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Original

Effects of food supplements on periodontal status and local and systemic inflammation after nonoperative periodontal treatment

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Abstract: The present study aimed to assess the effects of a multimicronutrient food supplement on periodontal clinical parameters and systemic/local inflammatory markers. Thirty patients with severe chronic periodontitis who adhered to the Mediterranean diet (MD) underwent non-surgical therapy and daily took either the micronutrient complex (group-A) or olive oil (group-B) from baseline (T0) to 3 months (T2). Supragingival debridement was performed at T0. One month later (T1), one-stage-full-mouth disinfection was performed. Periodontal clinical parameters were monitored and correlated with serum C-reactive protein (CRP) and salivary matrix metalloproteinase-8/9 (MMPs-8/9) quantified at each time point. Longitudinal analysis revealed that in group-A, the MMP-8/-9 levels were decreased at T2 compared with at T0 ($P = 0.013$ and $P = 0.004$, respectively) and that the MMP-9 levels were decreased at T1 ($P = 0.004$). These reductions were not significant in group-B. The CRP levels in both groups did not change over time. No between-group differences were noted for any parameter. The correlation between the full-mouth bleeding score and the MMP-8 level in both groups was significant ($P < 0.001$). The investigated local and systemic inflammatory parameters were not affected by the tested multimicronutrient supplement in patients who adhered to MD. In conclusion, MMP-8 is useful for assessing the reduction in periodontal inflammation.

Keywords: metalloproteinase; dietary supplements; periodontal therapy; gingival crevicular fluid; olive oil; inflammation.

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Introduction

Specific pathogenic bacteria that elicit host immune response and lead to resorption of the periodontal ligament, cementum, and alveolar bone cause periodontitis, an inflammatory disease; this destructive activity is mediated by clastic cells (osteoclasts), proinflammatory cytokines (i.e., receptor activator of nuclear κ ligand [RANKL] and interleukin-1 [IL-1]), and proteinases involved in extracellular matrix breakdown (i.e., metalloproteinases [MMPs]) (1). In periodontal disease, higher amounts of MMPs are released by polymorphonuclear leukocytes and osteoclasts to the gingival crevicular fluid (GCF) and saliva. Levels of these proteins assessed in

saliva and GCF have been closely related to the status of periodontal disease and to the clinical performance of periodontal regenerative treatment (2-4). A recent critical review of the literature concluded that the analysis of the MMP-8 levels in oral fluid is beneficial in periodontal disease diagnosis, disease progression prediction, as well as treatment and medication monitoring (5).

Periodontitis elicits local immune and inflammatory responses and contributes to increased systemic inflammation. Studies have reported that benefits in oral health occurring 6 months after periodontal treatment were associated with improved endothelial function related to decreased systemic inflammation and serum C-reactive protein (CRP) (6). Studies evaluating the beneficial effects of food supplements have used serum CRP as a biomarker of systemic inflammation.

Analysis of the levels of salivary MMP-8 and -9 as well as serum CRP is beneficial in the overall investigation of the local (oral) and systemic inflammation reduction that occurs after periodontal therapy as well as after the adjunctive administration of treatments directed at modulating inflammatory responses. Vitamins, calcium, and antioxidants reduce oxidative stress; improve cellular metabolism, integrity, and activity; and improve collagen synthesis. In periodontal tissue, micronutrients exert their beneficial effects by reducing tissue inflammation and the damages that follow after inflammatory disease (7). Apart from the suppression of infection via the application of correct oral hygiene procedures, the host immunocompetence and optimal epithelial/connective tissue healing activity play key roles in periodontal health maintenance. Several clinical studies have demonstrated this favorable association between the micronutrient levels and the periodontal health status (8-10), and awareness campaigns toward an healthy lifestyle and eating habits rich in fruits and vegetables have been conducted to promote and maintain systemic and periodontal health (11,12). Furthermore, the integration between non-surgical/surgical interventions and micronutritional approaches has been proposed for the treatment of periodontitis and tested with encouraging results in several clinical trials (13-16). After non-surgical periodontal therapy, clinical trials have revealed improved clinical outcomes when supplementation with a specific and single micronutrient (i.e., single vitamin or calcium) (13) as well as a combination of micronutrients (i.e., complexes containing fruits and vegetables) (15,16) were prescribed. Daily integration of a multimicronutrient complex may exert wider beneficial effects than a single one owing to its complete activity in the different tissues involved in periodontal healing (bone, epithelium, and connective tissue) and in different healing phases (inflammation and tissue neoformation).

