

ORIGINAL ARTICLE

Toll-like receptor-1, -2, and -6 genotypes in relation to salivary human beta-defensin-1, -2, -3 and human neutrophilic peptide-1

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Abstract

Aim: To examine whether functional gene polymorphisms of toll-like receptor (*TLR*)1, *TLR*2, and *TLR*6 are related to the salivary concentrations of human beta-defensins (hBDs)-1, -2, -3, and human neutrophilic peptide (HNP)-1.

Materials and Methods: Polymorphisms of *TLR*1 (rs5743618), *TLR*2 (rs5743708), and *TLR*6 (rs5743810) were genotyped by PCR-based pyrosequencing from the salivary samples of 230 adults. Salivary hBD-1, -2, -3, and HNP-1 concentrations were measured using enzyme-linked immunosorbent assay. General and periodontal health examinations, including panoramic radiography, were available for all participants.

Results: The genotype frequencies for wild types and variant types were as follows: 66.5% and 33.5% for *TLR*1, 95.5% and 4.5% for *TLR*2, and 25.1% and 74.9% for *TLR*6, respectively. The *TLR*2 heterozygote variant group exhibited higher salivary hBD-2 concentrations than the *TLR*2 wild-type group ($p = .038$). On the contrary, elevated hBD-2 concentrations were detected in the *TLR*6 wild-type group compared with the *TLR*6 heterozygote and homozygote variant group ($p = .028$). The associations between *TLR*6 genotypes and salivary hBD-2 concentrations remained significant after adjusting them for periodontal status, age, and smoking.

Conclusion: hBD-2 concentrations in saliva are related to *TLR*2 and *TLR*6 polymorphisms, but only the *TLR*6 genotype seems to exhibit an independent association with the salivary hBD-2 concentrations.

KEYWORDS

defensins, genetic, periodontium, polymorphism, saliva

Clinical Relevance

Scientific rationale for study: Toll-like receptor (TLR)-1, -2, and -6 recognize bacterial peptidoglycans and lipoproteins, and their heterodimer formation leads to improved recognition of various lipid moieties. In response to bacterial stimuli, gingival epithelial TLRs mediate pro-inflammatory cytokine and human beta-defensin (hBD) expression.

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Principal findings: Here, we demonstrated that salivary hBD-2 concentrations associate with the *TLR6* genotype.

Practical implications: Understanding the genetic background behind the activation mechanisms of salivary immune-regulatory peptides may help us define individuals who could have an increased risk of developing periodontal disease.

1 | INTRODUCTION

Toll-like receptors (TLRs) are eukaryotic pattern recognition receptors that are specialized to recognize microbe-associated molecular patterns and to initiate cellular response. At present, 10 TLRs have been identified in humans, and of those TLR 1–9 are expressed in human gingiva (Chang et al., 2021). TLRs are localized either on the cell surface (plasma membrane-localized TLRs: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) or at the cytosolic endosomal compartment (intracellular TLRs: TLR3, TLR7, TLR8, and TLR9). The TLR2 subfamily comprises TLR1, TLR2, TLR6, and TLR10 (Tapping et al., 2007). Indeed, TLR2 can recognize a broad spectrum of microbe-associated molecular patterns, as it is able to form heterodimers with TLR1 and TLR6 to identify peptidoglycan, lipopeptide, and lipoproteins (Groeger & Meyle, 2019).

