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Single nucleotide polymorphisms of pattern recognition receptors and chronic periodontitis

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Background and Objective: Periodontitis is a multifactorial disease influenced partly by genetics. Activation of pattern recognition receptors (PRRs) can lead to the up-regulation of inflammatory pathways, resulting in periodontal tissue destruction. Hence, functional polymorphisms located in PRRs can explain differences in host susceptibility to periodontitis. This study investigated single nucleotide polymorphisms of PRRs including toll-like receptor (*TLR*)*2* (G2408A), *TLR4* (A896G), *TLR9* (T1486C), *TLR9* (T1237C) and *CD14* (C260T) in patients with chronic periodontitis and in periodontally healthy subjects.

Methods: One-hundred and fourteen patients with chronic periodontitis and 77 periodontally healthy subjects were genotyped using TaqMan® allelic discrimination assays. Fisher's exact test and chi-square analyses were performed to compare genotype and allele frequencies.

Results: The frequency of subjects with the CC genotype of *CD14* (C260T) (24.6% in the chronic periodontitis group vs. 13% in the periodontally healthy group) and those expressing the T allele of *CD14* (C260T) (CT and TT) (75.4% in the chronic periodontitis group vs. 87% in the periodontally healthy group) was statistically different among groups ($p = 0.04$). Homozygocity for the C allele of the *CD14* (C260T) polymorphism (CC) was associated with a two–fold increased susceptibility to periodontitis ($p = 0.04$; odds ratio, 2.49; 95% confidence interval, 1.06–6.26). Individuals with the CC genotype of *TLR9* (T1486C) (14.9% in the chronic periodontitis group vs. 28.6% in the periodontally healthy group) and those expressing the T allele of *TLR9* (T1486C) (CT and TT) (85.1% in the chronic periodontitis group vs. 71.4% in the periodontally healthy group) were also significantly differently distributed between groups without adjustment ($p = 0.03$). Further analysis of nonsmokers revealed a significant difference in the distribution of genotypes between groups for *TLR9* (T1486C; $p = 0.017$) and *CD14* (C260T; $p = 0.03$), polymorphisms again without adjustment.

Conclusion: The CC genotype of *CD14* (C260T) is related to susceptibility to chronic periodontitis in Caucasians. In addition, differences observed in the distribution of *TLR9* (T1486C) genotypes between groups warrant further investigation.

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Periodontal diseases occur as a result of infection with gram-negative, anaerobic pathogenic bacteria that trigger destructive host immune responses (1,2). Variations in host susceptibility and clinical presentation of periodontal disease between individuals can partly be explained by genetic variation (3,4), and recent investigations have focused on identifying genetic markers that may modify immune responses and subsequently make individuals more susceptible to periodontal disease (5).

The host immune system responds to invading pathogens by detecting conserved pathogen-associated molecular patterns (PAMPs) through an array of receptors called pattern recognition receptors (PRR), including toll-like receptors (TLRs) and CD14. Engagement of PRRs with their ligands sets off a cascade of inflammatory reactions which, if not balanced, may exacerbate chronic inflammatory processes and possibly periodontal diseases (6). Single nucleotide polymorphisms (SNPs) in toll receptor and CD14 genes have been identified and found to be associated with various forms of infectious and immune diseases (7). Hence, PRR genes that are involved in the regulation of inflammatory processes represent plausible candidates for the elucidation of the genetic background of periodontal disease.

TLR4 is a transmembrane receptor with a leucine-rich extracellular domain and an intracellular domain with high homology to the interleukin-1 receptor. TLR4 was the first toll protein described in mammals and functions mainly as a receptor for bacterial lipopolysaccharide (LPS) (8). The *TLR4* gene is located on chromosome 9q32-q33. A missense mutation in the coding region of the *TLR4* gene, *TLR4* (A896G), is associated with hyporesponsiveness to LPS (9–11). The G allele of the *TLR4* (A896G) polymorphism is associated with an increased risk of urinary tract infection in children (12), impaired responses to respiratory syncytial virus bronchiolitis (13,14) and an altered chronic course of sarcoidosis (15). While some studies reported a positive association between chronic periodontitis and the *TLR4* (A896G) polymorphism (16,17), others failed to find any associations (18,19). One study reported a decreased risk for aggressive periodontitis associated with the *TLR4* (A896G) polymorphism (20), while another study reported that gingival epithelial cells heterozygous for the *TLR4* (A896G) polymorphism are hyporesponsive to *Porphyromonas gingivalis* infection (21). A recent meta-analysis of the studies investigating the association of TLR4 and periodontal disease revealed that the G allele of the *TLR4* (A896G) SNP may contribute to increased susceptibility to chronic periodontitis (22).

