EXPRESSION OF SERUM INTERLEUKIN 4, INTERLEUKIN 6 AND TNF-A IN SUBJECTS WITH CHRONIC ADULT PERIODONTITIS

ELENA-TEODORA TÂLVAN[1], VICTOR CRISTEA[2], CĂLIN MOHOR[3], DANIEL CHISNOIU[4], RADU-SEPTIMIU CÂMPIAN[5]

[1,4]PhD Candidate "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, [3]"Lucian Blaga" University of Sibiu, [2,5]"Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca

Keywords: *chronic periodontitis, interleukin 4, interleukin 6, TNF- α, serum, ELISA-test*

Abstract: *Chronic adult periodontitis results from complex interaction between microbial insult and the host inflammatory-immune responses. In periodontal disease, multiple virulence factors, derived from periodontopathic bacteria, trigger the immune cells to release pro-inflammatory mediators such as cytokines. The aim of the study was to evaluate associations between interleukin 4, interleukin 6 and TNF-α in patients with chronic periodontitis, in order to determine if there is a correlation between clinical stage of the disease and concentration levels of these cytokines. A total of 78 subjects, 47 females and 31 males, divided in two groups: control and generalized chronic periodontitis group, were included in the study. Clinical periodontal parameters were recorded. Blood samples were obtained from each patient for serum analysis of IL-4, IL-6 and TNF-α. Enzyme-linked immunosorbent assay (ELISA) test was used, for quantification of these proteins in the blood samples. Statistical analysis was performed, using parametrical and nonparametrical techniques. The findings in our study demonstrate that IL-4, IL-6 and TNF-α concentrations are different between the two groups. Chronic periodontitis along with high concentrations of the cytokines are more frequent in older patients. Il- 4 is found to be higher in the healthy group and in early stages of chronic periodontitis, being directly linked to periodontal health status. IL-6 concentration is significantly higher in chronic periodontitis group, supporting its role in advanced bone destruction. TNF-α increases according to each interleukin in the two groups, meaning that it has no specificity in chronic periodontitis, only in the acute phase of inflammation. No correlation was found whatsoever between IL-4 and IL-6 in the same group. IL-4, IL- 6 and TNF-α concentrations in the crevicular fluid o patients with chronic periodontitis, reported in literature, have the same correspondent in serum of patients with the same disease. However, further studies, and correlations with other cytokines are required, in order to demonstrate that interleukins may be used as indicators of chronic periodontal disease.*

INTRODUCTION

Periodontitis is an inflammatory disease of bacterial origin, that results in the progressive destruction of the tissues that support the teeth, specifically the gingiva, periodontal ligament, cementum and alveolar bone.(1) The two most prevalent and most investigated periodontal diseases are dental plaque-induced gingivitis and chronic periodontitis, always the former preceding the latter.(2,3) However, in some cases gingivitis never progresses to periodontitis.(3) While gingivitis is a reversible inflammatory reaction of gingiva, periodontitis, with progressing inflammation at deeper periodontal tissues, leads to irreversible connective and bone tissue breakdown.(4)

Periodontal diseases include a heterogeneous group of chronic inflammatory conditions, which are mainly due to specific oral bacteria, enriched in subgingival biofilms.(4) Traditionally, Gram-negative species, such as Porphyromonas Gingivalis, Tannarella Forsythia, Treponema Denticola, and Aggregatibacter Actinomycetemcomitans, are considered major culprits of periodontal destruction.(4) The principal determinant is a host susceptibility profile, mainly related with individual inflammatory response.(5) However, half of the variation of the disease within a population results from genetic factors.(5) As a result, the severity of periodontal disease progression depends on the interplay of several factors, including biofilm bacteria, host immune status, genetic and environmental factors.(5) Chronic adult periodontitis results from complex interaction between microbial insult and the host inflammatory-immune responses.(6) The cross-talk between periodontal pathogens and inflammatory process is regulated by a network of cytokines, which are essential for most periodontal tissue breakdown, leading to clinical signs of disease.(7,8) They are a group of immune-regulation molecules, which develop an important and central role in the immunopathology of periodontal disease.(5) The cytokine network takes control over inflammatory mechanisms, in order to amplify or suppress tissue reactions in periodontal pathogenesis.(6) Cytokines are produced by different cells, taking part in defense mechanisms, like macrophages, T-cells, B-cells, lymphocytes, monocytes, natural killer cells, etc.(3) Interleukin 4 is an anti-inflammatory cytokine, secreted by T helper 2 lymphocytes (TH2), with potent down-regulation of macrophage function.(9) In periodontal tissues, lack of IL 4 can cause accumulation of macrophages and high production of IL 1β and TNF-α, which lead to bone resorption.(9) Interleukin 6 is a predominant cytokine associated

