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Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque

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Introduction: Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth associated with bacteria. Diagnosis is achieved retrospectively by clinical observation of attachment loss. Predicting disease progression would allow for targeted preventive therapy. The aim of this study was to monitor disease progression in patients on a maintenance program and determine the levels of specific bacteria in subgingival plaque samples and then examine the ability of the clinical parameters of disease and levels of specific bacteria in the plaque samples to predict disease progression.
Methods: During a 12-month longitudinal study of 41 subjects, 25 sites in 21 subjects experienced disease progression indicated by at least 2 mm of clinical attachment loss. Real-time polymerase chain reaction was used to determine the levels of *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Fusobacterium nucleatum*, and *Prevotella intermedia* in subgingival plaque samples.
Results: No clinical parameters were able to predict periodontal disease progression. In sites undergoing imminent periodontal disease progression within the next 3 months, significant partial correlations were found between *P. gingivalis* and *T. forsythia* ($r = 0.55$, $P < 0.001$) and *T. denticola* and *T. forsythia* ($r = 0.43$, $P = 0.04$). The odds of a site undergoing imminent periodontal disease progression increased with increasing levels of *P. gingivalis* and *T. denticola*.
Conclusion: Monitoring the proportions of *P. gingivalis* and *T. denticola* in subgingival plaque has the potential to help identify sites at significant risk for progression of periodontitis, which would assist in the targeted treatment of disease.

Key words: bacterial pathogens; biomarkers; chronic periodontitis; clinical study; real-time PCR

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Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth associated with bacteria. It results in localized or generalized destruction of the supporting tissues of the teeth; the periodontal ligament, bone, and soft tissues (30). The disease if left untreated, can ultimately lead to tooth loss. Following diagnosis and initial treatment, a lifelong maintenance program involving regular examinations coupled with scaling, root planing, and oral hygiene instruction is necessary to monitor and attempt to prevent further disease progression. The maintenance therapy is generally non-specific and does not target active or at-risk sites. It is costly, time-consuming, can be unpleasant, and has been shown to result in both soft and hard tissue losses as the result of trauma (4, 8, 56). Chronic periodontitis affects approximately 35% of the population worldwide (1, 55), and poses a significant public-health challenge because it has also been suggested to be a

risk factor for cardiovascular diseases (12, 66) and possibly preterm births and low birth weight infants (29, 45).

Clinical and radiographic measures of inflammation and attachment loss are the most frequently used and widely accepted means to diagnose chronic periodontitis (53, 54). Measuring attachment loss provides only retrospective evidence of periodontal destruction. Sites exhibiting signs of tissue inflammation are not always associated with attachment loss, and clinical parameters do not indicate current disease activity or future attachment loss at a specific site (23). One way to improve the current approach to the diagnosis and maintenance of chronic periodontitis is by identifying sites in a patient's mouth that are at risk of undergoing periodontal destruction, thereby allowing site-specific preventive therapy. A rapid and accurate method to assist in the diagnosis and monitoring of chronic periodontitis has not only benefits in maintaining periodontal health but also implications in the reduction of the prevalence of related systemic conditions.

Subgingival dental plaque is a polymicrobial biofilm from which over 500 bacterial species have been isolated (42, 49). Studies of the microbiota of subgingival plaque have shown that while the levels of many bacterial species do not differ significantly between healthy subjects and those with periodontal disease (21, 69, 75), a relatively small number of gram-negative bacterial species are significantly associated with chronic periodontitis. In a 1998 study of 13,261 plaque samples taken from both healthy and periodontally diseased subjects, Socransky et al. (62) repeatedly observed that species abundance clustered into five major bacterial complexes. A complex consisting of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (formerly *Bacteroides forsythus*) was strongly related to clinical parameters of periodontitis such as periodontal pocket depth and bleeding on probing. This consortium of bacteria was termed the Red Complex, and has since been repeatedly associated with chronic periodontitis (21, 48, 65, 75). A second tightly related group of bacterial species, termed the Orange Complex and including *Fusobacterium nucleatum* and *Prevotella intermedia*, was also reported by Socransky et al. (62). The authors suggested that an ecological succession of bacterial species might be involved in the initiation and progression of chronic periodontitis, with colonization by the Orange Complex preceding colonization and proliferation of the Red Complex.

Most clinical studies providing evidence of associations between bacterial species implicated in chronic periodontitis have been cross-sectional or retrospective rather than prospective longitudinal studies and have employed semi-quantitative methods for bacterial enumeration. Associations between a bacterial species and clinical measures of chronic periodontitis at a single time-point, or retrospectively, fail to answer arguably the most important question in the determination of a bacterial etiology – was the species or combination of species present before the periodontal destruction occurred, or present as a result of the nutritional and environmental conditions provided during and following the periodontal tissue destruction? Associations between putative periodontal pathogens and chronic periodontitis have also tended to be reported at a subject level rather than at a site level. Although being able to determine which subjects are at greater risk of future periodontal breakdown is undoubtedly beneficial to the patient and clinician, determination of increased risk of disease at a specific site within a patient would be the ideal in periodontal diagnosis.

