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Gingival crevicular fluid levels of calprotectin and myeloperoxidase during therapy for generalized aggressive periodontitis

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Background: Levels of the inflammation marker calprotectin in gingival crevicular fluid correspond to clinical and biochemical parameters of periodontal inflammation. Neutrophil granulocytes (polymorphonuclear neutrophils: PMNs) are supposed to be the main source of calprotectin in gingival crevicular fluid, but evidence is still lacking. The influence of periodontal therapy on gingival crevicular fluid levels of calprotectin has not yet been determined.

Objectives: Gingival crevicular fluid levels of calprotectin were monitored during therapy for generalized aggressive periodontitis. Interrelations between calprotectin and the PMN marker myeloperoxidase (MPO) were evaluated.

Material and methods: Gingival crevicular fluid samples were collected from 23 patients with generalized aggressive periodontitis before and 3 months after non-surgical therapy with an adjunctive antimicrobial medication. Clinical parameters were recorded with a pressure-calibrated electronic probe. Levels of calprotectin and MPO in gingival crevicular fluid were analysed by enzyme-linked immunosorbent assay (ELISA) procedures.

Results: At baseline, levels of calprotectin and MPO were highly correlated. Bleeding and suppurating sites showed significantly higher levels of calprotectin and MPO than non-bleeding, non-suppurating sites. Therapy significantly decreased levels of both biomarkers. These changes of calprotectin and MPO were highly correlated and also related to probing-depth reduction. Three months after therapy, the levels of both markers still showed significant correlations in initially deep sites, whereas in initially shallow sites no significant correlation was found. After therapy, levels of markers in bleeding and non-bleeding sites were comparable.

Conclusion: The correlations between calprotectin and MPO indicate that PMNs are a major contributor to the calprotectin content in gingival crevicular fluid of severely affected sites. Calprotectin levels in gingival crevicular fluid and their changes reflect periodontal inflammation as well as the clinical treatment outcome. A prognostic potential of this marker substance remains to be determined.

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Calprotectin is a dimeric protein of leukocytes, consisting of one light (11 kDa) and one heavy (14 kDa) chain, and is particular abundant in the cytosol of polymorphonuclear neutrophils (PMNs), where it contributes up to 45% to the total protein content (1, 2). It is released during death or activation of PMNs and monocytes (3, 4). The calprotectin levels in plasma, serum, synovial fluid or faeces are markedly elevated in certain inflammatory conditions, such as rheumatoid arthritis, systemic lupus erythematosus, kidney allograft rejection, Crohn's disease and ulcerative colitis, as well as in some bacterial infections, indicating a role as a marker substance for these diseases (5–9). Despite many suggested biological functions, such as regulation of differentiation of myeloid lineage cells, regulation of intracellular calcium levels in neutrophils or regulation of protein kinases, calprotectin also displays antimicrobial properties against mucosal and other bacteria *in vitro* due to a chelation reaction with zinc, which is essential for growth of microbes (2, 10–12). Calprotectin is present in gingival crevicular fluid and its concentration has shown correlations with clinical and other biochemical markers of periodontal inflammation in cross-sectional studies (13, 14). As the levels of lactoferrin and calprotectin are positively correlated in the gingival crevicular fluid of children (15) and the release of calprotectin by PMNs can be stimulated *in vitro* by lipopolysaccharides of periodontal pathogens (16), PMNs are supposed to be the main source of calprotectin in gingival crevicular fluid. However, longitudinal data on the impact of therapy on calprotectin in gingival crevicular fluid have yet not been reported. Also, interrelations between calprotectin and known marker substances for PMNs have not been studied in the gingival crevicular fluid of patients suffering from periodontal disease.

Myeloperoxidase (MPO) is a major constituent of the azurophilic granules of PMNs and oxidizes chloride ions to the strong oxidant HOCl, the most bactericidal oxidant produced by neutrophils (17). As the content of azurophilic granules is mainly directed into phagolysosomes, extracellular release may occur only following neutrophil lysis (18). Being an indicator of PMN infiltration, gingival crevicular fluid MPO is associated with the severity of periodontal disease (19, 20). In generalized aggressive periodontitis, amplified activity of PMNs is linked with elevated levels of MPO in gingival crevicular fluid when compared to chronic periodontitis (21).

