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ESTIMATION OF PENTRAXIN-3 AND TUMOR NECROSIS ALPHA LEVELS IN GINGIVAL CREVICULAR FLUID OF CHRONIC PERIODONTITIS PATIENTS

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ABSTRACT

Chronic Periodontitis (CP) is a disease characterized by interactions between microbial pathogens and host immune response that have a crucial role in its initiation and progression via the release of inflammatory mediators such as tumor necrosis factor- α (TNF-α). Pentraxin-3 (PTX3), the first long pentraxin described, is produced in response to pro-inflammatory cytokines by a variety of cells including those abundant in periodontal tissues. The aim of the present study was to investigate the role of PTX3 compared to TNF -α as a diagnostic and prognostic marker for CP. Gingival Crevicular Fluid (GCF) samples were taken from 53 CP patients before and after scaling and root planing (SRP) as well as from 50 periodontally healthy subjects as a control group. The GCF samples were tested for PTX3 and TNF-α levels by Enzyme-Linked Immunosorbent Assay (ELISA). Differences between CP patients and healthy subjects regarding clinical parameters and tested biomarkers in GCF were assessed. Moreover, the correlations between them were calculated. Out of 465 female patients examined, 53 patients were diagnosed as CP with a prevalence rate of 11.4%. The mean values of periodontal parameters were significantly reduced after treatment. Mean TNF-α and PTX3 levels were higher in CP patients (935.27 ± 264.21) (2.49 ± 0.537) than in the healthy group (772.32 ±0.148) (1.077 ± 0.084) respectively, with a significant difference between both groups ($p < 0.05$). Furthermore, these levels were reduced after treatment with a highly statistically significant difference. Positive correlations between the mean values of TNF-α, PTX3 levels, and clinical parameters were found whereas, PTX3 was more positively correlated. In conclusion, both PTX3 and TNF-α in GCF, previously recommended to be used as diagnostic markers for CP, can be also used as prognostic markers for follow-up of CP patients in addition to clinical and radiological parameters. However, further large-scale studies on both genders are recommended to confirm their role as prognostic markers.

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INTRODUCTION

Chronic periodontitis is a microbial infection caused by a disproportion in the virulence factors of micro-organisms and host defense mechanisms, resulting in an immune-inflammatory response that can cause destruction of the periodontium[1].

Data on the prevalence of periodontitis; depending on how the disease is defined and the age group from which they were taken, showed that 5% to 15% of any population suffers from chronic periodontitis [2]. However accurate data on the periodontal status of people in the Arab world is scarce with very limited number of published studies about Saudi Arabia [3].

The chronic periodontitis inflammatory process is in harmony by a system of cytokines and chemokines [4]. After periodontal micro-organism gets into the connective tissue, the acute-phase proteins are formed in response to the bacterial virulence factors and successively trigger the inflammatory response [5].

Tumor Necrosis Factor alpha (TNF-α) is a potent pro-inflammatory cytokine that is released at the site of inflammation and plays a crucial role in the pathogenesis of periodontal disease and bone destruction [6]. The source of TNF-α has been shown to include many oral cell types, particularly infiltrating monocytes, gingival fibroblasts, gingival epithelial cells and polymorphonuclear leukocytes [7]. Recent research suggests interleukin-1β (IL-1β) and TNF-α are the most potent inducers of a new pentraxin (PTX) called Pentraxin-3[8].

Pentraxins are traditional acute phase proteins belonging to a super family of proteins that are considered important markers of inflammation. Pentraxins include two groups; short pentraxins as C-reactive protein (CRP), and long pentraxins as pentraxin-3 (PTX3) [9, 10].

PTX3, also known as TNF-stimulated gene 14[11], is a recently discovered, first-recognized archetype of long pentraxin group that was identified in the 1990s sharing structural similarities with classic pentraxins with a difference in the presence of a discrete long N- terminal domain joined to the C-terminal pentraxin domain. It is produced by both inhabitant and innate immune cells in peripheral tissues in response to inflammatory signals. Thus, it is suggested to have a significant role in innate immune response via regulation of inflammatory reactions and clearance of apoptotic cells. PTX3 can also activate the complement system, suggesting a role for PTX3 in the augmentation of inflammation and in the innate immune response. The plasma level of PTX3 is raised in inflammatory conditions. Moreover, because of its extrahepatic synthesis in contrast to CRP, PTX3 levels are supposed to be the proper independent indicator of disease activity [12, 13].

