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ORIGINAL ARTICLE

Fetuin-A, serum amyloid A and tumor necrosis factor alpha levels in periodontal health and disease

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OBJECTIVES: This study is evaluating fetuin-A, serum amyloid A (SAA) and tumor necrosis factor alpha (TNF-α) levels in gingival crevicular fluid (GCF) and serum samples in periodontal health and disease.
MATERIAL AND METHODS: Sixty patients were divided into three groups: Group 1 periodontal health (n = 20), Group 2 gingivitis (n = 20) and Group 3 chronic periodontitis (CP) (n = 20). GCF and serum samples were evaluated using enzyme-linked immunosorbent assay kit.
RESULTS: SAA and TNF-α levels in GCF and serum were significantly higher in patients with gingivitis and CP compared with controls ($P < 0.016$). Contrarily, fetuin-A levels in GCF and serum were significantly higher in controls than in patients with gingivitis and CP ($P < 0.016$). In CP group, a significant correlation was observed between GCF-SAA amount and the number of sites with $4 \leq PPD \leq 5$ mm ($P < 0.05$). There was a significant correlation between GCF-fetuin-A levels and the number of sites with PPD ≥ 6 mm ($P < 0.05$). GCF-TNF-α was found to have a significant relationship with the number of sites with $4 \leq PPD \leq 5$ mm and PPD ≥ 6 mm ($P < 0.05$).
CONCLUSIONS: In conclusion, serum and total levels of SAA significantly increased, whereas fetuin-A levels significantly decreased, with increasing severity of PD.

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Keywords: Fetuin-A; serum amyloid A; gingival crevicular fluid; serum; periodontal disease

Introduction

Periodontal disease (PD) is a pathological condition that occurs as a result of interactions between dental biofilm and the host response. Gingivitis is an inflammatory response to the accumulation of biofilm without any loss of periodontal supporting tissues, whereas periodontitis is characterized by tissue destruction (Sanz and van Winkelhoff, 2011). The acute-phase proteins (APPs) are a group of serum molecules synthesized and released by many cell types, especially hepatocytes, in response to infection and injury. They are termed positive or negative APPs based on the change in plasma concentrations during inflammation (Wang and Sama, 2012). Various APPs have been detected in PD in both gingival crevicular fluid (GCF) and plasma or serum and can be considered inflammatory markers in PD (Ebersole and Cappelli, 2000).

GCF is a serum exudate found in the gingival sulcus that can be readily collected in multiple sites within the oral cavity simultaneously (McCulloch, 1994; Kinney *et al*, 2014). In GCF, the inflammation-related molecules may give dependable information pertaining to the condition of PD activity and systemic diseases (Offenbacher *et al*, 2010; Buduneli and Kinane, 2011; Carneiro *et al*, 2014). Further, serum supplies information about periodontal pathogen-induced inflammation and response (Buduneli *et al*, 2011). In the literature, there is evidence that biomarkers investigated in serum may be associated with periodontal diseases (Caula *et al*, 2014; Rodrigues *et al*, 2014).

Serum amyloid A (SAA), expressed during the acute stage of inflammation, is involved in a number of chronic inflammatory conditions, including amyloidosis, atherosclerosis, and rheumatoid arthritis (RA). There are several forms of SAA: SAA1 and SAA2 are synthesized and stimulated mainly in the liver. Pro-inflammatory cytokines that are related to the pathogenesis of periodontal disease, such as IL-1, IL-6, and TNF-α, help to regulate the expression of SAA1 and SAA2 genes in liver cells (Zhang *et al*, 2005). Similar to C-reactive protein (CRP), SAA levels increase within a few hours after an inflammatory stimulus. Furthermore, SAA levels may increase more than CRP levels (Ahmed *et al*, 2012). In addition to other diseases, studies investigating SAA in periodontal diseases have yielded conflicting results. In patients with CP (chronic periodontitis), serum SAA levels were increased compared with those of individuals without

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CP (Ardila and Guzman, 2015). Also, serum SAA levels were increased within 24 h after non-surgical periodontal treatments compared with baseline results in periodontitis patients (Graziani *et al*, 2010). SAA levels were also increased in periodontal infection in studies using mouse polymicrobial PD models (Rivera *et al*, 2013; Aoki-Nonaka *et al*, 2014). In contrast, Glurich *et al* (2002) reported lower SAA levels in periodontitis patients than in healthy individuals. In this study, SAA levels were evaluated due to the similar proinflammatory cytokines that are related to SAA secretion and the pathogenesis of periodontitis. Moreover, to avoid confusion about SAA levels and periodontitis, both GCF (gingival crevicular fluid) and serum levels were examined to more fully elucidate the role of SAA in CP.

