J Clin Periodontol 2008; 35: 23–30 doi: 10.1111/j.1600-051X.2007.01163.x

Journal of Clinical Periodontology

Total protein concentration and total bacterial load as measures of residual interproximal plaque in comparative clinical trials

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Milanovich N, Wei J, Jenkins W, Hefti AF, de Jager M. Total protein concentration and total bacterial load as measures of residual interproximal plaque in comparative clinical trials. J Clin Periodontol 2008; 35: 23–30. doi: 10.1111/j.1600-051X.2007.01163.x.

Abstract

Aim: Establish total protein concentration and total bacterial load as quantitative measures of residual interproximal plaque (IPP) in a clinical model designed to evaluate oral hygiene interventions.

Material and Methods: This clinical model was a randomized, examiner and laboratory technician-blinded, parallel-design study whereby levels of residual IPP were compared for subjects using a manual toothbrush or a toothbrush+floss. Differences between interventions were compared after 7 and 21 days of use. Protein concentration was measured using 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde in a fluorescence microplate format and bacterial load was assessed by quantitative real-time PCR with universal primers specific for 16S rRNA and detected by SYBR Green. ANCOVA was used to assess the statistical significance of the differences between interventions while clinical relevance was evaluated by a statistical model described by Man-Son-Hing et al. 2002.

Results: Ninety-three subjects completed the study. Significant differences between interventions, using both outcome measures, were observed after 7 and 21 days. The difference between interventions by total protein concentration were further determined to be clinically relevant.

Conclusions: Only total protein concentration provided both statistically significant and clinically relevant differences between two clinically distinct oral hygiene interventions in this clinical model for evaluating IPP.

Key words: bacterial load; clinical model; interproximal plaque; oral hygiene; protein concentration

Accepted for publication 8 October 2007

Everyday solutions for managing dental plaque include combinations of chemical and mechanical (e.g., toothpaste, rinse, toothbrush, and floss) means to minimize or remove the accumulated plaque. Visual plaque indices (PI) have traditionally been used to clinically evaluate the effectiveness of these chemical and mechanical interventions (Silness & Löe 1964, Turesky et al. 1970, Rustogi et al. 1992). Provided the tooth surfaces of interest are visually accessible, such as surfaces of anterior teeth, these indices can be considered sufficient. However, scoring plaque in hard-to-see posterior interproximal areas is challenging, if not impossible with a visual index.

In response to the shortcomings of the visual indices for assessing interproximal plaque (IPP) accumulation, objective means by which to quantify IPP have been reported. Such alternatives include measurement of plaque weight (Ainamo et al. 1993, Sjögren et al. 2004, McCracken et al. 2006) as well as indirect assessments based on total DNA content (Stilwell et al. 2006) and total protein concentration (Altman et al. 1979, FDA 1996, Bellamy et al. 2004, Milanovich et al. 2005, 2007).

Our aim was to establish total protein concentration and total bacterial load in

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.
This study was funded by Philips Oral Healthcare, Inc.

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a clinical model to detect significant differences in residual IPP levels between two oral hygiene routines expected to provide different degrees of IPP removal. The current model was derived from previously reported models for measuring IPP whereby plaque levels were determined by measuring total protein concentration in the harvested plaque (Milanovich et al. 2007). Unique to the present study, we used an alternative outcome measure to assess differences in IPP levels using universal primers for 16S rRNA to measure the total bacterial load within the plaque samples. The advantage to the latter was that universal primers for 16S rRNA would overcome the inherent background signal associated with the protein assay (Milanovich et al. 2007). Differences in residual IPP were measured for brushing with a manual toothbrush (MTB) *versus* brushing with a MTB and flossing (MTB+F) following 7 and 21 days of use. The shorter intervention time confirmed the model as a practical clinical method for evaluating differences in IPP removal between cleaning routines while the longer intervention time demonstrated the suitability of the model for extended, true-to-life, use of an intervention.

Material and Methods

The study protocol was approved by the Western Institutional Review Board® (WIRB), Olympia, WA, USA.

