

Cyclooxygenase-2 and matrix metalloproteinase-9 expressions correlate with tissue inflammation degree in periodontal disease

LUMINIȚA LAZĂR¹⁾, ANDRADA LOGHIN²⁾, EUGEN-SILVIU BUD³⁾, DIANA CERGHIZAN⁴⁾, EMŐKE HORVÁTH⁵⁾, ELŐD ERNŐ NAGY⁶⁾

¹⁾Department of Periodontology and Oral Diagnosis, University of Medicine and Pharmacy of Tirgu Mures, Romania

²⁾Department of Histology, University of Medicine and Pharmacy of Tirgu Mures, Romania

³⁾Department of Orthodontics, University of Medicine and Pharmacy of Tirgu Mures, Romania

⁴⁾Department of Fixed Prosthodontics, University of Medicine and Pharmacy of Tirgu Mures, Romania

⁵⁾Department of Pathology, University of Medicine and Pharmacy of Tirgu Mures, Romania

⁶⁾Department of Pharmaceutical Biochemistry and Environmental Chemistry, University of Medicine and Pharmacy of Tirgu Mures, Romania

Abstract

Introduction: Cyclooxygenase-2 (Cox-2) and matrix metalloproteinase-9 (MMP-9) have synergistic effects in the degradation of the extracellular matrix. **Objective:** The aim of our study was to correlate the intensity of inflammation with MMP-9 and Cox-2 expression in the periodontal tissue of patients with chronic inflammatory disease (gingivitis and chronic periodontitis) in order to determine the role of these two biomarkers in the progression of periodontal disease. **Materials and Methods:** To conduct this study we analyzed the gingival biopsies taken from patients clinically divided into three study groups: Group I (control): Patients free of periodontal disease (seven biopsies); Group II: Patients with gingivitis (10 biopsies); Group III: Patients with chronic periodontitis (10 biopsies). In these three groups, we graded the intensity of inflammation in the lamina propria and the immunohistochemical expression of MMP-9 and Cox-2. **Results:** The presence of a large number of inflammatory cells in the lamina propria in patients with gingivitis or chronic periodontitis (Groups II and III) correlated with the clinically diagnosed inflammation of the gingival tissue. The expression of MMP-9 was higher in patients with chronic periodontitis than in those with gingivitis, showing a trend towards statistical significance ($p=0.07$, Mann-Whitney *U*-test). The expression of Cox-2 in periodontitis was also higher compared to gingivitis ($p=0.05$, Mann-Whitney *U*-test) and to controls ($p=0.001$, Mann-Whitney *U*-test). The inflammation score could be positively correlated to the MMP-9 and Cox-2 expression scores at the overall study group, but not separately on gingivitis and periodontitis patients. **Conclusions:** The presence of an intensive inflammatory infiltrate is characteristic both for periodontitis and gingivitis. MMP-9 and Cox-2 show higher expression in periodontitis, than in gingivitis and healthy controls, but MMP-9 and Cox-2 expression scores cannot be directly correlated to the grade of inflammatory infiltrate in the two different disease entities. As biomarkers of chronic inflammation activity, angiogenesis, and degradation of the extracellular matrix, MMP-9 and Cox-2 can be used in clinical practice for the detection of patients with chronic periodontitis risk, at whom treatment with Cox-2 and MMP-9 inhibitors may be considered.

Keywords: cyclooxygenase-2, matrix metalloproteinase-9, gingivitis, chronic periodontitis.

Introduction

Pathological changes in periodontal tissue are caused by the presence of periodontal pathogens that act by direct or indirect mechanisms. Indirect activity occurs through the action of immune or non-immune cells, which release pro-inflammatory factors under the influence of bacterial agents. The periodontal pathogens responsible for gingival inflammation cause not only the activation of non-specific immune response but also that of immune cells with the appearance of a large number of plasma cells involved in immunoglobulin synthesis.

Cyclooxygenase-2 (Cox-2) is an inducible enzyme that is not present in the healthy tissue and is expressed by cells involved in inflammatory processes. Cox-2 is involved in the conversion of arachidonic acid into prostaglandins and it plays an important role in periodontal tissue inflammatory responses [1]. There is ample evidence that this enzyme catalyzes the synthesis of prostaglandins, which

are important mediators of tissue and bone damage in addition to vasodilatation and increased capillary permeability [2]. Cox-2 secretion is induced by pro-inflammatory cytokines and bacterial compounds, while prostaglandins are important regulators of IL-6 production by gingival fibroblasts [3].

