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Clinical characteristics and microbiota of progressing slight chronic periodontitis in adults

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Abstract

Aim: This study sought clinical and microbial risk indicators for progressing slight periodontitis.

Material and Methods: One hundred and seventeen periodontally healthy or slight periodontitis adults (20–40 years) were monitored clinically at 6-month intervals followed by supragingival cleaning. Inter-proximal sites with >1.5 mm increase in clinical attachment over 18 months were considered disease active. Subgingival plaque was analysed by 78 16S rDNA and 38 whole-genomic DNA probes and by PCR to *Porphyromonas gingivalis* and *Tannerella forsythia*. Characteristics were compared between active and inactive subjects.

Results: Twenty-two subjects showed disease activity principally at molars. Mean baseline gingival and plaque indices, bleeding on probing, probing depth and clinical attachment level (CAL) were higher in active subjects. DNA probes detected species and not-yet-cultivated phylotypes from chronic periodontitis, although few species were associated with active subjects. By PCR *P. gingivalis* ($p = 0.007$) and *T. forsythia* ($p = 0.075$) were detected more frequently during monitoring in active subjects. Stepwise logistic analysis associated baseline levels of gingival index, clinical attachment and bleeding with subsequent clinical attachment loss.

Conclusions: Gingivitis and CAL were significantly associated with progressing slight periodontitis in 20–40-year-old adults. Species associated with moderate and advanced chronic periodontitis were detected in slight periodontitis.

Key words: DNA probes; gingivitis; microbiology; *P. gingivalis*; slight chronic periodontitis; *T. forsythia*

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One of the challenges of periodontal practice is determining which patients with low levels of periodontal clinical attachment loss are most at risk for developing additional clinical attachment loss. This challenge extends to public health dentistry where population-based strategies are needed to reduce risk of periodontal infections that affect the dentition and can impact systemic health. The classification of Armitage described low levels of chronic periodontitis as slight periodontitis (Armitage 2004), hence this term is used although other terms including mild, early and initial periodontitis have also been used. Clinical predictors of periodontal disease progression have included level of clinical attachment (Machtei et al. 1999, Craig et al. 2003) and gingivitis (Albandar et al. 1998, Craig et al. 2001). Because the primary aetiology of periodontal diseases is bacterial, we hypothesized that certain bacteria could indicate subjects at risk for future clinical attachment loss. The major species associated with moderate and advanced chronic adult periodontitis were originally detected using cultivation-based methods and include *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* (*T. forsythensis*, *Bacteroides forsythus*), *Treponema denticola* and *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* (Moore et al. 1991, Kamma et al. 1995, Haffajee et al. 1998, Mombelli et al. 1998, Machtei et al. 1999, van Winkelhoff et al. 2002, Dogan et al. 2003, Kumar et al. 2003, Socransky & Haffajee 2005). The

Conflict of interest and source of funding statement

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range of bacterial species detected in periodontitis has expanded following the use of non-cultural molecular techniques (Kroes et al. 1999, Sakamoto et al. 2000, Paster et al. 2001, Kumar et al. 2005, Aas et al. 2007). Rapid methods, including those based on identification from 16S rRNA genes, have indicated associations with an expanded set of cultivated and not-yet cultivated species, with moderate to advanced chronic periodontitis. Additional periodontitis-associated species included *Filifactor alocis*, *Porphyromonas endodontalis*, *Eubacterium saphenum*, *Eubacterium nodatum* in addition to not-yet cultivated phylotypes (Kumar et al. 2003, Dahlen & Leonhardt 2006, Haffajee et al. 2006).

The microbiota of slight chronic periodontitis in adults and adolescents has been associated with *P. gingivalis* and *T. forsythia* using rapid immunofluorescence (Riviere et al. 1996, Clerehugh et al. 1997, Hamlet et al. 2004) and DNA probe methods (Tran et al. 2001). In a longitudinal study to detect progressing slight periodontitis, a combination of anaerobic culture and DNA hybridization assays associated *T. forsythia*, *Campylobacter rectus*, *Selenomonas noxia* and *P. intermedia* with inter-proximal progressing slight (initial) chronic periodontitis, compared with health or gingivitis (Tanner et al. 1998). In the latter study *P. gingivalis* was not associated with slight progressing periodontitis by culture or by DNA probes. In contrast to inter-proximal sites, the microbiota of progressing buccal or lingual sites resembled that of gingival health, suggesting clinical attachment loss at these sites might be the result of trauma from toothbrushing, rather than from infection (Tanner et al. 1998, Page & Sturdivant 2002).

The *clinical relevance* of studying the bacteriology of slight periodontitis is to evaluate whether bacterial assessment can contribute to understanding disease aetiology and ultimately to help identify subjects who are at risk for additional clinical attachment loss. To achieve this, the present study divided subjects with <2 mm mean clinical attachment loss into active and inactive disease categories based on the detection of one or more inter-proximal sites losing >1.5 mm clinical attachment over longitudinal monitoring, as described previously (Tanner et al. 1998). Clinical and microbial characteristics were compared between active and inactive subjects. Three microbiological assays were used. 16S rDNA-based oligonucleotide probes were used in subject-based analyses to detect cultivated and not-yet-cultivated species. Whole-genomic DNA probes were used to quantify species detected and to evaluate bacterial colonization in different regions of dentition. Longitudinal microbial monitoring over 18 months was performed by PCR to the major periodontal pathogens *P. gingivalis* and *T. forsythia* because of their association with slight periodontitis in other studies (Riviere et al. 1996, Clerehugh et al. 1997, Tran et al. 2001, Hamlet et al. 2004, Tanner et al. 2006).

Material and Methods

Subject recruitment and clinical monitoring

Subjects in this study were a subset of 225 people for whom cross-sectional baseline clinical data were reported previously (Tanner et al. 2005). Study subjects were recruited to three dental clinics in the Boston area by newspaper, radio and inter-institutional email advertisements. Subjects completed a medical and dental history, and underwent a clinical screening examination to evaluate eligibility for the study. General entry requirements were age between 20 and 40 years, medically healthy, no previous periodontal treatment and no antibiotics in the previous 6-month period. Periodontal entry requirements were that subjects should have a mean periodontal clinical attachment level (CAL) of <2.0 mm, which was determined at the screening visit. Subjects were requested to undergo periodontal and microbial monitoring at 6-month intervals for 18 months to participate in this study.

The study design was explained to all subjects, and those willing to sign an informed consent approved by the Institutional Review Board of participating clinics in accordance with the Helsinki Declaration of 1975, as revised in 1983, were accepted into the study. Subjects were offered reimbursement for their participation at each clinical measurement visit. Subjects completed a questionnaire that included their race/ethnicity and cigarette smoking history.

Clinical measurements at each visit included duplicate measurements of probing depth (PD) and CAL and single assessments of plaque index, gingival index and bleeding on probing (BOP) as described previously (Tanner et al. 2005). Measurements were made at six sites on all teeth, except for third molars, at the mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual positions using a Hu Friedy PCP-UNC-15 probe (Hu Friedy Instruments, Chicago, IL, USA), and estimated to the nearest 0.5 mm (Tanner et al. 2005). Radiographs were not taken. At the completion of each visit, subjects received supragingival scaling with a Gracey curette (Hu Friedy Instruments) and teeth were polished with a rubber cup and prophylactic paste to remove only supragingival plaque. Clinicians making clinical measurements participated in pre-study training by the clinician at the Forsyth Institute (the primary examiner) and calibration exercises as described previously (Tanner et al. 2005), which were repeated at 6-month intervals during the study. The intra-class correlations (Fleiss 1986) for duplicate measurements between examiners were 0.87 for PD and 0.85 for CAL (Tanner et al. 2005). For each subject, longitudinal clinical measurements were made by the same examiner, except early in the study when there were changes in examiners. The new examiners were calibrated to the primary examiner.

