

Association of *CD14-260* polymorphisms, red-complex periodontopathogens and gingival crevicular fluid cytokine levels with cyclosporine A-induced gingival overgrowth in renal transplant patients

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Background and Objective: Genetic factors may influence the colonization of pathogenic bacteria, therefore increasing the risk for the initiation and development of periodontal disease. The present study was carried out to investigate the association of *CD14-260* polymorphisms, subgingival microbiota, and gingival crevicular fluid (GCF) cytokine levels with cyclosporine A (CsA)-induced gingival overgrowth (GO) in renal transplant patients.

Material and Methods: A total of 204 patients were dichotomized into two groups: 124 with GO and 80 without GO. The *CD14-260* polymorphisms were measured using an allele-specific PCR method. The levels of periodontal pathogens were determined by real-time PCR of subgingival samples. GCF levels of IL-1 β and sCD14 were detected by ELISA.

Results: The frequency of *CD14-260* genotype CT + TT was found to be similar in both groups. Patients with GO presented increased prevalence of *Pg*, *Td*, and *Tf* (red complex) and significantly higher levels of interleukin-1 β than those without GO. Patients with GO carrying CT + TT genotypes were found to have higher frequencies of *Pg*, *Td*, and *Tf* than those carrying the CC genotype. Furthermore, in the presence of red complex, CT + TT genotypes were associated with higher interleukin-1 β levels and severe GO. Multiple logistic regression analysis demonstrated that the severity of GO is not dependent on age, gender and pharmacological variables, being only associated with *CD14-260* genotype and red complex periodontopathogens.

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Conclusion: No association between *CD14-260* polymorphisms and the prevalence of GO was revealed in renal transplant patients administered CsA. However, *CD14-260* CT + TT genotypes are associated with the prevalence of red complex periodontopathogens in patients with GO, and may thus play some role in the development of severe CsA-induced GO.

The pathogenesis of cyclosporine (CsA)-induced gingival overgrowth (GO) is multifactorial. Plaque-induced gingival inflammation has been shown to play a pivotal role in the development of CsA-induced GO (1,2). CsA may override the inhibitory effect of lipopolysaccharide on cell proliferation and maintain a capacity to stimulate fibroblast DNA synthesis (3,4). In co-cultures of gingival fibroblasts and macrophages, CsA can inhabit the activities of matrix metalloproteinase in the presence of *Porphyromonas gingivalis* (*Pg*) lipopolysaccharide, promoting abnormal accumulation of extracellular matrix components in the gingival lamina propria (5). Lipopolysaccharide induces the proliferation of periodontal epithelial cells via the CD14 and Toll-like receptor (TLR) signaling pathway (6). CsA positively regulates TLR-mediated inflammatory responses of gingival fibroblasts to microbial components, enhancing the production of proinflammatory cytokines and potentially augmenting the proliferation of gingival fibroblasts (7). These results suggest that complex interactions between the lipopolysaccharide signaling pathway and tissue metabolism might be involved in the pathogenesis of CsA-induced GO.

CD14, a pattern recognition receptor on monocyte and macrophage, plays a critical role in innate immunity through recognition of bacterial lipopolysaccharide (8). Through interaction with both CD14 and lipopolysaccharide-binding protein, signal transduction on effector cells is then transferred via the TLR/MD-2 signaling complex (9). This results in activation of the innate immune response and release of proinflammatory cytokines, such as interleukin (IL)-1 β , IL-6 and transforming growth factor- β 1, which act synergistically with CsA in

the modulation of gingival fibroblast metabolism (10–12).

A $-260C > T$ polymorphism in the *CD14* gene promoter results in decreased affinity of DNA/protein interaction at a GC box that contains a binding site for SP proteins and modulates activity of the promoter (13). Carriage of the TT genotype has been associated with significantly higher serum levels of the soluble CD14 receptor (sCD14) and an increased density of CD14 in monocytes (14). Individuals carrying the TT genotype are susceptible to developing periodontal infections induced by opportunistic pathogens (15). The carriers of the T-containing genotype of *CD14-260* (CT + TT) have been shown to have a higher extent of periodontal disease compared to those carrying the CC genotype (16), while other studies did not find associations (17,18).

The concept of periodontal infection genomics is defined as the relationship between host genetic factors and the composition of the subgingiva microbiota (19). Genetic factors may influence the colonization of pathogenic bacteria, therefore increasing the risk for the initiation and development of periodontal disease. Subjects positive for the composite *IL-1* genotype had increased counts of red and orange complex species (20). Other studies also demonstrated an association between *IL-6* gene and Fc γ receptor variants and subgingival detection of *Pg* and *Aggregatibacter actinomycetemcomitans* (*Aa*) (21–24). Therefore, we hypothesized that *CD14-260* genotype may influence the composition of subgingival microbiota, contributing to the increase of proinflammatory cytokines within gingival tissues and hence be associated with the severity of CsA-induced GO. The aim of this study

was to investigate the possible association of *CD14-260* polymorphisms, subgingival microbiota, and gingival crevicular fluid (GCF) cytokine levels of IL-1 β and sCD14 with CsA-induced GO in renal transplant patients.

