Study on the TNF-α, IL-1β and IL-6 Levels in Patients with Chronic Periodontitis and Cardiovascular Diseases

OVIDIU NICOLAICIUC[1], CONSTANTIN MIHAI[1]*, IRINA GEORGETA SUFARU[1], IOANA MARTU[1], SORINA MIHAELA SOLOMON[1], DIANA TATARCIUC[2], CRISTIAN BUDACU[1], SILVIA MARTU[1]

[1]Grigore T. Popa University of Medicine and Pharmacy Iasi, Faculty of Dental Medicine, 16 Universitatii Str., 700115, Iasi, Romania
[2]Grigore T. Popa University of Medicine and Pharmacy Iasi, Faculty of Medicine, 16 Universitatii Str., 700115, Iasi, Romania

Studies on the link between periodontal disease and atherosclerosis have generated conflicting results and the mechanisms underlying this relationship are incompletely understood. Therefore, this study aimed to assess the levels in serum and in gingival crevicular fluid (GCF) of TNF-α, IL-1β and IL-6, to clarify the possible link between periodontitis and hyperlipidemia, as well as the effects of conventional periodontal treatment through scaling and root surfacing on these pro-inflammatory molecules. The study was carried out on a total of 40 subjects divided into two main groups: the study group (n=26) and control group (n=14). The cases included patients with atherosclerosis with prescribed diet (D) or antilipemic therapy with a drug from the statin group (S). Controls (C) were selected from systemically healthy subjects with chronic periodontitis. Samples were performed from crevicular fluid and serum, by determining the initial and post-treatment of TNF-α, IL-1β and IL-6. For all groups there were significant serum decreases in TNF-α, IL-1β and IL-6 from baseline, and the decreases were more significant IL-1β for statin group. Significant decreases were found in the crevicular fluid for all cytokines. The decrease was most evident for IL-6 in the statin group. Combining antilipemic periodontal therapy and treatment can provide beneficial effects on metabolism and control of inflammatory atherosclerosis by lowering serum pro-inflammatory cytokines.

Keywords: TNF-α, IL-1β, IL-6, chronic periodontitis, atherosclerosis

Cardiovascular diseases (CVD) have become the leading cause of chronic disease morbidity and mortality in industrialized countries [1]. CVD is the main driver of atherogenesis; it spans a number of decades and can eventually manifest with clinical events as myocardial infarction and stroke. Several factors such as hypercholesterolemia, oxidative stress, diabetes, smoking and infection, may increase the expression of adhesion molecules and cause endothelial dysfunction. Increased production of adhesion mediators leads to increased permeability of the intima and the initial formation of atheroma [2].

Possible etiologic role of acute or chronic infections on CVD has attracted great attention in recent years. The potential impact of various infectious agents on systemic inflammation and autoimmunity and subsequently the onset and progression of CVD has been studied extensively [3, 4].

Periodontal disease is one of the most common oral diseases and is characterized by destruction of the alveolar bone and connective tissue support, as a result of infection with secondary inflammatory response to periodontal bacteria [5-7]. Severe periodontitis, which may result in tooth loss, is found in 5-20% of the adult population in the world. Children and adolescents may show different forms of periodontal problems such as aggressive periodontitis, chronic periodontitis and periodontitis as a manifestation of systemic diseases.

A cohort study on CVD, conducted on 1400 men aged 60-70 years, showed that severe periodontal attachment loss conferred a statistically significant risk of death compared with controls (15.7% versus 7.9%) [8]. Oral infection may induce increased production of cytokines or indirectly acute phase proteins that enter the systemic circulation and reach remote sites of initial infection [9]. Numerous studies have focused on how the microbiome disruption itself could influence systemic proinflammatory cytokine production and to influence the systemic inflammatory diseases [10-12]. For example, disruption and low diversity of the intestinal microbiota were associated with conditions such as obesity and inflammatory bowel disease [13]. It becomes obvious that microbiome deregulation is associated with other systemic inflammatory diseases, including diabetes and atherosclerosis.

This study aimed to assess levels in serum and crevicular fluid (GCF) of TNF-α, IL-1β and IL-6, to clarify the possible link between periodontitis and hyperlipidemia, as well as the effects of conventional periodontal treatment by scaling and root planing on these pro-inflammatory molecules.

Experimental part

The study included a total of 40 subjects divided into two main groups: the study group (n=26) and control group (n=14). The cases included patients with atherosclerosis with prescribed diet (D) or antilipemic therapy with a drug from the statin group (S). Controls (C) were selected from systemically healthy subjects with chronic periodontitis.