Microbiological methods

Sample taking

Samples were taken after measurement of plaque and gingival indices but before PD and CAL measurements. Supragingival plaque was removed with a Gracey curette, then samples were taken with a fresh sterile curette from subgingival sites. At each clinical visit, pooled subgingival samples, usually distolingual of first or second molars, were placed in 100 μl of Tris-EDTA (ethylene-diamine-tetraacetic acid) buffer and stored frozen at -70°C (Tanner et al. 2006). These samples were analysed by either 16S rDNA probes or multiplex PCR to *P. gingivalis* and *T. forsythia*. Separate mesio-buccal subgingival samples from all teeth at baseline were collected individually from all teeth into 150 μl of Tris-EDTA, 150 μl of 0.5 M NaOH added and analysed using whole-genomic probes.

Microbial analysis

Three different approaches were used to measure the subgingival microbiota.

The first assay used 16S rDNA probes to survey cultivated and not-yet-cultivated species in a cross-sectional analysis of pooled subgingival molar samples. Pooled subgingival samples were used because preliminary data indicated that the sample size from individual subgingival sites was too small to reliably obtain a PCR product. The second assay used whole-genomic probes to include additional cultivated species, in a cross-sectional analysis to quantitate the level of species detected, and also to examine the microbiota of different segments of dentition. The third microbiological assay, multiplex PCR for *P. gingivalis* and *T. forsythia*, was adopted primarily to improve sensitivity of detection of *P. gingivalis* over the 16S rDNA probes, and provide a longitudinal analysis of the subgingival microbiota. Not all samples were available for all analyses either because there was not enough plaque, particularly for the PCR-based 16S rDNA probe and the subsequent multiplex PCR assays on the pooled plaque assays, or because samples were broken down over 3 years despite storage at -80°C for the whole-genomic probe assay. For the whole-genomic probe assay, inactive subjects were selected by matching to active subjects based on gender, race/ethnicity, baseline disease characteristics and clinical centre.

16S rDNA probe analysis was performed using the PCR-based reverse capture checkerboard assay (Paster et al. 1998, Becker et al. 2002) as we described previously (Tanner et al. 2006). Probe species included periodontally related species and phylotypes from PCR-cloning studies of human dental plaque (Paster et al. 2001). Reactions were scored as species detected or not detected. The detection limit for the assay was approximately 10^3 cells.

Whole-genomic DNA probe analyses were performed on separate, individual mesial site samples using the whole-genomic DNA probe checkerboard assay as described previously (Socransky et al. 2004). Two lanes in each nylon membrane were used for a mixed DNA standard of cells from all probe species at 10^5 and 10^6 cell equivalents. The 38 probe species were selected from species identified from our previous cultural study of slight periodontitis (Tanner et al. 1998) including *C. rectus*, *S. noxia* and *P. intermedia* that were not assayed using 16S rDNA probes. Signals were detected using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA), and reactions enumerated as described recently (Socransky et al. 2004). Briefly, signals were converted to absolute counts by comparison with the 10^5 and 10^6 standards on each membrane. The sensitivity of the assay was set to detect 10^4 cells by adjusting concentrations of DNA probes and readings below 10^4 were set to zero (Socransky et al. 2004).

The multiplex PCR assay to detect *P. gingivalis* and *T. forsythia* was performed as previously described (Tran & Rudney 1999) on pooled molar samples as described for the baseline population (Tanner et al. 2006). Samples from all clinical visits over 18 months of monitoring were analysed. The multiplex PCR assay used different primers than for the 16S rDNA probes with a similar detection threshold for *T. forsythia* at 10^3 cells but with a more sensitive detection limit for *P. gingivalis* at 10^2 cells. Samples negative for *P. gingivalis* and *T. forsythia* were tested using the broad-range universal primers used in the oligonucleotide probe assay, to evaluate whether the sample contained detectable bacterial DNA particularly since some of these samples had been stored for over 3 years. Negative samples using the universal primers were not considered valid. Reactions from this assay were scored as species detected or not detected.

Statistical analyses

Subjects were divided into active and inactive disease categories. Subjects with >1.5 mm increase in clinical attachment at one or more inter-proximal (mesiobuccal, mesiolingual, distobuccal and distolingual) sites over 18 months monitoring were considered active, whereas inactive subjects did not show any such increases. Mean differences between active and inactive subjects with respect to age and baseline clinical measurements were evaluated by *t*-test. Contingency tables were used to evaluate associations between disease category and, respectively, gender, smoking history and race–ethnicity. Mean values at 18 months in levels of clinical measures at inter-proximal sites were compared across the two groups by analysis of covariance adjusting for baseline values. Associations between baseline mean levels of BOP, gingival index and plaque at inter-proximal sites and subsequent change in subject mean CAL and PD at inter-proximal sites were evaluated by Spearman's rank correlations. Distributions of inter-proximal and buccal/lingual sites with >1.5 mm change (loss or gain) over 18 months were tabulated by tooth type.

Associations of active and inactive disease categories with the cross-sectional baseline categories (Tanner et al. 2005) of health (mean CAL $\leqslant$ 1.5 mm, no sites CAL >2 mm), and slight (early) periodontitis 1 (mean CAL <1.5 mm, $\geqslant 1$ sites with CAL $>$ 2 mm) and slight (early) periodontitis 2 (mean CAL $\geqslant 1.5$ mm) categories were evaluated using the Mantel–Haenszel χ^2 test for trend.

For analyses of microbiological data, subjects were grouped into active and inactive disease categories. A cross-sectional analysis was performed on DNA probe data using baseline data. Significance levels were not adjusted for multiple comparisons over species. For the 16S rDNA (oligonucleotide) probe reverse capture checkerboard data, species were compared between clinical groups by χ^2 test. For the individual tooth data analysed using whole-genomic probes, species levels (counts $\times 10^5$) and species as a per cent of total DNA count were averaged over subjects, and compared between active and inactive subjects by *t*-tests. Samples were grouped by tooth type as described for the clinical data, but mandibular and maxillary teeth were not separated. Total DNA count levels (counts $\times 10^5$) and species as per cent of total DNA were compared by tooth type between active and inactive subjects by *t*-tests. To evaluate whether bacteria were associated with clinical measurements, Spearman's rank correlations were determined between total DNA probe count and individual species, and plaque and gingival indices, PD and CAL.

A longitudinal analysis of the microbiota was performed using the multiplex PCR assay, and the frequencies of detection of *P. gingivalis*- and *T. forsythia*-positive subjects at one or more of three longitudinal samples were compared by χ^2 analysis.

Logistic regression analysis, for both specified and stepwise models, was used to evaluate the association of combinations of baseline clinical measures and indicator variables for *P. gingivalis* and *T. forsythia* detection by PCR with subsequent no loss or loss (>1.5 mm) of attachment during monitoring. Average values of clinical measures for

each subject were entered as continuous independent variables. Microbial measures were entered as 0 or 1 (not detected/detected).

Results

Clinical data by subject

One hundred and seventeen subjects completed the 18-month longitudinal monitoring. Twenty-two subjects demonstrated one or more inter-proximal sites with >1.5 mm clinical attachment loss during monitoring and were considered to show periodontal disease progression (active subjects). The distribution of inter-proximal progressing sites in 22 active subjects was as follows: seven subjects with one active site, eight subjects with two to four sites and seven subjects with five to 10 active sites. The general and demographic characteristics of active subjects at baseline were compared with those of inactive subjects having no sites with progressing inter-proximal clinical attachment loss at >1.5 mm (Table 1). More active subjects were male and current smokers, although there were no significant differences in subject age, gender or smoking experience between the disease categories. There were higher proportions of inactive Caucasian and Asian subjects, and a higher proportion of active Hispanic subjects, but this group had few subjects.

Means of gingival index, plaque index, per cent of sites BOP, PD and CAL at baseline were significantly higher in subjects subsequently showing periodontal clinical attachment loss in whole-mouth and inter-proximal measurements (Table 2). There was a positive association between baseline health and slight periodontitis categories with disease activity (Table 3), with a higher proportion of baseline slight periodontitis subjects in the disease-active group. Examining mean levels of clinical measurements at baseline and at 18 months (Fig. 1), the active group had a higher mean plaque index at baseline only, with no differences between active and inactive subjects at the final visit (Fig. 1a). Increases in per cent of sites BOP (Fig. 1b) were approximately 7% in inactive and 11% in active subjects. Inactive subjects showed slightly lower mean PD and CAL at the final visit compared with baseline, whereas active subjects had higher mean PDs and CAL at the final visit (Fig. 1c).