In the present study, we assessed the levels of calprotectin in gingival crevicular fluid prior to and after the therapy of patients suffering from generalized aggressive periodontitis. Levels of MPO were determined to confirm the contribution of PMNs to calprotectin content in gingival crevicular fluid.

Material and methods

Patients

Twenty-three patients (mean age 34 ± 6 years, nine men, 14 women, nine smokers) with previously untreated generalized aggressive periodontitis were recruited from the Institute for Periodontology and Synoptic Dentistry, Charité – Universitätsmedizin Berlin. Patients included in the study were less than 40 years of age, presented at least 20 teeth and showed a clinical attachment loss of > 5 mm at a minimum of 12 teeth at the screening examination. At least three teeth except first molars and incisors had to be involved. Exclusion criteria were pregnancy, lactation period, allergy to medications used in the study, intake of antibiotics or anti-inflammatory drugs in the previous 6 months, systemic diseases with influence on periodontitis, history of systematic periodontal therapy and any condition requiring premedication before dental treatment.

Written informed consent was obtained from each patient. The study protocol was approved by the Institutional Ethics Committee of the Charité – Universitätsmedizin Berlin.

Gingical crevicular fluid sampling and clinical measurements

After a hygiene phase including oral hygiene instructions, supragingival scaling and polishing, the baseline examination and the collection of gingival crevicular fluid were performed. The initially deepest site (according to the screening examination) and, as a 'healthy' control, one approximal shallow site with probing depth < 4 mm, when available, were selected for sampling. Sites were isolated with cotton rolls and gently air-dried. Then, a paper strip (PerioCol Collection Strip, Oraflow, Plainview, NY, USA) was inserted into the sulcus and removed after 10 s. Strips visibly contaminated with blood were discarded. The gingival crevicular fluid volume was determined using a calibrated moisture meter (Periotron 6000, Oraflow, Plainview, NY, USA) and calculated in µl from a standard curve. Samples were stored separately in 200 µl sterile phosphate-buffered saline with 1% bovine serum albumin at −80°C until further processing.

Subsequently, the periodontal parameters probing depth and clinical attachment level were measured to the nearest 0.2 mm by means of a pressure-calibrated electronic probe (Florida Probe, Gainesville, FL, USA) equipped with a hand piece to detect the cemento–enamel junction (22). Bleeding on probing and suppuration were recorded as present or absent. For evaluation of oral hygiene, the approximal space plaque index of the patient was recorded (23).

Sampling and measurements were repeated 3 months after completion of mechanical therapy. To avoid inaccurate probe readings because of newly formed calculus and false-positive results regarding bleeding on probing due to sulcular bleeding, patients received supragingival scaling, polishing and oral hygiene instructions 1 week prior to the recall measurements.

Therapeutic intervention

All patients participated in a hygiene phase, including supragingival scaling, polishing of all teeth and repeated oral

hygiene instructions, until a sufficient plaque control (approximal space plaque index < 30%) had been achieved. After the baseline examination, scaling/root planing was performed in all sites exhibiting a probing depth ≥ 4 mm under local anaesthesia in four visits within 2 weeks. During this period, patients rinsed twice a day for 1 min with 15 ml of a 0.2% chlorhexidine mouth rinse (Chlorhexamed Fluid, GSK, Munich, Germany). After the last scaling/root planing visit, patients received polishing of all teeth and oral hygiene instructions again. Systemic antibiotics were prescribed (amoxicillin 500 mg and metronidazole 250 mg combined, three times daily for 10 days).

