

Original Article

The effect of non-surgical therapy on C reactive protein and IL-6 serum levels in patients with periodontal disease and atherosclerosis

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Abstract: During the last years, a large number of epidemiologic studies indicated that subjects with periodontitis presented a high risk for cardio-vascular diseases. Irrespective of the described mechanisms, the systemic inflammation is the main explanation for the relationship between the chronic infection and atherosclerosis. This study proposes an assessment of the periodontal non-surgical therapy effect on the serum levels of C-reactive protein (CRP) and interleukin-6 (IL-6) in patients with atherosclerosis and periodontal disease. The periodontal status of 64 patients was evaluated and the serum levels of CRP, IL-6 and the lipid profile were determined. Afterwards, non-surgical periodontal therapy was conducted and the patients were re-assessed after 3 months. Reduced values for the periodontal parameters and serum levels of CRP and IL-6 were achieved. Subjects with an improved periodontal status after 3 months also presented low levels of CRP and IL-6, after the correction of other co-factors. A relationship between the non-surgical periodontal therapy and the systemic parameters was observed. Therefore, inter-disciplinary protocols can emerge, with a complex approach of the patient with periodontal disease and risk for atherosclerosis. After the non-surgical periodontal therapy, the values of periodontal parameters and the levels of CRP and IL-6 have improved.

Keywords: Atherosclerosis, periodontitis, non-surgical periodontal therapy, CRP, IL-6

Introduction

The cardiovascular disease still remains the main cause of morbidity and mortality on a global level. In spite of the new therapies and prophylactic approaches, the number of deaths associated to cardiovascular diseases remains high and constant in most countries [1]. In the last decades it was widely accepted the fact that inflammation plays an essential role in the onset and evolution of atherosclerosis. Epidemiological studies confirmed the association between high level of acute phase products, like C reactive protein (CRP), fibrinogen or soluble adhesion molecules (ICAM-1, E-Selectin, VCAM-1) and the evolution of atherosclerosis [2]. Certain studies support the hypothesis that the immune system has a contribution to the

onset and evolution of atherosclerosis [3]. Therefore, an immune aggression of the host could relate to the atherosclerosis pathogenesis [4, 5]; theories supporting the importance of certain infectious or inflammatory conditions as risk factors for cardiovascular diseases have emerged [6, 7].

In the last years many epidemiological studies indicated that the subjects with periodontitis had a high risk for cardiovascular events [8]. Still, the association between periodontitis and cardiovascular disease remains an issue of debate. Recently, the discussions were focused on the contradictory results obtained by different groups after the analysis of the material of the same study. The critics emphasized the fact that periodontitis and atherosclerosis have

common risk factors but, even if this relationship is established, it can be frequently false. In the point of view of a firmly established relationship, the questioned cause-effect relation is an important aspect in the periodontal disease research.

There are two hypotheses of etiologic mechanisms regarding the association between periodontitis and systemic inflammations and cardiovascular diseases:

1. The invasion of periodontal pathogens in the atherosclerotic plaques (direct pathway): The existence in the atherosclerosis plaques of certain periodontal pathogens was demonstrated (*P.gingivalis*, *A.actinomycetemcomitans*, *P.intermedia*, *T.denticola*, *E.corrodens*) [9, 10]. *P.gingivalis* induces the expression of cellular adhesion molecules, IL-8, IL-6, MCP-1 and TLR-4 [11-13].

2. The systemic consequences of periodontal infection (the indirect pathway): The patients with periodontal disease have high levels of CRP, fibrinogen, TNF- α , IL-1, IL-6 and other products of acute phase reaction, associated also with cardiovascular events [14-16]. The pro-inflammatory cytokines reduce the expression of nitroxide synthase (eNOS), elevate the NADPH oxidase and enhances the expression of endothelial adhesion molecules (e-Selectin, ICAM-1, VCAM-1) [17]. The low anti-atherogenic properties in the endothelium enhance the leukocyte transmigration in the atherosclerosis plaques [3].