Exclusion criteria were represented by any other systemic diseases that affect lipid metabolism (affected glucose tolerance, diabetes mellitus, metabolic syndrome, or other endocrine diseases, nephritic syndrome, chronic renal disease and cardiovascular disease), any treatment antilipemic drug for more than 1 month, any current hormone replacement therapy, three-fold elevation of liver enzymes, which received no periodontal treatment in the last 6 months, and any systemic antibiotic administration during the last 3 months. Smokers and former smokers were also excluded.

* email: drmihaiconstantin_2005@yahoo.com

Each patient completed a questionnaire that included data on oral hygiene, diet, medication history, current medication. All data obtained from clinical history and clinical and laboratory examinations were recorded in individual files.

Cases were submitted to a comprehensive periodontal examination which included radiographs and periodontal diagnosis. Oral health status of the control group was verified by clinical examination. Measurements of periodontal parameters, periodontal probing, BOP, plaque index, calculus index, tooth mobility and furcation lesions were standardized to the examiners.

The study was conducted according to the Helsinki Declaration. All participants gave their informed consent. All analyzes were performed in blind.

Metabolic parameters were analyzed at baseline and at 3 months after periodontal therapy. Blood samples were taken to measure levels of triglycerides (TRG), total cholesterol (TC), low density lipoproteins (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL). Samples were obtained after a fasting period of 12 hours from the ante-cubital vein. Serum lipid levels were determined by routine enzymatic methods.

Analysis of IL-1β, IL-6 and TNF-α in serum and in crevicular fluid was conducted at the beginning of the study and at 3 months after periodontal therapy.

For cytokine analysis 5ml of venous blood were drawn from an antecubital vein using a blood collection tube. Blood was allowed to clot for 30 min on ice and centrifuged for 10 min at 3000 rpm prior to placing the serum in 0.5 mL aliquots polypropylene tubes, which were stored at -40^{0}C until biochemical analysis. For laboratory assessments of serum the Enzyme-Linked Immunosorbent Assay (ELISA) was used. Results are expressed in pg/ mL. The lower limits of detection were <7, <2, and <9 pg/mL for IL-1β, IL-6 and TNF-α, respectively.

Crevicular fluid samples were collected from sites of disease (PPD ≥4 mm). Subjects were instructed not to eat, drink or brush teeth for 1 h prior to GCF sampling. Before sampling, the tooth was isolated with cotton rolls, supragingival plaque was carefully removed and the site was gently dried with air spray. A sterile paper cone (Dentsply Maillefer, Tulsa, OK, USA) was placed in each of the selected pockets, left in site for 30 s and then immediately placed in sterile Eppendorf tubes, which were stored at -20^{0}C until the analysis. In case of visible contamination with blood, paper cone was removed and a new site was selected.

For the cytokines determination, the cone paper were thawed, cut into 1cm in length and thawed in 50 μL 1X phosphate buffered saline solution [13 mM Na_2HPO_4, 7 mM $NaHPO_4$, 100 mM NaCl (*p*H 7.0)] at 4^{0}C. Further, the paper points were centrifuged at 13,000 x g for 10 min at 4^{0}C.

TNF-α, IL-1β and IL-6 concentrations were determined using ELISA (MyBioSource, San Diego, CA, USA) in a Luminex® 200™ analyzer (Luminex Corporation, Austin, TX, USA). The measurements were performed according to the manufacturer's instructions and standards and samples were measured in duplicate.

Periodontal therapy included manoeuvres of scaling and root planing (Full-mouth disinfection), with ultrasonic and manual instruments, performed in the Clinic of Periodontology, Faculty of Dental Medicine of the Grigore T. Popa UMPh, Ia°i.

Post-treatment evaluation of local and systemic inflammatory status was carried out at 3 months after periodontal therapy. At this stage, specialized clinical assessments of patients, with periodontal indices re-determination were performed. The quantification of cytokines was performed in serum by ELISA.

Statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY, USA) and $p < 0.05$ was considered to indicate a statistically significant difference. Continuous variables with a normal distribution are expressed as mean ± standard deviation of the mean and were analyzed using parametric tests (t-test for paired samples and independent). Since the cytokine levels were not normally distributed, the data is expressed as median (minimum and maximum) and they were analyzed using non-parametric tests (Mann Whitney U test or Wilcoxon test for unrelated samples related to samples). Fisher and McNemar tests were used to compare frequencies between related or unrelated samples. Ñ Spearman correlation coefficient was used to analyze correlations between clinical parameters and cytokines.

