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ORIGINAL ARTICLE

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Cytokine profile in gingival crevicular fluid and plasma of patients with aggressive periodontitis

Hager R. Zein Elabdeen[a], Manal Mustafa[a,b], Raouf Ali[c] and Anne Isine Bolstad[a]

[a]Department of Clinical Dentistry – Periodontics, University of Bergen, Bergen, Norway; [b]Oral Health Centre of Expertise in Western Norway, Bergen, Norway; [c]Faculty of Dentistry, University of Science and Technology, Omdurman, Sudan

ABSTRACT

Objective: This study aimed to determine the content of cytokines in gingival crevicular fluid (GCF) as well as in plasma of Sudanese patients with aggressive periodontitis (AgP) and healthy controls (HC).
Materials and methods: Nineteen AgP patients and 19 HC were included. The mean probing pocket depth and clinical attachment level of the GCF sampled sites in patients were both $\geq$5 mm. The GCF and plasma levels of 27 cytokines were determined using 27-multiplex fluorescent bead-based immunoassays. Ratios were calculated among cytokines of the T-helper cell subsets Th1 and Th2. Descriptive statistics, the Mann–Whitney U-test and Spearman's *rho* rank correlation coefficient analysis were used.
Results: Interferon-γ was the only cytokine found in significantly lower levels in GCF of patients compared with HC. Levels of interleukin (IL)-10, IL-13, IL-1Ra, monocyte chemoattractant protein-1 (MCP-1), regulated on activation normal T-cell expressed and secreted (RANTES), granulocyte-colony-stimulating factor (G-CSF), and granulocyte-macrophage-CSF (GM-CSF) were significantly lower in plasma of AgP compared with HC. The ratios of Th1:Th2 in GCF and Treg:Th17 in plasma were significantly lower in AgP.
Conclusions: The lower levels of cytokines detected systemically in plasma of AgP patients may have an impact on the immune response. The lower ratio of Th1:Th2 cytokines in GCF samples of AgP patients suggests a role for Th2 at the local site of disease.

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Introduction

Aggressive periodontitis (AgP) is characterized by rapid periodontal destruction in susceptible individuals at an early age, involving a.o. first molars and incisors [1]. The pathogenesis of periodontal disease has been regarded as a host-mediated response to the biofilm in individuals at risk. As a result of the host-biofilm interaction, inflammatory mediators, such as cytokines, chemokines, arachidonic acid metabolites and proteolytic enzymes are released and contribute to tissue damage and bone loss [2–4].

Cytokines play a vital role in the direction of inflammatory responses towards either protective or destructive processes [3,5]. A proper balance between pro- and anti-inflammatory cytokines may protect the periodontal tissue from destructions [6], hence an imbalance may result in disease progression [3,6]. The multifunctional cytokines Interleukin (IL)-1 β and IL-6 have been found at enhanced levels in GCF of patients with chronic inflammatory lesions [7]. Suzuki et al. [8] found no difference between AgP and chronic periodontitis patients regarding mean IL-1β concentrations in GCF samples. Higher GCF levels of IL-1β, and higher IL1-β/IL-10 ratio have been reported in generalized AgP (GAgP) compared with healthy controls (HC) [9]. Stimulation of peripheral blood from patients with localized AgP (LAgP) with lipopolysaccharide caused elevated levels of pro-inflammatory cytokines, and a hyper-responsive trait was demonstrated in this group [10]. In addition, the inflammatory mediators (e.g. IFN-γ) produced by T cells subsequently may activate the production of metalloproteinases, such as collagenase, leading to differentiation of osteoclasts and consequently resulting in connective tissue destruction and bone tissue loss [11].