Co-enzyme Q10 multicomposite, *Boswellia serrata*, fish oil as a source of n-3 polyunsaturated fatty acids (PUFAs), and vitamins are micronutrients with proven antioxidant properties; these can reduce inflammation by exerting immunomodulatory activity (17-19). Calcium, biphasic calcium phosphate, and magnesium play significant roles in bone metabolism and seem to improve bone density (13,14,20). The activity of these elements may increase oral mucosal trophism and soft/hard tissue healing and regeneration as well as reduce local and systemic inflammation; however, till date, no study has assessed the effects of a combination of these micronutrients.

The Mediterranean diet (MD) is characterized by the high consumption of olive oil, vegetables, and fruits rich in antioxidants and vitamins. Adherence to MD has been correlated with a lower risk of systemic and inflammatory diseases (21,22).

The effects of a multimicronutrient food supplement containing the co-enzyme Q10 multicomposite, docosahexaenoic acid (DHA), *B. serrata*, vitamins, mineral salts, and fish oil as source of n-3 PUFAs on salivary levels of MMP-8 and MMP-9, and serum levels of CRP were assessed in Italian patients that adhered to MD, affected by severe chronic generalized periodontal disease and treated with non-surgical therapy.

Materials and Methods

This double-blind clinical trial comprised a total of 30 patients who were enrolled and randomly assigned to one of two experimental groups. Each subject was informed about the study protocol, and written internal review board-approved informed consent to participate in the study was obtained from all patients. This study was conducted in accordance with the 1975 Declaration of Helsinki, as revised in 2013, and was registered at clinicaltrials.gov (NCT02315222). The patients were treated at the Unit of Periodontology, Dental Clinic, Department of Biomedical, Surgical, and Dental Sciences, University of Milan, IRCCS Foundation (Istituto di Ricovero e Cura a Carattere Scientifico-Scientific Institute of Hospitalization and Care) Ca' Granda Polyclinic Milan, Italy.

All the enrolled patients had severe chronic generalized periodontal disease requiring nonoperative periodontal therapy. The inclusion criteria were as follows: at least two sites with a probing pocket depth (PPD) >7 mm, bleeding on probing >25%, age 18-60 years, and adherence to MD. The exclusion criteria were: other local diseases that may affect the periodontal status (e.g., acute ulcerative

necrotizing gingivitis and gingival hyperplasia), smoking >15 cigarettes a day, cardiovascular diseases, metabolic disorders that may alter the formation and maturation of the connective tissue (e.g., diabetes mellitus and rheumatologic diseases), pregnancy or lactation, antibiotic intake <3 months before the study, and restrictive dietary regimens (limitation due to intolerance or philosophy).

After their inclusion in the study, according to predefined randomization tables, each patient was randomly assigned by an operator (SF) to either of the two experimental groups (Groups A and B). To reduce the chance of unfavorable splits regarding key prognostic factors between the treatment groups, smoking habits (presence/absence) were considered for the randomization process.

The patients assigned to group A consumed dietary supplements in the form of micronutrient complex tablets containing Co-enzyme Q10 multicomposite; *B. serrata*; vitamins B3, B5, C, and H; sodium fluoride; calcium; biphasic calcium phosphate; magnesium; xylitol; and liquid-filled capsules containing fish oil (DHA 70% + eicosapentaenoic acid [EPA] 10% and vitamins A and D).

