Subgingival Microbial and Inflammatory Cell Morphotypes Associated with Chronic Periodontitis Progression in Treated Adults

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Abstract

Objective: In a secondary data analysis, this pilot study evaluated the relationship between subgingival biofilm morphotypes and chronic periodontitis progression in treated adults.

Methods: Periodontal parameters in 47 adults with chronic periodontitis were assessed by a calibrated examiner at baseline and a mean 4.5 years after a non-surgical periodontal therapy regimen. Microbial and inflammatory cell morphotypes in subgingival biofilm specimens from each patient were evaluated with phase-contrast microscopy at baseline, and at post-treatment intervals. Chronic periodontitis progression in patients was defined as ≥ 2 teeth exhibiting ≥ 3 mm interproximal clinical periodontal attachment loss from baseline evaluations. Bivariate and odds ratio analysis assessed baseline and post-treatment variables relative to chronic periodontitis progression.

Results: Eight (17%) patients had chronic periodontitis progression. No baseline clinical, radiographic or microbiological variables, and no post-treatment clinical variables demonstrated statistically significant relationships with chronic periodontitis progression. Elevated post-treatment counts of subgingival spirochetes, medium to large-sized motile rods, and crevicular leukocytes, both alone and concurrently, appeared more frequently in patients experiencing chronic periodontitis progression. A post-treatment occurrence of high concurrent counts of subgingival spirochetes and crevicular leukocytes exhibited the strongest association with chronic periodontitis progression (odds ratio = 10.1; 95% CI = 2.2, 45.4; $p = 0.004$), which was greater than with either morphotype alone.

Conclusions: Joint morphotype analysis of subgingival spirochetes and crevicular leukocytes, as simplified biomarkers of pathogenic biofilm infection and host inflammatory responses in periodontal pockets, may be diagnostically useful in assessing risk of progressive disease in treated chronic periodontitis patients.

Key words: Chronic periodontitis, spirochetes, subgingival microbiota, leukocytes, phase-contrast microscopy

Introduction

Periodontitis likely reflects the outcome of frustrated pro-inflammatory host responses to pathogenic bacterial biofilm growth on teeth, and possibly to the presence of lytic herpesvirus in gingival tissues, leading to progressive destruction of tooth-supporting connective tissues and alveolar bone (Slots, 2010). Because traditional clinical examinations and radiographic imaging of the periodontium show only limited utility in predicting the future periodontal status from present-day host/bacterial/viral interactions (Mombelli, 2005; Brägger, 2005), there is a long-standing need for additional diagnostic methods and criteria that better identify patients at increased risk of periodontitis progression (Tonetti *et al.*, 2005).

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Microscopic analysis of dental plaque biofilm morphology, first described by Antoni van Leeuwenhoek in 1683 (Arnim, 1962), has been advocated at various times for use in periodontal risk assessment (Bass and Johns, 1915; Arnim, 1964; Keyes *et al.*, 1978a, b; Keyes and Rams, 1983a). Studies with darkfield microscopy found only mixed results in identifying subjects at elevated risk for progressive periodontitis when subgingival bacterial cell morphotypes alone were monitored in subgingival plaque biofilms (Listgarten, 1986). Whereas increased baseline proportions of subgingival spirochetes and motile rods were significantly associated with chronic periodontitis progression in treated patients when periodontal maintenance care was discontinued for a year (Listgarten and Levin, 1981), or administered after extended customized time intervals (Listgarten *et al.*, 1986), no significant subgingival bacterial morphotype relationships to progressive disease were found when a periodontal prophylaxis was performed at regular 3-month intervals (Listgarten *et al.*, 1986).