It has been proposed that it is not the mere presence of opportunistically pathogenic bacteria in subgingival plaque that causes periodontal breakdown, but the presence of one or multiple species above threshold levels, and that these may serve as predictors of periodontal disease progression (5, 6, 25, 47). There is, however, a lack of prospective longitudinal data regarding the association of levels of pathogenic species in subgingival plaque with the progression of disease at individual sites.

To determine if the presence or levels of specific bacteria in subgingival plaque could predict periodontal sites at risk of disease progression, we used real-time polymerase chain reaction (PCR) to measure the levels of *P. gingivalis*, *T. denticola*, *T. forsythia*, *F. nucleatum*, and *P. intermedia* in subgingival plaque samples taken during a longitudinal clinical study of chronic periodontitis.

Materials and methods

Ethics approval

Ethics approval was obtained from the University of Melbourne Health Sciences Human Ethics Subcommittee and informed consent was obtained from participants.

Subjects

Forty-one subjects from the Specialist Periodontics Department of the Royal Dental Hospital Melbourne, The University of Melbourne were enrolled into a 12-month clinical study. The subjects had been previously diagnosed with chronic periodontitis, had completed their treatment, and had been on a maintenance program for a minimum of 6 months. Subjects were included in the study if they were over 35 years of age, had at least 20 teeth, were not pregnant or breast-feeding, did not exhibit a systemic condition that either might affect the progression of periodontal disease or would require premedication for treatment. Subjects were excluded from the study if they were on long-term non-steroidal anti-inflammatory drug therapy or had received antibiotic therapy in the preceding 6 months. Subjects were exited from the study and returned to the care of the Specialist Periodontics Department if they undertook a course of antibiotic therapy during the study or if they wished to withdraw.

Clinical examination and subgingival plaque sampling

At the baseline examination, the periodontal probing depths at six sites around each tooth were examined (disto-buccal, mid-buccal, mesio-buccal, mesio-palatal, mid-palatal, and disto-palatal). Five sites in each subject (205 sites total) were then selected for clinical monitoring. The selected sites were the five sites with the deepest periodontal pockets anterior to and including the mesial surface of the first molars. The five sites in each subject were examined five times during the study; at baseline and at 3, 6, 9, and 12 months. At each examination, Modified Gingival Index (MGI) (35), Plaque Index (PI) (61), pocket depth (PD), recession (REC), clinical attachment level (CAL), bleeding on probing (BOP), and suppuration were recorded for each of the selected sites in each subject. PD, REC, and CAL were measured (to the nearest 0.2 mm) using a Florida probe (Florida Probe Corporation, Gainesville, FL) (17). For the measurement of CAL and REC, the occlusal or incisal tooth surfaces were used as the reference points. Following the clinical examination, supragingival plaque was carefully removed from each selected site. A subgingival plaque sample was then taken using a single stroke with a sterile curette. Each plaque sample was immediately placed into 200 μl of TE buffer

(10 mM tris[hydroxymethyl]-methylamine chloride, 1 mM ethylenediamine tetraacetic acid) and stored on ice until transferred to −70°C. Two calibrated clinical operators carried out the examinations and subgingival plaque sampling, and each operator examined the same subjects throughout the study. Periodontal disease progression at a site was defined as an increase in clinical attachment loss of at least 2 mm during the study. If at an examination a site exhibited periodontal disease progression, following collection of the clinical data and subgingival plaque, it was exited from the study and received immediate treatment, and no further clinical data or subgingival plaque was collected from that site. As sites were exited from the study once clinical attachment loss of at least 2 mm was observed, sites were monitored for differing periods. Data from sites and subjects who failed to complete the 12 months of the study were included in analyses providing these subjects did not meet any of the exclusion criteria.

Bacterial strains and culture conditions

All bacterial species were obtained from the culture collection of the Melbourne Dental School, The University of Melbourne and were grown in an anaerobic work station (Don Whitley Mk III, Sydney, Australia) at 37°C in an atmosphere of 80% N_2, 15% CO_2, 5% H_2. *P. gingivalis* W50 (13), *T. denticola* (ATCC 35405) (34), and *T. forsythia* (ATCC 43037) (40) were cultured as previously published. *F. nucleatum* subspecies *polymorphum* (ATCC 10953) and subspecies *nucleatum* (ATCC 25586) were cultured as for *P. gingivalis*, and *P. intermedia* (ATCC 25611) was maintained in tryptic soy broth (30 mg/l) supplemented with cysteine (10 mg/l), menadione (0.4 mg/l), and hemin (5 mg/l).