Naïve T helper cells may differentiate into the subgroups T helper type 1 (Th1) and T helper type 2 (Th2) cells. Th1 and Th2 immunity refer to both innate and adaptive immune responses, although not strictly synonymous [12,13]. Type 1 effector response involves Th1, Th17 and cytotoxic T cells, as well as immunoglobulins. Th1 cells secrete IL 2, IFN-γ and TNF-α. Type 2 immune response involves Th2 cells as well as IL-4, IL-5, IL-9 and IL-13. Treg1-type cytokines (e.g. IL-10) are responsible for suppression of periodontal destruction in established periodontitis lesions [14]. No difference was reported between plasma levels of Th1 and Th2 cytokines in AgP, chronic periodontitis and healthy groups by Mattuella et al. [15]. However, they observed a trend towards low levels of Th2 cytokines in AgP patients and suggested a contribution of Th2 to the development of AgP.

CONTACT Hager R. Zein Elabdeen hagerzeinelabdeen@gmail.com Department of Clinical Dentistry, Faculty of Medicine and Dentistry, University of Bergen, Arstadveien 19, Bergen, Norway

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Cytokines interact and function within a network. Consequently, understanding their role in the pathogenesis of periodontitis is not possible by studying a single cytokine [5]. The level of cytokines in GCF as well as in saliva may reflect the inflammatory situation of the local tissues [16,17], while cytokines in blood and plasma may reflect systemic influence of periodontal disease [15,18–20].

The aim of this study was to profile cytokines in plasma and GCF of AgP patients using a multiplex immunoassay method for the concurrent detection of cytokines. Furthermore, to determine the ratios and relationship between cytokines that might be involved in inflammation/resolution processes in periodontal tissues.

Materials and methods

Study population

Nineteen AgP patients were enrolled from patients seeking treatment at the University of Science and Technology (UST), Faculty of Dentistry, Omdurman, Khartoum, Sudan (December 2008 to July 2009). To be involved in the study, probing pocket depth (PPD) and clinical attachment level (CAL) ≥5 mm had to be present in at least one incisor and one first molar. The patients were all <35 years. Full-mouth clinical examination including PPD, CAL, bleeding on probing (BoP) was performed by the same dentist (HRZE). Intra-individual calibration of the examiner was undertaken before the clinical examination and the intra-examiner ICC ranged between 0.61 and 1.00. Radiographs were used for confirmation of the diagnosis [21]. Subjects were excluded if they had received periodontal or antibiotic treatment within three months before the examination, were pregnant, a smoker or had any systemic disease. The 19 control subjects were healthy employees and students at UST, with PPD ≤3 mm for all teeth, and in most cases all teeth were present. All subjects involved in the study provided written informed consent and ethical approvals were obtained from the Research Ethics Committee at UST, Omdurman, Sudan, and the Regional Committee for Medical Research Ethics (REK) Western Norway (REK 177.04).

GCF sampling and elution of proteins

GCF samples were collected using perio-paper (PERIOPAPER® Gingival Fluid Collection Strips, Oraflow Inc., New York, NY) from the mesio-buccal site of the first molar. If the first molar was not available, the 2nd molar or the 2nd pre-molar from the same quadrant was used. GCF was collected from only one site per individual. Prior to the GCF collection, the site was isolated with cotton rolls and exposed to a gentle air stream for five seconds. Then the perio-paper was placed subgingivally for 10 s, before being removed and stored in an empty tube in liquid nitrogen. A Tris–HCl buffer (230 µl) with a final concentration of 12 mM at pH 7.6, containing 0.05% Tween-20, was added to the microcentrifuge tubes for protein elution from the perio-paper. The tubes were shaken for 30 min at room temperature and centrifuged for 10 min at 4 °C and 14000 rpm. The eluates were stored at −80 °C for later cytokine analyses.

Plasma samples

Peripheral blood was collected in BD Vacutainer Tubes containing EDTA (BD Biosciences) (Puls AS, Oslo, Norway) and centrifuged for 10 min at 3000 × *g* (SUPERFIT centrifuge, Mumbai, India). The supernatant (plasma) was divided into aliquots and transferred to polypropylene tubes, which were stored in liquid nitrogen.

