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ORIGINAL ARTICLE

Microbiological profile and calprotectin expression in naturally occurring and experimentally induced gingivitis

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Abstract
Objectives This study was performed to evaluate the microbiological profile and the calprotectin expression in gingival crevicular fluid (GCF) in spontaneous and experimentally induced gingival inflammation.
Materials and methods Thirty-seven periodontally healthy subjects were evaluated in *real life* conditions (N-O gingivitis) as well as after 21 days of experimental gingivitis trial (E-I gingivitis). During the experimental gingivitis trial, in one maxillary quadrant (test quadrant), gingival inflammation was induced by oral hygiene abstention, while in the contralateral (control) quadrant, oral hygiene was routinely continued.
Results The results of the study showed that (1) the microbiological profile of quadrants where gingival inflammation was experimentally induced (i.e., E-I test quadrants) differed significantly from that of either quadrants where gingival inflammation was controlled by proper plaque control (i.e., E-I control quadrants) or quadrants with N-O gingivitis, and (2) GCF calprotectin was significantly higher at E-I test quadrants compared to either E-I control quadrants or quadrants with N-O gingivitis. A positive intrasubject correlation was found between GCF concentration of calprotectin at sites presenting N-O and E-I gingivitis.
Conclusions N-O and E-I gingivitis showed a different microbiological profile of the subgingival environment. GCF calprotectin is a reliable marker of gingival inflammation, and its concentration in N-O gingivitis is correlated with its expression in E-I gingivitis.
Clinical relevance The modality of plaque accumulation seems to affect the subgingival microbiological profile associated with a gingivitis condition. Calprotectin levels in GCF may be regarded as a promising marker of the individual susceptibility to develop gingival inflammation in response to experimentally induced plaque accumulation.

Keywords Calprotectin · Interleukin-1 beta · Bacteria · Experimental gingivitis · Gingival inflammation

Introduction

In a series of experimental gingivitis studies, we advanced the hypothesis that the individual susceptibility to gingival inflammation under experimental conditions (i.e., at completion of a 21-day experimental gingivitis trial) could be identified a priori (i.e., from naturally occurring gingival inflammation), and we identified some of these environmental and genetic susceptibility determinants [1–10].

It has been long-established that the level of gingival inflammation is correlated with the quantity of plaque accumulated on and around the gingival margin [11, 12]. Although qualitative differences in plaque have been shown in certain types of gingival inflammatory conditions, e.g., high

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levels of *Prevotella intermedia* in pregnancy gingivitis [13], only a limited number of studies comparing different (i.e., spontaneous vs. experimental) gingivitis conditions have reported microbiological differences [14]. In most of the experimental gingivitis studies evaluating differences in gingivitis susceptibility among individuals, the possibility of such microbiological qualitative differences has not been investigated [15–17]. Given the limited amount of information on this issue and within the intent of our series of studies to characterize the determinants affecting the plaque-induced gingivitis, it seems pertinent to explore the possibility that qualitative differences in subgingival plaque (at the species level) may account for the differences between naturally occurring (N-O) and experimentally induced (E-I) gingivitis conditions.

Calprotectin is a complex of two calcium-binding subunits, MRP 8 and MRP 14 [18–22], which is produced by leukocytes, macrophages, and epithelial cells and stored in their cytosol [23]. To date, two major functions of calprotectin have been demonstrated. Calprotectin actively contributes to the host defense against bacterial infections and may have a key regulatory role in inflammatory reactions. Calprotectin and its subunits have been identified in the gingival crevicular fluid (GCF), suggesting a role of calprotectin in the host response to subgingival bacterial colonization leading to periodontal inflammation [24–28]. Interestingly, a previous experimental gingivitis study suggested that the GCF levels of calprotectin in naturally occurring gingivitis could be a predictor of the severity of gingival inflammation in response to the acute plaque accumulation that results when oral hygiene measures are withdrawn [29]. There is, however, less evidence on the possible association between increased calprotectin levels and destructive periodontal disease. In GCF, calprotectin levels reflected the *collagenase* expression in deep pockets of patients affected by chronic periodontitis [26]. Overall, these data indicate clearly that calprotectin level in GCF is a biomarker of inflammation residing in the periodontal tissues; however, its association with gingivitis conditions as well as destructive periodontal disease still needs to be elucidated. Recently, it has been shown that calprotectin levels predicted periodontal disease activity at sites that had undergone nonsurgical treatment plus systemic or local antimicrobial administration [30].

