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ORIGINAL ARTICLE

Pellicle and early dental plaque in periodontitis patients before and after surgical pocket elimination

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Abstract

Objective. Gingival inflammation may affect the composition of the dental pellicle and initial acquisition of bacteria, which in turn could affect the healing of the periodontal pocket. The aim of this study was to examine the dental pellicle and early supragingival biofilms in periodontitis patients with an established subgingival infiltrate before and after surgical pocket elimination. ***Materials and methods.*** Eleven patients with remaining pockets were selected. Samples were taken before and after surgical pocket elimination and after subsequent experimental gingivitis. Pellicle proteins were analyzed by SDS-PAGE, immunoblotting and image analysis and 4-h supragingival plaque by culturing. ***Results.*** The inflammatory status affected to a greater extent the concentration of plasma proteins than salivary proteins in the dental pellicle. The highest plasma protein concentrations were observed at remaining periodontal pockets where also the highest bacterial counts were found. The TVC was reduced on the gingival tooth surfaces ($p < 0.05$) after pocket elimination and increased slightly during experimental gingivitis. The finding of streptococci was highest on the incisal tooth surfaces and increased after surgery. Gram-negative anaerobes were sparse but seen more often before than after pocket elimination and on gingival than on incisal surfaces. ***Conclusions.*** This study suggests that increased amounts of plasma proteins in the pellicle formed in the presence of remaining periodontal pockets may foster the acquisition of bacteria, including proteolytic Gram-negative species. This, in turn, results in an increased *de novo* plaque formation rate.

Key Words: *plasma proteins, dental biofilm, periodontal pocket, gingival crevicular fluid, experimental gingivitis*

Introduction

As inflammation of the gingival margins increases, so does the formation of dental plaque both in terms of thickness [1] and tooth surface area covered [2]. An increased growth of dental plaque has been observed for naturally occurring gingivitis [1] and at experimentally inflamed gingival margins [2,3]. A number of possible mechanisms for this clinical observation have been discussed. The swelling of the gingival margin might serve as an anatomical shelter for the growing plaque [4]. Further on, as bacteria start multiplying, plaque grows faster at inflamed gingival margins than at healthy ones due to an increased nutrient supply through the gingival crevicular fluid (GCF) [2]. The GCF flow may, however, not only foster dental plaque growth by increased nutrient supply but also by modifying the pellicle composition and thereby the bacterial adhesion and the early dental plaque formation. The binding capacities of plasma components in the pellicle deriving from the GCF might compete with those of salivary origin that had been considered the dominating components of the dental pellicle under healthy conditions. Salivary components, e.g. agglutinins, mucins, sIgA, amylase proline-rich proteins and glycoproteins, were reported to promote *in vitro* adhesion of *Streptococcus* spp. and *Actinomyces* spp. (for review see [5,6]). Plasma and plasma components were reported to have good binding properties to *A. naeslundii*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* [7,8], whereas they were shown to inhibit the adhesion of streptococci [9]. There are few studies on pellicle and early dental plaque formation performed *in vivo*, although *in vivo* and *in situ* studies were considered necessary to understand the complex processes of bacterial adhesion and dental plaque formation (for review see [10]). In a previous study *in vivo*

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on healthy individuals, we found modified pellicles and early dental plaque at increased GCF flow during experimental gingivitis [3]. In periodontally diseased individuals, the inflammatory response of the gingival margin to plaque accumulation was found to be more pronounced than in healthy individuals [11]. One reason could be a higher acquisition of periodontopathogenic micro-organisms on the tooth surface in the former. The aim of this study was to examine the composition of the dental pellicle and the formation of the early dental plaque *in vivo* in periodontitis patients before and after surgical pocket elimination and after subsequent experimental gingivitis.

Materials and methods

Subjects and sampling principles

Eleven subjects (aged 39–66 years, 10 males, one female) diagnosed with general chronic periodontitis in a remaining dentition of 23–31 teeth were recruited. All subjects were in good general health and none of them had used antimicrobials during the 6 months prior to the study. Six of them were smokers (10–20 cigarettes per day), two used snuff but did not smoke and three neither smoked nor used snuff. The study participants were recruited at re-evaluation after basic therapy (comprising information of the disease, motivation and instruction to proper oral hygiene as well as supra- and subgingival scaling under local anesthesia) when surgical pocket elimination was deemed necessary to reduce infection and to arrest the progression of the disease. Good oral hygiene (PlI <15%) was a pre-requisite for participation and was maintained throughout the study. At the time point of enrollment into the study, the mean percentage of all sites (four sites per tooth) with probing depths of 5 or 6 mm was 17% and 6% for sites >6 mm. Teeth having approximal pockets ≥5 mm with bleeding upon probing were selected for sampling (four-to-five teeth per participant). No furcation-involved tooth was included. Teeth with buccal or lingual restorations were excluded. After surgery, no pockets >5 mm remained at the teeth selected for sampling and only two sites had a probing depth of 5 mm. Bleeding upon probing was substantially reduced (Table I).

