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Potential interactions among single nucleotide polymorphisms in bone- and cartilage-related genes in skeletal malocclusions

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Abstract

Objective: To investigate SNPs in bone- and cartilage-related genes and their interaction in the aetiology of sagittal and vertical skeletal malocclusions.

Settings and sample population: This study included 143 patients and classified as follows: skeletal class I (n = 77), class II (n = 47) and class III (n = 19); maxillary retrusion (n = 39), protrusion (n = 52) and well-positioned maxilla (n = 52); mandibular retrognathism (n = 50), prognathism (n = 50) and well-positioned mandible (n = 43); normofacial (n = 72), dolichofacial (n = 55) and brachyfacial (n = 16).

Materials and methods: Steiner's ANB, SNA, SNB angles and Ricketts' NBa-PtGn angle were measured to determine the skeletal malocclusion and the vertical pattern. Nine SNPs in *BMP2*, *BMP4*, *SMAD6*, *RUNX2*, *WNT3A* and *WNT11* were genotyped. Chi-squared test was used to compare genotypes among the groups. Multifactor dimensionality reduction (MDR) and binary logistic regression analysis, both using gender and age as co-variables, were also used. We performed Bonferroni correction for multiple testing.

Results: Significant associations at $P < .05$ were observed for SNPs rs1005464 ($P = .042$) and rs235768 ($P = .021$) in *BMP2* with mandibular retrognathism and for rs59983488 (*RUNX2*) with maxillary protrusion ($P = .04$) as well as for rs708111 (*WNT3A*) with skeletal class III ($P = .02$; dominant model), rs1533767 (*WNT11*) with a brachyfacial skeletal pattern ($P = .01$, OR = 0.10; dominant model) and for rs3934908 (*SMAD6*) with prognathism ($P = .02$; recessive model). After the Bonferroni correction, none of the SNPs remained associated. The MDR predicted some interaction for skeletal class II, dolichofacial and brachyfacial phenotypes.

Conclusion: Our results suggest that SNPs in *BMP2*, *BMP4*, *SMAD6*, *RUNX2*, *WNT3A* and *WNT11* could be involved in the aetiology of sagittal and vertical malocclusions.

KEYWORDS

bone, genes, malocclusion, polymorphisms

1 | INTRODUCTION

Skeletal malocclusions are complex craniofacial growth and developmental problems.[1] They are a set of human craniofacial morphologic characteristics that either exceed or exhibit deficiency of maxillary and mandibular dimensions, resulting in an improper relationship of the jaws that distorts the balance of the face.[2] Evidence gained especially from family and twin studies has demonstrated that genetic factors are strongly involved in the aetiology of skeletal malocclusions[3,4]

Genes encoding proteins involved in bone and cartilage biology and skeletogenesis are candidate for skeletal malocclusions. The Bone Morphogenetic Protein (BMP) family is the largest subfamily of the structurally conserved transforming growth factor-beta (TGF-β) superfamily. BMPs are multi-functional growth factors that regulate the development, proliferation and differentiation[5,6] of mature osteoprogenitor cells into osteoblasts.[6] A review including several studies has shown the involvement of BMP2 in bone formation,[5] and BMP4 is involved in cell differentiation during skeletogenesis.[7] BMPs are also among the key pathways regulating craniofacial development and facial patterning. They regulate postnatal craniofacial growth and are associated with dental structures.[5] BMPs are involved during cartilaginous development, as are SMADs.[7,8] SMADs are important signalling pathway proteins that regulate the transcription of TGF-β superfamily genes. SMAD6 inhibits BMP signalling by interacting with transcription repressors.[9]

RUNX2 (Runt-related transcription factor) is a key transcription factor associated with osteoblast differentiation and is considered a master regulator of skeletogenesis. RUNX2 is essential for the differentiation of pluripotent mesenchymal cells into osteoblasts, but also acts in mature osteoblasts maintaining the expression of bone matrix protein genes.[10] RUNX2 is expressed in different craniofacial tissues such as cartilage during the proliferation and maturation of chondrocytes.[10,11] Wnt signalling, one of the key cascades regulating development, is involved in important aspects of organogenesis such as control of cell polarity, fate and migration,[12] during cartilage and bone development, repair and regeneration.[13] The expression of Wnt signalling pathway genes during craniofacial development has been extensively investigated.[14]

Genetic factors are involved in the aetiology of skeletal malocclusions and in vertical, sagittal, and transverse interrelationships of the dental arches.[2] Single-nucleotide polymorphisms (SNPs) are the most frequent variations in the human genome. SNPs in many genes were associated with different skeletal malocclusion phenotypes in different populations.[15-22] Therefore, in this study we investigated SNPs in bone- and cartilage-related genes in the aetiology of sagittal and vertical skeletal malocclusions.

