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Genetic influences on dental enamel that impact caries differ between the primary and permanent dentitions

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Clinically, primary and permanent teeth are distinct anatomically and the presentation of caries lesions differs between the two dentitions. Hence, the possibility exists that genetic contributions to tooth formation of the two dentitions are different. The purpose of this study was to test the hypothesis that genetic associations with an artificial caries model will not be the same between primary and permanent dentitions. Enamel samples from primary and permanent teeth were tested for microhardness at baseline, after carious lesion creation, and after fluoride application to verify association with genetic variants of selected genes. Associations were found between genetic variants of ameloblastin, amelogenin, enamelin, tuftelin, tuftelin interactive protein 11, and matrix metallopeptidase 20 and enamel from permanent teeth but not with enamel from primary teeth. In conclusion, our data continue to support that genetic variation may impact enamel development and consequently individual caries susceptibility. These effects may be distinct between primary and permanent dentitions.

Genetic association studies of caries have suggested that caries experience may be influenced by polymorphic variants in ameloblastin (1–3), amelogenin (1, 2, 4–6), enamelin (1, 2, 6, 7), matrix metalloproteinase 20 (8), tuftelin (1, 2, 4, 9), and tuftelin-interacting protein 11 (2, 6). However, the results are not consistent across the studies, and differences related to study design (how caries experience is defined, inclusion of covariates such as *Streptococcus mutans* data, sample sizes, genetic polymorphisms studied, geographical origin of the DNA samples, age and dentition of the population studied, and concomitant systemic conditions) probably contribute to the discrepancies seen in the reported findings.

Based on observations of *S. mutans* colonization data, our group previously suggested that genetic studies of caries should take into consideration the dentition of the subjects (10). Genome-wide association analyses of caries in the primary dentition (11) showed distinct results in comparison with similar analyses in the permanent dentition (12). We have also used an in vitro approach to create artificial initial caries lesions and have used these data as the phenotype for genetic association analysis (2). The initial results from analyses of a cohort of permanent teeth suggest that results vary depending on which tooth surface is tested. Hence, genetic variation in tuftelin-interacting protein 11 was associated with subclinical demineralization.

Here we expanded this work to a larger sample of permanent teeth and added a cohort of primary teeth to test the hypothesis that genetic associations with our initial caries model will not be the same between primary and permanent dentitions.

Material and methods

Permanent teeth collection

This part of the study was approved by the Ethics Committee of the Istanbul University, Medical Faculty, Istanbul, Turkey and the University of Pittsburgh Institutional

Review Board (IRB# 11070236). Informed consent was obtained from all participating individuals and parents/legal guardians. One-hundred orthodontic patients from Istanbul University, Faculty of Dentistry, Department of Orthodontics, participated in this study during the period 5 September 2011 to 30 November 2012.

Participants were seated in a dental chair, and one of the authors (M.B.) carried out the clinical examination after being calibrated by an experienced specialist (F.S.). The intra-examiner agreement was assessed by a second clinical examination in 10% of the sample after 2 wk, with a κ of 1.0 obtained. Subjects were examined using a flashlight, dental mirror, and probe. The sum of decayed, missing, and filled teeth (DMFT) was calculated for each subject (13). Teeth that had been extracted for orthodontic reasons were not included in the DMFT/decayed, missing, and filled surfaces (DMFS) scores. Dental photographs and panoramic radiographs were also obtained for all participants. One first premolar, extracted for orthodontic reasons, was obtained from each participant as a source of enamel.

Primary teeth collection

Enamel samples from 108 exfoliated primary teeth (74 molars, 27 incisors, and seven canines) and genomic DNA were used for this experiment. Biological samples were collected after subjects and their parents provided written informed consent. This part of the study was approved by the University of Pittsburgh Institutional Review Board (IRB# 11070236) and by the Federal University of Rio de Janeiro (#333.167).