Mean values of clinical variables at 18 months (or equivalent mean changes from baseline) between the active and inactive groups were compared by analysis of covariance, adjusting for baseline levels of each measure. Adjusted mean levels of plaque and gingival indices at 18 months were not significantly different between the groups. Adjusted for differences in baseline levels, the mean per cent of sites BOP was higher in active subjects relative to inactive subjects ($p=0.03$). Mean levels, adjusted for baseline differences, of PDs and CAL at 18 months were significantly higher in active than in inactive subjects (both $p<0.001$). The appropriateness of hypothesis testing on adjusted means for PD and clinical attachment could be questioned given the retrospective definition of the active and inactive groups (i.e. all subjects with one or more sites exhibiting clinical attachment loss >1.5 mm were defined as active), which would clearly contribute to a mean difference between the groups. Even with the removal of the 82 inter-proximal sites with >1.5 mm clinical attachment loss from the analysis, however, adjusted mean differences

Table 1. General and demographic characteristics of study population at baseline

	Inactive subjects* ($n=95$)	Active subjects† ($n=22$)
Mean age (± SEM) in years‡	30.2 ± 0.6	31.1 ± 1.6
Gender, *n* (% male)‡	38 (40%)	13 (59%)
Smoking history§		
Never smoker, *n* (%)	67 (71%)	14 (63%)
Former smoker, *n* (%)	13 (14%)	3 (14%)
Current smokers, *n* (%)	15 (16%)	5 (23%)
Race/ethnicity¶		
Asian, *n* (%)	10 (11%)	1 (5%)
African American, *n* (%)	14 (15%)	4 (18%)
Hispanic, *n* (%)	3 (3%)	4 (18%)
Caucasian, *n* (%)	65 (68%)	12 (54%)
Other, *n* (%)	3 (3%)	1 (5%)

*Inactive subjects: no sites showing >1.5 mm inter-proximal clinical attachment loss over 18 months monitoring.

†Active subjects: $\geqslant 1$ site with >1.5 mm inter-proximal clinical attachment loss over 18 months monitoring.

‡No difference between groups (*t*-test).

§No association between smoking and periodontal progression (Mantel–Haenszel χ^2).

¶$p=0.0430$ between disease progression and four major ethnic groups (χ^2 test).

SEM, standard error of the mean.

Table 2. Inter-proximal and whole mouth clinical characteristics of study population at baseline

	Inter-proximal measurements		Whole mouth measurements	
	inactive† ($n=95$)	active ‡ ($n=22$)	inactive† ($n=95$)	active ‡ ($n=22$)
Gingival index (mean ± SEM)	0.78 ± 0.05	1.38 ± 0.13***	0.72 ± 0.05	1.26 ± 0.13***
Plaque index (mean ± SEM)	0.74 ± 0.05	1.26 ± 0.13***	0.64 ± 0.05	1.17 ± 0.13***
Percent sites BOP (mean ± SEM)	17.3 ± 2.2	28.5 ± 5.2*	14.8 ± 1.9	26.0 ± 5.2*
Probing depth (mm, mean ± SEM)	2.36 ± 0.03	2.67 ± 0.09**	2.11 ± 0.03	2.36 ± 0.09*
Clinical attachment level (mm, mean ± SEM)	1.38 ± 0.03	1.69 ± 0.08**	1.31 ± 0.02	1.60 ± 0.07***

*$p<0.05$, **$p<0.01$, ***$p<0.001$.

†Inactive subjects: no sites showing >1.5 mm inter-proximal clinical attachment loss during monitoring.

‡Active subjects: $\geqslant 1$ sites with >1.5 mm inter-proximal clinical attachment loss during monitoring inactive *versus* active, *t*-test.

BOP, bleeding on probing; SEM, standard error of the mean.

Table 3. Baseline categories* of active and inactive subjects

	Inactive	Active
Health (*n* %)	25 (93%)	2 (7%)
Early periodontitis 1	50 (86%)	8 (14%)
Early periodontitis 2	20 (64%)	12 (37%)

*Baseline categories: health (mean CAL $\leqslant$ 1.5 mm, no sites CAL $>$ 2 mm).
Early (slight) periodontitis 1: mean CAL $<$ 1.5 mm, $\geqslant$ 1 sites with CAL $>$ 2 mm.
Early (slight) periodontitis 2: mean CAL $\geqslant$ 1.5 mm.
Association between baseline early periodontitis and activity $p = 0.0026$ Mantel–Hanszel χ^2 for trend.
CAL, clinical attachment level.

between the groups were only slightly reduced and remained highly significant. Spearman's rank correlation coefficients evaluating associations of baseline levels of gingival indices, plaque indices and BOP with mean changes in PD at inter-proximal sites were gingival index 0.286 ($p = 0.002$); plaque index 0.207 ($p = 0.026$); and per cent BOP 0.272 ($p = 0.003$). Spearman's rank correlations of these baseline measures with changes in CALs did not approach significance. The scatter plot between baseline gingival index and change in PD over 18 months clinical monitoring (Fig. 2) exemplifies these associations, indicating a tendency for those subjects with larger increases in PDs (primarily active subjects) to have higher gingival index levels at baseline.

Logistic regression analysis was used to further examine associations between baseline clinical parameters averaged over inter-proximal sites and subjects with or without progressing attachment loss (Table 2). All five clinical measures were statistically significant ($p = 0.04$ for per cent sites BOP; $p < 0.001$ for other measures) in separate logistic models. A stepwise logistic analysis selected baseline gingival index, CAL and per cent sites BOP as significant predictors of subsequent clinical status. Plaque index and PD were not selected.

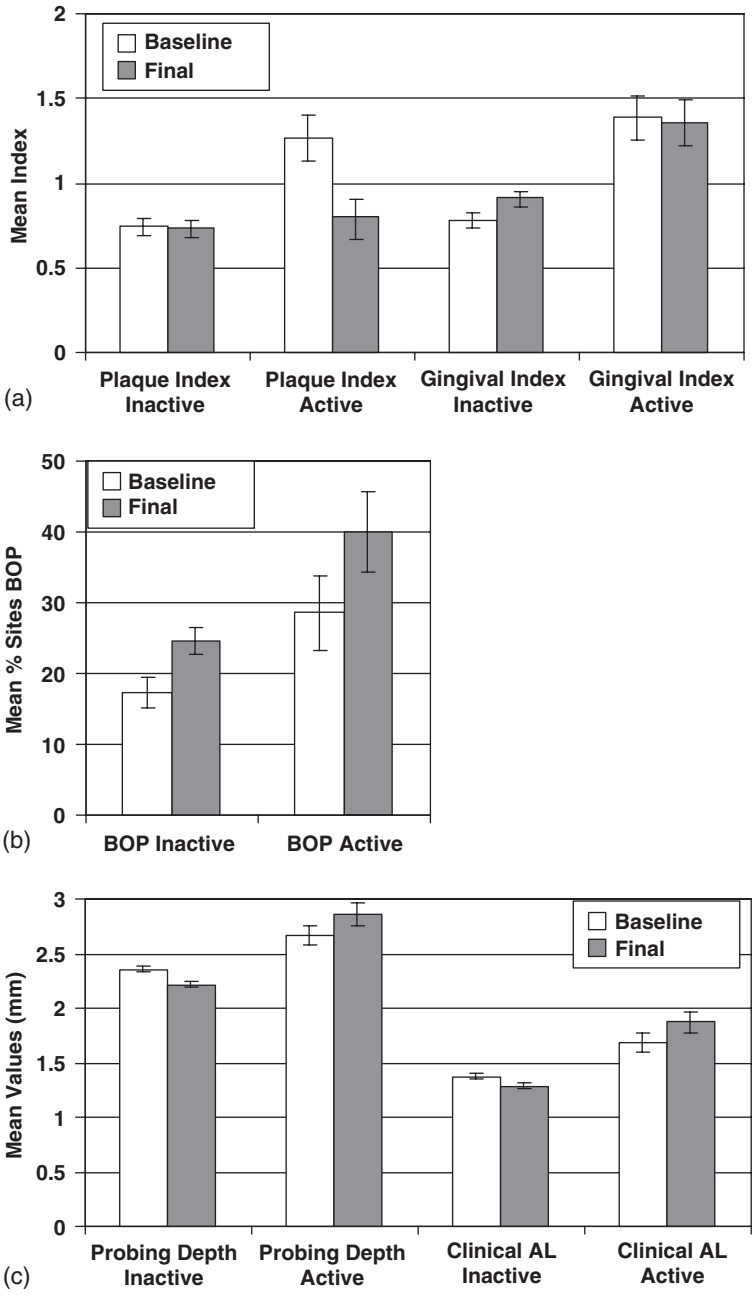

Fig. 1. Baseline and final mean inter-proximal clinical measures. Error bars are standard error of the mean. (a) Plaque and gingival indices. Baseline and final plaque and gingival indices in inactive and active subjects show an elevated plaque index at baseline in active subjects, and increase in gingival index in inactive subjects although differences were not significant. (b) Per cent sites bleeding on probing (BOP). There was increase in sites BOP of 7% in inactive subjects and a higher increase ($p = 0.03$), 11%, in active subjects. (c) Probing depth (PD) and clinical attachment level (CAL). PD and CAL decreased in inactive subjects between baseline and final visit, but increased in active subjects (both $p < 0.001$).

