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Association between genetic variants in key vitamin-D-pathway genes and external apical root resorption linked to orthodontic treatment

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Abstract

This study evaluated the association between single-nucleotide polymorphisms (SNPs) in vitamin-D-related genes and the amount of external apical root resorption linked to orthodontic treatment. One hundred and forty-three individuals were assessed. The amount of external apical root resorption of upper central incisors ($EARR_{inc}$) and lower first molars ($EARR_{mol}$) were evaluated in radiographs. Seven SNPs were genotyped across four genes including the vitamin D receptor [*VDR*], group-specific component [*GC*], cytochrome P450 family 27 subfamily B member 1 [*CYP27B1*], and cytochrome P450 family 24 subfamily A member 1 [*CYP24A1*]. Linear regressions were implemented to determine allele-effects on external apical root resorption. Individuals carrying the AA genotype in *VDR* rs2228570 had a 21% higher $EARR_{mol}$ than those having AG and GG genotypes (95% CI: 1.03,1.40). $EARR_{mol}$ in heterozygous rs2228570, was 12% lower than for homozygotes (95%CI: 0.78,0.99). Participants with the CCG haplotype (rs1544410-rs7975232-rs731236) in *VDR* had an $EARR_{mol}$ 16% lower than those who did not carry this haplotype. Regarding *CYP27B1* rs4646536, $EARR_{inc}$ in participants who had at least one G allele was 42% lower than for homozygotes AA (95%CI: 0.37,0.93). Although these results did not remain significant after multiple testing adjustment, potential associations may still be suggested. Further replication studies are needed to confirm or refute these findings.

KEYWORDS

25-hydroxyvitamin D3 1-alpha-hydroxylase, receptors, calcitriol, polymorphism, single nucleotide, vitamin D3 24-hydroxylase, vitamin D-binding protein

INTRODUCTION

External apical root resorption is a common adverse effect of orthodontic tooth movement [1]. External apical root resorption constitutes a destructive pathological process of the tooth apex mediated by inflammatory and resorptive cells [1, 2]. The severity of this condition among patients is variable following orthodontic treatment and difficult to predict before the start of therapy. In extremely severe

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cases, adverse outcomes such as dental hypermobility can be observed in post-treatment long-term evaluations [3].

External apical root resorption is considered a multifactorial condition, influenced by the environment (e.g., orthodontic-treatment-related factors) and variations in individual susceptibility [4, 5]. Evidence has shown a hereditary component to external apical root resorption explaining patients' predisposition to this outcome [6, 7]. Variants in genes that code for different biomarkers such as inflammatory mediators, osteoclastogenesis markers, or molecules that participate in tooth formation have been associated with this trait [8].

A previous study of Brazilian individuals diagnosed with a Class II Division 1 malocclusion suggested an association between rs731236 (*Taq*I) single-nucleotide polymorphism (SNP) in the *vitamin D receptor* (*VDR*) gene and external apical root resorption in orthodontic patients [9]. VDR is a transcription factor belonging to the nuclear receptor superfamily that, in a complex with an active form of vitamin D, regulates the expression of hundreds of target genes involved in various physiological processes [10, 11]. Several SNPs in *VDR* have been reported [12]. Among them, rs731236, rs7975232 (*Apa*I), rs1544410 (*Bsm*I), and rs2228570 (*Fok*I) are the four SNPs most studied for their possible association with a wide range of phenotypes. SNPs located at the 3′ end of *VDR* (rs731236, rs7975232 and rs1544410) could affect translational efficiency and gene expression by regulating mRNA stability [13]. On the other hand, rs2228570, located at the beginning of the *VDR* coding region, can result in an altered protein due to a missense alteration in the start codon [14, 15]. VDR, together with vitamin D, plays a prominent role in calcium homeostasis [16] and in the modulation of immunological activity [17]. It is assumed that SNPs in *VDR* could lead to a dysfunctional receptor, affecting the subsequent effects of vitamin D-VDR signaling.

Vitamin D needs to be metabolized into a biologically active product before binding to VDR [18]. Several molecules participate in the classical metabolic pathway of vitamin D (Figure 1). For example, the vitamin-D-binding protein (encoded by the group-specific component, *GC* gene) stabilizes and transports vitamin D and its metabolites to target tissues [19]. Two other essential molecules are the enzyme 25(OH)D 1α-hydroxylase (encoded by cytochrome P450 family 27 subfamily B member 1, *CYP27B1*), which metabolizes 25(OH)D (calcidiol) to 1α,25$(OH)_2$D (calcitriol, active form of vitamin D); and, the inactivating enzyme 25(OH)D 24-hydroxylase (encoded by cytochrome P450 family 24 subfamily A member 1, *CYP24A1*), which regulates the levels of both calcidiol and calcitriol [20, 21]. Variations in the genes encoding these molecules could also explain adverse outcomes related to vitamin D deficiency. Previous studies have suggested that the missense variant rs4588 in *GC* is associated with variations in vitamin D serum levels and, consequently, adverse health conditions [22–25]. The SNP rs4646536 in *CYP27B1* was indicated as a possible risk predictor of pathological fractures in the elderly [26]. Moreover, a potential interaction between rs927650 in *CYP24A1* and circulating plasma levels of 25(OH)D in women with breast cancer has been reported [27].

It is known that vitamin D plays a crucial role in the formation of teeth and bones and the regulation of the immune system [16, 17]. Having tooth structures (e.g., root cementum) with low-quality mineralized tissue or an exaggerated immune response could predispose to greater external apical root resorption. Assuming that the SNPs mentioned above could alter VDR-mediated vitamin D signaling or related metabolic responses, we hypothesized that these variants could be involved in the variability of the external apical root resorption phenotype. Thus, the objective of the present study was to evaluate the association between SNPs in *VDR*, *GC*, *CYP27B1*, and *CYP24A1* and the amount of external apical root resorption in patients who received orthodontic treatment.

MATERIAL AND METHODS

This genetic association study involved the retrospective evaluation of patient chart data and the collection of biological material (cheek cells). The Ethics Committee from the University Hospital Regensburg, Germany (ID 19-1549-101) approved the research protocol and supervised the proper conduct of this study. Once eligible subjects were identified, they were invited to participate in the research. All participants and/or legal guardians gave their written informed consent to participate in the study. Recommendations of the Strengthening the Reporting of Genetic Association Studies were followed for the research report [28].