Assessments of host inflammatory cells, and their constituents in crevicular sites, are also often proposed as potential periodontal diagnostic aids (Lamster and Ahlo, 2007). Elevated numbers of leukocytes, strongly correlated with bursts of destructive disease activity in experimental periodontitis (Zappa *et al.*, 1991), form a "leukocyte wall" between subgingival bacterial biofilms and gingival tissues, and likely contribute to both host protective and tissue-damaging outcomes in periodontal pockets (Delima and Van Dyke, 2003). Significantly higher neutrophil leukocyte counts are found in subgingival plaque biofilms and gingival crevicular fluid of chronic periodontitis patients as compared to persons with periodontal health (Bhadbhade *et al.*, 2012). Larger numbers of neutrophil leukocytes are also found in oral rinse samples, as a measure of oral inflammatory load, with increasing severity of gingival tissue inflammation and greater clinical periodontal breakdown (Bender *et al.*, 2006; Landzberg *et al.*, 2014).

In this regard, improvements in clinical periodontal attachment level at individual periodontal sites are associated with reduced numbers of crevicular leukocytes and subgingival spirochetes (Claffey *et al.*, 1985). Periodontitis patients favorably responding to non-surgical therapy exhibit significantly reduced numbers of crevicular leukocytes and motile bacterial morphotypes in subgingival dental plaque biofilms (Rams *et al.*, 1985; Malali *et al.*, 2012), reduced levels of vital leukocytes in crevicular lavages (Boretti *et al.*, 1995), and reduced oral rinse neutrophil leukocyte counts (Bender *et al.*, 2006). Analysis of crevicular leukocytes in subgingival biofilm samples with phase-contrast microscopy may improve initial diagnostic identification and characterization of periodontitis patients (Apsey *et al.*, 2006). This approach is also proposed as a rapid chair-side method to partially assess a patient's host response to periodontal bacterial pathogens, and characterize the inflammatory nature of their periodontal lesions (Apsey *et al.*, 2006).

However, subgingival morphotype analysis with phase-contrast microscopy has been characterized as an outmoded technology in periodontal diagnostics that "does not appear to have added diagnostic value over conventional clinical techniques for the assessment of disease or the monitoring of the progress of a case" (Kornman, 1998). Consequently, little attention has been given in recent years to subgingival morphotype analysis as other microbiological methods and diagnostic tools became available and the focus of research studies. An opportunity to further study the diagnostic potential of subgingival morphotype analysis may be obtained from data collected in a previously reported longitudinal clinical study of treated adults where some patients experienced chronic periodontitis progression (Keyes *et al.*, 1978a, b; Rams *et al.*, 1985; Rams and Keyes, 1990). In this prior study, microbial and inflammatory cell morphotypes in subgingival biofilms, as well as conventional periodontal diagnostic parameters, were assessed but not subjected to statistical analysis relative to their possible associations with chronic periodontitis progression. As a result, the purpose of the present pilot study was to use this prior clinical research data to evaluate the relationship between subgingival biofilm morphotypes and chronic periodontitis progression in 47 adults treated with a non-surgical periodontal therapy regimen.

Materials and methods

Patients

A secondary data analysis was performed on demographic, clinical, radiographic, and phase-contrast microscopic morphotype data from a prior clinical research study performed at the dental institute at the National Institutes of Health, Bethesda, MD, USA, on non-surgical periodontal treatment of 47 adults with chronic periodontitis (Keyes *et al.*, 1978a, b; Rams *et al.*, 1985; Rams and Keyes, 1990). Details of the non-surgical periodontal therapy performed on the study patients are previously described and published (Keyes *et al.*, 1978a, b; Rams *et al.*, 1985; Rams and Keyes, 1990). In brief, study patients with moderate to severe chronic periodontitis (Armitage, 2004) were treated with 1) subgingival tooth instrumentation until a smooth, hard root surface was clinically detected, 2) repeated professional pocket irrigation with a 1% chloramine-T antiseptic solution and/or saturated sodium bicarbonate or sodium chloride solutions, 3) short-term systemic tetracycline-HCl therapy (for 46 of the 47 study patients), 4) regular professional periodontal maintenance care and reinforcement of patient home care instructions at two- to four-month intervals, and 5) a daily patient home oral hygiene regimen involving sulcular brushing, flossing, oral irrigation with a saturated inorganic salt (sodium bicarbonate or sodium chloride) solution, and application of a sodium bicarbonate and 3% hydrogen peroxide paste to dentogingival surfaces with toothbrushes, interdental brushes, floss and rubber cone stimulators (Keyes *et al.*, 1978a, b; Rams *et al.*, 1985; Rams and Keyes, 1990).