DNA from exponentially growing cells was extracted using the DNeasy Tissue kit (Qiagen, Clifton Hill, Vic., Australia) following the manufacturer's instructions. The concentration of the DNA for real-time PCR standards was determined using the Quant-iT DNA Assay kit (Molecular Probes, Invitrogen, Mt Waverley, Vic., Australia), according to the manufacturer's instructions with fluorescence measured on a Wallac 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA). DNA concentration in ng/μl was then converted to the equivalent number of cells/μl for use as real-time PCR standards using genome sizes obtained from the Bioinformatics Resource for Oral Pathogens (www.brop.org) (7).

Real-time PCR

All oligonucleotide primers targeted the 16S ribosomal RNA (rRNA) gene (Table 1) and were as previously published for *P. gingivalis* and *T. denticola* (58), *T. forthysia* (16), *F. nucleatum* (10), *P. intermedia* (15), and universal (3). Primers were checked for specificity and cross-reactivity using BLAST searches (2). A single mismatched base was identified in the *P. gingivalis* reverse primer sequence of Sakamoto et al. (58) when the primer sequence was aligned with the *P. gingivalis* 16S rRNA sequence obtained from The Institute for Genomic Research website (50) (www.jcvi.org), and therefore was corrected. Real-time PCR conditions were optimized for the Corbett Rotor-Gene™ (Corbett Research, Mortlake, NSW, Australia). Reactions were carried out in triplicate, in a 25 μl reaction volume consisting of 12.5 μl Platinum® SYBR® Green qPCR Supermix UDG (Invitrogen), 9.5 μl DNase-free deionized water, 200 nM final concentration of forward and reverse primer, and 2 μl template. Real-time PCR conditions for all primer pairs consisted of an initial heating step at 50°C for 2 min, initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Fluorescence data were collected immediately following the extension step of each cycle. Specificity of the primer pairs was confirmed by melt curve analysis by heating from 72 to 95°C in 0.2°C increments. Melting peaks were compared with the bands obtained following agarose gel electrophoresis. Tenfold serial dilutions of DNA of known concentration were used to construct standard curves for quantification of each species. The same serial dilutions of *P. gingivalis* DNA were used to determine the standard curve for the Universal primer pair. *P. gingivalis* has four copies of the 16S rRNA gene per genome, which we determined to be representative of the average for the oral plaque bacteria with sequenced genomes and for the 502 bacterial species whose ribosomal RNA gene copy number was available in the rRNA gene copy number database (31). The lower limit for the accurate quantification of each individual species was 2×10^3 cells of a species per plaque sample. The level of each bacterial species was expressed as a percentage of the total number of bacterial cells in each subgingival plaque sample.

Effect of subgingival plaque on DNA extraction and real-time PCR

To determine any inhibitory effect of dental plaque on real-time PCR efficiency, plaque samples that had previously tested negative for each species using real-time PCR were spiked with known numbers of cells of each species before DNA extraction. Real-time PCR was then carried out on 10-fold serial dilutions of each sample. The presence of dental plaque had no effect on PCR efficiency or quantification.

Statistical analysis of data

For descriptive statistics of the clinical measures of periodontal disease and bacterial species, measurements made at the site level were used. The data were analysed using a matched case–control design and conditional logistic regression

Table 1. Sequence of the 16S ribosomal RNA oligonucleotide primers used for real-time polymerase chain reaction

Species	Forward primer	Reverse primer	Product size (base pairs)	GenBank ID	Reference
Porphyromonas gingivalis	aggcagcttgccatactgcg	actgttagtaactaccgatgt	404	AB035456	(58)
Treponema denticola	taataccgaatgtgctcatttacat	tcaaagaagcattccctcttcttctta	316	AF139203	(58)
Tannerella forsythia	aaaacaggggttccgcatgg	ttcaccgcggacttaacagc	426	AB035460	(16)
Fusobacterium nucleatum[1]	agagtttgatcctggctcag	gtcatcgtgcacacagaattgctg	360	AJ133496	(10)
Prevotella intermedia	cgtggaccaaagattcatcggtgga	ccgctttactccccaacaaa	259	X73965	(15)
Universal	gattagataccctggtagtccac	cccgggaacgtattcaccg	602	N/A	(3)

N/A, not applicable.
[1]*F. nucleatum* primers detect all *F. nucleatum* subspecies, *Fusobacterium mortiferum* and *Fusobacterium periodonticum* (10).

models (26). Case sites were those sites that experienced periodontal disease progression (defined as at least 2 mm clinical attachment loss). Each case site was matched with sites from the same subject that did not exhibit disease progression during the study and for which microbiological data were available (the control sites). This led to a variable number of control sites (one to three) being matched to each case site. Matching of sites within subjects meant that effects at the subject-level (such as age, smoking status, and oral hygiene) did not need to be incorporated into the model. To investigate the correlation between two bacterial species, a partial correlation coefficient was calculated, controlling for the effects of the other bacterial species.