The orthodontic records of 224 patients treated at the University Hospital Regensburg and two collaborating private orthodontic practices between 2009 and 2021 were screened to determine their eligibility. German biologically unrelated patients, who had panoramic and/or cephalometric radiographs taken at the beginning and end of orthodontic treatment, were selected by convenience sampling. The exclusion criteria were the following: diagnosed syndromes and metabolic diseases, having received orthognathic surgery, root canal treatment in upper central incisors or lower first molars, incomplete root formation of the mentioned teeth at the beginning of treatment, and low-quality radiographic images.

Upper central incisors (assessed together as a single unit) and lower first molars (each individually) were selected for analysis as these teeth represent the anterior and posterior segments of the dental arches and single- and multi-rooted teeth.

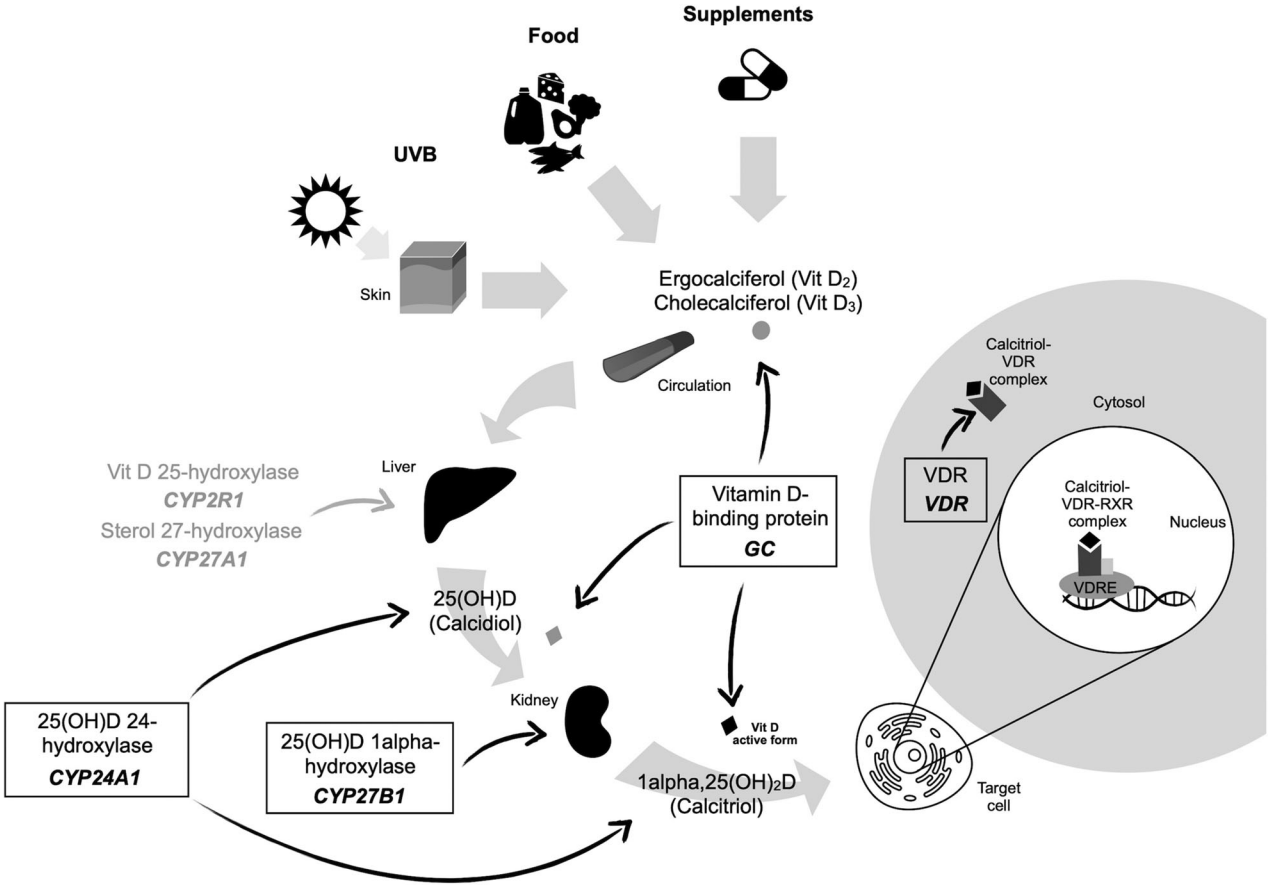

FIGURE 1 Classical metabolic pathway of vitamin D. Both vitamin D isoforms, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), bind to the vitamin-D binding protein (encoded by the *GC* gene) in the circulation that transports them to the organs where they will be metabolized and carry their products to the target cells/tissues. In the liver, vitamin D is metabolized by hydroxylases such as 25-hydroxylase and sterol 27-hydroxylase (encoded by the *CYP2R1* and *CYP27A1* genes, respectively) to 25(OH)D (calcidiol). Subsequently, 25(OH)D is further metabolized by 25(OH)D 1α-hydroxylase (encoded by the *CYP27B1* gene) in the kidney to 1α,25$(OH)_2$D (calcitriol, the active form of vitamin D). Calcidiol and calcitriol levels are regulated by 25(OH)D 24-hydroxylase (encoded by the *CYP24A1* gene). In target tissues, calcitriol binds to the vitamin D receptor (encoded by the *VDR* gene), and both subsequently bind to the retinoid-X receptor (RXR), forming a heterodimer. This new complex binds to the vitamin D response element (VDRE) in the promoter region of its target genes to regulate the mRNA expression.

In addition, the teeth had to be visualized with little distortion on radiographs. Upper central incisors are visualized in full extension on cephalometric radiographs without the limitations observed on panoramic radiographs (i.e., overlapping of the pharyngeal airspace over the roots and shortening due to variations in tooth inclination). On the other hand, the lower first molars are adequately visualized on panoramic radiographs without overlapping the contralateral teeth seen on cephalometric radiographs. Since radiographic images from secondary sources, acquired with different x-ray equipment and with the absence of a ruler in the images to calibrate the tooth size were used, it was not possible to estimate the amount of external apical root resorption in millimeters. Alternatively, distances of interest were measured in pixels and external apical root resorption proportions were used for phenotype evaluation.