Human beta-defensins (hBDs) are cysteine-rich cationic antimicrobial peptides that are expressed and secreted by epithelial cells. The second group of human defensins, α -defensins (human neutrophilic peptides [HNPs]), is produced by polymorphonuclear leukocytes and intestinal paneth cells (Gursoy & Könönen, 2012). Both types of defensins are detected widely in gingiva, in gingival crevicular fluid, and in saliva (Dale & Fredericks, 2005; Gursoy, Könönen, Luukkonen, & Uitto, 2013). hBD expression can be activated by bacteria, microbial molecules, and an inflammatory stimulate via TLRs and NOD-like receptors (Fruitwala et al., 2019). The stimulation of oral epithelial cell lines or primary culture oral epithelial cells with TLR2 agonists induces up-regulation of hBD-2 expression (Sugawara et al., 2006). *Fusobacterium nucleatum*, a well-known species in oral biofilms, activates hBD-2 expression via TLR2-mediated manner (Ji et al., 2009). Indeed, *F. nucleatum*-associated defensin inducer (FAD-I), a cell wall-associated lipoprotein, stimulates hBD-2 expression via heterodimerizations of TLR1/TLR2 and TLR2/TLR6 (Bhattacharyya et al., 2016). It was also proposed that TLRs regulate neutrophil antimicrobial functions (Sabroe et al., 2005); however, salivary antimicrobial peptide profiles in relation to TLR genetic disruptions are still unknown.

Previous studies demonstrated that *TLR1* (rs5743618) and *TLR6* (rs5743810) single-nucleotide polymorphisms (SNPs) associate with the colonization of a known periodontal pathogen *Treponema denticola* (Mehlotra et al., 2016), while *TLR2* (rs5743708) polymorphism is connected to risk of developing periodontitis (Shan et al., 2020). To date, however, salivary hBD or HNP concentrations in relation to *TLR1*, *TLR2*, and *TLR6* gene polymorphisms have not been examined. We hypothesized that polymorphisms in the *TLR2* gene and in the genes of TLR2 heterodimer pairs, *TLR1* and *TLR6*, have an impact on

defensin concentrations in saliva. Therefore, we aimed to determine the relation between *TLR1*, *TLR2*, and *TLR6* gene polymorphisms and salivary concentrations of hBD-1, -2, -3, and HNP-1.

2 | MATERIALS AND METHODS

2.1 | Study design and salivary samples

The salivary samples of the present study originated from the national Finnish health survey “Health 2000” sample collection (BRIF8901). A detailed information on the survey design and protocols can be found elsewhere (Gursoy, Könönen, Huuononen, et al., 2013; Liukkonen et al., 2017). Briefly, the study protocols of the survey had been approved by the Ethical Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland. All survey participants gave written informed consent. Information on general health originated from the questionnaires, interviews, and clinical health examinations. Oral health status of the participants had been assessed by specially trained dentists. In the periodontal examination, the probing pocket depths (PPD) were measured by the WHO periodontal probe (LM-Dental™, Parainen, Finland) around each tooth at four points, and the number of bleeding sextants was recorded.

Digital panoramic radiographs, which were available for all subjects, were used in the detection of alveolar bone loss on the mesial and distal surfaces of each tooth as well as on the furcation areas of multi-rooted teeth. The radiographical examination protocols had been approved by the Advisory Board for Radiation Safety, and the Radiation and Nuclear Safety Authority and the Ethics Committees for Human Studies of the National Public Health Institute and the Institute of Epidemiology and National Health, Finland granted the safety licences. The participation in the radiographical examination was voluntary, and pregnancy was the only exclusion criterium. Radiographs were taken with a dental panoramic X-ray device (PM 2002 CC proline apparatus; Planmeca, Helsinki, Finland), and the measurements were performed using an inbuilt measuring tool of the Dimaxis™ software (Planmeca). A detailed description of the measurements regarding alveolar bone loss has been previously described (Gursoy, Könönen, Huuononen, et al., 2013).

Paraffin-stimulated whole saliva samples were collected and placed in carbonic acid ice at the field, and then stored at -70°C until their further use. A subset of 230 salivary samples, collected from a population of 40–60 (mean of 48.7) years of age, having a minimum of 20 teeth, was included in the present study for genotyping and the determination of antimicrobial peptide concentrations. The selection

of saliva samples was based on the number of teeth with PPD ≥ 4 mm to form three periodontally distinct groups as follows: 81 participants had PPD ≥ 4 mm at 14 or more teeth; 65 participants had PPD ≥ 4 mm at two or seven teeth; and 84 participants had no teeth with PPD ≥ 4 mm.