Samples were collected by three examiners (E.C.K., A.L., and H.F.R.) and were calibrated by an experienced specialist (M.C.C.). The intra-examiner agreement was assessed by a second clinical examination in 10% of the sample after 2 wk, with a κ of 1.0 obtained. Cohen's kappa values for agreement between examiners was 0.91. The DMFT value was calculated for each subject (13), for both primary (dmft) and permanent (DMFT) dentitions. Teeth lost to trauma, or primary teeth lost to exfoliation, were not included in the final DMFT/dmft scores. When records indicated that teeth were extracted for orthodontic reasons, or treatments were performed in sound teeth, these situations were not included in the final DMFT/dmft scores.

Details regarding the characteristics of each population studied are presented in Table 1.

DNA samples and genotyping

Unstimulated saliva samples were obtained from all participants and stored in Oragene DNA Self-Collection kits (DNA Genotek, ON, Canada) at room temperature until processed. DNA was extracted according to the manufacturer's instructions. Fourteen single nucleotide polymorphisms (SNPs) were selected, including rs7526319, rs4970957, rs3828054, rs3790506, and rs2337360 in tuftelin (*TUFT1*), rs4694075 and rs34538475 in ameloblastin (*AMBN*), rs12640848 and rs3796704 in enamelin (*ENAM*), rs1784418 in matrix metallopeptidase 20 (*MMP20*), rs5997096 and rs134136 in tuftelin-interacting protein 11 (*TFIP11*), and rs17878486 and rs946252 in amelogenin (*AMELX*). These SNPs were chosen based on their locations relative to the genes, linkage disequilibrium relationships, and results of previous studies (1, 2, 4, 8). Table 2 summarizes linkage disequilibrium between markers in the two cohorts studied.

Polymerase chain reactions with TaqMan SNP Genotyping Assays from Applied Biosystems (Valencia, CA, USA), with a total volume of 3 μl per reaction and 3.0 ng of DNA per reaction, were used for genotyping all selected markers in a Tetrad PTC225 thermocycler from MJ Research (Waltham, MA, USA). Genotype detection and analysis were performed using the ABI 7900HT with ABI SDS software (Applied Biosystems, Valencia, CA, USA).

Specimen preparation and enamel microhardness analysis

One-hundred caries-free premolar teeth (one from each participant), extracted for orthodontic reasons, were studied. In addition, 108 exfoliated primary teeth were also studied. The tissue remnants were cleaned from the teeth and then teeth were stored in 10% buffered formalin (pH 7.0) solution at 4°C until required for initial laboratory manipulation. The crowns were separated from the roots, and then each crown was separated buccolingually and mesiodistally using a low-speed saw (Isomet; Buehler, Lake Bluff, IL, USA) under continuous water-cooling. Five surfaces (mesial, buccal, distal, occlusal, and lingual/palatine) were obtained from each crown. The enamel surfaces were sanded using abrasive papers of 320, 400, and 600 grit and then polished with 6, 1, and 0.25 μm polycrystalline diamond suspension on a Minimet 1000 Grinder-Polisher (Buehler) under water-cooling. After the polishing step, all samples were sonicated for 1 min with distilled water in an FS6 ultrasonic cleaner (Fisher Scientific, Waltham, MA, USA).

Samples were submitted to baseline microhardness analysis using an Indentamet 1100 Series microhardness tester (Buehler) with a knoop diamond under a load of 25 g for 5 s. Five indentations, with an intervening distance from each other of 100 μm, were made. Artificial caries lesions were created by immersing each enamel sample in 24 ml of demineralizing solution (1.3 mM of calcium, 0.78 mM of phosphorus, 0.05 M of acetate buffer, 0.03 μg mL^{-1} of fluoride, pH 5.0) at 37°C for 16 h (14). Surface microhardness was measured again by creating another five indentations directly beneath the initial indentations. Caries lesions were exposed for 10 min to a fluoride solution made from Aquafresh Extreme Clean toothpaste (GSK, Brentford, Middlesex, UK) containing 0.15% wt/vol fluoride ion. Surface microhardness was measured once more by creating five indentations underneath the previous ones.

