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Collagenase-2 (MMP-8) as a point-of-care biomarker in periodontitis and cardiovascular diseases. Therapeutic response to non-antimicrobial properties of tetracyclines

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ABSTRACT

Neutrophil collagenase or collagenase-2 (matrix metalloproteinase [MMP]-8) belongs to the collagenase subgroup of the MMP superfamily of calcium- and zinc-dependent neutral proteinases. MMP-8 is catalytically the most competent proteinase to initiate type I collagen and extracellular matrix degradation associated with periodontal and peri-implant tissue destruction leading to tooth and dental implant loss. Regarding cardiovascular diseases, pathologically excessive MMP-8 has been implicated in atherosclerotic plaque destabilization and rupture probably through its proteolytic ability to thin the protecting collagenous fibrous cap lining coronary and other arteries. During the initiation and course of inflammatory responses in periodontitis, peri-implantitis and cardiovascular diseases, proinflammatory mediators including especially MMP-8 are up-regulated not only in affected tissues but also in the secreted, disease-affected, oral fluids (gingival crevicular fluid [GCF], peri-implant sulcular fluid [PISF], mouthrinse and saliva) as well as in serum and plasma. Regarding periodontitis, peri-implantitis and cardiovascular diseases, the oral fluid and serum MMP-8 analysis has proven to be a sensitive and an objective biomarker as an indicator of health, pathologic processes and pharmacologic response to therapeutic intervention including doxycycline medication as an MMP inhibitor. Oral fluids, i.e., GCF, PISF, mouthrinse and saliva are easily and non-invasively collected for the site- and patient-specific diagnostic analysis in periodontitis and peri-implantitis, whereas serum and/or plasma sample collection is required for diagnosis and monitoring of cardiovascular diseases. Research in periodontology and cardiology has identified a need for the development of innovative point-of-care diagnostic tests for MMP-8. We summarize and review the recent studies on these topics.

1. Introduction

Periodontitis is a chronic bacterial infection-induced inflammatory condition affecting gingival and tooth-supporting structures including bone [1,2]. It is a polymicrobial infection involving predominantly anaerobic bacteria organized as a dental biofilm in the deepened periodontal pockets [1–3]. The prevalence of periodontitis in Western countries is 5–20% [3]. Untreated and persistent periodontal infections evoke host defense and destruction mechanisms that can lead to tooth and dental implant loss. Several epidemiological and clinical studies have revealed that untreated periodontitis may threaten general health by increasing the risk of cardiovascular diseases, preterm labour and pulmonary diseases [3]. These observations have raised the need for the development of chair-side diagnostic molecular point-of-care tools for both periodontitis and related systemic clinical conditions such as cardiovascular diseases [3,4].

In the clinical discipline of periodontics, the traditional diagnostic procedures involve probing pocket depths (mm) of the gingival crevice, bleeding on probing, clinical attachment levels, gingival index and radiographic analysis of alveolar (periodontal) bone loss [5,6]. These clinical measures can be supplemented by microbial analysis [5–7]. Although these conventional clinical and microbial measures are convenient and reliable to use, they require a highly trained clinician, expensive equipment, and time. They are also costly to the consumer and determine mainly the past history of the disease rather than disease activity [5–8]. In this regard, the conventional disease diagnostic techniques lack the ability to identify

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highly susceptible periodontitis sites or patients at risk for future progression of periodontitis [8].

As result of the interaction between the bacterial infection and immune (host) response, irreversible destruction of periodontal connective tissue and alveolar bone occurs [8,9]. Type I collagen is the main extracellular matrix component in the soft (gingiva and periodontal ligament) and hard (alveolar bone) periodontal tissues and thus, type I collagen degradation is regarded as one of the key factors in the uncontrolled destructive lesions [8,9]. The major collagenolytic MMPs associated with severity of periodontal inflammation and disease are collagenase-2 (MMP-8) and collagenase-3 (MMP-13), whereas collagenase-1 (MMP-1) is related to physiological periodontal tissue turnover [4,9–14]. Although these MMPs have been assumed to primarily play a matrix degradative role, a broader substrate degradome analysis shows that proteolytic susceptibility to MMPs includes cytokines, chemokines, complement factors, cell surface components, serpins, other MMPs and apoptotic signals; and, as a result of MMP-mediated modification of various bioactive non-matrix substrates, these neutral proteinases can also modulate inflammatory and immune responses [4,9,15–18]. Recent generation of MMP-8 deficient mice has confirmed the concept that MMP-8 is a central mediator in chronic infection-induced inflammatory conditions, and can exert, in addition to the classical surrogate tissue destructive properties, also anti-inflammatory and defensive properties [19–23]. MMP-8 is the major type of interstitial collagenase present in human periodontitis-affected gingival tissue, gingival crevicular fluid (GCF), peri-implant sulcular fluid (PISF), saliva and mouthrinse samples [4,9,10].