Clinical data by site

Eighty-two inter-proximal sites lost more than 1.5 mm periodontal clinical attachment. A higher proportion of these losing sites were around molars (particularly lower second molar) followed by premolars compared with incisor or canine teeth (Fig. 3a). At buccal/lingual sites, seven sites showed loss around mandibular teeth, and four sites showed loss around maxillary teeth of $>$ 1.5 mm (data not shown).

A higher proportion of active subjects had gaining sites compared with inactive subjects. The location of 33 inter-proximal sites that showed $>$ 1.5 mm periodontal gain (improvement) during the same monitoring period is shown in Fig. 3b. Eleven of the gaining inter-proximal sites were in the 22 active subjects mainly around incisors. None of these gaining sites showed BOP compared with 71% of the losing sites of active subjects that bled on probing. The remaining 22 gaining inter-proximal

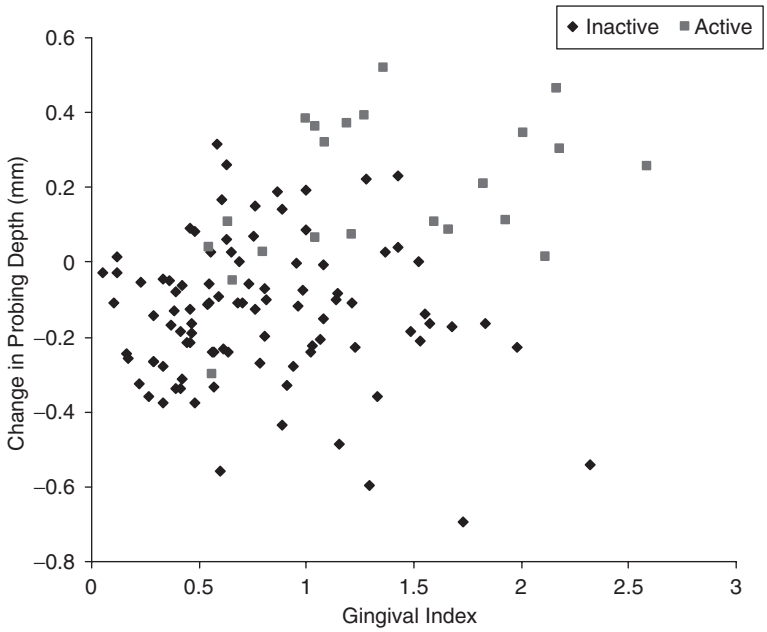

Fig. 2. Baseline gingival index and change in probing depth (PD). Scatter plot of baseline gingival index against change in PD over 18 months of clinical monitoring illustrating data in Table 3. Subjects showing >1.5 mm inter-proximal clinical attachment loss at $\geqslant 1$ sites (active) are plotted in red and inactive subjects in blue. Active subjects showed increases in PD and higher baseline gingival index more frequently than inactive subjects. Most of the inactive subjects who showed decrease in mean probing depth.

sites were in inactive subjects, 17 (77%) around molars, 50% of these sites bled on probing.

Thirty-two sites showed >1.5 mm gain at buccal/lingual locations around teeth (data not shown). The distribution of these sites over teeth was similar for active and inactive subjects and spread over all tooth types. Nineteen of these sites were in 22 active subjects, 26% BOP compared with 50% BOP for losing buccal/lingual clinical attachment. Thirteen gaining buccal lingual sites were in 95 inactive subjects, 31% gaining sites showed BOP compared with no BOP for losing sites.

Microbiological data using 16S rDNA oligonucleotide probes

The microbiota of samples assayed using 16S rDNA probes to pooled molar subgingival baseline samples from 78/95 inactive and 21/22 active subjects is shown in Fig. 4. Species are ordered by phylogenetic group, with phylotypes with no cultivable representative at the bottom. Species showing modest increased detection frequency in active relative to inactive subjects included *Streptococcus sanguinis*, *Streptococcus infantis*, *Peptostreptococcus micros*, *Dialister invisus*, *F. alocis*, *T. forsythia*, *Fusobacterium nucleatum* subsp. *polymorphum* and *T. denticola*. *P. gingivalis* was detected in two active and one inactive subjects using this DNA probe assay. Species or phylotypes showing modest increased detection frequency in inactive relative to active subjects included *Streptococcus parasanguinis*, *Actinomyces gerencseriae*, *Prevotella denticola*, *Bacteroidetes* clone AU126 and a clone of TM71025. Species not illustrated that showed $<5\%$ difference in detection between active and inactive subjects, respectively, included *Streptococcus mutans* (19%, 19%), *Streptococcus* phylotype 7A/H6 (14%, 18%), *Atopobium parvulum* (10%, 10%), *Actinomyces dentocariosa* (19%, 23%), *A. actinomycetemcomitans* (24%, 21%), *Campylobacter concisus* (10%, 8%) and *Cardiobacterium hominis* (14% 15%). Overall, there were few significant differences between disease categories (none significant if adjusted for multiple comparisons) in the microbiota assayed using 16S rDNA probes to pooled molar subgingival samples.

Microbiological data using whole-genomic probes

The microbiota of samples assayed using means of whole-genomic DNA probe counts from up to 28 mesiobuccal subgingival sites of 19 active and 17 matched inactive subjects is shown in Fig. 5. Species levels (counts) did not differ significantly between active and inactive subjects although there were modest increased levels of some periodontitis species including *E. saphenum*, *C. rectus* ($p = 0.06$ *t*-test), *P. intermedia* and *S. noxia*. Several species including *S. anginosus*, *S. sanguinis*, *C. sputigena*, *T. forsythia*, *F. nucleatum* and *T. denticola* that were detected more frequently by 16S rDNA probes from active subjects (Fig. 4) were also detected at higher mean counts from active subjects using the whole-genomic probes (Fig. 5).

When subgingival whole-genomic probe data were separated by incisor, canine, premolar and molar tooth types, there were differences in DNA counts (levels) by tooth type for total DNA count and for certain species (Fig. 6). In active subjects there were higher DNA probe counts samples from incisor, canine and molar teeth compared with premolar teeth ($p<0.05$) (Fig. 6a). In inactive subjects, counts from molar teeth were higher than from other tooth types ($p<0.05$). Total DNA counts by tooth type did not differ significantly between active and inactive subjects. Plaque index by tooth type showed a somewhat similar pattern as for total DNA probe counts (Fig. 6b). While correlations were positive between DNA probe count and plaque index by tooth type, this association was only statistically significant for molar teeth in active subjects (correlation 0.47, $p = 0.04$), and close to significance for incisor teeth in active subjects (correlation 0.43, $p = 0.07$).

Most individual species showed higher DNA probe levels from incisor samples of active compared with inactive subjects (although not significantly), and the pattern of species counts resembled that of total DNA probe counts (data not shown). However, *C. rectus* (Fig. 6c) from incisors and canines, and *E. saphenum* (Fig. 6d) from incisors, were detected in higher levels from active than inactive subjects ($p\leqslant 0.05$). *E. saphenum* comprised a higher proportion of the microbiota (per cent total DNA) from active than inactive subjects from molar teeth ($p = 0.0467$) (data not illustrated). The mean bacterial species counts for *T. forsythia* and *T. denticola* are illustrated in Fig. 6e and f, respectively. *P. gingivalis* had a similar pattern of counts by tooth type as *T. forsythia*.