The post-treatment external apical root resorption in upper central incisors ($EARR_{inc}$) and lower first molars ($EARR_{mol}$) were evaluated on digitized radiographs mentioned above, following previously described approaches [6, 29, 30]. All measurements were performed in Image J software (https://imagej.nih.gov/ij/). The root length (linear measurement in pixels) was obtained by subtracting the coronal height from the total length of the tooth, both in the initial treatment radiograph (T1) and the final one (T2). In the case of lower first molars, the lengths of the mesial and distal roots were obtained for both the right and left sides, and subsequently, a single average molar root measurement was obtained per individual. Coronal height was used to correct enlargement differences between T1 and T2 radiographic images, assuming this distance remained constant during treatment. Thus, the amount of external apical root resorption (EARR) was

calculated using the following formula:

$$EARR = Root\ length_{T1} - Root\ length_{T2} \times \left(Coronal\ height_{T1} \div Coronal\ height_{T2}\right)$$

Finally, the obtained value was then transformed into an external apical root resorption ratio (proportion of external apical root resorption or percentage of loss of the T1 root length) using the following calculation:

$$EARR\ ratio = EARR \times 100 \div Root\ length_{T1}$$

All evaluations were carried out by a single investigator (GAMV). Twenty cephalometric and twenty panoramic radiographs were randomly selected and evaluated twice within at least one-month interval. The reliability and agreement of the raw measures of root length were assessed using the intraclass correlation coefficient and the Bland-Altman limits of agreement (BA-LoA), respectively.

A sample of cheek cells (buccal cells) was collected for DNA analysis using sterile disposable cytobrushes Plus GT (Medscand; CooperSurgical). The samples were collected by swiping and rolling a cytobrush by the side (right and left), 2–3 times on the inner mucosa of the cheek and against the base of the tongue in each participant. Then, the samples were stored at −20˚C until processing. Genomic DNA was then extracted from the cells contained in the samples, following a previously published protocol [31]. The concentration and purity of the obtained DNA were evaluated using a spectrophotometer (NanoDrop 1000; Thermo Scientific). Seven SNPs across four vitamin-D-related genes (*VDR*, *GC*, *CYP27B1*, and *CYP24A1*) were selected for the present study based on previously published evidence suggesting the association of these SNPs with bone or dental phenotypes or biological activity during the formation of these tissues (Table 1). Genotyping was blindly performed by polymerase chain reactions (PCR) using Endpoint analysis [32] and TaqMan technology in a real-time PCR system (Mastercycler ep realplex-S thermocycler; Eppendorf). Primers, probes, and universal master mix were provided by Applied Biosystems.

Ten percent of the sample was randomly selected for duplicate genotyping. None of the SNPs examined exhibited a genotyping failure rate greater than 10%; hence, all SNPs were included in subsequent analyses.

Statistical analysis

Descriptive statistics were used to report the characteristics of the participants. The alleles and genotypes frequencies and the Hardy-Weinberg Equilibrium for the SNPs studied were calculated. External apical root resorption was analyzed as a quantitative trait (EARR ratio). The distribution and normality of the data were evaluated by employing histograms, the Shapiro-Wilk test, and skewness values.

Linear regression models for each SNP were fitted in PLINK (https://zzz.bwh.harvard.edu/plink/download.shtml) to determine the additive allele effects, deviation from dominance effects, and the combined effect of both on the post-treatment $EARR_{inc}$ and $EARR_{mol}$. In addition, regressions were also fitted in the dominant and recessive models for the lower-frequency alleles. The duration of orthodontic treatment was considered a covariate for the regression models. The implemented regression-based method works well with samples from approximately Gaussian distributions. Therefore, since the present outcome data were positively skewed, parametric transformations (log or square-root transformations) were performed on the EARR ratio data to approximate normality prior to running the analyses. Procedures for previous evaluation and processing of data were conducted in JAMOVI (Computed Software, https://www.jamovi.org/).

For the SNPs that showed a significant effect in the regression analyses, subsequent ANCOVA and Tukey's post hoc tests were applied in JAMOVI (Computed Software, https://www.jamovi.org/) to compare the EARR ratios between genotypes. The assumptions of normality of residuals and homogeneity of residual variances for these analyses were verified by the Shapiro-Wilk test and the Levene's test, respectively. The duration of orthodontic treatment was also considered a covariate for comparison between groups.

Moreover, pair-wise linkage disequilibrium measures (D' and r^2) were estimated in PLINK for the SNPs in *VDR*. Haplotypes formed by the markers showing some degree of linkage disequilibrium (D' > 0.5) were tested for their possible association with external apical root resorption. Haplotype analyses were performed using 2- and 3- SNP sliding windows.

Since the regressions were implemented with transformed variables, the effects are reported as exp(β). A p-value less than or equal to 0.05 was set as significant at the nominal level. p-values were also adjusted using the Benjamini-Hochberg procedure to decrease the false discovery rate due to multiple testing.

RESULTS

Method error

The reliability of the raw measures of root length was high. The intraclass correlation coefficient was 0.94 (95% CI: 0.87, 0.98) and 0.98 (95% CI: 0.94, 0.99) for measurements of

TABLE 1 Single-nucleotide polymorphisms studied

Gene	Band	Position (GRCh37)	Reference sequence	Functional consequence	Base Change (Context sequence)	MAF (ALFA)
VDR	12q13.11	48,238,757	rs731236	Synonymous variant	CTC[A/G]ATC	G = 0.398
VDR	12q13.11	48,238,837	rs7975232	Intron variant	GGC[A/C]CCT	C = 0.463
VDR	12q13.11	48,239,835	rs1544410	Intron variant	ATG[T/C]GCA	T = 0.399
VDR	12q13.11	48,272,895	rs2228570	Missense variant[a]	TCC[A/G]TCC	A = 0.387
GC	4q13.3	72,618,323	rs4588	Missense variant[b]	TCC[G/T]TGG	T = 0.281
CYP27B1	12q14.1	58,157,988	rs4646536	Intron variant	ACA[A/G]CAA	G = 0.317
CYP24A1	20q13.2	52,772,741	rs927650	Intron variant	AGA[C/T]CTC	T = 0.471

The reported MAF are specific to the European population and represent the aggregate allele frequency from dbGaP.

