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Calprotectin levels in gingival crevicular fluid predict disease activity in patients treated for generalized aggressive periodontitis

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Background and Objective: Clinical parameters such as probing depth and bleeding on probing are commonly used for monitoring after periodontal treatment. However, these parameters have poor prognostic utility. The biomarker calprotectin is used to monitor conditions such as inflammatory bowel disease because of its ability to predict disease activity. Levels of calprotectin in gingival crevicular fluid correlate with periodontal disease severity and treatment outcome. The validity of calprotectin as predictor for future periodontal disease activity has not yet been investigated.

Material and Methods: Thirty-six subjects with generalized aggressive periodontitis were treated with scaling and root planing (SRP), and with adjunctive antimicrobial medications. Probing depth, clinical attachment level and bleeding on probing were assessed at baseline, and 3 and 6 mo after SRP. A gingival crevicular fluid sample was collected from the initially deepest site in each patient 3 mo after SRP and analysed for calprotectin levels. Activity was defined as a probing depth increase of > 0.5 mm between 3 and 6 mo at the sample site. The ability of individual parameters to predict activity was analysed by construction of receiver operating characteristic curves.

Results: Nine active sites were identified. Clinical attachment level, probing depth, bleeding on probing and gingival crevicular fluid volume showed no predictive utility [area under the curve (AUC) < 0.6, $p > 0.05$]. However, calprotectin concentration (AUC $= 0.793$, $p = 0.01$) and the total amount/sample of calprotectin (AUC $= 0.776$, $p = 0.02$) significantly predicted activity. Patients with calprotectin levels above calculated cut-off values had significantly more active sites than patients with negative results.

Conclusion: Calprotectin levels were predictors of disease activity at both site and subject levels. The calculated cut-off values provide a dichotomous basis for prospective evaluation of calprotectin as a diagnostic marker for monitoring periodontal treatment.

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A pivotal issue in periodontal therapy is to re-evaluate previous treatment procedures to decide whether there is any need for further active therapy (1). At re-evaluation, detection of persisting or recurrent disease activity by monitoring may result in additional periodontal treatment, such as subgingival re-instrumentation, administration of adjunctive antimicrobial drugs or periodontal surgery.

In everyday practice, monitoring of periodontitis connotes that diagnosis, prognosis and the resulting therapeutic decisions are based on clinical findings generated using the periodontal probe. Probing depths and clinical attachment levels, or the magnitude of their changes assessed a short time after initial cause-related therapy, are the fundamentals of this well-established diagnostic practice. High proportions of persisting deep sites, residual suppuration and increases of probing depth are considered as indicators of a lack of periodontal stability and as predictors of future attachment loss on a subject level (2–5). Accordingly, the proportions of deep sites persisting after initial cause-related therapy are routinely used to assess the need for additional periodontal surgery (6,7).

However, a systematic review failed to confirm the validity of most clinical parameters for predicting future disease activity and provided only a small amount of evidence for the prognostic ability of persistent increased probing depths (8).

On the site level, the presence or absence of bleeding on probing is a common diagnostic determinant of periodontal disease activity, and bleeding on probing-positive sites are usually treated with subgingival instrumentation (9). However, although the presence of bleeding on probing is biologically linked to inflammation as a result of the loss of collagen and the presence of inflammatory cells in connective tissue (10), it may occur even in the absence of inflammation as a result of tissue trauma caused by a strong probing force (11,12). On the other hand, the suppressive effect of smoking on gingival bleeding may further hamper the diagnostic utility of bleeding on probing (13).

Given the low sensitivity (29%) and the very low positive predictive value (PPV) (6%) of presence of bleeding on probing to detect disease activity and the subsequent loss of clinical attachment, it has been calculated that routine instrumentation of bleeding on probing-positive sites will result in unnecessary treatment of four out of five sites (14). Overtreatment, however, may increase the incidence of unwanted effects of periodontal therapy, such as recession, noncarious cervical lesions, dentine hypersensitivity, and treatment costs (15).

Finally, the high level of disagreement on the need for further treatment even among expert clinicians suggests that the low diagnostic validity of the present clinical parameters may result in empirical treatment planning (16–18). Therefore, it must be admitted that the diagnostic validity of clinical periodontal parameters is modest at best (19); hence, patients who are likely to show disease activity are difficult to identify by clinical parameters. Consequently, a need for additional parameters to monitor periodontal disease (i.e. to objectively evaluate a patient's risk for further disease progression and the response to previous treatment) has been identified (15,19,20).

