Inflammatory biomarkers in saliva and serum of patients with rheumatoid arthritis with respect to periodontal status

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Abstract

Objective: To study prospectively the association of salivary and serum matrix metalloproteinase (MMP)-8, tissue inhibitor of MMPs (TIMP)-1 and interleukin (IL)-6 with periodontal and systemic inflammation in rheumatoid arthritis (RA). We hypothesized that biomarker concentrations reflect inflammation.

Methods: Fifty three early untreated RA (ERA) and 28 chronic RA (CRA) patients, underwent rheumatological and dental examinations at baseline and one year later after starting first conventional or biological disease modifying antirheumatic drug. We included 43 control subjects. Saliva and serum samples were analyzed for MMP-8, TIMP-1 and IL-6. Periodontal health was assessed by bleeding on probing (BOP), pocket depth (PD) and periodontal inflammatory burden index (PIBI); RA disease activity was assessed by disease activity score DAS28. Joint destruction was analyzed by the modified Sharp-van der Heijde (SHS) method.

Results: Serum MMP-8 ($p<0.001$; $p<0.001$) and IL-6 ($p<0.001$; $p=0.002$) were significantly higher in CRA *vs.* other study groups during the study. Salivary MMP-8 ($p=0.010$) and IL-6 ($p=0.010$) were significantly higher in ERA *vs.* other study groups at baseline. Salivary MMP-8 associated with periodontal parameters.

Conclusion: Elevated serum concentrations of MMP-8 and IL-6 in CRA patients reflected chronic RA, while elevated salivary concentrations of MMP-8 levels in ERA patients reflected increased periodontal inflammation.

Key messages

1. Concentrations of inflammatory biomarkers in serum and saliva were different between patients with RA and healthy controls.

2. Concentrations of MMP-8 and of IL-6 in serum were elevated in patients with chronic RA reflecting joint inflammation and the burden of established RA.

3. Concentrations of MMP-8 in saliva was elevated already at the early stage of RA and the level of salivary MMP-8 was associated with poor periodontal health both in patients with early and in those with chronic RA.

Introduction

Inflammatory destruction of connective tissue and surrounding bone is characteristic to both periodontitis and rheumatoid arthritis (RA). The destruction is a result of the actions of co-operative cytokines, reactive oxygen species and proteolytic enzymes together with other proinflammatory mediators. Several inflammatory biomarkers related to periodontitis and systemic diseases can be detected in saliva. These include, but are not limited to, interleukins-1β, -6, -8 and -18 (IL-1β, -6,-8, -18), tumor necrosis factor-α (TNF-α), matrix metalloproteinases (MMP)-8 and -9, and tissue inhibitors of MMPs (TIMP)-1 [1, 2]. Further, whole saliva of RA patients may contain serum-derived inflammatory biomarkers originating from inflammatory cells, collagen breakdown or bone remodeling [3].

MMP-8 is a collagenase and the major tissue destructing MMP present in periodontitis-affected gingiva produced mainly by polymorphonuclear neutrophils [4-6]. Gingival fibroblasts, endothelial cells, sulcular epithelial cells, plasma cells, and odontoblasts can also *de novo* express and produce MMP-8 [4, 7].

Increased levels of MMPs in serum have also been connected to many diseases such as development, growth and metastasis of tumors, atherosclerosis, osteoarthritis and RA [8–10]. MMP-1, -2, -3, -8, -9,-13 and -14 have been demonstrated in articular cartilage [11]. MMP-3, -8, and -9 are present mainly in the tissue destructive pathologic processes [12]. MMP-8 is the catalytically competent collagenase acting in the cartilage aggrecan and type II collagen degradation [5]. Increased MMP-8 levels in synovial fluid in inflamed joints and serum have been reported in patients with RA [13].

TIMPs control the activity of MMPs. The MMP-8/TIMP-1 ratio reflects the net inflammatory activity of MMP-8. A disturbed balance of MMPs and TIMPs is found in various pathologic

conditions, such as cancer, cardiovascular diseases, RA and periodontitis. In a study by Rathnayake et al. [14] the mean value of salivary MMP-8/TIMP-1 ratio in patients with no periodontitis was reported to be 0.46±0.56, while the ratio was even 0.97±1.80 in those with advanced periodontitis. TIMP-1 usually dominate in healthy periodontal tissue and fluids and protect from the effect of MMPs, but in inflamed periodontal tissue and fluids, MMP-8 are often pathologically elevated to the levels unbalanced by TIMP-1 leading to irreversible tissue destruction [6]. Repeatedly pathologically elevated concentrations of active MMP-8 in oral fluids reflect and associate with the severity of periodontal disease [4, 7].

IL-6 is a pleiotropic cytokine [15] with an important role in the regulation of immune process regulation and the sustained over production of this cytokine leads to inflammation. It is the main inducer of C-reactive protein (CRP), fibrinogen and serum amyloid A protein, as well as other factors [16]. Elevated values of IL-1β, IL-6, MMP-8 and TNF-α have been detected in inflamed joints and serum in RA patients. Earlier studies [17, 18] have shown that the overproduction of IL-6 (i.e. detected in synovial fluid) correlates to disease activity of RA. Further, IL-6 concentration in gingival crevicular fluid increases during periodontitis [19]. Increased concentrations of IL-6 in plasma have been found in patients with periodontitis [20, 21].

We have recently reported that RA patients with early (ERA) and chronic (CRA) have high frequency of periodontitis even at the time of diagnosis and before any antirheumatic therapy [22]. In the present study, our aim was to investigate the concentrations of MMP-8, TIMP-1 and IL-6 in serum and saliva in RA patients and control participants and to correlate the levels with the periodontal status. Furthermore, we evaluated the impact of disease activity of RA and treatment of RA on these biomarkers. The hypothesis was that inflammatory biomarkers differ between RA patients and controls and that, periodontal status as well as the disease activity of RA and its treatment affect their concentrations of the biomarkers in serum and saliva.