PTX3 opsonizes fungi, some bacteria, and viruses. Opsonization results in increased phagocytosis, killing and increased cytokine and nitric oxide production. PTX3 binds fibroblast growth factor and enhances the angiogenesis in different physiologic and pathologic conditions [14].

PTX3 is suggested to act as an acute-phase protein because its blood levels are low in normal circumstances, increase rapidly and significantly during inflammation and infection and correlate with the severity of the acute condition. Thus, PTX3 has been suggested to have the potential to be a new diagnostic marker for various inflammatory diseases [15].

Diagnosis of periodontal diseases is made by long-established measures like clinical examination as bleeding on probing, periodontal pocket depth, clinical attachment level, and radiographic assessment of the alveolar bone loss [16].

The presence of bleeding on probing is still the best disease activity predictor available, and its absence is considered as a negative predictor of periodontal disease activity, but it reveals too many

false positives. However, these measures cannot reliably identify sites with further periodontal destruction and does not provide any data about the cause of the condition or the prognosis of the disease [17].

Advances in periodontal disease diagnostic research are shifting to methods that clarify the periodontal risk as biomarkers in prognosis and diagnosis. Therefore, there is a need for an advanced marker with higher sensitivity to accurately identify the disease. Acute phase proteins make steps towards these criteria [18].

PTX3 is synthesized by a diversity of cells, mostly by cells in periodontal tissue such as neutrophils, fibroblasts, monocytes/macrophages, dendritic cells, epithelial cells and endothelial cells. So, the aim of the present study was to investigate the acute phase protein, PTX3, as a novel diagnostic and prognostic marker for CP patients in comparison with TNF-α using GCF as a non-invasive sampling procedure [13]. Also, the study aimed at collecting data about prevalence of CP among patients seeking dental treatment at Dental teaching hospital, Umm Al-Qura University, Makkah.

SUBJECTS, MATERIALS AND METHODS

Study population

The study was carried out over 12 months where 465 female patients seeking dental treatment at Dental teaching hospital, Umm Al-Qura University were examined for proper diagnosis and determination of their periodontal status. Each subject underwent periodontal examination and full mouth periapical radiographs to differentiate patients with CP from other groups based on bleeding index (BI), plaque index (PI), probing depth (PD), clinical attachment level (CAL), and radiographic evidence of bone loss.

Written informed consent was obtained from patients who agreed to participate voluntarily, and ethical clearances were obtained from the institution's ethical committee. Exclusion criteria included: patients with any systemic disease that could alter the course of the periodontal disease, history of smoking or tobacco use, taking medications like antibiotics during the last three months, history of periodontal therapy in the preceding six months and pregnant/lactating women.

Studied groups

Participants, aged from 40 to 55 years, were categorized based on the American Academy of Periodontology (AAP) criteria [19] into the following groups:

- Group (I): Periodontally healthy control group included 50 participants with clinically healthy periodontium BI = 0 PI = 0, PD < 3mm, CAL = 0, and no evidence of bone loss in radiographs.
- Group II a (before treatment): moderate to severe CP group included 53 participants who had signs of clinical inflammation, BI >1, PD ≥4 mm, CAL ≥3mm, and radiographic evidence of bone loss.
- Group II b (8 weeks after treatment) included the same CP patients who were subjected to SRP.

Clinical examination

PD and CAL were measured in view of a fixed reference point on the occlusal surface of the teeth and cemento-enamel junction. All the clinical assessments were done by a single examiner with a periodontal probe (Michigan 0 probe with Williams' markings).

Site Selection and GCF Collection

Samples from patient groups II a& b were selected from only one site per participant with CAL ≥3 mm that showed the highest (PD) together with signs of inflammation, in conjunction with radiographic evidence of bone loss. While, in group I

(control group), multiple sites with absence of inflammation were pooled to ensure the collection of an adequate amount of GCF. The GCF samples were collected after; gently drying the area, removing supragingival plaque without touching the marginal gingiva in CP patients and isolating the area using cotton rolls to avoid saliva contamination.

Samples of GCF were taken using standard paper strips (Periopaper; Oraflow, Plainview, NY, USA). Each paper strip was kept in the sulcus for 30s. Samples were visibly checked for blood contamination to discard contaminated ones. Non blood contaminated GCF samples were immediately transferred to airtight plastic Eppendorf tubes containing 500μL phosphate buffered saline (PBS) and were stored at -70ºC until assayed.