Biological diagnostic markers (biomarkers) are routinely used for monitoring diseases and treatment effects in many fields of medicine. For example, diagnosis and treatment of inflammatory bowel disease (IBD) were markedly improved by introduction of the biomarker calprotectin to the diagnostic procedures (21). Calprotectin [or myeloid related protein (MRP 8/14)] is a major leukocyte protein that consists of two subunits (MRP 8 and MRP 14) and it constitutes 40–60% of the soluble cytosolic protein in neutrophil granulocytes [polymorphonuclear neutrophils (PMNs)]. It is released during cell activation and turnover (22,23) and is a direct measure of gastrointestinal PMN excretion (24).

IBD is a chronic condition of intestinal neutrophil-dominated inflammation and is, for most patients, characterized by episodes of disease remission and intermittent relapses. Usually, a disease relapse is not clinically detectable in an early state and becomes manifest not until severe intestinal inflammation is fully established. Systemic laboratory parameters such as erythrocyte sedimentation rate and C-reactive protein levels show both low sensitivity and specificity for detection of a disease relapse, while clinical diagnostic methods, such as disease activity indices, do not perform better and do not correlate to mucosal inflammation (25). Endoscopy with histological assessment of mucosal biopsies is the gold standard for evaluating bowel inflammation. However, these techniques are expensive and not well tolerated by the patients because the repeated invasive examinations affect their quality of life (26). Conversely, assessment of faecal calprotectin levels is simple, rapid, inexpensive and noninvasive, and a high concentration of faecal calprotectin is an excellent predictor of relapse in IBD patients on remission (25,27).

Calprotectin is present in gingival crevicular fluid. PMNs are the main source of gingival crevicular fluid calprotectin (28) and its levels correlate positively with clinical and other biochemical parameters of periodontal inflammation (29). Furthermore, a highly significant correlation between calprotectin levels and the short-term clinical outcome of nonsurgical periodontal treatment has been reported (28). However, the potential of calprotectin to predict relapse (or progression) of periodontal disease has not been investigated.

Previously, we reported the results of a randomized controlled clinical trial comparing the efficacy of two antimicrobial medications as adjunct to nonsurgical therapy of generalized aggressive periodontitis over a period of 6 mo (30). In this study, adjunctive systemic amoxicillin/metronidazole was more efficacious than repeated subgingival application of controlled-delivery chlorhexidine chips (CHX chips) with regard to full-mouth probing depth reduction and 'gain' of clinical attachment level at the end of the study. In addition, disease activity – as shown by a significant increase of both probing depth and clinical attachment level between 3 and 6 mo after scaling and

root planing (SRP) – was detected in some patients.

The secondary analysis of this study, described in the present report, had two specific aims. First, to evaluate whether differences in the clinical parameters between the trial groups were reflected in gingival crevicular fluid parameters; and second, to evaluate the validity of calprotectin levels in gingival crevicular fluid as predictors for disease activity after nonsurgical periodontal therapy.

Material and methods

Patients and periodontal treatment

The inclusion/exclusion criteria, patient characteristics and the treatment regimen have been reported previously in detail (30). The study protocol was approved by the institutional Ethics Committee of the Charité –Universitätsmedizin Berlin. Written informed consent was obtained from all subjects.

Briefly, 36 patients displaying severe generalized aggressive periodontitis were treated with SRP after a hygiene phase consisting of repeated oral hygiene instructions and supragingival scaling. Following SRP, patients were randomly assigned to adjunctive antimicrobial treatment: either local application of CHX chips (PerioChip; Perio Products Ltd., Jerusalem, Israel) at every site with a baseline probing depth of ≥ 5 mm, but at most two per tooth [test group; 18 patients, median age 37 (range 21–39) years] or systemic amoxicillin/metronidazole (500 mg/250 mg, three times per day for 10 d) [control group; 18 patients, median age 38 (range 21–39) years]. At 3- and 6-mo time-points after SRP, patients were treated with supportive periodontal therapy (SPT), consisting of clinical measurements, supragingival scaling and polishing. Additionally, every site that still had a probing depth ≥ 5 mm or that had a probing depth of 4 mm plus bleeding on probing was again treated with subgingival instrumentation. In the test group, a CHX chip was again inserted at every site with a probing depth of ≥ 5 mm.

Gingival crevicular fluid sampling and clinical measurements

The initially deepest interproximal site at a single-rooted tooth or a molar flat surface was selected based on measurements obtained at a screening examination before treatment. Gingival crevicular fluid samples were obtained at these sites, before probing, at the SPT visit at the 3-mo time-point. For sampling, sites were isolated with cotton rolls and gently air-dried. A paper strip (PerioCol collection paper; Oraflow, Smithtown, NY, USA) was inserted into the site up to the reference edge or until mild resistance was felt and left in place for 10 s. Strips visibly contaminated with blood were discarded. The gingival crevicular fluid sample volume was determined immediately after removal of the strips, using a micro-moisture meter (Periotron 6000; Oraflow) and calculated in microlitres from a standard curve. Samples were stored at −80°C in 200 μL of sterile phosphate-buffered saline containing 1% bovine serum albumin, until further processing. After gingival crevicular fluid collection, the clinical parameters probing depth and clinical attachment level were recorded to the nearest 0.2 mm at all sites, using an automated periodontal probe, equipped with a handpiece to detect the cemento–enamel junction and with a constant probing force of 0.2 N [Florida Probe with 'PASHA' probe (Pressure-controlled, Automated, Standardised Handpiece); Florida Probe Corporation, Gainesville, FL, USA)]. Bleeding on probing was registered as present or absent.