In light of these results, we have carried out the present study with the aim to evaluate the microbiological profile, with particular emphasis on the subgingival presence of major periodontal pathogens, as well as the calprotectin expression in GCF in both N-O and E-I plaque-associated gingivitis. Moreover, the possible association between GCF calprotectin levels and the presence of periodontal pathogens was also investigated in both gingivitis conditions.

Material and methods

Experimental design and study population

The study protocol was approved by the Research Ethics Committee, University of Ferrara, and all participants provided written informed consent. The study design was described in detail in a previous publication [8]. Briefly, after a first randomized split-mouth localized experimental gingivitis clinical trial (first trial) conducted from October 2000 to November 2001, two subpopulations of periodontally healthy individuals were identified, defined as high responders (HR, $n=24$) and low responders (LR, $n=24$), and characterized by significantly different severity of gingivitis to similar amounts of plaque deposits [1]. On January 2002, all HR and LR individuals were recalled to verify their availability and eligibility for a second experimental gingivitis trial ("repeat" trial) [7]. Volunteers underwent the repeated trial between April and November 2002.

Subjects underwent a first evaluation for plaque and gingival status under real life conditions 2 weeks before the "repeat" experimental gingivitis trial had been initiated. This real life condition was regarded as naturally occurring (N-O) gingivitis [8]. The evaluation included the assessment of clinical, microbiological, and immunological parameters as described below. Then, subjects entered a 14-day period of professional and supervised tooth cleaning to achieve optimum gingival health (gingival index (GI)=0 at all the experimental sites) and to standardize gingival conditions at the beginning of the experimental gingivitis trial. Immediately after the assessments of study parameters for N-O gingivitis, a session of full-mouth professional scaling and polishing was performed. A medium toothbrush (Elmex Inter X, GABA International AG, Therwil, Switzerland), unwaxed floss (Elmex, GABA International AG), and standard toothpaste (Aronal, GABA International AG), along with oral hygiene instructions, were provided. In addition, to exclude the possibility of subclinical ascorbate deficiency, all subjects were provided with a vitamin C (ascorbic acid, 500 mg; Vitamina C Angelini, ACRAF, Ancona, Italy) supplement to be taken once daily for the entire pretrial and trial period (35 tablets per subject). Polishing and oral hygiene instructions were repeated 1 week before the experimental gingivitis trial.

Before the start of the experimental gingivitis trial, one maxillary quadrant was randomly assigned as "test," and the contralateral quadrant served as "control," according to a computer-generated randomization list. The experimental gingivitis was induced by oral hygiene abstention (E-I gingivitis) in test quadrants, while oral hygiene was continued in control quadrants [1, 7]. After 21 days of experimental gingivitis, all subjects were reevaluated for clinical, microbiological, and immunological parameters.

Therefore, each subject was evaluated in two different conditions of plaque accumulation and related gingival response: N-O and E-I gingivitis. To allow within-quadrant comparisons between the two gingivitis conditions, we also did refer to the "test" and "control" quadrants for N-O gingivitis. In other words, test and control quadrants were the same for both N-O and E-I gingivitis and were matched for the analysis.

Clinical parameters

At the first evaluation and after 21 days of experimental gingivitis, the following parameters were assessed at the buccal and the mesiobuccal aspects of the maxillary lateral incisors, first bicuspids, and first molars, in the order listed:

- GI, according to a modification of the method of Löe and Silness [31] without the bleeding on probing component.
- Plaque index (PlI), according to Silness and Löe [32] and modified as follows: Plaque formed on the various tooth surfaces was first categorized as either PlI score 0 or PlI score 2. Subsequently, plaque was stained by the use of topically applied erythrosine solution to distinguish between PlI score 0 and PlI score 1 [33].
- Gingival crevicular fluid volume (GCF), collected as previously described [8, 34], was measured with a calibrated Periotron 8000 (OraFlow Inc., Plainview, NY, USA) according to the manufacturer's instructions and was expressed in microliter.
- Angulated bleeding score (AngBS), which is a modification of the angulated bleeding index [35]. After lightly drying the gingiva with compressed air, a periodontal probe was held at an angle of approximately 60° to the longitudinal axis of the tooth and in contact with the sulcular gingival tissues. AngBS was scored as 0, no bleeding; 1, bleeding upon probe stimulation; and 2, spontaneous bleeding.

Two trained and calibrated examiners recorded all parameters with good to excellent intra- and interexaminer agreement [1, 7].