Biochemical analyses

For recovery of gingival crevicular fluid, the samples were vortexed for 1 min and centrifuged for 5 min at 3000 *g* after thawing. The concentrations of calprotectin and MPO were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Calprotectin: BMA Biomedicals, Augst, Switzerland; MPO: Immundiagnostik, Bensheim, Germany). All samples and standards were assayed twice. Data were presented in μg/μl for concentrations and in μg/sample for total amounts in the selected sites.

Statistical analyses

Statistics were performed with the site as the unit of analysis. Changes of the clinical parameters and the gingival crevicular fluid parameters were analyzed by non-parametric tests. Spearman's correlation coefficient was calculated to evaluate interrelations between both biomarkers. Connections between the gingival crevicular fluid parameters and clinical signs of inflammation (bleeding on probing and suppuration, respectively) as well as differences between deep sites and their shallow controls were analyzed by the Mann–Whitney *U*-test. Statistical significance was defined as $p < 0.05$. A software program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for all calculations.

Results

Clinical parameters

Periodontal treatment resulted in a significant improvement of clinical parameters at deep sites, as displayed by pronounced gain of clinical attachment, reduction of probing depth, reduction of bleeding on probing, elimination of suppuration and a reduced gingival crevicular fluid volume 3 months after therapy. However, slight significant reductions of probing depth and gingival crevicular fluid volume were observed in initially shallow sites, but other clinical parameters remained unchanged. Constantly low approximal space plaque index values throughout the study indicated maintenance of good oral hygiene (Tables 1 and 2).

Table 2. Clinical parameters: frequencies

Parameter	Category	Baseline	3 months
API	Patient	25	26
BoP	Deep	91	26***
(%)	Shallow	8	33
Pus	Deep	30	0
(%)	Shallow	–	–

API, approximal space plaque index; BoP, bleeding on probing.
Deep: $n = 23$; shallow: $n = 12$.
***Significantly different from baseline, $p < 0.001$ (chi-squared test).

Gingival crevicular fluid markers

Calprotectin — At baseline, the deep sites showed significant higher concentrations (2.9-fold) and total amounts/site (4.9-fold) of calprotectin than their shallow control sites ($p < 0.001$, Table 3). Three months after therapy, a significant decrease of calprotectin concentration and total amount/site in initially deep sites was detected ($p < 0.001$). In initially shallow sites, no significant changes to baseline were observed. Differences between initially deep and their shallow control sites were not significant 3 months after therapy.

Myeloperoxidase — At baseline, the total amount/site of MPO in deep sites was significantly elevated (1.7-fold) over that in shallow sites ($p < 0.001$, Table 4), whereas the difference between concentrations failed to reach statistical significance ($p = 0.068$). Therapy resulted in a significant decrease of both concentration and total amount/site of MPO in initially deep sites ($p < 0.01$). Three months after therapy, initially shallow sites showed a significant higher concentration of MPO in gingival crevicular fluid than initially deep sites (2.4-fold), whereas the total amount/site was not significantly different between both site categories.

Correlations of calprotectin and myeloperoxidase at baseline

At baseline, the concentrations ($r^2 = 0.618$, $p = 0.002$), as well as the total amounts/site ($r^2 = 0.757$, $p < 0.001$), of calprotectin and MPO in the gingival crevicular fluid of deep sites were highly correlated. For the gingival crevicular fluid of shallow sites, no significant correlations were found (data not shown).

Table 1. Clinical parameters and their changes: medians and ranges

Parameter	Category	Baseline	3 months	Change
PD	Deep	8.2 (6.0 to 9.2)	4.2*** (1.8 to 6.8)	−4.0 (−6.2 to −1.8)
(mm)	Shallow	2.1 (1.6 to 3.0)	1.7* (1.0 to 2.8)	−0.2 (−2.0 to 0.6)
CAL	Deep	9.2 (7.0 to 12.0)	6.0*** (2.8 to 10)	−2.4 (−5.4 to 0.0)
(mm)	Shallow	2.5 (1.8 to 4.0)	2.5 (1.4 to 4.4)	–
GCF vol.	Deep	0.47 (0.18 to 1.07)	0.25*** (0.06 to 0.6)	−0.19 (−0.62 to 0.02)
(μl)	Shallow	0.23 (0.07 to 0.86)	0.14* (0.06–0.39)	−0.1 (−0.67 to 0.12)

CAL, clinical attachment level; GCF, gingival crevicular fluid; PD, probing depth.
Deep: $n = 23$; shallow: $n = 12$.
*Significantly different from baseline, $p < 0.05$ (Wilcoxon signed rank test).
***Significantly different from baseline, $p < 0.001$ (Wilcoxon signed rank test).