To assess whether the clinical measures of periodontal disease (MGI, PI, PD, BOP), presence/absence of bacterial species, or the bacteria levels (defined as the percentage of total bacteria cells present in the subgingival plaque samples) were capable of predicting whether a site would undergo periodontal disease progression, univariate and multivariate conditional logistic regression models were fitted using the data collected at the examination 3 months before the disease progression being clinically detectable.

The level of statistical significance was set at $P = 0.05$. Multivariate conditional logistic regression models were fitted using a stepwise procedure. All analyses were carried out using the statistical package STATA (Stata Statistical Software: Release 10.0; Stata Corporation, College Station, TX). Variance estimates were adjusted for clustering of sites within subjects using robust variance estimation procedures.

Results

Subjects and sites

The mean age of subjects in the study was 60.1 ± 11.2 years (range 39–81) with the majority being female (28/41). The baseline clinical and bacterial species data for all monitored sites are summarized in Table 2. At the baseline examination, 34.6% (71/205) of sites had pocket depths less than 3 mm, 55.6% (114/205) had depths between 3 and 4.8 mm, and 9.8% (20/205) had depths of 5 mm or greater. Suppuration was recorded at the baseline examination in only one of the 205 sites monitored and so this variable was excluded from analyses. Based on the identification of sites that had clinical attachment loss of ≥ 1 mm over the course of the study a total of 475 plaque samples from 108 sites in 37 subjects were analysed to quantify the levels of *P. gingivalis*, *T. denticola*, *T. forthysia*, *F. nucleatum*, *P. intermedia*, and total bacteria. *F. nucleatum* was detected in 87.0% (94/108) of sites at baseline and comprised, on average, 21.7% of the total bacteria present in the subgingival plaque samples (range 0–100.0%). On average, the other species investigated tended to make up only a small proportion of the total bacteria and were often not detected in the plaque samples (Table 2). The data are indicative of the population from which the sites were drawn: a population of subjects that had been previously diagnosed with periodontal disease, had recently completed a course of active periodontal therapy, and who were on a maintenance program.

Site specificity of chronic periodontitis progression

Site-specific chronic periodontitis with variable rates of progression was observed in this subject population. Not only did disease progress with varying rates, but disease progression, clinical improvement, and stability could all be observed simultaneously within subjects. During the course of the study, 25 sites (12.2%) from 21 subjects experienced disease progression. Seven sites experienced periodontal progression within the first 3 months, another nine sites within 6 months, four within 9 months, and five within 12 months. One subject had two sites progress at 6 months (the same control site was matched to both sites). Another subject had one site progress at 9 months and one at 12 months and a third subject had one site progress at 9 months and two at 12 months. For these two subjects, the same sites were used for the control sites at both time-points. The mean age of subjects with sites that progressed was 61.6 ± 11.4 years (range 42–81) and 71.4% (15/21) were female. The baseline clinical and bacterial species data for sites that experienced periodontal disease progression and their matching control sites are shown in Table 3. There was a statistically significant difference in PD at baseline between sites that progressed and sites that remained stable ($P = 0.01$). There were no statistically significant differences at baseline for any of the other clinical parameters or for any of the bacteria species data.

Table 2. Clinical parameters and bacterial species data at baseline for all sites monitored during the study

	Mean ± SE	Range	Prevalence (%)
Clinical measures (n = 205)			
Modified Gingival Index	1.6 ± 0.1	0–4	–
Plaque Index	1.4 ± 0.1	0–3	–
Pocket depth (mm)	3.3 ± 0.1	1.2–9.8	–
Recession (mm)	7.9 ± 0.2	2.6–15.2	–
Clinical attachment level (mm)	11.3 ± 0.3	5.8–18.6	–
Bleeding on probing	–	–	25.8
Bacteria species (n = 108)			
Porphyromonas gingivalis	1.9 ± 0.6[1]	0.0–33.3	21.3
Treponema denticola	1.2 ± 0.3[1]	0.0–13.9	31.4
Tannerella forsythia	1.9 ± 0.5[1]	0.0–20.9	37.0
Fusobacterium nucleatum	21.7 ± 2.5[1]	0.0–100.0	87.0
Prevotella intermedia	0.5 ± 0.2[1]	0.0–11.7	22.2

The baseline bacterial species data shows the prevalence (percentage of sites positive) and the level (percentage of total bacteria at a site) of each bacterial species in subgingival plaque from the 108 sites selected for real-time polymerase chain reaction analysis.
[1]Defined as the percentage of cells per total bacterial cell number present in the subgingival plaque sample.