Microbiological assessments

At the first evaluation and after 21 days of experimental gingivitis, subgingival plaque was collected from the palatal surfaces (i.e., mesiopalatal, palatal, and distopalatal) of all experimental teeth in the experimental quadrants and was pooled. Saliva was blotted with a gauze or cotton roll, and the supragingival plaque was removed with a curette or scaler. Subgingival plaque was collected with a curette or scaler and wiped onto a sterile, coarse endodontic paper point. The sample was placed in a sterile microcentrifuge tube and frozen until lab processing.

Plaque samples were resuspended into 150 μl of sterile water and homogenized by vortexing for 3 min. DNA was then extracted using the boiling-lysis technique (103°C for 5 min followed by 10 min at −20°C and 5 min of centrifugation at 15,500×*g*). Supernatants were retrieved, purified, and concentrated using a commercial kit (illustra™, GE Healhcare, Buckinghamshire, UK). We first amplified the bacterial 16S rRNA sequence using broad-range eubacterial primers (EUB 1, 5′-GAG TTT GAT CCT GGC TCA G-3′; EUB 2, 5′-AGA AAG GAG GTG ATC CAG CC-3′). This PCR amplification was carried out in a master mix solution containing 2 units of Taq DNA polymerase, 1× polymerase buffer with 2 mM $MgCl_2$, 0.2 mM of a mixture of each deoxynucleoside triphosphate, 1 μM primers, and 5 μl template DNA, in a total volume of 50 μl. Samples were preheated at 95°C for 2 min followed by amplification under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. Thirty-six cycles were performed followed by an elongation step at 72°C for 10 min.

Secondly, a nested PCR with specific primers for putative periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Eikenella corrodens*, *Campylobacter rectus*, *Tannerella forsythia*, *Treponema denticola*, *Parvimonas micra*, and *Fusobacterium nucleatum*) was performed. All primers were designed on the basis of 16S rRNA sequence (Table 1). PCR amplification was performed in a master mix solution containing 1 unit of Taq DNA polymerase, 1× polymerase buffer with 2 mM $MgCl_2$, 0.2 mM of a mixture of each deoxynucleoside triphosphate, 1 μM primers, and 2 μl PCR products as template DNA, in a total volume of 25 μl. PCR conditions were the same as previously described for the broad-range eubacterial primers.

Negative and positive controls were included in each batch of samples. The negative control was sterile distilled water instead of template DNA. Positive controls consisted of DNA from pure bacterial cultures.

A 12-μl aliquot of the amplified sample from each PCR tube was electrophoresed through a 1% agarose gel (Agarose D-2, Pronadisa, CONDA, Torrejón de Ardoz, Madrid, Spain) in tris-acetate EDTA buffer. The gel was stained with ethidium bromide (10 mg/ml) and photographed under an ultraviolet light transilluminator (Gel Printer Plus TD, Alcobendas, Madrid, Spain). A DNA ladder (Invitrogen, Carlsbad, USA) was used as a molecular weight marker. The band position of the PCR products was in accordance with the length of the primers.

The detection limit of the nested PCR was assessed by determining the results (positive/negative) of serial 10-fold dilutions of extracted genomic DNA from each targeted bacteria (except for *T. denticola*, which could not be cultivated). Dilutions ranged from 10^9 UFC/ml to 10^1 UFC/ml.

Table 1 Specific primers used for nested PCR

Bacteria	Sequence (5′–3′)	Positions	Length (bp)
A. actinomycetemcomitans	AAACCC ATCTCTGAG TTCTTCTTC ATGCCA ACTTGA CGT TAA AT	F478 R1034	557
P. gingivalis	AGGCAG CTTGCC ATA CTGCG ACT GTT AGCAACTAC CGATGT	F729 R1132	404
P. intermedia	TTT GTTGGG GAG TAA AGC GGG TCA ACA TCT CTG TAT CCTGCGT	F458 R1032	575
E. corrodens	CTAATACCGCATACGTCCTAAG CTACTAAGCAATCAAGTTGCCC	F169 R856	688
C. rectus	TTTCGGAGCGTAAACTCCTTTTC TTTCTGCAAGCAGACACTCTT	F415 R1012	598
T. denticola	TAATACCGAATGTGCTCATTTACAT TCAAAGAAGCATTCCCTCTTCTTCTTA	F193 R508	316
T. forsythia	GCGTATGTAACCTGCCCGCA TGCTTCAGTGTCAGTTATACCT	F120 R760	641
P. micra	TCGAACGTGATTTTTGTGGA TCCAGAGTTCCCACCTCT	F54 R1128	1,074
F. nucleatum	TAAAGCGCGTCTAGGTGGTT ACGGCTTTGCAACTCTCTGT	F517 R1214	697

All of them (except for *F. nucleatum* and *P. micra*) were based on the design of Ashimoto et al. [53]

Irrespective of the pathogen, the methodology allowed for the amplification of 10^2 UFC/ml.