Analysis of calprotectin levels

The levels of calprotectin in gingival crevicular fluid were determined as reported previously (28). The samples were thawed, shaken for 1 min with a vortex mixer and centrifuged for 5 min at 3000 *g* to recover the gingival crevicular fluid from the collection paper. After removal of the paper strips, the levels of calprotectin in the supernatant were determined using a commercially available ELISA kit, according to the manufacturer's instructions (BMA Biomedicals, Augst, Switzerland). All samples and standards were assayed twice. Data were presented in μg/μL for concentration and in μg/sample for total amount of calprotectin per sample.

Statistical analyses

Statistics were performed with one site per patient as the unit of analysis. Because of the nonnormal distribution of the data (confirmed using the Kolmogorov–Smirnov test; data not shown), nonparametric statistical tests were used (Mann–Whitney *U*-test and Fisher's exact test for cross-sectional data, and Wilcoxon's signed rank test for longitudinal data). Medians and interquartiles (IQ) were calculated for metric parameters (probing depth, clinical attachment level, and concentration and total amount/sample of calprotectin) for each treatment group (test/control). Bleeding on probing as a dichotomous variable is presented as a frequency (%).

Activity at a sample site was dichotomously (yes/no) defined as an increase in probing depth of > 0.5 mm between the first and the second SPT visit (3 and 6 mo, respectively, after first-line SRP). In addition, the total number of active sites and the percentage of active sites/full mouth were calculated.

Receiver operating characteristic (ROC) curves for the ability to predict subsequent disease activity at a sample site between 3 and 6 mo after first-line SRP were constructed for both calprotectin concentration and total amount/sample in gingival crevicular fluid samples as well as for gingival crevicular fluid volume and bleeding on probing scores obtained at the 3-mo visit. The optimal cut-off values for calprotectin levels as dichotomous diagnostic tests (above threshold: positive; below threshold: negative) were determined by maximization of Youden's index (31).

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for the selected calprotectin cut-off values by construction of contingency tables. In addition, positive and

negative likelihood ratios (LRpos. and LRneg., respectively) and the corresponding pre- and post-test probabilities were calculated.

A statistical software program was used for all calculations (SPSS 14.0 for Windows, SPSS Inc., Chicago, IL, USA). Statistical significance was defined as a p-value of < 0.05.

Results

Clinical and laboratory parameters for both treatment groups

Baseline probing depth and clinical attachment level of the selected sites, and their changes throughout the study period, are shown in Table 1 for the test and control groups. The gingival crevicular fluid sample volume and the levels of calprotectin in gingival crevicular fluid are shown in Table 2. Three months after SRP and medication, both test and control groups showed highly significant reductions of clinical attachment level and probing depth ($p < 0.001$). At that time, no significant difference between the groups was found. Between 3 and 6 mo, neither probing depth nor clinical attachment level changed significantly in test patients. However, control patients showed an ongoing significant improvement of both probing depth (−0.5 mm; IQ: −1.1, −0.15; $p = 0.002$) and clinical attachment level (−0.2 mm; IQ: −1.25, −0.25; $p = 0.038$). Accordingly, control patients showed significantly more clinical attachment level 'gain' (0.5 mm; $p < 0.05$) between 3 and 6 mo. A simultaneous significant intergroup difference was observed for probing depth reduction (1.0 mm in favour of the control treatment; $p < 0.01$). Finally, after 6 mo, control patients presented 1.2 mm more probing depth reduction than did test patients ($p < 0.05$; Table 1).

The reduction of gingival crevicular fluid sample volume between baseline and 3 mo was highly significant in both groups ($p \leq 0.001$; Table 2), and an intergroup difference was not found at that time ($p > 0.05$). The gingival crevicular fluid volume continued to decrease significantly in control patients between months 3 and 6 ($p = 0.003$), whereas no further change occurred in test patients ($p > 0.05$). This difference significantly favoured the control group ($p < 0.01$), resulting in a significantly higher reduction of gingival crevicular fluid volume between baseline and the end of the study ($p < 0.05$).