CD14 is a 55-kDa glycoprotein that forms a complex with LPS and can be found in two forms – soluble CD14 and membrane-bound CD14 – and is mainly expressed on the surface of myeloid cells, especially on monocytes and macrophages. CD14 lacks transmembrane and cytoplasmic domains and therefore does not elicit intracellular signalling, but can form a complex with LPS and LPS-binding protein (LBP), playing a role as a co-receptor for TLRs. Binding of the LPS/LBP/CD14 complex to TLR4 triggers the downstream events in LPS signalling, resulting in the activation of nuclear factor-kappaB followed by the transcription of various proinflammatory cytokine genes (23). As well as its role in LPS-mediated signalling, CD14 also plays role in the recognition of PAMPs from mycobacteria and viruses and participates in signalling events involving TLR2 (24). The gene for the CD14 receptor is located on chromosome 5q31 and consists of a 3900-bp region organized into two exons encoding a protein of 375 amino acids. The SNP (C260T) is located in the promoter region of the *CD14* receptor gene and is associated with enhanced transcriptional activity leading to higher serum CD14 levels (25). The TT genotype of the *CD14* (C260T) SNP and the G allele of the *TLR4* (A896G) polymorphism were found to be associated with atherosclerosis (26). The T allele of the *CD14* (C260CT) polymorphism was also associated with reduced atopy (27). With regards to periodontal disease susceptibility, the *CD14* (C260T) polymorphism has been associated with chronic severe periodontitis in Caucasian subjects of north European origin, with 90% of patients vs. 72% of controls expressing the C allele (28). While one study reported an association of the C allele of the *CD14* (C260T) polymorphism with chronic periodontitis in female patients (29), another study reported an association between chronic periodontitis and the TT genotype of the *CD14* (C260T) polymorphism (19). No association of the *CD14* (C260T) SNP with periodontal disease was found in west European Caucasians (20) or in Japanese people (30). However, increased expression of the T allele in younger Japanese patients with periodontitis prompted the authors to suggest that carriage of the T allele may be a predisposing factor for early disease activity in this population (30). Again, no association of the *CD14* (C260T) polymorphism and chronic periodontitis in a Finnish population could be found, but the investigators reported a composite genotype effect of the T allele of the *CD14* (C260T) SNP and the G allele of the interleukin-6 (C174G) SNP in the susceptibility to periodontal diseases (31). Other studies also reported inconsistent results for the distribution of *CD14* (C260T) genotypes in periodontal disease (32–34).

TLR2 is involved in both gram-positive and gram-negative PAMP recognition (6). The major PAMPs associated with periodontal pathogens that elicit signalling through TLR2 include LPS and fimbriae from *P. gingivalis* (35,36), the BspA protein of *Tannerella forsythia* (37), as well as bacterial lipoproteins. The polymorphism *TLR2* (G2408A) is located within the coding region of the receptor, resulting in an amino acid change: arginine to glycine at position 753. The *TLR2* (G2408A) polymorphism has been associated with increased susceptibility to recurrent bacterial infections and rheumatic fever in children (38,39) and increased susceptibility to tuberculosis (40) as well as increased risk for preterm birth (41) and acute reactive arthritis (42). So far, studies investigating the effect of the *TLR2* (G2408A) polymorphism in periodontal disease

have failed to identify any association (18,43).