The saliva samples were centrifuged at 10,000 rpm for 5 min, and the pellets were used for the assays of genotyping and the supernatants for hBD 1–3 and HNP-1 measurements. The samples were analysed blindly in the laboratory.

2.2 | Exposure variable

TLR1, *TLR2*, and *TLR6* genotypes were defined as exposure variables. Genotyping of the selected TLRs was performed by an automated pyrosequencer (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden). The isolated genomic DNA was used for PCR.

The PCR and sequencing primers were designed as described elsewhere (Vuononvirta et al., 2011; Nuolivirta et al., 2013) and ordered from Sigma–Aldrich, Finland. To guarantee the specificity of pyrosequencing, three negative controls were included in each run. PCR products with potential SNPs were recognized as the template in the pyrosequencing reactions, using a commercial reagent kit (PSQ™96 Pyro Gold Q96 reagent kit) according to the manufacturer's protocol. The following polymorphisms of TLRs (SNP id) were examined: *TLR1* (rs5743618), *TLR2* (rs5743708), and *TLR6* (rs5743810).

2.3 | Outcome variables

Salivary hBD-1, hBD-2, hBD-3, and HNP-1 concentrations (pg/ml) were defined as outcome variables. hBD-3 and HNP-1 concentrations were measured by in-house sandwich-enzyme-linked immunosorbent

TABLE 1 The distribution of age, gender, the percentage of smokers, number of teeth, mean, number of teeth with probing pocket depth (PPD) of ≥ 4 mm, number of bleeding sextants, and total alveolar bone loss among genotypes of *TLR1*, *TLR2*, and *TLR6*

	<i>TLR1</i> wild type (G/G) (n = 147)	<i>TLR1</i> G/T and T/T variants (n = 74)	p	<i>TLR2</i> wild type (C/C) (n = 213)	<i>TLR2</i> C/T variant (n = 10)	p	<i>TLR6</i> wild type (C/C) (n = 55)	<i>TLR6</i> CT and T/T variants (n = 164)	p
Age (mean \pm SD)	48.7 \pm 5.3	48.8 \pm 5.7	.921	48.8 \pm 5.4	47.2 \pm 4.9	.376	48.9 \pm 5.9	48.5 \pm 5.2	.631
Male (%)	44.9	45.9	.883	45.1	60	.273	45.5	46.3	.909
Smokers (%)	37.4	39.2	.798	38.5	20	.424	34.5	39	.554
No. of tee (mean \pm SD)	26.8 \pm 2.4	27.2 \pm 2.4	.206	26.9 \pm 2.4	27.5 \pm 2.4	.449	27.2 \pm 2.2	26.9 \pm 2.5	.484
No. of teeth with PPD ≥ 4 mm (mean \pm SD)	8.3 \pm 9.2	8.1 \pm 8.7	.835	8.3 \pm 9.0	4.6 \pm 6.1	.199	6.4 \pm 8.6	8.9 \pm 9.2	.083
No. of bleeding sextants (mean \pm SD)	2.95 \pm 2.33	3.28 \pm 2.45	.338	3.08 \pm 2.35	2.20 \pm 2.78	.349	2.53 \pm 2.35	3.20 \pm 2.37	.069
Total alveolar bone loss (mm) (mean \pm SD)	17.4 \pm 30.5	19.3 \pm 31.4	.667	18.1 \pm 31.1	13.8 \pm 15.0	.660	12.9 \pm 19.3	19.3 \pm 33.6	.177

TABLE 2 Frequencies of toll-like receptor (*TLR1*), *TLR2*, and *TLR6* genotypes and human beta-defensin (HBD)-1, -2, -3 and human neutrophilic peptide (HNP)-1 concentrations among the 230 study participants, stratified according to their periodontal status