Cardiovascular diseases (CVD), which untreated periodontitis can affect as a risk factor [3,24], is the leading cause of mortality and disability in the Western world. The major cause of CVD is atherosclerosis, a disease characterized by accumulation of lipids and inflammation in vessel walls. Type I collagen is the major extracellular matrix component and load bearing molecule of the fibrous cap in the atherosclerotic lesions. Among collagenases and other MMPs, collagenase-2 (MMP-8) is catalytically the most efficient enzyme to initiate the degradation of type I collagen [9]. Recently MMP-8 has been implicated in atherosclerotic plaque destabilization though its capacity to thin the protecting fibrous cap thereby rendering it more susceptible to rupture [25].

With this background we summarize and review here our recent studies on collagenase-2 (MMP-8) as a potential oral fluid and serum point-of-care biomarker in periodontitis and cardiovascular diseases, and their response to non-antimicrobial property of tetracyclines, notably doxycycline.

2. Methodological background

We have analyzed GCF and mouth rinse samples collected and clinically characterized as described [26–29] with an immunofluorometric assay (IFMA) [28] in the research laboratory, and by one feasible method for chair-side diagnostic testing: the MMP-8 specific immunochromatographic chair-side dip-stick test [5,9,26,27] in which the result is provided in 5 min and graded by eye as positive or negative (the principle of the test was described by Sorsa et al. [4,31]; and clinical testing protocol by Mäntylä et al. [26,27]). The chair-side testing method, IFMA and dentoELISA use the same monoclonal antibodies [28–32]. Samples have also been analyzed with the commercial ELISA kit provided by Amersham for MMP-8 and TIMP-1 [29,32].

The dentoAnalyzer is designed as a portable, user-friendly bench-top instrument. It automatically conducts the whole assay process, that is, steps like liquid handling as well as readout based on a software program and a robust algorithm. (For a more detailed description of the dentoAnalyzer, its key components and the art of quantification of MMP-8 [as well as other biomarkers] see Ref. [32].) The key component is a cartridge consisting (i) of a liquid-handling module containing all relevant reagents for immunological reactions like clinical sample, conjugate, wash buffers, and substrate and (ii) a reaction chamber containing six filters including positive and negative controls, where the immunological reactions take place [32]. Two antibodies directed against specific epitopes of the antigen are used in a sandwich based immunoassay technology known as antibody immunocolumn for analytical process (ABICAB) which is based on an immunoaffinity filter design using flow through solid phase filters with extremely high binding potential [29].

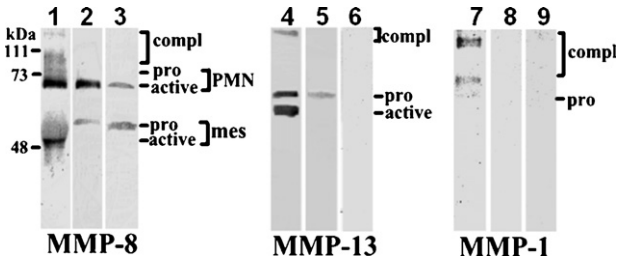

Fig. 1. Western immunoblot analysis periodontitis-affected gingival crevicular fluid (GCF) samples (1–3, 4–6, 7–9; lanes 1, 4, and 7 represent GCF from very diseased and lanes 2, 3, 5, 6, 8 and 9 GCF from mildly diseased pockets) for collagenase-2 (MMP-8), collagenase-3 (MMP-13) and collagenase-1 (MMP-1), respectively. Compl indicates high (>100 kDa) molecular size complexes, PMN neutrophil-type MMP-8 isoform, mes fibroblast-type MMP-8 isoform, pro proform and active activated form of MMPs. Mobilities of the molecular weight standards are indicated on the left.