Independently from the described mechanisms, the systemic inflammation remains the main explication for the relationship between the chronic infection and atherosclerosis.

Moreover, the patients present an unbalanced lipid profile (high serum levels of cholesterol); this fact can be explained by the lifestyle and diet but also by the repeated chronic exposures to bacterial aggression and endotoxins disseminations.

The purpose of this study is to establish if the resolution of the periodontal infection can have an impact on the serum markers of the systemic inflammation in patients with risk of atherosclerosis and to assess the systemic effects of the treatment by examination of the changes determined by the treatment on the systemic markers of inflammation and on the lipid pro-

file: total cholesterol (TC), low and high-density lipoprotein (LDL, HDL), triglycerides (TG).

Materials and methods

Patients

The research methodology respected the international standard. The experiments were conducted according to the ethical directives of the Helsinki Declaration and the methods were the ones certified for clinical and para-clinical use. The information and confirmation principles for research purposes were strictly respected; the signed informed consent for study inclusion was obtained from each patient.

The present study was conducted on a number of 64 subjects with generalized advanced periodontitis. The subjects were recruited from the patients referred to the Periodontology Clinic of the Faculty of Dental Medicine, University of Medicine and Pharmacy "Gr.T.Popa" Iasi, Romania.

The inclusion criteria for the patients were: presence of the generalized advanced periodontitis, without any other signs of infection. The subjects presented periodontal pocket depths higher than 6 mm and alveolar bone resorption higher than 30% on at least 50% from the present teeth.

The exclusion criteria were as follows: history and/or presence of infectious diseases of any type; antibiotic treatment in the last 3 months; treatment with any drugs which can influence the serum levels of inflammatory markers (non-steroidal anti-inflammatory drugs); periodontal treatment in the last 12 months; pregnancy or lactation.

The serum and periodontal parameters were assessed at baseline and at 3 months after the completion of non-surgical periodontal treatment. At every examination we conducted: periodontal probing, recession examination (from the free gingival margin to the amelocemental junction), bleeding on probing assessment in 6 sites for every tooth, bacterial plaque index evaluation, expressed in percentages of surfaces entirely covered (O'Leary).

The patients were submitted to non-surgical periodontal treatment and they were informed regarding the oral hygiene methods. We performed scaling (manual and ultrasonic) and

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root planing with Gracey curettes under local anesthesia. The therapy was not limited by time and sessions number, being completed after 1-3 months from the first session.

Venous blood was collected from the cubital fossa at baseline and at 3 months after the treatment. The serum was obtained by centrifugation at 200 rpm for 15 minutes. The samples were maintained at -70°C before the analysis and were submitted to a standard interpretation, to avoid inter-individual variations.

CRP

The CRP serum levels were determined by immunoturbidimetry with a minimum detection limit of 0.25 mg/l [CRP Detection kit (latex), Archem Diagnostik End. LTD, Istanbul, Turkey]. The principle of the assay consists in the fact that serum CRP causes agglutination of the latex particles coated with anti-human CRP. The agglutination of the latex particles is proportional to the CRP concentration and can be measured by turbidimetry; this process measures the loss of intensity of transmitted light due to the scattering effect of the suspended particles; light is passed through a filter, creating a light with a known wavelength which is then passed through a solution. A photoelectric cell collects the light, a measurement being given for the amount of the absorbed light. The assay is based on the reaction between the mix of two reagents (working reagent) and the sample; the first reagent contains glycine buffer and sodium azide and the second reagent suspension of latex particles coated with antibody CRP and sodium azide. Reagent volumes are prepared by mixing 4 ml of the first reagent with 1 ml of the second reagent. The kit also provides a CRP calibrator (standard). The working reagent and the samples are brought to 37°C; 1 ml of the working reagent and 7 µl of the sample/standard are pipetted in the cuvette. The absorbance at 540 nm is recorded after 10 seconds (A_1) and after 2 minutes (A_2). The CRP concentration in the sample is calculated after the following formula:

$$C_{\text{sample}} = [(A_2 - A_1)_{\text{sample}} / (A_2 - A_1)_{\text{standard}}] \times C_{\text{standard}}$$