	No teeth with PPD ≥ 4 mm (n = 81)	PPD ≥ 4 mm at two or seven teeth (n = 65)	PPD ≥ 4 mm at 14 or more teeth (n = 84)	p Value
<i>TLR1</i> wild type (G/G) (%)	69.2	64.5	65.4	.814 ^a
<i>TLR1</i> G/T and T/T variants (%)	30.8	35.5	34.6	
<i>TLR2</i> wild type (C/C) (%)	91.1	98.4	97.5	.063 ^a
<i>TLR2</i> C/T variant (%)	8.9	1.6	2.5	
<i>TLR6</i> wild type (C/C) (%)	29.5	28.3	18.5	.223 ^a
<i>TLR6</i> CT and T/T variants (%)	70.5	71.7	81.5	
hBD-1 (pg/ml) (median, min–max)	3616 (977–21,800)	3152 (1026–8186)	3781 (1147–17,300)	.014 ^b
hBD-2 (pg/ml) (median, min–max)	65.4 (0–1138)	87.1 (0–1575)	37.8 (0–382)	.002 ^b
hBD-3 (pg/ml) (median, min–max)	0 (0–2162)	726 (0–3420)	0 (0–2162)	<.001 ^b
HNP-1 (pg/ml) (median, min–max)	81.9 (17.4–184)	93.0 (21.8–361)	103 (17.4–305)	.004 ^b

Abbreviation: PPD, probing pocket depth.

^aChi-square test.

^bKruskal–Wallis test.

assay (ELISA) assays (Rabbit anti-hBD-3, cat# 500-P241; Biotinylated rabbit anti-hBD-3, cat# 500-P241; hBD-3, cat# 300-52; Goat anti-hNP-1, cat#500-P126G; Biotinylated goat anti-hNP-1, cat# 500-P126G and hNP-1, cat# 300-42; PeproTech®, Rocky Hill, CT). Commercial sandwich-ELISA kits (PeproTech®) were used for salivary hBD-1 and -2 detection. Absorbances were read with the Multiskan™ EX, and Ascent™ Software V. 2.1 (Thermo Scientific, Waltham, MA) was used in analyses. A detailed description of the ELISA assays can be found elsewhere (Gürsoy et al., 2016).

2.4 | Potential confounders

Sociodemographic variables were age and gender. Smoking status (current smokers and non-smokers, including former or never smokers) was used as a behavioural variable. Periodontal status, number of teeth with PPD \geq 4 mm, number of bleeding sextants, and alveolar bone loss were included as oral clinical variables.

2.5 | Statistical analyses

The SPSS statistical program (version 26.0; IBM Corp., Armonk, NY) was used in data analyses. The data distributions of hBD-1, -2, -3, and HNP-1 concentrations and alveolar bone loss were skewed, and thus, the non-parametric Kruskal-Wallis and Mann-Whitney *U* tests were applied. Sociodemographic, behavioural, and oral clinical differences among the TLR genotype groups were analysed with the independent samples *t*-test. Linear regression analysis was used to define the associations between the *TLR2* and *TLR6* variants and salivary hBD-2 concentrations, in the presence or absence of confounders. For linear regression analysis, hBD-2 concentrations in saliva were log₁₀-transformed. A statistical significance was defined as a *p* value <.05.

3 | RESULTS

Among the 230 samples, the examined genotypes were detected as follows: *TLR1* in 221 samples; *TLR2* in 223 samples; and *TLR6* in 219 samples. The variant frequencies were as follows: 66.5% for wild type (G/G) and 33.5% for heterozygote (G/T) and homozygote (T/T) types of *TLR1*; 95.5% for wild type (C/C) and 4.5% for heterozygote type (C/T) for *TLR2*; and 25.1% for wild type (C/C) and 74.9% heterozygote (C/T) and homozygote (T/T) types of *TLR6*.