The IFMA and dentoELISA methods have been described by Hanemaaijer et al. [30] and Leppilahti et al. [29], and the MMP-8 specific chair-side dip-stick test by Sorsa et al. [4,28,31], and Mäntylä et al. [26,27]. The MMP-8 and TIMP-1 analysis by Amersham ELISA kit have been performed according to manufacturer's instructions [29]. The treatment response of the periodontitis patients' lesion (i.e., periodontal pocket) sites is seen as a reduction of pocket probing depth (PPD) and attachment loss (AL) and indicated by the dip-stick test, IFMA, dentoELISA and Amersham ELISA levels have been analyzed. The MMP-8 levels of the GCF and mouthrinse samples in question have previously found to correlate with the periodontal status of the tested sites and patients in a larger scale study [26–30].

We have further analyzed the association of cardiovascular diseases (CVD) and subclinical atherosclerosis (carotid artery intima media thickness [IMT]) and serum MMP-8, TIMP-1 and MMP-8/TIMP-1 of 1018 men with the follow-up time of 10 years [33]. We also have studied the association of the serum levels of MMP-8 to the concentrations of C-reactive protein (CRP) and serum amyloid-A (SAA) [33].

3. Outcome of utilization of MMP-8 as a diagnostic biomarker of periodontitis and CVD

Oral fluids (GCF, PISF, mouthrinse and saliva) are known to contain large amounts of serum proteins, inflammatory mediators, host tissue and cell degradation products as well as microbial metabolites and enzymes [4,8,9,34]. In particular, host-derived proteinases such as MMP-8 are thought to play a major role in periodontitis and dental peri-implant health and diseases; Western immunoblot analysis reveals that MMP-8 is the major collagenase in periodontitis-affected GCF (Fig. 1). Thus the collection and analysis of oral fluid samples are considered to mirror periodontal and peri-implant health and disease [4,8,9,34].

Recently both qualitative and quantitative chair-side point-of-care technologies have been developed or are under development

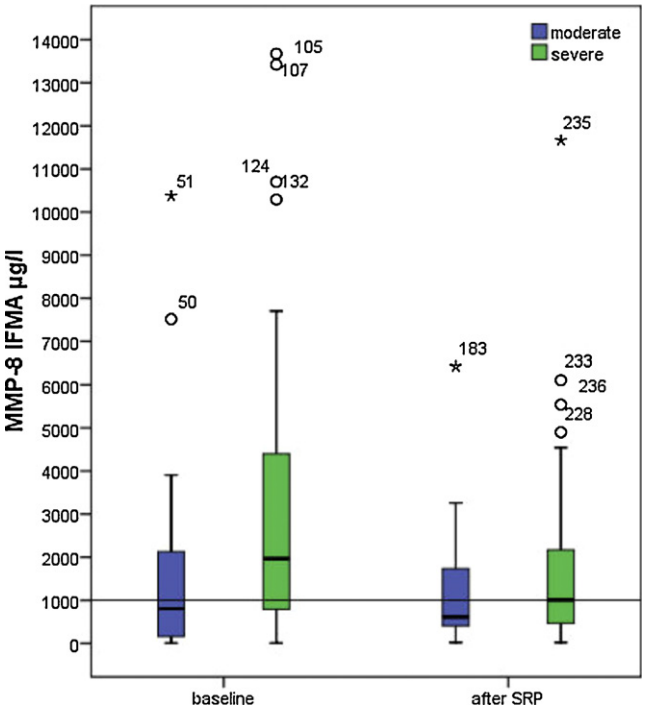

Fig. 2. Boxplot figure showing GCF MMP-8 IFMA levels at baseline and one month after periodontal treatment (scaling and root planing, SRP); 34 sites from four patients with moderate periodontitis and 81 sites from 10 patients with severe periodontitis. MMP-8 levels were significantly higher at baseline in severe compared to moderate periodontitis patients' sites ($p = 0.001$, Mann–Whitney test) but tended to reach similar low levels after SRP ($p = 0.110$). Decrease of MMP-8 levels after SRP in severe periodontitis patients' sites is significant ($p < 0.001$, Wilcoxon test) but not in moderate periodontitis patients' sites ($p = 0.516$) though a trend can be observed. Dip-stick test positive results percentage (cut-off level 1000 µg/l indicated in figure) at baseline and after SRP 67.9%, 44.4% for severe and 32.4%, 44.2% for moderate periodontitis patients' sites.