Fetuin-A protein, also known as alpha-2-HS-glycoprotein, consists of two polypeptide chains and is synthesized in the liver. It is negatively regulated by TNF, IL-1, IL-6, and IFN-γ, which are important cytokines that are stimulated during inflammation. Fetuin-A protein downregulates inflammation by inhibiting some pathological pathways, such as those related to TGF-β and insulin receptor tyrosine kinases (Ebersole and Cappelli, 2000; Zhang *et al*, 2005; Li *et al*, 2011; Ahmed *et al*, 2012; Wang and Sama, 2012). Recently, fetuin-A has been associated with many diseases and conditions, including arteriosclerosis, RA, systemic lupus erythematosus, and obesity and was shown to be relevant in dialysis patients (Sato *et al*, 2007; Chen *et al*, 2009; Mori *et al*, 2012; Vassalle and Mazzone, 2016). Fetuin-A prevents atherosclerosis by binding to newly formed calcium phosphate crystals, inhibiting crystal growth and mineral accumulation and thereby preventing calcification of blood vessel walls (Herrmann *et al*, 2012; Seto *et al*, 2012) These calcium phosphate crystals are also related to bone calcification and inflammation. The theoretical mechanism underlying these effects is as follows: digestion of crystals by macrophages causes the release of proinflammatory cytokines, inducing apoptosis (Herrmann *et al*, 2012). Contrarily, fetuin-A and calcium phosphate crystal compounds facilitate the elimination of these crystals, downregulating the inflammatory response (Herrmann *et al*, 2012; Smith *et al*, 2013). Therefore, circulating fetuin-A is strongly associated with bone mass and bone formation mediators (Vassalle and Mazzone, 2016). Additionally, it is thought that fetuin-A, as a negative acute-phase reactant, is probably associated with chronic inflammation (Sun *et al*, 2014). This is supported by the finding that fetuin-A levels are associated with a reduced risk of cardiovascular disease despite increased CRP levels. Fetuin-A is considered in this study because of its anti-inflammatory properties, which are thought to be relevant in periodontal disease (Schure *et al*, 2013; Sun *et al*, 2014).

Tumor necrosis factor (TNF) is a significant inflammatory mediator with two ligands. TNF-α, produced by phagocytes and T cells, occurs in response to bacteria and particularly bacterial products called lipopolysaccharide resulting in periodontal tissue destruction (Ebersole and Cappelli, 2000). TNF-α activation and induction is controlled by a cytokine complex. TNF-α shows the impact on periodontal tissue destruction by managing the expression of various inflammatory mediators including IL-1, PGE2, MMP (Ebersole and Cappelli, 2000; Cekici *et al*, 2014). As fetuin-A is examined for the first time in PD pathogenesis, we decided to evaluate another established inflammatory mediator in PD pathogenesis to confirm the presence of inflammatory condition.

Although some studies have evaluated serum levels of SAA (Glurich *et al*, 2002; Graziani *et al*, 2010; Ardila and Guzman, 2015), to our knowledge, no reported study has assessed SAA and fetuin-A levels as inflammatory biomarkers in the GCF of patients with PD. This study tested the hypothesis that GCF and serum levels of the negative acute-phase protein fetuin-A decrease, while the GCF and serum levels of the positive acute-phase protein SAA increase, in PD. This study also evaluated correlations between biomarkers and clinical parameters in periodontal health and disease.

Materials and methods

Study population and inclusion and exclusion criteria

The study population consisted of 60 individuals (aged 28–45 years; 33 men, 27 women) admitted to the Periodontology Department of Bulent Ecevit University between November 2013 and March 2014 (Figure 1). The study design was approved by the Ethics Committee on Human Research of Bulent Ecevit University and was conducted according to the Declaration of Helsinki (Protocol Number: 2013-95-03/09). Written informed consent was provided by every participant. The study was registered under NCT02433405 at ClinicalTrials.gov.