Subjects and methods

Study design

RA patients attending the Department of Rheumatology at the Helsinki University Hospital were invited to participate in this prospective follow-up study. The patients received oral and written information about this study by a doctor. We recruited 53 patients with untreated ERA and 28 CRA patients with active RA with inadequate response to synthetic disease modifying anti-rheumatic drugs (DMARDs). Age- and gender-matched control subjects living in the same geographic area as RA patients were selected using Statistics Finland, the national database. The control subjects were contacted by letter with a similar description of the study as received by the RA patients and were invited to participate the study. The 43 control subjects were examined once. The study protocol has been described previously [22]. In brief, rheumatological and oral examinations in RA patients were conducted twice, at baseline and after initiation of new DMARD treatments with a follow-up with a mean of 15.9±6.1 months. After the baseline examinations, ERA patients started treatment with synthetic DMARDs comprising methotrexate (MTX), leflunomide, sulfasalazine and hydroxychloroquine either as monotherapy or in various combinations. CRA patients started biological DMARDs consisting of TNF-α inhibitors or non-TNF-α biologicals mainly combined with MTX.

We included RA patients between 18-70 years of age. One rheumatologist and one dentist examined each patient independently and were blinded from each other regarding the rheumatological and dental conditions. The rheumatological examination was performed according to our hospital protocol at the Department of Rheumatology. The dental examinations were conducted in the Department of Oral and Maxillofacial Diseases. All patients and control participants gave written informed consent to participate in the study. The study protocol has been

approved by the independent review board of the Helsinki and Uusimaa Hospital District (no 240/2004, 16 June 2004) and the study was conducted according to the principles of the Declaration of Helsinki.

Study population

ERA patients were referred to the Department of Rheumatology for diagnosis and treatment. The patients were mostly women (85%) with a mean (± SD) age of 51±15 years and they had suffered from the symptoms of RA for a mean of 10.4±17.1 months. CRA patients also were mostly women (82%) with a mean age of 52±11 years and with a mean duration of the RA of 176±116.8 months. The patients fulfilled the 1987 classification criteria for RA [23]. The mean age of control participants (88% were women) was 56±13 years (Table 1). During the follow-up, two patients of ERA group moved to another district, four participants interrupted the study for personal reasons and one patient died between the dental and rheumatological examinations. Two patients of the CRA group refused to participate in the follow-up examination.

Clinical dental examinations

Complete dental status with panoramic jaw tomogram and bite-wing x-rays was recorded of all subjects. Natural teeth were examined. Dental implants and third molars were excluded in the recording. Periodontal parameters were recorded according to the WHO recommendations [24], degree of periodontitis was defined according to the Center for Disease Control and Prevention and the American Academy of Periodontology (CDC/AAP) [25, 26]. Details have been described previously [22].

Periodontal inflammatory burden index (PIBI) was calculated according to formula PIBI = Σ [Nmod PPD (probing pocket depth) + 2Nadv PPD], where Nmod= number of sites with moderate periodontal lesions (4-5mm) and Nadv= number of sites with advanced periodontal lesions (≥6mm)

[27]. Probing depth (PD) and clinical attachment loss (AL) were assessed at four sites per tooth from every tooth. Bleeding on probing (BOP) was reported as percentages (positive sites/all sites).

Unstimulated and stimulated saliva samples were collected for 5 minutes. Paraffin wax chewing was used for saliva stimulation [28, 29]. Flow rates were measured using graded test tubes and recorded as millimeters per minute. Saliva samples were available from all patients and controls at baseline. At follow-up examination, saliva samples were collected from 47 patients with ERA and 26 patients with CRA.

Analysis of MMP-8, TIMP-1 and IL-6

MMP-8 was analyzed by time-resolved immunofluorometric assay (IFMA) [14, 30, 31-33]. The monoclonal MMP-8- specific antibodies (8708 and 8706, Medix Biochemica, Espoo, Finland) were used as catching antibody and a tracer antibody. The tracer antibody was labeled with europium-chelate [34]. The assay buffer contained 20 mM Tris-hydrochloride (pH 7.5), 0.5 M sodium chloride, 5 mM calcium chloride, 50 μM zinc chloride , 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/L diethylenetriaminepenta-acetic acid. Saliva and serum samples were diluted in assay buffer and incubated for an 1 hour, followed by incubation for another hour with tracer antibody. Enhancement solution was added and after 5 minutes, fluorescence was measured using an IFMA (1234 Delfia Research Fluorometer, Wallac, Turku, Finland). The specificity of the monoclonal antibodies against MMP-8 corresponds to that of polyclonal MMP-8 [35]. The detection limits and inter-assay coefficients of variation were 0.08 ng/mL and 7.1% for MMP-8, respectively [36].

The concentrations of TIMP-1 and IL-6 in saliva and serum were analyzed by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Europe Ltd, Abingdon, UK for TIMP-1 and eBioscience Inc., San Diego, CA, USA for IL-6). The

detection limits and inter-assay coefficients of variation were 7.8 pg/mL and 3.4% for TIMP-1 and 0.39 pg/mL and 4.2% for IL-6, respectively [37].

Rheumatological examinations and serum analyses

The RA patients were examined by a rheumatologist according to our hospital protocol. The number of swollen (66 joint count and 28 joint count) and tender joints (68 joint count and 28 joint count) were recorded. The patient's global assessment of disease activity (PGA) was based on a 100 mm visual analogue scale. Disease Activity Score (DAS28) was calculated from the number of tender and swollen joints (28-joint count), patient global assessment (PGA) and erythrocyte sedimentation rate (ESR) [38]. Blood samples were collected from all participants and analyzed for rheumatoid factor (RF), anticyclic citrullinated peptide antibody (CCPAb), CRP, and ESR.
The treatment response was evaluated using the European League Against Rheumatism (EULAR) criteria [39, 40]. Radiology of hands and feet of the patients were examined at baseline and at follow-up. Radiographs were scored according to the modified Sharp-van der Heijde (SHS) method by an expert in joint radiology blinded with regard to the clinical data and treatments [41].