Prediction of chronic periodontitis progression

The clinical parameters and bacterial species data for sites that experienced periodontal disease progression and their matching control sites 3 months before detectable clinical progression are shown in Table 4. In sites that experienced periodontal disease progression, significant partial correlations were found between *P. gingivalis* and *T. forsythia* ($r = 0.55$, $P < 0.001$) and *T. denticola* and *T. forsythia* ($r = 0.43$, $P = 0.04$). For the control sites, significant partial correlations were found between *P. gingivalis* and *T. denticola* ($r = 0.56$, $P < 0.001$) and *T. denticola* and *T. forsythia* ($r = 0.40$, $P = 0.02$). To investigate the relationship between imminent periodontal disease progression and

Table 3. Clinical parameters and bacterial species data at baseline for sites that experienced periodontal disease progression and their matching control sites

	Sites with at least 2 mm attachment loss (n = 25)			Matched control sites (n = 36)		
	Mean ± SE	Range	Prevalence (%)	Mean ± SE	Range	Prevalence (%)
Clinical measure						
Modified Gingival Index	1.6 ± 0.2	1–4	–	1.6 ± 0.1	0–3	–
Plaque Index	1.4 ± 0.2	0–3	–	1.6 ± 0.1	0–3	–
Pocket depth	4.0 ± 0.4	1.4–9.8	–	3.2 ± 0.2	1.2–5.2	–
Recession	7.2 ± 0.4	3.2–10.4	–	7.5 ± 0.4	3.6–11.2	–
Clinical attachment level	11.2 ± 0.6	7.0–17.8	–	10.7 ± 0.6	6.2–15.8	–
Bleeding on probing	–	–	40.0	–	–	30.6
Bacterial species						
Porphyromonas gingivalis	3.1 ± 1.7	0.0–33.3	28.0	1.9 ± 0.8	0.0–16.1	25.0
Treponema denticola	2.3 ± 0.9	0.0–13.9	48.0	1.3 ± 0.5	0.0–11.6	38.9
Tannerella forsythia	2.2 ± 0.9	0.0–17.3	52.0	2.5 ± 1.0	0.0–16.3	47.2
Fusobacterium nucleatum	16.3 ± 3.4	0.0–44.6	76.0	21.1 ± 4.3	0.0–87.7	94.4
Prevotella intermedia	0.1 ± 0.1	0.0–1.3	20.0	0.7 ± 0.4	0.0–11.1	27.8

The baseline bacterial species data shows the prevalence (percentage of sites positive) and the level (percentage of total bacteria at a site) of each bacterial species in subgingival plaque from the selected sites. Modified Gingival Index is presented on a scale of 0–4 as described previously (35). Plaque index was assessed by using the 0–3 scale of Silness and Loe (61). Pocket depth, recession and clinical attachment level are expressed as mean ± SD in mm.

Table 4. Clinical parameters and bacterial species data for sites that experienced periodontal disease progression and their matching control sites 3 months before disease progression being clinically detected

	Sites with at least 2 mm attachment loss (n = 25)			Matched control sites (n = 38[1])		
	Mean ± SE	Range	Prevalence (%)	Mean ± SE	Range	Prevalence (%)
Clinical measure						
Modified Gingival Index	1.7 ± 0.1	1–4	–	1.6 ± 0.1	1–3	–
Plaque Index	1.2 ± 0.2	0–3	–	1.4 ± 0.1	0–3	–
Pocket depth	3.7 ± 0.4	1.4–9.6	–	3.0 ± 0.2	1.4–5.2	–
Recession	7.3 ± 0.5	3.2–11.0	–	7.7 ± 0.4	3.6–12.0	–
Clinical attachment level	11.0 ± 0.7	7.0–20.0	–	10.7 ± 0.4	6.2–15.0	–
Bleeding on probing	–	–	36.0	–	–	21.1
Bacterial species						
Porphyromonas gingivalis	2.4 ± 1.2	0.0–25.6	32.0	0.7 ± 0.4	0.0–13.8	15.8
Treponema denticola	1.8 ± 0.7	0.0–13.9	40.0	0.9 ± 0.4	0.0–11.6	28.9
Tannerella forsythia	2.1 ± 0.8	0.0–12.0	56.0	3.3 ± 1.6	0.0–36.2	34.2
Fusobacterium nucleatum	17.6 ± 2.8	0.0–63.2	92.0	22.6 ± 4.2	0.0–77.1	89.5
Prevotella intermedia	0.4 ± 0.3	0.0–8.3	20.0	0.2 ± 0.1	0.0–3.1	18.4

The bacterial species data shows the prevalence (percentage of sites positive) and the level (percentage of total bacteria at a site) of each bacterial species in subgingival plaque from the selected sites. Modified Gingival Index is presented on a scale of 0–4 as described previously (35). Plaque index was assessed by using the 0–3 scale of Silness and Loe (61). Pocket depth, recession and clinical attachment level are expressed as mean ± SD in mm.
[1]For the two subjects who had sites that progressed at different time-points, the same sites were used for the control sites at each time-point.

clinical measures of disease, univariate conditional logistic regression models were fitted (Table 5). Although none of these clinical measures were statistically significant, sites with deeper pocket depths [odds ratio (OR) = 1.58, P = 0.09] and sites which bled on probing (OR = 3.14, P = 0.11) had an increased risk of the site undergoing disease progression in the following 3 months.