The concentration of calprotectin in gingival crevicular fluid was significantly reduced between baseline and 3 mo in control patients ($p = 0.004$), whereas the corresponding reduction in the gingival crevicular fluid of test patients failed to reach statistical significance ($p = 0.063$). No further changes between 3 and 6 mo were detected in either group; however, both treatment arms presented significant reductions of calprotectin concentration after 6 mo ($p \leq 0.007$), without a detectable intergroup difference ($p > 0.05$).

The total amount/sample of calprotectin was significantly reduced between baseline and 3 mo in both test and control patients ($p = 0.02$ and 0.001, respectively) and there was no further change between 3 and 6 mo for test patients ($p > 0.05$). In control patients, however, the total amount/sample of calprotectin continued to decrease between 3 and 6 mo ($p = 0.038$). The reduction between baseline and the end of the study was highly significant in both groups ($p < 0.001$), and a significant intergroup difference was not detected ($p > 0.05$).

Characteristics of sites with or without activity

Nine active sites (with an increase in probing depth of > 0.5 mm between 3 and 6 mo) and 27 nonactive sites were identified (Table 3). Eight active sites belonged to test patients who had received adjunctive treatment with the CHX chip, whereas only one active site was found in the control group (significant difference in favour of the antibiotic treatment, $p < 0.01$). Active sites showed a median increase in probing depth of 1.0 mm between 3

Table 1. Clinical parameters and their changes according to test and control treatment groups (initially deepest site/patient, medians and 25 and 75 percentiles)

						p-value[a]		
Variable	Group	Baseline 0 mo	Difference 0–3 mo	Difference 3–6 mo	Difference 0–6 mo	Change 0–3 mo	Change 3–6 mo	Change 0–6 mo
PD	Test	7.5 (7.0, 8.45)	−3.8 (−4.45, −2.7)	0.5 (−0.35, 1.0)	−3.4 (−3.8, −2.45)	< 0.001	0.197	< 0.001
	Control	8.4 (6.75, 9.0)	−4.4 (−4.9, −2.35)	−0.5 (−1.1, −0.15)	−4.6 (−5.45, −3.35)	< 0.001	0.002	< 0.001
Difference between groups		–	–	1.0**	1.2*			
CAL	Test	9.0 (7.6, 10.0)	−2.2 (−2.65, −1.4)	0.3 (−0.25, 0.6)	−2.2 (−2.8, −1.15)	< 0.001	0.148	0.001
	Control	8.8 (7.5, 9.85)	−2.2 (−3.15, −0.8)	−0.2 (−1.25, −0.25)	−2.9 (−4.35, −1.55)	< 0.001	0.038	< 0.001
Difference between groups			–	0.5*	–			

[a]Wilcoxon's signed rank test.

CAL, clinical attachment level; PD, probing depth.

*Significant difference between groups favouring control treatment (Mann–Whitney U-test, $p < 0.05$).

**Significant difference between groups favouring control treatment (Mann–Whitney U-test, $p < 0.01$).

Table 2. Gingival crevicular fluid parameters and their changes according to test and control treatment groups (initially deepest site/patient, medians and 25 and 75 percentiles)

Variable	Group	Baseline 0 mo	Difference 0–3 mo	Difference 3–6 mo	Difference 0–6 mo	*p*-value[a] Change 0–3 mo	Change 3–6 mo	Change 0–6 mo
Gingival crevicular fluid volume (µl/10 s)	Test	0.41 (0.29, 0.52)	−0.15 (−0.26, −0.05)	0.03 (−0.06, 0.14)	−0.14 (−0.28, −0.01)	0.001	0.395	0.017
	Control	0.51 (0.36, 0.60)	−0.22 (−0.36, −0.05)	−0.10 (−0.18, −0.03)	−0.25 (−0.43, −0.18)	< 0.001	0.003	< 0.001
Difference between groups		–	–	0.13**	0.11*			
Calprotectin concentration (µg/µl)	Test	44.2 (22.5, 63.5)	−20.8 (−49.6, 6.9)	−7.0 (−40.8, 8.8)	−18.0 (−52.1, −12.1)	0.063	0.102	0.003
	Control	32.2 (18.3, 97.9)	−16.7 (−96.0, −3.0)	0.1 (−9.8, 8.5)	−15.0 (−84.3, 0.1)	0.004	0.918	0.007
Difference between groups		–	–	–	–			
Calprotectin total amount (µg/sample)	Test	20.8 (10.7, 29.5)	−12.0 (−23.5, 1.4)	−1.4 (−17.5, 2.2)	−12.9 (−23.8, −8.1)	0.02	0.287	< 0.001
	Control	17.1 (8.2, 47.0)	−11.7 (−54.2, −3.7)	−1.2 (−2.9, 0.7)	−13.3 (45.5, −4.7)	0.001	0.038	< 0.001
Difference between groups		–	–	–	–			