The patients assigned to group B were administered inert tablets (without the micronutrient complex) and olive oil-filled capsules.

Treatment

Immediately after inclusion in the study (T0), clinical measurements were taken, saliva samples were collected and processed as reported below, and serum analysis was performed. Furthermore, each patient received oral hygiene-related instructions and regular professional supragingival debridement. Supplementation with the micronutrient complex (group A) or the olive oil-filled capsules (group B) was assigned and initiated at T0.

Four weeks after initiating the therapy (T1), the clinical measurements, saliva sample collection and processing, and serum analysis were repeated. Next, one-stage full-mouth disinfection (FMD) was conducted in both groups as described further. Scaling and root planning (SRP) of all pockets was performed using ultrasonic devices and hand instruments (Gracey standard and mini-five curettes; Hu-Friedy, Chicago, IL, USA) within 24 h combined with extensive application of chlorhexidine (0.20%) to all intraoral niches (periodontal pockets, tongue dorsum, and tonsils) with systemic antibiotic treatment (1 g amoxicillin twice daily for 8 days + 250 mg metronidazole three times daily for 8 days) (23,24). Three months after T0 (T2), the clinical measurements, saliva sample collection and processing, and serum analysis were repeated again.

To summarize, the patients were administered the micronutrient complex (tablets/capsules) or the inert tablets/olive oil capsules for 3 months (from T0 and until 2 months after FMD-T2). In group A, each patient took one micronutrient complex tablet in the morning (with breakfast) and one capsule in the evening (with dinner). Group B patients took one inert tablet in the morning (with breakfast) and one capsule containing olive oil in the evening (with dinner) as prescribed in group A. Compliance was evaluated by counting the number of tablets and capsules taken at the end of the study.

Measurements and analysis

Clinical measurements

To evaluate inflammatory status, plaque control, and periodontal disease severity, the following clinical measurements were performed at T0, T1, and T2 by an examiner (SF) blinded to the treatment and assigned to each patient: full-mouth plaque score (FMPS) (25), full-mouth bleeding score (FMBS) (26), PPD, percentage of sites with PPD (%PPD) >3 mm, gingival recession (REC), and clinical attachment level (CAL) calculated as PPD+REC. These clinical measurements were obtained using a UNC-15 mm periodontal probe (Hu-Friedy).

X-rays were imaged via a parallel technique at baseline during the diagnostic process of periodontal disease.

Serum analysis

CRP was evaluated as a marker of systemic inflammation (6). In all patients, blood samples collected at T0, T1, and T2 were analyzed.

Saliva collection and analysis

Unstimulated whole saliva was collected from each subject at the beginning of the study, as described previously by Ramseier et al. (2). Briefly, the subjects rinsed their mouths with tap water for 20 s to remove gross debris and then expectorated the water. After 2 min, whole saliva was passively expectorated by the patients into the plastic funnel placed on a plastic tube. Each tube was labeled with the subject's initials, harvest date, and sample name. The collection was completed once 2 mL of the whole saliva was collected or a maximum of 15 min of sampling time was reached. The sample was then immediately placed on ice, supplemented with a proteinase inhibitor combination of 1% aprotinin (1 mg/mL) and 0.5% phenylmethylsulphonyl fluoride (200 mM in methanol; Sigma Chemical Company, St. Louis, MO, USA), and aliquoted before storage at −80°C until analysis. MMP-8 and -9 in the saliva samples were quantified using a custom human protein array (Quantibody