Material and methods

Study population and clinical examination

The cross-sectional study was conducted in the Department of Urology and Department of Stomatology in Zhongshan Hospital, Fudan University, Shanghai, China, where renal transplant recipients were seen on a regular basis to monitor drug therapy and graft survival. The immunosuppressive therapy consisted of a triple regimen, including CsA, mycophenolate mofetil and prednisolone. At the time of the study, all patients had been followed for a minimum of 6 months. No subjects had received periodontal therapy and no subjects had taken medication known to affect periodontal status (e.g., antibiotics and anticonvulsants) within 3 months before enrolment. Radiographic examination was also carried out to detect alveolar bone destruction. Subjects receiving CsA without any sign of alveolar bone loss were selected for the present study. Demographic and pharmacologic data were obtained from the medical documentation and recorded by a single investigator. The research protocol was approved by the Ethics Committee of Fudan University.

Clinical measurements included plaque index (PLI) (25), papillary bleeding index (PBI) (26) and probing depths (PD). The subjects were assessed using a clinical scoring method according to Pernu *et al.* (27). The subjects were ascribed a general whole-mouth score of between 0 and

3: 0 = no overgrowth seen; 1 = mild GO (thickening of the marginal gingival and/or lobular granulation of the gingival pocket as well as overgrowth covering the gingival third of the crown or less); 2 = moderate GO (overgrowth extending to the middle of the crown); and 3 = severe GO (overgrowth covering two-thirds of the crown or affecting the whole attached gingival). The patients were dichotomized into a gingival overgrowth-negative (GO-) group, those with no signs of GO (score 0), and a gingival overgrowth-positive (GO+) group, those with signs of overgrowth (score 1-3) for analysis. All subjects were examined by two trained and calibrated examiners with good to excellent intra- and inter-examiner agreement.

Genotyping

DNA was extracted from epithelial buccal cells with a sequential phenol-chloroform solution and precipitated with a salt/ethanol solution. The CD14-260 polymorphisms were measured using an allele-specific PCR method, as previously described (14). Amplification reactions were performed in a total volume of 25 µL containing 50-100 ng of DNA, 1 × PCR buffer (Mg²⁺ Plus), 0.2 mM of each dNTP, 0.75 U of HotStart TaqTM DNA polymerase (Takara, Dalian, China), and 5 pmol of each primer (Invitrogen, Shanghai, China). PCR was performed in a Mastercycler 5333 PCR system (Eppendorf, Wesseling-Berzdorf, Germany) as follows: 35 cycles at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min. The final extension step was at 72°C for 5 min. The amplified samples were visualized by electrophoresis in 2% (w/v) agarose gel stained with ethidium bromide. The assay thus yielded a 227-bp band for the C allele and a 381-bp band for the T allele. In each PCR series, samples with known genotypes were also included to show that the PCR works. Whenever the results were not clear, the analysis was repeated. If the result was still uncertain after repetition, no result was recorded for that polymorphism.

Gingival crevicular fluid and subgingival plaque collection

GCF samples and subgingival plaque samples were collected from one proximal site of one tooth in the lower anterior arch from each subject after measuring PLI and before measuring PBI and PD. For GO+ patients, one site presenting moderate to severe GO (score 2-3) was chosen to be the sample site, and for GO- patients, one clinically uninfamed site without overgrowth was chosen.

Following the careful removal of all supragingival plaque, areas were washed with a water spray, isolated with cotton rolls, and gently dried for 30 s. Subgingival plaque samples were taken with one sterile paper point no. 30 that was inserted into the bottom of the periodontal pocket for 30 s. Points with blood marks were discarded. The paper points were placed in sterile polypropylene tubes containing 1.5 mL of phosphate-buffered solution.

The teeth were washed again; the area was then isolated and gently dried. GCF was collected with paper strips (Whatman International Ltd, Maidstone UK) inserted into the gingival crevice and maintained for 30 s. Strips with blood marks were discarded. The GCF flow volume was measured by weighing the strips in sterile polypropylene tubes before and after sample collection (Mettler AE240 balance, Mettler-Toledo, Greifensee, Switzerland). This method was

used as an alternative method to direct GCF volume estimation with Periotron measurement (28). The absorbed fluid was eluted from each strip into 350 µL of phosphate-buffered solution (pH 7.2). All samples (subgingival plaque and GCF) were stored at -20°C until assayed.

Microbiological evaluation

The real-time PCR method is based on amplification of variable regions of 16S rRNA genes of *Pg*, *Aa*, *Prevotella intermedia* (*Pi*), *Treponema denticola* (*Td*) and *Tannerella forsythia* (*Tf*). Species-specific primers were selected using software (Primer Premier version 5.0; Premier Biosoft International, Palo Alto, CA, USA) based on the published 16S rRNA sequences (Table 1).