Immunological assessments

At the first evaluation and after 21 days of experimental gingivitis, sampling of GCF was performed at the buccal and the mesiobuccal aspect of the maxillary lateral incisors, first bicuspids, and first molars at test and control quadrants. GCF samples obtained from the same quadrant were pooled for storage and process. GCF was collected after the GI and PlI assessments. Once the area was isolated with a cotton roll or gauze and the supragingival plaque was removed with a curette or scaler, the GCF was collected on paper strips (Perio-paper®; IDE Interstate, Amityville, NY, USA), as previously described [8, 34]. The strips were placed in vials containing a transport medium (1 ml of PBS and bovine serum albumin) and stored at −70°C until GCF extraction. To perform GCF extraction, the vials were sonicated for 5 min and centrifuged at 1,000×*g* at 4°C for 15 min. The extracted supernatants were aliquoted in five Eppendorf vials (each containing 200 μl) and stored at −80°C until analysis. GCF samples were analyzed for calprotectin using commercially available enzyme-linked immunosorbent assays (calprotectin, BMA Biomedicals, Augst, Switzerland). Analyses were performed according to the manufacturer's instructions. All ELISA determinations were performed in duplicate. Results were calculated using the standard curves created in each assay. Concentrations of calprotectin corrected for GCF volume (cMRP 8/14) were calculated and expressed as microgram per milliliter. Lab procedures for the quantification of IL-1β in GCF have been described in details elsewhere [8].

Statistical analysis

The subject was the statistical unit. For each parameter at each observational period, the subject was represented by a single value for the test quadrant and a single value for the control quadrant. The clinical outcome values recorded from all the selected sites per quadrant were averaged in order to obtain the subject mean value for each test and control quadrant. For the microbiological outcome values, the quadrant was considered as positive for a specific bacterial species if such species was detected in the pooled microbiological sample of that quadrant.

In order to detect which parameters are associated with calprotectin expression in gingival inflammatory conditions, the study population was subdivided into different categories according to cMRP 8/14 levels at test quadrants under E-I gingivitis.

Data were expressed by median and interquartile range (IR). Microbiological profiles (i.e., the frequency of periodontal pathogens) were compared with χ^2 test. Comparisons of clinical and immunological parameters between test and control quadrants as well as between N-O and E-I gingivitis were performed with Wilcoxon test. Comparisons of clinical and immunological parameters between subjects with a low, medium, and high cMRP 8/14 expression at test

quadrants in E-I gingivitis were performed with Mann–Whitney *U* test. The strength of the correlation between variables was evaluated using the Spearman test. The level of significance was set at 5%.

Results

Study population

Of the originally identified 48 HR/LR subjects [1], 37 (20 males and 17 females; mean age, 23.7±1.8 years) volunteered for the repeat trial. Seventeen subjects belonged to the LR group (9 males and 8 females; mean age, 23.4± 2.0 years) and 20 belonged to the HR group (11 males and 9 females; mean age, 24.0±1.6 years). All the 37 subjects completed the repeat trial complying with the study instructions. In one subject, the immunological analysis was not possible due to technical reasons. Therefore, the statistical analysis was performed on 36 subjects.

Plaque accumulation and gingival inflammation

The values of PlI, GI, and AngBS in N-O and E-I gingivitis in the test and control quadrants of the present study population are reported in detail in a previous publication [8]. Briefly, in test quadrants, more severe supragingival plaque accumulation (PlI, $p<0.0001$) and gingival inflammation (GI and AngBS, $p<0.0001$) were observed in E-I gingivitis compared to N-O gingivitis. In contrast, significantly lower PlI ($p<0.01$) and GI ($p<0.001$) were observed in E-I gingivitis compared to N-O gingivitis at control quadrants. In N-O gingivitis, no significant differences in PlI, GI, and AngBS were observed between test and control quadrants. In E-I gingivitis, PlI, GI, and AngBS were significantly higher in test quadrants compared to control quadrants ($p<0.0001$).

Prevalence of periodontal pathogens in N-O and E-I gingivitis

The frequency distribution of each periodontal pathogen in test and control quadrants under N-O and E-I gingivitis is reported in Table 2.

