

## Matrix metalloproteinase -7, -8, -9 and -13 in gingival tissue of patients with type 1 diabetes and periodontitis

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### Abstract

There is scientific data to support the existence of a two-way relationship between diabetes and periodontitis, with diabetes increasing the risk for periodontitis, and periodontal inflammation negatively affecting the diabetic status. Our study aims to investigate the expression of MMP-7, -8, -9 and -13 in the gingiva of the young patients with aggressive periodontitis (AP) and type 1 diabetes mellitus (T1D). Gingival biopsies were harvested from five adult patients aged 19–29 years with T1D+AP with moderate (three cases) to severe (two cases) forms of AP and from four adult patients aged 18–28 years with moderate AP without T1D. The MMP-7 immunoreaction was positive in the five cases with T1D+AP with different staining patterns. The MMP-8 immunostaining was positive in all cases. The reaction was more intense in cases with T1D+AP, especially in those with severe periodontitis. The MMP-9 immunoreaction was present in all the structures of the gingival mucosa with different intensity, being frequently present surrounding the blood vessels of the chorion. In most of the patients, reaction to MMP-9 was intense, localized at the level of the cells in the superficial chorion and very rarely at the level of some dispersed cells in the connective vascular islands. MMP-13 was present in all cases, but it was more intense in the two cases with T1D+AP with probing depth (PD)>6 mm when it had similar patterns as MMP-9 staining and in one case with AP when the staining was observed strictly in the lamina propria associated with moderate chronic inflammatory infiltrate. The expression of MMP-7, -8, -9 and -13 in the gingiva of the young patients with aggressive periodontitis and T1D was positive in all studied cases supporting the hypothesis that both are inflammatory diseases with common pathogenic mechanisms involving inflammatory mediators and may be possible biomarkers of disease status.

**Keywords:** matrix metalloproteinases, aggressive periodontitis, type 1 diabetes mellitus.

### Introduction

There is scientific data to support the existence of a two-way relationship between diabetes and periodontitis, with diabetes increasing the risk for periodontitis, and periodontal inflammation negatively affecting the diabetic status [1].

Periodontitis and diabetes are inflammatory diseases with common pathogenic mechanisms involving inflammatory mediators, which have been investigated as possible biomarkers of disease status [2]. The most important inflammatory mediators linked to initiation and progression of periodontal disease is a complex network of pro-inflammatory cytokines, matrix metalloproteinases (MMPs) and prostaglandins [2]. There are a large number of studies of cytokines, adipokines and other mediators involvement in periodontitis and diabetes using GCF (gingival crevicular fluid), saliva or gingival tissues samples [1, 3].

Matrix metalloproteinases are a family of zinc-dependent endopeptidases that mediates the degradation of the extracellular matrix. All MMPs contain Zn<sup>2+</sup> at the

catalytic site and need Ca<sup>2+</sup> for stability and activation [4]. MMP-8 is known also as neutrophilic collagenase or collagenase 2. This enzyme degrades especially the fibrillar collagen type I, but also type II and III, serpins and alpha-2 macroglobulin. All collagenases cleaves the fibrillar collagen into a specific site resulting terminal N and C fragments, that denaturizes at body temperature (by other metalloproteinases) forming the gelatin. Substrate specificity of collagenases is variable, MMP-8 degrading especially the type I collagen, his action being more intense upon type II and III collagen [5]. MMP-8 was determined in patients with periodontal disease [6] and in patients with both diseases – periodontitis and diabetes [7].

MMP-9 exhibits a gelatin ligation domain, inserted between the catalytic and the active domain, reason for which the MMP-9 is known as gelatinase B. MMP-9 has gelatin as its preferred substrate, fibronectin, elastin and type IV, V, VI and X collagen and type I denaturized collagen, being involved in the inflammatory destructive processes in periodontal disease [8].