Phenotype definitions and statistical analysis

Based on DMFT/dmft distributions, subjects were classified as having 'low caries experience' (below the mean DMFT of the 100 Turkish subjects or below the mean dmft of the 108 Brazilian subjects), or having 'high caries experience' (above the mean DMFT of the 100 Turkish subjects or above the mean dmft of the 108 Brazilian subjects). The differences in genotype and allele frequencies between the 'high' and 'low' caries-experience groups were tested using the PLINK software package (15) with an established alpha of 0.05. Standard case/control association analysis using Fisher's exact test, as well as full model association tests (Cochran–Armitage trend test, genotypic 2-degrees-of-freedom test, dominant gene action 1-degree-

Table 1

Characteristics of the populations from whom the samples were obtained

Characteristic	Sample origin			
	Turkey (100 permanent premolars)	Brazil (108 primary teeth)	Brazilian White people	Brazilian Black people
Age (yr)	17.2 (3.0)	8.8 (2.5)	9.2 (3.3)	8.4 (2.1)
Sex				
Male	38	62	43	19
Female	62	46	23	23
Ethnicity				
White	100	65	–	–
Black	0	43	–	–
Caries status of the individuals studied				
Caries free	6	44	22	22
Caries affected	94	64	43	21
DMFT/dmft	5.19 (3.4)	3.17 (3.4)	3.0 (0.4)	4.2 (0.6)
Enamel microhardness (Knoop hardness)*				
Baseline				
Mesial	289.52 (48.68)	210.54 (81.08)	212.0 (81.16)	207.7 (83.2)
Distal	280.65 (48.23)	229.24 (64.95)	236.0 (71.64)	216.1 (48.6)
Buccal	284.04 (39.86)	235.04 (69.26)	238.2 (66.1)	230.6 (73.5)
Occlusal	260.27 (50.59)	235.86 (69.53)	256.4 (61.56)	205.8 (71.7)
Lingual/Palatine	281.04 (44.64)	239.99 (73.12)	231.7 (74.51)	248.2 (74.6)
After artificial caries creation				
Mesial	200.66 (79.16)	150.03 (76.48)	166.9 (75.7)	136.2 (84.3)
Distal	185.68 (73.31)	152.48 (78.29)	236.0 (71.6)	123.8 (73.2)
Buccal	201.82 (74.79)	143.27 (79.79)	147.8 (77.87)	136.5 (80.2)
Occlusal	172.15 (73.69)	140.31 (64.05)	134.0 (40.31)	144.9 (78.6)
Lingual/Palatine	186.88 (75.2)	151.12 (79.33)	139.5 (66.23)	162.4 (95.9)
After fluoride exposure				
Mesial	210.61 (81.47)	195.75 (89.69)	209.6 (89.37)	172.0 (76.07)
Distal	199.78 (81.01)	194.5 (73.82)	203.7 (74.3)	160.3 (85.10)
Buccal	221.1 (77.56)	161.23 (88.51)	167.3 (85.7)	151.5 (94.24)
Occlusal	187.5 (75.26)	146.5 (80.82)	160.0 (57.17)	136.0 (97.54)
Lingual/Palatine	199.33 (78.77)	179.38 (81.08)	158.7 (66.08)	203.0 (92.83)

Values are given as mean (SD) or *n*.
DMFT, decayed, missing, or filled teeth index for permanent dentition; dmft, decayed, missing, or filled teeth index for primary dentition.
*All surfaces studied were free of any clinical signs of caries or demineralization. Differences in enamel microhardness in the three experimental conditions (at baseline, after artificial caries creation, and after fluoride exposure) were statistically significant ($P < 0.05$).