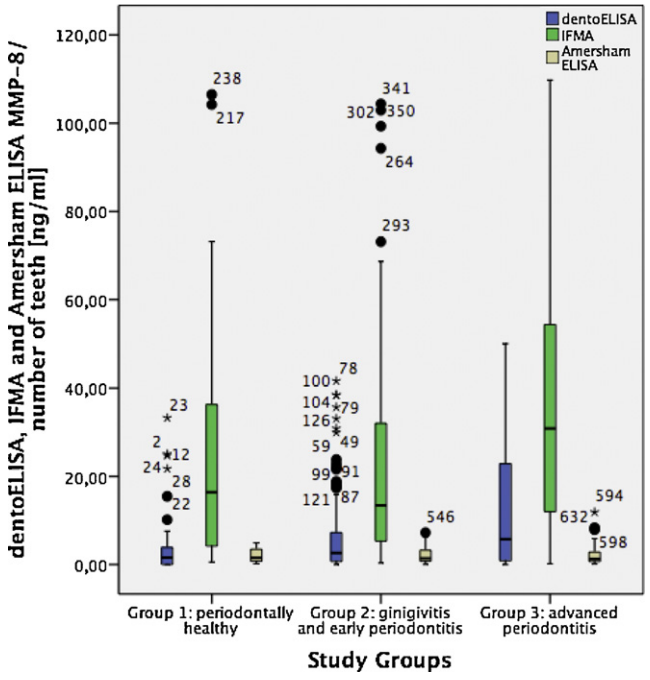

Fig. 3. Boxplots representing the distributions of the MMP-8 levels (ng/ml) in oral rinse samples adjusted to numbers of teeth detected by dentoELISA, IFMA and Amersham ELISA in three patient categories. Difference significant both for dentoELISA ($p = 0.002$) and IFMA ($p = 0.012$) but not for Amersham ELISA ($p = 0.950$) detection (Kruskall–Wallis test).

for the rapid detection of pathologically elevated levels of MMP-8 in oral fluids and serum [4,8,9,26–29,31–39]. We have developed monoclonal antibodies for MMP-8 [30] to be utilized in chair-side point-of-care immunotests for oral fluid and serum MMP-8 analysis [5,6,27,29]. One of these chair-side tests we developed, resembles pregnancy home test [5,9,26,27,31].

The MMP-8 stick-test can differentiate healthy and gingivitis (no alveolar bone loss) sites from periodontitis (detectable alveolar bone loss) sites, and is in a good agreement with quantitative laboratory immunofluorometric assay (IFMA) [26,27]. The dip-stick test's agreement was very good when assessed by the Kappa-statistics ($K = 0.81$) and provided specificity of 0.96 and sensitivity of 0.83 [26]. A repeated MMP-8 elevation in the GCF test-stick, IFMA and dentoELISA results preceded the clinical signs of progressive (i.e., "active") periodontal disease [9,27–29]. The chair-side test-stick can be used to monitor the beneficial effect of scaling and root planing (Fig. 2). These rapid point-of-care MMP-8 tests eventually are also useful tools for monitoring peri-implantitis [4,9,39].

We also compared different laboratory and chair-side methods to detect pathologically elevated MMP-8 concentrations in periodontitis-affected GCF and mouthrinse samples [28,29]. We compared IFMA, MMP-8 specific chair-side dip-stick test, dentoELISA and Amersham ELISA kit. IFMA and dentoELISA correlated with each other in good agreement, and the chair-side dip-stick test results were well in line with IFMA and dentoELISA assays [28,29]. Periodontitis sites with unstable characteristics and periodontitis patients with increased inflammatory burden could be identified and differentiated from the others by MMP-8 dip-stick, IFMA and dentoELISA, respectfully (Figs. 2 and 3). The Amersham ELISA results were not in line with findings obtained by the other methods [28,29,38].

Oral fluid and serum MMP-8 detection by methods using the same MMP-8 antibodies (dip-stick, dentoELISA and IFMA) differentiated the subjects with disease-affected sites, from the controls, with a higher accuracy than the traditional ELISA method [26–30,38]. This can likely be explained by the differences and sensitivities between the antibodies used in the IFMA, dentoELISA and Amersham ELISA [28,29,38]. The antibody used in IFMA and dentoELISA identifies the neutrophil- and fibroblast-type MMP-8 isotypes and especially their active forms [29,30]. In fact, this antibody identifies an active form of MMP-8 molecule which is mainly found in GCF from sites with active periodontitis [28], while a latent form of MMP-8 is associated with gingivitis [4,9,28]. The Amersham ELISA method detects all forms of MMP-8 [29,38].