The 33 female and 14 male study patients averaged 47.3 ± 9.8 (SD) years in age at baseline, were in good systemic health, had at least 22 teeth, and had not received any systemic antibiotic therapy within six months prior to baseline examinations. A total of six (12.8%) patients were current smokers. None of the patients had diabetes mellitus, hypertension, sodium intake restrictions, renal disorders, previous allergic reactions to tetracycline antibiotics, or any immunological diseases. The length of post-treatment follow-up observations averaged 4.5 ± 1.0 (SD) years (range 3 - 6.5 years) for the 47 study patients (Rams *et al.*, 1985).

The prior clinical research study was conducted with written human subject informed consent and protection oversight at the National Institutes of Health Clinical Center in Bethesda, Maryland, USA, in compliance with the Helsinki Declaration. Approval for the present secondary data analysis was provided by the Temple University Human Subjects Protections Institutional Review Board.

Clinical and radiographic variables

A single experienced and calibrated periodontist examiner, who was unaware of the course of the rendered periodontal therapy (single-blind evaluations), independently assessed clinical parameters on each study patient at pre-treatment baseline and a mean 4.5 years post-treatment, as previously described (Rams *et al.*, 1985; Rams and Keyes, 1990). These assessments included enumeration of missing teeth, teeth with grade 2 or 3 furcation involvements, probing depths, clinical periodontal attachment level, presence of suppuration, sulcular bleeding scores, tooth mobility, and occurrence of periodontal abscess formation. In addition, teeth with radiographic loss of ≥ 50% crestal alveolar bone support were noted from pre-treatment periapical radiographs. Patients maintaining excellent post-treatment plaque control were identified as yielding no or minimal levels (1-3 positive tooth surfaces assessed with a periodontal probe) of clinically detectable supragingival dental plaque at post-treatment maintenance care appointments.

Phase-contrast microscopic variables

At pre-treatment baseline and post-treatment intervals ranging from two to four months, disease-associated microbial morphotypes and crevicular leukocytes in direct wet-mount preparations of pooled subgingival biofilm specimens from each study patient were evaluated with phase-contrast microscopy, as previously described (Keyes *et al.*, 1978a,b; Keyes *et al.*, 1982; Keyes and Rams, 1983a; Rams *et al.*, 1985). In brief, after removal of supragingival plaque, subgingival biofilm samples were removed with a sterile curette from the most apical portions of two to five periodontal sites per patient with the greatest gingival inflammation, deepest residual probing depths and/or furcation involvements. The specimens were then pooled undispersed into 20 µl of sterile water on a microscopic slide, coverslipped with slight compression (no staining or fixing), and examined immediately at 400x and 600x with a high-quality phase-contrast microscope (Olympus BH series, Olympus America Inc., Center Valley, PA USA) equipped with a 1.25x to 1.5x intermediate magnification changer. The entire slide was examined, and at least ten fields containing the greatest concentrations of motile morphotypes and crevicular leukocytes were assessed quantitatively at 400x magnification. Subgingival counts of medium to large-sized motile rods, crevicular leukocytes, brush formations, amoeba (*Entamoeba gingivalis*), and trichomonads (*Trichomonas tenax*) were semi-quantitatively scored (not detected, 10±, 100± or ≥ 125 for motile morphotypes; ≤ 25, 50-100± or ≥ 125 for crevicular leukocytes; not detected or present for *E. gingivalis* and *T. tenax*) from the highest scoring microscopic fields per sample by a single dentist examiner (author PHK), who was experienced with phase-contrast microscopic analysis of subgingival dental plaque biofilm specimens (Keyes *et al.*, 1978a, b; Keyes *et al.*, 1982; Keyes and Rams, 1983; Rams *et al.*, 1985). A total of 1,978 post-treatment subgingival biofilm specimens were evaluated and scored from the 47 study patients (mean = 42.1 ± 14.7 (SD) per study patient).