Measurement of Pentraxin 3 and TNF-α level in GCF samples

Commercial enzyme-linked immunosorbent assay (ELISA) kits for both Pentraxin 3 (Human ab202537- Pentraxin 3 ELISA Kit, Abcam, UK) and TNF-α (ab181421 – TNF-alpha Human Simple Step ELISA Kit, Abcam, UK) were purchased, and testing was performed according to the manufacturers' instructions. The concentrations of PTX3 and TNF-α in the tested samples were estimated by comparing the average absorbance readings of each sample with the concentrations of standard curves in the assays. Reading the absorbance was done on a SPECTRO star Nano microplate reader (BMG LABTECH., Germany) set at a wavelength of 450 nm.

Statistical analysis

Data was analyzed using SPSS version 17. Differences in clinical parameters and biomarkers between diseased and healthy subjects were assessed using the Wilcoxon t-test and Mann-Whitney U-test. The correlations between biomarkers and clinical parameters were calculated using Spearman's rank correlation. The level of significance was set at $p < 0.05$ with a 90% confidence interval.

RESULTS

Out of 465 patients examined, 53 patients were diagnosed as CP with prevalence rate of 11.4%.

Clinical periodontal measurements and gingival parameters:

Analyses of the clinical periodontal measurements and gingival parameters of the study groups are outlined in Table (1&2).

Table (1) shows that mean values of periodontal measurements (PD, CAL) were lower in group I in comparison to group II a and II b with high significant difference between groups ($p = 0.001$). Also these measurements were significantly decreased in group II b after SRP compared to group II a before treatment ($p = 0.001$).

TABLE (1): Clinical periodontal measurements of the study groups

Periodontal measurements	Healthy group (group I) (Mean± SD)	Patient groups		t (p value)
		Group II [a] (Mean± SD)	Group II [b] (Mean± SD)	
Pocket depth (PD)	1.8±0.514[ab]	4.10 ± 0.823	2.54 ± 0.753	19.55 (0.001)*
Clinical Attachment Loss(CAL)	0	3.06 ± 0.752	2.00 ± 0.767	32.39 (0.001)*

**** significant difference among the periodontal disease group before and after the treatment***

a) significant difference between the healthy group and diseased group before treatment

b) significant difference between the healthy group and diseased group after treatment

The percentages of CP patients with clinical signs of gingival inflammation (PI and BI) showed statistically high significant decrease after treatment (23.79 % versus 55.38% and 31.31% versus 66.12% respectively) (p= 0.001) (table 2)

TABLE (2): Percentages of patient groups with clinical signs of gingival inflammation before and after treatment

Gingival parameters	Group II a	Group II b	Z (p value)
Plaque index	55.38 %	23.79 %	6.292 (0.001)*
Bleeding index	66.12 %	31.31%	6.287 (0.001)*

significant at p = 0.001

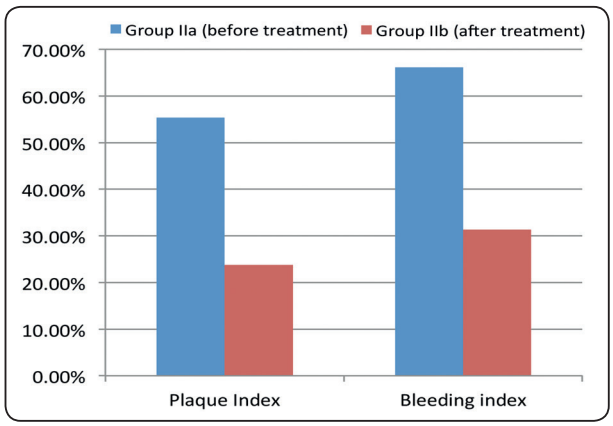

Fig. (1) Comparison of percentages of patient groups with clinical signs of gingival inflammation before and after treatment

Gingival Crevicular Fluid Biomarkers

Table 3 shows that mean TNF-α level was higher in group II a (935.27 ± 264.21) than in group I (772.32 ±0.148) with a significant difference between both groups (p < 0.05). On the other hand, the mean TNF-α level reduced in group II b after treatment (787.45 ± 152.31) with a highly statistically significant difference in comparison to group II a (<0.001).

The mean PTX3 level was higher in group II a (2.49±0.537) than group I (1.077 ± 0.084) with statistically significant difference between two groups (< 0.05). The mean PTX3 level was reduced in group II b after treatment (2.12 ± 0.217) with statistically significant difference in comparison to group I (<0.05).