We have found, in a ten year follow up, that elevated serum MMP-8 concentration is an independent risk factor for acute myocardial infarction, coronary heart diseases and cardiovascular diseases, and all can cause death [33]. Especially men with subclinical atherosclerosis (carotid artery intima media thickness [IMT] > 1 mm) had a 3-fold increased risk for cardiovascular death independent of other risk factors. Regarding increased serum MMP-8/TIMP-1 ratio, the risk was increased 2,5-fold, but was only of borderline significance in the multivariate model [33]. Fig. 4 demonstrates the associations between increasing serum concentration of MMP-8 with C-reactive protein (CRP) and serum amyloid-A (SAA). Fig. 5 demonstrates the cumulative survival rates for cardiovascular disease deaths with MMP-8, TIMP-1 and MMP-8/TIMP-1 concentrations. In cardiovascular diseases pathologically elevated MMP-8 expression has been found not only in neutrophils but also in endothelial cells, smooth muscle cells, plasma cells and macrophages after stimulation with CD40L, TNF-α and lipopolysaccharide (LPS) [3,25]. MMP-8 has been shown to be localized to the shoulder region of advanced atherosclerotic lesions and is excessively expressed in patients with cardiovascular dis-

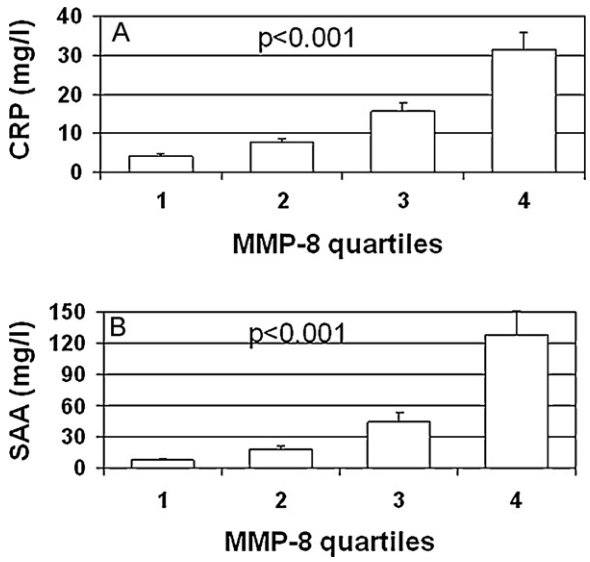

Fig. 4. The association of serum MMP-8 concentrations with CRP and SAA. The mean (A) CRP and (B) SAA concentrations were calculated in the quartiles of serum MMP-8 concentrations (increasing quartiles 1–4, $n = 669$). The error bars depict SEM and the p value is for linear trend by the ANOVA test.

eases characterized by plaque progression [25,33]. In fact, patients exhibiting subclinical atherosclerosis together with elevated serum MMP-8 levels may have ongoing systemic inflammatory process eventually leading to plaque rupture and acute manifestation of cardiovascular diseases [25,33]. Overall these findings are in good accordance for MMP-8 being an early player in cap remodeling preceded by an inflammatory condition leading to acute coronary syndromes [25,33].

The importance of the blood sample collection and preparation techniques for blood MMP-8 and TIMP-1 measurements has been addressed, and, in the same patients, serum samples had higher MMP-8 and TIMP-1 levels relative to the plasma samples [40]. Moreover, serum samples collected with a clot activation had higher MMP-8 levels in comparison to serum samples collected without any clot activator [40]. The rationale behind these findings was that MMP-8 and TIMP-1 may be released from platelets and leukocytes during the blood collection or coagulation [40]. In our study we used glass tubes since the blood clotting in them is more effective than in plastic tubes and no clot activations are needed [33,41]. Moreover, the differences between serum and plasma MMP-8 concentrations with the two different methods, i.e., IFMA and Amersham ELISA, haves been tested [41]. With both IFMA and Amersham ELISA serum, MMP-8 concentrations were significantly higher than in plasma [41]. Noteworthy, there were significant positive correlations between serum and plasma IFMA as well as serum and plasma ELISA results indicating the suitability of serum for these measurements [41]. In any case, we collected our serum samples in a uniform way from each patient and, irrespective of the origin of the MMP-8, the results demonstrate a positive association between elevated serum MMP-8 concentration and cardiovascular disease events [33,41]. Therefore serum analysis of MMP-8 may serve a predictive biomarker of future cardiovascular disease events [33,41].