Results and discussions

Twenty-six patients with atherosclerosis, aged 39-62 years (13 women and 13 men) and 14 systemically healthy control subjects aged between 30 and 51 years (7 women and 7 men) participated in the study. There were no significant differences related to age and gender ($P > 0.05$).

Eight patients in the test group received a dose of 10mg or 20 mg atorvastatin for 1 month. At the end of the first month, the lipid levels of statin patients were reassessed for possible need for dose adjustment. However, it has been found there is no need to change the dose of the statin. Thus, statin dose of the studied population was constant throughout the study. Also, all patients said they have complied with the doctor's recommendations during the study.

For groups of atherosclerosis, body mass index (BMI) ranged between 22.24 and 41.38 and for systemic group healthy BMI value ranged between 18.00 and 30.60 at baseline. Groups S and D had a greater value of BMI compared to the C group ($P < 0.05$ and $P < 0.01$, respectively) at baseline and at the end of the study period ($P < 0.05$ and $P < 0.01$, respectively).

Table 1 displays the periodontal clinical parameters and serum lipids in study groups according to the study periods. For the C, S and D groups there were significant differences in parameters between the baseline and 3 months evaluation, except CAL (table I).

GCF and serum levels of TNF-α, IL-1β and IL-6 of the study groups are shown in table 2. In all groups there was a significant decrease in TNF-α, IL-1β and IL-6 from baseline and the most significant decreases was recorded for IL-1βfor statin group ($P < 0.01$).

Significant decreases were found in the crevicular fluid for all cytokines (table 2). The most obvious decrease ($P = 0.001$) was for IL-6 in the group of statins.

Periodontitis is an infectious disease caused by predominantly anaerobic Gram-negative bacteria, and causes exacerbation of systemic and local proinflammatory molecules such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1 beta (IL-1β), RANKL and IL-6 [f, g, h]. These cytokines generate an increased mobilization of fats from the liver and adipose tissue [17] and enhance the binding of low density lipoprotein (LDL) to the endothelium and smooth muscles.

There is evidence that infections may be responsible for the accelerated development of atherosclerosis [18]. The association between periodontal infection and an increased risk of cardiovascular disease was highlighted by several researchers [13, 20].

Table 1

CLINICAL AND SERUM PARAMETERS ON GROUP STUDIES*

	C (n = 14)			D (n = 14)			S (n = 12)		
	Baseline	3M	P Value	Baseline	3M	P Value	Baseline	3M	P Value
PI	1.05 (0.38–3.20)	0.15 (0.13–1.90)	0.000	1.18 (0.31–2.90)	0.20 (0.11–2.01)	0.002	1.48 (0.28–2.84)	0.25 (0.11–1.20)	0.000
GI	1.10 (0.25–2.13)	0.12 (0.02–0.65)	0.000	0.91 (0.33–2.20)	0.12 (0.07–1.10)	0.000	0.90 (0.25–2.10)	0.16 (0.11–1.17)	0.001
PPD(mm)	2.64 (1.00–4.26)	2.10 (1.90–3.40)	0.015	3.06 (1.06–0.13)	1.31 (1.12–2.83)	0.049	1.92 (1.77–3.87)	1..30 (1.84–2.67)	0.000
BOP (%)	51.02 (8.38–100)	44.00 (0.23–100)	0.000	51.18 (3.34–100)	15.21 (6.25–44.00)	0.000	91.50 (0.60–100)	15.60 (5.79–32.69)	0.001
CAL(mm)	3.18 (1.16–7.13)	3.09 (2.06–5.60)	0.264	3.14 (1.25–4.38)	3.04 (1.94–4.05)	0.758	3.33 (1.21–4.89)	2.91 (1.93–4.95)	0.085
BMI (kg/m²)	24.90 (18.00–30.60)	25.40 (19.20–32.20)	0.777	28.65 (22.24–36.87)	28.50 (17.75–38.70)	0.218	28.77 (24.19–41.38)	29.57 (23.28–38.42)	0.983
TC/HDL	3.52 (2.09–4.75)	3.88 (2.26–5.88)	0.114	5.26 (3.16–7.46)	5.12 (2.20–7.26)	0.244	4.66 (2.97–9.10)	4.71 (2.89–8.54)	0.108
TC(mg/dl)	163.00 (90.00–190.00)	162.00 (133.00–209.00)	0.324	221.50 (130.00–271.00)	213.50 (144.00–269.00)	0.856	218.00 (144.00–347.0)	204.50 (123.0–298)	0.594
LDL(mg/dl)	96.50 (31.80–124.40)	98.60 (27.70–148.20)	0.717	150.00 (59.00–192.80)	138.20 (62.00–193.00)	0.834	146.90 (78.60–216.00)	130.10 (64.00–202.40)	0.348
HDL(mg/dl)	46.00 (33.00–72.00)	47.00 (31.00–62.00)	0.102	41.00 (30.00–61.00)	41.00 (27.00–64.00)	0.834	44.00 (19.00–67.00)	45.00 (34.00–77.00)	0.463
VLDL(mg/dl)	17.40 (8.00–43.20)	23.20 (10.50–41.00)	0.019	25.00 (10.20–74.00)	30.80 (11.00–77.00)	0.209	23.30 (9.00–147.00)	38.20 (13.80–114.60)	0.413
TG(mg/dl)	84.00 (38.00–203.00)	98.00 (44.00–210.00)	0.102	124.00 (50.00–367.50)	153.50 (53.00–286.00)	0.549	117.00 (45.00–686.00)	137.50 (71.00–307.0)	0.656