Statistical methods

The results are given as medians with IQRs (25–75%; non-parametric distribution) or in means with SDs (parametric distribution). Correlations for nonparametric data were analyzed by the Spearman rank correlation coefficients. Non-parametric Kruskal-Wallis and Mann-Whitney tests were used when comparing independent samples. The baseline and follow-up values were analyzed with non-parametric Wilcoxon test comparing distributions between related samples. No corrections were made for multiple testing. Statistical analyses were performed with SPSS V.24 and $p < 0.05$ was considered as statistically significant.

Results

Demographic data of the study groups are given in Table 1. Moderate or high disease activity (DAS≥3.2) of RA was present in 69.8% of the ERA and in 67.8% of the CRA patients.
RA patients had more periodontal findings assessed by PD≥4mm, BOP and PIBI compared to controls (Table 1).

Biomarkers in serum

At baseline, the concentration of MMP-8 was significantly higher in the patients with CRA compared with the patients with ERA and with the control subjects ($p<0.001$, Table 2, Figure 1). During the study, the MMP-8 concentration decreased in the patients with ERA ($p<0.001$). In contrast, there was no statistically significant change in MMP-8 concentrations from baseline to the follow-up in the patients with CRA (Figure 1 and Table 2). However, after follow-up, the serum concentration of MMP-8 was significantly higher in CRA patients *vs.* ERA patients (Table 2).

The concentration of MMP-8 (median, IQR) in RF-positive ERA patients was 44.8 ng/mL (16.0-121.8) and in RF- negative 16.0 ng/mL (9.2-48.4, $p=0.133$), respectively. In CCPAb-positive ERA patients the concentration of MMP-8 was 54.0 ng/mL (22.3-121.9) and in CCPAb-negative 9.2 ng/mL (7.6-22.4, $p<0.001$), respectively. In RF-positive CRA patients the respective MMP-8 values were 222.2 ng/mL (53.1-337.5) compared with 37.4 ng/mL (12.0-147.3, $p=0.022$) in RF negative, respectively. In CCPAb-positive *vs.* CCPAb-negative CRA patients the values were 130.0 ng/mL (40.8-280.6) and 144.4 ng/mL (10.8-144.0, $p=0.529$), respectively.

The concentration of TIMP-1 was significantly higher in the control participants compared to that in the RA patients at baseline, ($p<0.001$; Table 2) and decreased during the study in the CRA patients, ($p=0.016$; Table 2). The MMP-8/TIMP-1 ratio was significantly higher in the CRA patients both at baseline and at follow-up compared to the ERA patients (Figure 1, Table 2).

The concentrations of IL-6 were significantly higher in the ERA and CRA patients at baseline (Table 2), compared to that in the controls, (ERA *vs.* controls, $p<0.001$; CRA *vs.* controls, $p=<0.001$, Figure 2). In the CRA patients the IL-6 concentrations remained significantly increased compared to ERA group at follow-up ($p=0.002$, Table 2).

Serum MMP-8, TIMP-1, MMP-8/TIMP-1 ratio, IL-6 and periodontal parameters

The concentration of MMP-8 had no significant association with any periodontal parameter at baseline among the study groups (Table 3). The concentration of TIMP-1 correlated positively with the number of PD≥ 4mm and the PIBI scores in the controls and with BOP in the ERA patients at baseline (Table 3).

The MMP-8/TIMP-1 ratio associated significantly negatively with the BOP in the control group. In the ERA patients, the concentration of IL-6 associated significantly positively with BOP at baseline. After follow-up, the concentration of MMP-8 associated significantly with BOP in ERA group, but no other significant associations were observed between periodontal parameters and the concentrations of MMP-8-, TIMP-1-, and IL-6-values or the MMP-8/TIMP-1 ratio, respectively. The results are given in detail in Tables 3 and 4.

Serum MMP-8, TIMP-1, MMP-8/TIMP-1 ratio, IL-6 and RA activity

In the ERA patients at baseline, DAS28 associated significantly with MMP-8, TIMP-1 and IL-6 concentrations and TIMP-1 also associated with the erosion score. After follow-up, significant associations were observed between DAS28 and TIMP-1 and IL-6. In the CRA patients, significant association was observed between DAS28 and IL-6 both at baseline and follow-up. Furthermore,

the concentration of MMP-8 associated with erosion score at baseline (Table 3). MMP-8/TIMP-1 ratio had no significant association with RA activity in neither ERA nor CRA patients (Table 4).

Biomarkers in saliva

The concentration of MMP-8 was significantly higher in the ERA patients *vs.* the CRA patients and the controls at baseline (ERA *vs.* CRA, p=0.016; ERA *vs.* controls, p=0.008), but not anymore at follow-up (Figure 1, Table 2). However, a comparison of the follow-up levels of the ERA and CRA patients with those of the controls shows elevated MMP-8 level in ERA: ERA *vs.* controls [221.0 (128.1-452.8) *vs.* 113.6 (76.0-226.8), p=0.007] and CRA *vs.* controls [175.6 (75.0-391.8), p= 0.396], respectively.

In the RF-positive ERA patients, the concentration of MMP-8 was 345.8 ng/mL (152.1-703.3) *vs.* 180.0 ng/mL (24.4-235.2) in the RF-negative ERA patients (p=0.026). In the CCPAb-positive ERA patients the concentration of MMP-8 was 350.8 ng/mL (169.6-703.3) *vs.* 186.0 ng/mL (87.6-342.4, p= 0.113) in the CCPAb- negative ERA patients. In the CRA patients the corresponding values of MMP-8 in the RF-positive patients were 113.8 ng/mL (50.7-344.5) *vs.* 180.4 ng/mL (110.8-180.4, p=0.468) in the RF-negative CRA patients. In the CCPAb-positive CRA patients MMP-8 was 105.6 ng/mL (38.8-290.8) *vs.* 144.8 ng/mL (38.8-169.2, p=0.173) in the CCPAb –negative CRA patients.