Matrix metalloproteinase-9 (MMP-9) is a proteolytic enzyme, which cleaves the denatured collagen, in particular collagen type IV, the major component of the basement membrane. The secretion and release of MMP-9 by activated monocytes and lymphocytes is strictly regulated by cytokines. MMP-9 is secreted as a proenzyme and remains inactive until it is activated by removal of the propeptide moiety by proteolytic enzymes (stromelysin-1, MMP-2, and other MMPs) [4]. MMP-9 is usually secreted in conjunction with a specific inhibitor, TIMP-1, which controls its proteolytic activity. MMP-9 is produced by macrophages, polymorphonuclear (PMN) leukocytes, osteoclasts and it is responsible for bone formation.

membrane type-1 (MT1)-MMP is involved in angiogenesis and in connective tissue and bone formation [5]. The imbalance between MMP and TIMP (tissue inhibitor of MMP) leads to excessive tissue degradation, common in chronic inflammatory diseases including chronic periodontitis [6–9].

Our study aimed to correlate the histological score of inflammation intensity with MMP-9 and Cox-2 expression in the periodontal tissue sampled from patients with gingivitis and chronic periodontitis in order to determine the role of these two biomarkers in disease progression.

Materials and Methods

In order to conduct this study, we analyzed the gingival biopsies taken from patients who presented at the Department of Periodontology, Faculty of Dentistry, University of Medicine and Pharmacy of Țirgu Mureș, Romania.

Inclusion criteria were: the presence of dental units whose indication was extraction, cooperative patients, different clinical periodontal status to allow us to include the patient in one of the three groups. Exclusion criteria were: the presence of systemic diseases, gingival hypertrophy secondary to administration of drugs, smoking, pregnant or breastfeeding women, periodontal treatment in the last six months, antibiotic, immunosuppressive and steroidal or non-steroidal anti-inflammatory therapy in the last six months, data reported by the patients own declaration.

Patients were divided into three study groups: Group I (control) consisted of 10 patients free of periodontal disease, with no bleeding on probing and probing depth absence of ≥ 3 mm; Group II included 10 patients with gingivitis, bleeding on probing and absence of ≥ 3 mm probing depth; Group III comprised 10 patients with chronic periodontitis, with the presence of ≥ 3 mm attachment loss and ≥ 6 mm probing depth.

For each patient we performed a biopsy from the dental unit, which was to be extracted. For patients in Groups I and II, a biopsy was taken with a scalpel by the incision in the mesial or distal interdental papilla after anesthesia and prior to extraction. For patients in Group III, we sectioned the gingival wall of the periodontal pocket, each biopsy containing epithelium and lamina propria. The aim was to obtain a sufficient amount of tissue including epithelium and lamina propria for the histopathological and immunohistochemical examinations.

Personal data and clinical examination results were recorded in observation sheets for each group separately; subsequently, immunohistochemical and histopathological findings were added.

The study was conducted with the informed consent of the patients; they were informed about the working protocol, the benefits, and risks of participating in the study. The Scientific Research Ethics Committee of the University of Medicine and Pharmacy of Țirgu Mureș granted favorable approval.

The histopathological and immunohistochemical study was conducted independently by two senior pathologists in order to assess the intensity of inflammation and the expression of Cox-2 and MMP-9 in inflammatory cells, both having synergistic effects in the degradation of the extracellular matrix.

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and cut at 4–5 μ m. The quantitatively insufficient and incomplete biopsies were excluded from the study. The routine histology examination was conducted on Hematoxylin–Eosin (HE) staining, followed by grading the inflammation and immunohistochemistry analyzes in order to establish the correlation between the tissue destructive markers (MMP-9 and Cox-2) and the extent of inflammation. The source, clonal codes and dilution of the antibodies are shown in Table 1.