Sources of information: dbSNP from: https://www.ncbi.nlh.nih.gov/snp/; https://genome.uscs.edu/; and, https://www.thermofisher.com.

Abbreviations: ALFA, Allele Frequency Aggregator (NCBI database of Genotypes and Phenotypes [dbGaP]); MAF, minor allele frequency.

[a]Methionine → Threonine.

[b]Threonine → Lysine.

TABLE 2 Alleles and genotypes frequencies in the current sample

Gene SNP (1/2)[a]	Genotyping rate	MAF	Genotypes *n* (%)			H-W *p*-value[b]
			Homozygous 1	Heterozygous	Homozygous 2	
VDR rs731236 – *Taq*I (A/G)	99%	0.465	30 (21.1)	72 (50.7)	40 (28.2)	0.867
VDR rs7975232 – *Apa*I (C/A)	99%	0.404	24 (17.0)	66 (46.8)	51 (36.2)	0.729
VDR rs1544410 – *Bsm*I (T/C)	97%	0.417	22 (15.8)	72 (51.8)	45 (32.4)	0.490
VDR rs2228570 – *Fok*I (A/G)	97%	0.457	23 (16.5)	81 (58.3)	35 (25.2)	0.059
GC rs4588 (T/G)	99%	0.319	13 (9.2)	64 (45.4)	64 (45.4)	0.700
CYP27B1 rs4646536 (G/A)	98%	0.286	13 (9.3)	54 (38.6)	73 (52.1)	0.535
CYP24A1 rs927650 (T/C)	93%	0.436	24 (18.0)	68 (51.1)	41 (30.8)	0.726

Abbreviations: H-W, Hardy-Weinberg; MAF, minor allele frequency; SNP, single-nucleotide polymorphism.

[a](1 = minor allele/2 = major allele).

[b]Critical H-W *p*-value ($p \leq .001$).

the root length of upper central incisors and lower first molars, respectively. The BA-LoA did not show evidence of a difference between the measurements made with at least one-month interval for either upper central incisors (Bias estimate = 1.49; 95% CI: −0.48, 3.43; BA-LoA: −6.66, 9.64) or lower first molars (Bias estimate = −0.92; 95% CI: −3.91, 2.07; BA-LoA: −13.45, 11.61).

Quality control

One hundred percent agreement for duplicate genotyping of 10% of the sample with the original calls was observed. The total genotyping rate was 97% (Table 2). The frequencies of the minor alleles for the present sample were greater than 25% in all cases. None of the SNPs studied were excluded based on the Hardy-Weinberg Equilibrium test (Table 2).

Main findings

The characteristics of the 143 individuals, who were finally included in the study, are presented in Table 3. No differences were observed in the EARR ratio according to sex, skeletal malocclusion, and treatment type ($p > 0.05$; Table 3). Among the continuous variables, only treatment duration influenced the EARR ratio ($p < 0.05$), and therefore, the subsequent analyses were adjusted for this variable.

Genetic analyses only detected significant associations at the nominal level ($p < 0.05$). Linear regressions showed an effect of rs2228570 and rs4646536 on post-treatment

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TABLE 3 Characteristics of the individuals

Variable	(*n* = 143)	*p*-value
Sex—*n* (%)		
Male	71 (49.7)	—
Female	72 (50.3)	
Age—years (start of treatment)—mean ± SD	13.5 ± 4.5	—
Skeletal malocclusion[a]—*n* (%)		
Class I	60 (42.0)	—
Class II	76 (53.1)	
Class III	7 (4.9)	
Treatment type—*n* (%)		
1st phase treatment only	18 (12.6)	—
2nd phase treatment only	27 (18.9)	
1st plus 2nd phase treatment	98 (68.5)	
Treatment duration—months—mean ± SD	46.6 ± 13.6	—
$EARR_{inc}$ ratio (%)—min—max	0.0 – 33.5	—
$EARR_{inc}$ ratio (%) according to sex—mean ± SD		
Male	8.3 ± 7.4	0.584
Female	8.6 ± 6.7	
$EARR_{inc}$ ratio (%) according to skeletal malocclusion—mean ± SD		
Class I	10.1 ± 7.5	0.179
Class II	7.3 ± 6.3	
Class III	6.7 ± 8.0	
$EARR_{inc}$ ratio (%) according to treatment type—mean ± SD		
1st phase treatment only	5.0 ± 6.3	0.218
2nd phase treatment only	9.1 ± 6.4	
1st plus 2nd phase treatment	7.6 ± 6.6	
$EARR_{mol}$ ratio (%)—min—max	0.0 – 31.2	—
$EARR_{mol}$ ratio (%) according to sex—mean ± SD		
Male	5.3 ± 4.0	0.726
Female	5.9 ± 6.3	
$EARR_{mol}$ ratio (%) according to skeletal malocclusion—mean ± SD		
Class I	5.3 ± 6.1	0.123
Class II	5.9 ± 4.8	
Class III	4.9 ± 3.2	
$EARR_{mol}$ ratio (%) according to treatment type—mean ± SD		
1st phase treatment only	4.9 ± 4.4	0.810
2nd phase treatment only	4.0 ± 4.4	
1st plus 2nd phase treatment	4.6 ± 3.7	

Abbreviations: $EARR_{inc}$, amount of external apical root resorption in upper central incisors; $EARR_{mol}$, amount of external apical root resorption in lower first molars; SD, standard deviation.

[a]Skeletal malocclusion was determined based on ANB angle (individualized for Caucasians) [33], obtained from digital cephalometric tracings using Dolphin Imaging software (version 8.0; Dolphin Imaging and Management Solutions, Chatsworth, Calif).

EARR ratios. Regarding rs2228570, the recessive model analysis showed that individuals carrying two minor A alleles had a 1.21-fold increase in the $EARR_{mol}$ ratio compared to heterozygotes and common homozygotes ($\exp(\beta) = 1.21$; 95% CI: 1.03, 1.40; $p = 0.016$; allele + deviation from dominance effects test, $p = 0.038$; Table 4). On the other hand, deviation from the dominance effects test showed that $EARR_{mol}$ in rs2228570 heterozygotes was 12% lower than for homozygotes ($\exp(\beta) = 0.88$; 95% CI: 0.78, 0.99; $p = 0.032$; Table 4). Genotypes comparisons demonstrated a statistically significant difference between AG and AA genotypes ($p = 0.031$; Figure 2A) but not between the AG and GG genotypes. Regarding the rs4646536, the dominant model analysis showed that the $EARR_{inc}$ ratio in subjects carrying at least one G allele was 42% lower than for common homozygotes ($\exp(\beta) = 0.58$; 95% CI: 0.37, 0.93; $p = 0.025$; Table 4). Comparisons between the GG, GA, and AA genotypes for this SNP showed no significant differences in the EARR ratio (Figure 2B).