Within the MMP family, MMP-7 is the smallest of

the known MMPs [9]. Unlike other MMPs, which are produced and released only upon response to injury, MMP-7 is mainly produced in many non-injured exocrine and mucosal epithelia rather than connective tissue cells [10]. In GCF, it was shown that patients with periodontal disease had comparable GCF MMP-7 levels to the healthy ones. The lack of elevated GCF MMP-7 levels in diseased groups might provide evidence that this MMP is preferentially released into the GCF for early defensive purposes [11].

Collagenase 3 (MMP-13) is a member of the matrix metalloproteinase gene family that is expressed at high levels in diverse human carcinomas and in articular cartilage from arthritic patients. In addition to its expression in pathological conditions, collagenase 3 has been detected in osteoblasts and hypertrophic chondrocytes during fetal ossification [12, 13]. Some of the studies show that involvement of MMPs in pathogenesis of type 1 diabetes mellitus (T1D) and its renal complication [14] but not in the T1D–periodontal disease relationship.

Our study aims to investigate the expression of MMP-7, -8, -9 and -13 in the gingiva of the young patients with aggressive periodontitis (AP) and T1D.

## Materials and Methods

Gingival biopsies were harvested from five adult patients aged 19–29 years with T1D+PA with moderate [probing depth (PD)<6 mm] (three cases) to severe [probing depth (PD)>6 mm] (two cases) forms of AP and from four adult patients aged 18–28 years with moderate AP without T1D. All patients with T1D had a good glycemic control (HbA1c<7%). These adult patients were part of group test (GTA) and group control (GCA) of whom demographical, periodontal and diabetic clinical data were published elsewhere [15]. The Ethical Committee of University of Medicine and Pharmacy of Craiova, Romania approved this study.

The samples were fixated in 10% buffered formalin solution and paraffin wax embedded using the standard technique. The paraffin blocks thus obtained were cut to 5 µm thick sections using a Microm microtome. The sections were displayed on histologically slides previously treated with poly-L-Lysine on their surface.

The immunostaining was performed Dako EnVision™ 3.3'-diaminobenzidine (DAB) as chromogen. For the heat-mediated antigen retrieval, we preferred the incubation at 65°C for 18 hours citrate buffer solution, pH 6. We chose this method instead of microwave oven because given the dimensions of the harvested tissue it was a high risk for its separation from the slide during the boiling process.

The primary antibodies used were rabbit polyclonal to MMP-13 Dako dilution 1:100, MMP-7 Dako 1:50, MMP-8 (Abcam ab81286) Dako 1:100, MMP-9 (Abcam ab38898), dilution 1:200. All antibodies are rabbit anti-human. The slides were incubated with the primary antibody overnight at 4°C. The next day, the slides were incubated in Dako EnVision™ detection system for 30 minutes at room temperature. The nuclei were counterstained with Hematoxylin.

The samples were examined with Nikon Eclipse 90i microscope and the pictures were taken using a dedicated Nikon camera and NIS-Elements software.

## Results

The MMP-7 immunoreaction was positive in the five cases with T1D+AP with different staining patterns (Figures 1 and 2). It was present in few cells of the lamina propria. The epithelial staining had a cytoplasmatic or nuclear pattern in the cells of basal and intermediary layers of the hypertrophic epithelium. The immunoreaction was no present in cases with AP only.

The MMP-8 immunostaining was positive in all cases. The reaction was more intense in cases with T1D+AP, especially in those with severe periodontitis. Immunostaining was localized especially in the cells of the superficial lamina propria, being also observed in some connective tissue isles formed during the hyperplasia of the epithelial cells. In the chorion of the mucosa, the MMP-8 immunostaining was observed with a cytoplasmatic pattern in some cells that had fibroblastic morphology (Figure 3).

There was positive staining even with nuclear pattern in the cells of the intermediate layer of the epithelium.

The MMP-9 immunoreaction was present in all the structures of the gingival mucosa with different intensity, being frequently present surrounding the blood vessels of the chorion.