Table 1 – Antibodies and their applicability conditions used for immunophenotyping of inflammatory cells

Antibody	Dilution	Clone	Manufacturer	Staining in inflammatory cells
NCL-MMP9-439	1/40	15W2	Novocastra Laboratories Ltd., UK	Heterogeneous cytoplasmic expression
Monoclonal mouse anti-human Cox-2	1/100	CX-294	DAKO, Denmark	Heterogeneous cytoplasmic expression

The immunophenotype of proliferating cells was studied using a heat-induced antigen retrieval method (HIER). The antigen expression was visualized with EnVision™ Flex immunodetection system (K8010) and the reaction product with DAB (3,3'-diaminobenzidine) chromogen followed by Hematoxylin-counterstaining. Colorectal adenocarcinoma tissue samples were used an endogenous positive control for Cox-2 and normal kidney tissue (positive staining in tubules) for MMP-9. The primary antibody was replaced by normal serum (mouse IgG1, code X0931) in negative control tissues.

In order to determine the intensity of inflammation, the number of inflammatory cells (inflammation grade) in the lamina propria was taken into account. We examined five microscopic high-power fields (HPF, 40 \times) from surface to depth, calculating the mean number of cells on a progressive scale from 0–4: score 0 (no inflammation) – less than five inflammatory cells; score 1 (mild inflammation) – 5–20 inflammatory cells; score 2 (moderate inflammation) – 20–40 inflammatory cells; score 3 (severe inflammation) – more than 40 inflammatory cells.

The evaluation of the MMP-9 expression (target cells: monocytes/MF, PMN) and Cox-2 (target cells: fibroblasts, lymphocytes, plasma cells, PMN granulocytes, and endothelial cells) was assessed semiquantitatively by calculating the percentage of all positive mononuclear cells of all the inflammatory cells. These were examined in the lamina propria in five HPF (40 \times) classifying them also as score 0–3. Score 0 represented the absence of positive inflammatory cells, with expression only in the basal epithelial cells. Score 1 is characterized by expression in less than 20% of all the above-mentioned cells. Score 2 covers cases with 20–40% immunolabeled cells and score 3 cases with over 40% positive cells.

Statistical analysis

Taking into consideration the small size of the groups, we performed a statistical comparison by the non-parametric Mann–Whitney *U*-test. As cut-off level for statistical significance, a threshold level of $p=0.05$ was chosen.

Results

After the histopathological examination, three patients with incomplete biopsies (lamina propria reduced quantitatively) from the control group (Group I) were excluded. The age of remnant gingival tissue donors ranged from 20–72 years (mean age 51.57 ± 12.38), without statistically significant difference between the three groups.

Histological evaluation of the inflammatory infiltrate

Histopathology revealed the presence of qualitatively and quantitatively different inflammatory infiltrates in the two disease groups. In the tissue fragments of patients with periodontitis, the inflammatory infiltrates rich in lymphocytes, macrophages, fibroblasts and plasma cells interested the whole thickness of lamina propria. Subjacent to junctional epithelium, we observed increased capillary permeability, presenting leukocyte marginalization and migration into junctional epithelium. A proliferation of basal cells of the junctional epithelium and papilla elongation were also observed. In some cases, we detected

inflammatory cells in the epithelial thickness (exocytosis), even associated with surface ulceration. In gingivitis samples, the inflammatory infiltrates poor in plasma cells was localized predominantly in the junction, compared to the periodontitis group.

Inflammation in the healthy controls (Group I) was absent in three cases (score 0) and was minimal (score 1) in all other cases. The 10 tissue samples of the patients diagnosed with gingivitis (Group II) presented varying degrees of inflammation: three samples of score 1, five of score 2, and two of score 3. A moderate mixed inflammatory infiltrate with the presence of granulocytes (Figure 1) prevailed in this group.

All tissue samples in periodontitis patients (Group III) showed chronic inflammatory infiltrate, seven biopsies with score 3 while the other three were score 2, the chronic inflammatory infiltrates being mainly represented by mononuclear elements (lymphocytes, monocytes/macrophages, histiocytes, fibroblasts, and fibrocytes) and a few PMN granulocytes.

We also observed the presence of neoformed capillaries (Figure 2).