The exclusion criterion for this study was systemic disease, such as diabetes mellitus, RA, obesity or cancer; patients were also excluded if they were pregnant, lactating, had aggressive periodontitis, were a current smoker, were currently undergoing periodontal treatment, or had used antihypertensive and immunosuppressant medications, steroids, non-steroidal drugs, anti-TNF-α agents, or that affect bone metabolism (drugs including hormones or medications but excluding bisphosphonates) within the previous 6 months. All the conditions defined as exclusion criteria were obtained from the medical histories reported by the patients. All participants were divided into three groups based on periodontal parameters. Healthy individuals were placed in Group 1 ($n = 20$), patients with gingivitis formed Group 2 ($n = 20$), and patients with chronic periodontitis (CP) formed Group 3 ($n = 20$). Inclusion criteria were based on radiographic and full-mouth clinical periodontal examinations and included the gingival index (GI) (Loe and Silness, 1963), plaque index (PI) (Silness and Loe, 1964), bleeding on probing (BOP) (Ainamo and Bay, 1975), clinical attachment level (CAL), and probing pocket depth (PPD). All participants had at least 20 teeth, not including their third molars. Patients in Group 1 had no inflammation and no attachment or bone loss (GI = 0, PPD $\leq$ 3 mm, CAL $\leq$ 3 mm). Meanwhile, Group 2, comprised of patients who had signs of inflammation, such as gingival redness, higher BOP levels and edema, had no attachment or alveolar bone loss (GI $\geq$ 1, PPD and CAL $\leq$ 3 mm). For Group 3, the inclusion criteria were, in addition to clinical signs of inflammation, GI > 1, PPD $\geq$ 5 mm, and CAL $\geq$ 5 mm with alveolar bone loss affecting >30% of the existing teeth on clinical and radiographic examination. A periodontal probe was used to take all of the measurements (Williams periodontal probe; Hu-Friedy, Chicago, IL, USA), by one physician. All disease classifications were determined based on the periodontal disease classification system published in 1999 (Armitage, 1999).

Ten people were chosen at random for calibration before the study measurements were taken, and were assessed on two separate occasions 2 days apart. The findings from these measurements were deemed to be sufficiently reproducible ($\kappa = 0.982$), assuming those measured after 48 h and at baseline were within 10% of each other on the millimeter scale (Schwarz *et al*, 2006).

Site selection and sample collection

Samples were collected on the day following the measurements to avoid contamination of GCF with blood. Two sites (two teeth, one region of each tooth) were selected as the sampling sites for each individual. The

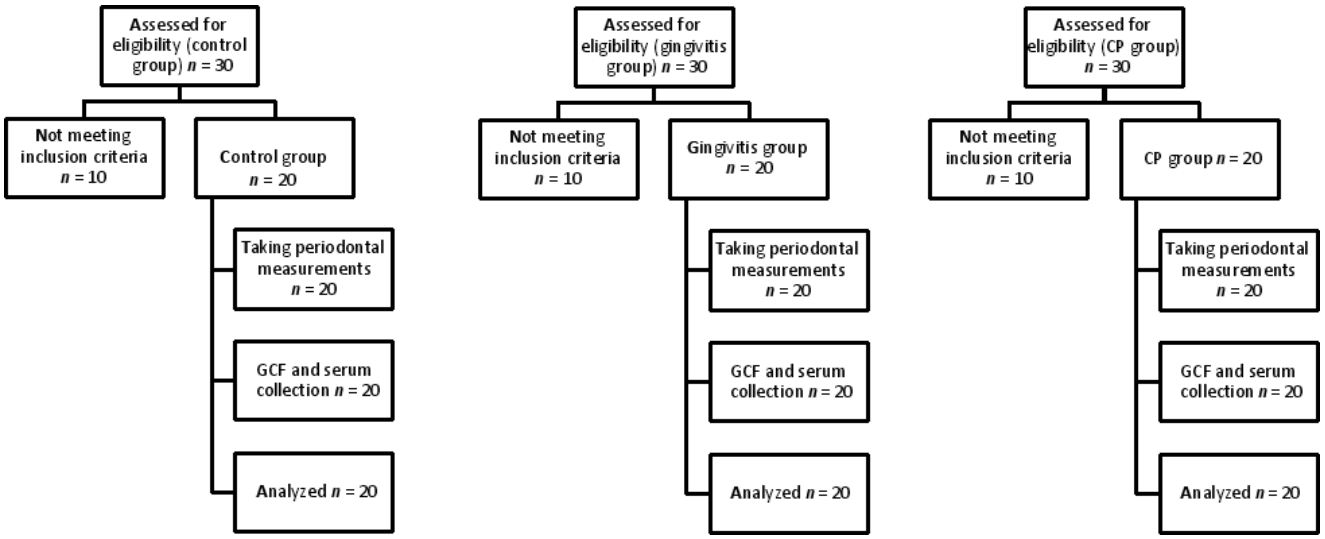

Figure 1 Flowchart of participation in the study

mesiobuccal or distobuccal areas of the single-rooted teeth were used to sample GCF to ensure standardization. Care was taken to avoid saliva contamination during GCF sampling. The area was then dried, and filter paper (Periopaper' ProFlow, Amityville, NY, USA) was used to collect samples of GCF via the intracrevicular method. This technique is quick and simple to use and is also possibly the least traumatic method (Griffiths, 2003). Paper strips were placed into the crevice until mild resistance was felt and left in position for 30 s. Electronic impedance was used to determine the GCF volume of each strip (Periotron 8000; ProFlow Inc.) and then placed in an empty microcentrifuge tube, where they were pooled to make one sample. Strips contaminated with saliva or blood were not used. Peripheral blood (2 ml) was collected from each individual from the antecubital fossa, and the serum component was separated under centrifugation (3000 ***g***, 5 min). Samples were immediately stored at −40°C until assay.