Microbiological profile in N-O gingivitis

In N-O gingivitis, the microbiological profile differed significantly between test and control quadrants ($p<0.001$). In particular, test quadrants showed a higher prevalence of *T. denticola*, *C. rectus*, and *P. micra* and a lower prevalence of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, and *F. nucleatum* compared to control quadrants.

Microbiological profile in E-I gingivitis

In E-I gingivitis, test and control quadrants showed a significantly different microbiological profile ($p<0.001$). *P. gingivalis*, *T. denticola*, *P. micra*, *F. nucleatum*, and *P. intermedia* were more prevalent at test quadrants compared to control quadrants.

Comparison between microbiological profiles in N-O and E-I gingivitis

A significantly different microbiological profile was observed at test quadrants in E-I gingivitis compared to test quadrants in N-O gingivitis ($p<0.001$). Test quadrants in E-I gingivitis showed a lower prevalence of *T. forsythia*, *T. denticola*, *C. rectus*, *P. micra*, *P. intermedia*, and *F. nucleatum* compared to N-O gingivitis test quadrants.

The microbiological profile of control quadrants in E-I gingivitis significantly differed from that observed at control quadrants in N-O gingivitis ($p<0.001$). A lower prevalence of most bacterial species was observed at control quadrants in E-I gingivitis compared to control quadrants in N-O gingivitis.

cMRP 8/14 in N-O and E-I gingivitis

cMRP 8/14 in N-O and E-I gingivitis is reported in Table 3. In N-O gingivitis, cMRP 8/14 was significantly higher in test quadrants compared to control quadrants ($p=0.004$). Under experimental conditions (E-I), a significantly higher cMRP 8/14 was observed in test compared to control quadrants ($p<0.001$). In test quadrants, cMRP 8/14 was also significantly higher in E-I compared to N-O gingivitis ($p=0.002$).

Intrasubject correlation between cMRP 8/14 expression in N-O and E-I gingivitis

A statistically significant positive correlation between cMRP 8/14 in N-O and E-I gingivitis was found in test quadrants (Spearman=0.17, $p=0.021$) (Fig. 1), but not in control quadrants.

Characterization of subjects with low, medium, or high cMRP 8/14 levels

We identified three groups of subjects with significantly different levels of cMRP 8/14 at test quadrants following E-I gingivitis: low (3.0 μg/ml; IR, 3.5–3.6), medium

Table 2 Microbiological profiles of test and control quadrants in naturally occurring (N-O) and experimentally induced (E-I) gingivitis

		N-O gingivitis								E-I gingivitis							
		Pg	Tf	Td	Aa	Pi	Ec	Cr	Fn	Pm	Pg	Tf	Td	Aa	Pi	Ec	Cr	Fn	Pm
Test quadrant	Positive	21	31	29	2	7	31	33	31	30	23	17	8	3	3	29	8	24	12
	Negative	12	5	7	34	29	5	3	5	6	12	18	27	32	32	5	27	11	23
	Not available	3	0	0	0	0	0	0	0	0	1	1	1	1	1	2	1	1	1
	% Positive	63.6	86.1	80.6	5.6	19.4	86.1	91.7	86.1	83.3	65.7	48.6[a]	22.9[a]	8.6	8.6[b]	85.3	22.9[a]	68.6[b]	34.3[a]
Control quadrant	Positive	32	28	5	30	33	30	22	32	18	3	13	1	2	1	23	10	15	3
	Negative	2	6	29	4	1	4	11	2	16	29	19	31	30	31	8	22	17	29
	Not available	2	2	2	2	2	2	3	2	2	4	4	4	4	4	5	4	4	4
	% Positive	94.1	82.4	14.7	88.2	97.1	88.2	66.7	94.1	52.9	9.4[a]	40.6[a]	3.1	6.3[a]	3.1[a]	74.2[b]	31.3[a]	46.9[a]	9.4[a]
	p value	<0.001	0.485	<0.001	<0.001	<0.001	0.595	0.001	0.033	<0.001	<0.001	0.257	<0.001	0.426	0.044	0.101	0.215	0.006	<0.001

Pg P. gingivalis, *Tf T. forsythia*, *Td T. denticola*, *Aa A. actinomycetemcomitans*, *Pi P. intermedia*, *Ec E. corrodens*, *Cr C. rectus*, *Fn F. nucleatum*, *Pm P. micra*

[a] Significantly different from the same quadrant as assessed in N-O gingivitis ($p<0.001$)

[b] Significantly different from the same quadrant as assessed in N-O gingivitis ($p<0.05$)

(4.8 μg/ml; IR, 4.6–5.1), and high (11.1 μg/ml; IR, 7.9–13.8). The distribution of different clinical and immunological parameters potentially associated with the cMRP 8/14 expression is reported in Table 4.