TLR9 represents another member of the toll-receptor family and is mainly involved in bacterial DNA recognition (44). More than one pathogen is implicated in the aetiology of periodontal disease, and the possible role of periodontal bacterial DNA as a PAMP initiating inflammatory responses in periodontal disease pathology has been suggested (45,46). Increased expression of *TLR9* has also been reported in periodontally diseased sites compared with gingivitis sites, implicating a role of TLR9-mediated immune responses in periodontal disease pathogenesis (47,48). The *TLR9* gene is located on chromosome 3p21.3, spanning approximately 5 kb and with two exons. In the *TLR9* gene-promoter region, two polymorphisms (T1486C) and (T1237C), were identified that create new binding sites for Sp-1 and nuclear factor-kappaB, respectively (49). *TLR9* polymorphisms were shown to be associated with susceptibility to asthma (50), Crohn's disease (51,52), atopic eczema (53), pulmonary tuberculosis (54), puerperal group A streptococcal sepsis (55), Hodgkin's lymphoma (56) and systemic lupus erythematosus (57). So far, only one study has investigated the involvement of *TLR9* polymorphisms in periodontal disease which reported increased expression of *TLR9* haplotypes (T1486/T1237) combined with the A allele of the *TLR2* (A2848G) SNP in chronic periodontitis in Caucasians (58).

There is no doubt that PRRs play major role in periodontal disease pathogenesis and that it is likely that functional polymorphisms in *PRR* genes may modify periodontal inflammation leading to either a resistant or a susceptible phenotype. To date, studies investigating the role of *TLR2*, *TLR4* and *CD14* gene polymorphisms in the periodontal disease process are not in agreement. This may be a result of the racial or geographical differences in the study populations or because of the low frequency of some *TLR* polymorphisms in the general population. *TLR9*, however, has not been adequately evaluated as to date there is only one report that has investigated the frequency of SNPs in the *TLR9* gene in periodontitis. The aim of this study was to determine the frequency of SNPs in *TLR2*, *TLR4*, *TLR9* and *CD14* genes in patients with chronic periodontitis and in periodontally healthy Caucasian subjects. Our results provide further evidence for the involvement of specific SNPs in various PRRs in periodontal disease susceptibility.

Material and methods

Study population and experimental design

Patients and control subjects were previously recruited to participate in studies involving genetic analysis to elucidate the role of the genetic background in periodontal disease in the Periodontal Disease Research Centers at the State University of New York at Buffalo and Virginia Commonwealth University. The protocol for genetic analysis was approved by an Institutional Review Board in both universities, and all subjects had signed consent forms to participate in a study involving genetic analysis. Clinical measurements included dental plaque index, periodontal pocket depth, bleeding on probing and clinical attachment level.

A total of 191 subjects with available DNA samples enrolled in this study. Subjects were assigned as having chronic periodontitis if they exhibited at least four periodontal pockets of ≥ 5 mm that bled on gentle probing and at least one pocket of ≥ 7 mm. A periodontally healthy reference group consisted of individuals who did not have any periodontal pockets of > 3 mm. Individuals were excluded who had, currently or previously, any systemic conditions including cancer, cardiovascular disease, any renal or hepatic disorders, diabetes, severe asthma, oral candidiasis or HIV. Also excluded were those with any systemic conditions requiring use of nonsteroidal anti-inflammatory drugs or any immunosuppressive drugs, and who used antibiotics, steroids or nonsteroidal anti-inflammatory drugs within 3 wk before the baseline examination, or who were pregnant or lactating. The smoking status was classified as current, former and nonsmoker. Subjects who had quit smoking 5 years or more before baseline were assigned to the nonsmoker group and the former smoker group included those who had quit smoking within 5 years.

Genetic analysis

Genotyping was performed on DNA samples that had been previously extracted from whole blood and stored at −70°C, for the following genes: *TLR2* (G2408A) [SNP identification (ID): rs5743708], *TLR4* (A896G) (SNP ID: rs4986790), *TLR9* (T1237C) (SNP ID: rs5743836), *TLR9* (T1486C) (SNP ID: rs187084) and *CD14* (C260T) (SNP ID: rs2569190). All screening methods were PCR based using TaqMan® allelic discrimination Assay-by-Design^{SM} SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Specific primers and probes were predesigned using the Primer Express program (Applied Biosystems) and labelled with VIC and FAM fluorescein dyes. PCR was performed using a Real Time thermocycler AB 7500 (Applied Biosystems) in a volume of 20 μL containing 2 μL of DNA (2–4 ng/μL), 13 μL of PCR mix containing primers, probes and 2× TaqMan Universal MasterMix, No AmpErase UNG (Applied Biosystems) and 5 μL of H_2O, following the manufacturer's instructions. The PCR conditions were as follows: a hold step at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and an annealing and extension step at 60°C for 1 min. Fluorescein signals were determined after completion of PCR amplification and, based on the signals generated from each well, specific alleles were determined using ABI PRISM Sequence Detection System (SDS) software. Positive controls with known genotypes, and negative controls without DNA template, were included in each PCR run. To further validate the results obtained from real-time analysis, PCR-restriction fragment length polymorphism analyses were performed on selected samples, following published protocols (19,59).