Evaluation of fetuin-A, SAA, and TNF-α protein

Phosphate-buffered saline (400 μl, pH 7.4) was included in the tubes that contained the sample strips. The tubes were vortexed and homogenized for 1 min and then centrifuged at 3000 ***g*** and 4°C for 15 min. The strips were removed, and the supernatants were divided into three aliquots for identifying each parameter. The concentrations of fetuin-A (BioVendor Research and Diagnostic Products, Brno, Czech Republic), SAA, (SunRed Biological Technology, Shanghai, China) and TNF-α (eBioscience, San Diego, CA, USA) in GCF and serum were identified via sandwich enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer. The calibration range of the fetuin-A assay was up to 100 ng ml^{-1}, and the analytical sensitivity was 2 ng ml^{-1}. The within- and total-run coefficient of variation (CV) values were <2.9% and <4.7%, respectively. The calibration range of the SAA assay was up to 35 μg ml^{-1}, and the analytical sensitivity was 2 μg ml^{-1}. The within- and total-run CV values were <9% and <11%, respectively. The calibration range of the TNF-α assay was up to 1000 pg ml^{-1}, and the analytical sensitivity was 1 pg ml^{-1}. The within- and total-run CV values were <6% and <7.4%, respectively. All samples and standards were assayed in duplicate, as suggested by the manufacturers. The optical density was ascertained following the cessation of color development. This was done using a microtiter plate computerized reader set to a wavelength of 450 nm. Total amounts of each biomarker were determined by multiplying concentrations and GCF volumes (Eley and Cox, 1992).

Statistical analysis

We used the primary outcome variable (fetuin-A levels in GCF) to calculate the sample size. Due to the nonexistence of accurate data concerning fetuin-A amounts, the sample size required for the study could not be calculated. Our estimates were determined based on the protocol of a pilot study that included 10 patients per group. We speculated that 14 individuals per group would allow a type I error level of $\alpha = 0.05$ (5% probability) and a type II error level of $\beta = 0.20$ (80% power). Power was determined retrospectively because the population size could not be determined a priori. A power of 85%, which was provided by a *posteriori* computation, can define the variation among groups. To account for possible dropouts, we included 20 patients in each group.

In this study, GCF samples were pooled to create one sample. The pooled sample from each individual was considered as the unit of observation. The Shapiro–Wilk test was performed to assess normal distribution. Intergroup comparisons of the biochemical and clinical parameters were analyzed by employing the Kruskal–Wallis nonparametric test, followed by *post hoc* group comparisons with the Bonferroni-adjusted Mann–Whitney U-test, because the data were not normally distributed. Regarding the Bonferroni correction, $\alpha = 0.05 / 3 = 0.016$ was taken to be statistically significant. An chi-square analysis was used to compare the gender distribution among the groups. To determine the association between GCF-SAA, fetuin-A and TNF-α levels and clinical periodontal parameters, Spearman's rank correlation test was employed. SPSS statistical software (ver. 19.0; SPSS Inc., Chicago, IL, USA) was used to carry out all statistical calculations, and $P < 0.05$ was considered to indicate statistical significance.

Results

Clinical findings

Table 1 presents the clinical and demographic findings. All clinical parameter values (full-mouth sample sites) were statistically greater in patients with CP than in those with gingivitis and healthy controls ($P < 0.016$). There were no significant differences in age or gender distribution among the study groups ($P > 0.05$).

Biochemical findings

Two samples were collected from the mesiobuccal or distobuccal areas (64 and 56 sites, respectively) of the single-rooted teeth. All GCF and serum samples contained SAA, fetuin-A, and TNF-α. Serum and GCF levels of SAA, fetuin-A, and TNF-α are shown in Table 2. Serum SAA and TNF-α levels were significantly elevated in the CP group compared with patients with gingivitis and healthy

Table 1 Clinical parameters (full-mouth and sampled sites' periodontal examination) and demographic results in the study groups