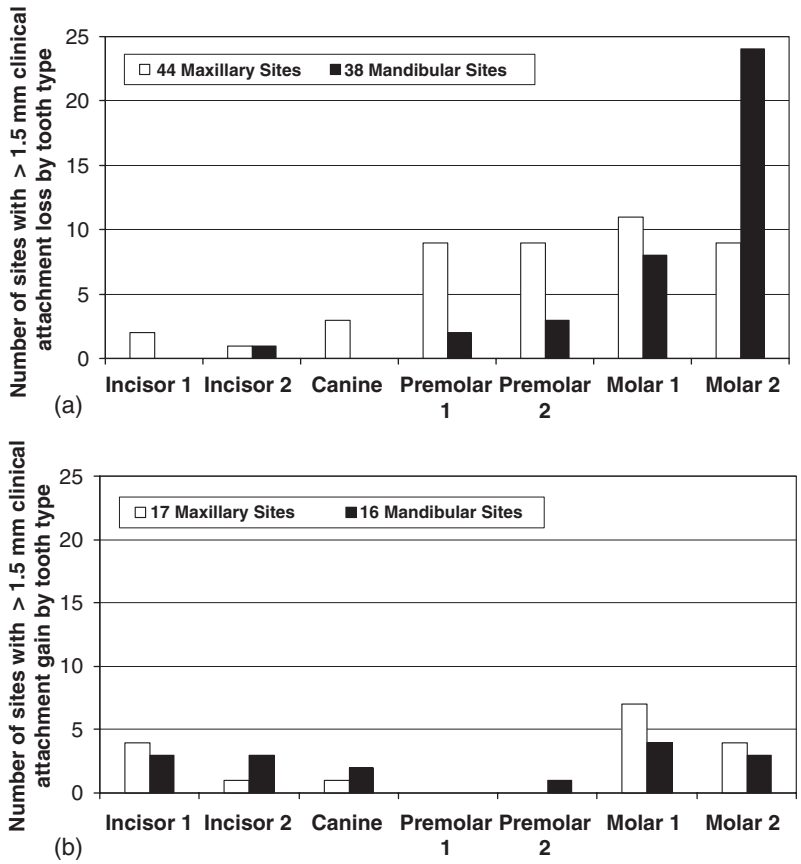

Fig. 3. (a) Inter-proximal sites showing clinical attachment loss. Per cents of inter-proximal sites by tooth type showing >1.5 mm periodontal clinical attachment loss over 18 months of longitudinal monitoring. Tooth sites are ordered: 1 = central incisor, to 7 = second molar. Posterior teeth were more often affected than anterior teeth, with lower second molars showing more sites with progressing loss than other teeth. (b) Inter-proximal sites showing clinical attachment gain. Per cents of inter-proximal sites by tooth type showing >1.5 mm clinical attachment gain over 18 months of longitudinal monitoring. Tooth sites are as in Fig. 1. Molars and incisors showed more sites with >1.5 mm reduction of clinical attachment level.

Microbiological data by multiplex PCR for *P. gingivalis* and *T. forsythia*

Data from 73 subjects, 11 active, with at least three longitudinal samples, indicated an increased detection frequency for *P. gingivalis* ($p = 0.0073$) and *T. forsythia* ($p = 0.075$) with the disease-active subjects (Fig. 7). Although detection frequencies of *P. gingivalis* and *T. forsythia* by multiplex analysis were individually significantly associated with disease progression, these variables did not add significantly to the stepwise logistic model based on the clinical parameters.

Discussion

This study of slight periodontitis indicated that low levels of progressing periodontal clinical attachment loss, primarily localized to posterior molar teeth, were detected in about 20% of subjects over 18-month follow-up. Progressing slight periodontitis was associated with baseline gingival inflammation and CAL. Bacterial species detected in this population included newly described species and not-yet-cultivated phylotypes. While there were few clear associations with any assayed species in adult subjects with slight chronic periodontitis using DNA probes in a cross-sectional analysis, species detected more frequently at higher counts from active subjects included *C. rectus* and *E. saphenum*. In longitudinal analysis *P. gingivalis* and *T. forsythia*, as detected by PCR, were significantly associated with subjects exhibiting clinical attachment loss.

Clinical findings

Over half of the baseline study population of 225 subjects (Tanner et al. 2005) was followed longitudinally for 18 months. The study population and disease activity measurement were selected to detect change consistent with slight chronic periodontitis. Increasing clinical attachment loss has frequently been used to identify progressing periodontitis (Haffajee et al. 1991, Beck et al. 1997b, Reddy et al. 2000, Cullinan et al. 2001, Tran et al. 2001, Craig et al. 2003, Hamlet et al. 2004). Previous study of slight periodontitis used duplicate probing measurements, estimated to 0.5 mm, to detect small increments of change (Tanner et al. 1998). This is a lower threshold of change than previously used to define disease activity in moderate to advanced periodontitis (Haffajee et al. 1983, Beck et al. 1997a). In the current study, 82 inter-proximal sites showed clinical attachment loss compared with 33 showing gain, suggesting disease progression in the study population consistent with a previous report (Tanner et al. 1998).

More inter-proximal than buccal/lingual sites showed clinical attachment loss. The molar, particularly mandibular molar, location of progressing sites (Fig. 3) reflected the disease pattern of loss in the baseline cross-sectional analyses (Tanner et al. 2005). Greater progressing clinical attachment loss at posterior maxillary inter-proximal sites compared with more anterior, mandibular and buccal sites was similar to that reported for older subjects with higher baseline levels of clinical attachment loss (Beck et al. 1997a, Reddy et al. 2000). We also examined the frequency of sites with CAL gain at the same 1.5 mm threshold (Fig. 3). Most of this variability was around posterior molars and central incisors.

Several demographic indicators of periodontitis, including age, male gender, smoking history and racial/ethnic category, were associated with slight periodontitis in our cross-sectional analysis (Tanner et al. 2005). These associations were not significant in the longitudinal analyses, except for an association between race/ethnicity and disease progression, which, while consistent with previous reports of increased periodontitis in Hispanic and decreased periodontitis in Asian subjects (Craig et al. 2003), was based on rather few subjects.

There was a positive association between cross-sectional baseline health and slight (early) periodontitis categories (Tanner et al. 2005) and subjects