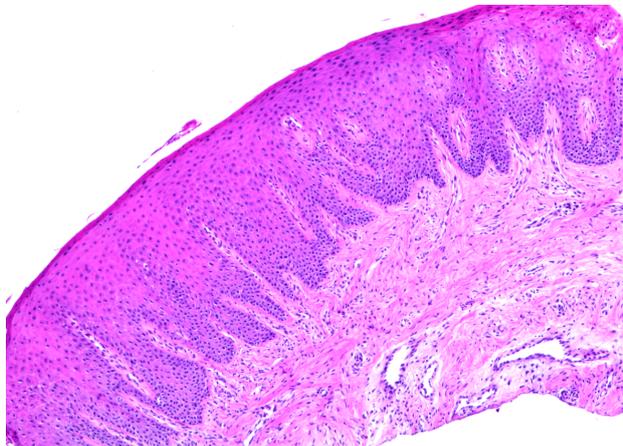


Figure 1 – Gingival biopsy: lamina propria with moderate chronic inflammatory infiltrate (grade 2). HE staining, $\times 40$.

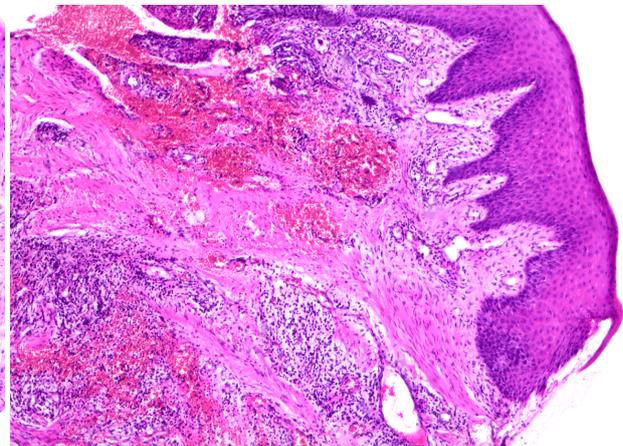


Figure 2 – Gingival biopsy: lamina propria with high level of chronic inflammation (grade 3). HE staining, $\times 40$.

The overall degree of inflammation analyzed on the basis of inflammation scores showed a statistically significant difference between the control group and the two groups with gingival lesions (control *versus* gingivitis $p=0.003$, control *versus* periodontitis $p=0.001$) and between the gingivitis and periodontitis groups ($p=0.023$) (Figure 3).

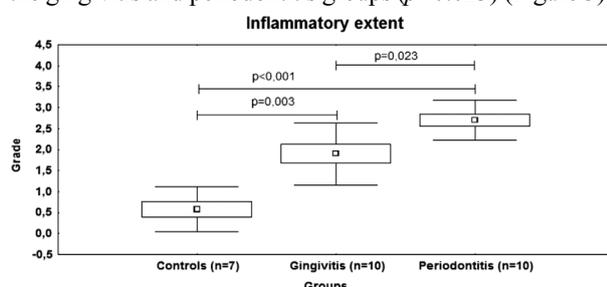


Figure 3 – Box-plot representation (mean, standard error and standard deviation) of the degree of inflammation in the three groups.

Evaluation of immunohistochemical reactions

The expression of the two biomarkers examined was reported in relation to the positive and negative controls,

taking into account only the cytoplasmic expression in the target cells. In the control group (Group I), MMP-9 expression was weak, four cases were classified as score 0 and the other three as score 1. Cox-2 expression presented a similar pattern.

The MMP-9 score 2 (Figure 4) was present in three biopsies belonging to Group III, only one case in Group II. Score 3 appeared only in Group III (one case). Cox-2 quantified as score 1 (Figure 5) appeared in all the three groups examined, but scores 2 and 3 were predominant in Group III.

The two biomarkers scores and their relationship to the degree of inflammation for each case studied are shown in Table 2.

Statistical analysis showed that the expression of MMP-9 does not differ significantly between healthy patients (Group I) and those with gingivitis (Group II) ($p=0.31$), however, it is significant between healthy subjects and those with periodontitis ($p=0.018$), with a trend toward statistical significance between the two groups of patients with gingival lesions (gingivitis *versus* periodontitis, $p=0.07$) (Figure 6).

In terms of Cox-2 expression, we obtained the following results: a statistically significant difference between periodontitis and control groups ($p=0.001$), periodontitis and

gingivitis ($p=0.05$), and higher scores with a trend toward statistical significance in patients with gingivitis compared to healthy subjects ($p=0.08$) (Figure 7).