The concentration of TIMP-1did not differ significantly between the study groups at baseline or after follow-up (Table 2).

The concentration of IL-6 was significantly higher in the ERA patients compared to the CRA patients and controls at baseline, (ERA *vs.* CRA, p=0.021; ERA *vs.* controls, p= 0.006, Figure 2). After the follow-up, no significant difference was observed between the RA groups regarding the salivary IL-6 concentrations (Table 2).

Salivary concentrations of MMP-8, TIMP-1, IL-6 and MMP-8/TIMP-1 ratio and periodontal parameters

The concentration of MMP-8 correlated significantly positively with the number of PD≥ 4mm and the value of PIBI in the ERA, CRA and control groups at baseline. Further, higher concentrations associated significantly with higher BOP per cent sites at baseline in the patients with CRA and in the controls (Table 5). TIMP-1 had no association with BOP, the number of PD≥4mm or PIBI values in any study groups at baseline.

At baseline, salivary MMP-8/TIMP-1 ratio was significantly higher in the ERA patients compared to the CRA patients and the controls (ERA *vs.* CRA, p=0.025; ERA *vs.* controls, p=0.030, Figure 1, Table 2). Further, the ERA patients had significantly elevated MMP-8/TIMP-1 ratio when compared the follow-up levels with those of the controls [1.9 (1.2-4.0) *vs.* 1.3 (0.8-2.7) p=0.018], Figure 1.

MMP-8/TIMP-1 ratio associated with the number of PD≥4mm and PIBI in both RA groups at baseline. After follow-up, a significant association was observed between MMP-8/TIMP-1 ratio and PD≥4mm, BOP and PIBI, in the patients with CRA, while in controls, the MMP-8/TIMP-1 ratio associated significantly with BOP, the number of PD≥4mm, and PIBI. The concentration of IL-6 had no association with periodontal parameters in the RA groups during the study. The results are given in detail in Tables 4 and 5.

Salivary concentrations of MMP-8, TIMP-1, IL-6 and MMP-8/TIMP-1 ratio, and RA activity

At baseline, no significant association was observed between the levels of MMP-8, IL-6 or MMP-8/TIMP-1 ratio and DAS28 and joint erosion score among the RA patients. The concentration of TIMP-1 associated significantly positively with the joint erosion score in the CRA patients (Table

5). After follow-up, a significantly positive association in the patients with ERA was observed between DAS28 and MMP-8 and also between erosions and the IL-6 levels (Table 5).

Discussion

We found that the serum concentrations of MMP-8 and IL-6 as a whole were significantly higher in patients with chronic RA consistently during the study. Further, the CCP antibody positivity associated with elevated serum MMP-8 levels in the ERA group, but not in the CRA group. MMP-8/TIMP-1 ratio in serum was also significantly higher in CRA patients during the study reflecting chronic activity of RA.

Salivary MMP-8 and IL-6 concentrations in general were higher in the patients with early RA when compared to the controls and the patients with chronic RA at baseline. The presence of RF associated with even higher levels of MMP-8 in the saliva of the ERA patients. Interestingly, CCP antibody positivity did not associate with the salivary MMP-8 levels in either RA group.

As expected, the disease activity of RA associated with serum concentrations of MMP-8 and IL-6 in the RA patients at baseline. Serum concentration of MMP-8 remained significantly elevated in patients with chronic RA during the study, which might indicate continuous inflammatory burden and joint destruction as has also been reported by Tchetverikov et al. [13]. Elevated serum MMP-8 levels can have prognostic significance, since these levels reflect progressive neutrophil activation predisposing to increased susceptibility to bacterial infections and systemic inflammation [10]. In earlier study by Mattey et al. [8], MMP-8 concentration in serum was reported to predict mortality in RA due to respiratory diseases. Furthermore, elevated serum MMP-8 concentration and especially serum MMP-8/TIMP-1 ratio, but not TIMP-1 alone, have often been connected to cardiovascular diseases [9, 10, 36].

During inflammatory process a variety of different cytokines control the production of acute phase proteins in hepatocytes in the liver. The main regulators are IL-1 and IL-6 type cytokines. IL-1β modifies the production of IL-6 induced acute phase proteins, such as CRP [42]. In our study, the serum IL-6 levels were significantly elevated in both RA groups when compared with the control group at baseline. The levels of serum IL-6 in patients with early RA were almost at the same level as in patients with chronic RA and serum IL-6 associated positively with the RA disease activity score DAS28 in both the RA groups reflecting the clinically active RA of both patient groups at baseline.

Of periodontal parameters, only BOP associated with the serum levels of IL-6 in ERA group at baseline. Thus, serum IL-6 is a marker of systemic inflammation and it seems to reflect RA disease activity more than that of oral inflammation. Antirheumatic medication as well as the use of glucocorticoids indeed suppress the production of IL-6 which then is reflected in lower serum concentrations [43-45]. Interestingly, Kobayashi et al. [46] suggested that the use of IL-6 receptor inhibition therapy (such as tocilizumab) might be advantageous for the periodontal health due to improvement in clinical periodontal condition when treating and suppressing the disease activity of RA with IL-6 inhibitors.