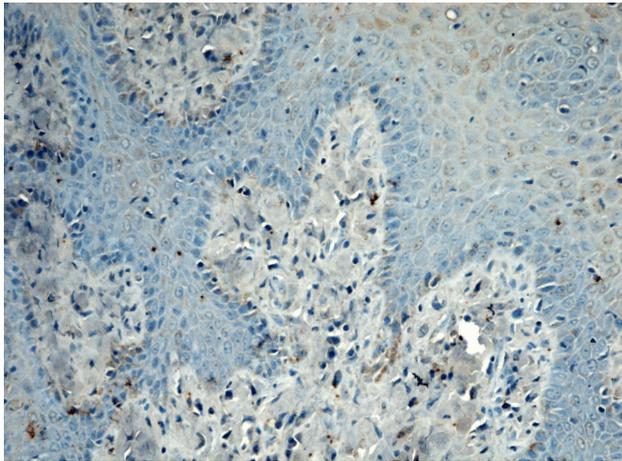
In most of the patients, reaction to MMP-9 was intense, localized at the level of the cells in the superficial chorion and very rarely at the level of some dispersed cells in the connective vascular islands. Detailed observation of this marking evidenced it in the cytoplasm of the fibroblasts in the gingival chorion (Figure 4).

In the two cases with deep forms of AP [probing depth (PD)>6 mm] from the five cases of T1D+AP it was noticed, when using an enhanced optic, the presence of an intense positive immunomarking at the level of intracellular spaces of the intermediate layer of the gingival mucosa. Desmosomes in the MMP-9 positive area were broken, leading to the disorganization of the intermediate layer cytoarchitecture.

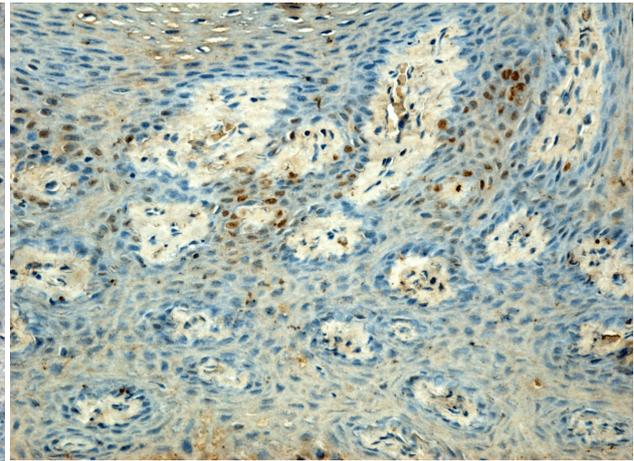
MMP-13 was present in all cases, but it was more intense in the two cases with T1D+AP with PD>6 mm when it had similar patterns as MMP-9 staining and in one case with AP when the staining was observed strictly in the lamina propria associated with moderate chronic inflammatory infiltrate (Figures 5 and 6).

## Discussion

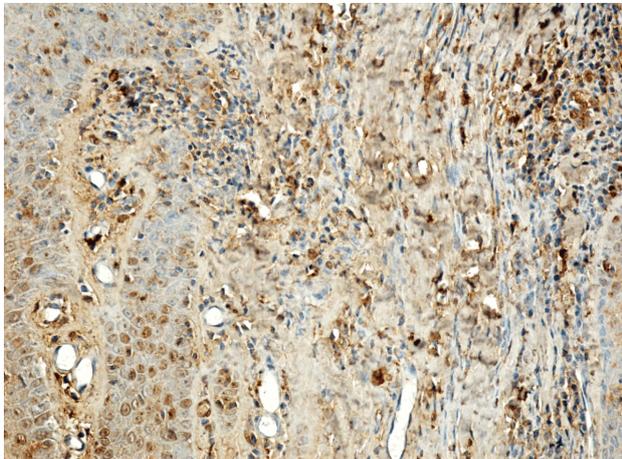
Periodontitis is a common chronic inflammatory disease characterized by destruction of the supporting structures of the teeth. It is highly prevalent (severe periodontitis affects 10–15% of adults) and has multiple negative impacts on quality of life. Epidemiological data confirm that diabetes is a major risk factor for periodontitis; susceptibility to periodontitis is increased by approximately threefold in people with diabetes. There is a clear relationship between degree of hyperglycemia and severity of periodontitis. The mechanisms that link these two conditions are not completely understood, but involve aspects of immune functioning, cytokines and metalloproteinases biology. Treatment of periodontitis is associated with HbA1c reductions. Oral and periodontal health should be promoted as integral components of diabetes management [1].