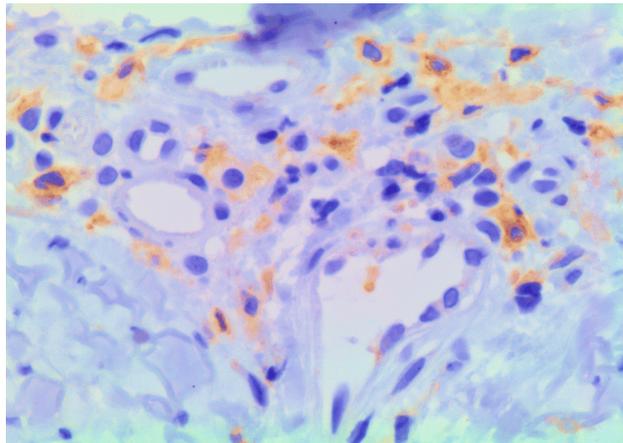


Figure 4 – MMP-9 immunolabeled cells representing more than 40% of the total inflammatory cells (score 3). DAB chromogen, $\times 400$.

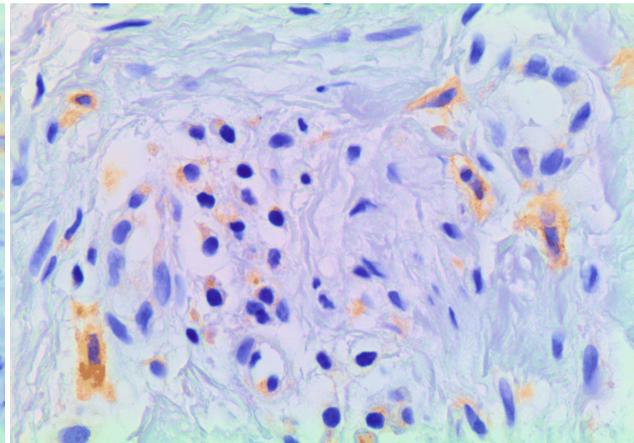


Figure 5 – Cytoplasmic Cox-2 expression in 20–40% of the inflammatory cells (score 2). DAB chromogen, $\times 400$.

Table 2 – The expression of the biomarkers investigated and their relationship to the degree of inflammation in the three groups studied

Groups	Inflammation score	MMP-9 score	Cox-2 score
Group I			
H1, H6, H7	0	0	0
H2	1	1	1
H3, H5	1	1	1
H4	1	0	0
Group II			
G1, G6	1	1	1
G2	2	0	2
G3, G5	2	1	1
G4	2	1	3
G7	2	0	0

Groups	Inflammation score	MMP-9 score	Cox-2 score
G8	3	1	0
G9	1	0	1
G10	3	2	2
Group III			
CP1, CP3	1	3	3
CP2	2	2	3
CP4	2	1	2
CP5	2	1	1
CP6	2	0	2
CP7	3	2	2
CP8, CP10	1	1	1
CP9	3	2	3

H: Healthy; G: Gingivitis; CP: Chronic periodontitis.

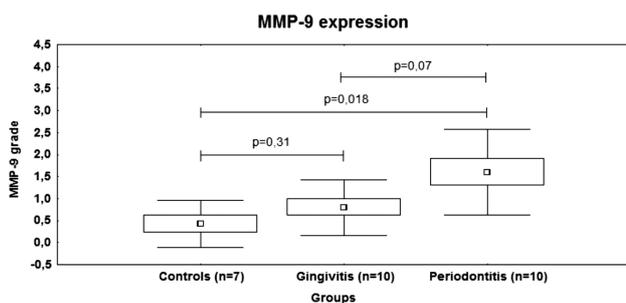


Figure 6 – Box-plot representation (mean, standard error and standard deviation) of MMP-9 expression in gingival tissue in the three groups.

The expression scores are shown in Table 3.