Table 2

Linkage disequilibrium (D') between the markers studied

Gene symbol	SNP combinations		Permanent dentition	Primary dentition
AMBN	rs4694075	rs34538475	0.02	0.15
AMELX	rs17878486	rs946252	0.01	0.68
ENAM	rs3796704	rs12640848	0.01	0.54
TFIP11	rs5997096	rs134136	0.16	0.16
TUFT1	rs7526319	rs4970957	0.13	0.01
	rs7526319	rs3828054	0.12	0.49
	rs7526319	rs3790506	0.14	0.13
	rs7526319	rs2337360	0.12	0.17
	rs4970957	rs3828054	0.12	0.06
	rs4970957	rs3790506	0.13	0.01
	rs4970957	rs2337360	0.14	0.12
	rs3828054	rs3790506	0.15	0.12
	rs3828054	rs2337360	0.17	0.12
	rs3790506	rs2337360	0.18	0.4

AMBN, ameloblastin; *AMELX*, amelogenin, X-linked; *ENAM*, enamelin; SNP, single nucleotide polymorphism; *TFIP11*, tuftelin interactive protein 11; *TUFT1*, tuftelin 1.

of-freedom test, and recessive gene action 1-degree-of-freedom test) were used to evaluate the data. Finally, linear and logistic models were used to allow the inclusion of sex and ethnic background (for the Brazilian cohort) as covariates.

Based on microhardness values, subjects were classified into dichotomous groups (baseline values or rate changes above or below the average of the group). Subjects were classified as having 'softer enamel' (below the average of the groups) and 'harder enamel' (above the average of the groups) for determination of microhardness phenotypes. Representative examples of the distribution of these values are shown in Fig. 1. Data were analysed according to surface because we are aware that enamel assessments differ between surfaces within the same tooth (2).

The following three differences of enamel microhardness values were compared using the Wilcoxon signed-rank test to confirm that the in vitro model showed the expected decrease in enamel microhardness between baseline and after artificial caries lesion creation and a subsequent increase in enamel microhardness after exposure to a fluoridated solution: between baseline and after artificial

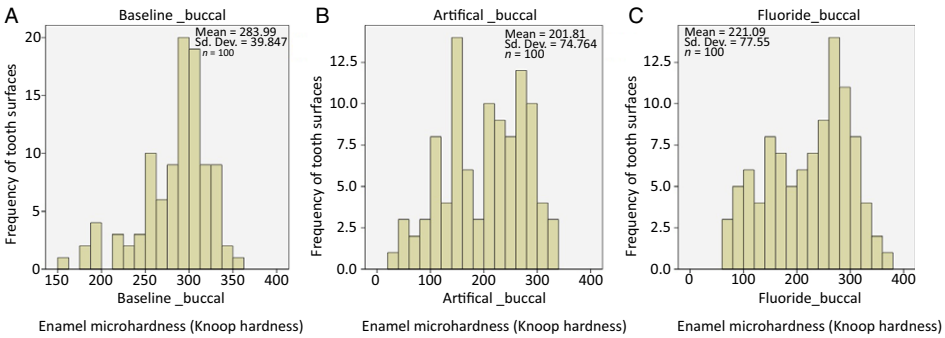

Fig. 1. Representative distribution of enamel microhardness values in the permanent teeth cohort (buccal surface) at baseline (A), after artificial caries creation (B), and after fluoride exposure (C).

caries lesion creation; after artificial caries lesion creation and after fluoride exposure; and between baseline and after fluoride exposure (Table 1). Chi-square and Fisher's exact tests were used to assess association between the SNPs and microhardness values by the use of the PLINK software package (15) with an established alpha of 0.05.

Results

Whereas associations between caries experience and markers in *AMBN*, *AMELX*, and *TFIP11* (*ENAM* was borderline associated) were found for the individuals who provided premolars for this study, only one marker in *TUFT1* showed association with caries experience among the children who donated their exfoliated primary teeth (Table 3, which only lists the models for which statistical evidence for differences in genotype or allele distributions was obtained). Linear and logistic models were used to allow the inclusion of sex and ethnic background (for the Brazilian cohort) as covariates; the results did not differ from those presented in Table 3, and therefore these data are not shown.

As expected, enamel microhardness decreased after creation of artificial caries and increased after fluoride exposure, for teeth of both dentitions (Table 1). Also, microhardness values were lower for primary teeth than for permanent teeth. When considering the results of the microhardness of the enamel at baseline, after artificial caries creation, and after fluoride exposure, in comparison with genetic variation, statistically significant differences could be seen in the permanent teeth but not in the primary teeth.