TABLE (3): Biomarkers mean levels in GCF of the study groups

Biomarker	Healthy group Group (I) (Mean± SD)	Patient groups	
		Group II a (Mean± SD)	Group II b (Mean± SD)
TNF -α (pg/ml)	772.32 ± 0.148[a]	935.27 ± 264.21	787.45 ± 152.31**
PTX3 (ng/ml)	1.077 ± 0.084[ab]	2.49 ± 0.537	2.12 ± 0.217*

significant difference among the periodontal disease group before and after the treatment at p level < 0.05

****** *significant difference among the periodontal disease group before and after the treatment at p level < 0.001***

a) significant difference between the control group and diseased group before treatment at p level < 0.05

b) significant difference between the control group and diseased group after treatment at p level < 0.05

Correlations between mean GCF biomarkers and clinical parameters:

Correlations between the levels of TNF-α, PTX3 and clinical parameters are presented in Table 4 and figure 2. There were significant positive correlations between the mean level of TNF-α, and the mean measurement of clinical parameters for PD, CAL and PI (P < 0.05), while there was a strong positive correlation between the mean values of TNF-α and BI (P < 0.01). However, strong positive correlations between the mean level of PTX3 and the mean clinical parameters of PD, CAL, PI and BI at P level < 0.01 were observed.

TABLE (4) Correlation between mean GCF biomarkers and clinical periodontal status measurements

Biomarker	Periodontal measures Spearman's rank correlation coefficients			
	Pocket depth (PD)	Clinical Attachment Loss (CAL)	Plaque index (PI)	Bleeding index (BI)
TNF -α (pg/ml)	0.255*	0.247*	0.357*	0.557**
PTX3 (ng/ml)	0.738**	0.593**	0.402**	0.774**

**** Significant at p level < 0.05 ** Significant at p level < 0.01***

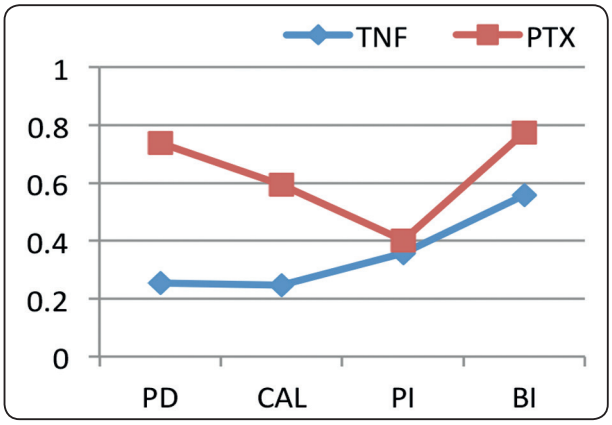

Fig. (2) Correlations between the levels of TNF-α, PTX3 and clinical parameters

DISCUSSION

Periodontitis is a chronic bacterial infection affects gums and bone supporting the teeth; if it remains untreated, it can lead to teeth loss. It remains difficult to make conclusive statements about its prevalence in Makkah community due to very limited number of epidemiological studies that have been carried out in the Kingdom of Saudi Arabia. Although there are advances in understanding periodontal diseases, we still rely upon traditional diagnostic procedures. Due to the heterogeneity of clinical presentation, there is a great challenge to determine immunological markers for screening and predicting the disease or evaluating the efficacy of therapy.

Tumor necrosis factor alpha is considered as a pro-inflammatory cytokine which is believed as stimuli for release of PTX3 and both of them participate in innate resistance to pathogens [11]. But, only a few studies have assessed the TNF-α and PTX3 level in the GCF and correlated them with the clinical parameters of the periodontal conditions.

Therefore, the aim of this study was to test the ability of TNF-α and PTX3 to be considered as diagnostic and prognostic markers for chronic periodontitis and further to correlate the TNF-α and PTX3 levels with the associated clinical parameters. The present study was carried out on patients attending to dental teaching hospital, Umm Al-Qura University, where PTX3 and TNF -α levels were measured in GCF samples of CP patients using ELISA, before and after SRP ,which is considered a traditional therapy and the most often treatment of choice that is widely used [20].

Gingival Crevicular Fluid sample was chosen for the study because analysis of its components showed that it contains a large number of proteins and peptides derived from inflamed host tissues thus it could recognize potential biomarkers of periodontitis [21].