Univariate conditional logistic regression models were also fitted using dichotomous indicators for the presence/absence of bacterial species and bacterial species levels to explore the relationships between imminent periodontal disease progression and subgingival bacterial data (Table 5). None of the dichotomous indicators of species presence were statistically significant predictors of periodontal progression in the following 3 months but *P. gingivalis*, *T. denticola*, and *T. forsythia* were

Table 5. Univariate conditional logistic regression analyses for a site undergoing disease progression in the following 3 months

	Odds ratio	95% confidence intervals	P-value
Clinical measures			
Modified Gingival Index	1.27	(0.69, 2.34)	0.44
Plaque Index	0.91	(0.45, 1.85)	0.80
Pocket depth	1.58	(0.94, 2.65)	0.09
Recession	0.87	(0.59, 1.29)	0.49
Bleeding on probing (%)	3.14	(0.77, 12.86)	0.11
Bacterial presence/absence			
Porphyromonas gingivalis	3.81	(0.63, 23.03)	0.15
Treponema denticola	2.71	(0.55, 13.25)	0.21
Tannerella forsythia	2.93	(0.79, 10.86)	0.11
Fusobacterium nucleatum	1.82	(0.15, 22.07)	0.64
Prevotella intermedia	1.17	(0.32, 4.36)	0.81
Bacterial levels[1]			
Porphyromonas gingivalis	1.18	(0.98, 1.42)	0.08
Treponema denticola	1.28	(0.92, 1.77)	0.14
Tannerella forsythia	0.98	(0.90, 1.06)	0.57
Fusobacterium nucleatum	0.98	(0.95, 1.01)	0.21
Prevotella intermedia	1.24	(0.85, 1.82)	0.26

[1]Defined as the percentage of cells per total bacterial cell number present in the subgingival plaque sample.

Table 6. Stepwise conditional logistic regression model for a site undergoing disease progression in the following 3 months

Bacteria levels	Odds ratio	95% confidence intervals	*P*-value (Wald test)
Porphyromonas gingivalis	1.62	(1.16, 2.26)	0.005
Treponema denticola	2.30	(1.23, 4.32)	0.009
Tannerella forsythia	0.74	(0.52, 1.05)	0.09[1]

[1]Removal of this term from the model significantly decreased the predictive power of the model (Likelihood ratio test, $P = 0.005$).

more likely to be detected in sites that experienced periodontal disease progression than in their matching control sites. The odds of a site experiencing periodontal progression increased as the levels of *P. gingivalis* and *T. denticola* in the plaque sample increased, but the odds ratios were not statistically significant (Table 5).

To investigate the joint effects of the clinical measures and the bacterial species levels, multivariate conditional logistic regression models were fitted using stepwise selection procedures. The most parsimonious model obtained included terms for levels of the three species *P. gingivalis*, *T. denticola*, and *T. forsythia* (Table 6). Although the Wald test for the *T. forsythia* term was not significant (OR = 0.74, $P = 0.09$), removal of this term from the model significantly decreased the predictive power of the model (Likelihood ratio test, $P = 0.005$). The odds of a site undergoing imminent periodontal disease progression within the next 3 months increased with increasing levels of *P. gingivalis* and *T. denticola* in the plaque sample. Similar probabilities of disease progression were obtained for various levels of *P. gingivalis*, *T. denticola*, and *T. forsythia*. The probability of a site experiencing disease progression for various levels of *P. gingivalis* and *T. denticola* when compared with a control site where *P. gingivalis*, *T. denticola*, and *T. forsythia* were absent is shown in Fig. 1.

Discussion

Currently, the initial diagnosis of periodontal disease and, following treatment, the monitoring for disease recurrence involves a clinical assessment of features associated with tissue inflammation such as erythema and edema, bleeding on probing, suppuration and supragingival plaque accumulation (53). The recording of clinical attachment at sequential examinations by periodontal probing is the most commonly used method to diagnose progressive periodontal disease (54).

The methods currently used to determine if disease has progressed are retrospective, and require a substantial amount of tissue loss to have occurred before disease progression can be accurately detected. Studies of clinical parameters used in the diagnosis and monitoring of chronic periodontitis such as measures of gingival inflammation, supragingival plaque accumulation, bleeding on probing, and periodontal pocket depth suggest that although the presence of deep periodontal pockets may be able to predict which individuals are more likely to have sites that will undergo future disease progression (24, 38, 39, 52, 68) none of these variables are able to predict periodontal disease activity at specific sites within an individual (23, 52, 68).

Chronic periodontitis has been proposed to progress in both a slow continuous manner and in short bursts of disease activity followed by periods of disease inactivity, or quiescence (37, 63). Both modalities were observed in the current study. Although diabetes (36), smoking (70), and genetic factors (41) can increase an individual's overall susceptibility to periodontal disease, it appears that within an individual not all sites are equally susceptible at one time. This site-specific difference in disease progression in an individual can be attributed to differences in the bacterial species composition of subgingival plaque.