[a]Wilcoxon's signed rank test.
*Significant difference between groups favouring control treatment (Mann–Whitney *U*-test, $p < 0.05$).
**Significant difference between groups favouring control treatment (Mann–Whitney *U*-test, $p < 0.01$).

and 6 mo (IQ: 0.8, 1.0; $p = 0.007$), whereas nonactive sites improved owing to a median probing depth decrease of 0.5 mm (IQ: −0.4, 0.0; $p = 0.001$). These intragroup changes resulted in a highly significant difference between active and nonactive sites (1.5 mm; $p < 0.001$; Table 3). The clinical attachment level did not change significantly for both categories between 3 and 6 mo. Regarding the clinical measurements obtained at 3 mo (probing depth, clinical attachment level, bleeding on probing and gingival crevicular fluid volume), no significant differences between future active and nonactive sites were found. Both calprotectin concentration and total amount/sample, however, were significantly elevated in future active sites, when compared with nonactive sites (concentration: 51.8 µg/µL vs. 13.9 µg/µL; total amount/sample: 22.3 µg vs. 3.8 µg; $p < 0.05$; Table 3).

Prediction of activity

ROC curves for bleeding on probing, gingival crevicular fluid volume, calprotectin concentration, and calprotectin total amount/sample as predictors of subsequent activity are shown in Figs 1–4. The presence or absence of bleeding on probing at 3 mo did not predict a subsequent increase of probing depth [area under the curve (AUC) = 0.537; $p = 0.74$; 95% confidence interval (CI): 0.315–0.759; Fig. 1], just as little as GCF volume (AUC = 0.558; p = 0.609; 95% CI: 0.349–0.766; Fig. 2). However, ROC curves constructed for calprotectin levels in gingival crevicular fluid showed that a subsequent increase of probing depth was significantly predicted by both calprotectin concentration (AUC = 0.793; $p = 0.013$; 95% CI: 0.571–1.015; Fig. 3) and total amount/sample (AUC = 0.776; $p = 0.02$; 95% CI: 0.551–1.002; Fig. 4).

Maximizing of Youden's index (J) showed that a concentration of 16.8 µg/µL was the best cut-off level for calprotectin concentration (J = 0.52). Construction of a contingency table for this cut-off level of choice revealed a sensitivity of 89%, a specificity of 63%, a PPV of 44% and an NPV of 94%

Table 3. Characteristics of active and nonactive sites

Progression	*n*	PD at 3 mo	PD change 3–6 mo	CAL at 3 mo	CAL change 3–6 mo	BoP at 3 mo, %	GCF volume at 3 mo, µL	Calprotectin		Treatment group	
								Concentration 3 mo, µg/µL	Total amount 3 mo, µg	Test	Control
Yes	9	3.8 (2.9, 4.6)	1.0 (0.8, 1.0)	7.0 (5.9, 8.8)	0.2 (−0.5, 0.6)	44	0.34 (0.20, 0.38)	51.8 (21.9, 92.3)	22.3 (4.6, 30.7)	8/18	1/18
No	27	4.0 (3.6, 4.6)	−0.5 (−0.4, 0.0)	6.2 (5.6, 7.2)	0.0 (−1.2, 0.4)	37	0.24 (0.19, 0.36)	13.9 (8.6, 25.1)	3.8 (1.6, 6.9)	1/18	17/18
Difference	–	–	1.5***	–	–	–	–	37.9*	18.5*	8 vs. 1**	

BoP, bleeding on probing; CAL, clinical attachment level; GCF, gingival crevicular fluid; PD, probing depth.
*Significant difference between sites with/without activity (Mann–Whitney *U*-test, $p < 0.05$).
**Significant difference between treatment groups favouring control treatment (Fisher's exact test, $p < 0.01$).
***Significant difference between sites with/without activity (Mann–Whitney *U*-test, $p < 0.001$).

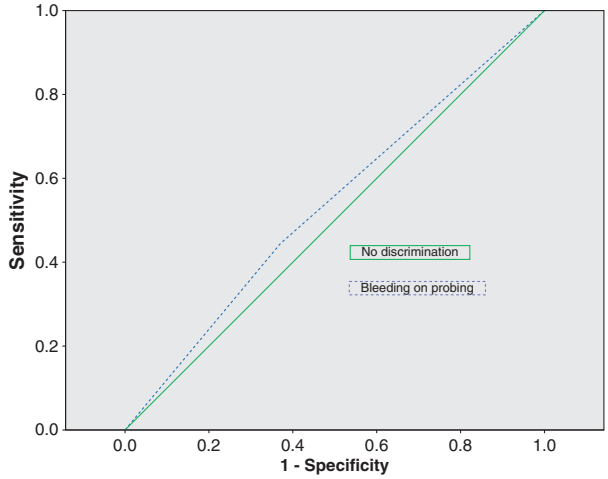

Fig. 1. Receiver operating characteristic curve for the ability of bleeding on probing, registered at 3 mo, to predict a subsequent probing depth increase of more than 0.5 mm between 3 and 6 mo. Area under the curve: 0.537; $p = 0.74$; 95% confidence interval: 0.315–0.759.