In this study RA patients in the early and chronic stage had more periodontal findings compared to controls, which is in line with previous reports as reviewed by Araújo et al. [47]. Salivary MMP-8 concentration associated significantly with the number of PD≥4mm and PIBI in RA patients and controls at baseline and also with bleeding on probing (BOP) in the CRA group and in controls. Further, salivary MMP-8 concentration and MMP-8/TIMP-1 ratio associated significantly with PD≥4mm and PIBI scores in patients with early RA, which finding might even suggest a pathogenic link between RA and periodontitis. This finding is also in line with a recent report by Kirchner et al. [48], who found a correlation between RA and periodontal disease with increased (active MMP-

8) aMMP-8 levels in saliva. Further, after follow-up, we observed significant association between salivary MMP-8 and periodontal parameters in the CRA group despite the long-term use of synthetic DMARDs and period of biological DMARDs. This confirmed our hypothesis in showing that periodontal inflammation is reflected in salivary biomarkers, such as MMP-8 in RA patients. The concentration of TIMP-1 in saliva did not differ significantly between the study groups during our study. Noteworthy, MMP-8/TIMP-1 ratio in saliva was significantly elevated in the ERA group at baseline reflecting the presence of periodontal inflammatory burden already in these DMARD naïve RA patients.

Salivary IL-6 concentration, on the other hand, did not associate with periodontal parameters in the present study which result differs from those of previous studies [49-51]. Our results are, however, in line with Gursoy et al. [52], Ramseier et al. [53] and Teles et al. [54], who observed no significant difference in the concentrations of salivary IL-6 between patients with periodontitis when compared with healthy controls.

The disease activity of RA and the joint erosion score did not associate significantly with salivary MMP-8 and IL-6 concentrations or with MMP-8/TIMP-1 ratio at baseline. After follow-up, an association was observed between salivary IL-6 and erosions and MMP-8 and DAS28 in patients with chronic RA. This might reflect high level of salivary IL-6 and MMP-8 in the RA group during the study.

Recent studies with aMMP-8 specific chairside tests have shown the association between MMP-8 levels in mouth rinse and severity of periodontitis [55-57]. Thus, in patients with RA it might be possible to use quantitative aMMP-8 chairside point-of-care immunoassay kits as an adjunctive diagnostic tool for detecting periodontitis as well as for active treatment and it`s evaluation in dental health care. This might help the clinician´s decision making when poor periodontal health should be taken into account among RA patients with various disease activity. Such diagnostic methods

would also provide useful screening tools in hands of non-dental medical professionals, *e.g.* rheumatologists and nurses [58].

Finally, we may conclude that in the present study serum concentrations of MMP-8 and IL-6 and the MMP-8/TIMP-1 ratio were higher in patients with chronic active RA compared to patients with early RA reflecting the chronic inflammatory condition of CRA. Disease activity and inflammation in the RA patients was reflected in serum MMP-8 and IL-6 concentrations. Further, RA patients had higher prevalence of periodontal findings compared to controls and this was most evident in early (DMARD-naïve) RA. This was reflected by increased MMP-8, MMP-8/TIMP-1 ratio (and IL-6) saliva levels in ERA, compared to CRA and controls and was further supported by significant correlations between salivary MMP-8 levels and the MMP-8/TIMP-1 ratio and periodontal parameters. These data could point to an etiological (causative) link between periodontal disease and RA, which has been discussed for a long time [59, 60].

As the concentrations of biomarkers in saliva and serum, such as MMP-8 and IL-6, differ between patients with RA compared to healthy controls and inflammation, originating from RA or periodontium, affects differently these inflammatory biomarkers in serum and in saliva differently, this finding might be useful in clinical decision making when treating patients with RA.

Disclosure statement

Dr Timo Sorsa is an inventor of US-patents 5652223, 5736341, 5866432 and 6143476.
No potential conflict of interest was reported by the authors.

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Table 1. Clinical characteristics of the study participants during the study.

	Early RA			Chronic RA			Controls	
	Baseline *n*=53	**Follow-up** *n*=47	*p*[¶]	**Baseline** *n*=28	**Follow-up** *n*=26	*p*[¶]	*n*=43	*p*[¶¶]
Age	51±15			52±11			56±13	0.160[*]
Women	45 (84.9)	40 (85.1)		23 (82.1)	22 (84.6)		38 (88.4)	0.758[*]
RF positive	42 (79.2)			18 (69.2)			3 (8.1)	**<0.001**[*]
CCPAb positive [‡]	37 (77.1)			15 (78.9)				0.574[*]
ESR (mm/h)	20 (11–34)	9 (5-16)	**<0.001**	20 (9-46)	16 (7-31)	**0.038**	2 (2-10)	**<0.001**
CRP (mg/L)	6 (3-14)	3 (2-6)	**0.001**	18 (5-30)	10 (2-21)	**0.012**	2 (2-3)	**<0.001**
DAS28	4.0 (3.2–4.8)	2.4 (1.7–2.9)	**<0.001**	4.1 (3.0–4.9)	3.1 (2.0–3.9)	**0.003**		0.974
Sharp Total [**]	3.9±11.9	4.8±12.9	**<0.001**	72.6±94.8	77.1±95.1	**0.001**		**<0.001**
Erosion score	2.7±7.7	3.5±8.2	**<0.001**	44.6±56.4	47.3±57.3	**0.001**		**<0.001**
Narrowing score	1.1±4.4	1.3±5.0	**0.041**	23.2±30.3	25.0±30.0	**0.002**		**<0.001**
BOP per cents sites	15 (10–26)	13 (6-21)	0.124	9 (5-19)	8 (3-22)	0.903	4 (2-8)	**<0.001**
PD≥4mm [‡‡]	45 (84.9)	43 (81.1)	0.250	25 (89.3)	21 (75.0)	1.000	28 (65.1)	**0.012**
PIBI	10 (3-18)	9 (6-19)	0.907	5 (3-15)	4 (1-16)	0.856	1 (0-3)	**<0.001**

The results are presented as *n* number of patients (%), mean ±SD or median with interquartile range (IQR).RA: rheumatoid arthritis; CRA: chronic RA; ERA: early RA; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: serum C-reactive protein; CCPAb: serum anticyclic citrullinated peptide antibody; DAS28: disease activity score (28-joint); BOP: bleeding on probing; PD: pocket depth; PIBI: periodontal inflammatory burden index.

[‡] data missing from 5 ERA patients and from 9 CRA patients.