2 | MATERIALS AND METHODS

2.1 | Sample

The Human Ethics Committee of the (identifying information) approved this study. Informed consent was obtained from all patients/children and/or their parents/legal guardians (in the case of minors).

Following the Strengthening the Reporting of Genetic Association study (STREGA) statement checklist,[23] we evaluated genomic DNA (gDNA) extracted from saliva samples and pre-treatment lateral cephalograms from self-reported Caucasians as previously described.[15] All the included patients were enrolled in the orthodontic treatment at the (identifying information).

Biologically unrelated patients with no underlying syndromes nor congenital alterations and those without previous orthodontic and/or orthopaedic treatments were consecutively included in this study from 2015 to 2017. All patients that met the inclusion criteria were invited to participate in the study. Among 146 assessed individuals, one oral cleft patient and two patients, whose siblings were already included in the study, were excluded, yielding a total of 143 included patients.

2.2 | Phenotypes definition

Pre-orthodontic lateral cephalograms with the mandible in centric relationship were used, and digital cephalometric tracings performed by a calibrated orthodontist using the software Dolphin Imaging version 8.0 (Dolphin Imaging, Chatsworth, CA, USA).[16] Steiner's ANB, SNA, SNB angles and Ricketts' NBa-PtGn angle were measured to determine the sagittal skeletal jaw relationship (skeletal malocclusion) and the vertical pattern. The following landmarks were used for cephalometric analysis: point A, point B, sella (S) and nasion (N). Sagittal skeletal discrepancies were assessed using angular measurements: SNA (sella, nasion and subspinale point A), SNB (sella, nasion and supramentale point B) and ANB (subspinale point A, nasion and supramentale point B). Vertical skeletal discrepancies were assessed using Nasion Basion-Pt Point Gnathion angle (NBa-PtGn). The sample was classified according to the ANB angle as class I (0°-4°), class II (>4°) or class III (<0°); according to the SNA angle as maxillary retrusion (<80°), well-positioned maxilla (80°-84°) or maxillary protrusion (>84°); according to the SNB angle as mandibular retrusion (<78°), well-positioned mandible (78°-82°) or mandibular prognathism (>82°) and according to the NBa-PtGn angle as mesofacial (87°-93°), dolichofacial (<87°) or brachyfacial (>93°).

gDNA was used for genotyping analysis. Nine SNPs, which were previously associated with diseases or development dysfunction in bone and/or cartilage, were selected for this study. Validated probes supplied by Applied Biosystems (Foster City, CA) were used: rs1005464 (A > G, C___8954270_20) and rs235768 (A > T, C___2244893_10) in *BMP2*, rs17563 (A > G, C___9597660_20) in *BMP4*; rs2119261 (C > T, C___9136214_10) and rs3934908 (C > T, C__27896468_10) in *SMAD6*, rs59983488 (G > T, C__27841338_10) and rs1200425 (A > G, C___1440244_10) in *RUNX2*, rs708111 (A > G, C___7543813_10) in *WNT3A*, and rs1533767 (A > G, C___7624882_10) in *WNT11*. The characteristics of each SNP are demonstrated in Supplementary Table S1. The genotyping was

blindly performed using the Taqman™ method for real-time PCR (ABI PRISM® 7900HT Sequence Detection System, Foster City, CA, USA). Additionally, 10% of the sample was genotyped twice and an agreement of 100% was observed. The reaction was previously described.[15]

2.3 | Statistical analysis

Chi-squared test was used to estimate the Hardy-Weinberg equilibrium and to compare genotype distribution among groups. Binary logistic regression analysis adjusted by gender and age was also performed. The established alpha for the exploratory results was $P < .05$. We also used the formal threshold for statistical significance after Bonferroni correction for multiple testing $P < .0055$ (0.05/9 SNPs).

Multifactor dimensionality reduction (MDR), a model-free and non-parametric method, was used to identify SNP-SNP interactions[24] using gender and age (before and after the growth spurt) as co-variables. MDR analysed all possible SNP combinations. A 10-fold cross-validation (CV) was performed, which calculated the ratio for each combination, separating 'high' or 'low' risk genotypes for each phenotype. The 1000 permutation test adjusted and determined statistical significance of the analysis. Models with 9/10 or 10/10 CV consistency, the testing balancing accuracy (TBA) >0.55 and $P \leq .05$ were considered best models. Entropy values were obtained by the Jakulin and Bratko (2003)[25] formula, and MDR created dendrograms and interaction graphs using these values.

3 | RESULTS

The mean age was 15.2 years (standard deviation = 7.3), 69 males and 74 females. The sample distribution according to the phenotypes is presented in Table 1.

The amplification rate of each SNP was the following: 90.2% for rs1005464 (n = 129), 91.6% for rs235768 (n = 131), 88.8% for rs17563 (n = 127), 90.9% for rs2119261 (n = 130), 91.6% for rs3934908 (n = 131), 89.5% for rs59983488 (n = 128), 88.1% for rs1200425 (n = 126), 91.6% rs708111 (n = 131), and 67.8% for rs1533767 (n = 97). The SNPs were within the Hardy-Weinberg equilibrium.

