Inflammatory Markers from Crevicular Fluid in Periodontal Disease

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In this study there were analysed the local pro-inflammatory, anti-inflammatory and immunomodulating cytokines in the gingival crevicular fluid (GCF) in periodontal diseases (PD). It has been used the ELISA method for the determination of the immune-inflammatory markers of periodontal disease in GCF (on 117 patients). A local increase of pro-inflammatory (IL-1α, IL-1αβ, IL-6, TNF-α) and immunomodulating (IFN-γ) cytokines in GCF has been observed in patients with different stages of periodontitis and gingivitis ($p<0.0001$, respectively $p<0.001$), compared to healthy subjects. The anti-inflammatory cytokines (IL-4) were significantly decreased in all periodontal diseases ($p<0.0001$, respectively $p<0.0004$), vs. control group. The IL-1α and IL-1β concentrations were significantly increased in aggressive periodontitis, in comparison with gingivitis. TNF-α levels showed the same ascendant evolution in quantity in patients with gingivitis and aggressive periodontitis. IL-6 and IFN-ã had an increasing evolution as the disease progressed. Our study demonstrates the increased production of IL-1α, IL-1β, IL-6, TNF-α, IFN-γ and decreased secretions of IL-4 in GCF in patients with periodontitis and gingivitis, as an expression of the inflammatory response in periodontal diseases. The imbalance favours the progressive inflammatory destruction in periodontal diseases.

Keywords: pro-inflammatory, anti-inflammatory and immunomodulating cytokines, periodontitis, gingivitis

Periodontitis is an inflammatory lesion of the sustainable soft tissues surrounding the tooth which appears as a consequence of the local salivary bacteria which accumulate as dental plaque. The periodontitis represents a multifactor pathological entity, which has as a major cause the *periodontopat germs* (*Actinobacillus Actinomycetemcomitans, Porphyromonas gingivalis, Bacteroides forsythus, Porphyromonas gingivalis, Prevotella intermedia*) that contribute to the generation of a local immune response. These germs induce an inflammatory local immune response. Local inflammation produced by bacterial invasion is considered to be the determinant factor in human periodontal disease [1, 2]. The interleukin 1 (IL-1) is a , *multifunctional cytokine* which belongs in the group of proinflammatory cytokines. There have been identified 2 forms of IL-1: IL-α and IL-β. The production of IL-1 is triggered by microorganisms, microbial residual products, inflammatory mediators, and antigens. IL-1 has an important effect on several target cells and tissues. Both forms of IL-1 are glycoprotein with 17 kDa and have related polypeptidic structure that show approximately 25% homology at the amino acid level. They have similar pro-inflammatory properties, IL-1β being the more active one [3, 4]. The interleukin-6 (IL-6) mimics the multifunctional effects of IL-1, especially the pro-inflammatory ones. IL-6 acts as a multifunctional cytokine which is secreted by several cells such as monocytes and macrophages induced by bacterial LPS, T cells, destroyed endothelial cells [3, 5]. Interleukin-4 (IL-4) promotes the progression of the inflammatory reaction in which Th_2 cell plays main roles through periodontal disease [2, 6]. The tumor necrosis factor alpha (TNF-α) is the most important regulator of the host responses to microbial infection. Also it is a major modulator of extra cellular matrix catabolism and bone resorption. The TNF-α is a mediator of inflammation, which stimulates phagocytosis, degranulation and antibody dependent cytotoxicity of polymorphonuclear cells (PMN), which represent the majority of the inflammatory infiltrate cells in the acute phase [2, 7]. Interferon γ (IFN-γ) is the immunomodulating cytokine placed in the middle of the cytokine cascade released in the cellular inflammatory immune response [2, 8].

Periodontal diseases (PD) are chronic inflammatory diseases produced by local infection and are characterized by the resorbtion of the tooth-supporting structures. Periodontal diseases are the common form of bone pathology in humans and an influencing factor of the systemic health of the patients [9, 10].

The aim of this study was to identify the local changes of the inflammatory and immune markers, in the gingival crevicular fluid (GCF), as an expression of the inflammatory response in periodontitis and gingivitis.