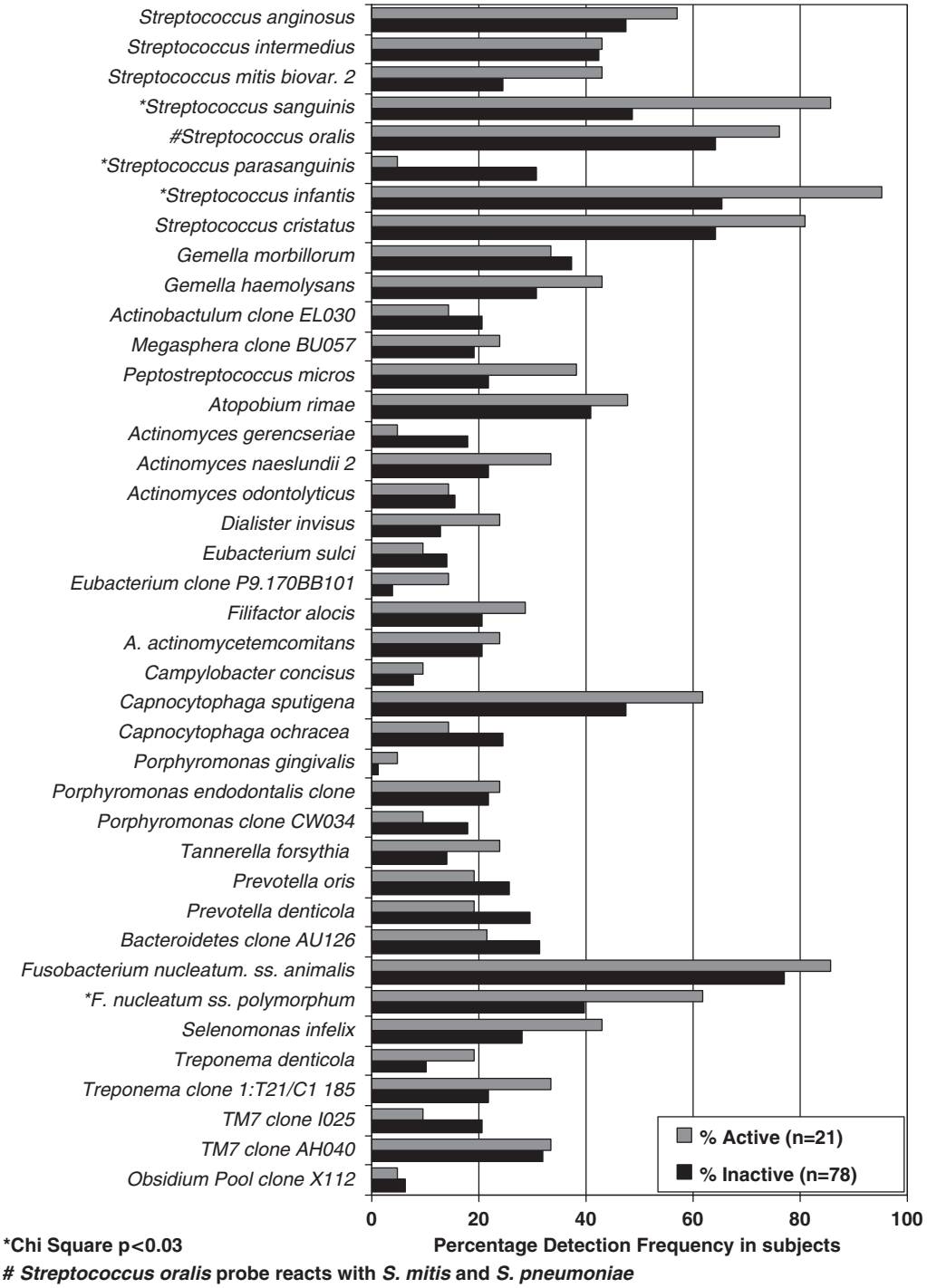

Fig. 4. Species detected using 16S rDNA oligonucleotide DNA probes. Bacterial species were assayed using the reverse capture checkerboard assay from pooled molar subgingival plaque samples from 78 inactive (blue bars) and 21 active (red bars) subjects. While several species were detected more frequently from active subjects, few differences were significant.

that did not or did show subsequent disease activity described in the current study (Table 3). All clinical measures were higher at baseline in active subjects. Gingival index was a major correlate of subsequent disease activity in the logistic model. This association between progressing slight periodontitis and measures of gingivitis has been observed in slight periodontitis (Tanner et al. 1998), in younger subjects with early-onset periodontitis (Albandar et al. 1998) and in urban minority populations (Craig et al. 2001).

The 6-monthly supragingival cleaning may have had an impact of the reduction in plaque index in active subjects during monitoring, as has been observed in other populations (Westfelt et al. 1998, Ximenez-Fyvie et al. 2000, Goodson et al. 2004). Subgingival

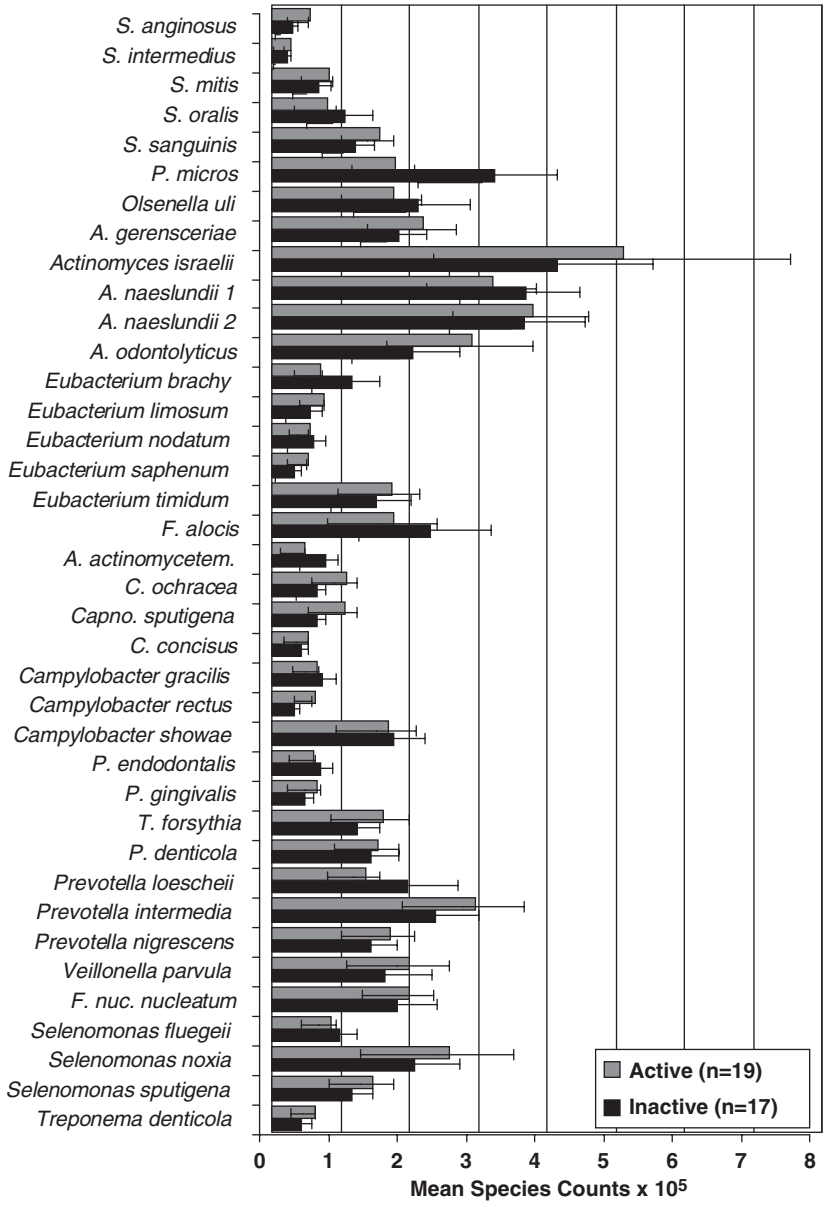

Fig. 5. Species counts using whole-genomic DNA probes. Error bars represent standard error of the mean. Bacterial species are listed to match the order of Fig. 4. Mean species levels (counts $\times 10^5$) from mesiobuccal subgingival sites of all teeth of each subject were compared. Many similar species were detected at elevated mean levels from active subjects as detected more frequently using the 16S rDNA oligonucleotide probes (Fig. 4). There were no significant differences in bacteria detected between active and inactive subjects when these pooled molar subgingival samples were compared.

(Rhemrev et al. 2006) and supragingival (Goodson et al. 2004) plaque removal can reduce the bacterial load. It is not clear, however, whether supragingival cleaning has an impact on disease progression (Pastagia et al. 2006). Furthermore, repeated professional supragingival plaque cleaning did not show improvement in CAL (Nogueira et al. 2000, Ximenez-Fyvie et al. 2000). These reports are consistent with the current study in which disease progression was observed despite the 6-monthly supragingival cleanings.

Microbiology findings

The 16S rDNA probe assay allowed the simultaneous detection of multiple species including not-yet-cultivated phylotypes. There were few differences in the microbiota of inactive and progressing subjects using the DNA probe assay, with only *S. infantis*, *S. sanguinis* and *F. nucleatum* subsp. *polymorphum* being detected more frequently from the active subjects. None of these species have been associated with moderate to advanced periodontitis. Species or phylotypes associated with periodontitis (Paster et al. 2001, Kumar et al. 2003, Dahlen & Leonhardt 2006) were, however, detected in similar or slightly higher proportions from slight periodontitis, including *P. micros*, *F. alocis*, *D. invisus* (*Dialister* strain GBA27), *T. forsythia* and *T. denticola*. While all these species have been cultured, for some fastidious species, particularly *F. alocis*, *D. invisus* and oral treponemes, use of more-sensitive molecular or immuno-assays has allowed associations with periodontitis to be made.