4. Point-of-care MMP-8 analysis to monitor tetracycline therapy

Several human and animal studies have demonstrated that the elevated MMP-8 associated with periodontal and inflammatory tissue destruction is sensitive to inhibition and reduction by sub-antimicrobial dose doxycycline medication (SDD) [42–47]. SDD by itself reduced collagenase and gelatinase activities (presum-

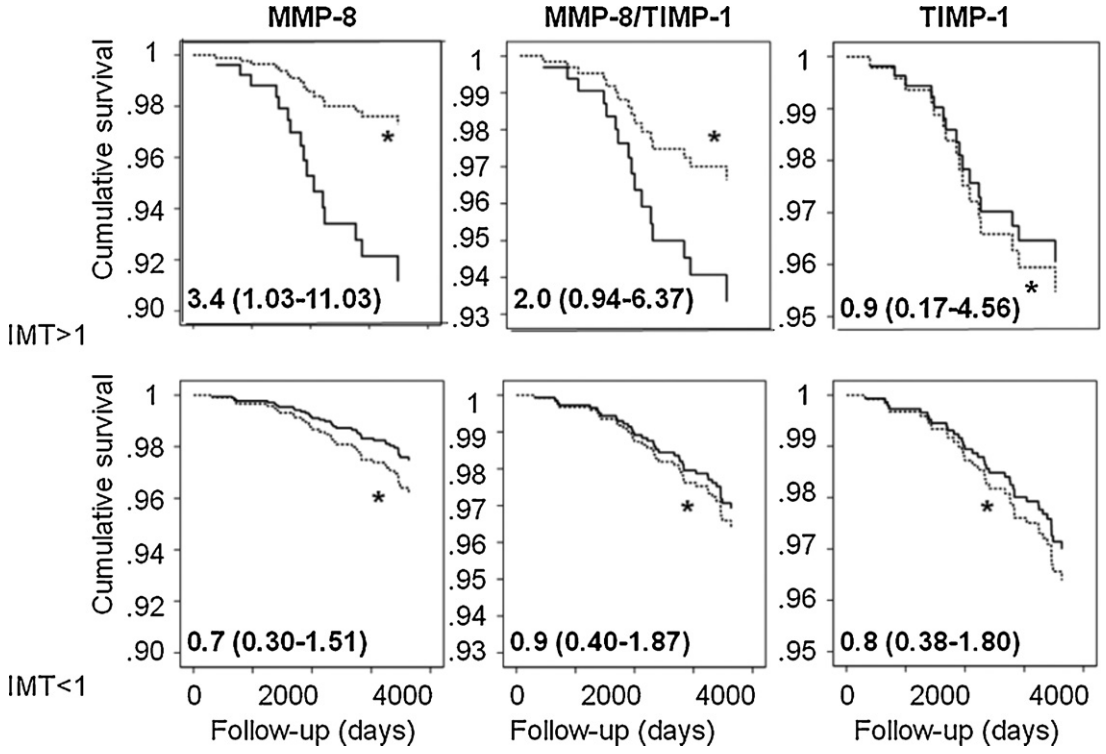

Fig. 5. Cumulative survival rates for CVD death. The men free from CVD at baseline ($n = 905$) were divided in those with IMT > 1 mm (upper three panels) or <1 mm (lower three panels). The cumulative survival rates of CVD death in the highest quartiles (*) of MMP-8, TIMP-1 concentration, and MMP-8/TIMP-1 ratio vs. the corresponding lower quartiles were analyzed by Cox regression model adjusted for age, BMI, smoking, diabetes, systolic blood pressure, serum cholesterol, triglyceride, and HDL cholesterol concentration, plasma fibrinogen concentration, serum antibody.