Study design

This was a randomized, parallel-design study with examiner and laboratory analysts blinded to the intervention assignments. Figure 1 summarizes the six visits included in this study. Enrolled subjects were placed on washout for 7–14 days, which consisted of using the Oral B P35 MTB and Crest Cool Mint Gel toothpaste twice daily (2 min./brushing). No other oral hygiene measures were permitted during washout. At visits 2, 5, and 6, subjects arrived with 12–18 h of plaque accumulation and used either the washout (visit 2) or their assigned intervention (visits 5 and 6). A new brush was given during each of these visits. Subjects were instructed to brush for 2 min. and, if assigned, floss the entire dentition. IPP samples were collected 60 min. after the intervention as a way of minimizing the contribution of proteins from stimulated saliva

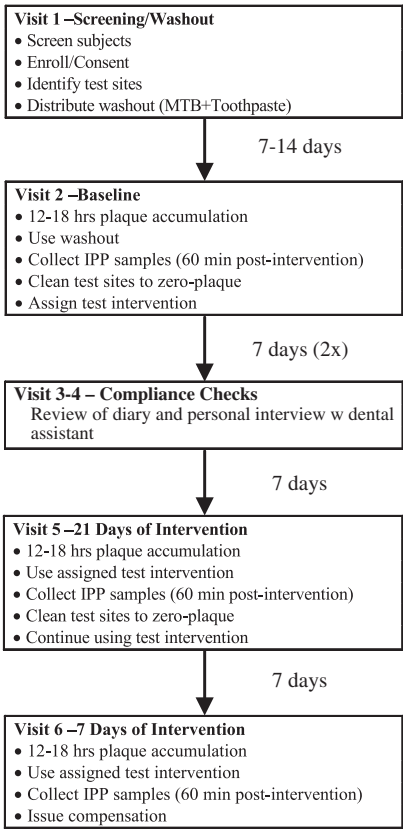

Fig. 1. Summary of visit sequences, associated tasks during each visit, and time between visits.

(Milanovich et al. 2007). Visits 2 and 5 concluded by having the examiner clean the test sites with floss (10 strokes/surface) before beginning the intervention phases. The 21-day intervention phase also included two weekly compliance visits (visits 3 and 4) to ensure that subjects continued to abide by the study instructions, particularly when flossing.

Oral hygiene interventions

- MTB: Oral B P35 MTB (Procter & Gamble, Cincinnati, OH, USA).
- MTB+F: Oral B P35 MTB and Oral B Ultrafloss (Procter & Gamble).

All subjects were instructed to brush twice daily (2 min./brushing) with a pea-sized dose of a standardized toothpaste (Crest Cool Mint Gel Toothpaste, Procter & Gamble, Cincinnati, OH, USA) and, if assigned, floss once daily. All subjects randomized to the MTB+F group were instructed on flossing technique. Subjects were prohibited from using any other oral hygiene measures during the entire study period.

Subject eligibility

Subjects qualifying for the study met the following key inclusion criteria:

- 18–65 years of age;
- $\geqslant 20$ natural teeth;
- $\geqslant 1$ interproximal site/quadrant with two adjacent, unrestored interproximal surfaces;
- <4 mm probing pocket depth for teeth comprising the test sites;
- average pre-brushing plaque score of $\geqslant 1.75$ (Turesky PI, full mouth) (Turesky et al. 1970);
- average gingival index score <2 (Löe & Silness Gingival Index, full mouth) (Löe & Silness 1963);
- absence of rampant decay and heavy deposits of calculus; and
- regular MTB users.

The study excluded individuals who:

- were undergoing or requiring extensive dental, periodontal or orthodontic interventions;
- had oral surgery within 6 months before enrollment in the study;
- had diabetes;
- had infectious or systemic diseases that may be unduly affected by participation in the study;
- were using prescription anti-inflammatory medications; and
- used antibiotics within 3 weeks before enrollment.

The use of antibiotics and antibacterials was not permitted during the study.

Sample size

A sample size of 45 subjects per intervention was determined. The estimate assumed 80% power, a common standard deviation of 0.868, an overall significance level of 0.05 (two-sided test), and a parallel design. Sample size was based on the difference between the mean bacterial load [expressed as $\log_{10}$(bacterial counts/ml)] for MTB (8.4 ± 0.9) and MTB+F (7.9 ± 1.0) observed in a pilot study (crossover design) after 7 days of standardized oral hygiene, followed by a single use of the assigned intervention.