The distribution of age, gender, the percentage of smokers, number of teeth with PPD \geq 4 mm, number of sextants with gingival bleeding, and alveolar bone loss did not differ between the genotypes of *TLR1*, *TLR2*, and *TLR6* (Table 1).

The frequencies of *TLR1*, *TLR2*, and *TLR6* genotypes among the 230 study participants are given in Table 2. No statistical difference in the *TLR* genotype frequency was observed when the study participants were stratified according to their periodontal status. The hBD

TABLE 3 Salivary human beta-defensin (hBD)-1, hBD-2, hBD-3, and human neutrophil peptide (HNP)-1 concentrations among genotypes of *TLR1*, *TLR2*, and *TLR6*

	<i>TLR1</i> wild type (G/G) (n = 147)	<i>TLR1</i> G/T and T/T variants (n = 74)	<i>p</i>	<i>TLR2</i> wild type (C/C) (n = 213)	<i>p</i>	<i>TLR2</i> C/T variant (n = 10)	<i>TLR6</i> wild type (C/C) (n = 55)	<i>p</i>	<i>TLR6</i> CT and T/T variants (n = 164)	<i>p</i>
hBD-1 (pg/ml)	3383 (977–21,800)	3468 (1316–10,800)	.675	3422 (977–21,800)	.636	3649 (2991–5537)	3568 (977–11,400)	.577	3415 (996–21,800)	.577
hBD-2 (pg/ml)	59.6 (0–1575)	66.2 (0–1009)	.993	58.7 (0–1575)	.038	201 (0–563)	97.6 (0–1009)	.028	52.9 (0–1575)	.028
hBD-3 (pg/ml)	382 (0–3420)	120 (0–2695)	.357	361 (0–3420)	.652	310 (0–1125)	404 (0–3420)	.239	323 (0–3005)	.239
HNP-1 (pg/ml)	91.99 (17.4–361)	95.7 (17.4–305)	.637	94.6 (17.4–361)	.548	80.5 (62.5–138.4)	92 (18.2–304)	.957	93.3 (17.4–361)	.957

Note: Data are presented as median (min–max). Significant differences (*p* < 0.05) are presented in bold.

TABLE 4 Salivary human beta-defensin (hBD)-1, hBD-2, hBD-3, and human neutrophil peptide (HNP)-1 concentrations in groups carrying either of the *TLR1/TLR2* or *TLR2/TLR6* variants

	<i>TLR1</i> and <i>TLR2</i> wild type (n = 139)	Being heterozygote or homozygote for either <i>TLR1</i> or <i>TLR2</i> (n = 82)	<i>p</i>	<i>TLR2</i> and <i>TLR6</i> wild type (n = 51)	Being heterozygote or homozygote for either <i>TLR2</i> or <i>TLR6</i> (n = 167)	<i>p</i>
hBD-1 (pg/ml)	3361 (977–21,800)	3474 (1316–10,800)	.657	3474 (977–11,400)	3443 (996–21,800)	.642
hBD-2 (pg/ml)	57.7 (0–1575)	66.7 (0–1009)	.420	86.5 (0–1009)	57.1 (0–1575)	.148
hBD-3 (pg/ml)	382 (0–3420)	249 (0–2696)	.359	404 (0–3420)	323 (0–3005)	.224
HNP-1 (pg/ml)	94.1 (17.4–361)	91.4 (17.4–305)	.949	93.5 (18.2–304)	93.2 (17.4–361)	.896

Note: Data are presented as median (min–max).

