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, TLR2, and TLR6 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 TLR1 (rs5743618), TLR2 (rs5743708), and TLR6 (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 TLR1, 95.5% and 4.5% for TLR2, and 25.1% and 74.9% for TLR6, respectively. The TLR2 heterozygote variant group exhibited higher salivary hBD-2 concentrations than the TLR2 wild-type group (p = .038). On the contrary, elevated hBD-2 concentrations were detected in the TLR6 wild-type group compared with the TLR6 heterozygote and homozygote variant group (p = .028). The associations between TLR6 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 TLR2 and TLR6 polymorphisms, but only the TLR6 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.
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 (intra-cellular 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 & Frederick, 2005; Gursoy, Könönen, Luukkonen, & Ulitto, 2013). hBD expression can be activated by bacteria, microbial molecules, and an inflammatory stimulus via TLRs and NOD-like receptors (Frutwala 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, Huumonen, 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 criterion. 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, Huumonen, 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

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.
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 assay.

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**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

|                    | TLR1 wild type (G/G) (n = 147) | TLR1 G/T and T/T variants (n = 74) | p  | TLR2 wild type (C/C) (n = 213) | TLR2 C/T variant (n = 10) | p  | TLR6 wild type (C/C) (n = 55) | TLR6 CT and T/T variants (n = 164) | p  |
|--------------------|---------------------------------|-----------------------------------|----|-------------------------------|--------------------------|----|-------------------------------|-----------------------------------|----|
| Age (mean ± SD)    | 48.7 ± 5.3                      | 48.8 ± 5.7                        | .921| 48.8 ± 5.4                    | 47.2 ± 4.9               | .376| 48.9 ± 5.9                    | 48.5 ± 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 teeth (mean ± SD) | 26.8 ± 2.4                     | 27.2 ± 2.4                       | .206| 26.9 ± 2.4                    | 27.5 ± 2.4               | .449| 27.2 ± 2.2                    | 26.9 ± 2.5                        | .484|
| No. of teeth with PPD ≥ 4 mm (mean ± SD) | 8.3 ± 9.2                      | 8.1 ± 8.7                        | .835| 8.3 ± 9.0                     | 4.6 ± 6.1                | .199| 6.4 ± 8.6                     | 8.9 ± 9.2                          | .083|
| No. of bleeding sextants (mean ± SD) | 2.95 ± 2.33                    | 3.28 ± 2.45                      | .338| 3.08 ± 2.35                   | 2.20 ± 2.78              | .349| 2.53 ± 2.35                   | 3.20 ± 2.37                        | .069|
| Total alveolar bone loss (mm) (mean ± SD) | 17.4 ± 30.5                   | 19.3 ± 31.4                       | .667| 18.1 ± 31.1                   | 13.8 ± 15.0              | .660| 12.9 ± 19.3                    | 19.3 ± 33.6                        | .177|

**TABLE 2** Frequencies of toll-like receptor (TLR)1, 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 |
|--------------------|-----------------------------------|-------------------------------------|--------------------------------------|---------|
| TLR1 wild type (G/G) (%) | 69.2                              | 64.5                                | 65.4                                 | .814^a  |
| TLR1 G/T and T/T variants (%) | 30.8                              | 35.5                                | 34.6                                 |         |
| TLR2 wild type (C/C) (%) | 91.1                              | 98.4                                | 97.5                                 | .063^a  |
| TLR2 C/T variant (%) | 8.9                                | 1.6                                 | 2.5                                  |         |
| TLR6 wild type (C/C) (%) | 29.5                              | 28.3                                | 18.5                                 | .223^a  |
| TLR6 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.

^a Chi-square test.

^b Kruskal–Wallis test.
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 ≥ 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 ≥ 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

| TLR1 wild type (n = 147) | TLR2 wild type (n = 213) | TLR6 wild type (n = 164) |
|--------------------------|--------------------------|--------------------------|
| hBD-1 (pg/ml)            | hBD-2 (pg/ml)            | HNP-1 (pg/ml)            |
| 3383 (1254–10,800)       | 59.6 (10–1575)           | 91.99 (17.4–361)         |
| 3468 (1316–10,800)       | 66.2 (10–1575)           | 95.7 (17.4–361)          |
| 3568 (977–11,000)        | 71.7 (10–1575)           | 94.6 (17.4–361)          |

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

GÜRSOY ET AL.
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

|                | TLR1 and TLR2 wild type (n = 139) | Being heterozygote or homozygote for either TLR1 or TLR2 (n = 82) | p     | TLR2 and TLR6 wild type (n = 51) | Being heterozygote or homozygote for either TLR2 or TLR6 (n = 167) | p   |
|----------------|-----------------------------------|---------------------------------------------------------------|-------|---------------------------------|---------------------------------------------------------------|-----|
| 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 4: Unadjusted and adjusted associations of salivary human beta-defensin-2 concentrations with TLR2 and TLR6 genotype variants

|                | Unadjusted | Model 1 | Model 2 | Model 3 |
|----------------|------------|---------|---------|---------|
| TLR2           | B = 0.409  | B = 0.372| B = 0.312| B = 0.292|
| p = .050       | p = .073   | p = .123| p = .151|
| TLR6           | B = −0.218 | B = −0.204| B = −0.221| B = −0.210|
| p = .034       | p = .046   | p = .026| p = .036|

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

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

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 ≥4 mm, whereas higher hBD-3 concentrations were measured only in the localized periodontitis group (PPD ≥4 mm at two or seven teeth) in comparison to the controls (no teeth with PPD ≥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 (Gurosoy 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 TRL6 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 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. Elja 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. Qiu-shui 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 Huumonen 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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