	Group 1 (Healthy)	*Group 2 (Gingivitis)*	*Group 3 (Periodontitis)*
Full-mouth periodontal examination			
PPD* (mm)	1.78 ± 0.50 (11.00)	2.34 ± 0.46 (20.00)	4.87 ± 0.52 (38.00)
CAL* (mm)	1.78 ± 0.50 (11.00)	2.34 ± 0.46 (20.00)	5.60 ± 0.55 (38.00)
GI*	0.30 ± 0.25 (8.00)	1.68 ± 0.30 (23.53)	2.25 ± 0.15 (37.47)
PI*	0.31 ± 0.16 (8.00)	1.68 ± 0.30 (23.53)	1.96 ± 0.13 (36.97)
BOP* (%)	2.05 ± 0.00 (8.00)	41.98 ± 9.49 (23.00)	78.63 ± 6.98 (38.00)
Sampled sites periodontal examination			
PPD* (mm)	1.75 ± 0.38 (12.80)	2.45 ± 0.39 (28.20)	6.40 ± 0.82 (50.50)
CAL* (mm)	1.75 ± 0.38 (12.80)	2.45 ± 0.39 (28.20)	7.48 ± 0.70 (50.50)
GI*	0.00 ± 0.00 (10.50)	1.75 ± 0.55 (34.45)	2.40 ± 0.45 (46.55)
PI*	0.00 ± 0.00 (10.50)	1.45 ± 0.46 (33.75)	2.25 ± 0.60 (47.25)
BOP* (%)	0.00 ± 0.00 (10.50)	100.00 ± 0.00† (40.50)	100.00 ± 0.00† (40.50)
Demographic results			
Age (years)‡	34.13 ± 3.31	34.60 ± 2.82	36.33 ± 2.61
Gender‡			
Males	9	9	11
Females	11	11	9

Data are expressed as the mean ± s.d. (Mean Rank).
Kruskal–Wallis/Bonferroni-adjusted Mann–Whitney
Bonferroni correction $\alpha = 0.05/3 = 0.016$
*Statistically significant difference between groups ($P < 0.05$).
†Statistically significant difference from Group 1 ($P < 0.05$).
‡No statistically significant difference among groups ($P > 0.05$).

Table 2 GCF and serum fetuin-A, SAA, and TNF-α levels in study groups

Parameter	*Group 1 (Control)*	*Group 2 (Gingivitis)*	*Group 3 (Chronic P)*
Total GCF-fetuin-A (ng)	2.02 ± 0.68*(1.95)	1.33 ± 0.25 (1.33)	1.11 ± 0.33‡ (1.13)
Serum Fetuin-A (ng ml^{-1})†	512.18 ± 99.26 (492.71)	407.28 ± 70.63 (391.84)	347.83 ± 83.16 (367.46)
Total GCF-SAA (μg)†	0.91 ± 0.10 (0.92)	1.08 ± 0.12 (1.06)	1.30 ± 0.11 (1.32)
Serum SAA (μg ml^{-1})†	4.81 ± 1.22 (4.73)	6.51 ± 1.47 (6.36)	9.04 ± 1.69 (9.11)
Total GCF-TNF-α (pg)†	0.14 ± 0.05 (0.12)	0.19 ± 0.05 (0.20)	0.25 ± 0.04 (0.25)
Serum TNF-α (pg ml^{-1})†	1.90 ± 1.60 (1.26)	6.05 ± 6.89 (3.33)	19.89 ± 18.35 (14.38)

GCF, gingival crevicular fluid. Data are expressed as the mean ± s.d. (median)
Kruskal–Wallis/Bonferroni-adjusted Mann–Whitney.
Bonferroni correction $\alpha = 0.05/3 = 0.016$.
*Statistically significant difference from Group 2 ($P < 0.016$).
†Statistically significant difference among groups ($P < 0.016$).
‡Statistically significant difference from Group 1 ($P < 0.016$).

individuals. Serum fetuin-A levels were significantly increased in healthy individuals compared with patients with gingivitis and CP ($P < 0.016$).

Table 2 shows the total levels of GCF. Total amounts of SAA and TNF-α were significantly decreased in the gingivitis group and the healthy controls compared with the CP group ($P < 0.016$). Moreover, total amounts of SAA and TNF-α were significantly increased in patients suffering from gingivitis compared with the healthy controls ($P < 0.016$). The total amount of fetuin-A was significantly higher in the control group than in the gingivitis and CP groups ($P < 0.016$). No significant difference in the total amount of fetuin-A between the gingivitis and CP groups was observed ($P > 0.016$).

Correlations

Table 3 lists the correlation coefficients. A positive correlation was observed between the total amount of SAA and CAL in the healthy control group ($P < 0.05$). Negative relationships were found between the total amount of SAA and fetuin-A, the total amount of fetuin-A and GI, and the total amount of fetuin-A and CAL in the gingivitis group ($P < 0.05$). Fetuin-A in GCF was negatively correlated with GCF-SAA levels, CAL, and GI, whereas GCF-SAA was positively correlated with GI and CAL, in the CP group ($P < 0.05$). A simultaneous examination of all clinical groups showed that the total amount of fetuin-A was negatively correlated with SAA, TNF-α, CAL, and GI ($P < 0.001$). Contrarily, there were positive correlations between the total amount of SAA and fetuin-A, TNF-α, CAL, and GI ($P < 0.001$). The total amount of TNF-α was positively correlated with fetuin-A, SAA, GI, and CAL ($P < 0.001$).