Neither cIL-1β nor clinical parameters of plaque accumulation and gingival inflammation were significantly different among groups (Table 4).

Discussion

The present study was performed to evaluate the subgingival microbiological profile and the calprotectin expression in GCF under either N-O or E-I plaque-associated gingivitis. Also, the possible association between GCF calprotectin levels and the presence of periodontal pathogens was investigated. The results indicated that (1) the microbiological profile of quadrants where gingival inflammation was experimentally induced (i.e., E-I test quadrants) differed significantly from that of either quadrants where gingival inflammation was controlled by proper plaque control (i.e., E-I control quadrants) or quadrants with N-O gingivitis, and (2) GCF calprotectin was significantly higher at E-I test quadrants compared to either E-I control quadrants or quadrants with N-O gingivitis. A positive intrasubject correlation was found between GCF concentration of calprotectin at sites presenting N-O and E-I gingivitis.

Previous studies reported on clinical and immunological aspects observed after 4 weeks of experimental gingivitis and persistent gingival inflammation in a naturalistic setting using a parallel arm, randomized controlled design [36]. In our experimental design [7, 8], clinical, microbiological, and immunological parameters related to experimental and spontaneous plaque-associated gingivitis were compared in the same individuals. Such design allowed for controlling bias derived from individual traits that may potentially affect the gingival inflammatory response to plaque [1–10, 37]. Moreover, to limit the influence of site-specific conditions, test and control quadrants, as randomly assigned before the start of the experimental gingivitis trial, were matched for analysis under N-O and E-I gingivitis.

In E-I gingivitis, a higher prevalence of specific bacterial species was observed at sites where rapid plaque accumulation and severe gingival inflammation were attained through oral hygiene abstention, compared to sites where plaque and gingivitis scores were maintained low by proper plaque control measures. Under experimental gingivitis conditions, qualitative differences in the composition of the subgingival microflora parallel the amount of plaque accumulated over time [12, 14]. Previous experimental gingivitis studies have also reinforced the concept that changes in the profile of the subgingival microflora are associated with symptoms of an advanced stage of gingival inflammation, such as gingival

Table 3 Concentration (μg/ml) of calprotectin in GCF (cMRP 8/14) at test and control quadrants in naturally occurring (N-O) and experimentally induced (E-I) gingivitis

	N-O gingivitis		E-I gingivitis		*p* value
	Median	Interquartile range	Median	Interquartile range	
cMRP 8/14 (μg/ml)					
Test quadrant	2.8	1.9–4.6	4.8	3.8–7.3	0.002
Control quadrant	2.1	1.6–2.9	2.0	1.0–2.9	0.868
p value	0.004		<0.001		

bleeding [11, 12, 14, 38, 39]. Therefore, it can be speculated that under experimental circumstances, differences in the microbiological profile, as observed between E-I test and control quadrants, may partly account for the difference in the severity of clinical signs of gingival inflammation between quadrants, beyond the quantitative aspects of plaque deposits [8]. However, due to the qualitative nature of the microbiological assessment, the causal role of a specific bacterial profile on the inflammatory response of gingival tissues should be interpreted with caution. Moreover, our analysis mainly included gram-negative, putative periodontal pathogens. Previous data have shown that in conditions of experimental gingivitis, the most striking microbial change is the shift from a *Streptococcus*-dominated plaque to an *Actinomyces*-dominated plaque, the gram-negative species being only less than 5% of the total microflora [14].

Differences in the microbiological profile may also be ascribed to differences in the plaque accumulation rate under either spontaneous or experimental conditions. In a cohort of volunteers abstained from oral hygiene procedures for a period of 14 days, sites showing a "rapid" formation of plaque deposits revealed higher proportions of gram-negative species compared to sites with a "slow" plaque accumulation rate [40]. Previous studies have also demonstrated that the severity of the gingival inflammatory status may affect the early rate of plaque formation [41] as well as the composition of the dental biofilm [42]. Even though we have no data on the rate of plaque accumulation in N-O compared to E-I gingivitis, it can be speculated that the two gingivitis conditions as well as the two quadrants in E-I gingivitis are characterized by substantial variations in the rate of plaque formation. Such variations may have eventually influenced the profile of the subgingival microflora.