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Experimental part

Material and method

Patient selection

In this study were included 117 patients, aged between 21 to 65, males and females, investigated during 2012-2015 in the Dentoalveolar Surgery office, Victor Babes University of Medicine and Pharmacy Timisoara. The patients were diagnosed according to the classifications recommended by the American Academy of Periodontology in 1999 [11].

All patients presented a generalized inflammation of the periodontal tissues. Inclusion criteria for the diagnosis of chronic periodontitis were attachment loss of 5 mm at more than 30% of sites. For the positive diagnosis of aggressive periodontitis patients had respected the following criteria: bone loss 50% determined radiographic for at least two different teeth. The subjects included in the study were clinically healthy (no systemic diseases, e.g. diabetes mellitus) and were not on any medication. The patients were nonsmokers for at least 5 years. Clinical examinations included a plaque record index [12, 13], a bleeding index, and measurements of probing depths and of attachment loss at six sites per tooth. The patients were divided into three cohorts, according to their dental status: the *cohort 1* of aggressive periodontitis (AgP; n=42; 18 women and 24 men) and a clinical diagnosis of aggressive periodontitis according to World Workshop in Periodontology criteria [14]; the *cohort 2* of chronic periodontitis (CP; n=31; 14 women and 17 men) and a clinical diagnosis of chronic periodontitis according to World Workshop in Periodontology criteria [15]; the *cohort 3* of gingivitis (G; n=22; 16 women and 6 men), the *control group* included healthy patients (HC; n=22; 12 women and 10 men). All participants to the study have provided a written informed consent. The samples of GCF were collected during the morning, 2-3h after the last meal, 1h after the last drink. The sites to be sampled were isolated with cotton rolls and gently air-dried, so the probes would not be contaminated with saliva. Crevicular washes were obtained using a previously described method [13]. The gingival crevicular fluid samples were provided from the periodontal pockets (1-4 pockets for each patient in the deepest sites, about 5mm in depths). The crevicular gingival fluid was collected on Periopaper GCF strips which were inserted inside gingival space (for 30-40 s), in order to be soaked with fluid. The samples were incubated for 60 min in 500μL phosphate-buffered saline (PBS) with *p*H=7.4. Then the solution was centrifuged at 300 rpm for 10 min and the supernatant was separated for further tests.

Immune-inflammatory markers determination

In this study there were measured by a sandwich ELISA (enzyme-linked immunosorbent assay) technique different levels of the cytokines in gingival crevicular fluid (GCF). We used the protocol indications of the ELISA Quantikine® kits from R&D Systems Inc., Minneapolis, USA: the human IL-1α/IL-1F1 Immunoassay (sensibility <1.0pg/mL, 8standard calibrators with range 0 to 250.0pg/mL, and reproducibility precision intra-assay CV=1.5-3.5% and inter-assay CV=4.3-8.3%); the human IL-1β/IL-1F2 Immunoassay (sensibility <1.0pg/mL, 8standard calibrators with range 0 to 250.0pg/mL, and reproducibility precision intra-assay CV=2.3-3.4% and inter-assay CV=3.4-7.1%); the human IL-6 Immunoassay (sensibility <0.7pg/mL, 8standard calibrators with range 0 to 300.0pg/mL, and reproducibility precision intra-assay CV=1.7-4.4% and inter-assay CV=2.0-3.7%); the human IL-4 Immunoassay (sensibility <0.03pg/mL, 8standard calibrators with range 0 to 16.0pg/mL, and reproducibility precision intra-assay CV=7.4-7.9% and inter-assay CV=8.1-10.1%); the human TNF-α Quantikine (sensibility < 1.6pg/mL, 8standard calibrators with range 0 to 1000.0pg/mL, and reproducibility precision intra-assay CV=4.4-5.3% and inter-assay CV=6.8-8.7%); the human INF-γ Quantikine (sensibility <8.0pg/mL, 8standard calibrators with range 0 to 1000.0 pg/mL, and reproducibility precision intra-assay CV=2.6-4.7% and inter-assay CV=3.7-7.8%).