Table 3. Calprotectin concentration and total amount/sample: medians and ranges

Calprotectin	Category	Baseline	3 months	Change
Concentration	Deep	27.3[a] (3.6 to 144.6)	12.6*** (2.9 to 63.2)	−12.7 (−136.6 to 18.0)
(μg/μl)	Shallow	9.5 (2.6 to 19.9)	11.2 (6.2 to 34.9)	–
Total amount	Deep	10.4[a] (1.7 to 75.2)	3.3*** (0.7 to 26.6)	−6.3 (−71.4 to 4.67)
(μg)	Shallow	2.1 (0.7 to 4.9)	2.3 (0.4 to 3.8)	–

***Significantly different from baseline, $p < 0.001$, Wilcoxon signed rank test.
[a]Significantly elevated over shallow, $p < 0.001$, Mann–Whitney *U*-test.

Table 4. Myeloperoxidase concentration and total amount/sample: medians and ranges

MPO	Category	Baseline	3 months	Change
Concentration	Deep	0.66 (0.16 to 6.73)	0.28** (0.01 to 1.10)	−0.35 (−5.96 to 0.43)
(μg/μl)	Shallow	0.38 (0.04 to 1.80)	0.67[b] (0.27 to 1.00)	–
Total amount	Deep	0.30[a] (0.05 to 3.10)	0.08*** (0.01 to 0.39)	−0.21 (−2.96 to 0.17)
(μg)	Shallow	0.12 (0.01 to 0.22)	0.07 (0.05 to 0.21)	–

MPO, myeloperoxidase.
**Significantly different from baseline, $p < 0.01$, Wilcoxon signed rank test.
***Significantly different from baseline, $p < 0.001$, Wilcoxon signed rank test.
[a]Significantly elevated over shallow, $p < 0.001$, Mann–Whitney *U*-test.
[b]Significantly elevated over deep, $p < 0.05$, Mann–Whitney *U*-test.

Correlations of calprotectin and myeloperoxidase after therapy

Three months after therapy, the concentrations of calprotectin and MPO in the gingival crevicular fluid of initially deep sites were significantly correlated ($r^2 = 0.646$, $p = 0.001$). Also, the total amounts/site of both substances were significantly correlated ($r^2 = 0.694$, $p < 0.001$). No significant association between both parameters was found at initially shallow sites (data not shown).

Correlations of longitudinal changes

The decrease of calprotectin and MPO in initially deep sites was significantly correlated both for concentrations and for total amounts/site (Fig. 1).

Interrelations of biomarkers and bleeding on probing or suppuration

At baseline, bleeding-on-probing positive sites secreted significantly higher amounts of calprotectin and MPO than bleeding-on-probing negative sites (Figs 2a and b). Suppuration was significantly associated with higher amounts of calprotectin and MPO, respectively (Figs 2c and d). The calprotectin concentration at suppurating sites was markedly elevated (44.2 μg/μl) compared with non-suppurating sites (16.4 μg/μl, $p < 0.001$). Three months after therapy, the association between both biomarkers and these clinical parameters was not longer present (data not shown).

Biomarkers and clinical outcome

Gingival crevicular fluid volume, concentrations and total amounts/site of calprotectin and MPO did not show significant correlations to probing depth either at baseline or after 3 months (data not shown). The clinical treatment outcome as measured by probing-depth reduction was significantly correlated with the reduction of gingival crevicular fluid volume and the reduction of calprotectin and MPO levels (r^2 from 0.422 to 0.635, Table 5). In particular the correlation between probing-depth reduction and the reduction of the total amount/site of calprotectin was highly significant.