Table 3

Single nucleotide polymorphisms (SNPs) and summary P-values for association tests between caries experience and genetic variants in the two study samples

			Minor allele frequency			Summary *P*-value	
				Brazil			
Gene symbol	Marker	Alleles	Turkey	White people	Black people	Permanent teeth	Primary teeth
TUFT1	rs7526319	CT	0.338	0.481	0.469	NS	NS
	rs4970957	AG	0.241	0.133	0.152	NS	0.009*
	rs3828054	AG	0.105	0.448	0.393	NS	NS
	rs3790506	AG	0.249	0.4	0.357	NS	NS
	rs2337360	AG	0.25	0.366	0.414	NS	NS
AMBN	rs4694075	CT	0.478	0.264	0.424	0.004†	NS
	rs34538475	GT	0.187	0.255	0.387	NS	NS
ENAM	rs12640848	AG	0.357	0.451	0.471	0.06*	NS
	rs3796704	AG	0.12	0.173	0.133	NS	NS
MMP20	rs1784418	CT	0.407	0.388	0.414	NS	NS
TFIP11	rs5997096	CT	0.47	0.416	0.444	0.006*	NS
	rs134136	CT	0.335	0.258	0.291	0.002*	NS
AMELX	rs17878486	CT	0.111	0.148	0.136	0.03*	NS
	rs946252	AG	0.3	0.183	0.2	0.025*	NS

AMBN, ameloblastin; *AMELX*, amelogenin, X-linked; *ENAM*, enamelin; NS, not statistically significant; *TFIP11*, tuftelin interactive protein 11; *TUFT1*, tuftelin 1.
*Fisher's exact test on the distribution of alleles.
†Genotypic two-degrees-of-freedom test.

Table 4

Summary of the positive associations of the genotype and allele frequency comparisons of baseline enamel microhardness assessments in the permanent teeth

Gene symbol	SNP	Enamel microhardness: Above the mean [*n* (%)]	Enamel microhardness: Below the mean [*n* (%)]	*P*
TUFT1	rs7526319 (occlusal)			
	Genotype			
	CC	**8 (14.2)**	1 (2.9)	**0.01**
	CT	27 (48.2)	27 (79.4)	
	TT	21 (37.5)	6 (17.6)	
	Allele			
	C	43 (38.3)	29 (42.6)	0.57
	T	69 (61.6)	39 (57.3)	
	rs7526319 (lingual/palatine)			
	Genotype			
	CC	2 (3.6)	**7 (10.7)**	**0.03**
	CT	37 (67.2)	17 (48.5)	
	TT	16 (29.09)	11 (31.4)	
	Allele			
	C	41 (37.2)	31 (44.2)	0.34
	T	69 (62.7)	39 (55.7)	
	rs2337360 (occlusal)			
	Genotype			
	AA	9 (14.7)	5 (12.8)	**0.03**
	AG	27 (44.2)	27 (69.2)	
	GG	**25 (40.9)**	7 (17.9)	
	Allele			
	A	45 (36.8)	37 (47.4)	0.13
	G	77 (63.1)	41 (52.5)	
	Genotype			
	AA	4 (6.4)	**10 (26.3)**	**0.01**
	AG	38 (61.2)	16 (42.1)	
	GG	20 (32.2)	12 (31.5)	
	Allele			
	A	46 (37.09)	36 (47.3)	0.15
	G	78 (62.9)	40 (52.6)	
ENAM	rs3796704 (distal)			
	Genotype			
	AA	0 (0)	0 (0)	**0.04**
	AG	3 (7.6)	**7 (25)**	
	GG	36 (92.3)	21 (75)	
	Allele			
	A	3 (3.8)	7 (12.5)	0.06
	G	75 (96.1)	49 (87.5)	
MMP20	rs1784418 (buccal)			
	Genotype			
	CC	11 (17.7)	6 (15.7)	**0.003**
	CT	44 (70.9)	17 (44.7)	
	TT	7 (11.2)	**15 (39.4)**	
	Allele			
	C	**66 (53.2)**	29 (38.1)	**0.03**
	T	58 (46.7)	47 (61.8)	
TFIP11	rs134136 (buccal)			
	Genotype			
	CC	9 (14.5)	2 (5.2)	0.9
	CT	30 (48.3)	14 (36.8)	
	TT	23 (37.09)	22 (57.8)	
	Allele			
	C	**48 (38.7)**	18 (23.6)	**0.02**
	T	76 (61.2)	58 (76.3)	
AMELX	rs17878486 (mesial)			
	Genotype			
	CC	1 (2.5)	1 (4.5)	0.05
	CT	10 (25)	12 (54.5)	
	TT	29 (72.5)	9 (40.9)	
	Allele			
	C	12 (15)	**14 (31.8)**	**0.02**
	T	68 (85)	30 (68.1)	