*Values are expressed as median (min–max)

C=control group; D=diet group; S=statin group; 3M= evaluation at 3 months after periodontal treatment; PI=plaque index; GI=gingival index; PPD=periodontal probing depth; BOP=bleeding on probing index; CAL=clinical attachment loss; BMI=body mass index; TC=total cholesterol; LDL=low density lipoproteins; HDL=high density lipoproteins; VLDL= very low density lipoproteins; TG=triglycerides.

Impaired lipid metabolism may play an important role in the association between periodontitis and cardiovascular diseases. Patients diagnosed with atherosclerosis presented significantly higher periodontal parameters values than subjects with of a normal metabolic status [21].

Furthermore, it has been shown that an improved periodontal health may affect the metabolic control of hyperlipidemia and can be considered as an adjunct to the standard of care of the patient with atherosclerosis [22]. Because the pro-inflammatory cytokines in the serum and GCF, such as TNF-α, IL-1b and IL-6 may play an important role in the association between periodontal disease and atherosclerosis [22], we have tried in this study to evaluate the effects of periodontal treatment on the metabolic control of hyperlipidemia and inflammatory components.

This study shows that inflammation plays a key role in the association between periodontal disease and atherosclerosis. At baseline, lifestyle changes such as diet modification and exercise - which are important components of treatment of lipid disorders - have been recommended in addition to antilipemic drug therapy. All patients said they have complied with the doctor's recommendations during the study, but further monitoring was not performed. Because it was not possible to measure fat mass during routine medical consultation, it was decided to use BMI as a substitute for changes in body composition. According to current results, periodontal treatment generally resulted in a slight decrease in atherogenic lipid profile with diet group (D).

In the statin group (S), there were also decreases in serum total cholesterol and LDL. In agreement with these findings, a study [14], which was conducted in hyperlipidemic patients, suggested that a group of antilipemic drugs resulted in significant decreases in serum total cholesterol and LDL, 3 months after completion of periodontal treatment.

Table 2

SERUM AND GCF CYTOKINE VALUES

	C (n = 14)			D (n = 14)			S (n = 12)		
	Baseline	3M	P Value	Baseline	3M	P Value	Baseline	3M	P Value
Serum (pg/ml)									
TNF-α	15.92 (1.70–178.94)	10.61 (0.75–93.06)	0.043	16.07 (1.70–732.66)	10.16 (0.14–130.63)	0.039	18.39 (2.52–177.51)	9.73 (4.90–68.92)	0.029
IL-1β	2.95 (0.90–27.08)	1.85 (0.53–20.40)	0.020	6.52 (0.73–31.40)	3.02 (1.00–15.34)	0.015	4.35 (0.23–30.75)	1.62 (0.11–12.35)	0.007
IL-6	5.83 (3.41–62.43)	4.12 (3.52–36.15)	0.050	8.26 (3.41–117.18)	5.12 (3.46–7.38)	0.001	8.01 (0.49–107.24)	6.05 (3.21–10.71)	0.023
GCF (pg/ml)									
TNF-α	0.51 (0.42–0.62)	0.49 (0.34–0.50)	0.050	2.41 (2.00–2.42)	0.52 (0.31–1.22)	0.005	0.56 (0.1–5.23)	0.20 (0.1–1.12)	0.039
IL-1β	2.11 (0.54–63.49)	2.79 (1.53–22.35)	0.230	2.35 (0.98–100.27)	3.00 (0.63–33.44)	0.968	3.91 (1.16–62.2)	2.36 (1.32–48.54)	0.180
IL-6	0.82 (0.12–2.57)	0.61 (0.49–1.43)	0.039	1.06 (0.98–1.14)	0.20 (0.81–1.67)	0.021	1.47 (1.01–1.85)	0.14 (0.07–0.19)	0.001