TABLE 5 Unadjusted and adjusted associations of salivary human beta-defensin-2 concentrations with *TLR2* and *TLR6* genotype variants

	Unadjusted	Model 1	Model 2	Model 3
<i>TLR2</i>	<i>B</i> = 0.409 <i>p</i> = .050	<i>B</i> = 0.372 <i>p</i> = .073	<i>B</i> = 0.312 <i>p</i> = .123	<i>B</i> = 0.292 <i>p</i> = .151
<i>TLR6</i>	<i>B</i> = –0.218 <i>p</i> = .034	<i>B</i> = –0.204 <i>p</i> = .046	<i>B</i> = –0.221 <i>p</i> = .026	<i>B</i> = –0.210 <i>p</i> = .036

Note: Model 1 was adjusted for smoking, model 2 was further adjusted for age, and model 3 was further adjusted for periodontal status.

1–3 and HNP-1 concentrations differed significantly between the periodontally healthy and two diseased groups (Table 2). HNP-1 concentrations were elevated along with increased number of teeth with PPD \geq 4 mm, whereas higher hBD-3 concentrations were measured only in the localized periodontitis group (PPD \geq 4 mm at two or seven teeth) in comparison to the controls (no teeth with PPD \geq 4 mm).

Table 3 presents the defensin concentrations in saliva according to the *TLR* genotypes. Salivary hBD-2 concentrations were significantly lower among *TLR2* wild types in comparison to the *TLR2* heterozygote and homozygote group (*p* = .038). In the *TLR6* wild-type group, in contrast, salivary hBD-2 concentrations were higher than those in the *TLR6* heterozygote and homozygote group (*p* = .028) (Table 3). A simultaneous presence of *TLR1/TLR2* or *TLR2/TLR6* genotypic variants did not affect salivary hBD 1–3 or HNP-1 concentrations (Table 4).

According to linear regression analysis, the implementation of periodontal status, age, and smoking in the regression model diminished the association between *TLR2* variants and salivary hBD-2 concentrations. The association between *TLR6* variants and salivary hBD-2 concentrations remained significant after adjustments (Table 5).

4 | DISCUSSION

It was previously demonstrated that the simultaneous presence of microbial pattern recognition receptor polymorphisms and the colonization of periodontitis-associated bacteria is associated with

periodontitis (Gursoy et al., 2016; Liukkonen et al., 2017), but the possible mechanism behind this outcome was not explained. To our knowledge, this study is the first to demonstrate a relationship between *TLR* polymorphisms and salivary hBD concentrations. Based on the current findings, *TLR2* (rs5743708) and *TLR6* (rs5743810) polymorphisms influence salivary concentration of hBD-2; however, only *TLR6* has an independent association with hBD-2 concentrations.

Genotype frequencies of *TLR1* (wild type 67.8%), *TLR2* (wild type 95.5%), and *TLR6* (wild type 28.7%) were almost identical to the frequencies reported in another Finnish population study (*TLR1* wild type 70%, *TLR2* wild type 95%, and *TLR6* wild type 28.7%) (Nuolivirta et al., 2013). Genotype variations between populations have been previously described for *TLR1* (rs5743618) (Barreiro et al., 2009). According to Nuolivirta et al. (2013), low (<3%) *TLR2* (rs5743708) and common (~50%) *TLR6* (rs5743810) frequencies are general among European populations. The outline of the present study was hBD 1–3 and HNP-1 concentrations in saliva. Further studies are needed to demonstrate disturbances of the hBD 1–3 and HNP-1 activation at mRNA and protein levels in individuals with different *TLR* polymorphisms.

