefts from Curitiba, Paraná, Brazil [29].

MIH and HSPM are qualitative enamel defects resulting from disturbances in the mineralization of the enamel matrix during amelogenesis [30]. Dental mineralization depends on complex mechanisms in which calcium plays a central role, contributing both to the formation of hydroxyapatite crystals and to the regulation of ameloblast activity [31]. Given the importance of calcium-related pathways in enamel mineralization, the present study also investigated the association between MIH/HSPM and polymorphisms in genes involved in this metabolic pathway among children with and without NSCL ± P. Combined phenotype groups were considered, as previous evidence has suggested a potential biological interplay between MIH/HSPM and NSCL ± P, possibly reflecting shared multifactorial genetic and environmental mechanisms underlying their concomitant occurrence [6, 32].

Calcium (Ca) homeostasis is regulated by hormones such as vitamin D, parathyroid hormone, and calcitonin [12]. Vitamin D promotes intestinal Ca absorption and renal Ca reabsorption; parathyroid hormone is secreted in response to hypocalcemia and acts by mobilizing Ca from bone and increasing its renal reabsorption; whereas calcitonin, released in response to hypercalcemia, inhibits osteoclastic bone resorption, thereby contributing to a reduction in serum Ca levels [12, 33]. The effects of these hormones are mediated by genes such as *VDR*, *CYP24A1*, *PTH*, and *CALCR*, which may also exert local effects during odontogenesis. Among these genes, *VDR* appears to have an important role in the later stages of tooth maturation and in the regulation of enamel mineralization. Its expression in ameloblasts has been associated with cell differentiation and with the regulation of enamel-related genes, including amelogenin [13, 14, 34]. *CYP24A1*, in turn, regulates vitamin D homeostasis through calcitriol catabolism and has been implicated in hard-tissue mineralization [35, 36]. *PTH* has been associated with odontoblast activity during dentin formation and with Ca transport during amelogenesis [17, 37, 38]. *CALCR* expression in the dental papilla of developing tooth germs further supports a potential local role in this process [16]. These biological functions provide a plausible basis for the involvement of genetic variants in these genes in alterations of enamel development and mineralization.

Among the individually evaluated variants, *PTH* rs694 was the only SNP associated with the combined phenotype groups. Under the recessive model, individuals with the CC genotype had 71% lower odds of presenting the combined phenotype. Notably, no significant association was observed for controls with MIH/HSPM or for individuals with NSCL ± P without MIH/HSPM. This pattern suggests that the association with rs694 may not reflect susceptibility to either condition independently, but rather may be related to the phenotypic context in which NSCL ± P and MIH/HSPM occur concomitantly.

Although evidence regarding *PTH* variants and enamel hypomineralization remains limited, previous studies support a role for this gene in dental development. The rs694 and rs307247 variants have been associated with variations in permanent tooth crown size [39], and *PTH* and vitamin D-related genes have also been investigated in relation to dental maturation and craniofacial traits [21, 40]. In contrast, previous studies investigating MIH have predominantly identified associations with variants in *VDR*, including rs739837 and rs78783628. In the present study, however, none of the evaluated *VDR* variants were associated with the combined phenotype

groups. These differences may reflect the distinct outcome investigated here, since the present analysis considered the concomitant occurrence of NSCL ± P and MIH/HSPM rather than enamel hypomineralization as an isolated phenotype.

When investigating pairwise interactions between SNPs in genes related to Ca homeostasis, a significant interaction was identified between rs6256 (*PTH*) and rs1801197 (*CALCR*) in relation to the concomitant presence of NSCL ± P and MIH/HSPM. This finding suggests that the combined effect of these variants may be associated to this specific phenotypic presentation, even though no individual association was observed for either SNP across the combined phenotype groups. Although no direct physical interactions between PTH and CALCR were identified in the STRING database analysis, evidence of co-expression between *PTH* and *CALCR* was observed (Fig. 1).