Table 3 – The mean and standard error of the degree of inflammation, of MMP-9 and Cox-2 expression in the three groups of patients

Diagnosis	Inflammation score	MMP-9 score	Cox-2 score
Control (n=7)	0.57 \pm 0.20	0.43 \pm 0.20	0.42 \pm 0.20
Gingivitis (n=10)	1.90 \pm 0.23	0.80 \pm 0.20	1.20 \pm 0.29
Periodontitis (n=10)	2.70 \pm 0.15	1.60 \pm 0.30	2.10 \pm 0.28

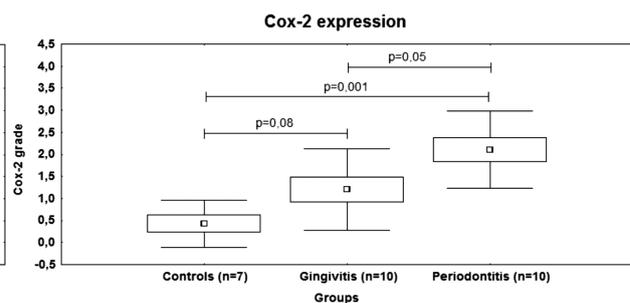


Figure 7 – Box-plot representation (mean, standard error and standard deviation) of Cox-2 expression in gingival tissue in the three groups.

Correlation analysis of the histological score revealed that both MMP-9 and Cox-2 scores could be significantly correlated to the inflammation score on the overall group. MMP-9 and Cox-2 scores also showed a strong significant positive correlation. In the healthy control group, MMP-9 and Cox-2 scores could be positively associated to the inflammation score (with borderline significance). No significant relationship of the two immunohistochemical scores and inflammation score could be highlighted. MMP-9 and Cox-2 expression scores correlated positively in the periodontitis group (Table 4).

Table 4 – Correlation results of inflammation score, MMP-9 and Cox-2 scores

	ISc × MMP-9 Sc		ISc × Cox-2 Sc		MMP-9 Sc × Cox-2 Sc	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Overall group (n=27)	0.65	<0.001	0.58	0.001	0.68	<0.001
Controls (n=7)	0.75	0.052	0.75	0.052	1.00	<0.001
Gingivitis (n=10)	0.38	0.27	0.03	0.92	0.26	0.47
Periodontitis (n=10)	0.39	0.25	0.08	0.82	0.76	0.001

r: Spearman's correlation coefficient; ISc: Inflammation score; MMP-9 Sc: MMP-9 score; Cox-2 Sc: Cox-2 score.

Discussion

Human gingival tissue, continuously exposed to bacterial infection (biofilm of bacteria on the teeth and below the gum-line) develops a consequent chronic inflammation, activating the pathogen-associated molecular pattern receptors. At the histological level, due to epithelial and mesenchymal components an adaptive response can be seen proinflammatory signals triggering Cox-2 and the prostaglandin E₂ (PGE₂) synthesis pathway. Functional gene polymorphisms of effectors like IL-2, Cox-2, and MMPs, have been found to play important roles in the development of pathological process leading to loss of teeth [10]. As a proinflammatory mediator, vasodilatation and capillary permeability inductor, PGE₂ stimulates osteoclast bone resorption [2]. The initial pathogenesis of periodontitis is mediated by Cox-2 via PGE₂, the latter being controlled by IL-1 and IL-6, also inductors of MMP-9 secretion in monocytes. MMP-9 plays an important role in the degradation of type IV collagen, the major component of the basal membrane and gingival tissue matrix. Elevated levels of active MMP-9 in the saliva, gingival crevicular fluid and gingival tissue, being secreted by activated lymphocytes and monocytes were described in several studies, and the molecule is considered as a biomarker of chronic periodontitis (CP).

The microscopic analysis of gingival biopsies taken from healthy subjects allowed us to notice known structural aspects of the stratified squamous epithelium covering the gums and of the underlying connective tissue [11, 12]. Although during the clinical examination the appearance of the gingiva was similar to that of healthy tissue, in four biopsies taken from patients in the control group, we observed the presence of inflammatory cells. Similar to our results, Şurlin *et al.* observed the presence of inflammatory infiltrate in the lamina propria, highly represented in patients with positive clinical signs of gingival inflammation while underrepresented in those showing no inflammation [13].