Data analysis

Post-treatment progression of chronic periodontitis in patients was defined in this secondary data analysis as the presence of two or more teeth exhibiting ≥ 3 mm interproximal clinical periodontal attachment loss from baseline evaluations, as recommended by the 5th European Workshop in Periodontology for risk factor research studies (Tonetti *et al.*, 2005).

Descriptive analysis, with patients as the unit of analysis, was used to determine the distribution of baseline and post-treatment demographic, clinical, radiographic and phase-contrast microscopic morphotype variables in study patients positive and negative for post-treatment progression of chronic periodontitis. Sulcular bleeding scores were dichotomized into bleeding on probing being either present or not present. Motile morphotype scores were dichotomized for statistical analysis as either elevated (for scores of 100± and ≥ 125±/highest scoring microscopic fields) or not elevated (for scores of not detected and 10±/highest scoring microscopic fields), similar to the coding scheme for phase-contrast microscopic findings employed by Apsey *et al.* (2006). Crevicular leukocytes scores were similarly dichotomized as either elevated (for scores of ≥ 125/highest scoring microscopic fields) or not elevated (for scores of ≤ 25 and 50 – 100±/highest scoring microscopic fields). The proportion of elevated scores for spirochetes,

medium to large-sized motile rods, and crevicular leukocytes, both individually and concurrently, among all post-treatment microscopic scores, were averaged for each patient, and then averaged across patients with and without post-treatment progression of chronic periodontitis.

Bivariate analysis, using the Student's *t*-test to evaluate differences in means, the Fisher's exact test to evaluate differences in proportions, and a two-tailed *p* - value of ≤ 0.05 as a critical threshold for statistical significance, assessed the relationship between various baseline and post-treatment variables to progression of chronic periodontitis in patients.

Because of the occurrence of zero event cells in 2x2 contingency table analysis, Peto odds ratios and their 95% confidence intervals (CI; Brockhaus *et al.*, 2014), as determined using an on-line calculator (http://www.hutchon.net), were used to estimate true odds ratios in assessing the relationship of binary post-treatment phase-contrast microscopic variables with progression of chronic periodontitis. Sensitivity, specificity, positive predictive value, and negative predictive values (McNeil *et al.*, 1975) for the binary post-treatment phase-contrast microscopic variable with the highest odds ratio relationship with chronic periodontitis progression were calculated to estimate its prognostic capability. A PC-based, 64-bit, statistical software package (JMP Pro 10.0.2, SAS Institute, Inc., Cary, NC USA) was used in the data analysis.

Results

A total of 8 (17%) of the study patients exhibited post-treatment progression of chronic periodontitis.

Table 1 shows that none of the pre-treatment demographic, clinical, radiographic or phase-contrast microscopic morphotype variables studied demonstrated statistically significant bivariate relationships to progression of chronic periodontitis in patients (all *p* - values > 0.05).

Table 1. Bivariate analysis of baseline variables with post-treatment progression of chronic periodontitis in treated adults.