Evidence of linkage disequilibrium was identified between rs1544410 and rs7975232 (D' = 0.800), rs1544410 and rs731236 (D' = 0.725) and to a lesser degree between rs7975232 and rs731236 (D' = 0.548). Based on the low r^2 values, the correlation between these SNPs was only considered weak (Figure 3). There was no evidence of linkage disequilibrium between rs2228570 and the other SNPs in *VDR* (Figure 3). Haplotype-phenotype association analysis showed that the $EARR_{mol}$ ratio in subjects carrying the CCG haplotype (rs1544410-rs7975232-rs731236) was 16% lower than for those who did not carry it ($\exp(\beta) = 0.84$; $p = 0.030$; Table 5). None of the results remained significant after adjustment for multiple testing.

DISCUSSION

External apical root resorption associated with orthodontic treatment is a multifactorial trait. Its severity could be influenced by epistatic interactions of several genes and environmental factors [4, 5]. A recent systematic review showed that different SNPs in some genes had been associated with this condition in many populations [8]. A previous study evaluated the SNP rs731236 in *VDR* [9], suggesting a possible

TABLE 4 Linear regression analyses to determine the effect of the studied SNPs on the EARR ratio

		$EARR_{inc}$ ratio				$EARR_{mol}$ ratio			
***Gene* SNP (1/2)**[a]	**Test**	**exp(β)**	**95% CI**	***p*-value**	**B-H**	**exp(β)**	**95% CI**	***p*-value**	**B-H**
VDR rs731236 - *Taq*I	Allele effects	0.90	0.62, 1.28	0.541	0.789	1.02	0.94, 1.11	0.690	0.779
(A/G)	Deviation from dominance effects	0.93	0.58, 1.52	0.789	0.889	1.05	0.94, 1.19	0.372	0.779
	Allele + deviation from dominance	—	—	0.781	0.889	—	—	0.593	0.779
	Dominant	0.83	0.48, 1.42	0.492	0.779	1.06	0.93, 1.20	0.366	0.779
	Recessive	0.90	0.49, 1.67	0.739	0.889	0.99	0.86, 1.14	0.882	0.908
VDR rs7975232 - *Apa*I	Allele effects	0.96	0.68, 1.35	0.813	0.889	0.97	0.89, 1.05	0.463	0.779
(C/A)	Deviation from dominance effects	1.45	0.90, 2.34	0.131	0.732	0.98	0.87, 1.09	0.688	0.779
	Allele + deviation from dominance	—	—	0.312	0.751	—	—	0.628	0.779
	Dominant	1.25	0.76, 2.03	0.386	0.751	0.94	0.84, 1.06	0.337	0.779
	Recessive	0.76	0.41, 1.40	0.383	0.751	0.97	0.83, 1.13	0.686	0.779
VDR rs1544410 - *Bsm*I	Allele effects	0.79	0.54, 1.15	0.227	0.732	1.04	0.95, 1.14	0.348	0.779
(T/C)	Deviation from dominance effects	0.97	0.59, 1.60	0.897	0.923	0.96	0.85, 1.08	0.503	0.779
	Allele + deviation from dominance	—	—	0.429	0.751	—	—	0.575	0.779
	Dominant	0.73	0.44, 1.23	0.242	0.732	1.02	0.90, 1.16	0.735	0.804
	Recessive	0.74	0.38, 1.45	0.388	0.751	1.08	0.93, 1.27	0.293	0.779
VDR rs2228570 - *Fok*I	Allele effects	0.78	0.54, 1.11	0.165	0.732	1.08	0.99, 1.19	0.092	0.537
(A/G)	Deviation from dominance effects	0.85	0.53, 1.38	0.512	0.779	0.88	0.78, 0.99	0.032[b]	0.443
	Allele + deviation from dominance	—	—	0.251	0.732	—	—	0.038[b]	0.443
	Dominant	0.64	0.38, 1.08	0.103	0.732	1.00	0.87, 1.14	0.944	0.944
	Recessive	0.79	0.43, 1.46	0.457	0.762	1.21	1.03, 1.40	0.016[b]	0.443

(Continues)

TABLE 4 (Continued)

		$EARR_{inc}$ ratio				$EARR_{mol}$ ratio			
***Gene* SNP (1/2)**[a]	**Test**	**exp(*β*)**	**95% CI**	***p*-value**	**B-H**	**exp(*β*)**	**95% CI**	***p*-value**	**B-H**
GC rs4588	Allele effects	1.04	0.69, 1.58	0.842	0.893	1.04	0.94, 1.15	0.476	0.779
(T/G)	Deviation from dominance effects	0.80	0.46, 1.38	0.424	0.751	0.92	0.80, 1.05	0.216	0.779
	Allele + deviation from dominance	—	—	0.798	0.889	—	—	0.458	0.779
	Dominant	0.88	0.54, 1.42	0.589	0.825	0.97	0.87, 1.09	0.662	0.779
	Recessive	1.20	0.54, 2.64	0.658	0.870	1.11	0.90, 1.34	0.326	0.779
CYP27B1 rs4646536	Allele effects	0.81	0.53, 1.23	0.327	0.751	1.11	1.00, 1.23	0.057	0.499
(G/A)	Deviation from dominance effects	0.70	0.40, 1.23	0.219	0.732	0.94	0.82, 1.08	0.404	0.779
	Allele + deviation from dominance	—	—	0.074	0.732	—	—	0.152	0.760
	Dominant	0.58	0.37, 0.93	0.025[b]	0.732	1.07	0.96, 1.21	0.213	0.779
	Recessive	0.84	0.37, 1.90	0.671	0.870	1.21	0.98, 1.49	0.074	0.518
CYP24A1 rs927650	Allele effects	1.01	0.71, 1.43	0.958	0.958	1.04	0.95, 1.14	0.361	0.779
(T/C)	Deviation from dominance effects	0.62	0.38, 1.01	0.055	0.732	0.98	0.87, 1.11	0.768	0.815
	Allele + deviation from dominance	—	—	0.141	0.732	—	—	0.650	0.779
	Dominant	0.70	0.43, 1.17	0.183	0.732	1.04	0.91, 1.19	0.562	0.779
	Recessive	1.35	0.72, 2.56	0.351	0.751	1.07	0.92, 1.25	0.386	0.779

Abbreviations: B-H, *p*-value adjusted by the Benjamini-Hochberg procedure; CI, confidence interval; $EARR_{inc}$, amount of external apical root resorption in upper central incisors; $EARR_{mol}$, amount of external apical root resorption in lower first molars; SNP, single-nucleotide polymorphism.