Surgical pocket elimination was performed as was required by the patients' individual treatment plan. After local anesthesia (Xylocain® Dental adrenalin 20 mg/ml + 12.5 μg/ml, Dentsply, Addlestone, Surrey, UK) and intracrevicular incision, a full-thickness flap was raised and granulation tissue removed. Osseous surgery was performed and the soft tissues adjusted as necessary to achieve immediate post-operative pocket elimination (pocket probing depth ≤4 mm). The flaps were repositioned with non-resorbable sutures (Supramid DS 19 4/0, Aesculap, Tuttlingen, Germany). No periodontal dressings were used. Tissues were allowed to heal for an average of 5 months (range 3–7 months) before samples were taken again.

Table I. Clinical variables at the teeth selected for sampling before and after surgical pocket elimination. Frequency distribution of pocket probing depths and bleeding upon probing (percentage of sites (four sites/tooth); mean and range).

	Surgical pocket elimination	
	Before	After
Pockets of 5–6 mm	28 (13–56)	1 (0–6)[a]
Pockets of 6–8 mm	8 (0–31)	0
Bleeding upon probing	39 (19–69)	10 (6–15)[b]

[a]After surgery no remaining pocket >5 mm.
[b]$p < 0.01$ for all differences before and after surgery.

Sample collection and registrations were made three times, before and after surgical pocket elimination as well as during experimental gingivitis (Figure 1). To create healthy conditions in the gingival margins before and after surgery and as a baseline for the experimental gingivitis period, all teeth were professionally cleaned every other day for a 2-week period. Experimental gingivitis was allowed to develop by asking the participants to abstain from oral hygiene for 5 days. The GCF flow has previously been shown to increase steadily during the first 5 days of experimental gingivitis [12]. After the last sampling, patients received repeated oral prophylaxis and hygiene instructions, as individually required.

The study protocol was approved by the Ethical Committee at Lund University (LU 580-03; 030924).

Pellicle collection and analysis

The dental pellicles were collected and analyzed as previously described [13]. In brief, pellicle formation was allowed for 60 min after polishing of the teeth using a rubber cup and fine-grade pumice. After rinsing with water the selected teeth were isolated with cotton rolls and air-dried. Pellicles were collected separately from equally sized areas of the gingival and incisal parts of the buccal and lingual surfaces, respectively, by alternately rubbing the surface with fiber-pellets (Quick-sticks™, Dentonova AB, Huddinge, Sweden), soaked in 2% SDS-solution and dry pellets. All fiber-pellets from the gingival and incisal surfaces, respectively, were pooled and, thus, two samples were forwarded for analysis after each sampling occasion. In the analysis, the pellicle proteins were desolved from the fiber-pellets by heat treatment in SDS-PAGE sample buffer and recovered by centrifugation. The samples were run on pre-cast 4–15% gradient gels, silver-stained or blotted onto nitrocellulose membranes using the Pharmacia automated Phast-System® (Pharmacia/LKB, Biotechnology,