	Post-treatment progression of chronic periodontitis		
	Yes (n = 8)	**No (n = 39)**	***p* - value***
Demographic variables - baseline			
Mean age (SD)	47.5 (9.8)	46.9 (10.0)	NS
Number (%) male gender	1 (12.5)	13 (33.3)	NS
Number (%) Caucasian	7 (87.5)	38 (97.4)	NS
Number (%) current smokers	1 (12.5)	5 (12.8)	NS
Clinical variables - baseline			
Mean number of missing teeth/patient (SE)	2.4 (0.8)	2.3 (0.3)	NS
Mean number of teeth/patient with furcation involvement (SE)	3.3 (0.5)	2.4 (0.4)	NS
Mean patient whole-mouth probing depth (mm) (SE)	3.8 (0.2)	3.8 (0.1)	NS
Mean % teeth/patient with ≥ 5 mm probing depth (SE)	63.8 (6.1)	60.0 (3.2)	NS
Mean % teeth/patient with suppuration (SE)	6.7 (6.7)	1.4 (0.6)	NS
Mean % teeth/patient with bleeding on probing (SE)	47.3 (15.3)	47.7 (4.7)	NS
Mean number of teeth/patient with mobility (SE)	1.1 (0.7)	0.8 (0.2)	NS
Number (%) patients with recent periodontal abscess	2 (25.0)	9 (23.1)	NS
Radiographic variable - baseline			
Mean number of teeth with ≥ 50% alveolar bone loss (SE)	5.6 (1.8)	6.1 (1.0)	NS
Phase-contrast microscopic variables - baseline			
Number (%) patients with elevated spirochetes	7 (87.5)	33 (84.6)	NS
Number (%) patients with elevated medium- to large-sized motile rods	8 (100)	39 (100)	NS
Number (%) patients with elevated crevicular leukocytes	8 (100)	37 (94.9)	NS
Number (%) patients with concurrently elevated spirochetes and crevicular leukocytes	7 (87.5)	31 (79.5)	NS
Number (%) patients with concurrently elevated medium- to large-sized motile rods and crevicular leukocytes	8 (100)	37 (94.9)	NS
Number (%) patients with concurrently elevated spirochetes and medium- to large-sized motile rods	7 (87.5)	33 (84.6)	NS
Number (%) patients positive for brush formations	3 (37.5)	17 (43.6)	NS
Number (%) patients positive for *Entamoeba gingivalis*	2 (25.0)	17 (43.6)	NS
Number (%) patients positive for *Trichomonas tenax*	0	2 (5.1)	NS

*Student's *t*-test or Fisher's exact test for statistically significant differences between post-treatment progression of chronic periodontitis-positive vs. -negative patients; NS, not statistically significant ($p > 0.05$); SD, standard deviation; SE, standard error of the mean.

Table 2 reveals that no post-treatment clinical variables were statistically associated with progression of chronic periodontitis. However, several post-treatment phase-contrast microscopic morphotype variables showed statistically significant bivariate differences between patients with and without progression of chronic periodontitis. Significantly greater mean proportions of subgingival biofilm specimens per patient during all post-treatment visits were positive with elevated counts of spirochetes, medium to large-sized motile rods, and crevicular leukocytes, both alone and concurrently, in patients with, as compared to those without, progression of chronic periodontitis. On a patient level, significantly greater proportions of patients with progression of chronic periodontitis had ≥ 1 post-treatment subgingival biofilm specimens with elevated counts of either spirochetes alone, or concurrent with elevated counts of either medium- to large-sized motile rods or crevicular leukocytes, as compared to patients without progression of chronic periodontitis (*Table 2*). No statistically significant differences were found in the post-treatment occurrence of subgingival *E. gingivalis*, *T. tenax*, or brush formations between patients with and without progression of chronic periodontitis (*Table 2*).

Table 3 presents odds ratio analysis of the relationship between selected post-treatment phase-contrast microscopic variables and progression of chronic periodontitis in patients. High concurrent counts of subgingival spirochetes and crevicular leukocytes in ≥ 1 post-treatment subgingival biofilm specimens provided the strongest association with progression of chronic periodontitis (odds ratio = 10.1; 95% CI = 2.2, 45.4). A post-treatment occurrence of jointly elevated subgingival counts of spirochetes and crevicular leukocytes displayed a greater odds ratio relationship with progression of chronic periodontitis than did elevated subgingival counts of either of the two morphotypes alone (*Table 3*). High concurrent post-treatment counts of subgingival spirochetes and crevicular leukocytes were found to exhibit a sensitivity = 100%, specificity = 59.0%, positive predictive value = 33.3%, and negative predictive value = 100%, relative to progression of chronic periodontitis in treated adults.