Immunohistological determinations

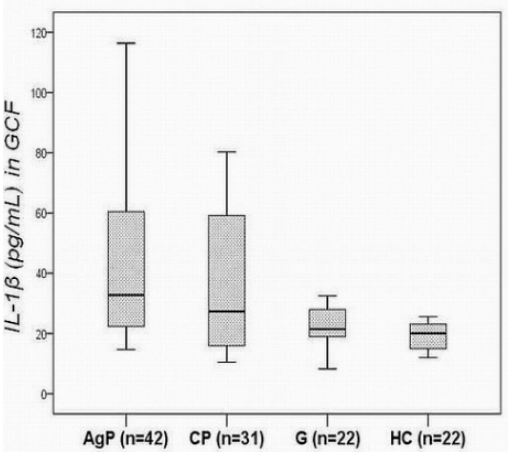
From the patients with AgP and CP it was performed a gingival biopsy in order to determine the expression for KI-67 and CD31 antibody and to investigate if there is a correlation between these markers and periodontal lesions. The obtained material were gingival fragments which were immunohistochemically examined. There were used anti-Ki-67 (clone MIB-1, Dako) and anti CD-31 (clone JC/70A, Dako) monoclonal antibodies and the visualization technique LSAB. For negative control the antibodies were substituted with buffer solution, and for positive control we used uvula tissue. In order to visualize them, the samples were stained with DAB and H&E.

Statistical analysis

All the values were reported as a medium ± standard deviation (mean±SD). The values were compared using Student's test for paired data, and values of $p<0.05$ were considered statistically significant. The strength and direction of a linear relationship between two random variables were determined using the r-Pearson correlation coefficient. Differences between mean values of variables were tested by one-way ANOVA, when more than 4 groups were compared.

Results and discussions

In periodontal disease, the difference between the mean ages of females and/or males versus those of the control group was not statistically significant (in AgP and G groups $p^*>0.05$) (table 1). These results constitute an important argument for making correlations between the levels of gingival crevicular fluid cytokines independent of the patient's sex. In the GCF of AgP group there were observed significantly increased levels of IL-1α (+53.1%, $p<0.0001$), IL-1β (+62.5%, $p<0.0001$), IL-6 (+48.5%, $p<0.0001$) and TNF-βα (+62.7%, $p<0.0003$) vs. the healthy cohorts. In this group, the levels of IL-4 in GCF



AgP-aggressive periodontitis, CP-chronic periodontitis, G-gingivitis, HC- healthy cohort (r-Pearson correlation coefficient,r>-0.700-higher significantly negative value tested by one-way ANOVA)

Fig. 1. Levels of IL-1β in gingival crevicular fluid in the periodontal disease compared to the healthy patients

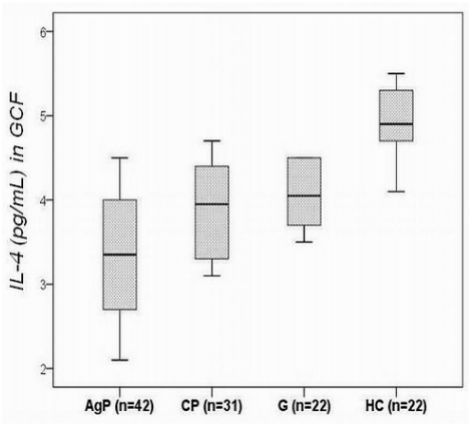


Fig. 2. Levels of IL-4 in gingival crevicular fluid in the periodontal disease, compared to the healthy patients

AgP-aggressive periodontitis, CP-chronic periodontitis, G-gingivitis, HC- healthy cohort (r-Pearson correlation coefficient,r>-0.700-higher significantly negative value tested by one-way ANOVA)