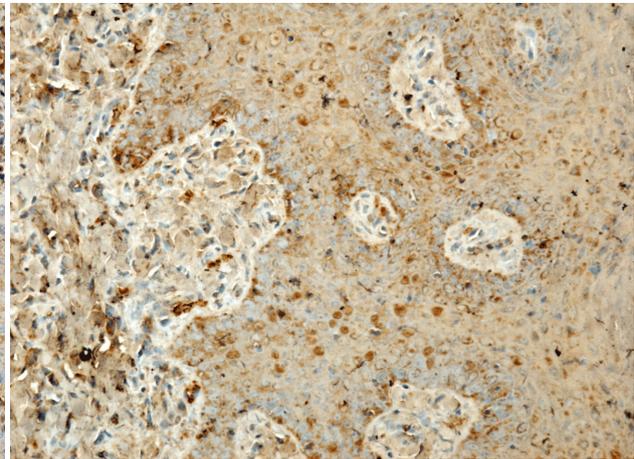
**Figure 1 – Positive MMP-7 immunoreaction in the immunostaining in the connective tissue and lamina propria papillae and hypertrophic epithelium in a T1D + moderate AP case. Immunostaining with antibodies anti-MMP-7,  $\times 100$ .**



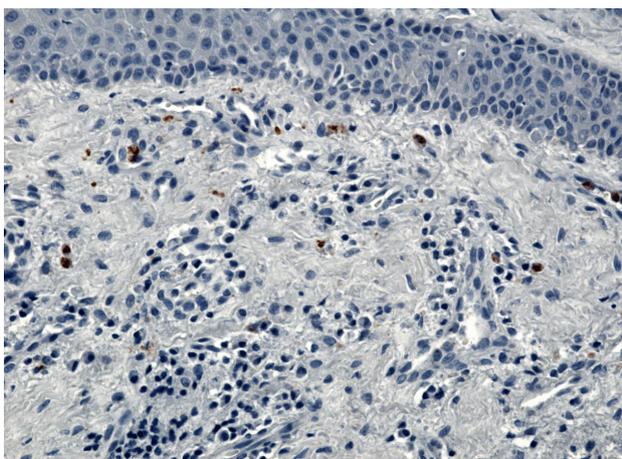
**Figure 2 – Positive MMP-7 immunoreaction in the immunostaining in the connective tissue and lamina propria papillae and hypertrophic epithelium in a T1D + severe AP case. Immunostaining with antibodies anti-MMP-7,  $\times 200$ .**



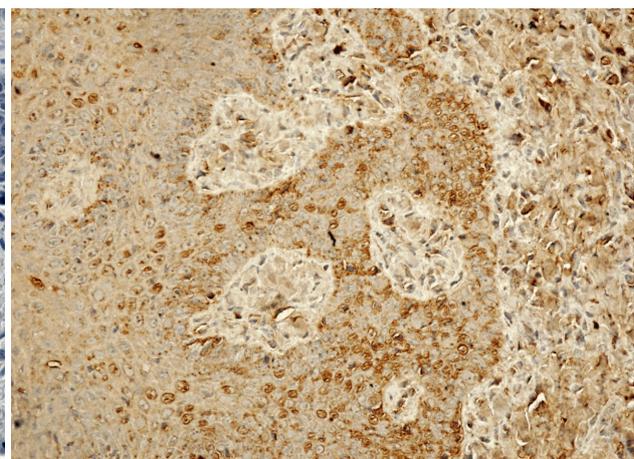
**Figure 3 – MMP-8 immunostaining positive in the gingival mucosa in a T1D + moderate AP. Immunostaining with antibodies anti-MMP-8,  $\times 100$ .**



**Figure 4 – MMP-9 present in the entire gingival epithelium and lamina propria of the mucosa, in a severe AP case. Immunostaining with antibodies anti-MMP-9,  $\times 100$ .**