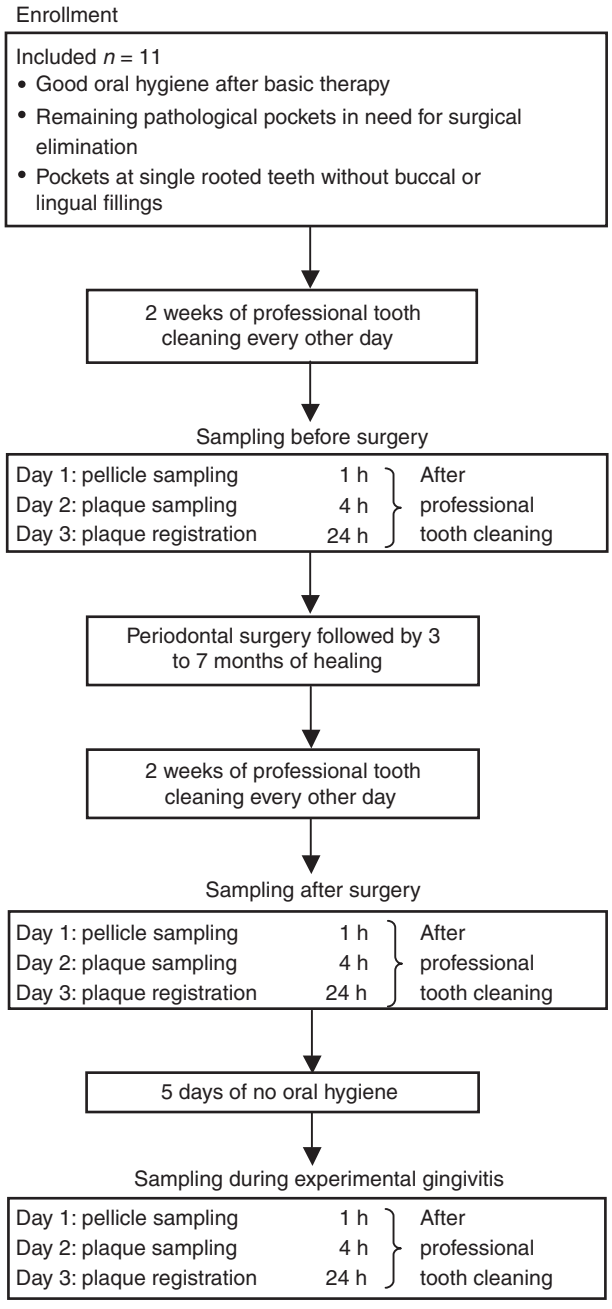

Figure 1. Flow-chart for the three sampling occasions.

Stockholm, Sweden) according to the manufacturer's description and recommendations.

A panel of antibodies to IgA and typical plasma (IgG, albumin, fibronectin and fibrinogen) and salivary proteins (amylase and proline-rich proteins (PRP)) was used to identify specific proteins by immunoblotting (all antibodies were from Sigma-Aldrich Sweden AB, Stockholm, Sweden). As staining intensity could vary between different gels and different immunoblot membranes, samples to be compared were run on the same gel. The detection limits for all proteins had previously been determined from the staining of purified, serially diluted proteins in the immunoblots [3].

Stained gels and membranes (Figure 2) were scanned and digitized prior to semi-quantification of the proteins by densitometric analysis (ImageJ 1.40 g, public domain program; NIH).

Plaque sampling

The selected teeth were polished using a pumice, as above, and plaque was allowed to form for 4 h, undisturbed from food and fluid except water. The teeth were rinsed with water, isolated with cotton rolls and air-dried prior to collection of plaque by scraping the respective teeth surfaces with sterile scalers (Scaler N°2 REF 418 04, Produits Dentaires S.A. Vevey 1800, Switzerland). Two samples, from the gingival and incisal surfaces, respectively, were collected in VMGA III transport media [14,15].

Bacterial examination

Plaque samples were vortexed prior to serial dilution in PBS (50 mM K-phosphate buffer, 0.4% KCl, pH 7). Diluted samples were plated on MS (Mitis salivarius) agar for growth of *Streptococcus* spp. and on CFAT (Cadmium sulfate-fluoride-acridine tryptcase)-Agar [16] for growth of *Actinomyces* spp., which were further characterized by their rod-shaped or filamentous appearance after Gram-staining. Samples were cultured on TSBV (Trypticase soy blood vancomycin) -agar [17] for growth of *Aggregatibacter actinomycetemcomitans* and on Brucella-agar (enriched