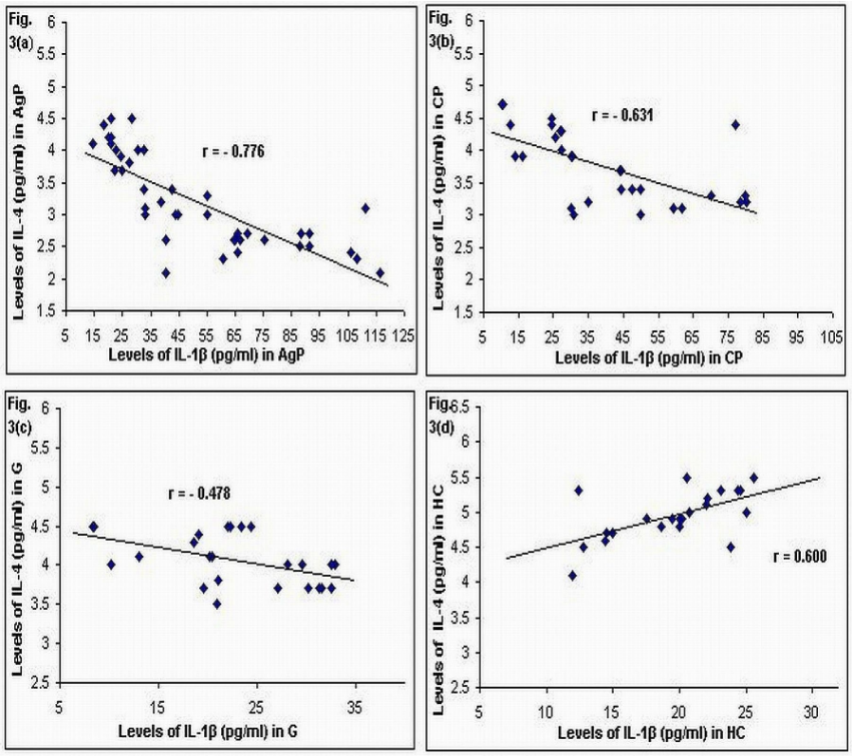


Fig. 3. Correlation between levels of IL-1β and IL-4 in aggressive periodontitis (a), chronic periodontitis (b), gingivitis (c), and healthy cohort (d)

PARAMETER (p)	AgP (mean±SD) (range)	CP (mean±SD) (range)	G (mean±SD) (range)	HC (mean±SD) (range)
Age patients (yrs)	**35.6±12.4** (18 - 55.5) p*=0.218	**51.7±7.8** (39.5 - 65) p<0.01	**38.2±14.2** (22 - 61) p*=0.315	**40.8±15.3** (18 - 62)
Age women (yrs)	**37.3±13.7** (18 - 52) p*=0.391	**51.9±10.1** (38 - 65) p<0.01	**38.7±16.1** (20 - 61) p*=0.342	**42.4±15.1** (18.5 - 61.5)
Age males (yrs)	**34.6±12.4** (18.5 - 55) p*=0.482	**51.4±8.9** (39.5 - 63.5) p<0.05	**37.6±13.5** (22 - 59.5) p*=0.374	**38.9±16.2** (18 - 62)

Tables 1
DEMOGRAPHIC CHARCATERISTICS IN PERIODONTAL DISEASE

AgP-aggressive periodontitis, CP-chronic periodontitis, G-gingivitis, HC-healthy cohort;
p<0.05-significantly different from control group; were determined using the Student t' test).

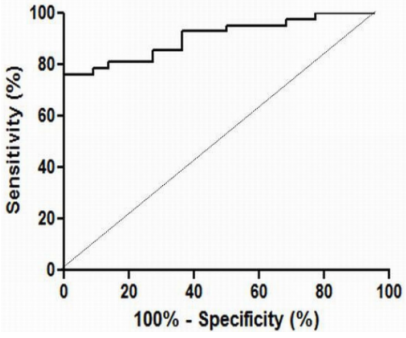
were significantly decreased (-24.3%, p<0.0001), compared to healthy subjects. The levels of IFN-γ were increased (+79.5%, p<0.001) (table 2, figs. 1-2). Also, the increased levels of IL-1β were not in strong negative correlations (r= -0.776) with the decreased level of IL-4 (fig. 3a).

Analyzing the obtained results, in CP group there were significantly higher levels in GCF of: IL-1α (+47%, p<0.001), IL-1β (+59.3%, p<0.0004), IL-6 (+74.9%, p<0.0001), TNF-α (+60.3%, p<0.0005) and IFN-ã (77.6%, p<0.0001) vs. the healthy subjects. Also, the levels of IL-4 in GCF were decreased (-23.9%, p<0.0001), compared to the control group (table 2, figs. 1-2). In CP disease this negative correlations between the increased levels of IL-1β and the decreased levels of IL-4 maintains the high value (r= -0.631) (fig. 3b).