[2]Corresponding author: Victor Cristea, Str. Aleea Firiza, Nr. 10, Ap. 11, Cod poştal 400659, Cluj-Napoca, România, E-mail: victor_cristea@yahoo.com, Phone: +40740 216722

Article received on 05.05.2016 and accepted for publication on 03.06.2016
ACTA MEDICA TRANSILVANICA June 2016;21(2):117-121

with bone destruction in advanced periodontal disease.(10) It is a T-cell and macrophage secreted interleukin that stimulates osteoclast formation.(11) TNF-α, a cell signalling cytokine, is involved in acute phase reaction of systemic inflammation. It is produced mainly by activated macrophages, but also by many other cell types such as CD4+ lymphocytes, NK cells and neutrophils.(10) TNF-α is seen in the bone destruction stage or advanced stage of periodontitis.(12) Although there have been significant advances in the understanding of the cause and pathogenesis of periodontal disease over the last 40 years, the traditional methods by which clinicians diagnose periodontal disease have remained virtually unchanged. The diagnosis of periodontal disease relies almost exclusively on clinical parameters and traditional dental radiography.(13) Cytokines, being of great importance in the progression of periodontal disease, may be used as markers in diagnosis.(14)

PURPOSE

The aim of the study was to evaluate associations between interleukin 4, interleukin 6 and TNF-α in patients with chronic periodontitis, in order to determine if there is a correlation between clinical stage of the disease and concentration levels of these cytokines.

MATERIALS AND METHODS

Study population

A total of 78 subjects, with generalized chronic periodontitis, were included in the study. All subjects were recruited from the department of General and Maxillofacial Surgery, Sibiu University Hospital, Romania. The purpose of the study was completely explained to each subject before entering the study and informed consent was obtained from each patient. Complete medical and dental histories were taken from all subjects. None of the patients underwent any nonsurgical or surgical periodontal treatment within the past 12 months. All patients presented clinical signs of periodontitis such as: halitosis, gingival recession, periodontal pockets or tooth loss in advanced stages of the disease. Selection of the patients was made according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for the Classification of Periodontal Diseases. The clinical attachment loss (CAL) was measured, classifying the severity of the disease in slight: CAL 1-2 mm, moderate: CAL 3-4 mm and severe: CAL 5 mm or more. At the screening stage, to determine the clinical periodontal status, all subjects had a clinical periodontal examination, including the measurement of pocket depth and CAL, by one examiner. Patients were divided into two groups: chronic periodontitis and healthy subjects. The generalized chronic periodontitis group consisted of 64 patients, 40 females and 24 males between the ages of 38 and 87 (mean of 65.3 ± 11.48 years). They had moderate to severe alveolar bone loss, CAL of ≥3 mm and probing depth of gingival sulcus (PD) of ≥ 4 mm, in multiple sites of all four quadrants of the mouth. The healthy group consisted of 14 patients, 7 females and 7 males, ranged in age from 35-52, with a mean age of 44.21 ± 5.04 years, who exhibited no CAL, PD of 1-2 mm, no clinical inflammation or sulcular bleeding and minimal radiographic evidence of bone loss.

Collection of blood samples

Venous blood samples were collected from each subject, from the antecubital vein, in 4 ml vacutainer glass blood collection tubes for coagulation, buffered with sodium citrate 3.2 %. All samples were centrifuged after clotting, to separate the serum from the cells, for 20-30 min at 2000-3000 g. The obtained blood serum was placed into sterile Eppendorf vials and kept at −40 °C until being analyzed.