Table 2. Bivariate analysis of post-treatment variables with post-treatment progression of chronic periodontitis in treated

	Post-treatment progression of chronic periodontitis		
	Yes (n = 8)	**No (n = 39)**	***p* - value***
Clinical variables - post-treatment			
Mean patient whole-mouth probing depth (mm) (SE)	3.5 (0.6)	3.1 (0.1)	NS
Mean % teeth/patient with ≥ 5 mm probing depth (SE)	48.6 (7.8)	34.9 (3.1)	NS
Mean % teeth/patient with suppuration (SE)	0	0	NS
Mean % teeth/patient with bleeding on probing (SE)	25.4 (6.8)	14.3 (2.1)	NS
Mean number of teeth/patient with mobility (SE)	0.5 (0.4)	0.1 (0.1)	NS
Number (%) patients with excellent supragingival plaque control	1 (12.5)	21 (53.8)	NS
Phase-contrast microscopic variables - post-treatment			
Mean % specimens/patient with elevated spirochetes (SE)	11.2 (2.4)	4.8 (1.0)	0.012
Mean % specimens/patient with elevated medium- to large- sized motile rods (SE)	25.0 (1.9)	14.0 (1.9)	0.014
Mean % specimens/patient with elevated crevicular leukocytes (SE):	25.9 (4.5)	14.0 (1.8)	0.011
Mean % specimens/patient with concurrently elevated spirochetes and crevicular leukocytes (SE)	8.2 (2.5)	2.7 (0.6)	0.003
Mean % specimens/patient with concurrently elevated medium- to large-sized motile rods and crevicular leukocytes (SE):	15.5 (2.6)	6.7 (1.1)	0.002
Mean % specimens/patient with concurrently elevated spirochetes and medium to large-sized motile rods (SE)	11.2 (2.4)	4.5 (0.9)	0.005
Number (%) patients with elevated spirochetes in ≥ 1 specimen	8 (100)	19 (48.7)	0.014
Number (%) patients with elevated medium to large-sized motile rods in ≥ 1 specimen	8 (100)	33 (84.5)	NS
Number (%) patients with elevated crevicular leukocytes in ≥ 1 specimen	8 (100)	34 (87.2)	NS
Number (%) patients with concurrently elevated spirochetes and crevicular leukocytes in ≥ 1 specimen	8 (100)	16 (41.0)	0.004
Number (%) patients with concurrently elevated medium- to large-sized motile rods and crevicular leukocytes in ≥ 1 specimen	8 (100)	28 (71.8)	NS
Number (%) patients with concurrently elevated spirochetes and medium- to large-sized motile rods in ≥ 1 specimen	8 (100)	18 (46.2)	0.006
Number (%) patients positive for brush formations	7 (87.5)	9 (23.1)	NS
Number (%) patients positive for *Entamoeba gingivalis*	5 (62.5)	12 (30.8)	NS
Number (%) patients positive for *Trichomonas tenax*	0	2 (5.1)	NS

*Student's *t*-test or Fisher's exact test for statistically significant differences between post-treatment progression of chronic periodontitis-positive vs. -negative patients; NS, not statistically significant ($p > 0.05$); SE, standard error of the mean.

Table 3. Odds ratio analysis of selected post-treatment phase-contrast microscopic variables with post-treatment progression of chronic periodontitis in treated adults.

Post-treatment phase-contrast microscopic variable	Odds ratio [95% CI] for post-treatment progression of chronic periodontitis	*p* - value*
Elevated counts of spirochetes and crevicular leukocytes concurrently detected	10.1 [2.2, 45.4]	0.004
Elevated counts of spirochetes and medium- to large-sized motile rods concurrently detected	8.4 [1.9, 38.3]	0.006
Elevated counts of spirochetes alone detected	7.8 [1.7, 35.7]	0.014
Elevated counts of medium- to large-sized motile rods and crevicular leukocytes concurrently detected	4.7 [0.8, 27.6]	NS
Elevated counts of medium- to large-sized motile rods alone detected	3.9 [0.4, 36.9]	NS
Elevated counts of crevicular leukocytes alone detected	3.7 [0.3, 43.0]	NS