Bacterial DNA was extracted as described previously (29). A primer concentration of 0.25 µM was ultimately used for the five species. Real-time PCR reaction was carried out using a Mastercycler system (Eppendorf, Wesseling-Berzdorf, Germany) with SYBR Green Mix (Ruicheng Biotech, Shanghai, China). Samples were assayed in duplicate in 25-µL reaction mixtures containing 2 µL template DNA, 0.5 µL forward primer and reverse primer, and 12.5 µL SYBR Green Mix. The cycling conditions used were (i) 95°C/15 min, 95°C/30 s, 53°C/30 s, 72°C/30 s and 40 cycles for *Pg* and *Tf*, and (ii) 95°C/15 min, 95°C/30 s, 54°C/30 s, 72°C/

Table 1. Species-specific primers used for real-time PCR

Primer	Sequence(5'-3')	Size of amplicon (bp)	Accession numbers
<i>Pg</i>	F1:GGAATAACGGGCGATACGA F2:CACCGCTGACTACCGAACA	155	X73964
<i>Aa</i>	F1:ATTGGGCATAAAGGGCATCT F2:TCGCACATCAGCGTCAGTA	204	X90833
<i>Pi</i>	F1:GCCTAATACCGATGTTGTCC F2:ACTTGGCTGGTTCAGACTTCC	237	L16468
<i>Td</i>	F1:CTGAGGACTCTGGCGGA F2:ACCGTGCTGATGTGTGCGATTA	228	D85438
<i>Tf</i>	F1:AGAGCCTGAACCGCCAAGT F2:ACAGCCCCACCTACGCACC	208	L16495

Pg, *Porphyromonas gingivalis*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Pi*, *Prevotella intermedia*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

30 s and 40 cycles for *Aa*, *Pi*, and *Td*. Melting peaks were used to determine the specificity of the PCR.

The absolute quantification of target bacteria in subgingival samples was performed using *Pg* (ATCC 33,277), *Aa* (Y4), *Pi* (ATCC 23,256), *Td* (ATCC 33,520), and *Tf* (ATCC 43,037) as controls. Standard curves were established with the controls, which could be used to convert cycle threshold values into the number of bacterial cells using controls with known amounts of bacterial-specific DNA. The level of detection was set to 10^3 bacteria/subgingival plaque sample for real-time PCR. The determination of DNA content in the controls was based on the genome size of each bacteria and the mean weight of one nucleotide pair (30,31).

Immunological analysis

GCF IL-1 β and sCD14 levels were analyzed after centrifugal elution, by using a human IL-1 β enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, CA, USA) and a human sCD14 ELISA kit (Yusen, Shanghai, China) according to the manufacturers' instructions. The results for IL-1 β and sCD14 were expressed as pg/site for total cytokine levels, which has been suggested to be a better indicator of relative GCF constituent activity than GCF volume, which might result in a decrease of the cytokine concentration (32).

Statistical analysis

A comparison of the demographic, pharmacological, and periodontal data between the two groups was made using an independent sample *t*-test, the chi-squared test, or the Mann-Whitney *U* test as appropriate.

The distribution of genotypes and allele frequencies were compared by the Fisher's exact test. Odds ratio (OR) and 95% confidence interval (95% CI) were also assessed. Genotypes were grouped according to the allele T carrier status, as this allele is known to be associated with a higher level of sCD14 (14). The Mann-Whit-

ney *U* test was used to assess statistical significance in the levels of GCF cytokine between GO status and between *CD14-260* genotypes.

The association between bacterial prevalence and GO status and *CD14-260* genotypes were analyzed by Fisher's exact test. Logarithmic transformations were performed for the amounts of bacteria to improve normality. Bacterial amounts in both groups were analyzed by the independent *t*-test. The Spearman rank correlation coefficient was used to explore associations between levels of GCF cytokines and bacterial amounts. A multiple logistic regression analysis was performed to evaluate possible associations between these variables and the severity of GO. $p < 0.05$ was considered statistically significant. All data analysis was performed using a statistical package (spss 13.0; SPSS Inc., Chicago, IL, USA).

Results

Clinical parameters and genotype profiles in groups with and without gingival overgrowth

A total of 204 renal transplant patients (132 males and 72 females)

aged 16–72 years (mean of 45.0 ± 11.0 years) were recruited for statistical analysis (three subjects were excluded because of their unclear genotype results, even after analysis was repeated). Eighty subjects were classified as score 0 of GO, 69 patients were ascribed score 1, 39 subjects scored 2 and 16 scored 3. Table 2 shows that GO+ patients presented significantly higher PLI, PBI, and PD levels compared with GO– patients ($p < 0.001$). Concomitant CCB use was also higher in GO+ patients; however, the differences were statistically not significant ($p > 0.05$). The cause of end-stage renal disease, such as glomerulonephritis, diabetes, hypertension, and chronic pyelonephritis, did not show significant differences in patients with and without GO (Table 3). The proportion of polycystic kidney disease was significantly higher in GO+ patients than in GO– patients ($p < 0.05$).

The distribution of *CD14-260* genotypes in the two groups did not differ significantly from the Hardy-Weinberg equilibrium. The frequency of genotypes CT + TT and of the allele T was found to be similar in the GO+ and GO– group (Table 4).