The same cytokines were determined in GCF for the pacients with gingivitis and were found different levels of IL-1α (+19.4%, p<0.0004), IL-1β (+15.3%, p<0.05), IL-6 (+37.4%, p<0.0004), TNF-α (+35.2%, p<0.0002) vs. the control group. The GCF levels of IFN-α were significantly increased (+72%, p<0.0002) and IL-4 levels were decreased (-17.8%, p<0.0004), compared to healthy cohort (table 2, figs .1-2). The correlation between level of IL-1 β and level of IL-4 decreased significantly in gingivitis (r= -0.478, negative medium values) (fig. 3c). The control group had a positive correlation (r= 0.600, significantly positive value) (fig. 3d).

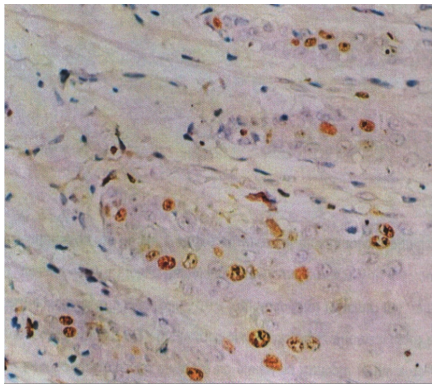
The levels of IL-1β were significantly increased in aggressive periodontitis. At a cutoff point of 22.4pg/mL, IL-1βrevealed a sensitivity of 85.7% (95% CI 71.4-94.5%) and specificity of 72.7% (95% CI 49.7-89.2%). However, when the cutoff value increased to 26.4 pg/mL, the specificity rised to 100% (95% CI 84.5-100%), but the sensitivity declined to 76.1% (95% CI 60.5-87.9%) (the area under ROC 0.909, 95% CI 0.840-0.977) (table 1, fig. 4).

In the inflammatory lesions from the gingival chorion extended to gingival mucosa, we have noticed an intensification of proliferative activity in superjacent basal layer (Image 1).

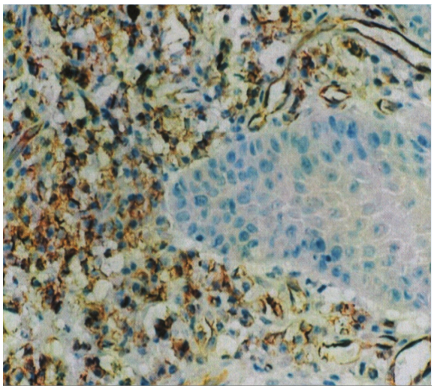


AgP-aggressive periodontitis (significantly sensitivity and specificity values > 70%)

Fig. 4. Sensitivity and specificity activity of levels IL-1β in aggressive periodontitis



Imagine 1. The expression for KI-67 antibody



Imagine 2. The expression for CD31 antibody

The immunohistochemical examined anti-Ki-67 and anti CD-31 monoclonal antibody and the visualization technique LSAB.

MARKERS (mean±SD) (range)	AgP (n=42)	CP (n=31)	G (n=22)	HC (n=22)
IL-1α (pg/ml)	**37.7±10.4** (17.5 - 88.4) p<0.0001	**33.3±14.6** (11.2 - 66.2) p<0.001	**19.8±3.8** (6.6 - 26.5) p<0.0004	**17.7±3.5** (10.3 - 21.4)
IL-1β (pg/ml)	**51.4±24.2** (14.7 - 116.4) p<0.0001	**37.9±16.4** (10.5 - 81.8) p<0.0004	**21.4±4.9** (8.3 - 32.5) p<0.05	**19.5±4.3** (7.2 - 25.6)
IL-6 (pg/ml)	**10.2±2.7** (6.5 - 16.1) p<0.0001	**21.3±6.9** (10.2 - 29.6) p<0.001	**8.4±1.2** (6.8 - 10.5) p<0.0004	**5.3±0.8** (4 - 6.4)
IL-4 (pg/ml)	**3.2±0.7** (2.1 - 4.5) p<0.0001	**3.8±0.5** (3.1 - 4.8) p<0.0001	**4.1±0.3** (3.7 - 5) p<0.0004	**4.9±0.3** (4.1 - 5.5)
TNF-α (pg/ml)	**19.6±2.1** (16 - 30.5) p<0.0003	**18.5±2.8** (14.6 - 23) p<0.0005	**11.3±0.8** (10 - 12.5) p<0.0002	**7.3±0.5** (6.2 - 8.2)
IFN-γ (pg/ml)	**36.2±9.4** (21 - 50) p<0.001	**31.4±6.8** (29.5 - 46.4) p<0.0001	**25.1±3.9** (10.2 - 30.7) p<0.0002	**7.1±1.1** (5 - 8.7)