Bold indicates statistical significance.
AMELX, amelogenin, X-linked; *ENAM*, enamelin; *MMP20*, matrix metallopeptidase 20; SNP, single nucleotide polymorphism; *TFIP11*, tuftelin interactive protein 11; *TUFT1*, tuftelin 1.

Enamel microhardness baseline values below the mean were significantly associated with rs7526319 ($P = 0.03$; lingual/palatine surface) and rs2337360 ($P = 0.01$; lingual/palatine surface) of *TUFT1*, with rs3796704 of *ENAM* ($P = 0.04$; distal surface), with rs1784418 of *MMP20* ($P = 0.003$; buccal surface), and with rs17878486 of *AMELX* ($P = 0.02$; mesial surface) (Table 4). Softer enamel was significantly associated with the CC genotype (lingual/palatine surface) in rs7526319 and with the AA genotype (lingual/palatine surface) in rs2337360 (both of *TUFT1*), with the AG genotype in rs3796704 of *ENAM* (distal surface), with the TT genotype in rs1784418 of *MMP20* (buccal surface), and with the C allele in rs17878486 of *AMELX* (mesial surface) (Table 4). Enamel microhardness baseline values above the mean were significantly associated with rs7526319 ($P = 0.01$; occlusal surface) and rs2337360 ($P = 0.03$; occlusal surface) of *TUFT1,* with rs1784418 of *MMP20* ($P = 0.03$; buccal surface), and with rs134136 ($P = 0.02$; buccal surface) of *TFIP11* (Table 4). Enamel microhardness values above the mean were significantly associated with the CC genotype in rs7526319 (occlusal surface) and the GG genotype in rs2337360 (occlusal surface), both of *TUFT1*, with the C allele in rs1784418 of *MMP20* (buccal surface), and with the C allele in rs134136 of *TFIP11* (buccal surface) (Table 4).

After creation of artificial caries lesions, enamel microhardness values above the mean were significantly associated with rs134136 of *TFIP11* ($P = 0.006$; buccal surface) and with rs946252 of *AMELX* ($P = 0.03$ for the distal surface and $P = 0.006$ for the buccal surface) (Table 5). More demineralization was significantly associated with the T allele in rs134136 of *TFIP11* (buccal surface), and with the T allele (distal and buccal surfaces) and the TT genotype (buccal surface) in rs946252 of *AMELX* (Table 5). After creation of artificial caries lesions, enamel microhardness values below the mean were significantly associated with rs134136 of *TFIP11* ($P = 0.009$; buccal surface) (Table 4). More demineralization was significantly associated with the

CC genotype in rs134136 of *TFIP11* (buccal surface) (Table 5).

After fluoride treatment, enamel microhardness values above the mean were significantly associated with rs2337360 of *TUFT1* ($P = 0.03$; lingual/palatine surface), with rs4694075 of *AMBN* ($P = 0.01$; distal surface), and with rs1784418 of *MMP20* ($P = 0.04$; mesial surface) (Table 6). A larger amount of enamel remineralization was significantly associated with the AA genotype in rs2337360 of *TUFT1* (lingual/palatine surface), with the T allele in rs4694075 of *AMBN* (distal surface), and with the TT genotype in rs1784418 of *MMP20* (mesial surface) (Table 6). After fluoride treatment, lower microhardness was significantly associated with rs4694075 of *AMBN* ($P = 0.01$; distal surface), and with rs5997096 ($P = 0.01$; mesial surface) and rs134136 ($P = 0.01$; mesial surface), both of *TFIP11* (Table 6). A lower degree of remineralization was associated with the CC genotype in rs4694075 of *AMBN* (distal surface), and the T allele in rs5997096 (mesial surface), and the TT genotype and T allele in rs134136 (mesial surface), all of *TFIP11* (Table 6).