**Figure 5 – MMP-13 present in the chorion of the gingival mucosa a T1D + moderate AP case. Chronic inflammatory infiltrate. Immunostaining with antibodies anti-MMP-13,  $\times 200$ .**



**Figure 6 – MMP-13 immunostaining in all structures of the hypertrophic mucosa in a severe AP case. Immunostaining with antibodies anti-MMP-13,  $\times 100$ .**

Lappin *et al.* explored plasma concentrations of bone metabolism markers comparatively between type 1 diabetes mellitus and non-diabetic patients and tried to

evaluate the influence of periodontitis on biomarkers of bone formation. They found that diabetics had significantly lower osteocalcin concentrations, lower RANKL

to OPG ratios and higher OPG concentrations than non-diabetics. The ratio of RANKL to OPG was altered by the periodontal status [16].

Periodontal MMP-8 expression is associated with periodontal disease but the information concerning the periodontal MMP-8 expression in T1D patients with periodontal disease is insufficient. In a comparative study upon nine adults with well-controlled T1DM and nine healthy adult controls, using a model of experimental gingivitis with cessation of oral hygiene for three weeks and monitoring for a further two weeks, Salvi *et al.* [17] found that MMP-8 and MMP-9 increased in both groups in parallel but were higher in T1D group.

Dysregulation of MMP may contribute to the development of diabetic complications, including diabetic retinopathy and coronary and peripheral arterial disease. Thrailkill *et al.* [18] explore how MMP-2 concentration relates to disease status of T1D. Authors measured MMP-2 concentrations and MMP-2 activity were measured in plasma and timed urine samples from 93 T1D and 50 healthy control subjects, aged 14–40 years. Findings showed that urine and plasma MMP-2 concentrations and plasma MMP-2 activity were all significantly elevated in T1D subjects, compared with control subjects. Urine MMP-2 concentrations, were correlated with several clinical parameters which infer increased risk for diabetic co-morbidity, and specifically for diabetic nephropathy, including higher HbA1c, longer duration of disease, evidence of renal hyperfiltration, and the presence of microalbuminuria.

During periodontal inflammation, matrix metalloproteinases are under the control of several regulatory mechanisms including the upregulation of expression by inducers and downregulation by inhibitors. Emingil *et al.* [11] aimed to examine the levels and molecular forms of MMP-7, tissue inhibitor of MMP (TIMP)-1, and extracellular matrix metalloproteinase inducer (EMMPRIN) in GCF from patients with different periodontal diseases. A total of 80 subjects – 20 patients with generalized AP, 20 with chronic periodontitis (CP), 20 with gingivitis, and 20 periodontally healthy subjects – were included. Periodontal status was evaluated by measuring probing depth, clinical attachment loss, presence of bleeding on probing, and plaque. GCF MMP-7, TIMP-1, and EMMPRIN levels and molecular forms were analyzed by enzyme-linked immunosorbent assay (ELISA) and western immunoblot techniques using specific antibodies. Results showed that total amounts of GCF MMP-7 were found to be similar between the study groups. Generalized AP, CP, and gingivitis groups had significantly higher total amounts of GCF EMMPRIN compared to healthy subjects. Among the patient groups, the generalized AP group had the highest total amount of GCF EMMPRIN relative to the gingivitis group. Soluble EMMPRIN existed in GCF in multiple molecular-weight species especially in periodontitis-affected GCF under non-reducing conditions. All patient groups had significantly elevated total amounts of GCF TIMP-1 relative to the healthy group. Generalized AP and CP groups also had a higher total amount of GCF TIMP-1 compared to the gingivitis group. These results indicate that MMP-7 is associated with the innate host defense in periodontal tissues. Increased EMMPRIN and TIMP-1 levels in GCF are associated with the enhanced

severity of periodontal inflammation, indicating that these molecules can participate in the regulation of progression of periodontal diseases.