Table 2
THE LEVELS OF INFLAMMATORY CYTOKINES IN GINGIVAL CREVICULAR FLUID

AgP-aggressive periodontitis, CP-chronic periodontitis, G-gingivitis, HC-healthy cohort; $p<0.05$-significantly different from control group; were determined using the Student t' test).

In patients with AgP, we observed an intense process of acute intraepithelial inflammation, which was significantly correlated with increased levels of IL-1β ($p<0.0001$) and IFN-γ ($p<0.001$), and decreased level of IL-4 ($p<0.0001$) in CGF. In patients with CP, we observed chronic inflammation present in the superficial chorion of the gingival lining, lymphoproliferation, increased levels of IL-1β and IFN-γ ($p<0.001$, respectively $p<0.0001$), and lower level of IL-4 ($p<0.0001$) in CGF.

After CD31 staining examination there were oberved cytoplasmic and membrane positive reactions in the endothelial cells, but also in lymphocytes, plasmocytes and macrophages. On all examined samples were identified vascular structures and capillaries. Also there were noticed endothelial cells which tend to form vascular lumens. In CP the expression of CD31 was moderate. In AgP an increased number of inflammatory cells were intense positive for CD31 in gingival chorion from superficial layers of the epithelia. It was observed that the number of inflammatory positive cells has decreased in the deepness of chorionic mucosa (Image 2).

Periodontal disease is characterized by inflammatory destruction of the supporting tissues of the tooth, the epithelial attachment which descent from cervix to the apex, periodontal ligament which fall off the cementum, and alveolar bone. This often results in tooth mobility which aggravates in tooth loss [15, 16]. Although periodontitis cannot occur in the absence of bacterial infection, less than 20% of the causality in periodontal disease can be related with the presence or absence of bacterial plaque. This indicates a significant contribution of the host response to periodontal disease expression [17, 18].

The study was a pilot in our University in which we determined several inflammation markers. The data were compared with other published data. The results were not significantly different in relation with demographic characteristics (age, sex) as shown in table I. There were used gingival crevicular fluid samples (GCF) and were determined the levels of proinflammatory cytokines (IL-1α, IL-1β; IL-6; TNF-α), immunomodulating cytokines (IFN-γ), anti-inflammatory cytokines (IL-4) measured by sandwich ELISA technique. In our study, in concordance with other published data, a local increase of proinflammatory IL-1á cytokine in gingival crevicular fluid has been observed in the patients with different stages of periodontitis ($p<0.0001$ for AgP, respectively $p<0.001$ for CP), and gingivitis ($p<0.0004$) compared to the control group. Similar results were obtained for proinflammatory cytokine IL-1β. Citokine IL-1β revealed a higher sensitivity of 85.7% and specificity of 72.7% in aggressive periodontitis being the most reliable of the proinflammatory local markers (table 2, figs. 1 and 4).

This study has proved a significant increase of inflammatory cytokines IL-1α, IL-1β in periodontitis (aggressive periodontitis and chronic periodontitis). This

demonstrates the gradual implication of these cytokines in the immune inflammatory response. The fact which must be pointed out is that the increased values of these cytokines were determined in the gingival crevicular fluid, which represents exudates of the local inflammatory cellular infiltrate and is a reflection of the local immune response. The cytokine cascade elaborated in inflammation, beside IL-1α and IL-1β, continues with IL-6, another proinflammatory cytokine. It has been suggested that IL-1α and β have a crucial role in the pathology associated with chronic inflammatory diseases, including periodontitis, and play an important role in the regulation of the immune response [19-21].

In our study we found that the value of IL-6 was increased in parodontitis and gingivitis compared with the control group. The level of IL-6 was significantly increased in chronic periodontitis ($p<0.001$) vs. control group, IL-6 being a specific chronic disease proinflammatory cytokine (table 2).