Statistical analysis

Fisher's exact test and chi-square analyses were performed to compare genotype and allele frequencies. The chi-square test was employed if the homozygote and heterozygote frequencies for each SNP deviated from Hardy–Weinberg equilibrium. As there were differences between the groups in the prevalence of smoking, gender distribution and age distribution, logistic regression was used to adjust the significance levels for these factors.

The meta-analysis of the *CD14* (C260T) polymorphism was conducted using Comprehensive Meta Analysis Version 2.0 (Biostat, Englewood, NJ, USA). A comprehensive literature search (until May 2010) was carried out on MEDLINE with language restriction to English using the key words 'CD14 polymorphism' and 'periodontitis'. Out of 19 manuscripts that met the criteria, 10 were included based on the following inclusion criteria: original investigations; and cross-sectional studies investigating the association of the *CD14* polymorphism with chronic periodontitis and aggressive periodontitis. Random-effect methods were used for the analysis. We calculated the specific odds ratios along with their 95% confidence intervals to compare the distribution of alleles and genotypes between cases and controls.

Results

The characteristics of the study population are shown in Table 1. There was a statistically significant difference between groups regarding age and smoking status, and therefore all analyses were adjusted taking into account these differences as well as gender. Distribution of specific genotypes and alleles among groups are presented in Table 2. There was no significant difference in the frequency of specific genotypes among groups; however, the distribution of genotypes was marginally different for the *TLR9* (T1486C) polymorphism among groups ($p = 0.06$) (Table 2). We then investigated the differences based on allelic expression. Table 3 summarizes the distribution of *TLR9* (T1486C) and *CD14* (C260T) polymorphisms among the diseased group (chronic periodontitis) vs. the healthy group (periodontally healthy), comparing homozygocity for the C allele of both SNPs (CC genotype) with the T-allele carrier status (CT and TT). The frequency of subjects who were homozygous for the C allele of *CD14* (C260T) (CC) (24.6% in the chronic periodontitis group vs. 13% in the periodontally healthy group) and those expressing the T allele of *CD14* (C260T) (CT and TT) (75.4% in the chronic periodontitis group vs. 87% in the periodontally healthy group) was statistically different among groups after adjusting for confounding factors ($p = 0.04$) (Table 3). Individuals who were homozygous for the C allele of the *TLR9* (T1486C) SNP (CC) (14.9% in the chronic periodontitis group vs. 28.6% in the periodontally healthy group) and who expressed the T allele of the *TLR9* (T1486C) (CT and TT) (85.1% in the chronic periodontitis group vs. 71.4% in the periodontally healthy group) were also significantly differently distributed between groups without adjustment ($p = 0.03$); however, the significance did not remain after adjusting for age, gender and smoking status ($p = 0.06$) (Table 3). Further analyses of the association of *CD14* (C260T) polymorphisms with periodontal disease revealed that being homozygous for the C allele of the *CD14* (C260T) polymorphism was associated with increased susceptibility to periodontitis ($p = 0.04$; odds ratio, 2.49; 95% confidence interval, 1.06–6.26) (Table 3). There are conflicting reports regarding the association of the *CD14* (C260T) polymorphism with periodontal disease. Therefore, we also performed a meta-analysis including the studies that investigated the association of the *CD14* (C260T) polymorphism with periodontal disease. While our results were consistent with a number of previously published studies that reported an increased frequency of the C allele in subjects with periodontal disease compared to healthy controls, the results of the meta-analysis did not determine any statistically significant differences in the distribution of genotypes of the *CD14* (C260T) polymorphism among patients with chronic periodontitis vs. periodontally healthy subjects ($p = 0.08$) (Table 4). Our analyses using a subgroup of nonsmokers from each group (chronic periodontitis and periodontally healthy) revealed a significant difference in the distribution of genotypes between chronic periodontitis and periodontally healthy groups for *CD14* (C260T) ($p = 0.03$) (Table 5) and *TLR9* (T1486C) ($p = 0.017$) (Table 6) polymorphisms but the significance was lost after adjusting for confounders (Tables 5 and 6). Consistent with the previous reports, the presence of polymorphic alleles for certain genes was low in the study population. There were no individuals homozygous for the G allele of *TLR4* (A896) and the G allele of *TLR2* (G2408A) SNPs in our study population (Table 2). All genotyping was performed using real-time PCR and the results were further confirmed by