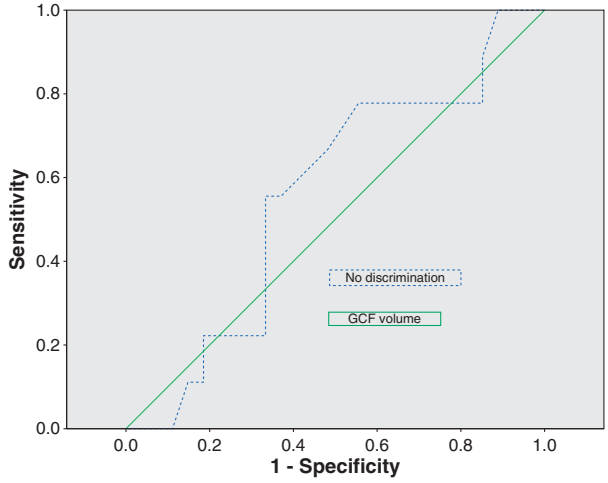

Fig. 2. Receiver operating characteristic curve for the ability of gingival crevicular fluid volume (GCF volume) at 3 mo to predict a subsequent probing depth increase of more than 0.5 mm between 3 and 6 mo. Area under the curve: 0.558; $p = 0.609$; 95% confidence interval: 0.349–0.766.

(Table 4). Calculation of LRpos. and LRneg. showed an LRpos. of 2.4 and an LRneg. of 0.18.

A total amount of 4.2 µg was the best cut-off level for total amount of calprotectin per sample (J = 0.41). A sensitivity of 78%, a specificity of 63%, a PPV of 41% and a NPV of 94% were found (Table 5). The LRpos. was 2.1 and the LRneg. was 0.35.

In patients with positive calprotectin results (above cut-off levels), a median number of 34 (IQ: 29, 38) or a median percentage of 23% (IQ: 20, 32) active sites was found. Patients with negative calprotectin results (below cut-off levels) showed significantly fewer active sites [median = 20 (IQ: 12, 28) or median = 14% (IQ: 10, 17); $p \leq 0.004$].

As the prevalence of disease activity at sample sites was 25% (nine active, 27 nonactive), the pretest probability for disease activity was 25%. Calculation of post-test probabilities resulted in positive test result values of 60% and 52.5% for calprotectin concentration and total amount/sample of calprotectin, respectively. For negative results, post-test probabilities of 4.5% and 8.8% were found.

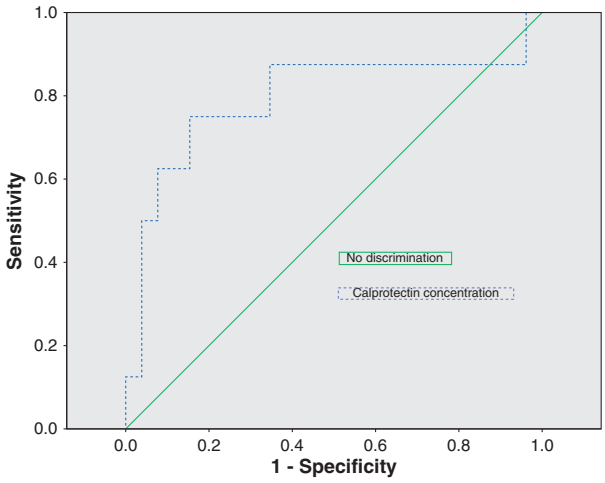

Fig. 3. Receiver operating characteristic curve for the ability of calprotectin concentration in gingival crevicular fluid at 3 mo to predict a subsequent probing depth increase of more than 0.5 mm between 3 and 6 mo. Area under the curve: 0.793; $p = 0.013$; 95% confidence interval: 0.571–1.015.