The findings of the Spearman's rank correlation (r) for the CP group, regarding total amounts of fetuin-A, SAA, TNF-α, and the number of deep sites, are provided in Table 4. The total amount of SAA was positively correlated with the number of sites that had $4 \leq PPD \leq 5$ mm ($P < 0.05$). A negative and significant relationship was observed between the total amount of fetuin-A and the number of sites with PPD ≥ 6 mm ($P < 0.05$). The total amount of TNF-α was positively correlated with the

Table 3 The Spearman's rank correlation (r) among groups with respect to GCF-fetuin-A, GCF-SAA, GCF-TNF-α, and sampled sites CAL and GI

	Group 1 (Control)		*Group 2 (Gingivitis)*		*Group 3 (Chronic P)*		*All groups*	
Parameter	r	P	r	P	r	P	r	P
GCF-fetuin-A to CAL	−0.123	0.605	−0.451*	0.046*	−0.635*	0.003*	−0.711*	0.000*
GCF-fetuin-A to GI	NA	NA	−0.572*	0.008*	−0.626*	0.003*	−0.776*	0.000*
GCF-SAA to CAL	0.651*	0.002*	0.384	0.095	0.522*	0.018*	0.851*	0.000*
GCF-SAA to GI	NA	NA	0.370	0.108	0.472*	0.036*	0.816*	0.000*
GCF-TNF-α to CAL	0.354	0.126	0.016	0.948	−0.106	0.658	0.620*	0.000*
GCF-TNF-α to GI	NA	NA	0.264	0.260	−0.110	0.646	0.596*	0.000*
GCF-fetuin-A to GCF-SAA	−0.106	0.657	−0.465*	0.039*	−0.582*	0.007*	−0.703*	0.000*
GCF-fetuin-A to GCF-TNF-α	0.076	0.749	0.044	0.855	−0.044	0.854	−0.466*	0.000*
GCF-SAA to GCF-TNF-α	0.337	0.146	−0.056	0.814	−0.034	0.888	0.569*	0.000*

GCF, gingival crevicular fluid; NA, Not applicable; GCF-SAA, total amount of SAA in GCF; GCF-FA, total amount of fetuin-A in GCF; GCF-TNF-α, total amount of TNF-α in GCF; GI, gingival index; CAL, clinical attachment level.
*Statistically significant ($P < 0.05$).

Table 4 The Spearman's rank correlation (r) with respect to GCF and number of deep sites in CP group

	Group 3 (Chronic P)	
	r	p
GCF-TNF to $4 \leq PPD \leq 5$	0.512*	0.021*
GCF-TNF to $PPD \geq 6$	0.719*	0.000*
GCF-SAA to $4 \leq PPD \leq 5$	0.500*	0.025*
GCF-SAA to $PPD \geq 6$	0.377	0.101
GCF-FA to $4 \leq PPD \leq 5$	−0.320	0.169
GCF-FA to $PPD \geq 6$	−0.550*	0.012*

GCF-SAA, Total amount of SAA in GCF; GCF-FA, total amount of fetuin-A in GCF; GCF-TNF, total amount of TNF in GCF.
*Statistically significant ($P < 0.05$).

number of sites with $PPD \geq 6$ mm and $4 \leq PPD \leq 5$ ($P < 0.05$). No correlation was found between the total amount of SAA and the number of sites having $PPD \geq 6$ mm, or between the total amount of fetuin-A and the number of sites with $4 \leq PPD \leq 5$ mm ($P > 0.05$).

Discussion

During the destructive period of periodontitis, gingival inflammation increases pro-inflammatory cytokines and mediators, including various acute-phase proteins such as TNF-α, α-2 macroglobulin, α-1 antitrypsin, and CRP in GCF (Ebersole and Cappelli, 2000). With the discovery of new acute-phase proteins, their potential relationship with periodontal disease has provoked scientific interest. Studies have reported that APPs may be inflammatory markers of PD (Pradeep *et al*, 2011; Keles *et al*, 2014; Balli *et al*, 2015).