Table 1 Clinical data (mean and standard deviation) of group-A and -B patients

Group A			
(mean ± SD)	T0	T1	T2
FMPS (%)	51.6 ± 21.4	41.1 ± 17.2	31.3 ± 18.8
FMBS (%)	41.7 ± 8.8	29.5 ± 8.9	15.3 ± 6.7
PPD (mm)	3.6 ± 0.6	3.4 ± 0.6	2.8 ± 0.5
PPD >3 (%)	43 ± 16.1	36.6 ± 16.4	18.6 ± 16.4
CAL (mm)	4.0 ± 0.7	3.8 ± 0.8	3.3 ± 0.7
Group B			
(mean ± SD)	T0	T1	T2
FMPS (%)	63.1 ± 19.2	38.1 ± 14	28.1 ± 12.4
FMBS (%)	42.4 ± 12.9	31.8 ± 12.8	15.5 ± 8.1
PPD (mm)	3.7 ± 0.7	3.4 ± 0.5	2.9 ± 0.6
PPD >3 (%)	46.2 ± 19.9	38.3 ± 13.9	23.6 ± 15.7
CAL (mm)	4.3 ± 0.8	4.0 ± 0.6	3.6 ± 0.6

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PPD: probing pocket depth; CAL: clinical attachment level; SD: standard deviation.

Custom Array; RayBiotech, Inc., Norcross, GA, USA). Before each assay, whole saliva samples were thawed and microcentrifuged at 500 g for 5 min at 5°C to obtain a cell-free supernatant for analysis. Each well contained cytokine standards used for making known serial dilutions, with sample diluent serving as the negative control. The experimental samples were then incubated overnight at 4°C followed by washing with wash buffer to remove the unbound material. The detection antibody was then bound to the antigens within each well. Cy3 equivalent dye-conjugated streptavidin, which binds to the detection antibody associated with the immune complexes, was pipetted to each well. The wells were incubated and covered with aluminum foil to avoid exposure to light, and fluorescence from each well was detected using a laser scanner (Axon Gene Pix; RayBiotech, Inc.). The resultant sample signals were compared against the standard curve for each cytokine to determine the cytokine concentrations within the samples. Data were extracted and analyzed using the microarray analysis software (RayBio Q Analyzer software; RayBiotech, Inc.).

Statistical analysis

The sample size was calculated using $\alpha = 0.05$ (5%) and a power of 80%. For variability, the value of 28.40 ng/mL (standard deviation of MMP-8 concentration in unstimulated saliva) obtained in previous reports was considered (3). The minimum significant value considered was 29.29 ng/mL (3). Based on these data, the number of patients required to be enrolled was 15 for group A and 15 for group B. In both groups, the mean and standard deviation were computed for all data at each time point: (1) clinical: FMPS (%), FMBS (%), mean PPD (mm), sites with %PPD > 3 mm (%), CAL (mm); (2) serum: CRP (mg/dL); and (3) saliva: MMP-8 and-9 (ng/mL).

The Friedman test was applied to study the trends over time within each group. The Mann-Whitney *U*-test was used to calculate the between-group differences for each time point. The Wilcoxon signed-rank test was used to study differences in the data between different time points in the same group. The Spearman's rank correlation was used to assess correlations between all (clinical, serum, and saliva) data. The data were analyzed with SAS PROC MIXED (SAS Institute, Inc. 2008. SAS/STATVR 9.2 User's guide; SAS Institute, Inc., Cary, NC, USA). A 5% level of significance ($P < 0.05$) was considered.

Results

Of the 30 patients (15, group A; 15, group B) enrolled in the study between June 2014 and January 2016, two from group A and four from group B discontinued participation, leaving only 24 patients available for analysis (six males and seven females in group A [mean age, 50.2 years] and five males and six females in group B [mean age, 49.9 years]). There were four smokers (44.4%) in group A and three (37.5%) in group B (P = not significant between the groups).