Biochemical analysis

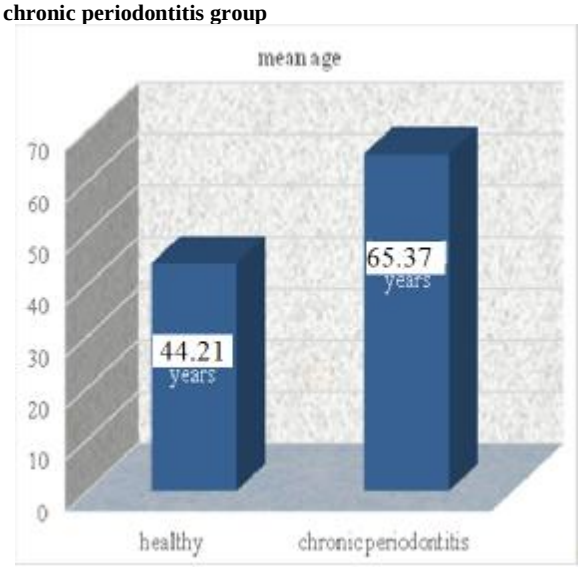
The IL-4 (Boster Biological Technology Co., Ltd. Fremont, CA, USA), IL-6 (Boster Biological Technology Co., Ltd. Pleasanton, CA, USA) and TNF-α (Boster Biological Technology Co., Ltd. Pleasanton, CA, USA) were analyzed by enzyme-linked immunosorbent assay (ELISA), for quantification of these proteins in the blood samples. Manufacturers' guidelines were followed for each assay and 96-well plates, precoated with appropriate antibodies, were used. The lower detection thresholds for the IL-4 and IL-6 and TNF-α assays were 15.6 pg/ml, 4.69 pg/ml and 15.6 pg/ml, respectively.

Statistical analysis

Statistical analysis was performed, using parametrical and nonparametric techniques. Comparison between the study groups and single interleukin testing between the groups were performed, using T-Student test, $p<0.05$ being considered to be statistically significant. Parametrical Pearson rank correlation and nonparametric Spearman rank correlation analysis were used to analyze the correlation between serum IL-4, IL-6 and TNF-α, for healthy and chronic periodontitis group and $p<0,05$ was considered to be statistically significant.

RESULTS

Figure no. 1. Mean ages of subjects in healthy group and chronic periodontitis group



There is a significant statistical difference ($p<0.001$) regarding age, between the chronic periodontitis group (mean age 65.37 years) and healthy group (mean age 44.21 years), meaning that chronic periodontitis is more frequent in older patients.

Figure no. 2. Correlation of clinical stage of PD between healthy and chronic periodontitis groups

Study group	N	Min.	Max.	Mean		Std. Deviation
	Stat.	Stat.	Stat.	Stat.	Std. Error	Stat.
Healthy subjects group	13	1.00	3.00	2.0000	0.16013	0.57735
Chronic periodontitis group	63	2.00	4.00	3.0476	0.08595	0.68223

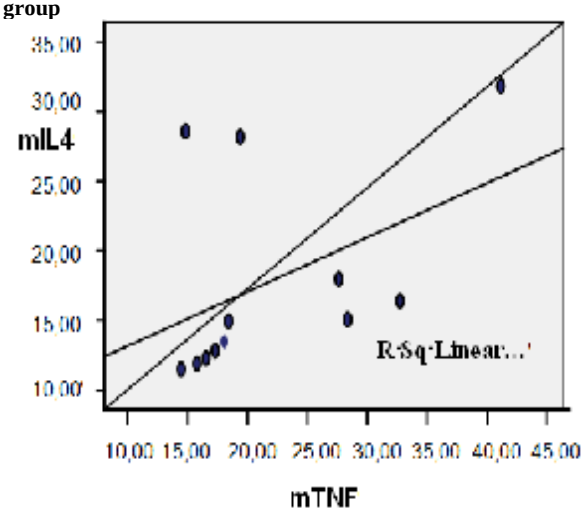
Regarding the clinical stage of periodontal disease, there is a significant statistical difference between the 2 groups (p<0,001). IL-4, IL-6 and TNF-α concentration are higher in chronic periodontitis group.

Figure no. 3. Mean, Std. Deviation, Std. Error, maximum and minimum results of IL-4, IL-6 and TNF-α testing between the 2 groups

Study group	N	Min.	Max.	Mean		Std. Deviation
	Stat.	Stat.	Stat.	Stat.	Std. Error	Stat.
Healthy subjects IL4	13	11.50	31.81	16.6334	1.74734	6.30011
Chronic periodontitis IL4	63	10.84	115.35	18.0521	2.07167	16.44334
Healthy subjects IL-6	13	7.54	14.04	10.1767	0.47234	1.70305
Chronic periodontitis IL-6	63	6.24	108.25	14.6084	2.25663	17.91145
Healthy group TNF	13	14.46	41.17	21.7876	2.26488	8.16615
Chronic periodontitis TNF	63	14.65	75.00	20.3013	1.10957	8.80693

Although IL-4 concentrations are slightly higher in periodontitis group (18.05 pg/ml) than in healthy group (16.63 pg/ml), the difference is not statistically important, p=0.761 (p>0.05). Similar to IL-4 testing results, the differences between the healthy and chronic periodontits group in IL-6 testing are not statistically significant, p=0.37818249 (p>0.05). The difference in TNF-α testing between the two groups are also not statistically significant, p=0.57913 (p>0.05).