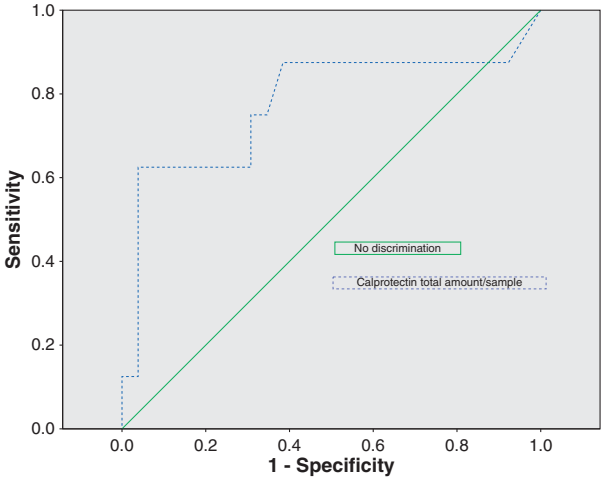

Fig. 4. Receiver operating characteristic curve for the ability of calprotectin total amount/sample at 3 mo in gingival crevicular fluid to predict a subsequent probing depth increase of more than 0.5 mm between 3 and 6 mo. Area under the curve: 0.776; $p = 0.02$; 95% confidence interval: 0.551–1.002).

Discussion

The clinical outcome of the sites selected for this secondary analysis was in good agreement with the results found on patient level for both CHX chip and amoxicillin/metronidazole groups (30). Three months after SRP, both groups showed a comparable reduction of probing depth and 'gain' of clinical attachment level. Likewise, the reductions of gingival crevicular fluid volume and calprotectin levels were similar for both treatments at that time-point. Control patients showed ongoing significant improvements of probing depth, clinical attachment level and gingival crevicular fluid volume between 3 and 6 mo. After 6 mo, significant differences for reduction of probing depth and gingival crevicular fluid volume were found in favour of the control group.

Prediction of disease activity between 3 and 6 mo after SRP was carried out by determining the calprotectin levels in gingival crevicular fluid after 3 mo. Ideally, a test would detect periodontal inflammation before irreversible tissue destruction (i.e. before loss of clinical attachment). Following the natural course of periodontal inflammation in so far that increasing probing depth precedes the result of periodontal disease progression in terms of loss of clinical attachment, we chose probing depth increase as diagnostic criterion for disease activity. Differences between repeated probing depth measurements with the type of constant-force electronic probe used in our study have been shown to be consistently < 0.5 mm (32). Accordingly, a probing depth increase of at least 0.5 mm between 3 and 6 mo after SRP was selected as a predefined threshold for disease activity.

Following this threshold, nine active sites and 27 stable sites were identified, and the test group displayed significantly more active sites than the control group. Interestingly, calprotectin levels in gingival crevicular fluid did not reflect these intergroup differences 3 mo after SRP, when both treatment groups were compared. However, calprotectin levels predicted periodontal disease activity. The concentration and total amount/sample of calprotectin could significantly predict increases of probing depth with a moderate PPV and an excellent NPV (Tables 4 and 5).

Likelihood ratios summarize how many times more or less likely patients with disease are to have a particular test result than patients without disease (33). Thus, a patient with an active site was 2.4 times more likely to have a positive test result for calprotectin concentration (LRpos. = 2.4) and 2.1 times more likely to have a positive test result for calprotectin total amount/sample (LRpos. = 2.1). Conversely, a patient with a nonactive site was only 0.18 times more likely to have a positive test result for calprotectin concentration (LRneg. = 0.18) and only 0.35 times more likely to have a positive test result for the calprotectin total amount/sample (LRneg. = 0.35).

The prevalence of disease activity at sample sites in the entire study population was 25% (nine active sites and 27 nonactive sites); consequently,

Table 4. Contingency table for calprotectin concentration (cut-off 16.8 μg/μL)

Calprotectin concentration above threshold	Probing depth increase		
	Yes	No	
Yes	8	10	44.44% positive predictive value
No	1	17	94.44% negative predictive value
	88.89% Sensitivity	62.96% Specificity	

PD, probing depth.

Table 5. Contingency table for calprotectin total amount/sample (cut-off: 4.2 μg/10 s)

Calprotectin total amount above threshold	Probing depth increase		
	Yes	No	
Yes	7	10	41.17% positive predictive value
No	2	17	89.47% negative predictive value
	77.78% sensitivity	62.96% specificity	

any study patient without calprotectin test would have a risk of 25% for disease activity (pretest probability). After calprotectin testing, the calculation of post-test probabilities (LRpos. × prevalence, LRneg. × prevalence) showed that the risk of subsequent disease activity of a patient with a positive calprotectin test result increased to 60% (calprotectin concentration) and 52.5% (calprotectin total amount/sample). On the other hand, a negative test result indicated a decreased risk for disease activity (4.5%, calprotectin concentration; 8.8% calprotectin total amount/sample).

In contrast to the diagnostic performance of calprotectin levels, clinical parameters (probing depth, clinical attachment level, bleeding on probing, and gingival crevicular fluid volume) did not prove to be useful predictors of disease activity.