Beyond the individual association observed for *PTH* rs694, the SNP–SNP interaction analysis provided additional evidence of a potential contribution of calcium homeostasis-related genes to the combined phenotype. The co-expression of *PTH* and *CYP24A1* may be explained by the coordinated role of these genes in modulating vitamin D and calcium metabolism. PTH, secreted in response to decreased calcium ion levels, stimulates enzymes such as CYP27B1, thereby activating vitamin D, whereas CYP24A1 promotes its inactivation, allowing balanced regulation of active vitamin D levels, particularly in tissues such as the kidney [41]. The observed co-expression of *PTH* and *CALCR*, in turn, reflects calcium regulation through these hormonal pathways: *PTH* increases calcium levels through bone resorption and renal reabsorption, whereas *CALCR* inhibits osteoclastic bone resorption. Evidence indicates that the PTH receptor is expressed in osteoblasts and osteoclasts, as is CALCR in these tissues, suggesting functional co-occurrence that may enable a physiological counter-regulatory mechanism essential for mineral homeostasis [42].

Although the individual and interaction analyses evaluated distinct genetic effects and involved different *PTH* variants, both findings point to *PTH* as a potentially relevant gene in the genetic background associated with the concomitant occurrence of NSCL ± P and MIH/HSPM. Importantly, these findings should be interpreted as complementary rather than directly related, since the analyses addressed different genetic effects. The present findings are consistent with the hypothesis that epistatic effects between genetic variants involved in mineral regulation may contribute to susceptibility to the combined NSCL ± P and MIH/HSPM phenotype. However, the biological mechanisms underlying the concomitant occurrence of these conditions remain poorly understood. Both conditions have complex etiologies involving interactions between genetic and environmental factors, which makes it difficult to identify isolated causal mechanisms and reinforces the need for further investigations that account for their multifactorial nature.

This study has some limitations that should be considered when interpreting the findings. Although the overall sample size was adequate for the initial comparison between participants with NSCL ± P and controls, classification into four combined phenotype groups resulted in relatively small numbers of individuals in some categories, which may have limited the statistical power to detect more subtle genetic associations and reduced the precision of some estimates. MIH and HSPM were therefore analyzed jointly to avoid further fragmentation of the sample into smaller subgroups. Despite these limitations, important strengths include the use of a control group, the evaluation of distinct genetic inheritance models, and the combined analysis of NSCL ± P and MIH/HSPM phenotypes. Furthermore, to the best of our knowledge, this is one of the first studies to investigate both individual genetic variants and gene–gene interactions involving mineral homeostasis-related genes in the context of the concomitant occurrence of NSCL ± P and MIH/HSPM.

5 Conclusion

The findings of this study suggest that genetic variants in genes related to calcium homeostasis may be involved in the concomitant occurrence of NSCL ± P and MIH/HSPM. Among the individually evaluated SNPs, *PTH* rs694 was associated with the combined NSCL ± P and MIH/HSPM phenotype under the recessive model, with the CC genotype associated with lower odds of belonging to this phenotype group relative to controls without MIH/HSPM. In addition, the interaction between *PTH* rs6256 and *CALCR* rs1801197 was significantly associated with the concomitant presence of NSCL ± P and MIH/HSPM. Together, these findings highlight the potential relevance of both individual genetic effects and gene-gene interactions in the genetic background of complex phenotypes. Future studies with larger samples and multicenter designs are needed to replicate these findings. A better understanding of the genetic factors associated with the concomitant occurrence of these conditions may ultimately contribute to the identification of susceptible individuals and to more individualized preventive and dental care strategies.

Disclosures

and declarations

Declarations

Conflict of Interest:

The authors declare that they have no conflict of interest.

Ethics approval:

The study protocol was approved by the Research Ethics Committee of the Paraná State Health Department (protocol no. 5,100,185) and by the Research Ethics Committee for Human Studies in the Health Sciences Sector of the Federal University of Paraná (protocol no. 3,752,172). The study was conducted in accordance with the principles of the Declaration of Helsinki.

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Author Contribution

All authors contributed to the study conception and design. G.F.S. and V.S.T. performed data collection. G.F.S., J.F.S., and E.C.K. conducted data analyses. R.A.M. and R.D.C. performed the interaction analyses. E.C.K. and C.K.

contributed to the laboratory analyses. G.F.S. wrote the first draft of the manuscript. All authors critically reviewed the manuscript, contributed to its final version, and read and approved the final manuscript.