[a](1 = minor allele/2 = major allele).

[b]Indicates statistical significance at the nominal level.

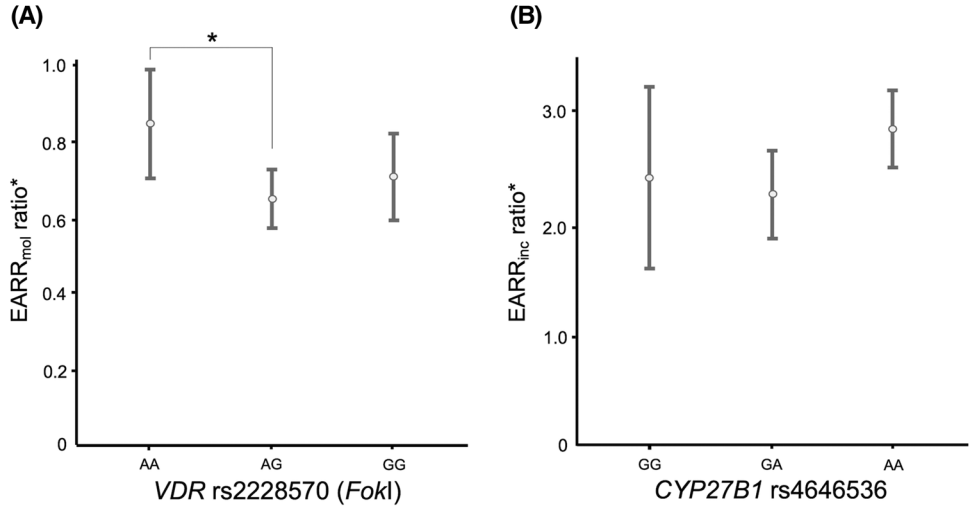

FIGURE 2 Plots showing marginal means of EARR ratios and their corresponding 95% confidence intervals for the genotypes in *VDR* rs2228570 (A) and *CYP27B1* rs4646536 (B). Tukey's post hoc test evidenced a significant difference between AA and AG genotypes for rs2228570 ($p = 0.031$). *EARR ratios represent transformed data.

TABLE 5 Haplotype-phenotype association analyses for SNPs in VDR

			$EARR_{inc}$ ratio			$EARR_{mol}$ ratio		
SNPs	**Haplotype**	**Freq**	**exp(*β*)**	***p*-value**	**B-H**	**exp(*β*)**	***p*-value**	**B-H**
rs7975232-rs731236	CA	0.304	0.90	0.606	0.808	1.01	0.883	0.893
*Apa*I-*Taq*I	CG	0.096	1.40	0.248	0.794	0.87	0.079	0.632
	AA	0.167	0.93	0.768	0.936	1.02	0.793	0.893
	AG	0.433	1.00	0.984	0.984	1.03	0.521	0.893
rs1544410-rs7975232	TC	0.033	0.65	0.443	0.808	0.96	0.802	0.893
*Bsm*I-*Apa*I	TA	0.379	0.79	0.212	0.794	1.04	0.328	0.893
	CC	0.368	1.15	0.459	0.808	0.96	0.408	0.893
	CA	0.219	1.21	0.374	0.808	0.99	0.893	0.893
rs1544410-rs7975232-rs731236	TCA	0.017	1.15	0.883	0.942	0.81	0.305	0.893
*Bsm*I-*Apa*I-*Taq*I	TCG	0.015	0.29	0.155	0.794	1.27	0.343	0.893
	TAA	0.038	0.77	0.514	0.808	1.15	0.150	0.800
	TAG	0.348	0.90	0.590	0.808	1.01	0.752	0.893
	CCA	0.290	0.87	0.497	0.808	1.02	0.672	0.893
	CCG	0.079	1.70	0.095	0.794	0.84	0.030[a]	0.480
	CAA	0.123	1.06	0.819	0.936	0.96	0.618	0.893
	CAG	0.091	1.57	0.231	0.794	1.02	0.750	0.893

Abbreviations: B-H, *p*-value adjusted by the Benjamini-Hochberg procedure; $EARR_{inc}$, amount of external apical root resorption in upper central incisors; $EARR_{mol}$, amount of external apical root resorption in lower first molars; Freq, frequency; SNP, single nucleotide polymorphism.
[a]Indicates statistical significance at the nominal level.

effect of this gene on the severity of external apical root resorption. The present study aimed to expand this evaluation to other SNPs in crucial genes related to vitamin D metabolism. Although our findings should be interpreted with caution because results did not remain significant after adjustment for multiple testing, a potential association between the assessed SNPs and external apical root resorption should be considered and discussed. Our suggestion, on the one hand, is that the AA genotype for rs2228570 might be a risk factor; and that the AG genotype for rs2228570, the G allele for rs4646536 and the CCG haplotype for rs1544410-rs7975232-rs731236 would be protective factors for a more significant