Randomization of subjects and balance of test groups

At visit 2, subjects were randomly assigned to one of the two intervention groups. Randomization was balanced by

gender and smoking to ensure equivalent representation of these classes in each group. This was necessary to account for potential differences in responses between males and females and smokers *versus* non-smokers. The randomization schedule was generated using SAS version 9.1.3, with access restricted to the statisticians. A copy of the randomization schedule was sent to the CRO personnel responsible for issuing interventions and training of subjects.

Blinding

The examiner collecting the plaque samples and the laboratory analyst undertaking the assays for protein concentration and bacterial load were blinded to the intervention assignments. The dental assistant and subjects were not blinded.

Sample collection and preparation for analysis

Four test sites, one site/quadrant, were selected during screening (visit 1) from which to collect IPP samples throughout the study. Sites were selected, in order of preference, as follows: first molar/second pre-molar, second pre-molar/first pre-molar and first pre-molar/canine; test sites were required to have closed, yet flossable contacts. Plaque collection from the four quadrants was taken in the following order: upper right, upper left, lower left and lower right.

Before the sample collection, test sites were rinsed with water to remove loose plaque or debris, and then dried with an air syringe. To minimize sample contamination with saliva, test sites were isolated individually by placing a cotton roll in the vestibule and, for mandibular sites, between the tongue and the test teeth. Dryness was maintained with an air syringe. To collect a sample, floss was pre-cut and marked, with the markings ~ 10 mm apart. These markings aided the examiner with placing the floss between the teeth. The floss was then slipped through the contacts, with care taken to remain above the gingival margin, thus collecting only supra-gingival plaque. The floss was bent into a ''C'' shape and slid upward along the tooth surface with a single motion up to the contact point. This procedure was then repeated on the adjacent surface. Once a sample was collected, the floss was drawn through the inter-dental space and the segment with a sample on it was cut and placed into a vial containing 1.0 ml of sterile Ringer's solution (Baxter Healthcare Co., Deerfield, IL, USA). The samples were stored at -80°C until the study was completed. This procedure was repeated for the remaining three test sites, placing the sample from each site in separate vials. Subsequently, the test sites were cleaned with floss using 10 vertical strokes between the subgingival area and the contact to ensure essentially plaque-free interproximal test surfaces.

Before conducting the analyses, thawed samples were removed from the floss and dispersed by adding 0.3 g of 200 μm glass beads (G-1277, Sigma Chemicals, St Louis, MO, USA) to each sample tube, and then shaking samples with a Mini-Bead Beater 8 (BioSpec Products Inc., Bartlesville, OK, USA) for 45 s at maximum speed. Dispersed samples were split into two halves: one to be used for the total protein analysis and the other for bacterial load determination.

Protein analysis

The protein-specific probe, 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) (Invitrogen Inc., Carlsbad, CA, USA), was chosen because its linear range was the most appropriate given the typical sample readings, compatibility with other sample components, and sensitivity compared with other commercially available protein assay kits (You et al. 1997, Haugland 2005). The general protocol used with CBQCA was according to the manufacturer's instructions, with two notable changes meant to better manage the processing of the large number of samples collected in this study. First, KCN and the Na Borate buffer were premixed, producing a 20 mM KCN/0.1 M Na Borate solution (pH 9.3). The bovine serum albumin (BSA) standards (final concentrations of 100, 50, 25, 12.5, 6.25, and 0.0 μg/ml) were prepared by dissolving BSA powder in this premixture. Second, samples and reagents were incubated for 2 h at ambient temperature to ensure complete reaction of the samples and reagents. Standards and clinical samples were prepared in triplicate on Costar 96-well flat bottom/black side microplates (Corning Inc., Corning, NY, USA). Each plate contained its own set of BSA calibration standards. Plaque samples were thawed completely, then sonicated for 10 s, followed by vortexing for 5 s. Fluorescence was read with a Bio-Tek FL600 Fluorescence Plate Reader and KC4 v.3.1 software (Bio-Tek® Instruments Inc, Winoski, VT, USA).

16S rRNA analysis

Total bacterial load was determined by quantitative, real-time PCR (qPCR) using a MyiQ PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), IQ – SYBR Green Supermix (Bio-Rad Laboratories), and universal primers 341F/519R (Muyzer et al. 1993, Ovreas et al. 1997, Casamayor et al. 2000) for 16S rRNA (Sigma-Genesys, St Louis, MO, USA). Extraction of DNA from the bacteria was completed using the DNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Standard curves for determining the quantity of the PCR product were established using the United States Environmental Protection Agency's method for Mold Detection (http://www.epa.gov/nerlcwww/moldtech.htm). *Bacillus subtilis* (ATCC # 9372) was used as the reference organism; standard bacterial levels were 10^3, 10^4, 10^5, and 10^6 counts/ml.