IL-6

The IL-6 serum levels were determined by enzyme-linked immunosorbent assay (ELISA) (Human IL-6 ELISA Kit, Sigma-Aldrich Co. LLC, USA). This assay consists in the adsorption of an anti-human IL-6 coating antibody onto the

microwells from the provided kit. The human IL-6 present in the sample and in the standard (calibrator) binds to the adsorbed antibodies. A biotin-conjugated anti-human IL-6 antibody is added and binds to the human IL-6 captured by the first antibody. Following incubation, unbound biotin-conjugated anti-human IL-6 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human IL-6 antibody. Following incubation, unbound streptavidin-HRP is removed during a wash step and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of human IL-6 present in the sample and in the standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IL-6 standard dilutions and human IL-6 sample concentration is determined.

TC, LDL, HDL, TG

The levels of TC (Cholesterol Quantitation Kit, Sigma-Aldrich Co. LLC, USA), LDL and HDL (HDL and LDL/VLDL Quantitation Kit, Sigma-Aldrich Co. LLC, USA) and TG (Serum Triglyceride Determination Kit, Sigma-Aldrich Co. LLC, USA), were determined by enzymatic spectrophotometry (Synergy HTX, BioTek Instruments Inc., USA), according to the instructions provided by the manufacturer.

Statistical investigation

All the obtained data were registered and statistically analyzed. Normally distributed variables are reported as mean \pm standard deviation, with 95% confidence intervals (95% CI). Changes in serum concentrations of CRP were tested by one-way analysis of covariance as primary outcomes. Changes in IL-6, triglycerides and total/LDL/HDL cholesterol were similarly tested as secondary outcomes. Age, gender, body mass index and cigarette smoking were subsequently included as covariates. For the data analysis we used the PASW 18 Statistics software ($P < 0.05$ being considered statistically significant). The laboratory results were reported to clinical changes of periodontal probing depths and gingival recessions.

Results

The initial number of patients was 79; only 64 subjects agreed to the treatment plan and fol-

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Table 1. The demographic data of the included subjects

Age* (years)	Female 47±5	Male 46±8
Gender	Female 36 (56-25%)	Male 28 (43-75%)
Provenience environment	Urban 43 (67-18%)	Rural 21 (32-82%)
Smoking	Yes 41 (64-06%)	No 23 (35-94%)
BMI* (kg/m ²)	27.5±2.3	
Family history of CVD	Yes 27 (42-18%)	No 37 (57-82%)
Periodontal diagnosis	Chronic periodontitis 61 (95-31%)	Aggressive periodontitis 3 (4-69%)

*The values are expressed in Mean value ± Standard Deviation. BMI: Body mass index; CVD: cardio-vascular diseases.

Table 2. Periodontal parameters (baseline and at 3 months after treatment)

Periodontal parameter	Baseline	At 3 months
Plaque Index	62.05±19.54	23.80±12.34 [#]
BOP sites	64.47±15.36	17.41±12.37 [#]
Periodontal pockets >4 mm (mm)	78.23±12.33	31.42±13.37 [#]
Probing depth	4.59±0.48	3.15±0.27 [#]
Recession (mm)	2.56±0.47	2.55±0.74 [§]
CAL (mm)	5.03±2.43	3.76±1.54 [*]

The values are expressed in Mean value ± Standard Deviation. *P<0.05; [#]P<0.001; [§]P>0.05.