Table 1. Characteristics of the study population

	Chronic periodontitis ($n = 114$)	No periodontitis ($n = 77$)	*p*-value
Mean age (yr)	52.9 ± 1.0	45.7 ± 1.7	< 0.0003
Gender (female)	56 (49)	34 (45)	NS
Smoking status			< 0.0001
Current	40 (35)	4 (5)	
Former	31 (27)	17 (22)	
Never	43 (37)	56 (73)	
Mean probing depth (mm)	3.22 ± 0.1	1.79 ± 0.0	< 0.0001
Mean attachment loss (mm)	5.8 ± 0.1	1.1 ± 0.1	< 0.0001
Mean plaque index	1.46 ± 0.06	0.40 ± 0.03	< 0.0001

Data are given as *n* (%) or as mean ± standard error.
NS, not significant.

Table 2. Genotype and allele frequency of toll-like receptor *TLR4*, *TLR2*, *TLR9* and *CD14* gene polymorphisms in patients with chronic periodontitis and periodontally healthy subjects

Polymorphism	Genotype	Chronic periodontitis *n* (%) (*n* = 114)	Periodontally healthy *n* (%) (*n* = 77)	Allele	Chronic periodontitis 2n (%) (*n* = 228)	Periodontally healthy 2*n* (%) (*n* = 154)	*p*-value
TLR4 (A896G)	AA	95 (83.3)	59 (76.6)	A	209	135 (87.6)	NS
	AG	19 (16.6)	17 (22.1)	G	(91.6)	19 (12.4)	
	GG	0 (0.0)	1 (1.3)		19 (8.3)		
TLR2 (G2408A)	GG	105 (92.1)	96.1 (74)	G	219 (96)	151 (98)	NS
	GA	9 (7.9)	3.9 (3)	A	9 (4)	3 (2)	
	AA	0 (0.0)	0.0				
TLR9 (T1486C)	TT	38.6 (44)	36.4 (28)	T	141 (61.8)	83 (53.8)	0.06
	TC	46.5 (53)	35.1 (27)	C	87 (38.2)	71 (46.2)	
	CC	14.9 (17)	28.6 (22)				
TLR9 (T1237C)	TT	71.1 (81)	72.7 (56)	T	193	131 (85)	NS
	TC	27.2 (31)	24.7 (19)	C	(84.6)	23 (15)	
	CC	1.8 (2)	2.6 (2)		35 (15.4)		
CD14 (C260T)	CC	24.6 (28)	13.0 (10)	C	117 (51.3)	68 (44.1)	NS
	CT	53.5 (61)	62.3 (48)	T	111 (48.6)	86 (55.9)	
	TT	21.9 (25)	24.7 (19)				

NS, not significant.

Table 3. Frequency of the CC genotype toll-like receptor (*TLR*)*9* (T1486C) and *CD14* (C260T) polymorphisms vs. expression of the T allele (CT and TT) among patients with chronic periodontitis and periodontally healthy subjects

Polymorphism	Genotype	Chronic periodontitis (%) (*n* = 114)	Periodontally healthy (%) (*n* = 77)	*p*-value	Odds ratio (95% confidence interval)[b]
TLR9 (T1486C)	CC	14.9	28.6	0.03[a]	0.48 (0.20–2.08)
	CT and TT	85.1	71.4	0.1[b]	
CD14 (C260T)	CC	24.6	13.0	0.06[a]	
	CT and TT	75.4	87.0	0.04[b]	2.49 (1.06–6.26)

[a] Bivariate.
[b] Adjusted for age, race and smoking.

restriction fragment length polymorphism analyses of selected samples. Figure 1 is an agarose gel showing different genotypes of the *CD14* (C260T) polymorphism after digestion with the restriction enzyme *Hae*III.