The cut-off levels of calprotectin concentration (16.8 μg/μL) and calprotectin total amount/sample (4.2 μg) used to predict disease activity were determined by constructing ROC curves and maximizing Youden's index. It should be emphasized that these values are not cut-off levels for 'normality'; rather, they represent the values that give the highest sum of sensitivity and specificity for predicting disease activity (i.e. increasing probing depth). These levels are distinctly higher than the values measured in patients with chronic and generalized aggressive periodontitis after treatment (28,34). Also, they clearly exceed the values found for experimental gingivitis (35) and for sites considered healthy (29,36).

The association between periodontal disease activity and elevated levels of calprotectin is biologically plausible. Periodontal inflammation is strongly related to increased numbers of neutrophil granulocytes (PMNs). PMNs are an important part of the innate immune system, and their ability to clear potentially pathogenic bacteria is crucial for periodontal health (37). However, although their primary role is protective, activated PMNs release mediators that essentially contribute to the inflammatory process that finally results in the breakdown of periodontal tissue (38,39). Given that PMNs are the main source of calprotectin in gingival crevicular fluid (28,40), calprotectin appears as a direct rather than as an indirect measure of inflammatory activity associated with tissue breakdown, when compared with clinical parameters. The finding that there was no difference between the treatment groups in regard to median calprotectin levels at 3 mo, but an association of calprotectin with future disease activity, further illustrates the association of this biomarker with acute inflammation and subsequent tissue breakdown. On the contrary, clinical parameters rather reflect the course of disease (or treatment) in the past, as shown by their poor prognostic validity found in our data and in other studies (5,8,41,42). Thus, a high gingival crevicular fluid calprotectin level in a patient after nonsurgical periodontal therapy may correspond to a persistent increased number of PMNs, thereby representing a stage of inflammation that will soon cause progression of periodontal disease.

A diagnostic test for monitoring and targeted treatment of periodontitis should be useful on the subject level, and samples of a few periodontal sites should reflect a patient's state of disease, rather than that of a single site (43). In the first instance, a positive calprotectin test result identified disease activity at the individual sample site. However, patients with a positive calprotectin test result showed significantly more active sites than patients with a negative calprotectin test result. These results correspond to the finding that calprotectin levels decrease in both diseased and clinically 'healthy' sites in chronic periodontitis patients after successful periodontal therapy (44) and indicate that calprotectin may indeed be a biomarker on subject level.

A variety of biomarkers are predictive for periodontitis progression, e.g. elevated gingival crevicular fluid levels of PMN components such as elastase and collagenase indicate future disease activity (45–49). In these studies, irrevocable consequences of periodontal inflammation were used as definitions for disease progression, i.e. radiographic loss of bone or loss of clinical attachment over longer periods of time. Short-term increase of probing depth, as used in our study, may be advantageous over these parameters because disease activity is detected earlier and before the occurrence of irreversible tissue destruction. Correspondingly, calprotectin testing detected subtle increases of probing depth and may therefore be more useful for diagnostics than other gingival crevicular fluid components.

Identification of the risk of imminent periodontal tissue breakdown

suggests the possibility of targeted treatment at a presymptomatic stage. Treatment at this particular stage of disease might avert destruction of periodontal tissue before it occurs. Conversely, it may be that SPT could be carried out less intensively or less frequently at a point where low gingival crevicular fluid calprotectin levels in a patient suggest that disease progression is unlikely to occur within a given period. Targeted treatment might cause fewer side effects with regard to soft and hard tissue damage, and might be of shorter duration and less expensive.

Although the performance of calprotectin as a diagnostic test was encouraging in our study, it must be considered that these results are valid at the given prevalence of disease activity in the study population (25%). Prevalence of activity may be associated with the specific entity of periodontal disease investigated here (generalized aggressive periodontitis); hence, the generalizability of the results should be clarified in other periodontal disease conditions.

The present study is a secondary analysis of clinical trial data, and the sample size was calculated for the primary endpoint of the main trial and not for the evaluation of a diagnostic test. Accordingly, the sample size was small and therefore the results should be validated in a larger study. Moreover, it has been shown that the *post-hoc* choice of cut-off levels introduces bias into diagnostic test validity studies (50) and these cut-off levels should be subjected to prospective investigation. Finally, an interventional study will have to be carried out to evaluate the validity of a dichotomous calprotectin test for risk assessment and treatment planning.

Conclusion

Our data show that calprotectin levels in gingival crevicular fluid were able to predict site-specific periodontal disease activity in the study population. Detection of site-specific activity was related to disease activity on subject basis. The calculated cut-off levels provide a dichotomous basis for prospective evaluation of calprotectin as a diagnostic marker for monitoring periodontal treatment.

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