Several species, including *T. denticola*, *F. nucleatum* subsp. *polymorphum*, *F. alocis*, *P. micros and S. mitis*, were detected more frequently from the baseline slight periodontitis categories than from health (Tanner & Izard 2006) and were also detected more frequently from progressing than from inactive subjects although not significantly. Other species detected more frequently in health, including *P. denticola*, *P. oris* and *C. ochracea*, were detected more frequently from inactive subjects.

The Obsidian Pool (Hugenholtz et al. 1998) and TM7 phylotypes belong to bacterial divisions with no known cultivated representatives. The reported association of TM7 1025 phenotypes with periodontitis (Brinig et al. 2003) was not observed for slight periodontitis in the population studied. The detection of these phylotypes and others with cultured representatives, and the detection of members of *Archaea* in subgingival samples (Lepp et al. 2004), however, reinforce the extent of complexity of the subgingival microbiota not appreciated from cultural studies.

Individual subgingival sites, rather than pooled molar samples as for the 16S rDNA probes, were assayed using whole-genomic probes. Additional species assayed using whole-genomic probes included *C. rectus*, *S. noxia* and *P. intermedia* that had been detected in our previous study (Tanner et al. 1998) also other cultivable species (Kumar et al. 2003) for which 16S rDNA probes had not been available. When comparing the different DNA probe assays, the whole-genomic probe assay showed increased detection of *Actinomyces*, compared with the PCR-based 16S

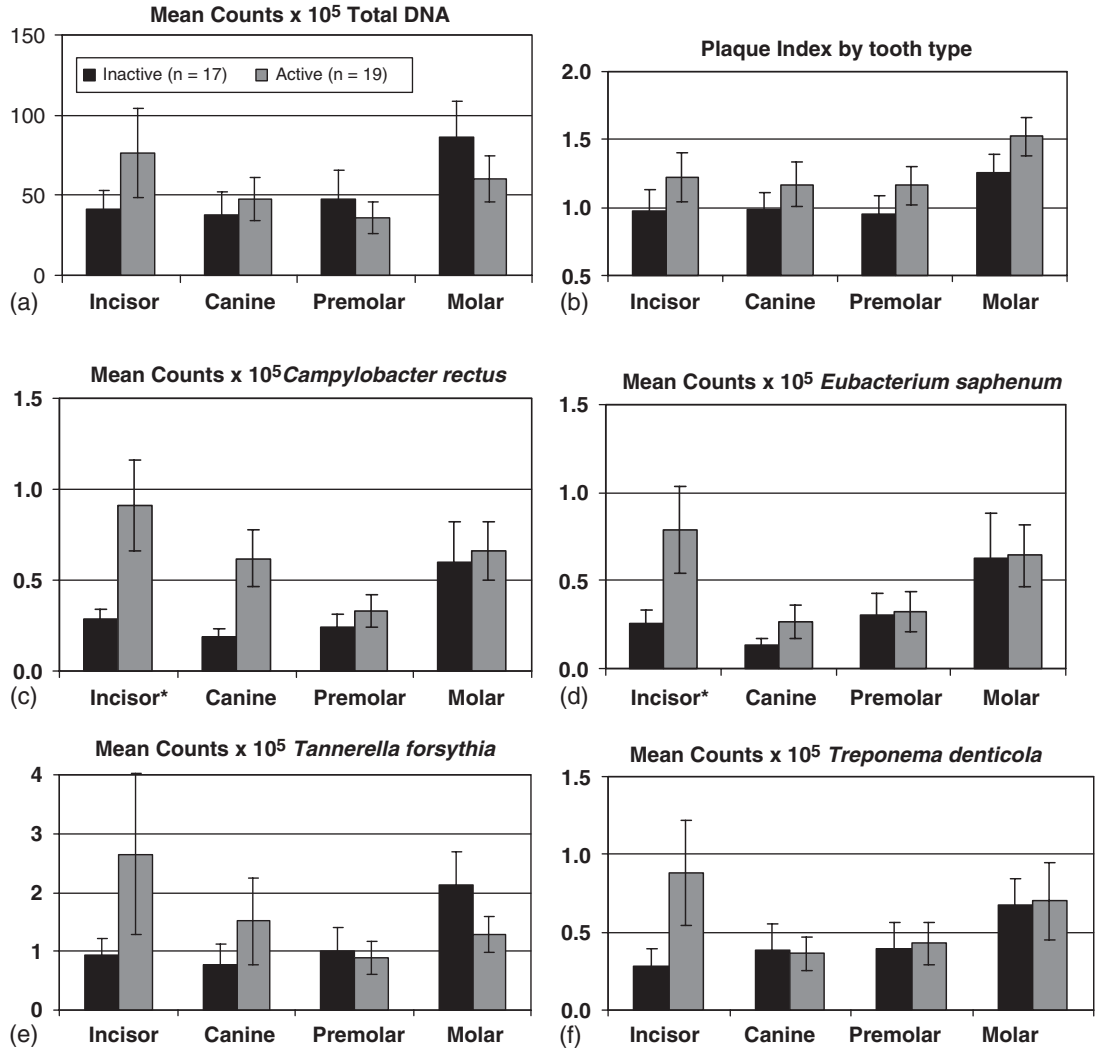

Fig. 6. Species counts using whole-genomic DNA probes, and plaque index by tooth types. Tooth types compared were incisor, canine, premolar and molar. Error bars represent standard error of the mean. (a) Mean counts $\times 10^5$ of total DNA probe count of 38 species. Higher mean species counts were observed from incisors but lower mean levels from molars from active compared with inactive subjects, although differences between active and inactive subjects were not significant. In inactive subjects counts from molar teeth were higher than the other tooth types, and in active subjects, counts were lower from premolars than other tooth types. (b) Plaque index by tooth type showed a somewhat similar pattern as for total DNA probe counts. There were positive Spearman's rank correlations between total DNA probe count and plaque index by tooth type, which was significant for molar teeth in active subjects, and close to significance for incisor teeth in active subjects. (c) Mean counts $\times 10^5$ of *Campylobacter rectus*. Higher levels of *C. rectus* were detected from incisor and canine teeth from active compared with inactive subjects. *C. rectus* comprised a higher mean percentage of the total DNA probe count from molar active compared with inactive subjects although not significantly different (data not shown). (d) Mean counts $\times 10^5$ of *Eubacterium saphenum*. Higher levels of *E. saphenum* were detected from incisor teeth from active compared with inactive subjects. *E. saphenum* comprised a higher mean percentage of the total DNA probe count from molar active compared with inactive subjects ($p = 0.046$) (data not shown). (e) Mean counts $\times 10^5$ of *Tannerella forsythia*. There was a similar pattern of species levels from different tooth types as for the total DNA probe count (a). *Porphyromonas gingivalis* and *Selenomonas noxia* showed a similar pattern of colonization as *T. forsythia*. (f) Mean counts $\times 10^5$ of *Treponema denticola*. As *T. forsythia* (e) there were higher counts of *T. denticola* from incisor teeth, although not significant. *T. denticola* comprised a higher mean percentage of the total DNA probe count from molar active compared with inactive subjects although not significant ($p = 0.08$), data not shown.

rDNA probe assay. This was possibly due, in part, to improved cell lysis using sodium hydroxide for the whole-genomic assay and in part by bias using universal or broad-range primers in the PCR reaction that do not equivalently amplify all segments of the bacteria in samples (Horz et al. 2005). Other species including *Streptococcus*, *Campylobacter*, *Capnocytophaga*, *Prevotella* species, *F. nucleatum*, *T. forsythia*, *P. gingivalis* and *T. denticola* that were detected more frequently in active than inactive subjects using the 16S rDNA probe assay were generally detected again at higher levels using the whole-genomic probes, indicating a degree of concordance between assays.