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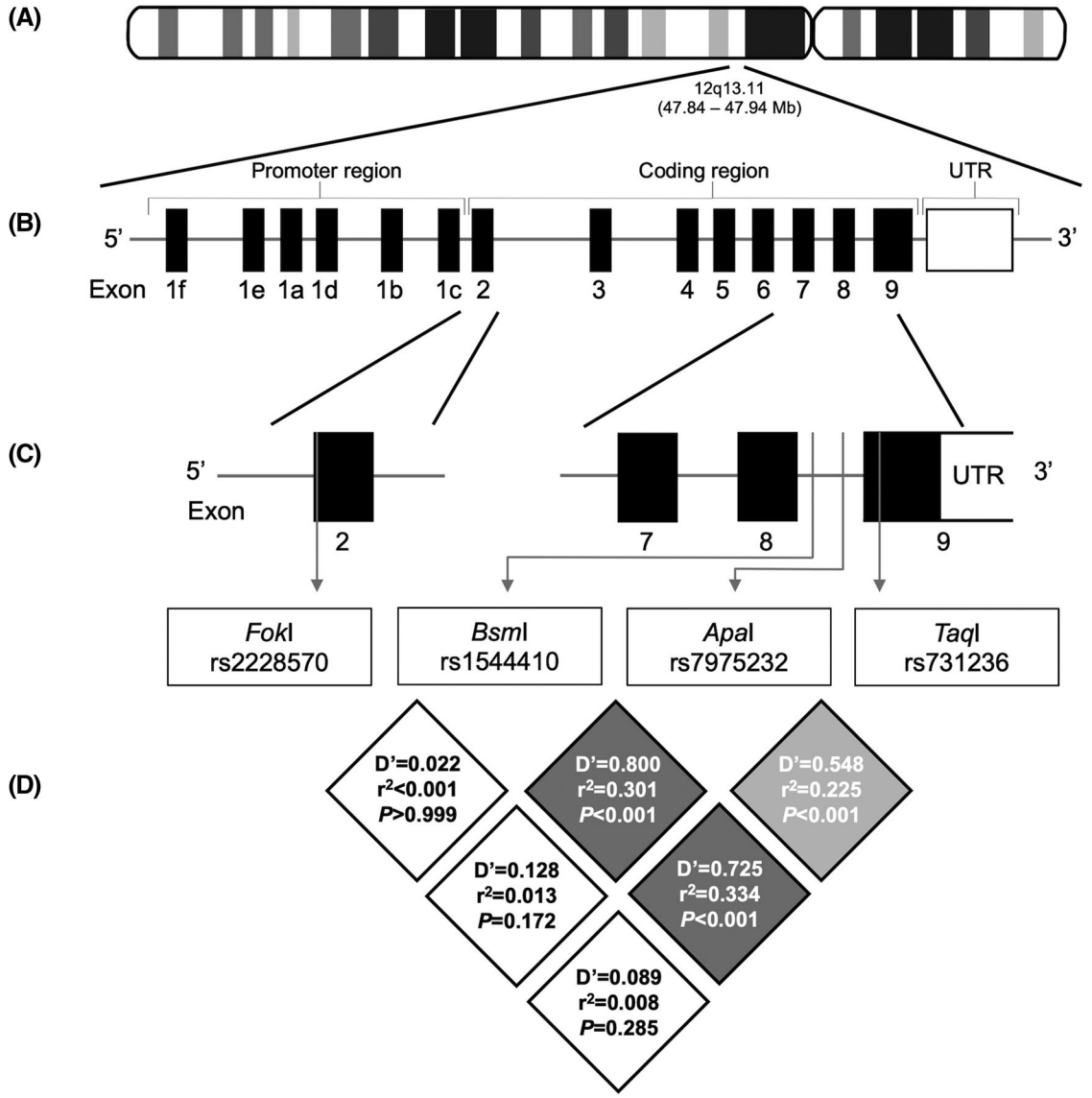

FIGURE 3 Schematic representation of SNPs evaluated in *VDR* and linkage disequilibrium patterns. (A) Chromosome 12 and cytogenetic location of range in Mb for *VDR*. (B) *VDR* genomic structure. (C) Location of the assessed SNPs in *VDR*. (D) Pair-wise linkage disequilibrium pattern. Darker boxes indicate higher D' values. The lighter the box, the lower the D' values. The reported D' and r^2 values are specific to the German sample examined.

amount of external apical root resorption in orthodontically treated German subjects.

rs2228570 is a missense SNP located in exon 2 of the *VDR* coding region that depending on the thymine to cytosine substitution at the first translation start codon (ATG → ACG; Met → Thr), can originate two structurally different isoforms: a longer f-VDR (also called ff genotype; 427 amino acids) and a shorter truncated F-VDR (also called FF genotype; 424 amino acids) [12, 14]. Heterozygous rs2228570 genotype (Ff) could produce both types of the VDR protein [34, 35]. Our results suggest that being homozygote AA (ff) or heterozygous AG (Ff) could influence having a greater or a lesser amount of post-treatment $EARR_{mol}$, respectively. Although controversial, some research has shown that ff individuals for rs2228570, those with low vitamin-D-related diseases and healthy ones, even children, have lower bone mineral density than subjects with the FF genotype [36–41]. Considering that VDR is strongly expressed in the development of dentoalveolar tissues [42, 43], and that it plays a pivotal role in the vitamin-D-dependent signaling pathways during the formation of these structures [44, 45], we hypothesized that a different activity of f-VDR could cause alterations during odontogenesis, generating a low-quality mineralized tissue more prone to be reabsorbed in the face of an environmental stimulus, in this specific case orthodontic tooth movement.

The immunomodulatory function of vitamin D and VDR has already been well documented [17]. Low vitamin D levels would result in the persistence and hyperactivation of

B and T cells and a failure to maintain immunoregulatory networks, leading to diseases in which the immunity has an exaggerated response [46]. The evidence is somewhat consistent in showing that the FF rs2228570 genotype, not the ff, is commonly associated with lower vitamin D levels [47–50]. Moreover, it has been shown that, regardless of its binding status with vitamin D, the shorter F-VDR is more active in transactivation capacity as a transcription factor than its longer version [14, 15, 34]. F-VDR results in higher NFkB-, NFAT-, and IL-12p40-promoter-driven transcription, as well as higher expression of IL-12 in monocytes and dendritic cells [15]. This information explains why FF is a risk genotype for conditions mediated by the immune system.