[‡‡] at baseline data missing from 3 ERA patients, after follow-up data missing from 7 ERA and from 3 CRA patients.

[*] *p* value by Chi-Square crosstabulation; [**] score by modified Sharp van der Heijde method.

[¶] *p* value by nonparametric Wilcoxon test comparing related samples between baseline and follow-up.

[¶¶] *p* value by nonparametric Mann-Whitney U-test or Kruskal-Wallis test for other variables comparing study groups at baseline.

Statistically significant *p* values are shown in bold

Table 2. MMP-8, TIMP-1, MMP-8/TIMP-1 ratio and IL-6 concentrations in saliva and serum from the study participants during the study.

		Early RA			Chronic RA			Controls		
		Baseline *n*=53	Follow-up *n*=47	*p*[¶]	Baseline *n*=28	Follow-up *n*=26	*p*[¶]	*n*=43	*p*[¶¶]	*p*[¶¶¶]
MMP-8 ng/mL [¶¶¶¶]	**serum**	39.6 (12.6–115.6)	9.4 (6.5–26.3)	**<0.001**	140.8 (44.0–257.0)	90.8 (19.4–176.8)	0.124	23.2 (14.8–42.4)	**<0.001**	**<0.001**
	saliva	311.2 (105.6–524.8)	221.0 (128.1–452.8)	0.800	114.8 (40.8–290.8)	175.6 (75.0–391.8)	0.221	113.6 (76.0–226.8)	**0.010**	0.236
TIMP-1 ng/mL	**serum**	97.7 (76.9–121.2)	97.1 (82.9–117.4)	0.823	122.0 (96.8–145.3)	96.6 (85.0–136.0)	**0.016**	177.0 (150.5–208.1)	**<0.001**	0.439
	saliva	119.8 (87.3–149.7)	111.3 (83.3–157.4)	0.260	110.6 (80.0–163.9)	133.5 (90.3–177.5)	0.069	104.5 (90.5–147.1)	0.917	0.351
MMP-8/TIMP-1 ratio	**serum**	0.5 (0.2-1.0)	0.1 (0.1-0.3)	**<0.001**	1.5 (0.3-2.0)	1.1 (0.2-1.3)	0.266	0.1 (0.1-0.2)	**<0.001**	**<0.001**
	saliva	2.5 (0.9-5.5)	1.9 (1.2–4.0)	0.775	1.0 (0.6-2.3)	1.2 (0.7-2.8)	1.000	1.3 (0.8-2.7)	**<0.001**	0.145
IL-6 pg/mL	**serum**	5.3 (2.3–17.2)	0.9 (0.4-1.7)	**<0.001**	6.1 (2.7–17.5)	2.6 (0.8-10.1)	0.727	0.4 (0.4-1.1)	**<0.001**	**0.002**
	saliva	4.2 (2.4–6.6)	3.0 (2.2–7.1)	0.385	2.7 (1.6–4.7)	4.3 (1.5–8.2)	0.076	2.4 (1.4–5.6)	**0.010**	0.824

The results are presented as *n* number of patients, mean ±SD or median with interquartile range (IQR).
RA: rheumatoid arthritis; CRA: chronic RA; ERA: early RA; IL: interleukin;
MMP: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinase.
[¶] *p* value by nonparametric Wilcoxon test comparing related samples between baseline and follow-up.
[¶¶] *p* value by nonparametric Kruskal-Wallis test comparing study groups at baseline.
[¶¶¶] *p* value by nonparametric Kruskal-Wallis test comparing ERA and CRA patients after follow-up.
[¶¶¶¶] salivary biomarkers available from 51 ERA, 27 CRA and 43 control participants at baseline, from 46 ERA and 26 CRA after follow-up.
Serum biomarkers from 52 ERA, 24 CRA and 31 control participants at baseline, from 40 ERA and 26 CRA after follow-up.
Statistically significant *p* values are shown in bold.

Table 3. Correlations of serum MMP-8, TIMP-1, IL-6 and periodontal and rheumatological parameters during the study.

	ERA at baseline			ERA after follow-up			CRA at baseline			CRA after follow-up			Controls		
	MMP-8	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**
	R_s	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**	**R_s**
	p	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***
BOP	0.268	0.398	0.278	0.422	0.105	0.128	– 0.157	0.052	– 0.264	– 0.140	– 0.154	– 0.384	– 0.329	0.002	– 0.353
	0.062	**0.004**	**0.048**	**0.007**	0.523	0.439	0.464	0.808	0.213	0.545	0.505	0.085	0.071	0.990	0.052
PD	0.120	0.047	0.190	0.273	0.229	– 0.053	0.190	– 0.123	– 0.001	0.180	– 0.125	– 0.104	0.062	0.395	– 0.087
≥4mm	0.403	0.745	0.181	0.092	0.161	0.749	0.374	0.567	0.997	0.435	0.590	0.655	0.741	**0.028**	0.640
PIBI	0.117	0.037	0.191	0.272	0.212	– 0.052	0.192	– 0.120	– 0.003	0.279	– 0.012	– 0.065	0.062	0.395	– 0.087
	0.414	0.799	0.180	0.094	0.195	0.755	0.368	0.576	0.987	0.234	0.960	0.787	0.741	**0.028**	0.640
DAS28	0.307	0.310	0.391	0.130	0.372	0.401	0.360	0.154	0.613	0.411	0.045	0.527			
	0.032	**0.030**.	**0.005**	0.437	**0.021**	**0.013**	0.092	0.482	**0.002**	0.071	0.850	**0.017**			
Erosion	0.228	0.284	0.217	0.303	0.024	0.256	0.413	– 0.133	0.397	0.345	– 0.352	0.002			
score	0.112	**0.046**	0.129	0.057	0.883	0.110	**0.050**	0.544	0.061	0.148	0.139	0.993			

RA: rheumatoid arthritis; ERA: early RA; CRA: chronic RA; DAS28: disease activity score (28-joint); Erosion score by modified van der Heijde method;
IL: interleukin; MMP: matrix metalloproteinase; TIMP: tissue inhibitor for matrix metalloproteinase; BOP: bleeding on probing; PD: pocket depth;
PIBI: periodontal inflammation burden index.
R_s: correlation, Spearman correlation, 2-tailed *p* value significant at the 0.05 level.
Statistically significant *p* values are shown in bold.