Discussion

An expanding body of evidence implicates the importance of PRRs in periodontal diseases. Here we report genotype and allele frequencies of different PRRs, including *TLR2*, *TLR4*, *TLR9* and *CD14*, in Caucasians with chronic periodontitis and in periodontally healthy subjects. The only SNP that holds significant association with periodontal disease after adjusting for the confounding factors is the C allele of the *CD14* (C260T) polymorphism. In addition, a marginal difference was observed in the distribution of *TLR9* (T1486C) genotypes between chronic periodontitis and periodontally healthy subjects.

In our study, the frequency of the subjects who were homozygous for the C allele of *CD14* (C260T) (CC) (24.6% in the chronic periodontitis group vs. 13% in the periodontally healthy group) and those expressing the T allele of *CD14* (C260T) (CT and TT) (75.4% in the chronic periodontitis group vs. 87% in the periodontally healthy group) was statistically different among groups ($p = 0.04$), and being homozygous for the C allele of the *CD14* (C260T) polymorphism was associated with a twofold increased susceptibility to the disease ($p = 0.04$, odds ratio, 2.49; 95% confidence interval, 1.06–6.26) (Table 3). The *CD14* (C260T) polymorphism is located in the promoter region of the gene within the Sp1 transcription factor-binding site. The T allele has been shown to cause increased transcriptional activity, resulting in the up-regulation of soluble CD14 levels by decreasing the affinity of Sp-1 (60). Although it is plausible to assume that T-allele carriage would increase susceptibility for periodontal disease by up-regulating CD14 expression, a number of previous studies reported increased frequency of the C allele in periodontitis patients (Table 4), and the distribution of specific CD14 (C260T) genotypes among chronic periodontitis and periodontally healthy groups in our study population was also consistent with these investigations. Yet, some of the previous studies also reported a higher frequency of the CD14 T allele combined with other genotypes of other genes, explaining the differences in disease severity (19,31). In contrast to the hypothesis that the T allele may be associated with increased susceptibility to periodontitis by increasing soluble CD14 levels in localized tissues, there are also reports

Table 4. Meta-analysis of the studies reporting the *CD14* (C260T) polymorphism in chronic periodontitis and periodontally healthy subjects in different populations: *CD14* (C260T) homozygous CC vs. CT and TT