Statistical analysis

The comparability of demographics and baseline characteristics between intervention groups were analysed by the overall *F*-test of the one-way analysis of variance (ANOVA) for age, mean plaque and gingival index scores and by Fisher's exact test for gender, smoking and site location for sampling.

The efficacy variables were total protein concentration and total bacterial load in IPP, expressed in microgram per millilitre and $\log_{10}$(bacterial counts/ml), respectively. The subject was the unit of analysis. Raw data for bacterial load were transformed to a $\log_{10}$ scale and treated as a continuous variable. Differences in protein concentration and bacterial load between interventions after 7 and 21 days were analysed using ANCOVA, with the baseline results used as the covariate (CPMP 2003). Statistical differences between interventions were evaluated using a two-sided *F*-test. Blinding was maintained throughout the clinical and laboratory phases of

the study. The randomization schedule was merged with the database after completing a consistency check of the data from both assays.

In addition, a number of post-hoc analyses were executed. They included calculating mean protein concentration and bacterial load as a function of test site location, and an assessment of the potential clinical relevance to the observed differences between interventions was made according to the method described by Man-Son-Hing et al. (2002).

Table 1. Baseline demographic and clinical characteristics of each intervention group (mean ± SD)

	MTB	MTB+F	*p*-value
Subjects	47	49	
Mean age (years)	33.8 ± 10.9	37.2 ± 10.5	0.121
Male/female	13/34	14/35	1.000
Tobacco users	5	5	1.000
Mean plaque score	2.70 ± 0.53	2.65 ± 0.48	0.601
Mean gingival index score	1.03 ± 0.18	1.01 ± 0.17	0.593

MTB, manual toothbrush; MTB+F, manual toothbrush and use of floss.

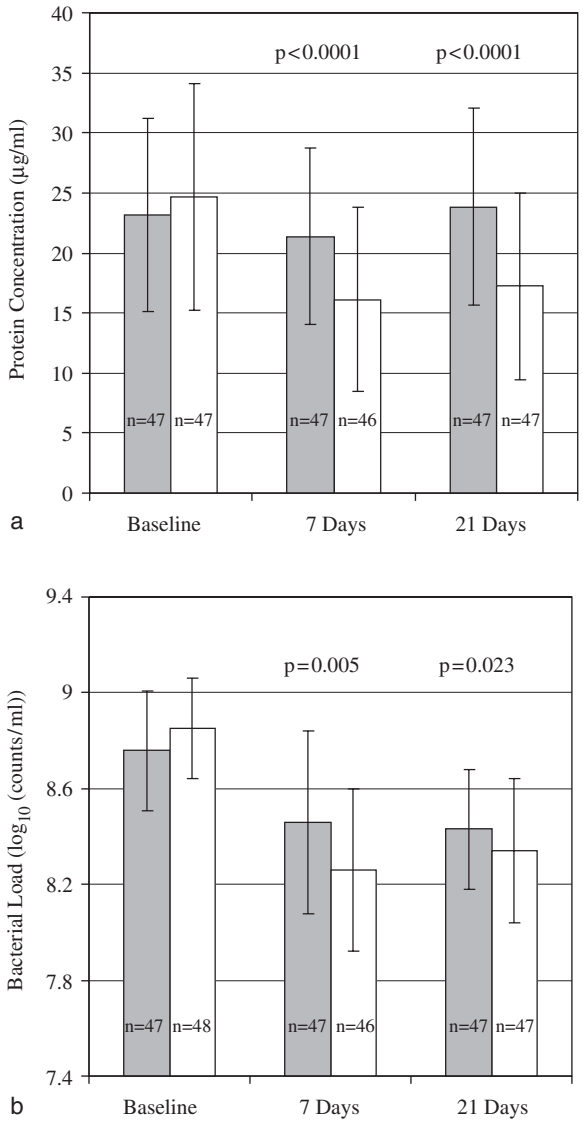

Fig. 2. Residual IPP levels determined by (a) total protein concentration and (b) bacterial load at baseline and after 7 and 21 days of intervention with MTB (■) or MTB+F (□). Results were analyzed by ANCOVA (MTB, manual toothbrush; MTB+F, manual toothbrush and use of floss), and expressed as mean and SD.