Table 3. Values for CRP, IL-6, TC, LDL, HDL and TG at baseline and after 3 months

Parameter	Baseline	After treatment
CRP (mg/l)	1.9±1.1	1.5±0.9 [#]
IL-6 (ng/l)	1.2	0.8 [#]
TC (mg/dl)	208.8±27.1	204.9±27.1 [§]
LDL (mg/dl)	127.6±23.2	119.9±23.2 [§]
HDL (mg/dl)	54.1±19.3	50.3±15.5 [§]
TG (mg/dl)	115.1±97.4	106.3±70.8 [§]

The values are expressed in Mean value ± Standard Deviation. [#]P<0.001; [§]P>0.05.

lowed it until the end-point of the study. The mean age was of 47±5 and 46±8 years old for female and male subjects, respectively. The demographic data are presented in **Table 1**.

The mean value for the Plaque Index at baseline was 62.05±19.54%; the mean value of sites with BOP (bleeding on probing): 64.47±15.36%; the mean number of deep periodontal pockets was 78.23±12.33, with a mean probing depth of 4.59±0.48 mm; the mean value of CAL (clinical attachment loss) was 5.03±2.43.

At 3 months after the treatment we observed that the oral hygiene had significantly improved; the Plaque Index was 23.80±12.34%

(P<0.001); the BOP sites were 17.41±12.37% (P<0.001); the mean number of deep periodontal pockets was 31.42±13.37 (P<0.001); the mean value of periodontal probing depth decreased to 3.15±0.27 mm (P<0.001) and the CAL to 3.76±1.54 mm (P<0.05). The gingival recessions did not present any significant changes (P=0.758) (**Table 2**).

The mean serum value of CRP at baseline was 1.9±1.1 mg/l, with no differences regarding the age, gender, periodontal diagnosis or smoking. The CRP values were significantly associated to BMI and mean bone loss. The mean value of CRP at 3 months after treatment was significantly reduced (P<0.001) to 1.5±0.9 mg/l (**Table 3**). The serum levels of IL-6 also presented a significant reduction (P<0.001), from 1.2 ng/l to 0.8 ng/l.

The lipid profile remained almost unchanged (P=0.461 for TC; P=0.320 for LDL; P=0.205 for HDL; P=0.563 for TG).

Discussion

Atherosclerosis and periodontitis are multifactorial diseases with an inception in early childhood, even though first symptoms may appear in adulthood [18, 19]. It has been demonstrated that the prevalence of cardiovascular diseases in patients with periodontitis is 25-50% higher than in healthy subjects [19].

In the present study at the 3 months after the treatment assessment of the patients there were no changes observed regarding the diet, the drug intake or the smoking habits.

The periodontal parameters emphasize the severity and the extent of the periodontal infection. The values of the periodontal parameters

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(BOP, probing depth, CAL) significantly improved after the periodontal treatment. This aspect can be related also to the improvement of the oral hygiene.

CRP is a pentameric plasma protein of about 23 kDa molecular mass that was first reported by William Tillet and Thomas Francis Jr. in 1930 [20, 21]. This member of the superfamily of pentraxins although it is mainly synthesized by hepatocytes has also been reported in renal cortical tubular epithelial cells, respiratory epithelium, arterial tissue, neuronal cells, adipocytes, and leukocytes [20, 21]. CRP is an acute phase reactant and its blood levels increase as a non-specific response to infections and non-infectious inflammatory processes [22].

CRP expression is regulated by cytokines like IL-6, IL-1 β and TNF- α [20, 21]. CRP levels are positively correlated with obesity, smoking, triglycerides, periodontal disease and diabetes [20].

CRP's blood levels are normally defined as <10 mg/L and in most healthy subjects is usually 1 mg/L. Its levels grow within 4-6 h after initial tissue injury and continue to rise several hundred fold within 24-48 h, remaining elevated during the acute phase response and returning to normal with rehabilitation of tissue structure and function. The increase in CRP is exponential, doubling every 8-9 h, and the half-life is less than 24 h. CRP's clearance rate is constant, hence CRP's blood level is regulated merely by synthesis [23].

The serum levels of CRP at baseline were at the superior level of normal values, being associated to an acute infection or a systemic inflammatory impairment. This results must be taken into consideration: the specialist has to evaluate the serum levels of CRP in infectious context, as an indicator of systemic infection and of therapy possibilities.