All genotype distributions are demonstrated in Table 2. The SNPs rs1005464 and rs235768 in *BMP2* were associated with mandibular retrognathism ($P = .042$, OR = 0.29, CI 95% = 0.10-0.82 and $P = .021$, OR = 3.54, CI 95% = 1.32-8.84, respectively). The rs59983488 SNP in *RUNX2* was associated with maxillary protrusion ($P = .04$, OR = 0.11, CI 95% = 0.01-0.88). In the dominant model, the SNP rs708111 in *WNT3A* was associated with skeletal class III ($P = .02$, OR = 0.30, CI 95% = 0.10-0.91). In the dominant model, the SNP rs1533767 in *WNT11* was associated with a brachyfacial phenotype ($P = .01$, OR = 0.10, CI 95% = 0.00-0.68). In the recessive model, rs3934908 in *SMAD6* was associated with prognathism ($P = .02$, OR = 0.29, CI 95% = 0.09-0.83). After the Bonferroni correction for multiple testing, none of the SNPs remained associated. For the SNPs and phenotypes with suggestive association ($P < .05$), a logistic regression analysis was also performed using age and gender as co-variates (Table 3).

TABLE 1 Population characteristics for each phenotype

Phenotypes	N (%)	Male (%)	Female (%)	*P*-value[a]	Mean of age (SD)	*P*-value[b]
Skeletal class I	77 (53.8)	40 (51.9)	37 (48.1)	.22	15.2 (7.1)	.43
Skeletal class II	47 (32.9)	18 (38.3)	29 (61.7)		13.8 (5.3)	
Skeletal class III	19 (13.3)	11 (57.9)	8 (42.1)		18.1 (11.2)	
Well-positioned maxilla	52 (36.4)	24 (46.1)	28 (53.8)	.80	15.3 (8.3)	.99
Maxillary retrusion	39 (27.2)	18 (46.1)	21 (53.8)		15.4 (7.3)	
Maxillary protrusion	52 (36.4)	27 (51.9)	25 (48.1)		14.7 (6.4)	
Well-positioned mandible	43 (30.0)	17 (39.5)	26 (60.5)	.31	15.0 (7.6)	.91
Mandibular retrognathism	50 (35.0)	27 (54.0)	23 (46.0)		15.4 (7.6)	
Mandibular prognathism	50 (35.0)	21 (42.0)	29 (58.0)		15.0 (7.0)	
Normofacial	72 (50.3)	37 (51.4)	35 (48.6)	.21	15.0 (6.8)	.46
Dolichofacial	55 (38.5)	22 (40.0)	33 (60.0)		15.0 (7.5)	
Brachyfacial	16 (11.2)	10 (62.5)	6 (37.5)		15.8 (9.6)	

[a]Chi-squared test.

[b]Kruskal-Wallis.

TABLE 2 Genotype distribution of each SNP according to each phenotype in sagittal and vertical patterns