The inflammatory cell score characterizing the lamina propria in patients with gingivitis and chronic periodontitis (Groups II and III) was directly correlated to the presence of clinically diagnosed inflammation in the gingival tissue. Our results showed a statistically significant correlation between the inflammation score and MMP-9 and Cox-2 scores in the overall study group, relationship reflected in the healthy controls. The positive correlation was slightly stronger for the MMP-9 in the overall group. The lack of correlation between the inflammation score and the two-immunohistochemical scores was observed in the gingivitis and periodontitis groups. Periodontitis patients also showed a positive correlation MMP-9 and Cox-2 scores. The results showed that by our scoring approach,

MMP-9 expression reflex better the grade of inflammatory infiltrate. Correlation of MMP-9 and Cox-2 in the periodontitis group showed the possible presence of persistent trigger in the mononuclear infiltrate. Similarly, a moderate-to-severe chronic inflammatory infiltrate was observed in the lamina propria of patients with gingivitis and chronic periodontitis, mainly composed of the mature plasma cell, macrophages, and B- and T-lymphocytes, more prominent near the sulcular epithelium [14].

MMP-9 is stored in secretory granules of neutrophils, eosinophils, macrophages as inactive proenzyme that require activation to exert its function. MMP-9 expression is induced by the presence of periodontal pathogens and inflammatory mediators. Its role in the progression of periodontal lesions is that they cause degradation of connective tissue proteins, such as types IV, V, and XI collagen, proteoglycans and elastin. In addition, MMP-9 can cause degradation of the bone matrix.

In our study, expression of MMP-9 was higher in patients with chronic periodontitis than in those with gingivitis, with a trend towards statistical significance. Smith *et al.* observed that the gingival tissue from patients with periodontitis contains MMP-9 in the junctional and sulcular epithelium as a deposit scattered throughout the connective tissue while low levels of MMP-9 were found in the epithelium unexposed to inflammation [15].

Cox-2 is the enzyme responsible for prostaglandin synthesis in inflamed sites in epithelial cells, endothelial cells, fibroblasts, and macrophages. Prostaglandin E₂ is a mediator of tissue damage which appears in periodontitis and which, besides vasodilatation and increased capillary permeability, stimulates osteoclastic bone resorption. Alveolar bone resorption is the main element proving the evolution from gingivitis to periodontitis, which can ultimately lead to tooth loss.

The histological samples of patients with pathological changes in the periodontal tissue detected on clinical examination showed. Therefore, we can state that there is a direct correlation between the degree of periodontal tissue destruction and Cox-2 expression.

Mesa *et al.* [14] found that Cox-2 expression in patients with gingivitis and chronic periodontitis is higher than in patients free of periodontal disease and is negatively correlated with the amount of connective tissue in the chorion. This finding led the authors to state that Cox-2 is involved in the mechanism of destruction of fibrillar structures of the periodontal support. Schaefer *et al.* showed that the Cox-2 plays an important role in periodontitis in mediating the inflammatory responses of periodontal tissues [16]. Cox-2 expression is stimulated significantly in tissues with elevated levels of inflammatory infiltrate, detected at the level of gingival epithelium, both in endothelial cells and in cells with fibroblast appearance. Elevated levels

of Cox-2 may play a key role in generating high levels of prostaglandin E, with the occurrence of tissue destruction [17].

The evidence that periodontal tissue destruction is caused by high levels of Cox-2 and MMPs led researchers to conduct numerous studies related to the introduction of medicinal products that inhibit these enzymes in periodontal therapy [18–24].

☒ Conclusions

Correlation of MMP-9 and Cox-2 in the periodontitis group showed the possible presence of persistent trigger in the mononuclear infiltrate. The expression of Cox-2 and MMP-9 in the inflamed gingival tissue does not discriminate the two-histological disease subtypes (gingivitis and chronic periodontitis) but characterizes true the severity of the lesion. They can be used in clinical practice for the detection of high-risk patients with chronic periodontitis as a consequence treatment with Cox-2 inhibitors *versus* non-selective non-steroidal anti-inflammatory drugs and inhibitors of MMP (chemically modified tetracyclines) may be considered.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding author

Eugen-Silviu Bud, Lecturer, PhD, Department of Orthodontics, University of Medicine and Pharmacy of Tîrgu Mureș, 38 Gheorghe Marinescu Street, 540139 Tîrgu Mureș, Romania; Phone +40265–215 551, Fax +40265–210 407, Mobile +40744–437 661, e-mail: eugen.bud@gmail.com