Table 2. Demographic, periodontal, and bacterial variables of CsA-treated patients in GO+ and GO– groups

	GO+ (n = 124)	GO– (n = 80)	p value
Age (years)	45.0 \pm 10.1	45.0 \pm 12.2	0.98
Gender distribution (F/M)	40 : 84	32 : 48	0.26
Duration of CsA therapy (years)	4.2 \pm 4.0	4.1 \pm 4.6	0.74
CsA dosage (mg/day)	190 \pm 48	189 \pm 46	0.84
CsA serum concentration (μ g/L)	705 \pm 233	764 \pm 269	0.10
Calcium channel blocker use (%)	64 (52%)	36 (45%)	0.36
PLI	1.44 \pm 0.65	0.58 \pm 0.41	< 0.001
PBI	1.21 \pm 0.75	0.42 \pm 0.36	< 0.001
PD (mm)	3.44 \pm 0.87	1.90 \pm 0.49	< 0.001
<i>Pg</i>	106 (85.5)	45 (56.3)	< 0.001
<i>Aa</i>	92 (74.2)	57 (71.3)	0.747
<i>Pi</i>	72 (58.1)	36 (45.0)	0.085
<i>Td</i>	108 (87.1)	43 (53.8)	< 0.001
<i>Tf</i>	97 (78.2)	49 (61.3)	0.011
<i>Pg</i> + <i>Td</i> + <i>Tf</i>	81 (65.3)	20 (25.0)	< 0.001

CsA, cyclosporine A; GO, gingival overgrowth; PLI, plaque index; PBI, papillary bleeding index; PD, probing depth; *Pg*, *Porphyromonas gingivalis*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Pi*, *Prevotella intermedia*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

Bold represents a significant difference ($p < 0.05$).

Table 3. Cause of end-stage renal disease in CsA-treated patients with and without GO

Cause of end-stage renal disease	GO+ (n = 124)	GO- (n = 80)	p value
Glomerulonephritis	34 (27.4)	26 (32.5)	0.437
Diabetes	38 (30.6)	27 (33.8)	0.648
Hypertension	23 (18.5)	13 (16.3)	0.711
Chronic pyelonephritis	6 (4.8)	4 (5.0)	1.000
Polycystic kidney disease	9 (7.3)	2 (2.5)	0.008
Other	14 (11.3)	8 (10.0)	

CsA, cyclosporine A; GO, gingival overgrowth.
Bold represents a significant difference ($p < 0.05$).

Association of genotype and gingival crevicular fluid cytokine levels with periodontal parameters

The total amount and concentration of GCF IL-1 β , but not sCD14, were statistically higher in the GO+ group than those in the GO- group (Fig. 1A,B). No difference were found between IL-1 β , sCD14 levels, and periodontal parameters in the different genotype of the GO- group, whereas CT + TT carriers from the GO+ group presented significantly increased GCF IL-1 β levels (Fig. 1G). We found that in the GO+ group, CT + TT carriers were also associated with higher levels of PLI, PD, and GO scores (Fig. 1C,E,F). In view of the absence of any association of sCD14 with genotype and/or clinical parameters in both groups, sCD14 was not included in the subsequent figures.

Association of periodontopathogens and gingival crevicular fluid interleukin-1 β levels with gingival overgrowth status

The frequencies of *Pg*, *Td*, and *Tf*, and the prevalence of the simulta-

neous occurrence of red complex pathogens (*Pg* + *Td* + *Tf*) were significantly more prevalent in the GO+ group than in the GO- group (Table 2). The amounts of red complex and *Pi* were found to be markedly higher in the GO+ group (Fig. 2A). In the GO+ group, the presence of red complex and *Pi* were associated with significantly high levels of GCF IL-1 β (Fig. 2B-F). The amounts of red complex and *Pi* were also positively associated with the total amounts of IL-1 β in GCF (Fig. 3).

Association of genotype, periodontopathogens, and gingival crevicular fluid interleukin-1 β levels with the severity of gingival overgrowth

In the GO- group, no differences were found in the frequency of periodontopathogens in the different *CD14-260* genotype groups. However, in the GO+ group, CT + TT genotype carriers presented higher frequencies of *Pg*, *Td*, and *Tf* and red complex (Table 5). Interestingly, in the absence of *Pg*, *Td*, and *Tf*, patients with GO bearing CT + TT genotypes showed

similar levels of GCF IL-1 β levels, whereas in the presence of *Pg*, *Td*, and *Tf*, CT + TT genotype groups presented significantly higher levels of IL-1 β (Fig. 4). In addition, in the presence of red complex, CT + TT carriers were also found to be associated with higher GO scores ($p < 0.05$) (data not shown).

For further comparison, subjects in the GO+ group were categorized as patients with mild GO (score 1) and moderate to severe GO (score 2–3). Patients with moderate to severe GO were found to have significantly higher levels of GCF IL-1 β and red complex than patients with mild GO ($p < 0.05$) (data not shown). The frequency of genotypes CT + TT was also significantly higher in patients with moderate to severe GO (76.4%) than in patients with mild GO (47.8%, $p = 0.001$, OR = 3.5, 95% CI = 1.6–7.7). Multiple logistic regression analysis demonstrated that the severity of GO is not dependent on age, gender, and pharmacological variables, including duration of CsA therapy, CsA dosage, CsA serum concentration (peak), and concomitant calcium channel blocker use, being only associated with *CD14-260* genotype ($p = 0.012$, OR = 3.0, 95% CI = 1.3–7.0) and red complex periodontopathogens ($p = 0.009$, OR = 3.3, 95% CI = 1.3–8.2).