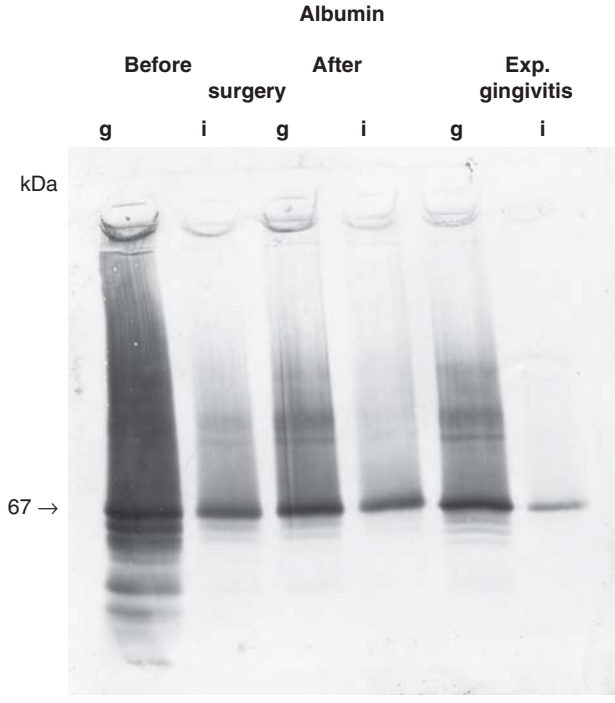

Figure 2. Immunoblot with bands of albumin (~ 67 kDa) in gingival (g) and incisal (i) pellicles before and after surgical pocket elimination and in the presence of experimental gingivitis after pocket elimination.

with 5% horseblood, 0.5% hemolyzed horseblood and 5 mg/l menadione) for determination of the total viable counts (TVC) and numbers of black-pigmented *Prevotella* spp., *P. gingivalis*, *Capnocytophaga* spp. and *Fusobacterium nucleatum* from their typical morphological appearance [18]. The MS and TSBV agar plates were incubated for 2, 3 and 5 days, respectively, in 90% N_2 and 10% CO_2 at 37°C. The CFAT and Brucella-agar plates were incubated in 95% H_2 and 5% CO_2 at 37°C for 5 and 7 days, respectively.

Clinical variables

Gingival crevicular fluid flow was measured using Perio papers® and a Periotron 8000® device (Oraflow, Smithtown, NY). The Periotron value was transformed into a fluid volume (µl) using a standard curve generated by calibration with standardized fluid volumes. The plaque formation rate was registered as a percentage of surfaces covered by plaque 24 h after professional tooth cleaning and abstaining from oral hygiene. Plaque was disclosed (Rondell röd, DAB Dental, Upplands Väsby, Sweden) and single-blinded registration of the presence or absence of plaque was performed at six sites per tooth.

Statistics

To allow for comparison to our previous study [3], calculations on proteins and bacteria that were detected on a majority of the surfaces (>60%) were performed on the basis of quantitative data (pixel values from image analyses and percentage of TVC, respectively). Otherwise, the detection frequencies were used. Wilcoxon matched pairs signed rank sum test was applied for comparative tests.

Results

Pellicle composition

IgG, albumin, amylase and IgA were detected in the vast majority of the samples. Fibronectin, fibrinogen and PRP were on average seen in less than 60% of all samples and therefore their analyses were based on detection frequencies (Table II).

Semi-quantitative amounts of total protein (from silver-stained gels) and specific proteins (from immunoblots; Figure 2) were obtained from the pixel values (Table III). The amounts of total protein, IgG and albumin, and the detection frequencies of fibronectin and fibrinogen in the pellicles displayed an overall pattern of decreased values after surgical pocket elimination followed by increased values after subsequent experimental gingivitis.

Bacteria in biofilms

The bacterial counts per sample varied considerably and few statistically significant differences were obtained. Higher total viable counts (TVC) were, however, seen on the gingival than on the incisal parts at all occasions. The highest counts were observed at remaining pockets where TVC was reduced after pocket elimination (gingival surfaces, $p < 0.05$) and slightly increased during subsequent experimental gingivitis (Table IV).

Streptococcus spp. were identified in 97% of all samples. Their percentage of TVC was higher on the incisal than on the gingival part of the tooth and the highest percentage was seen after pocket elimination (Table IV).

Actinomyces spp. were detected in 32% of all samples. The mean number was $1.1 \pm 3.0 \times 10^4$ (SD) on gingival surfaces before surgery. In all other samples the mean number was <100. Except for samples collected during experimental gingivitis, where they were found to similar extent on the gingival and incisal surfaces, they were more often found on the gingival ones (Table IV).

Other bacteria were less common and found in <10% of all samples. Gram-negative species (*Campylobacter* spp., *Capnocytophaga* spp. or *F. nucleatum*) were more often found on gingival than on incisal surfaces and more often before than after

Table II. Detection frequencies[a] of proteins in gingival and incisal pellicles formed at the different sampling time points (see Figure 1).

	Before surgery		After surgery		Experimental gingivitis	
	Gingival	Incisal	Gingival	Incisal	Gingival	Incisal
IgG	90.9	90.9	100.0	90.9	100.0	100.0
Albumin	100.0	100.0	100.0	100.0	100.0	100.0
Fibronectin	63.6	36.4	54.5	9.1	72.7	18.2
Fibrinogen	54.5	27.3	54.5	18.2	63.6	45.5
Amylase	100.0	81.8	100.0	100.0	100.0	100.0
IgA	81.8	81.8	100.0	90.9	90.9	90.9
PRP	45.5	63.6	81.8	72.7	54.5	36.4

[a]Percentage of the samples where the protein was detected.