Results

Ninety-six subjects were enrolled in the study, with 93 subjects completing all phases (baseline, 7 and 21 days) of the study. Table 1 summarizes the baseline demographics and characteristics of the enrolled subjects. Two subjects withdrew consent for the study (one before completing the 21-day intervention phase and the other before the 7-day intervention phase). A third subject began taking antibiotics during the study and was, thus, excluded from analysis. Sample sizes at baseline for the MTB+F group were different for total protein concentration ($n = 47$) and bacterial load ($n = 48$) due to a laboratory sampling error during the protein analysis.

Figure 2 summarizes the results for post-intervention assessments of residual IPP by total protein concentration (Fig. 2a) and total bacterial load (Fig. 2b). Differences in protein concentration for MTB *versus* MTB+F were statistically significant at both 7 and 21 days ($p < 0.0001$ at both time points). Furthermore, a reduction in protein concentration was observed for MTB+F relative to baseline at 7 days and increased only slightly at 21 days. In contrast, the protein concentration for MTB nearly returned to baseline levels by 7 days, and only a slight increase from 7 to 21 days.

For total bacterial load (Fig. 2b), expressed on a $\log_{10}$ scale, analysis showed that the differences between the two interventions were significant after 7 and 21 days ($p = 0.005$ and 0.023, respectively). In general, both interventions decreased the total bacterial load relative to baseline, with MTB+F demonstrating a greater reduction than the MTB group. Comparing the results at 7 days *versus* 21 days, MTB showed virtually no change in bacterial load while the total load for MTB+F increased over time.

A majority of the samples was collected from molar/pre-molar or pre-molar/pre-molar sites, while only a small number of samples were taken from pre-molar/canine sites (Table 2). The distribution of site locations was similar for both intervention groups ($p = 0.131$). The mean protein concentrations for molar/pre-molar and

Table 2. Total protein concentration (in μg/ml) and bacterial load (in 10^6 counts/ml) for MTB and MTB+F for the types of interproximal test sites from which plaque samples were collected throughout the study (mean and SD)

	Protein concentration (μg/ml)							
	MTB				MTB+F			
Interproximal site	# of sites	baseline	7 days	21 days	# of sites	baseline	7 days	21 days
Molar/pre-molar	138	23.4 (10.0)	22.2 (10.5)	25.1 (11.0)	127	25.4 (12.9)	16.5 (10.1)	18.5 (11.6)
Pre-molar/pre-molar	42	23.3 (12.7)	20.5 (9.7)	21.5 (9.6)	48	24.9 (11.8)	16.0 (8.4)	14.9 (7.41)
Pre-molar/canine	8	17.9 (15.5)	12.9 (7.2)	15.7 (6.9)	17	17.9 (11.3)	13.3 (7.4)	14.6 (10.7)
	Bacterial load (10^6 counts/ml)							
	MTB				MTB+F			
Interproximal site	# of sites	baseline	7 days	21 days	# of sites	baseline	7 days	21 days
Molar/pre-molar	138	760.6 (564.8)	461.1 (553.7)	372.6 (284.3)	127	915.8 (654.0)	275.5 (235.3)	329.5 (256.2)
Pre-molar/pre-molar	42	850.9 (851.0)	423.5 (459.0)	309.9 (268.7)	48	925.5 (632.8)	354.6 (391.5)	279.2 (247.2)
Pre-molar/canine	8	446.4 (324.3)	544.7 (931.5)	211.2 (87.9)	17	627.7 (300.5)	218.6 (166.0)	289.7 (313.9)

MTB, manual toothbrush; MTB+F, manual toothbrush and use of floss.

pre-molar/pre-molar sites showed that protein concentrations at baseline, 7 and 21 days were, overall, close to one another, while the concentrations at pre-molar/canine sites were typically less than for the other two interproximal sites (the exception to this trend was for MTB+F at 21 days). A similar trend was observed with bacterial load at baseline, but was tenuous at 7 and 21 days.