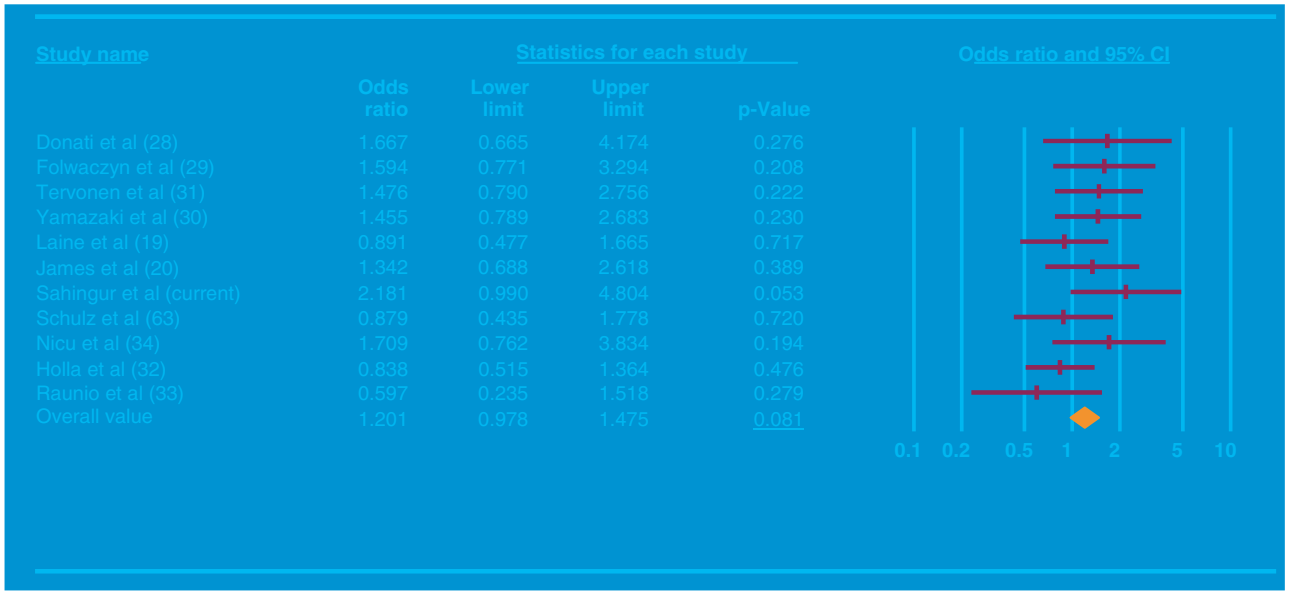

Table 5. Distribution of CD14 (C260T) genotypes in non-smokers between periodontal disease (CP) versus periodontally-healthy subjects (NP)

Study Groups (N)	CC N (%)	CT & TT N (%)	*p* value
CP (43)	15 (34.9)	28 (65.1)	
NP (56)	8 (14.3)	48 (85.7)	
			$p = 0.03$ (Bivariate)
			$p = 0.09$ (*Adjusted)

*Adjusted for age, race and smoking.

Table 6. Distribution of TLR9(T1486C) genotypes in non-smokers between periodontal disease (CP) vs. periodontally-healthy subjects

Study Groups (N)	CC N (%)	CT & TT N (%)	*p* value
CP (43)	5 (11.6)	38 (88.4)	
NP (56)	19 (33.9)	37 (66.1)	
			$p = 0.01$ (Bivariate)
			$p = 0.06$ (*Adjusted)

*Adjusted for age, race and smoking.

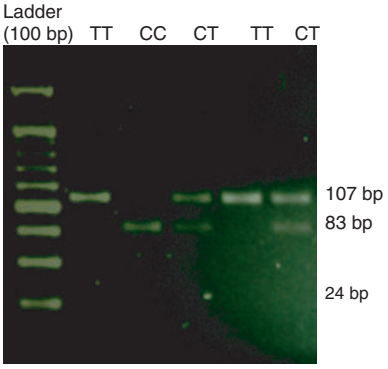

Fig. 1. Restriction fragment length analysis of the *CD14* (C260T) polymorphism. Genotyping was performed using the forward primer 5′-TCACCTCCCCACCT-CTCTT-3′ and the reverse primer 5′-CCT-GCAGAATCCTTCCTGTT-3′. PCR amplification was performed as follows: cycle 1 (×1), 94°C for 2 min; followed by cycle 2 (×35), 94°C for 20 s, 60°C for 20 s and 68°C for 1 min. *Hae*III (Fermentas) digestion of the PCR products resulted in two fragments of 83 bp and 24 bp for the C allele and 107 bp for the T allele. The 4% agarose gel shows CC, CT and TT genotypes of the *CD14* (C260T) polymorphism.

which demonstrated decreased expression of membrane-bound CD14 and of soluble CD14 that were associated with advanced attachment loss (61,62). Furthermore, reduced expression of CD14 on monocytes was also associated with increased susceptibility for aggressive periodontitis (63). Hence, it is likely that increased expression of CD14, associated with the presence of the T allele, may be protective in periodontal disease and this may explain the significantly higher prevalence of the CC genotype in the periodontitis group and T-allele carriage in control subjects reported in previous publications, as well as in this study (Table 4). In addition, decreased prevalence of *Prevotella intermedia* in patients expressing the TT genotype further supports the notion that T-allele carriage may be protective in periodontitis

(64). Considering the variations in different studies regarding the distribution of specific genotypes of *CD14* polymorphism in periodontitis, we also performed a meta-analysis that failed to identify any statistically significant differences in the distribution of genotypes of the *CD14* (C260T) polymorphism ($p = 0.08$) (Table 4). However, the inconsistent findings among studies warrant further investigation.