Table 4. Correlations of serum and salivary MMP-8/TIMP-1 ratio and periodontal and rheumatological parameters during the study

	Early RA				**Chronic RA**				**Controls**	
	baseline serum	**saliva**	**follow-up serum**	**saliva**	**baseline serum**	**saliva**	**follow-up serum**	**saliva**	**baseline serum**	**saliva**
	R_s	R_s	R_s	R_s	R_s	R_s	R_s	R_s	R_s	R_s
	p	*p*	*p*	*p*	*p*	*p*	*p*	*p*	*p*	*p*
BOP	0.178	0.155	0.417	0.067	– 0.072	0.344	– 0.171	0.483	-0.380	0.394
	0.217	0.283	**0.009**	0.667	0.739	0.079	0.458	**0.017**	**0.035**	**0.009**
PD≥4mm	0.122	0.307	0.213	0.233	0.283	0.684	0.156	0.585	-0.110	0.391
	0.393	**0.030**	0.200	0.133	0.180	**<0.001**	0.500	**0.003**	0.557	**0.010**
PIBI	0.121	0.301	0.218	0.220	0.287	0.685	0.234	0.610	-0.110	0.391
	0.403	**0.034**	0.189	0.156	0.174	**<0.001**	0.321	**0.002**	0.557	**0.010**
DAS28	0.243	0.108	0.028	0.298	0.357	– 0.123	0.391	– 0.220		
	0.096	0.464	0.867	0.074	0.095	0.550	0.089	0.326		
Erosion score	0.144	0.024	0.292	0.235	0.292	– 0.007	0.359	– 0.053		
	0.322	0.870	0.072	0.130	0.072	0.973	0.131	0.819		

CRA chronic RA; ERA early RA; RA rheumatoid arthritis; DAS28 disease activity score (28-joint); Erosion score by modified van der Heijde method;
IL interleukin; MMP matrix metalloproteinase; TIMP tissue inhibitor for matrix metalloproteinase;
BOP bleeding on probing; PD pocket depth; PIBI periodontal inflammation burden index
correlation R_s, Spearman correlation, 2-tailed *p* value significant at the 0.05 level.
Statistically significant *p* values are shown in bold.

Table 5. Correlations of salivary MMP-8, TIMP-1, IL-6 and periodontal and rheumatological parameters during the study.

	ERA at baseline			**ERA after follow-up**			**CRA at baseline**			**CRA after follow-up**			**Controls**		
	MMP-8	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**	**MMP-8**	**TIMP-1**	**IL-6**
	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s *p*	R_s p
BOP	0.191	– 0.017	0.067	0.111	– 0.101	0.055	0.399	0.153	0.286	0.501	0.229	0.122	0.382	0.063	0.195
	0.185	0.905	0.645	0.467	0.509	0.721	**0.039**	0.445	0.148	**0.011**	0.272	0.560	**0.011**	0.687	0.209
PD	0.305	– 0.117	– 0.038	0.198	– 0.196	0.249	0.720	0.378	0.354	0.585	0.244	0.245	0.391	– 0.058	0.206
≥4mm	**0.031**	0.419	0.792	0.197	0.203	0.104	<**0.001**	0.052	0.070	**0.002**	0.240	0.239	**0.010**	0.713	0.184
PIBI	0.298	– 0.119	– 0.035	0.185	– 0.200	0.229	0.720	0.379	0.355	0.618	0.298	0.278	0.391	– 0.058	0.206
	0.035	0.410	0.807	0.229	0.194	0.136	<**0.001**	0.051	0.069	**0.001**	0.157	0.188	**0.010**	0.713	0.184
DAS28	0.182	0.159	– 0.009	0.359	0.171	0.070	– 0.123	– 0.102	0.249	– 0.321	– 0.153	0.292			
	0.217	0.281	0.953	**0.027**	0.305	0.677	0.550	0.619	0.219	0.145	0.496	0.187			
Erosion	0.008	0.010	0.169	0.238	0.118	0.308	0.072	0.482	0.022	– 0.150	– 0.092	– 0.308			
score	0.957	0.450	0.247	0.120	0.447	**0.042**	0.726	**0.013**	0.917	0.516	0.693	0.175			

RA: rheumatoid arthritis; ERA: early RA; CRA: chronic RA; DAS28: disease activity score (28-joint); Erosion score by modified van der Heijde method; IL interleukin; MMP: matrix metalloproteinase; TIMP: tissue inhibitor for matrix metalloproteinase; BOP: bleeding on probing; PD pocket depth; PIBI periodontal inflammatory burden index; R_s: correlation Spearman correlation, 2-tailed *p* value significant at the 0.05 level.
Statistically significant *p* values are shown in bold.