Enamel microhardness values (for all surfaces) did not correlate with caries experience of the individuals who provided samples (data not shown).

Table 5

Summary of the positive associations of the genotype and allele frequency comparisons of enamel microhardness assessments in the permanent teeth after artificial caries lesion creation

Gene symbol	SNP	Enamel microhardness: Above the mean [*n* (%)]	Enamel microhardness: Below the mean [*n* (%)]	*P*
TFIP11	rs134136 (buccal)			
	Genotype			
	TT	6 (14.6)	5 (8.4)	**0.009**
	CT	24 (58.5)	20 (33.8)	
	CC	11 (26.8)	**34 (57.6)**	
	Allele			
	T	**36 (43.9)**	30 (25.4)	**0.006**
	C	46 (56.09)	88 (74.5)	
AMELX	rs946252 (distal)			
	Genotype			
	TT	6 (21.4)	2 (5.8)	0.15
	CT	8 (28.5)	9 (26.4)	
	CC	14 (50)	23 (67.6)	
	Allele			
	T	**20 (35.7)**	13 (19.1)	**0.03**
	C	36 (64.2)	55 (80.8)	
	rs946252 (buccal)			
	Genotype			
	TT	**7 (25.9)**	1 (2.8)	**0.02**
	CT	7 (25.9)	10 (28.5)	
	CC	13 (48.1)	24 (68.5)	
	Allele			
	T	**21 (38.8)**	12 (17.1)	**0.006**
	C	33 (61.1)	58 (82.8)	

Bold indicates statistical significance.
AMELX, amelogenin, X-linked; SNP, single nucleotide polymorphism; *TFIP11*, tuftelin interactive protein 11.

Table 6

Summary of the positive associations of the genotype and allele frequency comparisons of enamel microhardness assessments in permanent teeth after fluoride exposure

Gene symbol	SNP	Enamel microhardness: Above the mean [*n* (%)]	Enamel microhardness: Below the mean [*n* (%)]	*P*
TUFT1	rs2337360 (lingual/palatine)			
	Genotype			
	AA	**10 (20.8)**	4 (7.6)	**0.03**
	AG	20 (41.6)	34 (65.3)	
	GG	18 (37.5)	14 (26.9)	
	Allele			
	A	40 (41.6)	42 (40.3)	0.85
	G	56 (58.3)	62 (59.6)	
AMBN	rs4694075 (distal)			
	Genotype			
	TT	4 (10.2)	4 (7.5)	**0.01**
	CT	24 (61.5)	18 (33.9)	
	CC	11 (28.2)	**31 (58.4)**	
	Allele			
	T	**32 (41.02)**	26 (24.5)	**0.01**
	C	46 (58.9)	80 (75.4)	
MMP20	rs1784418 (mesial)			
	Genotype			
	CC	10 (21.7)	7 (12.9)	**0.04**
	CT	22 (47.8)	39 (72.2)	
	TT	**14 (30.4)**	8 (14.8)	
	Allele			
	C	42 (45.6)	53 (49.07)	0.62
	T	50 (54.3)	55 (50.9)	
	C	66 (78.5)	61 (64.8)	
TFIP11	rs5997096 (mesial)			
	Genotype			
	CC	16 (37.2)	8 (18.1)	0.06
	CT	19 (44.1)	20 (45.4)	
	TT	8 (18.6)	16 (36.3)	
	Allele			
	C	51 (59.3)	36 (40.9)	**0.01**
	T	35 (40.6)	**52 (59.09)**	
	rs134136 (mesial)			
	Genotype			
	TT	3 (6.5)	**8 (14.8)**	**0.03**
	CT	16 (34.7)	28 (51.8)	
	CC	27 (58.6)	18 (33.3)	
	Allele			
	T	22 (23.9)	**44 (40.7)**	**0.01**
	C	70 (76.08)	64 (59.2)	

Bold indicates statistical significance.
AMBN, ameloblastin; *MMP20*, matrix metallopeptidase 20; SNP, single nucleotide polymorphism; *TFIP11*, tuftelin interactive protein 11; *TUFT1*, tuftelin 1.