Table III. Amounts of protein (pixel values) and ratios thereof at the different sampling time points (see Figure 1) in gingival and incisal pellicles. Pixel values (mean ± SD) obtained from image analysis of the silver-stained gels and immunoblots.

	Gingival pellicle			Incisal pellicle		
	Before surgery	After surgery	Experimental gingivitis	Before surgery	After surgery	Experimental gingivitis
Total proteins[a]	4790 ± 3896	1872 ± 1139	3684 ± 2853	2460 ± 3183	1397 ± 1368	1147 ± 1325
	2.6[b]		2.0[c]	1.8		0.8
IgG	8570 ± 6107	3993 ± 2462	5907 ± 5054	3390 ± 3921	1386 ± 1384	1559 ± 987
	2.1[b]		1.5	2.4[b]		1.1
Albumin	11795 ± 6577	7545 ± 4563	9660 ± 5644	4606 ± 3654	2700 ± 2290	3077 ± 1853
	1.6[b]		1.3	1.7		1.1
Amylase	1896 ± 1208	3921 ± 3323	4521 ± 3315	2835 ± 3574	3630 ± 3534	4011 ± 2436
	0.5[b]		1.2	0.8		1.1
IgA	4429 ± 5076	3516 ± 2865	2781 ± 2419	2639 ± 4427	2393 ± 4461	1196 ± 931
	1.3		0.8	1.1		0.5

[a]Total amount of proteins as determined from silver-stained gels.
[b]ratio $p < 0.05$.
[c]ratio $p < 0.01$.

surgical pocket elimination. None was found on incisal parts after surgery (Table IV). In no case were *A. actinomycetemcomitans*, *P. gingivalis* or black-pigmented *Prevotella* spp. detected.

Experimental clinical variables

Gingival crevicular fluid significantly ($p < 0.05$) decreased at the experimental teeth from 0.115 ± 0.025 µl/10 s before to 0.043 ± 0.023 µl/10 s after the surgical pocket elimination. This observation was paralleled by a significantly ($p < 0.05$) lower post-operative plaque formation rate on these teeth (pre-operative 40 ± 28% vs post-operative 27 ± 20% tooth surfaces covered by plaque after 24 h of no oral hygiene). At experimental gingivitis, a slight increase was observed for both these clinical variables (0.055 ± 0.023 µl/10 s and 29 ± 19%).

Discussion

In the present study, dental pellicles and early dental plaque were formed under the influence of either an established subgingival infiltrate or an experimentally induced marginal infiltrate after surgical pocket elimination. The status after surgical pocket elimination and repeated oral hygiene instructions served as a comparison. Plasma proteins were found in higher amounts and bacteria were identified in higher numbers before than after surgery, which is in line with substantial reduction or elimination of the subgingival infiltrate by surgery. This paralleled our previous

Table IV. Bacteria in early gingival and incisal plaque at the different sampling time points (see Figure 1) (mean ± SD; $n = 11$).

	Before surgery		After surgery		Exp. gingivitis	
	Gingival	Incisal	Gingival	Incisal	Gingival	Incisal
Log (TVC[a])	5.51 ± 2.18[b]	4.45 ± 2.28	4.35 ± 1.42	3.63 ± 1.50	4.87 ± 1.23	3.96 ± 1.88
Streptococcus spp.[c]	76.6 ± 33.9	79.8 ± 30.7	65.1 ± 28.8	89.4 ± 14.1[d]	55.3 ± 34.4	70.0 ± 27.7
Actinomyces spp.[e]	36.7%	27.7%	45.5%	27.7%	27.7%	27.7%
Gram-negative species[e,f]	36.4%	9.1%	18.2%	n.d.[g]	9.1%	n.d.[g]

[a]Total viable counts.
[b]$p < 0.05$ before vs after surgery.
[c]percentage of TVC.
[d]$p < 0.05$ after surgery vs experimental gingivitis.
[e]detection frequency.
[f]*Campylobacter*, *Capnocytophaga* spp. or *F. nucleatum*.
[g]not detected.

findings of plasma proteins being incorporated into dental pellicles *in vitro* and *in vivo* and being increased in the presence of experimental gingival inflammation in healthy subjects [3,7,13].