Clinical data

The clinical data of both groups are reported in Table 1. At the inter-time point analysis, the FMPS and FMBS levels, mean PPD and CAL, and sites with %PPD >3 mm were significantly reduced for all time points (Wilcoxon signed-rank test, $P < 0.05$) in both groups. For each time

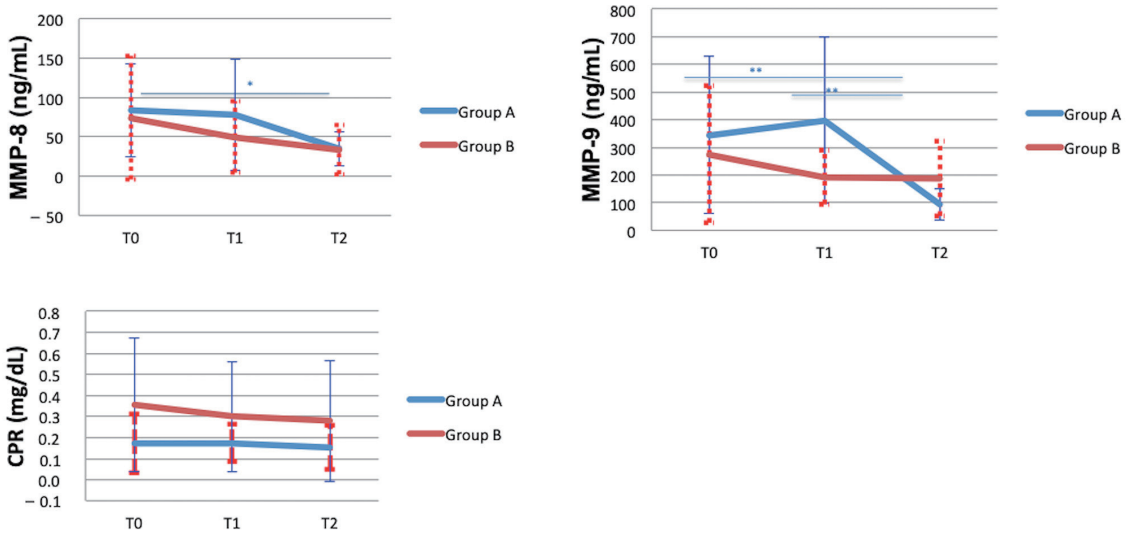

Fig. 1 Metalloproteinases-8 (MMP-8) and -9 (MMP-9) levels in the saliva and C-reactive protein (CRP) in the serum of group A and B patients.
*$P < 0.05$. **$P < 0.005$ (Wilcoxon signed-rank test).

point, no significant differences were observed between the groups (Mann-Whitney *U*-test).

Saliva and serum proteins

At the analysis for each time point (baseline, 1 and 3 months) of differences between groups on salivary and serum MMP-8/-9 levels, no significant differences were found.

At the intra-group longitudinal analysis, in the saliva of group A patients, both analyzed proteins decreased significantly (Friedman test, $P = 0.008$ for MMP-8 and $P = 0.003$ for MMP-9). In particular, compared with baseline, the MMP-8 and -9 levels were significantly reduced at 3 months (Wilcoxon signed-rank test, $P = 0.013$ and $P = 0.004$, respectively) and the MMP-9 levels were significantly reduced at 1 month ($P = 0.004$). In group B, the reduction of both proteins in the saliva samples was not significant at 1 or 3 months (Fig. 1).

The serum CRP levels of the patients in both groups were reduced at 3 months compared with baseline, but not significantly (Fig. 1).

Analysis of correlations

Significant correlations (Spearman's rank correlation) were found in both groups between FMPS and FMBS ($P < 0.05$ for group A, $P < 0.000001$ for group B), MMP-8 and MMP-9 ($P < 0.0001$ and $P < 0.00001$, respectively), FMBS and MMP-8 ($P < 0.001$ and $P < 0.001$, respectively), %PPD >3 mm and FMPS ($P < 0.05$ and $P < 0.001$, respectively), %PPD >3 mm and FMBS ($P < 0.0001$ and $P < 0.001$, respectively), and PPD and FMBS ($P < 0.0001$ and $P < 0.001$, respectively).

Correlations were found in group A between FMPS and MMP-9 ($P < 0.05$), PPD and MPP-8 ($P < 0.05$), and PPD and MMP-9 ($P < 0.05$).