Discussion

The present longitudinal study evaluated the gingival crevicular fluid levels of calprotectin and MPO in the course of treatment of generalized aggressive periodontitis patients.

At the baseline examination, diseased sites displayed higher concentrations and total amounts/site of calprotectin and MPO than sites considered healthy. Bleeding-on-probing positive sites showed significantly higher total amounts/site of both marker substances than bleeding-on-probing negative sites, indicating a connection between both biomarkers and clinical parameters of inflammation and confirming results for calprotectin achieved in cross-sectional studies (13, 14). However, the calprotectin levels (concentration: 2.6–144.6 μg/μl; total amounts/site: 0.4–75.2 μg) in our study with generalized aggressive periodontitis patients were up to 12-fold elevated when compared to the reports mentioned above investigating samples of chronic periodontitis patients or healthy controls (concentration: 0.15–12.4 μg/μl; total amount/site: 0.24–10 μg). Besides methodological issues (differing sampling and elution protocols, different ELISA kits), the fact that aggressive periodontitis can be characterized by amplified PMN activity measured in gingival crevicular fluid may contribute to these differences (21).

Suppurating sites showed significantly higher total amounts of calprotectin and MPO than non-suppurating sites. Furthermore, a pronounced elevation of median calprotectin concentration (44.2 μg/μl) was noted. These findings correspond to markedly elevated levels of calprotectin in exudates and abscess fluids, where components of PMNs are released by dying cells and essentially contribute to the antimicrobial activity of these fluids (24, 25). Elevated levels of gingival crevicular fluid MPO regarding total activity and concentration of the enzyme have been reported for periodontitis and gingivitis sites, when measured with enzymological assays (20, 26, 27). Immunological methods showed increasing total amounts/site of MPO with increasing severity of gingival and periodontal inflammation (28), presenting a range of mean total amount/site from 0.09 ± 0.06 μg for healthy

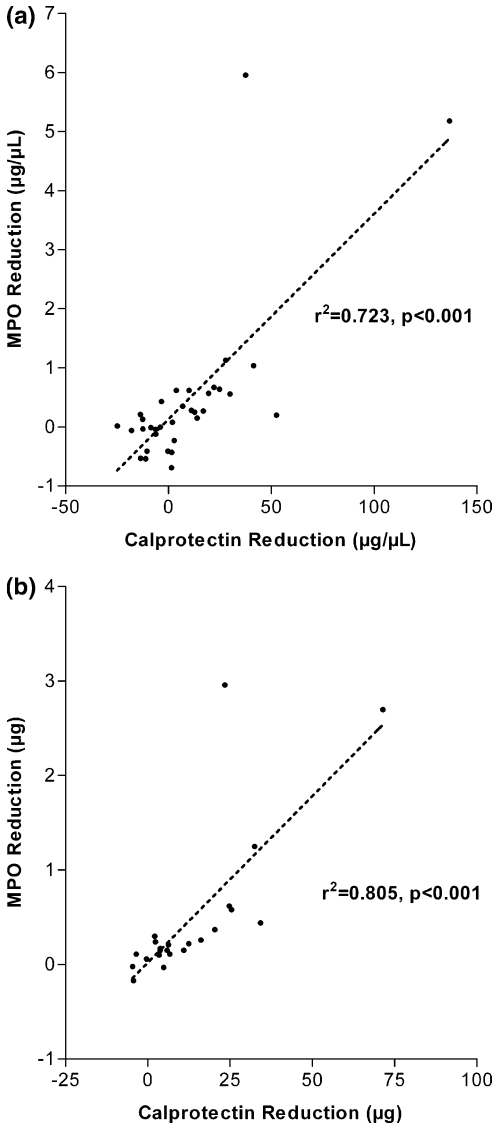

Fig. 1. Correlation of changes of calprotectin and myeloperoxidase (MPO). (a) Concentrations in initially deep sites. (b) Total amount/site in initially deep sites.