*Values are expressed as median (min–max)

C=control group; D=diet group; S=statin group; 3M= evaluation at 3 months after periodontal treatment; GCF=gingival crevicular fluid; TNF-α = tumor necrosis factor -alpha; IL-1β=interleukine-1 beta, IL-6= interleukine-6.

Bleeding on probing (BOP) was significantly higher in S group than C and D. This result can be interpreted by the fact that the extent of damage to lipid metabolism may be associated with periodontitis when taking into account both inflammatory components.

According to current findings, the S group had serum and GCF IL-6 levels higher than D and control groups. In addition, increased levels of BOP were observed in S group compared to groups C and D.

According to the results, there have been significant decreases in IL-6 serum levels at the 3 months assessment of atherosclerosis groups. When taking into account that IL-6 could play a key role in the development of coronary artery disease (through a number of different mechanisms: metabolic, endothelial and coagulation) and there is a close relationship between circulating concentrations of CRP, IL -6 and TNF-á and serum lipid components [23], the role of periodontal treatment in patients with atherosclerosis becomes important.

Several cytokines play a role in the pathogenesis of both coronary heart disease and periodontitis. These include interleukin-1, interleukin-6, interleukin-8, tumor necrosis factor, intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin. Interleukin-6 is involved in promoting coagulation, which may lead to the development of atherosclerosis. In a prospective study of 14 916 apparently healthy subjects, levels of IL-6 in 202 subjects who had a subsequent heart attack were higher than those of 202 control subjects, during a follow-up of 6 years (1, 8 to 1.5 pg/ml, P = 0.002) [24]. This indicates that the levels of interleukin-6 may be a predictor of future risk of heart attack in apparently healthy subjects.

Severe forms of periodontitis can lead to a state of systemic inflammation characterized by elevated serum levels of interleukin-6. One study reported that both subjects with coronary artery disease and periodontitis had significantly higher serum levels of interleukin-6 in comparison to subjects with coronary artery disease who did not have periodontitis (P <0.05) [25]. The results of studies on the effect of periodontal intervention therapy on serum interleukin-6 are not consistent. Most studies have taken confounding factors such as gender, age, smoking habits and medical history into account.

Gingival fluid (GCF) is a serum exudate found in gingival sulcus. GCF is an attractive oral liquid due to its ease of collection and capacity for the clinician to evaluate several sites in the oral cavity simultaneously. In a molecular epidemiological study, Offenbacher et al. described new clinical categories represented by different biological phenotypes based on clinical measurements, microbial and inflammatory response of the host to identify periodontal disease. Interestingly, the authors found that subjects with deeper probing depths and more severe bleeding had elevated levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6) [26, 27].

The immune system plays an important role in the pathology of periodontal disease, because most of the genes responsible for the development of periodontitis are related to the immune response. These include genes that affect IL-1, IL-6, TNF-α, IL-10, Fc-gamma receptor, CD14. The effects of IL-6 and TNF-a on lipid metabolism could still affect endothelial nitric oxide generation as a result of elevated non-esterified fatty acids [27]. The effect of IL-6 on platelets, fibrinogen and coagulation and of TNF-α in the expression of endothelial cells, adipose tissue and hepatocytes plasminogen activator inhibitor, can lead to a pro-coagulant state in such subjects. Therefore, the reduction of inflammatory mediators such as TNF-α and

IL-6 (serum and/or GCF), which are also associated with atherosclerosis and periodontitis, may provide an additional contribution to the bidirectional relation between periodontitis and atherosclerosis.

Conclusions

The levels of TNF-α, IL-1β and IL-6 in serum and GCF decreased significantly in the groups of atherosclerosis. A significant decrease was found in the level of IL-6 in GCF at the end of the study in the group of statins. Combining periodontal therapy and antilipemic treatment can provide beneficial effects on metabolism and control of inflammatory atherosclerosis by lowering serum proinflammatory cytokines.

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