Our data showed that GCF calprotectin was more expressed in conditions of experimental versus spontaneous gingivitis. In this respect, differences in GCF calprotectin levels between E-I and N-O gingivitis paralleled the differences in the severity of clinically assessed gingival inflammation [8]. Consistently, previous studies have shown that the calprotectin concentration in GCF was positively correlated with clinical parameters of gingival inflammation including GCF volume [26]. Calprotectin was found significantly higher at bleeding sites compared to nonbleeding sites of different probing depth in patients with different periodontal status [24], as well as in patients affected by generalized aggressive periodontitis [27]. On the other hand, the reduction of gingival inflammation by mechanical root

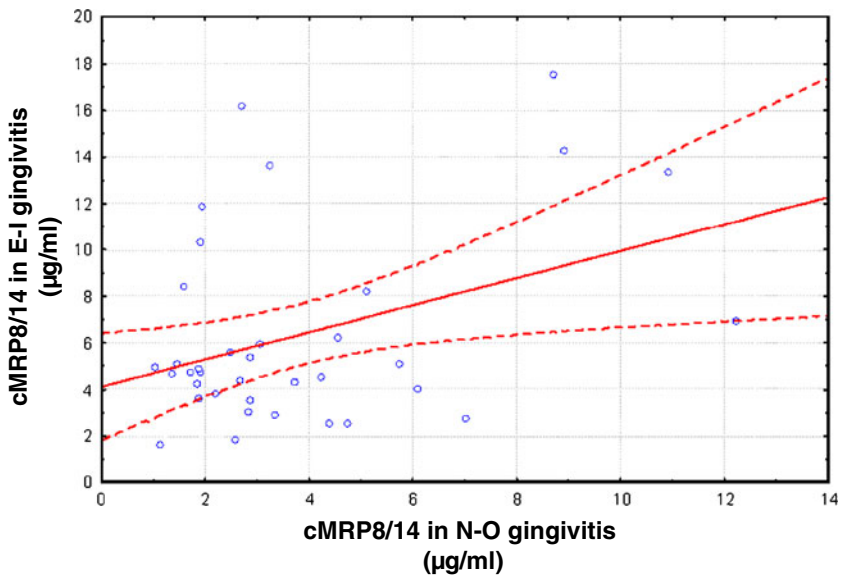

Fig. 1 Intrasubject correlation between calprotectin expression in GCF (cMRP 8/14) at test quadrants in naturally occurring (N-O) and experimentally induced (E-I) gingivitis. r=0.3830, p=0.0211

Table 4 Clinical, microbiological, and immunological characterization of subjects with low, medium, or high levels of calprotectin in GCF (cMRP 8/14) at test quadrants in experimentally induced (E-I) gingivitis

	cMRP 8/14 (μg/ml) (E-I gingivitis, test quadrants)			
	Low (n=12) 1.601 ≤ cMRP 8/14 ≤ 4.254	Moderate (n=12) 4.317 ≤ cMRP 8/14 ≤ 5.584	High (n=11) 5.940 ≤ cMRP 8/14 ≤ 17.556	p value for intergroup comparisons
cMRP 8/14 (μg/ml) (E-I gingivitis, testquadrant)	3.0 (2.5–3.6)	4.8 (4.6–5.1)	11.1 (7.9–13.8)	l vs m, 0.016 m vs h, 0.016 l vs h, <0.001
cIL-1β (pg/ml) (E-I gingivitis, test quadrant)	25.9 (21.6–31.0)	24.4 (22.4–29.6)	27.2 (24.7–31.7)	NS
PlI (E-I gingivitis, test quadrant)	1.8 (1.5–2.0)	1.8 (1.7– 2.0)	1.8 (1.8–2.0)	NS
GI (E-I gingivitis, test quadrant)	0.9 (0.8–1.0)	0.8 (0.8–1.0)	0.8 (0.7–1.0)	NS
AngBS (E-I gingivitis, test quadrant)	0.5 (0.3–0.8)	0.4 (0.3–0.5)	0.3 (0.2–0.5)	NS

cIL-1β concentration of IL-1β in GCF, *PlI* plaque index, *GI* gingival index, *AngBS* angulated bleeding score, *l* low, *m* moderate, *h* high, *NS* not significant

instrumentation is associated with a reduction of calprotectin levels in GCF [27, 28]. Given that PMNs are the main source of calprotectin in GCF [27, 43], calprotectin appears as a direct measure of inflammatory activity. Recently, calprotectin has been shown as a marker of disease progression at sites treated with scaling and root planing in association with local/systemic antimicrobials [30]. The findings suggest that nonsurgical treatment resulted in a significant decrease of calprotectin levels at 3 months and that the relationship of calprotectin with future disease activity may support the association of this biomarker with an acute phase of periodontal inflammation eventually leading to potential tissue breakdown [30]. Overall, these observations reinforce the role of GCF calprotectin as a reliable marker of plaque-induced gingival inflammation. In contrast with our findings, a short-term experimental gingivitis trial reported that the GCF expression of MRP 8/14 and its subunits did not generally increase after 10 days of undisturbed plaque accumulation compared to the beginning of the experimental phase [29].