In the present study, we evaluated the serum and GCF levels of fetuin-A and SAA proteins in healthy patients, and in those with periodontal disease. The study included three groups to evaluate the role of fetuin-A and SAA proteins in different stages of PD. GCF samples, oral rinse samples, and saliva samples were used for full-mouth investigation of oral biomarkers (Taba *et al*, 2005). Some studies have suggested that, similar to saliva, GCF also contains locally and systemically derived markers of periodontal disease and may be used to evaluate biomarkers of periodontitis and other systemic diseases at a patient-specific level (Kaufman and Lamster, 2000; Ozmeric, 2004). The severity of periodontitis is associated with local increases in biomaterials in GCF or tissue (Taba *et al*, 2005). Therefore, the teeth with the highest PPD and CAL were used as GCF-collecting sites, considering that these sites might show periodontitis. In addition, it has been reported that the GCF volume differs among sites and cannot be collected from the entire mouth (Lamster *et al*, 1991). To standardize collection of the GCF, only single-rooted teeth were used. Additionally, to eliminate confusion we supported the GCF amounts with serum levels. Our results showed that both the total amount of GCF and serum levels of fetuin-A were clearly decreased in the gingivitis and CP groups compared with the control group, whereas the total GCF levels were similar between the gingivitis and CP groups. In contrast, SAA protein levels were clearly increased in the gingivitis and CP groups compared with the healthy control group. In addition, GCF and serum levels of SAA were greater in the CP group compared with the gingivitis group. As expected, serum and total amounts of GCF-TNF-α were increased based on disease severity. It is important to reduce the impact of extraneous variables in research. In determining the level of the investigated mediators in periodontal diseases, total amount of data was used primarily to reduce the impact of GCF volume. Previous studies instead analyzed total amounts of GCF per sampling site, rather than the total concentration, when considering the association between GCF elements and PD (Lamster *et al*, 1988; Lin *et al*, 2005). It may be more appropriate to use all available data to prevent any inconsistency.

Due to the multifactorial etiology of PD and the relation between APPs and various systemic diseases, the individuals were chosen from a non-smoking and systematically healthy population, thus eliminating the effect of systemic diseases on fetuin-A and SAA levels. Because of the chronic nature of disease, the individuals were selected according to a specified age group (28–45 years). To minimize the influence of gender on fetuin-A and SAA

protein concentrations, the male and female numbers were approximately equal in each group.

APPs may be correlated with the inflammatory status of the periodontium. Increased levels of proinflammatory cytokines that have a key role in PD can alter the concentrations of APPs based on negative and positive APP characterization (Kaysen, 2009). Circulating fetuin-A levels are negatively correlated with levels of cytokines, such as IL-6 (Wang and Sama, 2012). Other studies have reported that early proinflammatory cytokines such as TNF, IL-6 and interferon-γ (IFN-γ) inhibit fetuin-A expression (Daveau *et al*, 1988; Li *et al*, 2011). Thus, fetuin-A generally may be regarded as anti-inflammatory (Chertov *et al*, 1994). Clinical evidence indicates that fetuin-A protein levels in RA contribute to disease pathogenesis. Additionally, some studies have found decreased fetuin-A protein levels in RA (Sato *et al*, 2007; Saroha *et al*, 2012). Calcified tissues such as bone contain large amounts of fetuin-A due to its strong binding affinity to hydroxyapatite (Kazama *et al*, 2005). Although fetuin-A metabolism is believed to be closely related to inflammatory conditions, it may also be related with inflammatory bone resorption (Sato *et al*, 2007). Periodontitis and RA are chronic inflammatory conditions with similar pathological features (Kobayashi and Yoshie, 2015). As expected, in the present study, we demonstrated a direct negative relationship between total amount of fetuin-A and CP. Significantly lower GCF and serum fetuin-A levels were found in the CP and gingivitis groups compared with the control group. The absence of statistically significant differences in GCF-fetuin-A between gingivitis and CP patients may be due to the inflammatory processes that exist in both conditions.

SAA is released in response to inflammation or infection. Production of SAA is stimulated by proinflammatory cytokines such as IL-6, IL-1, TNF, IFN-γ, and transforming growth factor-β (TGF-β) (Migita *et al*, 2011; Nakamura, 2011). SAA may play an important, pathogenic role in the proinflammatory process of RA (Connolly *et al*, 2012). Moreover, studies have indicated increased serum levels of SAA in RA and found it to be strongly associated with disease activity (Targonska-Stepniak and Majdan, 2014). In the current study, increased levels of serum SAA were demonstrated in the CP group. Moreover, the gingivitis group showed higher levels of SAA than the periodontal healthy group. Our results are consistent with data in the literature. Ardila and Guzman (2015) reported that SAA levels were significantly higher in patients with CP than in individuals without periodontitis. In another study, Graziani *et al* (2010) assessed serum SAA levels in periodontitis patients before and after non-surgical therapy and reported that serum levels of SAA were elevated within 24 h of treatment, but normalized after 30 days. Similarly, another study showed that SAA levels were reduced 3 months after full-mouth tooth extraction (Vuletic *et al*, 2008). It is not possible to compare our results with these studies due to the lack of a periodontal healthy control group. In contrast, Glurich *et al* (2002) reported higher levels of SAA in healthy individuals than in periodontal patients. Earlier polymicrobial PD models in mice showed that periodontal infection produced elevated SAA levels (Rivera *et al*, 2013; Aoki-Nonaka *et al*, 2014). To our knowledge, there is no reported study assessing SAA levels in GCF samples. The present study showed higher SAA total amounts in GCF samples in patients with CP and gingivitis than in healthy individuals.