Discussion

Partial-mouth sampling

Partial-mouth sampling, limited to one posterior interproximal space per quadrant, was used to collect samples for determination of bacterial load and protein concentration. A similar methodology has been used previously, although in a somewhat different context (Gmür & Guggenheim 1994, Sjögren et al. 1996). The selection of the method used was made for a number of reasons and occurred after careful consideration of the advantages and disadvantages over full-mouth sampling. First, plaque sampling in clinical trials of moderate to large size is a very time-consuming endeavour. Subject throughput must be taken into account when methodology is developed that could later be adopted in Phase III or IV clinical trials. Second, due to spatial limitations, proper sampling of the most posterior interproximal spaces is quite difficult to accomplish clinically, just adding to the time concern. Third, inclusion criteria in this study, e.g., specified that interproximal surfaces should be natural tooth substance, i.e., no restorations, and in an effort to control the experimental conditions while developing this method. It would have been nearly impossible to qualify enough subjects without restorations in entire posterior regions, let alone in the whole mouth. Finally, interproximal spaces in posterior regions are more difficult to clean using a toothbrush (and floss) than in anterior regions and therefore of greater interest to the clinical community.

Protein as an outcome measure

Total protein concentration has been shown to be a reasonable outcome measure for objectively evaluating the efficacy of oral hygiene measures (Altman et al. 1979, FDA 1996, Bellamy et al. 2004, Milanovich et al. 2005, 2007). Evidence suggested that protein was a better discriminator of IPP than the Rustogi (Rustogi et al. 1992) and Turesky (Turesky et al. 1970) PIs (Milanovich et al. 2005). Distinct differences in protein concentration between MTB and MTB+F were consistent with previous findings (Bellamy et al. 2004, Milanovich et al. 2005, 2007). In addition, the relative concentrations of protein for MTB were similar to baseline after 7 and 21 days while the concentrations for MTB+F were consistently lower than baseline and MTB. For the former relationships, the baseline protein concentrations and the interventions were expected because MTB was effectively a continuation of the routine required during washout. The latter relationship was also anticipated because the action of flossing was expected to be more effective in removing IPP than brushing alone. The current results, therefore, confirmed the use of total protein concentration as a viable means by which to assess differences in residual IPP levels between oral hygiene interventions. The 21-day results demonstrated that significant differences between interventions were detectable after long-term use. Moreover, findings after 7 days of intervention confirmed this model as a short-term method for comparing and evaluating oral hygiene interventions. The validity of the 7-day results was further supported by its similarity in results to the 21-day time point.

The results summarized in Table 2 suggested that the protein concentrations at the molar/pre-molar and pre-molar/pre-molar sites were essentially the same. Furthermore, protein concentration at pre-molar/canine sites was consistently less than for the other sites. Having relatively less protein at pre-molar/canine sites was not surprising because the surface area was expected to be less than for the other two sites. Nonetheless, this observation indicated that had there been substantially more pre-molar/canine sites from which to sample in one of the study arms *versus* the other, then it is conceivable that the difference in residual plaque would be smaller between two interventions, thus, losing the ability to distinguish between two oral hygiene products for removing IPP. Therefore, future studies using protein concentration to evaluate interproximal cleaning should be restricted to molar/pre-molar and pre-molar/

pre-molar sites. In addition, because the molar/pre-molar and pre-molar/pre-molar sites had similar concentrations of protein, this might allow pre- and post-hygiene evaluations.

Bacterial load based on 16S rRNA as an outcome measure

Protein-specific markers are unable to discriminate between plaque and non-plaque proteins (e.g., salivary, gingival crevicular, and pellicle proteins), thus resulting in an inherent background signal when trying to measure residual IPP (Milanovich et al. 2007). As a solution to the background signal, the bacterial component of the plaque mass was investigated as another possible surrogate measure for residual IPP. The assumption was that universal primers for 16S rRNA were selective for the bacteria in the plaque mass, in turn, avoiding the background signal. Universal probes and primers for 16S rRNA have been used previously to estimate the total bacterial load, in studies on periodontal health and disease (Moncla et al. 1991, Lyons et al. 2000, Hutter et al. 2003, Maeda et al. 2003, Rowshani et al. 2004), caries (Nadkarni et al. 2002), and endodontic infections and therapy (Horz et al. 2005). That total bacterial load resulted in statistically significant differences in residual IPP between MTB and MTB+F after 7 and 21 days of intervention suggested that 16S rRNA might be used as an alternative outcome measure to total protein concentration for evaluating oral hygiene devices. It was also noted that the bacterial load for MTB and MTB+F at both 7 and 21 days declined relative to baseline. This observation for MTB+F was consistent with the results from protein concentration, but not for MTB. The relationship for MTB was unexpected because this intervention essentially continued the oral hygiene regimen the subjects followed during washout through baseline. Errors in sample analysis for bacterial load that might explain this relationship for MTB could not be found and, therefore, we have no plausible explanation at this time. Nonetheless, the bacterial load for MTB+F was significantly less than for MTB at both time points. Furthermore, we estimated the consistency between the two outcome measures by calculating bacterial load from the total protein concentration results, assuming 8.7×10^{-11} mg protein/bacterial cell (Teather et al. 1984), with the results using 16S rRNA. The calculated and actual bacterial load values were always of the same order of magnitude ($\sim 10^8$), with the bacterial loads measured by 16S rRNA typically greater than the calculated values by an $\sim$ 1.4–3 times for analogous data points.