Gene	SNP	Phenotypes	Genotypes n (%)			*P*-value
BMP2	rs1005464		**GG**	**GA**	**AA**	
		Skeletal class I	50 (70.4)	20 (28.2)	1 (1.4)	Ref.
		Skeletal class II	26 (63.4)	13 (31.7)	2 (4.9)	.480
		Skeletal class III	10 (58.8)	5 (29.4)	2 (11.8)	.100
		Well-positioned maxilla	33 (73.3)	12 (26.7)	0 (0)	Ref.
		Maxillary retrusion	25 (69.4)	9 (25.0)	2 (5.6)	.277
		Maxillary protrusion	28 (58.3)	17 (35.4)	3 (6.3)	.123
		Well-positioned mandible	20 (54.0)	15 (40.5)	2 (5.5)	Ref.
		Mandibular retrognathism	36 (80.0)	8 (17.8)	1 (2.2)	.042*
		Mandibular prognathism	30 (63.8)	15 (31.9)	2 (4.3)	.663
		Normofacial	40 (62.5)	23 (35.9)	1 (1.6)	Ref.
		Dolichofacial	37 (74.0)	11 (22.0)	2 (4.0)	.221
		Brachyfacial	9 (60.0)	4 (26.7)	2 (13.3)	.092
	rs235768		**TT**	**TA**	**AA**	
		Skeletal class I	37 (51.4)	33 (45.8)	2 (2.8)	Ref.
		Skeletal class II	16 (39.0)	23 (56.1)	2 (4.9)	.420
		Skeletal class III	10 (58.8)	6 (35.3)	1 (5.9)	.642
		Well-positioned maxilla	21 (45.7)	23 (50.0)	2 (4.3)	Ref.
		Maxillary retrusion	15 (41.7)	19 (52.8)	2 (5.5)	.921
		Maxillary protrusion	27 (55.1)	20 (40.8)	2 (4.1)	.648
		Well-positioned mandible	22 (59.5)	12 (32.4)	3 (8.1)	Ref.
		Mandibular retrognathism	15 (32.6)	29 (63.0)	2 (4.4)	.021*
		Mandibular prognathism	26 (54.2)	21 (43.7)	1 (2.1)	.300
		Normofacial	32 (50.0)	27 (42.2)	5 (7.8)	Ref.
		Dolichofacial	20 (38.5)	31 (59.6)	1 (2.0)	.104
		Brachyfacial	11 (73.3)	4 (26.7)	0 (0)	.208
BMP4	rs17563		**AA**	**AG**	**GG**	
		Skeletal class I	28 (40.6)	32 (46.4)	9 (13.0)	Ref.
		Skeletal class II	18 (43.9)	17 (41.5)	6 (14.6)	.880
		Skeletal class III	4 (23.5)	7 (41.2)	6 (35.3)	.081
		Well-positioned maxilla	17 (37.8)	22 (48.9)	6 (13.3)	Ref.
		Maxillary retrusion	10 (29.4)	18 (52.9)	6 (17.7)	.705
		Maxillary protrusion	23 (47.9)	16 (33.3)	9 (18.8)	.308
		Well-positioned mandible	15 (40.5)	16 (43.2)	6 (16.3)	Ref.
		Mandibular retrognathism	14 (32.6)	24 (55.8)	5 (11.6)	.526
		Mandibular prognathism	21 (44.7)	16 (34.0)	10 (21.3)	.663
		Normofacial	24 (38.1)	29 (46.0)	10 (15.9)	Ref.
		Dolichofacial	19 (38.8)	24 (49.0)	6 (12.2)	.857
		Brachyfacial	7 (46.7)	3 (20.0)	5 (33.3)	.125
SMAD6	rs2119261		**CC**	**CT**	**TT**	
		Skeletal class I	23 (32.4)	43 (60.6)	5 (7.0)	Ref.
		Skeletal class II	14 (33.3)	22 (52.4)	6 (14.3)	.419
		Skeletal class III	4 (23.5)	12 (70.6)	1 (5.9)	.741
		Well-positioned maxilla	15 (33.3)	24 (53.3)	6 (13.4)	Ref.
		Maxillary retrusion	7 (18.9)	28 (75.7)	2 (5.4)	.106

(Continues)

TABLE 2 (Continued)

Gene	SNP	Phenotypes	Genotypes n (%)			*P*-value
		Maxillary protrusion	19 (39.6)	25 (52.1)	4 (8.3)	.672
		Well-positioned mandible	9 (24.3)	23 (62.2)	5 (13.5)	Ref.
		Mandibular retrognathism	14 (30.4)	28 (60.9)	4 (8.7)	.697
		Mandibular prognathism	18 (38.3)	26 (55.3)	3 (6.4)	.282
		Normofacial	18 (28.1)	39 (60.9)	7 (11.0)	Ref
		Dolichofacial	19 (37.2)	28 (54.9)	4 (7.9)	.549
		Brachyfacial	4 (26.7)	10 (66.7)	1 (6.6)	.864
	rs3934908		**CC**	**CT**	**TT**	
		Skeletal class I	22 (30.1)	36 (49.3)	15 (20.6)	Ref.
		Skeletal class II	10 (24.4)	21 (51.2)	10 (24.4)	.777
		Skeletal class III	5 (29.4)	9 (52.9)	3 (17.7)	.952
		Well-positioned maxilla	9 (19.6)	26 (56.5)	11 (23.9)	Ref.
		Maxillary retrusion	9 (25.0)	18 (50.0)	9 (25.0)	.801
		Maxillary protrusion	19 (38.8)	22 (44.9)	8 (16.3)	.117
		Well-positioned mandible	11 (29.7)	14 (37.8)	12 (32.5)	Ref.
		Mandibular retrognathism	10 (21.7)	26 (56.6)	10 (21.7)	.236
		Mandibular prognathism	16 (33.3)	26 (54.2)	6 (12.5)	.074
		Normofacial	16 (25.0)	36 (56.2)	12 (18.8)	Ref.
		Dolichofacial	14 (26.9)	26 (50.0)	12 (23.1)	.774
		Brachyfacial	7 (38.9)	7 (38.9)	4 (22.2)	.394
RUNX2	rs59983488		**GG**	**GT**	**TT**	
		Skeletal class I	52 (74.3)	17 (24.3)	1 (1.4)	Ref.
		Skeletal class II	30 (73.2)	9 (21.9)	2 (4.9)	.548
		Skeletal class III	13 (76.5)	4 (23.5)	0 (0)	.880
		Well-positioned maxilla	28 (62.2)	14 (31.1)	3 (6.7)	Ref.
		Maxillary retrusion	28 (77.8)	8 (22.2)	0 (0)	.158
		Maxillary protrusion	39 (83.0)	8 (17.0)	0 (0)	.040*
		Well-positioned mandible	27 (73.0)	9 (24.3)	1 (2.7)	Ref.
		Mandibular retrognathism	33 (73.3)	10 (22.2)	2 (4.5)	.901
		Mandibular prognathism	35 (76.1)	11 (23.9)	0 (0)	.529
		Normofacial	44 (69.8)	18 (28.6)	1 (1.6)	Ref.
		Dolichofacial	39 (78.0)	9 (18.0)	2 (4.0)	.338
		Brachyfacial	12 (80.0)	3 (20.0)	0 (0)	.689
	rs1200425		**GG**	**GA**	**AA**	
		Skeletal class I	29 (40.8)	29 (40.8)	13 (18.4)	Ref.
		Skeletal class II	13 (32.5)	20 (50.0)	7 (17.5)	.966
		Skeletal class III	7 (46.7)	7 (46.7)	1 (6.6)	.540
		Well-positioned maxilla	15 (34.1)	22 (50.0)	7 (15.9)	Ref.
		Maxillary retrusion	17 (48.6)	14 (40.0)	4 (11.4)	.423
		Maxillary protrusion	17 (36.2)	20 (42.5)	10 (21.3)	.722
		Well-positioned mandible	15 (41.7)	16 (44.4)	5 (13.9)	Ref.
		Mandibular retrognathism	17 (38.6)	20 (45.4)	7 (16.0)	.949
		Mandibular prognathism	17 (37.0)	20 (43.5)	9 (19.5)	.778
		Normofacial	25 (40.3)	25 (40.3)	12 (19.4)	Ref.
		Dolichofacial	20 (40.0)	25 (50.0)	5 (10.0)	.336
		Brachyfacial	4 (28.6)	6 (42.8)	4 (28.6)	.640