Discussion

Limited data were available to delineate the effect of microbial profiles on the pathogenesis of CsA-induced GO, and even these findings are varied. Romito *et al.* demonstrated a positive association between *Micromonas micros* and the GO+ group in patients with heart transplant receiving CsA (33). Animal research indicated a higher proportion of *Pg* in the test sites of CsA-mediated beagle dogs (34). A recent study showed that GO subjects presented a higher frequency of *Tf* in the salivary samples of immunosuppressed patients under the administration of CsA, tacrolimus, or sirolimus (35). The results of the present study demonstrated a relationship between the

Table 4. Genotypes and allele frequencies of *CD14-260* polymorphisms in CsA-treated patients with and without GO

	GO+ group (n = 124)	GO- group (n = 80)	p value
Genotype			
-260CC	49 (39.5)	28 (35.0)	
-260CT	50 (40.3)	39 (48.8)	$p = 0.413$, OR = 1.365, CI = 0.730–2.551
-260TT	25 (20.2)	13 (16.3)	$p = 0.984$, OR = 0.910, CI = 0.403–2.057
-260CT + TT	75 (60.4)	52 (65.0)	$p = 0.616$, OR = 1.213, CI = 0.677–2.175
Allele			
-260C allele	148 (59.7)	95 (59.4)	
-260T allele	100 (40.3)	65 (40.6)	$p = 1.000$, OR = 1.013, CI = 0.676–1.518

CsA, cyclosporine A; GO, gingival overgrowth; OR, odds ratio; CI, confidence interval.