Expressions of gingival hBDs are regulated by infectious or inflammatory stimuli (Özdemir et al., 2020). The activation of various *TLRs*, such as *TLR2*, *TLR3*, and *TLR4*, has been linked to the hBD2 secretion in uterine, vaginal, or in bronchial epithelial cells (Fruitwala et al., 2019). It is notable that the functioning receptors and pathways related to hBD response of epithelial cells vary in different tissues and demonstrate a cell-type dependency (Fruitwala et al., 2019). In periodontal tissues, *F. nucleatum* stimulates gene and protein expressions of hBD-2 via its protein FAD-I through *TLR1/TLR2* and *TLR2/TLR6* heterodimerizations (Bhattacharyya et al., 2016). These heterodimers are thought to be pre-formed on the cell surface (Oliveira-Nascimento et al., 2012). In the present study, we hypothesized that single or simultaneous polymorphisms in *TLR1*, *TLR2*, or *TLR6* genes lead to alterations in pattern recognition and hBD activation. There is some evidence that *TLR2* polymorphisms are linked to the extent and severity of urinary tract infections or rheumatoid arthritis; however, these relations are prone to confounding factors, such as ethnicity, environmental exposures, or level of infection (Corr & O'Neill, 2009). These assumptions are in line with our study, where the association observed between the *TLR2* polymorphism and

hBD-2 concentration in saliva was disrupted when the confounding factors (periodontal status, age, and smoking) were included in the analyses. Although this observation does not rule out the effect of the *TLR2* genotype on the hBD-2 protein profile, it provides evidence that the hBD-2 expression profile is multifactorial. A few studies exist in the literature as regards to the association between TLR activation in neutrophils and their HNP expression. Over-expressions of genes *TLR2* and *TLR4* were related to suppressed gene expression of *hBD-1*, *hBD-2*, and *HNP-1* in children with adenoid hypertrophy (Gankovskaya et al., 2018). Surprisingly, it has been shown that Vitamin D increased pneumococcal killing of neutrophils by stimulating the HNP protein expression via *TLR2* activation (Subramanian et al., 2017). Although these findings indicate that the TLR-mediated HNP expression is dependent on the level of TLR activation, it is still unclear how much various *TLR* polymorphisms may modify the HNP production and secretions by neutrophils.

A novel finding in our study was the association between *TLR6* polymorphism and salivary hBD-2 concentration. hBD-2 expression in human colorectal adenocarcinoma cells is regulated by *TLR6* (Vora et al., 2004). In primary human oral epithelial cells, hBD-2 expression stimulated by FAD-I was mediated by *TLR2/TLR6* heterodimerization, but the role of *TLR6* alone in hBD expression was not defined (Bhattacharyya et al., 2016). There are controversial results in terms of hBD levels in saliva samples collected from periodontitis patients from whom elevated, steady, or suppressed hBD levels were reported (Yilmaz et al., 2020). One explanation could be the effect of *TLR6* polymorphisms on hBD-2 concentrations, as this polymorphism is widely (around 50%) present in populations. Another explanation may be due to the different bacterial profile in individuals with *TLR6* polymorphisms, indirectly affecting salivary hBD-2 concentrations. Unfortunately, the present study design does not allow us to test these potential explanations or to clarify the connection between hBD-2 expression and *TLR6* pattern recognition.

In conclusion, while *TLR2* (rs5743708) and *TLR6* (rs5743810) polymorphisms relate to salivary hBD-2 concentration, only *TLR6* (rs5743810) polymorphism has an independent association with it. Further studies are required to examine this association in terms of the bacterial colonization profiles, dysbiosis, and initiation of periodontal disease.

AUTHOR CONTRIBUTIONS

Mervi Gürsoy conducted the statistical analyses and analysed the results with co-authors, wrote the first drafts, and prepared the final manuscript with the co-authors. Eija Könönen prepared the research design and research plan in collaboration with the third and last authors and prepared the final manuscript with the first author. Qiushui He prepared the research design and research plan in collaboration with the second and last author, analysed the results with the first author, and prepared the final manuscript with the first author. Anna Liukkonen analysed the results with co-authors. Sisko Huuonen analysed the radiographic results with co-authors and prepared the final manuscript with the first author. Ulvi Kahraman Gürsoy prepared the research design and research plan in collaboration with the

authors, analysed the results with the first author, and prepared the final manuscript with the first author.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All participants were diagnosed in accordance with the ethical standards of the Ethical Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland and with the 1964 Helsinki declaration and its later amendments.

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