The prevalence rate of CP in the present study was 11.4% which is significantly lower than that reported in a retrospective study done at College of Dentistry outpatient clinic department of King Khalid University, Abha, Kingdom of Saudi Arabia

(36.8%) in 2009[22]. The difference between two studies may be attributed to the greater number of patients examined in their study (2739), their prevalence rate was done on various forms of periodontitis and the majority of their patients were males (36.8%) with different age groups ranging from 11 to 82 years. While the present study was restricted to female only aged from 40 to 55 years that may have different risk factors. The prevalence of CP in the present study was also lower than other studies conducted at the dental screening clinics of the College of Dentistry, King Saud University in Riyadh between 1990 &1992 (51%)[23] and in Sebha, Libya (>50%)[24] but higher than that recorded in North Jordan (5.5%)where participants were of younger age (20-29 years) [25].

The present study revealed that periodontal measurements (PD, CAL) and clinical signs of gingival inflammation (PI, BI) were highly significantly increased in CP patient group than in the healthy group with a significant decrease after treatment. These results seems representative of the normal response as BI, that measured clinically by bleeding on probing, occurs during the active phase of periodontitis where sulcular epithelium is ulcerated, being more permeable to bacterial byproducts that increase the severity of gingival inflammation.

Treatment by SRP leads to decrease in severity of gingival inflammation by decreasing the amount of bacteria and their products in the gingival sulcus. Moreover, reduction in PD score after treatment means that periodontal health is achieved as it gives an indication about the surface area of the gingiva where bacteria can invade the tissues and its reduction results in an environment that is less favorable for the establishment of anaerobic periodontopathic microorganisms leading the healing of periodontal tissues [26].

This study demonstrated that PTX3 and TNF-α levels were statistically significantly higher in patients with CP compared to periodontally healthy subjects. Positive correlations were found between mean clinical parameters (PD, CAL, PI and BI,) and the mean levels of PTX3 and TNF-α.These findings are in accordance with a previous study that investigated the cytokine levels including TNF-α and PTX3 in GCF from CP patients with more obvious correlations between PTX3 and periodontal status [27]. These results are also in agreement with previous studies which showed elevated levels of PTX3 in GCF [28] and in gingival tissues [29] of CP patients where positive correlations with periodontal parameters were observed.

The TNF-α level in GCF was also found to be progressively increased from periodontal health to periodontal disease [30]. Explained by the suggestion of Graves et al., [31]that the production of cytokines at deeper levels within the gingival connective tissue leads to an inflammatory cascade in this area until a critical level of pro-inflammatory cytokine, including TNF- α, is reached, then physiologic response becomes a pathologic response if this inflammatory response occurs predominantly in the area of attachment to cementum. Ertugrulet al.,[32] added that inflammatory mediators, including TNF-α, triggering destruction of periodontal tissues, increases as the severity of periodontal diseases increases that confirmed in the present study by positive correlation found between level of TNF- α in GCF and clinical periodontal measurements as well as gingival parameters.

The significantly higher levels of PTX3 in CP patients was previously explained by Mathews et al., [33] who stated that it is triggered by the release of cytokines with early arrival of neutrophils at sites of injury and infection, these neutrophils exhibit hyper-reactivity following stimulation by cytokines. Mathew et al.,[34] further described details that these neutrophils represent a reservoir of pre-stored PTX3 into specific granules that are ready for rapid release in response to inflammatory signals.

The significant reduction in PTX3 levels after treatment observed in the present study confirms the work of Mathew et al., [34] who suggested that

this reduction could be due to reduced number of neutrophils as a result of reduction in inflammation. This assumption could be confirmed by the work of Bender et al.,[35] who concluded that the level of oral neutrophils was reduced after treatment.

The present study showed a significant decrease in TNF- α levels in CP patients after treatment, compared to the pretreatment. This result supports that of Dag et al.,[36] who reported decreased circulating TNF- α concentration three months after the non-surgical periodontal therapy. In contrast to the present work, Yamazaki et al.,[37] did not find a decrease in TNF-α level after periodontal therapy. The difference from the present work may be attributed to different sample used as they examined serum levels while the present work examine GCF levels of TNF- α that could better reflect the cell activity and mediators of inflammation in periodontal tissues.

Clear observation in the present study was that, PTX3 was more positively correlated with clinical parameters than TNF – α which reflects its importance in diagnosis and prognosis of periodontal diseases. With the limitations of the present study that all the subjects examined were females and small sample, it could be concluded that the prevalence of CP in the present study is closely similar to its prevalence worldwide and much lower than that recorded in published studies in other areas of Saudi Arabia. Both PTX3 and TNF-α in GCF, previously recommended to be used as a diagnostic marker, could be also used as prognostic marker for follow up of CP patients in addition to clinical and radiological parameters. However, further large scale studies on both genders are recommended to confirm its role as a prognostic marker.

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