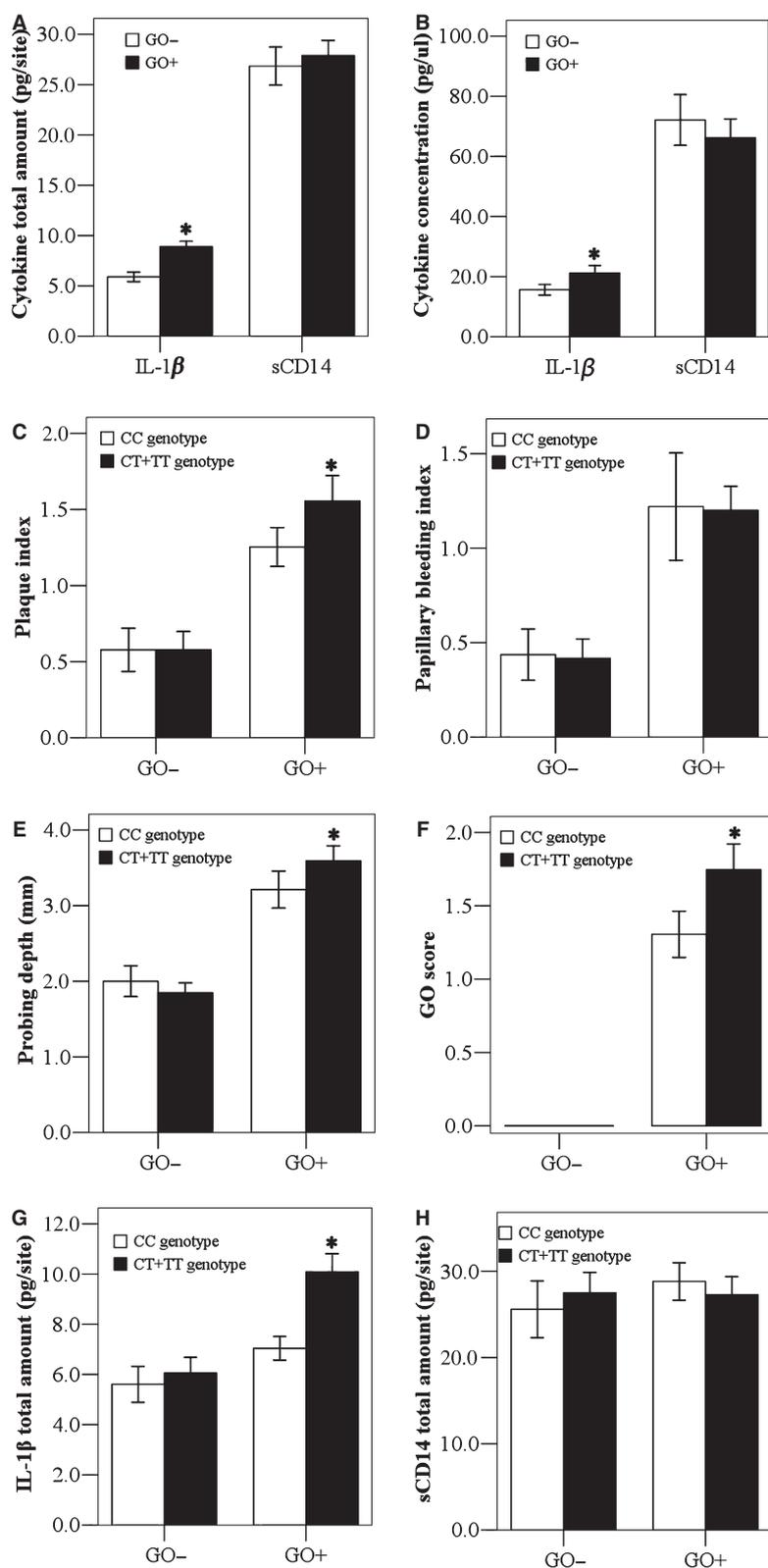


Fig. 1. Association of genotype with gingival crevicular fluid cytokine levels and periodontal parameters in patients with and without GO. * denotes $p < 0.05$ by Mann-Whitney U test. GO, gingival overgrowth; sCD14, soluble CD14; IL-1 β , interleukin-1 β .

prevalence of red complex and CsA-induced GO. These findings differed from the study of Vieira *et al.* (36) showing that renal transplant patients taking immunosuppressive medications had a lower frequency of *Pg*, *Td*, and *Tf* in their dental plaque using a BANA test. The reason for such contrary results is not clear and may be due to different methods of sample collection and analysis.

Because of gingival enlargement, gingival swelling and edema cause pseudopockets that accelerate accumulation of dental plaque, which can act as a reservoir, slowly releasing CsA and sustaining the deleterious effects on gingival tissues (37). The red complex periodontal pathogens detected in the present study have been shown to coexist as a consortium in deeper periodontal pockets (38). Enlarged gingival tissues may disrupt the normal symbiotic relationship between the host and its resident microbes, creating an environment that is more appropriate for the proliferation of these species (39). In addition, the host-impaired response due to immunosuppressive medications may also interfere in the alteration of microbial flora, favoring the growth of strictly anaerobic periodontal pathogens (40,41).

Pg, *Td*, and *Tf* have been associated with IL-1 β production *in vitro* and *in vivo* (42). The levels of IL-1 β are thought to be a critical determinant of periodontal disease outcome (43). IL-1 β is capable of synergizing with CsA to upregulate the secretion of IL-6 in human gingival fibroblasts, exerting a positive modulation on collagen and glycoaminoglycan synthesis (11). Atilla *et al.* reported that subjects with CsA-induced GO had higher levels of GCF IL-1 β than those without overgrowth (10), which is confirmed by our results. In the GO+ group, red complex-positive patients presented high levels of IL-1 β than red complex-negative patients, whereas no association could be found in the GO- group. Furthermore, the amounts of red complex were also positively associated with the levels of IL-1 β in the GO+ group,

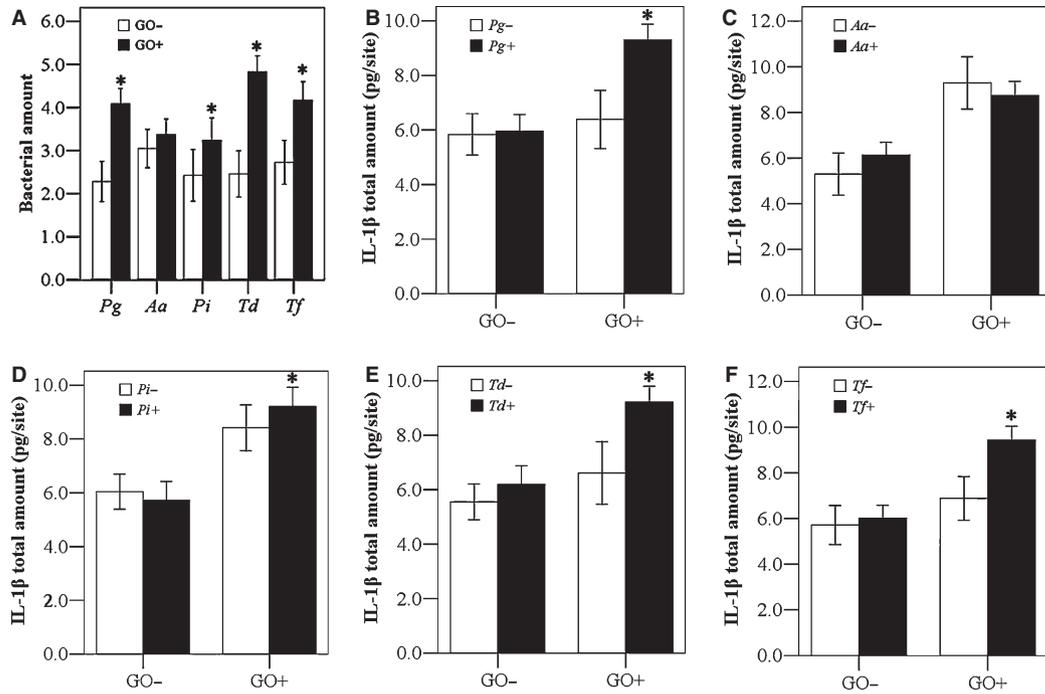


Fig. 2. Association of the presence and amount of periodontopathogens and gingival crevicular fluid IL-1 β levels with GO status. * $p < 0.05$ by Mann-Whitney U test or independent t -test. IL-1 β , interleukin-1 β ; GO, gingival overgrowth; *Pg*, *Porphyromonas gingivalis*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Pi*, *Prevotella intermedia*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