Data availability statements:

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

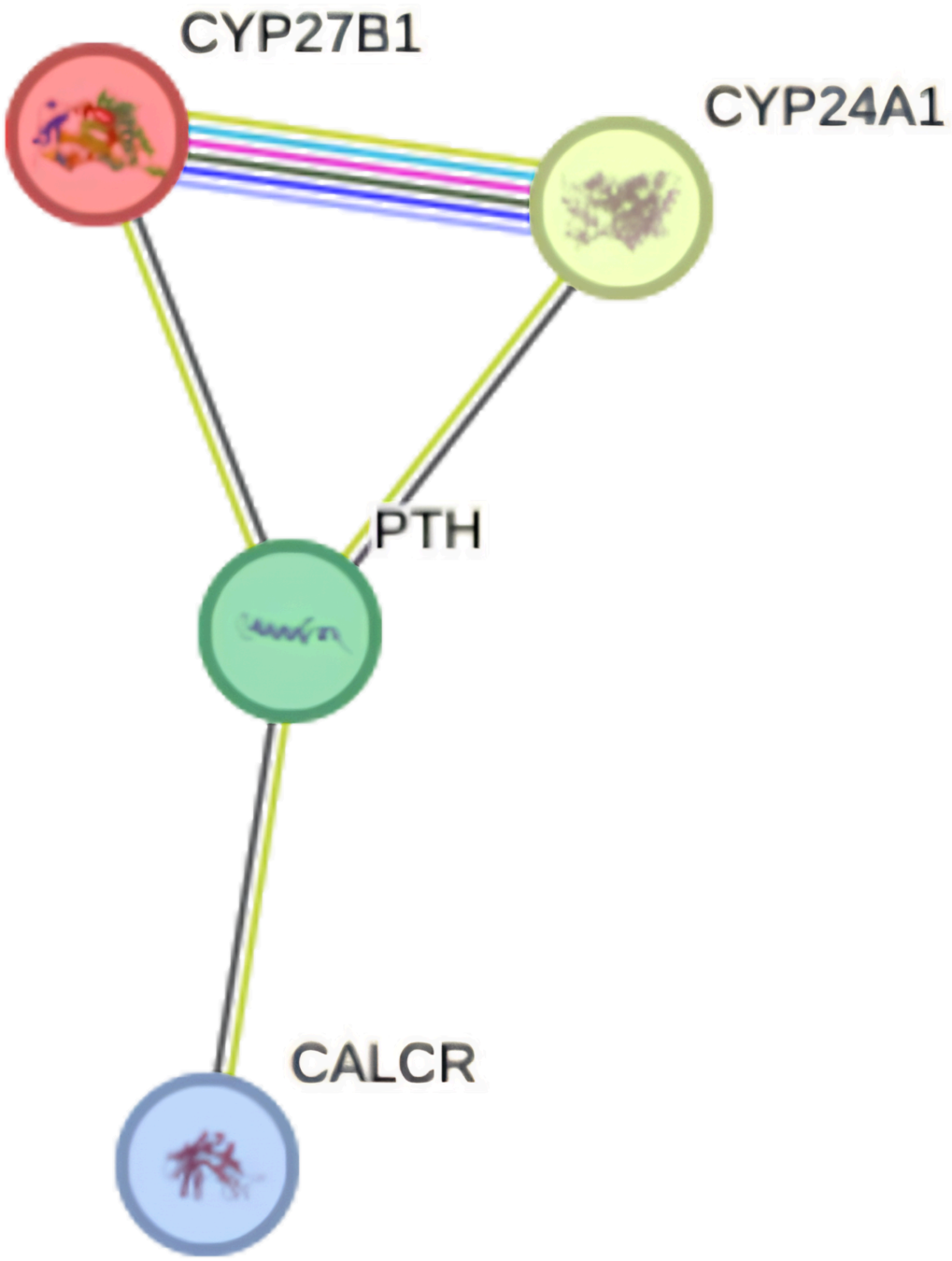


Figure 1

Interaction network among the PTH, CALCR, and CYP24A1 genes generated using STRING. The connections indicate potential functional relationships among the genes: black lines represent gene co-expression, whereas yellow lines represent associations based on co-occurrence in scientific literature.

Supplementary Files

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