Quantification of cytokines by multiplex bead immunoassays

Cytokine levels in the GCF and plasma samples were analysed using a multiplex fluorescent bead-based immunoassay. The Bio-Plex Pro Human Cytokine 27-plex Assay Group 1 (Bio-Rad Laboratories, Hercules, CA) identified 27 cytokines [pro-inflammatory cytokines as IL-1β, IL-2, IL-6, IL-7, IL-9, IL-12 (p70), IL-15, IL-17A; the colony stimulating factors G-CSF and GM-CSF; the chemokines eotaxin, IL-8, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted); TNF-α and anti-inflammatory cytokines as IL-1Ra, IL-4, IL-5, IL-10, IL-13, as well as growth factors, such as PDGF-BB, basic FGF and VEGF] with a workflow as described by Houser [22]. The Bio-Plex® 200 Systems processed by Luminex were used, including microplate platform and Bio-Plex Manager™ 6 software. The analysis was done according to the manufacturer's recommendations based on the Luminex xMAP technology. The amount of protein in each sample was extrapolated compared with standard curve ranges with concentrations reported in pg/ml, with results reported in pg/ml in plasma samples and reported as quantities of cytokines per 10 s (pg/10 s) in GCF [23,24]. The maximum detection limit for all cytokines ranged between 80,280 and 937 pg/ml, and the minimum detection limit ranged between 0.66 and 0.01 pg/ml (Table S1).

Statistical analysis

Statistical analyses were performed using IBM SPSS analytics software version 19 (IBM SPSS Statistics, Armonk, NY). Descriptive statistics (mean, standard deviation (SD) and median) were used to describe the cytokine concentrations in GCF and plasma from both patients and healthy controls. To include all individuals in the analysis the values below the detection limit were replaced by the lowest value that was extrapolated from the standard curve [25]. The Mann–Whitney U-test was used to compare the cytokine levels between AgP patients and healthy controls, as well as to compare the ratios between the different T-helper subset cytokines. Values of $p < .05$ were considered statistically significant. Spearman's *rho* rank correlation coefficient analysis was used to detect the relation between the different cytokines in the two groups. Correction for multiple comparisons was not applied to limit the risk of type II errors [26], and

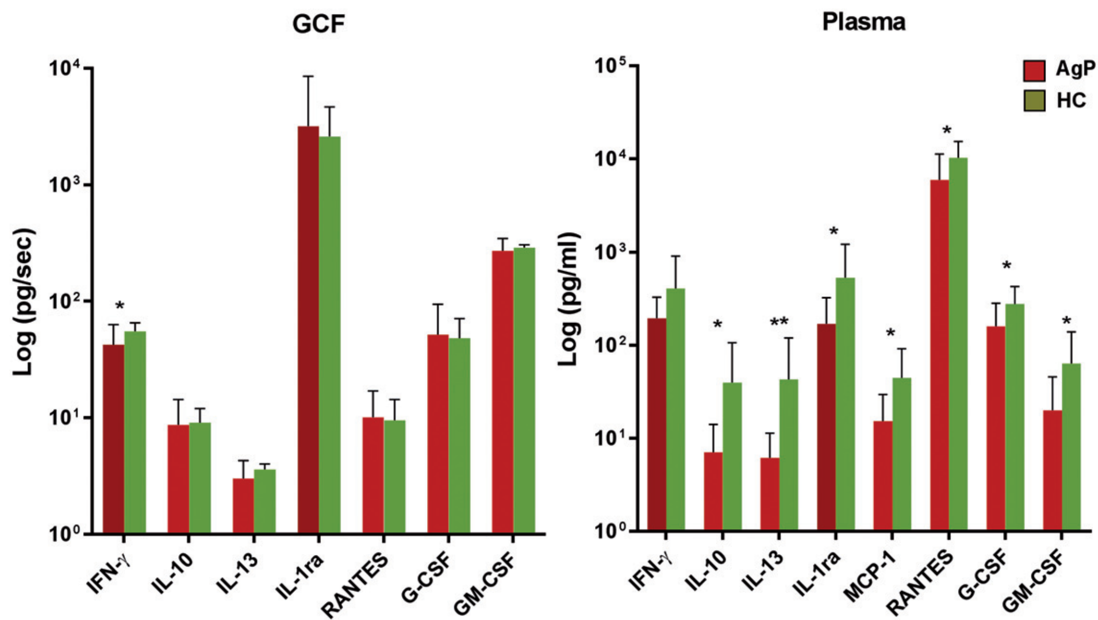

Figure 1. Comparison of cytokines in GCF and plasma of patients with aggressive periodontitis (AgP) and healthy controls (HC). A base-10 log scale is used for the Y axis. Standard deviation is visualized as error bars. Significant differences between groups are indicated as *($p < .05$) and **($p < .01$). MCP-1 was not detected in GCF.