(Continues)

TABLE 2 (Continued)

Gene	SNP	Phenotypes	Genotypes n (%)			*P*-value
WNT3A	rs708111		**AA**	**AG**	**GG**	
		Skeletal class I	17 (23.6)	34 (47.2)	21 (29.2)	Ref.
		Skeletal class II	9 (22.0)	21 (51.2)	11 (26.8)	.919
		Skeletal class III	9 (50.0)	7 (38.9)	2 (11.1)	.063
		Well-positioned maxilla	10 (20.8)	25 (52.1)	13 (27.1)	Ref.
		Maxillary retrusion	15 (40.6)	16 (42.2)	6 (16.2)	.122
		Maxillary protrusion	10 (21.7)	21 (45.7)	15 (32.6)	.799
		Well-positioned mandible	12 (29.3)	22 (53.6)	7 (17.1)	Ref.
		Mandibular retrognathism	13 (28.9)	20 (44.4)	12 (26.7)	.530
		Mandibular prognathism	10 (22.2)	20 (44.5)	15 (33.3)	.222
		Normofacial	18 (26.5)	28 (41.2)	22 (32.3)	Ref.
		Dolichofacial	12 (25.0)	26 (54.2)	10 (20.8)	.301
		Brachyfacial	5 (33.3)	8 (53.3)	2 (13.4)	.338
WNT11	rs1533767		**GG**	**GA**	**AA**	
		Skeletal class I	28 (51.9)	22 (40.7)	4 (7.4)	Ref.
		Skeletal class II	19 (59.4)	13 (40.6)	0 (0)	.275
		Skeletal class III	4 (36.4)	7 (63.6)	0 (0)	.311
		Well-positioned maxilla	17 (50.0)	15 (44.1)	2 (5.9)	Ref.
		Maxillary retrusion	15 (55.6)	11 (40.7)	1 (3.7)	.871
		Maxillary protrusion	19 (52.8)	16 (44.4)	1 (2.8)	.810
		Well-positioned mandible	12 (46.1)	12 (46.1)	2 (7.8)	Ref.
		Mandibular retrognathism	23 (60.6)	14 (36.8)	1 (2.6)	.415
		Mandibular prognathism	16 (48.5)	16 (48.5)	1 (3.0)	.720
		Normofacial	20 (41.7)	24 (50.0)	4 (8.3)	Ref.
		Dolichofacial	24 (58.5)	17 (41.5)	0 (0)	.080
		Brachyfacial	7 (87.5)	1 (12.5)	0 (0)	.054

Note: Chi-squared test was performed for this analysis.

*Means $P < .05$.