Comparison between outcome measures

That the differences between interventions for the two outcome measures were statistically significant implied that total protein concentration and total bacterial load based on universal primers of 16S rRNA can be used as surrogate measures for detecting differences in residual IPP after short-term (7 days) and long-term (21 days) use of oral hygiene interventions. However, as a clinical model, evaluating the efficacy of oral hygiene routines for interproximal cleaning by measuring protein concentration is preferable to bacterial load in spite of the inherent background signal from non-plaque proteins ever present in the plaque samples (Milanovich et al. 2007). One reason for this preference was that the bacterial load measurements had greater variability than the protein measurements, thus requiring a larger sample size in this clinical model. For example, to achieve a comparable effect size with the two outcome measures, evaluations based on bacterial load would require at least twice as many subjects as would be needed with protein concentration. The current study was, in fact, designed based on bacterial load because it was the more variable outcome measure. Second, the assay for total protein concentration was simpler and easier to execute, and less expensive *versus* the method for bacterial load. Third, the clinical relevance of the observed differences between interventions for the two outcome measures was in (apparent) contrast with one another, vide infra.

Clinical relevance and minimum clinically important difference (MCID)

This clinical model has clearly demonstrated the ability to resolve intervention-related differences in removal of IPP that were statistically significant. However, can we also consider these differences to be clinically relevant? Qualitatively, the inclusion of flossing in a daily oral hygiene routine has already been clinically shown to confer gingival health (Finkelstein & Grossman 1979, Lobene et al. 1982, Graves et al. 1989, Rich et al. 1989, Ciancio et al. 1992, Anderson et al. 1995, Pucher et al. 1995, Carter-Hanson et al. 1996, Cronin & Dembling 1996, Halla-Junior & Oppermann 2004) and anti-caries benefits (Wright et al. 1977) through the daily removal of (supragingival) IPP. Therefore, the reduction of plaque biofilm due to flossing, as measured in this study, was expected to be clinically relevant. The results, in fact, suggested that differences in total protein and bacteria (for four posterior interproximal sites) ranging from ~ 6 to 7.5 μg of protein or $\sim 0.6 \times 10^8$ to 1.80×10^8 bacterial counts might be sufficient to affect gingival health at those sites. Moreover, the residual plaque that remained after flossing still contained $\sim 3 \times 10^8$ bacteria, i.e, $\sim 3\times$ the amount removed. This implies, in an extension of a postulate recently put forth (Marsh 2006), that regularly disrupting the plaque biofilm might be a sufficient prerequisite for sustainable gingival health and that complete removal of plaque bacteria would be unnecessary.

To assess whether the intervention-related differences in protein concentration or bacterial load observed with this clinical model were, in fact, clinically relevant, we followed the approach described by Man-Son-Hing et al. (2002). (Henceforth, the term *clinically important* will be used in the remaining discussion on clinical relevance to be consistent with the method of Man-Son-Hing et al.) Given the health benefit of IPP removal by floss, the smallest intervention efficacies that would lead to a change in a patient's management, defined as the MCID, were assumed to be 5.0 μg/ml and 0.5 [$\log_{10}$(counts/ml)] for total protein concentration and bacterial load, respectively, as determined from previous clinical trials (internal results). Clinical importance can then be assessed by first comparing the point estimate (i.e., difference between interventions) and the associated confidence interval (CI) to the MCID. Based on the relationship between these parameters and the statistical significance of the difference between interventions, the clinical importance can then be classified as definite, probable, possible, and definitely not.