The pro-inflammatory cytokines activate the effectors functions of the phagocytes from the inflammatory infiltrate and thus are responsible for periodontal lesions [12, 21, 22]. The TNF-α is a mediator of inflammation, which stimulates phagocytosis, degranulation and antibody dependent cytotoxicity of polymorphonuclear cells [2, 18]. The higher values of TNF-α were found in aggressive parodontitis which could be the cause of an acute immune inflammatory process in development. TNF-α showed the same ascendant evolution in gingivitis and aggressive periodontitis as the other proinflammatory cytokines (table 2).

Anti-inflammatory cytokines IL-4 have multiple immune response-modulating functions on a variety of cell types, produced initially by activated T lymphocytes [16, 21].

In this study the levels of IL-4 in GCF were decreased in periodontal diseases with different stages of periodontitis (aggressive periodontitis -24.3%, $p<0.0001$ and chronic periodontitis -23.9%, $p<0.0001$) and in gingivitis (-17.8%, $p<0.0004$) vs. healthy group (table 2, fig. 2). This data suggest that the local defense mechanisms are overcomed by the bacterial citotoxicity and the inflammation occurs. The persisting local inflammation produces the initiation of the periodontal disease. The negative correlation between increased levels of IL-1β (proinflammatory cytokine) and decreased level of IL-4 (anti-inflammatory cytokine) decreased significantly from aggressive parodontitis to gingivitis (fig. 3a, b, c), compared to the control group (fig. 3d). The IFN-γ is an immunomodulating cytokine which inhibits even more the activity of IL-4 and activates IL-1α, IL-1β, IL-6 and TNF-α. This fact was demonstrated in our study because IFN-γ has increased as the disease progressed (table 2).

A local increase of proinflammatory (IL-1α, IL-1β, IL-6 and TNF-α) and immunomodulating (IFN-γ) cytokines in GCF has been observed in patients with different stages of periodontitis and gingivitis, compared to healthy subjects. The concentrations of both IL-1 were significantly increased in aggressive periodontitis compared to gingivitis. IL-6 mostly increases in chronic parodontitis.

In inflammatory lesions which extend in the deepness of the mucosa the proliferative activity increases. This was assessed in our study by Ki-67 immunostaining in AgP. The Ki-67 expression suggests an intense process of epithelial regeneration at this level (Image 1). CD31 immunostaining in AgP suggested an intense vascular inflammatory reaction localized in sub-epithelial structures (image 2).

Conclusions

Our study demonstrates the increased production of IL-1α, IL-1β IL-6, TNF-α and IFN-γ in gingival crevicular fluid (GCF) of patients with periodontitis (aggressive periodontitis and chronic periodontitis) compared to those with gingivitis and to the control group. Anti-inflammatory cytokines (IL-4) were progressive decreased in correlation with the gravity of periodontal disease.The increased levels of the proinflammatory markers are an expression of the inflammatory response in periodontitis and gingivitis. The decrease of this negative correlation between pro/anti-inflammatory cytokines attesting aggressive local inflammations is correlated with stages of periodontal disease. The intensity of the immune response is corelated with the increase of these mediators and can represent the evolution and monitoring markers of the periodontitis progression.

Abbreviations

- AgP = aggressive periodontitis;
- CD31= monoclonal antibody staining;
- CP = chronic periodontitis;
- DAB = 3,3'-Diaminobenzidine;
- E LISA = enzyme-linked immunosorbent assay;
- GCF = gingival crevicular fluid;
- G = gingivitis;
- HC = healthy cohorts;
- H&E = Hematoxylin and Eosin Staining;
- IFNγ = Interferon γ;
- IL-1α = Interleukin 1α;
- IL-1β = Interleukin 1β;
- IL-4 = Interleukin 4;
- IL-6 = Interleukin 6;
- Ki-67= monoclonal antibody staining;
- LPS = lipopolysaccharides;
- LSAB= Labeled streptavidin biotin method;
- PD = periodontal diseases;
- PMN = polymorphonuclear cells;
- TNF-α = tumor necrosis factor α;
- SD = standard deviation;

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