Figure no. 4. Pearson and Spearman rank correlation analysis between IL-4, IL-6 and TNF-α results in healthy group



Pearson rank correlation analysis in healthy group shows no significant statistical correlations between IL-4, IL-6 and TNF-α. In contrast to Pearson analysis, nonparametric Spearman rank corellation analysis demonstrates a statistical significance r=,647 (p<0.05) between IL-4 and TNF-α in the healthy group, meaning that TNF-α increases according to IL-4 in this group. No correlation was found whatsoever between IL-4 and IL-6.

Figure no. 5. Pearson rank correlation analysis between IL-4, IL-6 and TNF-α results in chronic periodontitis group

		pIL4	pIL6	pTNF
pIL4	Pearson Correlation	1	0.066	0.227
	Sig. (2-tailed)		0.607	0.073
	N	63	63	63
pIL6	Pearson Correlation	0.066	1	0.321(*)
	Sig. (2-tailed)	0.607		0.010
	N	63	63	63
pTNF	Pearson Correlation	0.227	0.321(*)	1
	Sig. (2-tailed)	0.073	.010	
	N	63	63	63

In chronic periodontitis group, Pearson rank correlation analysis between IL-4, IL-6, TNF-α concentrations, reveals statistically significant results between IL-6 and TNF-α concentration (p<0.05), meaning that IL-6 and TNF-α concentration levels are high in chronic periodontitis group.

Figure no. 6. Spearman rank correlation analysis between IL-4, IL-6 and TNF-α results in chronic periodontitis group

			pIL4	pIL6	pTNF
Spearman's rho	pIL4	Correlation Coefficient	1.000	0.120	-0.095
		Sig. (2-tailed)	.	0.349	0.457
		N	63	63	63
	pIL6	Correlation Coefficient	0.120	1.000	0.454(**)
		Sig. (2-tailed)	0.349	.	0.000
		N	63	63	63
	pTNF	Correlation Coefficient	-0.095	0.454(**)	1.000
		Sig. (2-tailed)	0.457	0.000	.
		N	63	63	63

Correlation is significant at the 0.01 level (2-tailed).

Nonparametric Spearman rank correlation analysis, also shows a statistically significant correlation (p<0.01) between IL-6 and TNF-α concentration. It is interesting to notice that studying correlations between IL-4, IL-6, TNF-α concentration in chronic periodontitis group show different results. Pearson rank correlation analysis (r=0.321) as well as nonparametric Spearman rank corellation (r=0.454) show statistically significant results in TNF-α and IL-6 concentrations, which means that TNF-α concentration increases according to IL-6 in this group.

DISCUSSIONS

Periodontitis is a chronic destructive infectious disease, in which periodontal pathogens are required for disease initiation, but not sufficient to define the complete outcome. In this context, the host response plays a critical role in periodontal tissue breakdown, which characterizes periodontal disease progression and severity.(15) It has been well documented that the activity of several cytokines increased and activated during the progression of periodontal disease.(6) It is well known that the quality of the host immune-inflammatory response against bacterial challenge determines the severity and extent of the disease.(6)

The main objective of this research has been to correlate clinical stage of chronic periodontal disease with serum expression of cytokine IL-4, IL-6 and TNF-α, in order to determine if a diagnosis is possible.

Our results demonstrate that effect of periodontal status on markers of systemic inflammation is significant.(16)

We observed that serum cytokine levels of IL-4, IL-6, TNF-α differ between the two groups. Analyzed independently, IL-4 concentrations were raised in early stages of chronic periodontitis, IL-6 in subjects with advanced chronic periodontitis and TNF-α in both of the groups. Most important is that association of IL-6 and TNF-α were significantly higher in chronic periodontitis patients, as well as IL-4 and TNF-α in the control group.

The study supports the well-known fact that autoimmune reactivity varies according to the stage of periodontal disease.(3) IL-4, IL-6 and TNF-α levels are different according to our analysis in advanced stages of periodontal disease, compared to levels of these cytokines in the control group. Also a significant high rate of advanced chronic periodontal disease is evident among older patients. Interleukin concentrations are high in advanced stages of chronic periodontitis. Therefore, we can conclude that increased levels of these cytokines are consistent with advanced ages.