A correlation was found in group B between PPD and FMPS ($P < 0.001$).

Discussion

Micronutrients play key roles in maintaining systemic as well as periodontal health (2). In the literature, the adjunctive effect of micronutrients on improved periodontal status after the treatment of periodontitis remains uncertain. The purpose of the present study was to assess the effect, on periodontal clinical status, local tissue remodeling and systemic inflammation, of a multimicronutrient dietary supplement formulated to improve the oral mucosa trophism as well as soft and hard tissue healing response in a population that adhere to the Mediterranean diet. In particular, salivary MMP-8 and -9, a serologic marker of systemic inflammation (CRP) and clinical parameters of periodontal status were assessed in patients with severe periodontal disease who underwent to non-surgical periodontal therapy and were daily administered with either the micronutrient complex or olive oil. Olive oil is a common ingredient in MD and contains monounsaturated fatty acids (MUFA) and polyphenols. These micronutrients exert beneficial effects on systemic markers of inflammation and endothelial function, and have shown promising activities in different inflammatory and autoimmune diseases (27,28). A study conducted in rats, that reproduced an age-dependent model of the periodontium, reported that fish and olive oils seem to prevent age-related alveolar bone resorption

through the contrast of oxidative stress. In the Italian population, olive oil is largely and spontaneously integrated to the diet since it is daily added as dressing for food. As a consequence of its wide use, the supplementation of a minimal dose of this component to the diet may not determine additional effects thus, in this study, it was chosen as a control.

The data obtained demonstrated that after non-surgical periodontal therapy, the clinical parameters, including local tissue inflammation (FMBS), were significantly enhanced in both groups. The improvement of periodontal clinical status after therapy has been widely illustrated in literature, and results of the current study seem to demonstrate that micronutrient complex does not provide additional benefits on the clinical outcomes.

The effect of micronutrients on immune system modulation and decreased tissue inflammation has been reported (18,19). Co-enzyme Q10 plays a key role in ATP synthesis and energetic metabolism and has a strong antioxidant function. A recent study reported the function of Co-enzyme Q10 as an inhibitor of osteoclast differentiation (19). *B. serrata* demonstrated beneficial effects on immune system modulation (18). This micronutrient reduces leukotriene synthesis in intact neutrophils by inhibiting 5-lipoxygenase, the key enzyme involved in leukotriene biosynthesis and the inflammatory process; thus, it exerts beneficial effects on some chronic inflammatory diseases, such as rheumatoid arthritis (18). DHA Omega 3 (Resolvine) has important antioxidant functions, protects cells from aging and moderates anti-inflammatory mechanisms reducing the protein levels of inflammatory cytokines, including tumor necrosis factor-α, interferon-γ, IL-1, IL-2, and vascular cell adhesion molecule-1 (17). In patients with chronic periodontal disease treated with a non-surgical approach, the adjunctive administration of omega-3 PUFAs and low-dose aspirin improved periodontal clinical parameters and induced a significant reduction of the salivary RANKL and MMP-8 levels compared with the control treatment (SRP + placebo) (29). Vitamins play important roles in systemic as well as oral health maintenance and promotion owing to their potential antioxidant roles in cell metabolism, repair, and proliferation; collagen synthesis; immune function modulation; and inflammation (7). Olive oil, with its MUFA content and polyphenols, significantly decreased the markers of inflammation, including CRP and IL-6 (27). In particular, olive oil polyphenols exert strong antioxidant activity, reduce vascular endothelial growth factor-induced angiogenic responses, and decrease MMP-9 and -2 expression (30). This may explain the beneficial effects of this food on chronic degenerative diseases, including cardiovascular and autoimmune diseases (28,31,32).