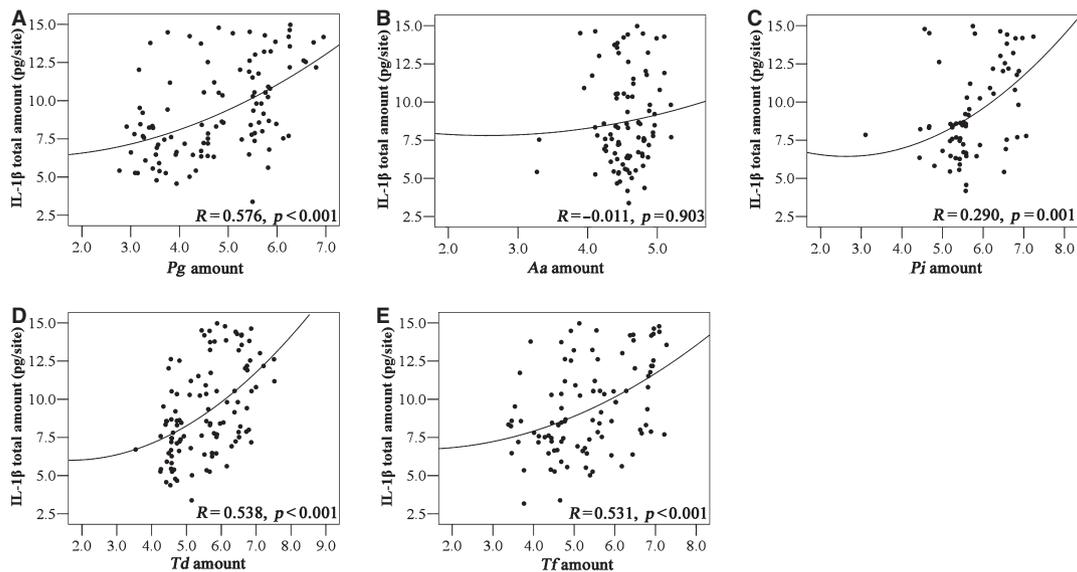


Fig. 3. Association between the amounts of periodontal pathogens and gingival crevicular fluid IL-1 β in patients with gingival overgrowth. * $p < 0.05$ by Spearman rank correlation coefficient. IL-1 β , interleukin-1 β . *Pg*, *Porphyromonas gingivalis*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Pi*, *Prevotella intermedia*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

suggesting these elevated red complex periodontal pathogens may represent an important role in the modulation of IL-1 β , which in turn synergizes with CsA to upregulate fibroblast proliferation and extracellular matrix production.

Several studies have identified specific polymorphisms involved in the inflammatory immune responses as risk factors for CsA-induced GO, including IL-1A, IL-6, transforming growth factor- β 1, and cytotoxic T-lymphocyte antigen-4 (44–47).

However, their findings are inconsistent. In the present study, we failed to demonstrate an association between *CD14-260* genotype and the prevalence of GO, which is inconsistent with some of the previous studies that *CD14-260* polymorphism was associ-

Table 5. Frequencies of periodontal pathogens in the GO+ and GO− group with regard to *CD14-260* genotype

Bacteria	GO+ group			GO− group		
	CC (n = 49)	CT + TT (n = 75)	p- value	CC (n = 28)	CT + TT (n = 52)	p- value
<i>Pg</i>	36 (73.4)	70 (93.3)	0.003	15 (53.6)	30 (57.7)	0.815
<i>Aa</i>	38 (77.6)	54 (72.0)	0.535	22 (78.6)	35 (67.3)	0.316
<i>Pi</i>	32 (65.3)	40 (53.3)	0.199	15 (53.6)	21 (40.4)	0.347
<i>Td</i>	39 (79.6)	69 (92.0)	0.042	16 (57.1)	27 (51.9)	0.815
<i>Tf</i>	32 (65.3)	65 (86.7)	0.007	18 (64.3)	31 (59.6)	0.811
Red complex	24 (49.0)	57 (76.0)	0.004	6 (21.4)	14 (26.9)	0.787

Fisher's exact test was performed to determine the association of the prevalence of the five periodontal pathogens and different genotype subgroups (CC vs. CT + TT). Bold represents a significant difference ($p < 0.05$).

GO, gingival overgrowth; *Pg*, *Porphyromonas gingivalis*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Pi*, *Prevotella intermedia*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

ated with chronic periodontitis in Caucasians (48,49). The ethnic background of the study populations and the different clinical selection criteria may explain these discrepancies. However, our data demonstrated that patients with moderate to severe GO presented higher proportion of CT + TT genotype compared with patients with mild GO. Multiple logistic regression analysis also demonstrated an association between the CT + TT genotype and GO severity. Our data are partly in agreement with the study of Tervonen *et al.* that carriers of the T-containing genotype of *CD14-260* have a greater extent of periodontal disease compared with those carrying the CC genotype (16), although the etiology of drug-induced GO and periodontitis are quite different. In contrast, there are also reports

which demonstrated that presence of the T allele, associated with increased expression of CD14, might be protective in periodontal disease (50).