ably MMP-8 and -9, respectively) in human gingival tissue (not just GCF) surgically excised for therapeutic purposes; low-dose NSAID (flurbiprofen) by itself had no effect on these gingival tissue MMPs. Importantly a combination ("COMBO") of the SDD and low-dose flurbiprofen produced a dramatic synergistic reduction in these gingival tissue MMPs [47]. In postmenopausal women who exhibited both periodontitis and systemic osteopenia, daily oral administration of SDD over a 2-year time period reduced MMP-8 levels, collagenase activity, IL-Iβ and a carboxyterminal telopeptide cross-link fragment of type I collagen (ICTP) in GCF indicating that SDD reduction of collagenase (about 80–90% of total collagenase is MMP-8) is indicative not only of decreased collagen degradation but also decreased bone resorption (ICTP) [47,48]. Furthermore, SDD can reduce systemic bone resorption biomarkers in serum of these osteopenic postmenopausal women as well [48]. In this regard physiological but not pathologically elevated MMP-8 levels seem to exert protective and anti-inflammatory functions due to the selective processing of bioactive non-matrix substrates [20–23,50] as recently evidenced by MMP-8 knock-out model studies in periodontal and lung diseases as well as cancer studies [19–23,51]. Therefore, "leaky" or less efficient MMP-inhibitors such as SDD obviously exert safe or beneficial effects because they only reduce pathologically excessive oral fluid, serum or tissue MMP-8 but not below the levels required for normal physiological or anti-inflammatory functions [52]. SDD can reduce both pathologically excessive GCF collagenase activity and MMP-8 levels close to the physiological levels found in healthy control sites and patients [4,9,42–48,53–56]. Therefore, the beneficial effects that associate with clinical improvement in periodontitis can be monitored by MMP-8 assays [4,9,46,47]. SDD can further reduce systemic serum and plasma levels of a set of proinflammatory biomarkers including MMP-8, MMP-9, MMP-8, -9/TIMP-1-ratios, CRP, TNF-α and IL-6 that can be utilized as biomarker predictors of future cardiovascular disease events [9,53,57,58] and future systemic bone loss [47–49]. Recently, doxycycline treatment reduced systemic plasma MMP-9 levels and resulted in less atherosclerosis in animals infected with periodontopathogen, and also prevented vascular remodeling in animal model of hypertension [59,60]. Earlier studies showed similar improvements in serum and plasma levels at MMP-8, MMP-8/TIMP-1 and -9 in human clinical trials [48,53,57].

5. Concluding remarks

In summary, collagenase-2 or MMP-8 appears to provide a clinically useful oral fluid (GCF, mouthrinse or saliva) biomarker in periodontal and peri-implant diseases and serum biomarker in cardiovascular diseases [4,9,10,28,29,33,38,39]. Point-of-care technologies utilizing MMP-8 may prove to be useful in diagnosis, treatment and follow-up of periodontal, peri-implant and cardiovascular diseases [4,9,26–30,35–38]. The effects of subantimicrobial doxycycline medication can be monitored by MMP-8 immunoassays in oral fluids and serum [4,9,42,46,48,55–58]. Differentiation of periodontitis and peri-implantitis sites and subjects, as well as cardiovascular disease-affected subjects, from controls seems to be highly dependent on the selected antibodies and assay techniques regarding oral fluid and serum MMP-8 assays [28–30,38]. MMP-8 antibodies being specific or exerting high affinity for active forms of MMP-8 would be most optimal and useful in the assays [28–30,38]. In this regard Golub et al. [48] using MMP-immunoassays [28–30,38] demonstrated that a 2-year SDD regimen in postmenopausal women significantly decreased GCF MMP-8 in their periodontal pockets, and Slepian et al. [58] found that serum levels of MMP-8/TIMP-1 and MMP-9 in these women were reduced as well. Further investigations with larger number of subjects and longer duration are in progress to better understand the MMP-8 point-of-care diagnostics in periodontitis, peri-implantitis and cardiovascular diseases. Overall, since there is no significant elevation in oral fluid MMP-8 without periodontitis [4,9], and untreated periodontitis can be considered as a potential risk factor for various systemic diseases such as, but not limited to, cardiovascular diseases, diabetes, stroke, arthritides and pulmonary diseases [3], it appears that oral fluid MMP-8 point-of-care diagnostics [4,9,26–29,31,32,35–38] have a huge potential to build up a diagnostic bridge from mouth to systemic conditions. In fact, a recent study on oral fluid (salivary) MMP-8 levels assessed by IFMA [26–30,38] in patients with or without coronary heart disease (CHD), revealed that elevated salivary MMP-8 is associated with CHD [61].

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