MMP-8 and -9 are released during periodontal inflammation, and their levels in saliva seem to be positively associated with clinical signs of gingival inflammation (33). Their analysis has been proposed for the assessment of periodontal treatment outcomes. Analysis of clinical parameters revealed that plaque reduction was correlated with the clinical indicator of periodontal inflammation (FMBS), thereby confirming the improvement of the tissue health by removal of the causal agent in accordance with the literature (24). Likewise, the correlation between the salivary MMP-8 amount and FMBS in both groups denotes the overall reduction of the clinical and cellular inflammatory activity, and confirms this protein as a marker for monitoring periodontal treatment outcomes. Furthermore, the correlation between these clinical and salivary data indicates that the tested food supplement does not selectively affect the two analyzed inflammatory parameters (FMBS and salivary protein). In the present study, at 3 months after baseline, the levels of MMP-8 and -9 decreased and, at each timepoint, no differences were found between the groups. However, the lapse of 3 months may be too short to indicate the benefits of an improved and still ongoing connective tissue healing process by means of clinical investigation. Infact, the ability to predict the periodontal clinical stability by analyzing levels of these proteins in GCF of patients has been demonstrated in a trial with 6 months of follow-up (34). Indeed, after injury, the connective tissue is actively remodeled and takes 6-12 months to mature (35). It would be interesting to observe the periodontal status of the treated patients at a mid-long-term time point.

The differences between the groups were found in the decreasing trend of MMPs. In patients who received the micronutrient complex supplementation, at 1 month after baseline, the levels of both MMP-8 and -9 remained almost stable. However, at 3 months after baseline, these levels significantly decreased. In contrast, in patients taking olive oil, the levels of these proteins were already reduced at 1 month after baseline, despite the lack of significance, and between 1 and 3 months were stabilized. These data indicate that in control patients, the soft-tissue healing response may occur faster but with less intensity than in patients taking the micronutrient complex, in whom this response seems to be delayed. It is not possible to establish the nature of this different behavior owing to the complex composition of the supplement investigated in the present study. The factors that accelerate tissue response to non-surgical therapy must be assessed in

future studies.

After baseline, although not significantly, the serum CRP levels decreased, and no differences were found between the groups. These data corroborate with those of clinical studies that analyzed the effects of periodontal therapy on systemic inflammation and endothelial function (6,36,37). The literature reports a significant increase in the CRP levels as well as in the blood markers of systemic inflammation in response to non-surgical periodontal therapy only immediately after debridement (within the first week) (37). One month after the treatment, the serum CRP levels decreased to lower than baseline, although not significantly (37).

The analyzed serum and salivary parameters (MMPs and CRP) showed large variability in the patients of the current study, and this may be due to the different eating habits of the subjects in this study. Because no particular diet was prescribed and only patients with restrictive dietary regimens were excluded, it was hypothesized that the food intake masked the effects of the investigated supplement. In a population that adheres to MD, a food supplement comprising a complex of micronutrients does not seem to provide adjunctive benefits compared with olive oil. Furthermore, variability in an individual's metabolism may also induce a hyper- or hyporesponse to the food supplement. Further studies including a larger population and prescribing a particular diet regimen should be designed to investigate the effects of such food supplements. Because the present study aimed to compare the effects of two different micronutrient supplements on oral wound healing and not to assess the beneficial effects of those supplements on the physiologic healing process, a control group treated with placebo was not included in the study design.

In conclusion, the data reported herein confirm that salivary MMP-8, but not serum CRP, is a useful marker for assessing the reduction in periodontal local inflammation and improving the clinical outcomes. Despite the limitations of the present study regarding the limited sample size and the absence of a prescribed dietary regimen, the investigated micronutrient complex did not affect the salivary levels of MMP-8 and -9, serum CRP, and the clinical outcomes after nonoperative periodontal therapy in patients who adhered to MD. However, the decreased levels of the analyzed MMPs appeared more pronounced, although delayed, in patients treated with the micronutrient complex than controls. Future studies on a larger number of patients with a longer follow-up are needed to confirm this trend and to verify the additional benefits of such dietary supplements.

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Conflict of interest

The authors have no conflict of interest to declare.

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