TABLE 3 Multiple logistic regression analysis with SNPs and phenotypes associated in genotype distribution

Phenotype	Genes	SNPs	Reference	Genotype	Odds Ratio (CI[a] 95%)	*P*-value
Mandibular retrognathism	*BMP2*	rs1005464	GG	GA	0.30 (0.10-0.85)	.024*
				AA	0.30 (0.02-3.66)	.346
		rs235768	TT	TA	3.98 (1.47-10.77)	.006*
				AA	1.06 (0.13-8.45)	.949
Mandibular prognathism	*SMAD6*	rs3934908	CC	CT	1.44 (0.50-4.11)	.487
				TT	0.36 (0.10-1.30)	.122
Maxillary protrusion	*RUNX2*	rs59983488	GG	GT	0.38 (0.14-1.07)	.068
Skeletal class III	*WNT3A*	rs708111	AA	AG	0.37 (0.11-1.20)	.100
				GG	0.17 (0.03-0.94)	.042*
Brachyfacial	*WNT11*	rs1533767	GG	GA	0.11 (0.01-1.04)	.055

Note: The analysis was performed with each genotype individually and adjusted by age and gender.

[a]C.I. means confidence interval.

*Means $P < .05$.

TABLE 4 Summary results of the best combination models of MDR analysis

Phenotype	Best Combination model	CVC[a]	TBA[b]	*P*-value[c]
Skeletal class II	rs708111-*WNT3A*, rs1533767-*WNT11*, rs235768-*BMP2*, rs1005464-*BMP2*, rs17563-*BMP4*, rs59983488-*RUNX2*, rs1200425-*RUNX2*, rs3934908-*SMAD6*, rs2119261-*SMAD6*	10/10	0.7091	.003*
Mandibular retrusion	rs235768-*BMP2*, rs1200425-*RUNX2*	9/10	0.7056	.029*
Dolichofacial	rs708111-*WNT3A*, rs1533767-*WNT11*, rs1005464-*BMP2*, rs1200425-*RUNX2*, rs3934908-*SMAD6*	9/10	0.6774	.014*
Brachyfacial	rs708111-*WNT3A*, rs1533767-*WNT11*, rs3934908-*SMAD6*	10/10	0.7718	.007*

[a]Cross-validation consistency.

[b]Testing balanced accuracy.

[c]*P*-values were based on 1000 permutations test. Adjusted by age and gender. Best combinations models were selected based on highest TBA and highest CVC.

*Means statistical significance difference ($P < .05$).

To explore high-order SNP-SNP interactions for each phenotype, we performed MDR analyses (Supplementary Table S2).

Table 4 summarizes the MDR analysis and demonstrates the best MDR-predicted interaction models for the phenotypes that present significant models. Entropy measures among SNPs were calculated to obtain epistatic effects. Figure 1 shows the interactions between SNPs (dendrogram and interaction map) for phenotypes with interaction models.

4 | DISCUSSION

Studies in different models point out that the proper postnatal growth and development of craniofacial structures (including bone, muscles and teeth) requires the coordination of many mechanisms. The growth and development of craniofacial structures involves the precise timing of migration of different cell types, coordinated displays of differentiation development and growth of tissues and also the interaction of different molecules.[21,26-28]

Results provided by twin studies have been increasing our knowledge on hereditability and genetically determined variables of maxilla and mandible position, shape, size and their relationship with the cranial base.[3,4] In the past decades, many genetic studies have been evaluating the association between different genes and maxillary/mandibular discrepancies as well as face morphology[15-22] These molecular genetic studies have mainly focused on skeletal class III and prognathism phenotypes.[17] More recently, some studies expanded the craniofacial phenotypes evaluated, including other sagittal and vertical patterns[16,18-22] Therefore, in the present study, we decided to evaluate sagittal and vertical craniofacial phenotypes and each dental arch separately, in order to evaluate whether any gene/SNP acts in the maxilla, mandible or both jaws discrepancies. Here, we also decided to perform MDR analysis to evaluate SNP-SNP interactions. This approach has proven to be a powerful tool for a variety of medical genetic studies.[29-31] In our study, MDR analysis allowed us to identify some interactions potentially involved in different craniofacial phenotypes.

Although non-syndromic mandibular retrognathism is a relatively common type of malocclusion, which refers to an abnormal posterior position of the mandible as a result of a developmental alteration, few studies explored the genetic aetiology of this condition.[32,33] Muscles are known to have extensive mutual effects on bones, and associated genes are candidate genes for skeletal malocclusions. Arun et al (2016)[32] identified a SNP in the myosin 1H (*MYO1H*) gene associated with retrognathism. More recently, in the same population, Balkhande et al (2018)[33] showed that SNPs in *MATN1*, a gene that encodes the matrilin-1 cartilage extracellular matrix protein, were associated with mandibular retrognathism. Our results suggest that *BMP2* is involved in mandibular retrognathism. Interestingly, both clinical[34] and animal[35] studies have provided evidence that *BMP2* is involved in abnormal mandibular development.