sites up to 0.19 ± 0.19 µg for sites displaying chronic periodontal disease. According to the amplification of MPO levels in generalized aggressive periodontitis, which is still detectable after successful therapy (21), our data show up to 16-fold elevated total amounts/site of MPO in this disease category. Although MPO is an important part of antimicrobial defence, increased formation of hypochlorous acid may aggravate tissue breakdown due to inactivation of protease inhibitors and a subsequent elevation of elastase activity (29). In fact, a correlation between MPO and elastase-like activity in gingival crevicular fluid has been reported (27). As PMN elastase can be used as a marker substance for differentiation between acute pyogenic infections and other exudates (30), our finding of highest levels of MPO at acute deteriorating, i.e. suppurating, sites (median total amount/site: pus positive: 0.61 µg; pus negative: 0.17 µg; Fig. 2d) appears not to be surprising.

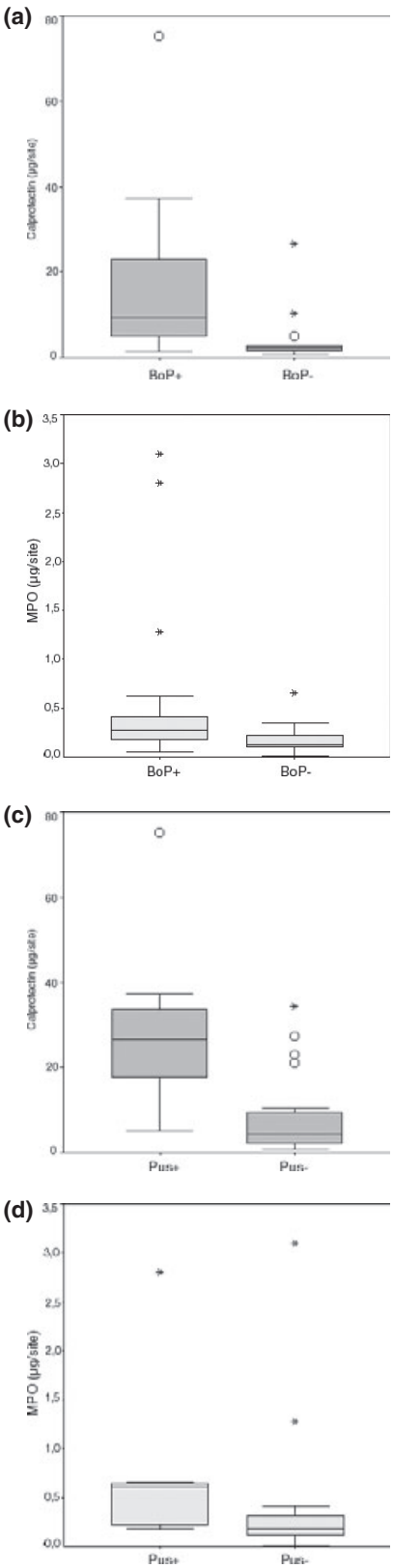

Fig. 2. Association of total amounts/site of calprotectin and myeloperoxidase (MPO) with bleeding on probing (BoP)/suppuration (Pus) at baseline. (a) Elevated calprotectin levels at BoP + sites ($p = 0.001$). (b) Elevated MPO levels at BoP + sites ($p = 0.016$). (c) Elevated calprotectin levels at Pus + sites ($p = 0.001$). (d) Elevated MPO levels at Pus + sites ($p = 0.025$).

Table 5. Correlation of gingival crevicular fluid parameters and clinical outcome (probing depth reduction)

	ΔGCF volume	ΔCalprotectin (concentration)	ΔCalprotectin (total amount)	ΔMPO (concentration)	ΔMPO (total amount)
ΔPD	0.422*	0.458**	0.635***	0.461**	0.552**

GCF, gingival crevicular fluid; PD, probing depth.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Significant and strong correlations were observed at baseline between calprotectin and MPO regarding the median concentration and median total amount/site. Initially, deep sites still showed significant correlations between calprotectin and MPO after successful therapy, whereas the levels of both marker substances were not significantly correlated at shallow sites.