adjusted p values for multiple comparisons were not calculated directly because these conservative p values alter the original calculated p values for the direct comparisons. Publications by Rothman [26] and Duarte et al. [27] discuss this issue further, and based on their reasoning we believe the original p values are of particular interest.

Results

The demographics of the participants have been described previously [21]. Briefly, the mean age of the AgP patients was 23.74 ± 6.84 years and of the HC 24.40 ± 3.54 years. The females represented 79 and 63% of the AgP patients and healthy controls, respectively.

The mean PPD and CAL scores of the sampled sites in the AgP patients were 6.1 ± 1.0 and 6.4 ± 1.7 mm, respectively, and the median was 6 mm for both parameters. BoP from all sites examined in each patient ranged between 17 and 100% [21]. In the sampled sites of the HC both PPD and CAL were ≤3 mm, which was in line with the inclusion criteria.

Of the collected GCF and plasma samples (76 in total), 11 samples [six GCF samples (two from AgP patients and four from controls) and five plasma samples (all from controls)] were not successfully analysed. Seven cytokines (IL-2, IL-5, IL-9, MCP-1, eotaxin, PDGFb and IL-15) were not detected in any of the analysed GCF samples, and FGFb was found in only one AgP patient. The pattern of cytokines in GCF and plasma samples was highly variable among the study subjects, although all cytokines were detected in the majority of plasma samples (Figure 1, Table S2).

There was significantly less IFN-γ in GCF samples from AgP patients compared with HC ($p < .05$). In addition, there was a trend towards an enhanced level of eight cytokines (IL-1β, IL-1ra, IL-8, IL-12 (p70), IL-17A, G-CSF, RANTES and VEGF) in the AgP patients, although the differences between patients and controls were not statistically significant ($p > .05$) (Figure 1).

In plasma samples, AgP patients had lower levels of all cytokines compared with healthy controls. The levels of IL-10, IL-13, IL-1ra, MCP-1, RANTES, G-CSF and GM-CSF were significantly different between the two groups ($p < .05$) (Figure 1).

Ratios between cytokines in GCF and plasma

The molecules under investigation were divided into subgroups belonging to Th1 (IFN-γ, IL-2), Th2 (IL-4, IL-5, IL-6, IL-9, IL-13), Th17 (IL-17, TNF- α) and Treg (IL-10) subsets. Ratios were assessed among cytokines subgrouped by T helper cell subsets (Th1:Th2 and Treg:Th17) in AgP patients compared with HC (Figure 2). Cytokines of the subsets Th1:Th2 in GCF and Treg:Th17 in plasma were significantly lower in AgP patients than in healthy controls. Further, the molecules were also subdivided into pro-inflammatory cytokines (IL-12, IFN-γ, IL-2, TNF-α, IL-6, IL-9, IL-1β, IL-17, IP-10, MCP-1, MIP-1 α, RANTES, IL-8, eotaxin, IL-7, IL-15, G-CSF, GM-CSF) and anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13, IL-1ra). In GCF, there were essentially no differences seen, while in plasma, there was a tendency towards a lower anti-inflammatory to pro-inflammatory ratio in AgP patients compared with HC (Figure 2).