IL-6 is a 26 kDa glycoprotein involved in inflammation, cell growth, cell survival, neurogenesis, gliogenesis, myelination, and demyelination in the CNS [24]. IL-6 signaling is mediated through two functional membrane proteins: an 80 kDa ligand-binding chain and a 130 kDa non-ligand-binding signal-transducing chain [25].

This dual-property cytokine, with pro-inflammatory and anti-inflammatory roles, is secreted at

local sites and released into the blood circulation when homeostatic perturbation occur like trauma, endotoxemia, endotoxic lung, and acute infections [26].

Inflammatory reaction is activated by the surgical procedures, and high levels of IL-6 are positively associated with the severity of surgical trauma, loss of blood, tissue damage and surgical duration [26]. High serum levels of IL-6 are also linked with poor prognosis in a wide range of cancers such as gastric cancer, bladder cancer, prostate cancer, ovarian cancer and colorectal cancer [26].

The subjects with an improved periodontal status after 3 months, with correction of other cofactors, also presented low levels of CRP and IL-6; these observations demonstrate the relationship between the therapy and systemic parameters. These results are consistent with those of other recent studies suggesting that periodontal treatment diminishes the risk of cardiovascular disease by reducing blood levels of inflammatory markers, such as CRP and IL-6 [22, 23, 27].

Moreover, an incomplete control of the periodontal disease (persistence of periodontal pockets and BOP) is associated to high levels of CRP and IL-6. These observations demonstrate new perspectives for further studies regarding the therapy approach, preventing the systemic inflammation and atherosclerosis. The degree of improvement of CRP and IL-6 values is noticeable. The decrease in CRP and IL-6 values is comparable to the one after anti-inflammatory drugs intake. Data from other longitudinal studies show that CRP values are related to atherogenesis and other cardio-vascular events [28-30]. There is a major interest regarding also the chronic infection and its association to the inflammatory response.

Biologically, smoking can exert an adverse effect on the fibroblasts function, chemotaxis and phagocytosis of neutrophils, immunoglobulins production and peripheral vasoconstriction. In this study the number of smokers was high (64.06%). The immune response is impaired in smokers, this fact being related to the affected functions. Therefore, smoking and systemic and local infections can determine endothelial irritation and/or pro-inflammatory cytokines stimulation, affecting the immune response of the host, with tissue lesions.

Smoking presents possible pathogenic properties in periodontal diseases and atherosclerosis, being recognized as a risk factor [31].

In this study no important changes in the lipid profile were registered. Bacterial lipopolysaccharides can significantly influence the lipid profile, determining also changes in the insulin secretion. These aspects require supplementary investigation.

The present study presents certain limitations; further studies are necessary to assess the global level of other inflammatory systemic markers (such as IL-1 and TNF α) and the genetic susceptibility of the host.

Conclusions

Following the periodontal non-surgical therapy (scaling, root planning), the periodontal parameters presented a noticeable improvement (reduced bleeding on probing, clinical attachment loss and probing depths); the diminished clinical signs of inflammation and tissue loss are the clear localized image of an improved periodontal status.

Furthermore, on a generalized image, the periodontal treatment determined decreased values of serum CRP and IL-6. The positive serum response was observed in patients with a good response to the periodontal therapy. This fact is of critical importance, suggesting that an improved local (periodontal) status could reflect on a systemic level of risk; the patients with reduced levels of pro-inflammatory CRP and IL-6 could exert also a reduced risk for cardiovascular events.

Changes of the lipid profile between baseline and the evaluation step could not be determined; this fact could be related to the lack of diet changes or to the bacterial metabolism, further researches being necessary.

The main conclusion of this study is that interdisciplinary protocols can emerge, with a complex approach of the patient with periodontal disease and atherosclerosis.

Acknowledgements

All authors contributed equally.

Disclosure of conflict of interest

None.

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