BMP2 haploinsufficiency results in severe craniofacial defects including mandibular retrognathism (micrognathism). In an animal investigation, *Bmp2* mutation caused the Pierre Robin sequence, which is a condition that includes mandibular retrognathism (micrognathism).[35] Thus, it is plausible to assume that both SNPs in *BMP2* studied here, the intronic and the missense (Arginine > Serine) variants, are involved in non-syndromic mandibular retrognathism. The MDR analysis also suggested an interaction between rs235768 (*BMP2*) and rs1200425 (*RUNX2*). Interestingly, Runx2 was identified to be an important mediator of Bmp2 expression during cranial bone development[36.] Furthermore, an association between rs59983488 in *RUNX2* and maxillary protrusion was suggested in the genotypic

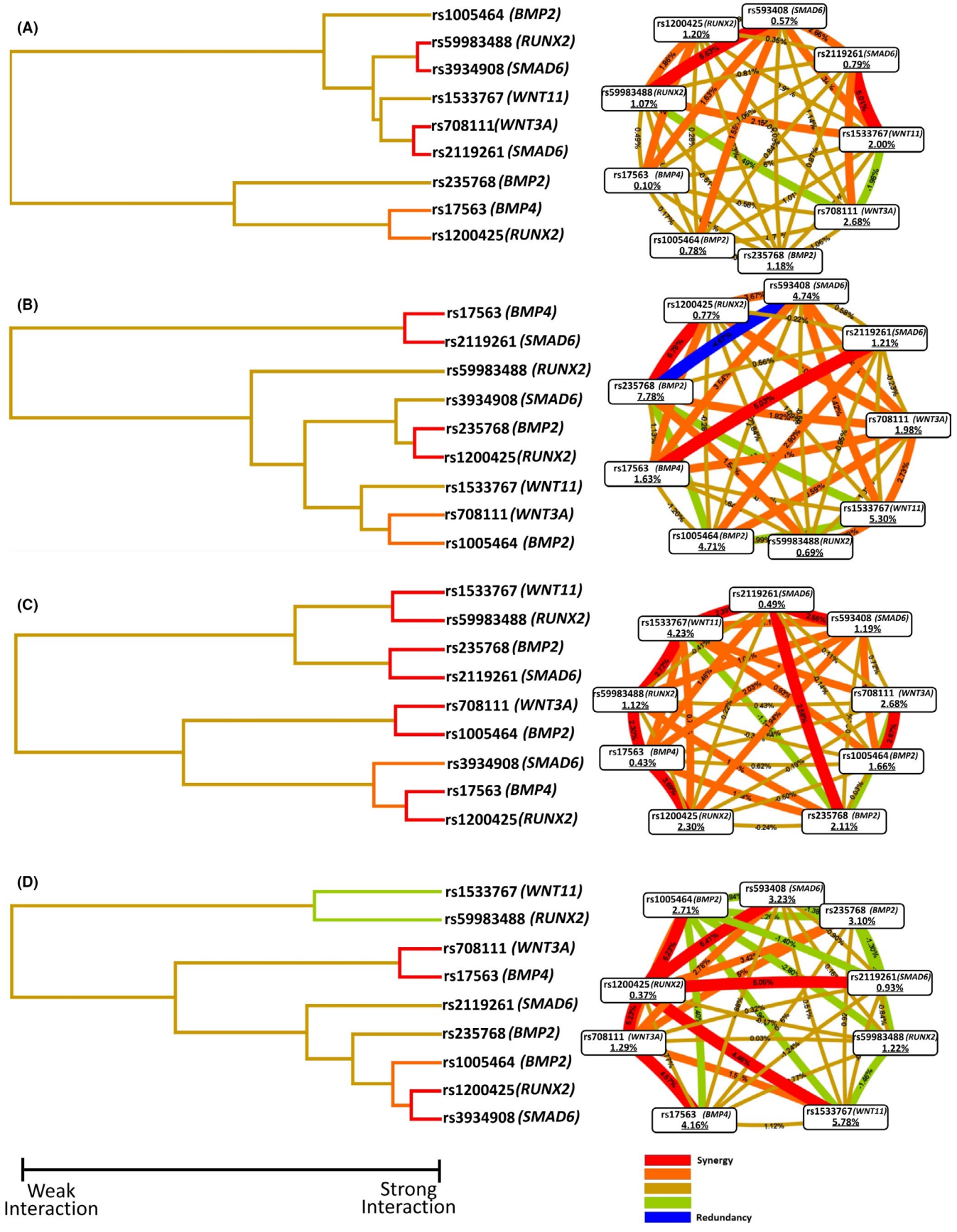

FIGURE 1 Entropy Analysis (EA). Dendrograms and interaction maps of the EA for each outcome statistically significant in MDR analysis. The keys farther to the right in the dendrogram represent a strong interaction between the SNPs, and the keys farther to the left represent a weak interaction. In the interaction map, the values inside the box are the percentage of entropy of each SNP individually, and the values connecting the boxes indicate percentage entropy resulting from the combination. When the SNP-SNP entropy effect is bigger than the sum of values of each SNP, this indicates synergy, represented by the red and orange connections. Green and blue connections on the other hand indicate a redundancy effect, when the SNP-SNP entropy effect is smaller than the sum of values of each SNP. A, EA for skeletal class II. B, EA for mandibular retrusion. C, EA for dolichofacial phenotype. D, EA for brachyfacial phenotype [Colour figure can be viewed at wileyonlinelibrary.com]

distribution. Mandibular retrognathism and maxillary protrusion are traits of the skeletal class II phenotype.