Furthermore, a more significant induction in the expression of vitamin-D-responsive genes (e.g., *ALP*, *OCN*, *RANKL*) has been reported in human gingival fibroblasts and human periodontal ligament cells carrying the FF genotype compared to the ff and Ff ones [51, 52]. Although both immunological processes and vitamin-D-responsive genes participate in the pathophysiology of external apical root resorption, the mechanisms mentioned above would not explain our findings since it was the AA (ff) genotype associated with higher amounts of root resorption. When interpreting the results, it is essential to consider that there could be an interaction between genotype and orthodontic tooth movement [53, 54], resulting in a different effect from that observed in other contexts. It is known that orthodontic tooth movement alters gene expression in periodontal ligament cells, even time-dependent during treatment [55, 56]. In addition, a recent study in cell culture showed that the higher the supply of $25(OH)D_3$ in periodontal ligament fibroblasts submitted to simulated orthodontic compressive strain, the higher the RANKL:OPG ratio expression levels [54]. Even though this situation would not necessarily occur in vivo, this could explain why ff subjects for rs2228570, associated with higher vitamin D levels, would show more external apical root resorption during orthodontic treatment. Besides, it was shown that other SNPs in *VDR* influence *VDR* mRNA expression under conditions of simulated pressure and $25(OH)D_3$ supply [54]. These findings would provide new insights into the molecular mechanisms of how SNPs in *VDR* would influence the amount of external apical root resorption related to orthodontic treatment.

The other SNPs evaluated in *VDR* that did not show an independent relationship with the EARR ratio evidenced a certain degree of linkage disequilibrium. Linkage disequilibrium is the non-random association of neighboring alleles with a higher probability of being inherited together. In a dependent manner, alleles in linkage disequilibrium (i.e., as haplotypes) would contribute to the phenotypic variation instead of doing it individually. Haplotypes formed by rs1544410 (T/C or also designated as B/b), rs7975232 (A/C, also known as A/a), and rs731236 (A/G or T/t) have been associated with vitamin-D-level-related health outcomes [57–60]. The most frequent haplotypes in our sample were TAG (BAt), CCA (baT), and CAA (bAT), which is in line with previous evidence in Caucasian individuals [57, 58]. These haplotypes did not show an association with external apical root resorption. Our results revealed a protective effect of the CCG (bat) haplotype on the amount of root resorption in lower first molars after orthodontic treatment. Although the evidence is still elusive on the molecular mechanisms that would explain this finding, it has been pointed out that different haplotypes in these SNPs would have functional effects related to vitamin D signaling with the consequent adverse outcomes on bone metabolism and quality [57, 59, 60], as well as on the immune response [58]. On the other hand, we also detected a protective effect of *CYP27B1* rs4646536 on the amount of $EARR_{inc}$. *CYP27B1* encodes 1-α-hydroxylase, which converts 25(OH)D into $1,25(OH)_2D_3$. Mutations in the coding region of this gene cause type I vitamin-D-dependent Rickets disease [61], and its consequent effects on teeth. Genotypic variation in the *CYP27B1* promoter is associated with different immunity-mediated disorders [62, 63]. Mechanisms similar to those explained for rs2228570 are likely to explain this result.

Our findings revealed different effects depending on the tooth type analyzed. Although we did not find a molecular explanation directly linked to the SNPs and genes studied, it is important to consider that differences in the mechanisms that regulate the formation of molars and incisors have been reported. It has been shown in animals that several regulatory networks are possibly responsible for shape and tissue organization differences between these tooth types [64]. On the other hand, there is likely a biomechanical explanation related to orthodontic treatment for different outcomes in these teeth. Although the evidence is inconclusive, it has been suggested that heavy forces and a greater amount of apical displacement over a long treatment time could favor a higher amount of external apical root resorption [65]. Multirooted teeth like molars may better withstand orthodontic forces than single-rooted teeth. In this sample, different treatment strategies would have generated different types and amounts of dental movement and mechanical stresses for each tooth type. Unfortunately, in the present research, it was impossible to obtain all the information related to orthodontic treatment for inclusion in the analyses. Furthermore, we considered not including the amount of sagittal apical displacement obtained from the pre-and post-treatment radiographs since this measure would be partially biased by the external apical root resorption existing in these teeth plus the fact that bi-dimensional radiographs do not reliably depict actual apical displacements.

We consider the information provided by this study may be relevant for orthodontic clinical practice in the near future

since these SNPs could be reasonable prognostic markers of variability in the amount of external apical root resorption. However, it should also be stated that external apical root resorption is probably polygenic. At this time, there is not an easily accessible, widely available genetic test to analyze any individual's predisposition for external apical root resorption before orthodontic treatment. This is likely to change in the short term. Nevertheless, the relative infrequency of severe external apical root resorption needs to be emphasized. It is unlikely that every prospective orthodontic patient will undergo such testing. More likely, with our increased knowledge in this area, specific patient phenotypes (e.g., a particular root morphology) with a greater predisposition to develop external apical root resorption (due to environmental factors) will benefit.

The present study has some limitations: (a) no sample size calculation was performed, therefore, both the precision of our estimates and the power of the study may be limited; (b) since the research was conducted on a convenience sample, the introduction of selection bias should not be ruled out; (c) because secondary sources of information (i.e., orthodontic records) were retrospectively evaluated, it was not possible to obtain all the detailed information on treatment, consequently, relevant orthodontic data (e.g., extracted teeth, detail of the orthodontic mechanics implemented) were not considered in the analyses; (d) none of the evaluated subjects presented EARR greater than one-third of the root length, therefore, these results do not necessarily apply to severe EARR cases; (e) since the regression-based analyses were performed with data from normal distribution approximations, biased parameter estimators, inflated type I error, and loss of power cannot be ruled out; and (f) these results would not necessarily apply to all ethnicities, since it has been suggested that there might be differences in haplotype structures for the *VDR* genotype between different populations, especially admixed populations [66]. Although our study confirmed previous results and added some novel potential associations, more research is still needed to confirm these findings further and better explain the underlying molecular mechanisms.

AUTHOR CONTRIBUTIONS

Conceptualization: Küchler E, Kirschneck C; **Methodology**: Marañón-Vásquez G, Küchler E, Kirschneck C; **Investigation**: Marañón-Vásquez G, Küchler E, Hermann S, Paddenberg E, Schröder A; **Formal analysis**: Marañón-Vásquez G, Küchler E, Baratto-Filho F, Flores-Mir C, Proff P; **Writing—original draft preparation**: Marañón-Vásquez G; **Writing—review and editing**: Küchler E, Hermann S, Paddenberg E, Schröder A, Baratto-Filho F, Flores-Mir C, Proff P, Kirschneck C; **Supervision**: Küchler E, Proff P, Kirschneck C; **Funding acquisition**: Küchler E, Proff P, Kirschneck C.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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