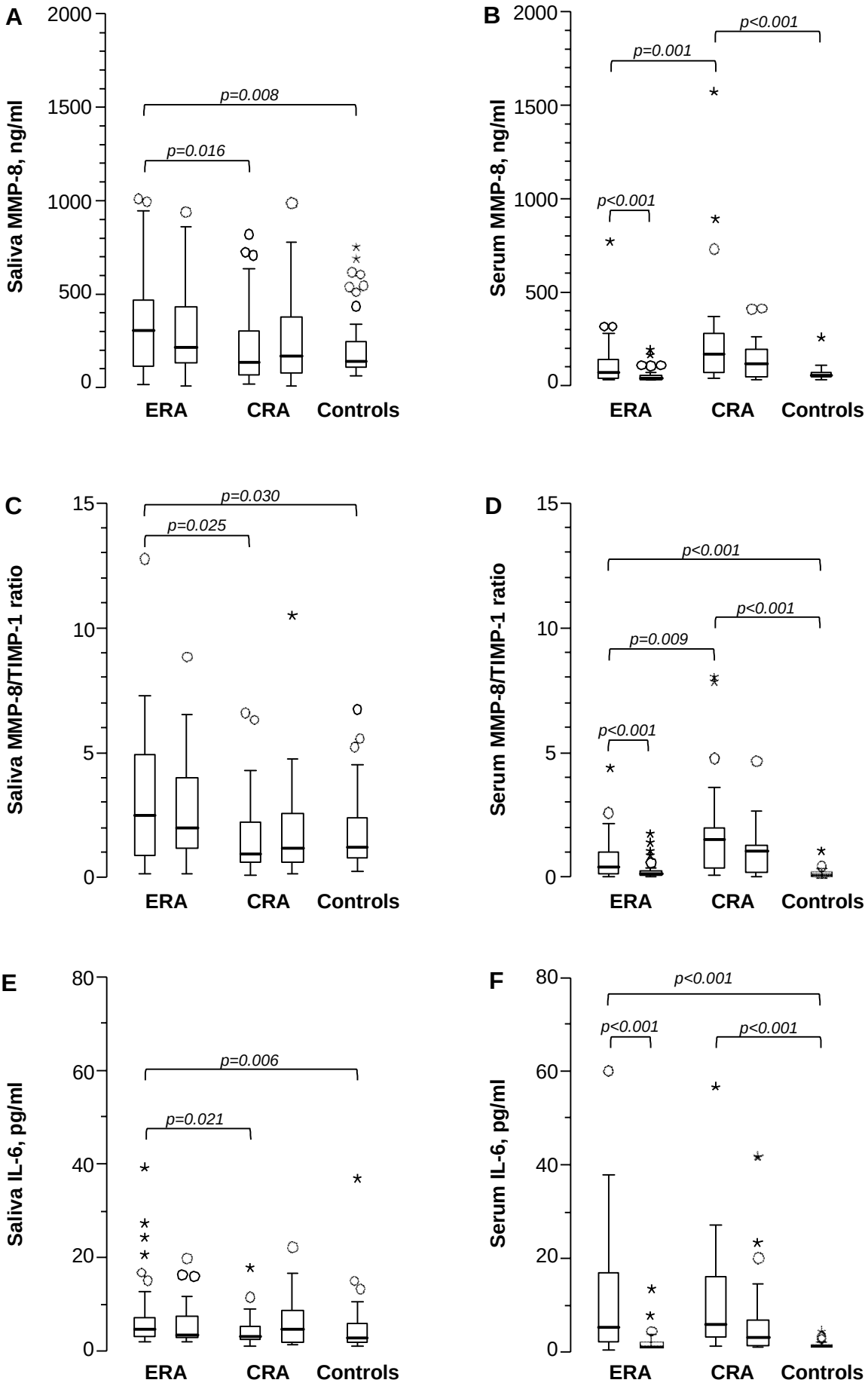
Titles and Legends to Figures

Figure 1.
Title: MMP-8 and MMP-8/TIMP-1 concentrations in saliva and serum at baseline and after follow-up in the study groups
Legend:

MMP-8 levels in (A) serum and (B) saliva, and MMP-8/TIMP-1 ratios in (C) serum and (D) saliva of patients with early rheumatoid arthritis (ERA), chronic rheumatoid arthritis (CRA), and healthy control subjects (Controls). The boxes denote the interquartile range (IQR; 25^{th} to 75^{th} percentiles), and the horizontal lines within the boxes denote the medians (50^{th} percentile). Outliers (values more than 1.5 times IQR from the median) are shown as circles, and extreme values (values more than 3 times IQR from the median) are shown as asterisks, with the minima and maxima of the remaining values shown as whiskers. Statistically significant *p*-values (<0.05) are shown corresponding to Mann-Whitney test (*post hoc* Kruskal-Wallis test) comparing ERA, CRA, and Controls groups, or corresponding to Wilcoxon test comparing baseline and follow-up values.

Figure 2.
Title: Il-6 concentrations in saliva and serum at baseline and after follow-up in the study groups
Legend:

Interleukin-6 (IL-6) levels in (A) serum and (B) saliva of patients with early rheumatoid arthritis (ERA), chronic rheumatoid arthritis (CRA), and healthy control subjects (Controls). The boxes denote the interquartile range (IQR; 25^{th} to 75^{th} percentiles), and the horizontal lines within the boxes denote the medians (50^{th} percentile). Outliers (values more than 1.5 times IQR from the median) are shown as circles, and extreme values (values more than 3 times IQR from the median) are shown as asterisks, with the minima and maxima of the remaining values shown as whiskers. Statistically significant *p*-values (<0.05) are shown corresponding to Mann-Whitney test (*post hoc* Kruskal-Wallis test) comparing ERA, CRA, and Controls groups, or corresponding to Wilcoxon test comparing baseline and follow-up values.


A
Saliva MMP-8, ng/ml
2000
1500
1000
500
0
p=0.008
p=0.016
ERA
CRA
Controls
B
Serum MMP-8, ng/ml
2000
1500
1000
500
0
p=0.001
p<0.001
p<0.001
ERA
CRA
Controls
C
Saliva MMP-8/TIMP-1 ratio
15
10
5
0
p=0.030
p=0.025
ERA
CRA
Controls
D
Serum MMP-8/TIMP-1 ratio
15
10
5
0
p<0.001
p<0.001
p=0.009
p<0.001
ERA
CRA
Controls
E
Saliva IL-6, pg/ml
80
60
40
20
0
p=0.006
p=0.021
ERA
CRA
Controls
F
Serum IL-6, pg/ml
80
60
40
20
0
p<0.001
p<0.001
p<0.001
ERA
CRA
Controls