In this study we monitored 41 subjects and observed periodontal disease progression in 21 individuals over a 12-month period. Of the 205 sites (five sites per subject) monitored over the course of this study 25 experienced disease progression, and this prevalence of progression is consistent with that seen in other studies in similar populations (27, 28). To determine if any of the clinical or microbiological parameters were capable of predicting which sites would undergo disease progression in the following 3 months, a nested case–control design was used and conditional logistic regression models were fitted to the data collected from the 25 sites that experienced disease progression and matching stable sites in the same subjects 3 months before disease progression being clinically detectable. An advantage of the matched case–control approach used in this study to compare sites within the same subject for disease progression is that subject factors (such as age, smoking status, and oral hygiene) factors are the same for case and control sites and so do not need to be incorporated into the model. However, a limitation of this approach is that the sites to be monitored for disease progression needed to be determined at the

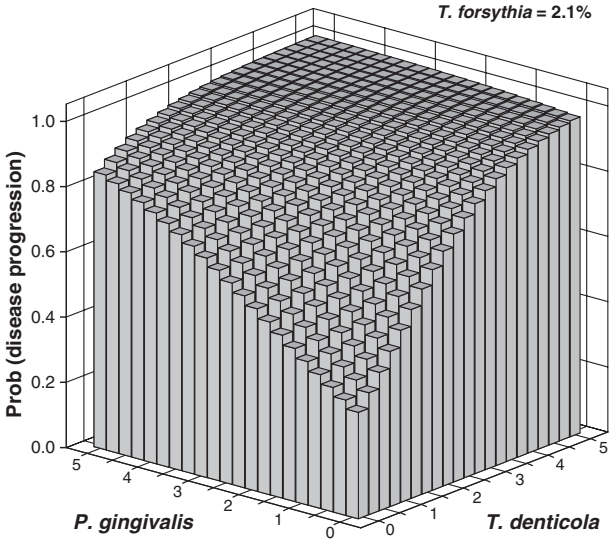

Fig. 1. The probability of a site undergoing disease progression for various levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque samples when compared with a control site where *P. gingivalis*, *T. denticola*, and *Tannerella forsythia* were not detected. In this figure the level of *T. forsythia* was set at 2.1%, which was the average level of *T. forsythia* in sites experiencing disease progression (Table 4); similar figures are obtained for other levels of *T. forsythia*.

baseline examination. For each subject the five sites with the deepest periodontal pockets were chosen to be monitored because previous studies had shown that these sites were more likely to experience disease progression. The costs associated with real-time PCR analysis for the five bacterial species were a limiting factor in the number of sites able to be monitored longitudinally. There was also considerable heterogeneity in periodontal pocket depth between the five monitored sites within subjects so it was not possible to use periodontal pocket depth to match control sites to case sites.

P. gingivalis, *T. denticola*, *T. forsythia*, *F. nucleatum*, and *P. intermedia* exist as part of a complex polymicrobial biofilm accreted onto the surface of the tooth root and are components of the normal oral microbiota. The finding of species such as *T. forsythia* in populations such as dental hygienists with excellent oral hygiene illustrates that it is possible to harbor these species and maintain a state of periodontal health (19). Chronic periodontitis is a disease that is initiated by changes in the species composition of subgingival plaque, and the subsequent alteration of the host immune response. *P. gingivalis* and *T. forsythia* were designated etiological agents of chronic periodontitis in a consensus report from the 1996 World Workshop on Clinical Periodontics (11) and are considered to be the lead candidates in causing the progression of disease because of their presence in subgingival plaque in increasing levels with increasing periodontal pocket depth (64, 75). In addition, *T. denticola* has also been consistently associated with diseased sites (47, 59, 67). Further, these three species are found associated together as members of a consortium (Red Complex) that has been strongly associated with the clinical measurements of chronic periodontitis such as periodontal pocket depth and bleeding on probing (62). Extracellular proteolytic enzymes capable of degrading host proteins and dysregulating the immune response are common to *P. gingivalis* (44), *T. forsythia* (57), and *T. denticola* (72) and are thought to play a vital role in the pathogenesis of chronic periodontitis (43, 51). *P. gingivalis*, *T. denticola*, *T. forsythia*, and *P. intermedia* have been demonstrated to have at least some pathogenic potential in animal models of disease. Of particular note *P. gingivalis* (14, 18), *T. forsythia* (60), and *T. denticola* (33) have been shown to cause alveolar bone loss in murine models of disease. *F. nucleatum* is considered to be a ubiquitous member of subgingival plaque and this is consistent with results from this study, where we found this species in the majority of subgingival plaque samples. There is limited evidence of its ability to cause tissue damage in animal models of disease (78), therefore it is most likely a commensal in regard to periodontal disease progression. However, it has been proposed to act as a scaffold in subgingival plaque aiding the colonization and proliferation of more pathogenic species (32). Interestingly, in the current study the levels of *F. nucleatum* in subgingival plaque samples were negatively correlated with pocket depth (data not shown), indicating that although this species may initially aid the colonization of sites by *P. intermedia* and members of the Red Complex, it appears not to thrive in their presence. Although associations between *P. intermedia* and chronic periodontitis have been reported (9, 75), these have not been as consistent as for *P. gingivalis*, *T. denticola*, and *T. forsythia* (21), and therefore the role of *P. intermedia* in chronic periodontitis is unclear.