Correlation analysis

The correlation coefficient Spearman's *rho* rank analysis was used to estimate the relationship between the 27-plex cytokines in the GCF of AgP patients and healthy controls, in order to understand the interaction between cytokines at the local site of the disease. In the GCF of AgP patients, IL-1β and TNF-α were positively correlated with most of the cytokines tested including the pro-inflammatory cytokines (IL-7,

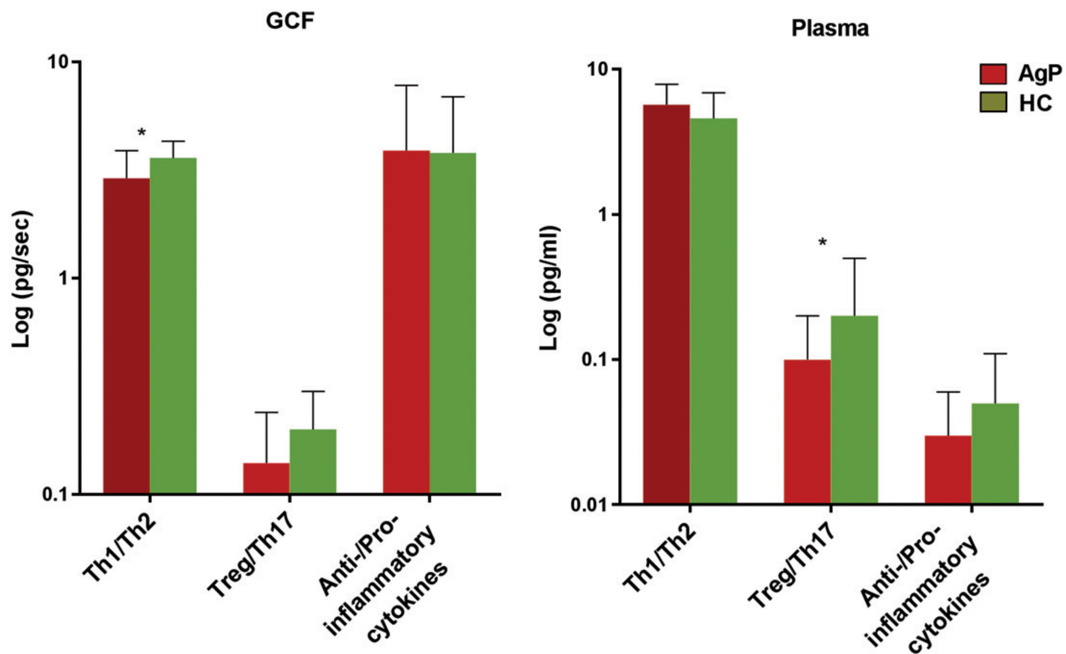

Figure 2. Th1 vs. Th2, Treg vs. Th17 and anti- vs. pro-inflammatory cytokines in GCF and plasma of patients with aggressive periodontitis (AgP) and healthy controls (HC). A base-10 log scale is used for the Y axis. Standard deviation is visualized as error bars. Significant differences between groups are indicated as *($p<.05$).