Deeper sites contain higher numbers of PMNs, which are significantly lowered after successful therapy (31, 32). Also, the protein composition and the cell-bound proteolytic activity reflect the number of PMNs in gingival crevicular fluid before and after therapy (33). As other markers for periodontal disease activity such as interleukin-1α do not show correlations to the count of crevicular PMNs (34), our results indicate that an increasing infiltration of PMNs may lead to an increasing proportion of calprotectin of PMN origin in gingival crevicular fluid, whereas the contribution of other cells to the calprotectin content in gingival crevicular fluid is pushed into the background. Beyond cross-sectional interrelations, this interpretation is supported by our findings of correlations of longitudinal changes, i.e. reductions of calprotectin and MPO levels. However, the significance of this concurrent change in relation to marker substances derived from other cell types should be the subject of further investigations. Pronounced correlations of calprotectin and MPO changes with clinical treatment outcome as measured by probing-depth reduction were noted (Table 5), which may reflect that probing-depth changes are also associated with changes of PMN activity within the gingival crevice (31). In summary, we conclude that PMNs are a major source of calprotectin in gingival crevicular fluid; however, the proportion may depend on the inflammatory status of the periodontium.

Interestingly, bleeding-on-probing positive sites presented elevated levels of calprotectin and MPO only at baseline. Three months after therapy, no significant difference between bleeding-on-probing positive and bleeding-on-probing negative sites was observed regarding the concentrations as well as the total amounts/site of both markers. Bleeding on probing is commonly recorded to evaluate periodontal inflammation (35) and is used especially in supportive periodontal therapy to determine sites at risk for further disease progression. Bleeding-on-probing positive sites usually undergo active treatment with subgingival instrumentation (36). However, only absence of bleeding on probing indicates periodontal stability with a reasonably high predictive value, whereas even repeated positive bleeding on probing shows a very low sensitivity of only 30% to detect future attachment loss (37, 38). In addition, in these classical studies the suppressive effect of cigarette smoking on gingival bleeding was not considered and therefore the diagnostic potential of bleeding on probing underlies further limitations (39). Despite persistent gingival inflammation representing one of the major risk factors for tooth loss (40), determination of the surrogate marker bleeding on probing cannot predict future periodontal disease activity (41).

Analysis of gingival crevicular fluid constituents is considered as a good approach for evaluation of a patient's risk for disease or disease progression (42). More than 80 substances have been investigated in the gingival crevicular fluid up to now (43). However, most of these studies were performed in a cross-sectional design and therefore the results may be of unknown significance for risk assessment in periodontology.

A first longitudinal and prospective study monitored the levels of calprotectin and its subunits during a short period of experimentally induced gingivitis (44). Two distinct patterns of calprotectin expression were found. Furthermore, for one expression pattern the initial levels of calprotectin estimated in part the extent of gingival inflammation at the end of the observation period, indicating a prognostic potential of this marker substance.

As calprotectin is extremely resistant to degradation in the presence of calcium, stool samples, for example, are routinely sent to the laboratory by mail (45). Handling of calprotectin samples therefore may be less difficult than of other gingival crevicular fluid components, which may require deep freezing, presence of protease inhibitors in storage liquid or cooling during further processing.

Summarizing our results and findings of previous studies, it was demonstrated that calprotectin in gingival crevicular fluid is mainly related to the PMNs, the dominating cells in the periodontal host response. Significant correlations exist with clinical parameters of periodontal inflammation. Longitudinally, calprotectin levels and their changes are associated with the clinical treatment outcome of non-surgical and adjunctive antimicrobial periodontal therapy. Prognostic possibilities have been described regarding development of gingival inflammation.

Further studies are necessary to elucidate the potential of calprotectin in gingival crevicular fluid as a predictor of development or progression of periodontitis. Long-term studies with adequately high numbers of patients are needed to investigate the interrelations between this interesting marker of inflammation and periodontal disease.

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