IL-4, measured by enzyme- linked immunoabsorbent assay, shows a slight increase of the cytokine in chronic periodontitis group, especially in early stages and significant high levels associated with TNF-α in the control group of healthy patients. This may support previous studies of low ratio concentrations of IL-4 in gingival crevicular fluid of patients with periodontal tissue destruction, whereas increased ratio of IL-4 in patients with improved periodontal health.(17) The study is in agreement with the hypothesis that absence of IL-4 might trigger periodontal disease and that presence of IL-4 is important to the health of periodontal sites. In other words, increased levels of IL-4 along with the absence or early stages of periodontal disease, is in agreement with the literature. Many studies have demonstrated that lower concentrations of IL-4 in the periodontal tissues were related with the progression of the disease. As a result, we agree that IL-4 is an anti-inflammatory cytokine related to periodontal health status.(9)

IL-6 analysis in our study shows increased levels of this cytokine in patients with chronic periodontitis and significant high levels of IL-6 and TNF-α association in advanced stages of the disease. Some variations of the IL-6 gene have been associated with human PD susceptibility. IL-6 is fundamental in the inflammatory response against infectious agents and influences bone resorption in periodontal lesions.(18) IL-6 is known to be a pleiotropic cytokine associated with the destruction of bone and connective tissues in periodontitis.(19)

Results for IL-6 testing support its implication in bone destruction of advanced stages of chronic periodontal disease.
TNF-α is directly involved in cell migration and plays a key role in alveolar bone resorption and loss of connective tissue, due to its correlation with the expression of metalloproteinases and the osteoclast differentiating and activating factor in periodontal tissues. TNF-α is a key mediator during periodontal disease, given that it recruits leukocytes, amplifies the inflammatory burden and recruits and activates bone-resorbing osteoclasts.(20) In periodontal disease multiple virulence factors derived from periodontopathic bacteria trigger the immune cells to release pro-inflammatory mediators, particularly TNF-α, IL-1 and IL-6. These cytokines further initiate a sequential cascade of inflammation, resulting in bone resorption and connective tissue degradation.(21)

In the present study, serum TNF-α concentration does not have statistical significance in the chronic periodontitis group, compared with the healthy group. Instead, studying correlations between IL-4, IL-6, TNF-α concentration in chronic periodontitis and healthy group, show different results. Pearson rank correlation analysis (r=0.321) as well as nonparametric Spearman rank correlation (r=0.454) show statistically significant results in TNF-α and IL-6 concentrations in chronic periodontitis group, which means that TNF-α concentration increases according to IL-6 in this group. Furthermore the same analysis in the healthy group shows significant results in correlation between IL-4 and TNF-α, meaning that TNF-α increases according to IL-4 in the healthy group. According to our study TNF-α has no specificity in chronic periodontal disease, considering that high concentrations of this interleukin are found in both of the study groups simultaneous, increased levels of IL-4 are evident in the healthy group and IL-6 in the periodontitis group. These findings may support the fact that high TNF-α concentrations only occur in acute stages of inflammation.

CONCLUSIONS

In conclusion, the paper supports literature data regarding role and significance of IL-4, IL-6 and TNF-α in chronic periodontal disease. The study demonstrates that IL-4, I- 6 and TNF-α concentrations in the crevicular fluid, found in literature, have the same correspondent in serum of patients with chronic periodontal disease. However, further studies, and correlations with other cytokines are required, in order to demonstrate that these interleukins may be used as indicators of chronic periodontitis.