Discussion

Our data support the hypothesis that genetic factors affecting dental caries, and involved in the structure of enamel, impact the primary and permanent dentitions differently. This comes as no surprise because previous genome-wide association studies (11, 12) and follow-up fine-mapping studies of loci of interest (16) provided very distinct results between primary and permanent

dentitions. Additional evidence supporting differences between genetic influences of caries in permanent vs. primary dentitions comes from analysis of the keratin75 polymorphism rs2232387 (alanine to threonine substitution at position 161), which is associated with a higher number of carious tooth surfaces in adults, but not in children (17). Also, the clinical patterns we observe in early childhood caries, related to progression of the disease, are clearly very distinct from the typical chronic development of caries in the permanent dentition. This is true, even in more severe cases, suggesting that both dentitions are distinct, not only in the number of units and anatomical features, but also at the microscopic level.

While concerned about multiple testing, we avoided to apply the strict Bonferroni correction and increase type II error. If we had used Bonferroni correction, we would have lowered the alpha to 0.0000595 (0.05/840). We have demonstrated previously (18) that known true associations are missed when correction for multiple testing is implemented. The results of our work should be considered with caution and serve to generate a hypothesis to be directly tested in larger and more homogeneous samples. On the other hand, simply disregarding the nominal associations presented here may delay discovery by misleading the field to believe that no true biological relationships exist.

Another limitation of our study is that the outcome 'dental caries' was first analysed as caries experience (DMFT/dmft, Tables 1 and 3), which represents the dental caries accumulated over time. This is not the same phenotype as the one analysed in Tables 4–6 concerning genotype associations with enamel microhardness. This phenotype is better characterized as a 'subclinical caries lesion', which is obviously clinically not detectable by the typical dental examination. The DMFT/dmft values and experimental variations in enamel microhardness observed here do not correlate, as expected from our previous preliminary work (2). Also, the few SNPs identified as associated have no clear functional implications and we are assuming that they may reflect changes in enamel that are relevant to the mechanism(s) of disease. It is still worth mentioning that the caries process in humans is complex and influenced by a large number of other factors that are not studied here or controlled for.

The present study follows our preliminary work suggesting that enamel microhardness might be a more sensitive way to define caries in comparison with the traditional DMFT/dmft scores (2). We collected additional enamel samples from both dentitions and repeated the original studies. Similarly to our preliminary data, we found that some individuals had lower enamel microhardness to begin with. In general, enamel microhardness decreases after creation of an artificial caries lesion and increases after fluoride exposure. It is not apparent that some individuals have enamel that demineralizes at a faster rate and that caries susceptibility is linked to baseline mineralization levels of the enamel. However, it is not possible to determine if the variation we see in our data is biologically relevant, and to conclude that some individuals may be more susceptible to caries as a result of their original enamel structure or mineralization levels. Variation in the enamel microhardness data according to tooth surface brings an additional layer of complication, making it almost impossible to compile the data in any way that can convincingly provide a direction for further analyses. In other words, independently from the innate genetic background that may protect the enamel against acidic conditions, clinically, if the enamel is under unfavourable conditions for long enough, it will develop a carious lesion. We recently showed that genetic variation in the genes studied here may influence the calcium and magnesium concentrations of teeth (19), and that biochemical, rather than mechanical, analyses of enamel might be more relevant to determine if particular individuals are more susceptible to enamel demineralization as a result of the acidic conditions created by biofilm formation.

In conclusion, our data continue to support that genetic variation may impact enamel development, which might be more prone to demineralization under acidic conditions. These effects may be distinct between primary and permanent dentitions.

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Conflicts of interest – The authors declare no conflicts of interest.

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