There was no significant difference in the frequency of *TLR2*, *TLR4* and *TLR9* genotypes among groups in this study; however, the distribution of genotypes was marginally different for the *TLR9* (T1486C) polymorphism among groups ($p = 0.06$) (Table 2). Further analyses, based on allelic expression, revealed differences in the distribution of *TLR9* (T1486C) alleles among groups without adjustment ($p = 0.03$) (Table 3). Analysis that included nonsmoking subjects again revealed a significant difference in the distribution of genotypes between chronic periodontitis and periodontally healthy groups for *TLR9* (T1486C) ($p = 0.017$) (Table 6) without adjusting for confounders. *In silico* analysis showed that the *TLR9* (T1486C) polymorphism is located within the Sp-1-binding site in the promoter region of the gene (49). It was also determined that C-allele carriage is responsible for decreased transcriptional activity (57). It is possible that decreased transcriptional activity resulting in decreased expression of *TLR9* may result in a protective phenotype that explains the abundance of the CC allele in periodontally healthy subjects. *TLR9* expression is increased in periodontally diseased tissues compared with gingivitis sites (47,48), and periodontal bacterial DNA from various periodontal pathogens can trigger the production of inflammatory cytokines from human fibroblasts and monocytes as well as from mouse macrophages (45,46) mainly via *TLR9* signalling. Considering the multibacterial aetiology of periodontal diseases, it is likely that periodontal pathogenic DNA that exists in the tissues can contribute to periodontal inflammation by activating *TLR9* signalling pathways. Hence, T-allele carriage of the *TLR9* (T1486C) SNP may predispose individuals to periodontal disease by creating a phenotype that is more prone to increased expression of *TLR9*, which might lead to a hyperinflammatory response. Considering the limited amount of available data on the interaction of TLR9 with periodontal disease, this might be a little speculative. However, cross-talk between different PPRs and signalling pathways exists (65–67) and it is certainly plausible to consider that TLR9 may contribute to periodontal inflammation either individually or by interacting with other receptors and signalling pathways. In fact, TLR9 expression is increased at sites with periodontitis compared to gingivitis sites (47,48). Moreover, increased frequency of the T allele of the *TLR9* (T1486C) SNP in patients with chronic periodontitis combined with the T allele of *TLR9* (T1237C) and the G allele of *TLR2* (G2848A), was also recently reported by another group (58). Our study did not include the *TLR2* (G2848A) SNP, but we did not detect any significant combined effect of different alleles in other polymorphic sites in other genes with periodontal disease (data not shown). We believe that our results, combined with previous reports, warrant further investigations to determine the importance of *TLR9* polymorphisms and their functional relevance to periodontal disease pathology.

Periodontal diseases are multifactorial and it is highly likely that more than one genotypic locus will influence an individual's susceptibility to periodontitis. This study was designed to identify SNPs that are located at potentially important functional sites in the PRR genes which are most frequently implicated in periodontal inflammation, including *TLR2*, *TLR4* and *CD14*. *TLR9*, in contrast, although not studied as extensively as the others in periodontal disease pathology, is a newly emerging receptor implicated in several systemic conditions as well as in periodontal inflammation. So far, studies investigating the frequency of different polymorphic sites in *TLR2*, *TLR4* and *CD14* genes in periodontitis reported inconsistent results and there is only one report that investigated the involvement of the *TLR9* polymorphism in periodontal disease. The lack of replication might be a result of the differences in populations or in disease definition, or may be caused by the complex interactions between multiple population-specific genetic and environmental factors. It is important to point out that there are other PRRs, as well as accessory molecules, besides the ones included in this study, that represent potential candidates to be evaluated in genetic susceptibility to periodontitis. Currently, the availability of genome-wide arrays allows scanning of a wide range of genes and identification of their association with the disease processes. Although such methods were not employed in this study and only a limited number of genes was investigated, our results provide promising evidence to design future studies with increased sample sizes to determine genetic susceptibility to periodontitis in PRRs. Future studies will continue to improve our understanding of the relationships among PRR signalling, genetic polymorphisms and periodontal disease, revealing new approaches to modulate the biological systems that will contribute to favourable clinical outcomes.

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