Table 3 summarizes the evaluation of clinical importance for the present study. Applying Man-Son-Hing's scheme to the protein data showed that the point estimate for the difference between interventions (1) had a CI that included the MCID, (2) was greater than the MCID, and (3) was statistically

Table 3. Determination of the clinical importance of treatment effects based on the criteria outlined by Man-Son-Hing et al. (2002). Statistical significance, point estimates of treatment effects, and associated 95% confidence intervals (95% CI) were determined from ANCOVA. Clinical importance is classified as definite, probable, possible, or definitely not, depending on the relationship between the expected minimum clinically important difference (MCID) and the estimated treatment effect

Outcome measure	Days	Statistically significant?	MCID	Point estimate	95% CI	Clinically important
Protein concentration	7	Yes	5.0*	6.12	3.7–8.54	Probable
	21	Yes	5.0*	7.40	4.72–10.09	Probable
Bacterial load	7	Yes	0.5†	0.21	0.07–0.36	Definitely not
	21	Yes	0.5†	0.13	0.02–0.23	Definitely not

*Expressed in units of μg/ml.
†Expressed as $\log_{10}$ (counts/ml).

significant. Therefore, it was concluded that the difference in protein concentration due to flossing was of *probable clinical importance*. This conclusion is reasonable and in agreement with evidence from numerous clinical trials. In contrast, the point estimate for bacterial load (1) had a CI that did not include the MCID, (2) was smaller than the MCID, and (3) was statistically significant. This scenario was determined to be *definitely not clinically important*. However, the assumption that flossing would reduce plaque bacterial load by 0.5 ($\log_{10}$ scale, MCID), or $\sim$ 60%, as observed in the internal pilot study, was overly optimistic. Instead, an MCID of 0.1 ($\log_{10}$ scale) would have been more realistic and in line with the assumed MCID for protein concentration, which had an estimated reduction in plaque proteins of $\sim$ 25%. In fact, substituting an MCID of 0.1 for 0.5 for the bacterial load analysis would lead to a similar conclusion about clinical importance as did protein concentration. This result emphasizes the importance of properly selecting an MCID in clinical trials. In general, the evaluation of clinical importance by the above method relied on the current data (point estimate and CI) and data from previous clinical trials (MCID). Disproportionately more historical data were available by which to accurately define MCID for protein concentration than for bacterial load. This meant that the intervention-related differences measured by protein concentration being of probable clinical importance were, in fact, accurate and reasonable. However, the limited data from which MCID was determined for bacterial load suggested that, at best, the clinical importance of the study results based on bacterial load was inconclusive.

Conclusions

This study established total protein concentration as a valid surrogate measure for assessing intervention-related differences in residual IPP for two oral hygiene routines in a clinical model. This held true for differences between interventions after as little as 7 days of use and as long as 21 days. Furthermore, the differences in protein concentration were determined to be clinically relevant. Intervention-related differences in total bacterial load were also statistically significant, but were determined not to be clinically meaningful. In addition, total protein measurements were less variable than total bacterial load, as suggested by calculation of effect sizes for each; the former was also simpler and easier to conduct than the latter. For all three reasons, we preferred total protein concentration over total bacterial load for assessment of residual IPP.

Acknowledgements

The authors thank Mark Putt, Jeffery and Kim Millemann from University Park Research Center (Fort Wayne, IN, USA) for conducting the study, Robert Brooks from Microbac Laboratories (Maryville, TN, USA) for conducting the real-time PCR analyses, Jackie Stilwell and Nick Elliott from Philips Oral Healthcare, and Sonia Souza from Nastech Pharmaceuticals Inc. for assistance with the protein assay and statistical analyses. The authors also thank Prof. Peter Heasmann from University of Newcastle upon Tyne for many helpful discussions and suggestions in the preparation of this manuscript.

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Clinical Relevance

Scientific rationale for the study: A recently described clinical model used total protein concentration in residual plaque to assess interproximal plaque removal. Bacterial load was proposed as an alternative measure to address shortcomings to using protein concentration.

Principal findings: Both outcome measures showed statistically significant differences between brushing and brushing+flossing. Further analysis concluded that protein concentration produced clinically relevant differences between interventions, while the clinical relevance of these differences was not established for bacterial load.

Practical implications: Protein concentration is a robust outcome measure, providing clinically relevant differences between oral hygiene interventions in a clinical model evaluating interproximal plaque removal efficacy.