In comparison with literature data obtained using the same DNA probe assay of health and periodontitis from

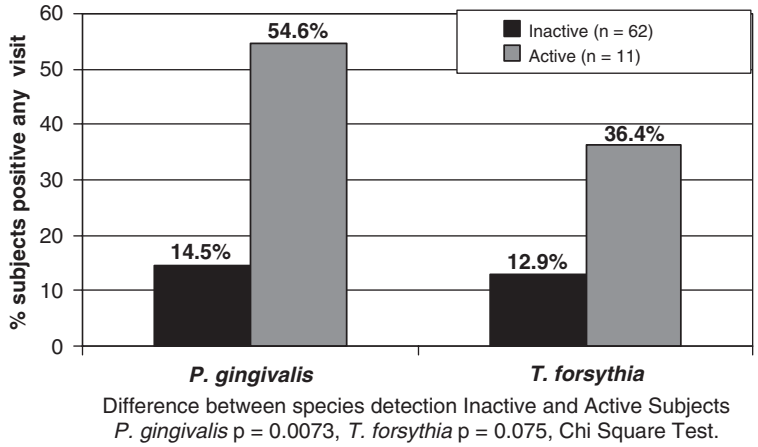

Fig. 7. Detection of *Porphyromonas gingivalis* and *Tannerella forsythia* using multiplex PCR. Species detection at any of three longitudinal visits was compared between 62 inactive (blue bars) and 11 active (red bars) subjects. In this longitudinal analysis, *P. gingivalis* ($p = 0.0073$) and *T. forsythia* ($p = 0.075$) were detected in active subjects more frequently than inactive subjects.

subgingival and supragingival samples (Socransky & Haffajee 2005), species levels for the inactive subjects were generally higher than those reported of healthy subgingival samples, but lower than those of supragingival samples. The inactive subject microbiota was, however, closer to subgingival levels from health for *A. naeslundii* 2, *A. gerensceriae* and *V. parvula*. The microbiota of active subjects more closely resembled that of subgingival compared with supragingival plaque in periodontitis subjects. Species at similar levels from slight and more-advanced periodontitis subgingival samples included *F. nucleatum* subsp. *nucleatum*, *P. intermedia*, *Campylobacter showae*, *Capnocytophaga* species, *T. forsythia* and *A. actinomycetemcomitans*. Species detected at higher levels from moderate to advanced chronic periodontitis than active slight periodontitis included *E. nodatum*, *Campylobacter gracilis*, *C. rectus*, *P. gingivalis*, *P. nigrescens*, *V. parvula* and *T. denticola* (Socransky & Haffajee 2005). *S. noxia* was detected at higher mean levels from subjects in the current study population than from the population in the healthy and periodontitis report (Socransky & Haffajee 2005). When comparing slight and more-advanced periodontitis, data suggest that subjects with earlier stages of disease progression have lower levels of putative pathogens than periodontitis subjects, perhaps reflecting the shallower depths of pockets from which samples were taken.

Relatively few reports have described the subgingival microbiota around different tooth types. Our data indicated a pattern of higher total, and individual species counts in incisor and molar teeth for the active subjects, with increased total DNA probe counts only in the molar teeth of inactive subjects. Most literature reports are limited to the detection of *A. actinomycetemcomitans*. A colonization pattern of elevated species detection from incisor and molar teeth was previously observed in periodontitis for *A. actinomycetemcomitans* (Ebersole et al. 1994), and for *P. gingivalis* and *A. actinomycetemcomitans*, and to a lesser extent for *T. forsythia* and *P. intermedia* (Christersson et al. 1992). In subjects with minimal disease, a molar, incisor colonization pattern was also observed for *A. actinomycetemcomitans* (Muller et al. 2001). Increased plaque may in part explain the distribution of bacterial counts around teeth. In another report, *P. gingivalis* was detected more frequently and in higher mean proportions of the microbiota from molar tooth samples compared with more anterior teeth from adult periodontitis, pre- (Mombelli et al. 1991) and post-therapy (Mombelli et al. 1996), which was a detection pattern similar to that of the inactive subjects in the current investigation. The different distribution of species in active and inactive subjects might have resulted from more effective plaque control by inactive subjects, and subjects under periodontal therapy (Mombelli et al. 1996) for the more easily reached anterior teeth.

Species detected at higher levels from active than inactive subjects from anterior teeth included *C. rectus*, which we previously associated with slight periodontitis (Tanner et al. 1998), and *E. saphenum*, previously associated with chronic periodontitis using PCR (Kumar et al. 2003). Finding higher levels of species from active subjects from incisor teeth was unexpected as the sites with most frequent increases in clinical attachment loss at a 1.5 mm threshold were around molars. Because in other populations progressing periodontitis over longer periods than 18 months was detected in sites not showing previous clinical attachment loss (Beck et al. 1997c), one might speculate that the detection of an increased load of periodontal species in the anterior shallow sites might be associated with a clinical attachment loss below the 1.5 mm threshold of this study and detectable only after longer periods of monitoring.

The lack of differentiation between active and inactive subjects using the DNA probe assays was disappointing but may have reflected the modest differences in PD and CAL between the active and inactive disease categories despite there being significant differences between the clinical categories.

Using the multiplex PCR assay, *P. gingivalis* and *T. forsythia* were associated with periodontitis in both the baseline cross-sectional (Tanner et al. 2006) and the longitudinal analysis of the current report. This assay improved detection of *P. gingivalis* compared with the PCR-based 16S rDNA probe assay. The association of *P. gingivalis* and *T. forsythia* with progressing (Grossi et al. 1995, Socransky & Haffajee 2005), advanced and refractory periodontitis (Lai et al. 1987, Grossi et al. 1995, van Winkelhoff et al. 2002, Kumar et al. 2003, Kumar et al. 2005, Socransky & Haffajee 2005, Tanner & Izard 2006) has been previously shown. Further, *P. gingivalis* was detected in slight periodontitis in adults (Riviere et al. 1996), and in early periodontitis of adolescents (Clerehugh et al. 1997) and in 11–13-year-old children (Ellwood et al. 1997). *T. forsythia* was associated with early periodontitis in adolescents (Hamlet et al. 2004) and adults with progressing slight periodontitis (Tanner et al. 1998, Machtei et al. 1999).

There were strengths and limitations in the molecular microbiological

methods used. The 16S rDNA probe assay could detect not-yet-cultivated species or phylotypes. For small samples, however, as from individual subgingival sites, it frequently proved difficult to obtain a good PCR amplicon with label for the 16S rDNA probe assay, which led to the use of pooled subgingival samples for the PCR-based assays. The whole-genomic checkerboard is a sensitive technique, which allows quantification. This method, however, is unable to detect not-yet-cultivated species, and requires sample size to be within the range for the assay (10^4–10^7 cells) to avoid non-specific binding and cross-reactions between probes to closely related species (Socransky et al. 2004).

Conclusions

Progressing slight periodontitis, detected most frequently around molar teeth, was associated with baseline gingival inflammation and CAL. These findings suggest a strong gingivitis component to progressing slight periodontitis in young adults. No species in baseline microbial samples alone were strongly associated with progressing slight periodontitis. The strongest microbial associations with slight periodontitis were with *P. gingivalis* and *T. forsythia* determined using PCR and detected in at least one of three longitudinal visits suggesting that these traditional pathogens are harder to detect in slight compared with more-advanced chronic periodontitis. While *P. gingivalis* from the longitudinal assay was associated with attachment loss by logistic regression, the species did not significantly improve a model based on baseline clinical measures. An alternative explanation for the relationship between bacteria and periodontitis is that the organisms are there *because* of the disease, which raises the possibility that periodontal pathogens may not predict future disease. Host inflammation may create the microenvironment necessary to allow overgrowth of the organisms; once established, the organisms promote further inflammation and tissue damage as has been suggested for Crohn's disease (Balfour 2007), cystic fibrosis (Chmiel & Davis 2003) and an animal model of tuberculosis (Karakousis et al. 2004). Future studies focusing on the interplay between the host inflammatory response and bacteria colonization and growth should clarify the interactions between host and periodontal pathogens in clinical attachment loss.

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Clinical Relevance

Scientific rationale for the study: One challenge of periodontal practice is identifying individuals who are at risk of losing additional clinical attachment. This study sought characteristics associated with progression of slight chronic periodontitis.

Principal findings: Gingivitis, CAL, PD with BOP were associated with periodontitis progression (activity) detected primarily around molar teeth. Periodontal bacteria, particularly *P. gingivalis*, were associated with disease progression.

Practical implications: Young adults with gingival inflammation, with clinical attachment loss and with BOP are at risk for disease progression.