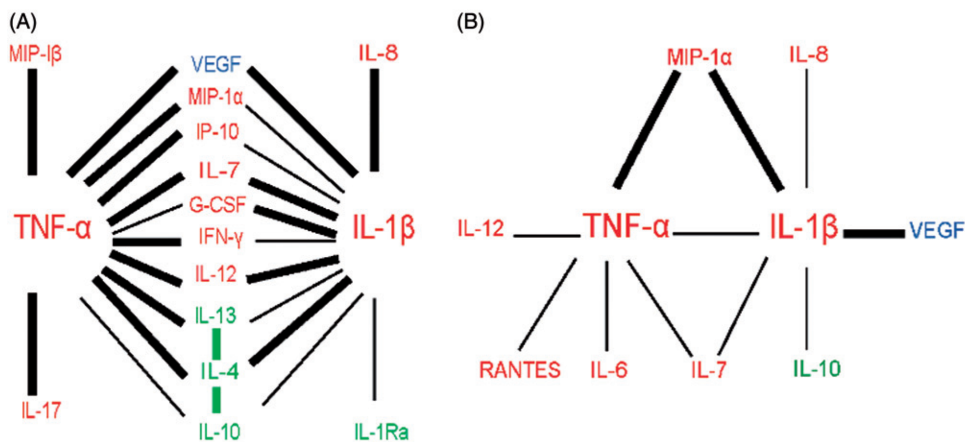

Figure 3. Cytokine networks in gingival crevicular fluid (GCF) of patients with aggressive periodontitis (AgP) and healthy controls (HC). (A) AgP and (B) HC. Bold lines represent significance at the 0.01 level (2-tailed) and the normal line represents correlation significant at 0.05 level (2- tailed) using Spearman's rho rank correlation coefficient analysis. (Pro-inflammatory cytokines: red, anti-inflammatory cytokines: green, growth factors: blue).

IL-12, IFN-γ, IP-10, G-CSF and MIP- 1α), anti-inflammatory cytokines (IL-4, IL-10 and IL-13) and VEGF. IL-1β also correlated with IL-8 and IL-1Ra, while TNF-α correlated with IL-17 and MIP-1β (Figure 3(A)). In healthy controls, IL-1β and TNF-α, however, were found to correlate with fewer numbers of cytokines compared to AgP patients. IL-10 was the only anti-inflammatory cytokine that showed a positive correlation with IL-1β in HC (Figure 3(B)). All correlations were statistically significant at the level $p<.05$ and the level $p<.01$ (2-tailed) (Figure 3).

Discussion

In an attempt to understand the impact of cytokines in AgP disease, 27 cytokines were profiled via multiplex analysis of GCF and plasma samples from AgP patients and healthy controls. Whereas IFN-γ was the only cytokine that measured in significantly lower levels in GCF of AgP patients compared with healthy controls, several cytokines were detected in significantly lower levels in patient plasma than in controls. The regulatory or anti-inflammatory cytokines IL-10, IL-13 and IL-1Ra, as well as the chemokines MCP-1, RANTES, and the colony stimulating factors G-CSF and GM-CSF were all present in significantly lower levels in plasma of patients than in controls. Further, the ratios of Th1:Th2 cytokines in GCF samples and Treg:Th17 cytokines in plasma samples were significantly lower in AgP patients compared to controls, suggesting a role for Th2 and Th17 at the local site of disease and at the systemic level, respectively.

Lower levels of IFN-γ were seen in both GCF and plasma of AgP patients compared to controls, but only reached significance in GCF. Low levels of IFN-γ have previously been

reported in peripheral blood mononuclear cells in early onset periodontitis patients [28]. In a recent study, cluster analyses were used to find patterns of cytokine/chemokine expression among participants and to subdivide them into clusters [29]. Three distinct clusters emerged among AgP participants stimulated by lipopolysaccharides: a high, mixed and low responder group [29]. Parallel to our results, the mixed and low responder groups had lower release of IFN-γ. However, higher levels of IFN-γ in GCF of patients with chronic periodontitis were found in active sites compared to inactive sites within the same patient [30]. It has also been suggested that IFN-γ levels in GCF depend more on the clinical stage of the site than on the disease stage of the patient [31]. Collectively these reports present conflicting data, possibly reflecting the influence of the stage of periodontal inflammation on the levels of cytokines present (initial process, progression and resolution) [32].

In this study, except for IFN-γ, no significant differences were found between AgP and HC regarding the cytokine levels in GCF, which is partially in agreement with a recent study investigating cytokine levels in plasma and GCF from chronic periodontitis patients [19]. A previous study on GAgP suggested a key role for RANTES and MCP-1 in the biological mechanism underlying the progression of GAgP [33]. In parallel, this study showed an enhanced, though not significant GCF level of RANTES among AgP patients compared to control.

Plasma samples of AgP patients contained lower levels of the cytokines IL-10, IL-13 and IL-1Ra, the chemokines MCP-1 and RANTES, and the colony stimulating factors G-CSF and GM-CSF, compared with healthy controls. The great individual variability of cytokines observed in plasma may also be due to differences in the ability to establish an adequate inflammatory response. As previously reported, TNF-α level in GCF was not different between AgP and HC [34]. This is in parallel with previous findings in saliva, where TNF-α level in AgP patients was not different to that of chronic periodontitis and healthy subjects [35]. In contrast, a previous study has reported higher levels of ILs, IFN-γ and TNF-α in GCF and saliva among AgP patients and HC [36]. In a recent review, it was reported that there is not sufficient data available to prove that there are distinctive GCF cytokine/chemokine markers for patients with AgP and chronic periodontitis [27]. Further studies are needed in this field.