Complex interactions between the microbiota and host genetic factor are at the basis of susceptibility to periodontal disease. In the present study, patients with GO carrying CT + TT genotypes presented higher frequencies of *Pg*, *Td*, and *Tf* compared with CC genotype carriers, whereas no difference could be found in patients without GO. Moreover, CT + TT genotype and the presence of red complex were shown to be associated with the severity of GO by multiple logistic regression analysis after adjusting for age, gender, and pharmacological variables. Considering the multifactorial etiology of periodontal disease, the two pathways for

periodontal infectogenomics can explain why the predominant periodontal pathogens preferably develop in GO subjects with the T allele of *CD14-260*. First, genetic factor coding for pattern recognition receptors (TLRs and CD14) involved in recognizing and killing bacteria may affect bacterial clearance (19). *In vitro* studies have shown that *TLR4* polymorphisms can affect responsiveness to *Pg* from gingival epithelial cells (51). Secondly, subjects carrying the T allele may increase the chance of overgrowth of periodontal pathogens due to increased inflammatory response. Monocytes with TT genotype of *CD14-260* have been shown to present elevated tumor necrosis factor- α production in response to *Pg* lipopolysaccharide stimulation (18). A high concentration of IL-1 β and IL-10 in culture supernatants were observed in the peripheral blood mononuclear cells of asthmatic children with the TT genotype on the response to endotoxin (52). The results of our study also demonstrate a close relationship between CT + TT genotype and higher levels of IL-1 β in patients with GO. These elevated pro-inflammatory cytokines might directly lead to an increased inflammatory response and affect the proliferation of red complex pathogens in patients with GO carrying CT + TT genotype.

When the putative role of these variables were evaluated individually, our results demonstrated that in the absence of red complex pathogens, CT + TT carriers showed similar lev-

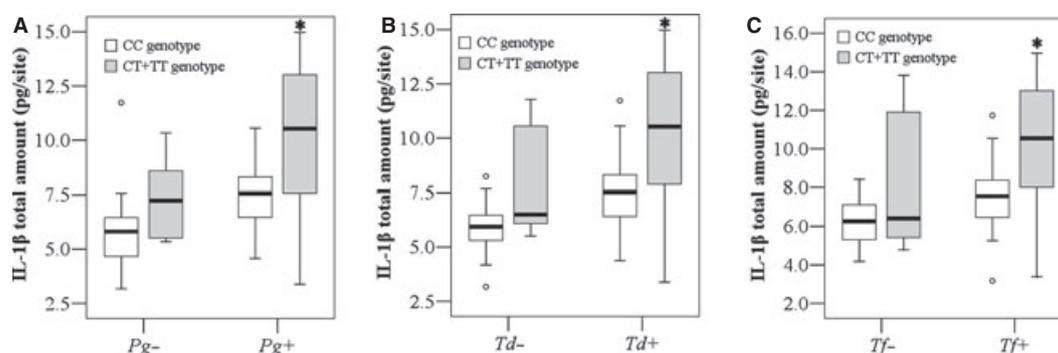


Fig. 4. Association between genotype, the prevalence of red complex periodontopathogens, and gingival crevicular fluid IL-1 β levels in patients with gingival overgrowth. The box represents the first and the third quartiles (rectangular boxes); the line within the box is the median and the little circles represent atypical values that were plotted separately. * $p < 0.05$ by Mann-Whitney *U* test. IL-1 β , interleukin-1 β . *Pg*, *Porphyromonas gingivalis*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

els of GCF IL-1 β levels, whereas in the presence of red complex pathogens, CT + TT genotype groups presented significantly higher levels of IL-1 β and were positively associated with the severity of GO. These positive associations between CT + TT genotype and red complex pathogens in patients with severe GO reinforce the concept of periodontal infectogenomics, and may help uncover the complex etiology of CsA-induced GO, possibly assisting in the prevention and management of this disease.

There are some limitations to this study that need to be considered. With the nature of cross-sectional study, it is difficult to identify whether the gingival inflammation was present before onset of GO or was a consequence of the gingival changes. On the other hand, some susceptible individuals without gingival changes may develop severe overgrowth in the future. This may result in selection biases in the interpretation of results (53). Additionally, in cross-sectional studies, it is hard to identify whether the gingival inflammation was present before the onset of GO or was a consequence of gingival changes. The increased levels of red complex and IL-1 β in patients with GO might be more a consequence of deeper and more inflamed pockets than a cause of GO. Further extensive studies are needed to analyze this putative relevance.

Conclusions

Taken together, no association between CD14-260 polymorphisms and the prevalence of GO was revealed in renal transplant patients administered CsA. However, CD14-260 CT + TT genotypes are found to be associated with the prevalence of red complex periodontopathogens in patients with GO, and may thus play some role in the development of severe CsA-induced GO.

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