TNF-α, an important pro-inflammatory mediator, is associated with periodontal pathogenesis (Page, 1991; Van Dyke *et al*, 1993; Ebersole and Cappelli, 2000). It is evident that TNF-α levels increase with the severity of PD (Page, 1991; Van Dyke *et al*, 1993). The findings of the current study agreed with those of previous studies showing that TNF-α levels increase significantly with greater disease severity. Serum and total amounts of TNF-α were found to be increased in patients with CP compared with those with gingivitis and healthy controls. As the current study was the first to examine fetuin-A in PD pathogenesis, we decided to evaluate TNF-α as an established inflammatory mediator in PD to confirm the presence of an inflammatory condition. Hence, increased levels of TNF-α in patients with gingivitis and CP, when evaluated along with clinical periodontal parameters, indicate an ongoing inflammatory process.

In the current study, consideration of all groups simultaneously showed a negative and significant correlation between the total amount of fetuin-A and SAA, TNF-α, CAL, and GI. There is evidence that fetuin-A is negatively associated with systemic inflammatory mediators (Sindhu *et al*, 2016). To evaluate the relationship between fetuin-A and inflammatory markers, we examined the correlations between fetuin-A, TNF-α, and SAA. As previously mentioned, TNF-α is an established inflammatory mediator in PD pathogenesis and the production of SAA, one of the most studied biomarkers in various systemic diseases, is stimulated by proinflammatory cytokines such as IL-6, IL-1, TNF, IFN-γ, and transforming growth factor-β (TGF-β) (Page, 1991; Van Dyke *et al*, 1993; Migita *et al*, 2011). The results demonstrated that SAA and fetuin-A are negatively correlated. This finding indicates that decreased fetuin-A levels can be considered as an indicator of disease onset in CP patients. The positive and significant correlations for all groups between SAA and TNF-α, and CAL and GI, supports the finding that similar to TNF-α, SAA levels may be increased in CP patients compared with healthy patients and those with gingivitis.

The correlation between GCF-fetuin-A, SAA, and TNF-α levels with PPD was also evaluated. There was a positive and significant relationship between the total amount of TNF-α and the number of sites that had $4 \leq$ PPD ≤ 5 mm and PPD ≥ 6 mm. These findings support the association of TNF-α with periodontal pathogenesis in both moderate and severe stages of inflammation. A positive and significant correlation was found between SAA and the number of sites that had $4 \leq$ PPD ≤ 5 mm, while there was no significant relationship with the number of sites that had PPD ≥ 6 mm. This can be explained theoretically by stating that SAA is involved in the moderate stages of inflammation, but cannot be detected in patients with severe periodontitis. SAA is also an important early predictor of inflammation. The negative and significant correlation between fetuin-A and the number of sites that had PPD ≥ 6 mm supports the finding that fetuin-A can

be detected in patients with severe periodontitis. According to our results, there was no significant correlation between fetuin-A and the number of sites that had $4 \leq PPD \leq 5$ mm, suggesting that fetuin-A may not be involved in moderate stages of inflammation.

In conclusion, serum and total levels of SAA significantly increased, whereas fetuin-A levels significantly decreased, with increasing severity of PD. Furthermore, serum and total TNF-α levels were significantly increased with disease severity. Increased levels of serum and total TNF-α in patients with gingivitis and CP supports the presence of an inflammatory process. Within the limitations of this study, we suggest that periodontal disease causes increased GCF and serum SAA levels in addition to decreased GCF and serum fetuin-A levels. Additional longitudinal studies are needed to confirm these findings.

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Conflict of interest

No potential conflict of interest relevant to this article was reported.

Author contribution

Study was designed by Çiğdem Coşkun TÜRER, Umut BALLI was responsible from data acquisition. The analysis and interpretation of data were done by Berrak GUVEN and Çiğdem Coşkun TÜRER. Çiğdem Coşkun TÜRER was responsible from drafting of manuscript: Critical revision from tha manuscript was done by Çiğdem Coşkun TÜRER, Umut BALLI and Berrak GUVEN.

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