Our own and other studies have demonstrated that fibronectin and fibrinogen readily adhere to hydroxyapatit *in vitro* [13,19]. The prevalence of fibronectin and fibrinogen but not of IgG and albumin *in vivo* was, however, notably lower in the present than in our previous study on periodontally healthy—and mostly non-smoking—subjects [3]. In the present study, the majority of the patients were smokers who are known to have a less gingival crevicular fluid flow than non-smokers [20], which may result in reduced amounts of plasma proteins available for pellicle formation. The difference may also be explained by the gingival recessions known to occur during the course of periodontal disease. Exposed root surfaces present surfaces that may bind these components better than enamel. Smaller molecules of the crevicular fluid may also more easily than larger ones reach and cover the tooth surface areas distant from the gingival margin. This is in line with a consistently high prevalence of especially albumin but also of IgG and IgA in the pellicles at all three occasions.

The limited number of samples containing minor amounts of protein did not allow the determination of the absolute protein concentrations in each sample. However, the aim was to examine possible relative differences due to the state of gingival inflammation. We believe this could be described by the relative staining intensity of proteins in pairwise individual samples on the same immunoblot. Pairwise individual samples were similarly used for comparisons of plaque bacteria. The plaque samples like pellicle samples were taken by the same investigator (SGR) in both our present and previous study [3]. Variations seen for the bacterial counts in the samples could be due to several reasons. Besides differences in degree of periodontal inflammation and to individual variations in plaque formation rate [21], the surface area of the sampled teeth could vary due to individual variations and type of tooth examined.

Gram-negative species associated with periodontal disease were less frequently found in this study than in our previous study on healthy subjects [3]. Low numbers of periodontopathogenic bacteria might reflect that the patients are repeatedly and forcefully instructed to optimal oral hygiene. The Gram-negative species identified were, however, most often found at the inflamed gingival margin before surgical pocket elimination. Similar to our previous study [3] the percentage of TVC of streptococci was highest in the incisal plaque in the absence of periodontal infection.

Our findings of a significantly higher plaque formation rate before than after pocket elimination suggest that the presence of remaining periodontal pockets has a high impact on the formation of the supragingival biofilm. Experimental gingivitis after resective periodontal surgery did not influence initial biofilm formation to the same extent as the remaining pockets. Previous studies support our notion that this clinical observation is paralleled by the size of the inflammatory infiltrate. Surgical periodontal treatment resulted in a remarkable reduction of the subgingival infiltrated connective tissue and the infiltrate after subsequent experimental gingivitis did not reach pre-surgical dimensions [22,23]. Also, experimental gingivitis did not notably influence the plaque formation rate, which is in contrast to our study on healthy subjects [3]. This observation might be explained by the fact that it is almost impossible to avoid some remaining infiltrated connective tissue after periodontal surgery [22]. Another explanation could be the longer distance to the enamel surface in the periodontitis patients affecting the ability of plasma components to reach the tooth surface as discussed above.

The predictive value of plaque scores for periodontal disease progression has since long been shown to be poor [24]. However, in a recent study, Eickholz et al. [25] showed that consistently higher plaque scores during periodontal supportive therapy were associated with a long-term deterioration of the periodontal status. Our findings of higher bacterial counts and increased plaque formation rate at periodontal pockets might be seen as one reason for long-term deterioration at sites constantly covered by plaque.

In the present study, a group of 11 individuals was regarded sufficient to allow for intra-individual pairwise comparisons. This was based on our previous study on eight healthy subjects [3]. A larger study population might have allowed us to show more statistically significant intra-individual differences and possible effects of smoking on pellicle and early plaque formation. Previous studies show that clinical periodontal conditions and the gingival microflora could differ due to smoking (for review see [26]).

It could, however, be stated that, even if few statistically significant differences were found, our study clearly demonstrated reduced plasma components in the gingival pellicle as well as reduced GCF flow and plaque formation rate on periodontitis teeth after surgery. A tendency to reduced findings of Gram-negative micro-organisms associated with periodontal disease on the teeth after surgery and, opposite to health-related *Streptococcus* spp., more of these bacteria on the gingival than on the incisal part of the teeth were also seen.

Our data suggest that the composition of the pellicle and the formation of early dental plaque is affected by the GCF flow in different states of periodontal inflammation. Increased GCF flow at the remaining pockets may foster the acquisition of bacteria including Gram-negative, proteolytic species. These findings may be of

interest in the discussion on when to surgically eliminate periodontal pockets.

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