In the 108 baseline subgingival plaque samples analysed, *P. gingivalis* was only found in the presence of *T. forsythia* and *T. denticola*, whereas both *T. denticola* and *T. forsythia* were frequently found in the absence of *P. gingivalis*. This suggests that *T. denticola* and *T. forsythia* may facilitate the emergence of *P. gingivalis* by providing an environment that is conducive to its colonization and proliferation. Whether this is via the provision of specific attachment sites, micronutrients, or protection from the host immune response is not yet clearly defined. However, *T. denticola* and *P. gingivalis* have been demonstrated to coaggregate (46) and exhibit a mutualistic enhancement of growth *in vitro* with each producing a fatty acid that stimulates the growth of the other (20). The growth of *P. gingivalis* has also been shown to be stimulated by the presence of cell extracts of *T. forsythia* (77) and a recent study has demonstrated a positive synergy between *T. denticola* and *P. gingivalis* in biofilm formation (76).

The study presented here is the first prospective longitudinal study employing quantitative real-time PCR to examine the effect of levels of bacterial species in plaque samples on the likelihood of imminent disease progression of chronic periodontitis. The development of quantitative real-time PCR has enabled the sensitive and accurate determination of the cell number of individual species in subgingival plaque samples. This technology is not without limitations, particularly with regard to the analysis of complex microbial communities such as subgingival plaque with its substantial species diversity (73). However, because it does not rely upon the cultivation of fastidious and uncultivatable organisms, real-time PCR currently allows for the best estimation of the total number of bacterial cells present in a complex sample, enabling the level of a specific bacterial species to be determined and expressed as a percentage of the total bacterial cell number.

The mere presence in subgingival plaque of any of the bacterial species investigated in this study at a specific site was not predictive of periodontal disease progression at that site. As these species are members of the normal oral microbiota and real-time PCR is a very sensitive technique for the detection of bacteria this is an understandable finding and one that has been reported previously (71, 74). The use of quantitative RT-PCR data for the bacterial species rather than dichotomous presence/absence data provided more statistical power for detecting any relationship with imminent disease progression. With presence/absence data, only subjects in whom the bacterial species were present (or absent) in either the case or the matching control sites, but not both, contribute to the conditional logistic regression analysis. For example, in the univariate analysis of the presence/absence of the bacterial species *P. gingivalis*, *T. denticola*, and *F. nucleatum* only 10 (40%), 10 (40%), and 4 (15%) of the case–control sets, respectively, were discordant and so informative for the conditional likelihood regression analysis. This also applied to dichotomous clinical measures such as bleeding on probing where only 11 (44%) of the case–controls sets were discordant.

It has been proposed that pathogenic bacteria need to reach specific threshold levels in subgingival plaque before they can cause disease and that detection of these species above these thresholds may serve as predictors of periodontal disease progression (5, 6, 22, 25, 47). This study shows that the levels of *P. gingivalis*, *T. denticola*, and *T. forsythia* in a subgingival plaque sample taken from a site enabled the prediction of the likelihood of imminent disease progression at that site (Table 6). An interaction between *P. gingivalis* and *T. denticola* in increasing the probability of a site undergoing imminent disease progression in this population was evident (Fig. 1). These data support the concept that the Red Complex of bacterial species is intimately involved in disease

progression and that the threshold proportion of one bacterial species that is associated with a significantly increased risk of future disease at a site is dependent upon the levels of other bacterial species at that site. The results strongly suggest that *T. forsythia* and/or *T. denticola* facilitate the emergence of *P. gingivalis* by providing an environment necessary for its colonization and proliferation.

For the determination of the specific bacterial composition of subgingival plaque to be of value to a clinician it must have an impact on the diagnosis of disease and/or overall treatment planning. Previous studies of bacterial levels in subgingival plaque samples as predictors of disease progression have tended to investigate which subjects are at greater risk of having a site or sites that will experience future disease progression (25, 52). In addition to this, increased risk has either been reported retrospectively or with time lines of 5–10 years (5, 47). The ability to predict that an individual with chronic periodontitis will exhibit future disease progression somewhere in their mouth at some time in the next 10 years would have little effect upon the clinician's treatment planning for that patient. The accurate chairside monitoring of the levels of *P. gingivalis*, *T. denticola*, and *T. forsythia* in subgingival plaque samples, as reported here, may aid clinicians in the identification of sites at significant risk for imminent periodontal breakdown, which would assist in the targeted treatment of disease.

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