As expected, a large interindividual variation in GCF expression of calprotectin was observed in both gingivitis conditions. Interestingly, a positive correlation was found between individual GCF concentrations of calprotectin under N-O and E-I gingivitis conditions (test quadrants). In other words, subjects who showed either high or low levels of GCF calprotectin in N-O conditions tended to consistently show high or low levels of GCF calprotectin following a boost in the bacterial challenge due to neglected oral hygiene. These observations indicate that the calprotectin GCF level detected in a spontaneous state of gingival inflammation is a reliable predictor of the calprotectin GCF expression in response to an acute, experimentally induced plaque accumulation. Our data are consistent with those of Que et al. [29] using a similar experimental approach. Subjects who showed higher levels of calprotectin at baseline (preexperimental) conditions did show higher calprotectin levels after 10 days of experimental gingivitis [29]. When comparing the GCF expression of calprotectin with IL-1β in N-O versus E-I gingivitis [8], calprotectin seems to be a more promising marker of the individual susceptibility to develop gingival inflammation in response to experimentally induced plaque accumulation.

In the present study, we characterized three groups of subjects with a significantly different cMRP 8/14 expression (low, moderate, high) at test quadrants in E-I gingivitis in order to assess the potential association between clinical and immunological parameters and GCF calprotectin levels (Table 4). The assumption was that E-I gingivitis is characterized by a marked gingival inflammatory response to plaque, thus representing a favorable condition to explore the potential effect of modulators on the local expression of calprotectin. Interestingly, the difference in cMRP 8/14 levels among groups was not significantly associated with either quantitative differences in plaque deposits (i.e., PlI) or parameters of gingival inflammation (i.e., GI, AngBS). Consistent with our observations, previous experimental gingivitis trial showed that the increase in plaque amounts was correlated with variable (either increased or decreased) expression of GCF calprotectin [29]. However, previous studies seem to suggest that qualitative aspects in plaque composition may exert some influence on the expression of GCF calprotectin. For instance, the lipopolysaccharide of *P. gingivalis* appears to positively modulate the local calprotectin expression [44–47]. Unfortunately, the association between different bacterial species and GCF calprotectin levels could not be

explored in our study since the sites used for microbiological assessments were topographically different from those used for GCF sampling. Our data also indicated no association between cMRP 8/14 and cIL-1ß levels (Table 4). In contrast, previous studies indicated a positive modulation of calprotectin expression by locally produced IL-1ß [24, 45, 47]. The observation that different levels of cMRP 8/14 were detected in patients showing similar clinical signs of plaque-induced gingival inflammation seems to reinforce the concept that calprotectin in GCF is a sensitive, reliable marker of inflammation of periodontal tissues [27–30]. Whether and to what extent GCF levels of calprotectin in naïve as well as experimental gingivitis conditions may be correlated with the individual susceptibility to plaque-induced gingival inflammation needs to be further clarified.

It may be hypothesized that the quantitative and qualitative changes in the subgingival dental biofilm, as induced by undisturbed plaque accumulation in the experimental gingivitis model, may represent a condition for the genetic modulation in GCF calprotectin production to become manifest, similar to what we have previously observed for IL-1ß [8]. At present, data regarding genetic variances affecting the expression of calprotectin or its subunits are restricted to a limited number of studies [48, 49]. Interestingly, previous studies suggested that specific single nucleotide polymorphisms on the genes encoding for calprotectin subunits might be associated with susceptibility to aggressive periodontitis [50–52]. Specifically designed studies are therefore needed to evaluate the genetic basis of the individual susceptibility to calprotectin production in response to plaque accumulation and its implications on the development of gingival inflammation and destructive periodontal disease.

In conclusion, the results of the present study demonstrated that N-O and E-I gingivitis showed a different microbiological profile of the subgingival environment. Calprotectin seems a reliable marker of gingival inflammation, commonly expressed in both gingivitis conditions. cMRP 8/14 levels under N-O gingivitis are correlated with GCF calprotectin expression under E-I gingivitis.

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Conflicts of interest The authors have no conflicts of interest to declare.

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