Skeletal class II and skeletal class III are both anteroposterior discrepancies between the maxilla and mandible. In our study, the rs708111 in *WNT3A* was suggested as a protective factor for skeletal class III phenotype, which was previously associated with the palatal rugae pattern.[37] The rs708111 in *WNT3A* was also involved with skeletal class II, when SNP-SNP interaction was analysed via MDR analysis. The Wnt signalling pathways interact in an extensive network during bone formation regulated by a variety of molecules.[38] Our MDR results of skeletal class II phenotype reflect this complex interaction. A recent study identified SNPs associated with skeletal class II and skeletal class III phenotypes, two of these contained binding sites of *RUNX2*; however, the association was observed for skeletal class III.[18] Although we were not able to observe SNP-SNP interactions for skeletal class III, it is important to mention that the sample size of this group could be a limitation to identify such interactions.

In fact, the sample size is an important limitation of our study, which used a convenience sample to explore the genetic background of maxillary and mandibular discrepancies. Although this convenience sample allowed us to perform an exploratory study, the association of some SNPs with uncommon phenotypes may not be observed due to sample size limitations. This is particularly true in low penetrance SNPs. After performing a Bonferroni correction, many SNP associations became statistically insignificant. Although a correction for multiple variables reduces the chance of a type I error, it also increases type II error in a small sample and SNPs with small effects. For these reasons, our results should be interpreted with caution, but warrant and should prompt future investigations. However, the 1.000 permutation test performed in MDR analysis is also an approach to adjust multiple tests, like Bonferroni correction, estimating type error I and power at 0.05 significance level.[39] Another limitation that should be highlighted is the fact that the population stratification correction was not performed to analyse the genetic association of our self-reported Caucasian population. In admixed populations, this could lead to associations with SNPs unlinked to the condition. Therefore, independent replication studies in different populations should be performed.

The twin-method study performed by Šidlauskas et al 2016,[3] suggested that the shape and sagittal position of the dental arches are under stronger genetic control. However, heritability is also involved in vertical morphology of the face3·.[4] In our study, the vertical morphology of the face was also evaluated here and some interesting interactions were suggested for both dolichofacial and brachyfacial phenotypes. MDR analysis is a data approach that aims to identify multi-locus combinations of genotypes that are associated with either high-risk or low-risk combinations. Therefore, it is also possible that the same SNPs/ genes may be involved in both dolichofacial and brachyfacial phenotypes, however, with different risk genotypes. This was observed in the MDR analysis, which elected the same SNPs in *WNT11* and *WNT3A* and also in *SMAD6* for both dolichofacial and brachyfacial phenotypes. *SMAD6* is essential to regulate BMPs during cartilage development.[8] BMP signalling is complex, and there are multiple potential cross-talks, including Smad signalling[8] and Wnt signalling,[38] different BMPs either enhance or antagonize Wnt-induced osteogenic differentiation.[40] The *RUNX2* rs1200425 was also included in the MDR model for the dolichofacial phenotype. Haploinsufficiency of *RUNX2* causes cleidocranial dysplasia in humans, which is characterized by vertical morphology alteration and heterozygous *Runx2*-deficient mice present a similar phenotype, suggesting that the expression level of *Runx2* influences skeletal facial phenotype.[41]

A more detailed understanding of the cross-talk between the signalling important for postnatal craniofacial growth and development will help to elucidate the SNP-SNP interactions involved in the facial phenotypes, and further studies are necessary in order to investigate whether these SNPs are involved in the aetiology of sagittal and vertical skeletal malocclusions in humans.

5 | CONCLUSION

Our results suggest that SNPs in *BMP2*, *BMP4*, *SMAD6*, *RUNX2*, *WNT3A* and *WNT11* genes and their interaction could be involved in the aetiology of both sagittal and vertical skeletal malocclusions.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

ECK, PNF, PP and CK involved in conceptualization and designed the study. ECK, PP, RDC, RS and CK involved in funding support. MANM collected the sample and determined the orthodontic phenotypes.

MANM and PNF performed the cephalometric analysis. ECK, AOP, JC and RDC performed the laboratorial analysis. CLBR and RS performed the statistical analysis. ECK, CLBR and CK wrote the manuscript. All authors corrected and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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