REFERENCES

1. Wolf DL, Lamster IB. Contemporary Concepts in the Diagnosis of Periodontal Disease. Dental Clinics of North America. 2011;55(1):47-61.
2. Tatakis DN, Kumar PS. Etiology and Pathogenesis of Periodontal Diseases. Dental Clinics of North America. 2005;49(3):491-516.
3. Javed A, Pramod K, Abu Tahir A, Ansari SH. Autoimmune responses in periodontal diseases. Autoimmunity Reviews. 2011;10(7):426-431.
4. Könönen E, Kumar PS. Bacteriology of Periodontal Diseases, Molecular Medical Microbiology .Second Edition. 2015;(2):957-968.
5. Albuquerque C, Morinha F, Requicha J, Dias I, Guedes-Pinto H, Viegas C, Bastos E. A case–control study between interleukin-10 gene variants and periodontal disease in dogs. Gene. 2014;539(1):75-81.
6. Kurşunlu SF, Öztürk VO, Han B, Atmaca H, Emingil G. Gingival crevicular fluid interleukin-36β (-1F8), interleukin-36γ (-1F9) and interleukin-33 (-1F11) levels in different periodontal disease. Archives of Oral Biology. 2015;(60)1:77-83.
7. Kornman KS, Page RC, Tonetti MS. The host response to the microbial challenge in periodontitis: assembling the players. Periodontol. 2000;14(1997):33-53.
8. Preshaw PM, Taylor JJ. How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? J Clin Periodontol. 2011;38:60-84.
9. Anovazzi G, Finoti LS, Sâmia CT, Yeon JK, Marcaccini AM, Gerlach RF, Capela MV, Orrico RP, Cirelli JA, Scarel-Caminaga RM. Interleukin 4 haplotypes of susceptibility to chronic periodontitis are associated with IL-4 protein levels but not with clinical outcomes of periodontal therapy. Human Immunology. 2013;74(12):1688-1695.
10. Wikipedia.
11. Shimizu N, Ogura N, Yamaguchi M, Goseki T, Shibata Y, Abiko T, Iwasawa H Takiguchi. Stimulation by interleukin-1 of interleukin-6 production by human periodontal ligament cells. Archives of Oral Biology. 1992;37:(9):743-

748.
12. Gümüş P, Buduneli E, Bıyıkoğlu B, Aksu K, Saraç F, Buduneli N, Lappin DF. .Gingival crevicular fluid and serum levels of APRIL, BAFF and TNF-alpha in rheumatoid arthritis and osteoporosis patients with periodontal disease. Archives of Oral Biology. 2013;58(10):1302-1308.
13. Santos J, Menegat B, Lira-Junior R, Siqueira MA, Brito F, Carvalho AT, Fischer R G, Figueredo CM. Cytokine expression in gingival and intestinal tissues of patients with periodontitis and inflammatory bowel disease: An exploratory study. Archives of Oral Biology. 2016;66:141-146.
14. Mogi M, Otogoto J, Ota N, Inagaki H, Minami M, Kojima K. Interleukin 1β, interleukin 6, β2-microglobulin, and transforming growth factor-α in gingival crevicular fluid from human periodontal disease. Archives of Oral Biology. 1999;44(6):535-539.
15. Ribeiro Braosi AP, Machado de Souza C, Luczyszyn SM, Dirschnabel AJ, Claudino M, Olandoski M, Probst CM, Garlet GP, Pecoits-Filho R, Trevilatto PC. Analysis of IL1 gene polymorphisms and transcript levels in periodontal and chronic kidneydisease, Cytokine. 2012;60(1):76-82.
16. Satpathy A, Ravindra S, Thakur S, Kulkarni S, Porwal A, Panda S. Serum interleukin-1β in subjects with abdominal obesity and periodontitis. Obesity Research & Clinical Practice. 2015;9(5):513-521.
17. Chi-Cheng Tsai, Chia-Hsin Ku, Ya-Ping Ho, Kun-Yen Ho, Yi-Min Wu, Chun-Cheng Hung. Changes in Gingival Crevicular Fluid Interleukin-4 and Interferon-gamma in Patients with Chronic Periodontitis Before and After Periodontal Initial Therapy. The Kaohsiung Journal of Medical Sciences. 2007;23(1):1-7.
18. Morinha F, Albuquerque C, Requicha J, Dias I, Leitão, Ivo J, Guedes-Pinto H, Viegas C, Bastos E. Detection and characterization of interleukin-6 gene variants in Canis familiaris: Association studies with periodontal disease. 2011;485(2):139-145.
19. Stefani FA, Viana MB, Dupim AC, Ricieri Brito JA, Gomez RS, Eustáquio da Costa J, Moreira PR. Expression, polymorphism and methylation pattern of interleukin-6 in periodontal tissues. Immunobiology. 2013;218:(7):1012-1017.
20. Queiroz-Junior CM, Bessoni RL, Costa VV, Souza DG, Teixeira MM, Silva TA. Preventive and therapeutic anti-TNF-α therapy with pentoxifylline decreases arthritis and the associated periodontal co-morbidity in mice. Life Sciences. 2013;93(9-11):423-428.
21. Sanchavanakit N, Saengtong W, Manokawinchoke J, Pavasant P. TNF-α stimulates MMP-3 production via PGE2 signalling through the NF-kB and p38 MAPK pathway ina murine cementoblast cell line. Archives of Oral Biology. 2015;60(7):1066-1074.