*Fisher's exact test; NS, not statistically significant ($p > 0.05$)

Discussion

This study provides the first longitudinal data analysis assessing both microbial and inflammatory cell morphotypes in subgingival biofilms as an aid in assessing the risk of progression of chronic periodontitis in treated adult patients. The study findings suggest a potential clinical value in periodontal diagnostics with joint analysis of subgingival spirochetes and crevicular leukocytes with phase-contrast microscopy. Elevated post-treatment counts of spirochetes, medium- to large-sized motile rods, and crevicular leukocytes, both alone and concurrently, appeared more frequently in subgingival biofilm specimens of patients experiencing chronic periodontitis progression. However, the occurrence in patients of high concurrent counts of subgingival spirochetes and crevicular leukocytes in ≥ 1 post-treatment subgingival biofilm specimens provided the strongest association with clinical progression of chronic periodontitis measured over a mean 4.5 year post-treatment period (odds ratio = 10.1; 95% CI = 2.2, 45.4; $p = 0.004$).

The joint association of elevated spirochetes and crevicular leukocytes with progression of chronic periodontitis was greater than the association with either of the two morphotypes alone. This suggests that subgingival periodontopathic microbial communities that elicit heightened host inflammatory cell responses, or proliferate in their presence (Van Dyke, 2014), in terms of elevated crevicular leukocyte counts, may be more important in periodontal risk assessment than the mere presence of putative periodontal bacterial pathogens in uninflamed periodontal pockets. Whereas high subgingival spirochetes counts alone demonstrated a statistically significant relationship with progressive chronic periodontitis, the greatest added diagnostic value was found with additional analysis of crevicular leukocyte counts, and less so with medium- to large-sized motile rods.

Importantly, the significant association of high concurrent subgingival spirochete and crevicular leukocyte counts with chronic periodontitis progression was in contrast to various baseline and post-treatment clinical and radiographic parameters, which were unable to significantly differentiate between patients with and without chronic periodontitis progression. These study findings are consistent with prior research demonstrating the relatively poor capability of conventional clinical and radiographic evaluations of the periodontium, with the possible exception of radiographic crestal lamina dura (Rams *et al.*, 1994), to reliably predict future episodes of periodontitis disease activity in patients (Mombelli, 2005; Brägger, 2005).

Previous microbiological research observations are also consistent with our study findings. With 454-pyrosequencing of 16S rRNA genes in subgingival plaque biofilms, seven spirochete species of the *Treponema* genus, including *Treponema denticola*, were identified as belonging to the core microbiome in human periodontitis lesions, but not in the core microbiome of periodontal health (Abusleme *et al.,* 2013). Reviere *et al.* (1997) found 14 of 55 (25%) healthy periodontal sites positive for subgingival spirochetes subsequently developed ≥ 2 mm periodontal attachment loss over the next 12 months (odds ratio = 3.1 for subgingival spirochetes in initially periodontally healthy sites that developed periodontitis). It was concluded that some periodontally healthy sites are microbiologically distinct about 6-12 months before clinical signs of periodontal attachment loss appear, with subgingival spirochete colonization preceding clinical deterioration, and that presence of subgingival spirochetes in periodontally healthy sites increased susceptibility to periodontitis development (Reviere *et al.*, 1997). In treated chronic periodontitis patients, Slots *et al.* (1985) reported post-treatment clinical periodontal attachment loss significantly associated with persistence of subgingival spirochetes monitored with phase-contrast microscopy. Similarly, Byrne *et al.* (2009) noted increased subgingival levels of *T. denticola*, monitored with quantitative real-time PCR methodology, to be associated with a 130% excess risk of clinical periodontal breakdown within a subsequent 3-month time period in treated chronic periodontitis