It was found that CP and generalized AP patients had similar GCF MMP-7 levels compared to gingivitis and healthy patients and that MMP-7 might play an important role in the early defense of periodontal tissues. Similar GCF MMP-7 levels among diseased groups might indicate that this enzyme does not have an important role in the pathogenesis of periodontitis. These findings suggest that MMP-7 is associated with the innate host defense in periodontal tissues. It is possible that it may be for this reason that it was not found an elevated MMP-7 level in GCF in patients with CP and generalized AP. In addition to destructive events, similar GCF MMP-7 levels between diseased and healthy groups suggest that defensive events are more prominent than tissue remodeling events in generalized AP and CP patients. It is usually accepted that the balance between activated MMPs and TIMPs controls the extent of extracellular matrix remodeling, and tissue degradation is caused by the disruption of this balance in favor of proteinases. Under pathological conditions associated with unbalanced MMP/TIMP activity, changes in TIMP-1 levels could be important in the regulation of the destruction of periodontal tissues by affecting the MMP levels in periodontal tissue [11]. Sustaining these findings, in our study, MMP-7 was not expressed in AP cases both moderate and severe, but the reaction was positive in all cases with T1D suggesting the involvement of this enzyme in GCF. Constituents such as MMP-8 can be diagnostically used to monitor the course, treatment, and medication (any drug that was given to patients for the supporting of periodontal treatment) of periodontal diseases [6, 19].

Hardy *et al.* [7] compared patients without it, patients with periodontal disease alone and patients with both periodontal disease and diabetes. Gingiva specimens were collected and examined in frozen sections and MMP-8 protein expression was detected using immunohistochemistry and quantified. Results showed that the difference in MMP-8 protein levels among the three groups were statistically significant. There was a tendency of increase in periodontal MMP-8 levels across the three groups. *In vitro* studies indicated that high glucose and LPS had a synergistic effect on MMP-8 expression [3].

In the present study, the MMP-8 expression was more intense in the gingiva of diabetic patients than in those non-diabetics, suggesting that the T1D have affected the collagen remodeling by increasing the production of MMP-8.

In the study of Hernández *et al.* (2007), a group of 76 patients with a diagnosis of moderate to severe chronic periodontitis and, besides, nine healthy patients with no clinical and radiographic periodontal changes were selected and consecutively enrolled. MMP-13 and TIMP-1 were determined from active and inactive sites. It was not detected a differences in total MMP-13 and TIMP-1 determinations between active and inactive sites from periodontitis progression patients, but it was observed a tendency towards an inverse correlation between MMP-13 and TIMP-1 in active samples [20]. The authors stated that collagenolytic activity of MMP-13 could play a central role in regulating this process and the balance

between MMP-13 and TIMP-1 expression could reflect the onset of periodontal activity [20].

In a prospective study, GCF samples from 21 subjects undergoing clinical progression of chronic periodontitis and 11 healthy controls were screened for carboxy-terminal telopeptide of collagen I (ICTP) levels, MMP-13 activity and TIMP-1. Diseased gingival explants were cultured, treated or not with MMP-13 adding or not CL-82198, a synthetic MMP-13 selective inhibitor, and assayed by gelatin zymography and densitometric analysis.

Active sites showed increased ICTP levels and MMP-13 activity in progression subjects. The MMP-9 activation rate was elevated in MMP-13-treated explants and MMP-13 inhibitor prevented MMP-9 activation.

MMP-13 and -9 could potentially form an activation cascade overcoming the protective TIMP-1 shield, which may become useful for diagnostic aims and a target for drug development [21]. In our study, MMP-13 and -9 had a similar pattern, the immunostaining being positive in all cases with more intense reaction in cases with T1D+AP, especially in those with severe periodontitis suggesting a more intense inflammation and collagen breakdown.

## Conclusions

The expression of MMP-7, -8, -9 and -13 in the gingiva of the young patients with aggressive periodontitis and T1D was positive in all studied cases supporting the hypothesis that both are inflammatory diseases with common pathogenic mechanisms involving inflammatory mediators and may be possible biomarkers of disease status.

## Author contribution

All authors made equal contribution to the paper, to that of first authors.

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