In this study, there was a trend towards a decreased anti-inflammatory to pro-inflammatory ratio in plasma of AgP patients, but in GCF there was virtually no difference between the AgP and healthy controls. This could be due to an attempt by the immune cells at the local diseased site to decrease the inflammatory response and augment the molecules involved in the healing process [37]. Given that a cytokine may behave as both a pro- as well as an anti-inflammatory cytokine, classification of cytokines into these two categories is an oversimplification [17,38,39].

The presence of low Th1:Th2 response in GCF of AgP patients suggests a Th2 dominant reaction at the site of periodontal destruction. The low ratio of Th1 to Th2 in AgP patients is in accordance with an early hypothesis that Th2, rather than Th1, is related to disease progression [28,37]. There was no difference in the ratio of Th1 to Th2 response in plasma samples, which is in agreement with the results of a recent study of AgP plasma samples [15]. Similarly, ratios of precursors of pro-resolution to pro-inflammatory lipid mediators was recently reported to be more relevant for describing the disease status of AgP patients than the concentration of specific lipid mediators [4]. Although T cells act as the main regulatory group of cells, the critical role of B cells and plasma cells in periodontitis should not be overlooked [40]. The host response to bacteria involves activation of T and B cells in the inflammatory infiltrate which bear an abundance of receptor activator of nuclear factor kappa-B ligand (RANKL) that promotes osteoclastic bone resorption [41]. There is evidence that B cells are a potent source of IL-1 and there is an interaction between T and B cells through number of cytokines associated with severe periodontitis [42]. Berglundh et al. [40] reviewed reports on distribution of cell proportions in periodontitis lesions, and concluded that B cells and their subset plasma cells represented about 60% of cells in the lesion, while T helper and T cytotoxic cells presented with fractions of 13 and 4%, respectively. Furthermore, several studies reported elevated levels and proportions of inflammatory markers and receptors, such as MMPs (MMP 1, 2, 3 and 13), NF-kB and IL-2 receptor-positive Th cell in periodontitis lesions [40].

In this study, the GCF correlation analysis suggests that cytokine networks may be involved in strengthening the inflammatory reaction in AgP patients. The positive correlations found here between pro- and anti-inflammatory cytokines at the site of the disease may reflect the positive and negative feedback between these cytokines, as discussed by Preshaw and Taylor [5].

Plasma was used in this study for analyses using multiplex immunoassays instead of serum. This was based on previous studies that found important differences in measurements of biomarkers between serum and plasma [43–45]. It has been argued that plasma is more sensitive than serum for cytokine detection [44].

GCF volume was not measured in our study and in an attempt to standardize GCF sampling; identical sampling time was used, in accordance with the study of Giannopoulou et al. [46]. In a recent review, the presentation of results in both volume and quantity was recommended [47]. Our results are presented only as amount/10 s, which may be a limitation in our study [27]. Further, the cross-sectional design and the relatively small number of participants as well as low power of this study also limit generalization of the current findings.

Conclusions

AgP patients had lower levels of IFN-γ and Th1:Th2 ratios in GCF as well as lower ratios of Treg:Th17 in plasma compared with healthy controls. In addition, lower levels of the anti-inflammatory cytokines IL-10, IL-13 and IL-1Ra, the chemokines MCP-1 and RANTES, as well as the colony stimulating factors G-CSF and GM-CSF were detected in plasma samples from AgP patients compared with plasma

of healthy controls. The lower levels of cytokines detected in GCF and systemically in plasma of AgP patients compared with HC may have an impact on the immune responses among AgP patients.

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Disclosure statement

The authors declare that they have no conflict of interest.

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