subjects on maintenance care. The study also found that clinical periodontal parameters, and the mere presence or absence of subgingival bacterial pathogens without quantification, failed to predict periodontitis disease activity (Byrne *et al.*, 2009). Loesche *et al.* (1985) found chronic periodontitis patients with severe periodontal attachment loss, who were treated and their clinical status stabilized for at least one year, had a strong likelihood of subgingival spirochetes being absent or only present in low levels (< 10% of direct microscopic counts) in comparison to untreated patients (odds ratio = 69.1 calculated from data tables presented). With studies of host leukocytes, chronic periodontitis sites undergoing clinical probing attachment loss yielded significantly more vital crevicular leukocytes than clinically stable periodontitis sites (Boretti *et al.*, 1995). Additionally, chronic periodontitis patients with poor clinical periodontal treatment responses demonstrated persistently high oral rinse neutrophil leukocyte counts, which mostly originate from periodontal pockets (Bender *et al.*, 2006).

Several phase-contrast microscopic morphotype parameters evaluated in the present study did not significantly correspond with post-treatment progression of chronic periodontitis. *E. gingivalis* and *T. tenax* in subgingival plaque biofilms have been related with periodontitis lesions in cross-sectional studies, and postulated to contribute to their etiology (Bass and Johns, 1915; Gottlieb and Miller, 1971; Lucht *et al.*, 1998; Ghabanchi *et al.*, 2010; Bonner *et al.*, 2014). In the first longitudinal study of these protozoa in periodontal pockets, the present study did not find the occurrence of subgingival *E. gingivalis* and *T. tenax* to be significantly related to chronic periodontitis disease activity. Subgingival brush formations, with organized metachronal wave movement by closely-packed spirochetes co-aggregating the outer surfaces of brush formation monofilaments (Keyes and Rams, 1993), were detected in seven of eight study patients with chronic periodontitis progression, but also in nearly one-fourth of treated patients without progression of chronic periodontitis. Medium- to large-sized motile rods alone in subgingival plaque biofilms were less enlightening than spirochetes in assessing risk of chronic periodontitis progression. This is likely due to varying periodontopathic potentials associated with the heterologous array of microbial species presenting as motile rod morphotypes in periodontal pockets.

The present pilot study has several limitations. A more detailed evaluation and identification of specific microbial species in subgingival plaque biofilms of the study patients, such as with cultivation or molecular techniques, as well as a more comprehensive analysis of host immunoinflammatory responses beyond enumeration of crevicular leukocytes, was not performed. The post-treatment follow-up observation time periods were not uniform among the 47 study patients, and no intermediate clinical data measurements prior to the final post-treatment evaluations were available. An unconventional post-treatment plaque control assessment method was employed. No comparisons in prognostic performance were made between the microbial-inflammatory cell microscopic morphotypes identified in this study and other microbial biomarkers proposed for predicting future periodontal breakdown, such as elevated cultivable subgingival proportions of major putative bacterial pathogenic species (Rams *et al.*, 1996). The data utilized to identify subgingival spirochetes and crevicular leukocytes as potential microbial-inflammatory cell biomarkers for progression of chronic periodontitis was too small to analyze with multivariate statistical methods, or to randomly split into two separate databases – one for biomarker discovery/development, and another for independent biomarker performance evaluation. The positive predictive value of only 33.3% provided by the post-treatment occurrence of elevated subgingival spirochete and crevicular leukocyte counts for progression of chronic periodontitis limits its overall clinical utility. In contrast, the 100% negative predictive value associated with this phase-contrast microscopic variable suggests that if no or only low spirochete and crevicular leukocyte counts are attained and maintained by periodontal treatment procedures, then the risk of chronic periodontitis disease progression appears to be minimal.

Conclusions

Consistent with prior clinical research studies, no pretreatment clinical, radiographic or microbiological variables, and no post-treatment clinical variables demonstrated statistically significant relationships with chronic periodontitis progression. However, joint phase-contrast microscopic morphotype analysis of post-treatment subgingival spirochete and crevicular leukocyte levels, as simplified biomarkers of subgingival plaque biofilm pathogenicity and host-derived inflammatory responses in periodontal pockets, respectively, appeared to provide added value over the use of conventional periodontal parameters alone in post-treatment risk assessment of chronic periodontitis patients, and is worthy of further research attention.

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