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Array analysis for T-cell associated cytokines in gingival crevicular fluid: Identifying altered profiles associated with periodontal disease status

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ABSTRACT

Objective: Cytokine networks regulate innate and adaptive immune responses, which in turn are recognised to direct the progression or arrest of periodontal disease. This study aimed to compare the profile of seven cytokines, implicated in regulating T-cell networks, in gingival crevicular fluid (GCF) samples with differing classification of periodontal status.

Methods: GCF samples were collected from patients with strong clinical evidence for chronic periodontitis, aggressive periodontitis, gingivitis or no gingival inflammation. Cytokines IL-6, IFN-ɣ, IL-4, IL-2, IL-17 A, IL10 and TNFα were measured in each sample using a commercial cytometric bead array assay. Descriptive statistics were used to indicate central tendency, data scatter and analysis of variance for each cytokine concentrations between respective patient groups. Heat maps with dendrograms were produced to visualise hierarchical clustering and trends within the data.

Results: Median concentrations for all cytokines analysed were highest for gingivitis samples and lowest for aggressive periodontitis samples. The median concentration of IL-6 in gingivitis samples was observed to be 10.5 fold higher (˜17,300 pg/μl) than IL-6 in aggressive periodontitis samples (˜1600 pg/μl). Median concentrations of IL-10, IL-17 A and TNFα were also 2–2.2 fold higher in gingivitis samples compared to aggressive periodontitis.

Conclusions: Descriptive statistical analysis noted raised concentrations of IL-6, IL-17 A and TNFα associated with gingivitis; pro-inflammatory cytokines usually associated with periodontal tissue destruction, including bone. Our results would suggest that these cytokines can additionally provide protective roles in preventing progression to advanced forms of periodontal disease. Potential for how these cytokines contribute to providing this role is discussed.

Clinical significance: Defining the roles for the many cytokines involved in the pathogenesis of periodontal disease is far from complete. Consequently the results of this study serve to evidence proposals that cytokines can exhibit both pro- and anti-inflammatory effects, which is dependent on the signalling environment within which they exist and the antagonizing or modifying actions of other cytokines. Whilst future research is necessary to explore mechanistic action, our study contributes new knowledge suggesting that IL-6 and IL-17 A can provide roles in stabilising the lesion to limit disease progression, which does not preclude alternative roles in promoting periodontal bone loss in advanced forms of disease progression, which is also documented in the literature.

1. Introduction

Periodontal diseases encompass a group of inflammatory diseases that have recently been re-classified to recognize the complex poly immuno-inflammatory nature of the disease pathology (reviewed by [1]). Gingivitis is hence defined as involving mild inflammation of the gingival tissues that may be plaque induced, modified by systemic factors, or initiated by specific bacterial species. Periodontal disease associated with extensive tissue destruction is classified as either chronic periodontitis involving slow to moderate rates of disease progression, which is modifiable by the presence of local risk factors such as smoking and stress; or aggressive periodontitis characterised by rapid loss of attachment and alveolar bone, and usually associated in patients under 30 years old. Various epidemiological studies have broadly suggested that prevalence of gingivitis can be considered high, affecting greater than 75% of any population [2]. Although precise data is difficult to obtain there is a consensus that only 5–15% of patients continue to develop chronic forms of periodontitis [2], whilst the prevalence of aggressive periodontitis is between 0.1–5%, which additionally appears to be ethnicity dependent [3]. Such trends in the data

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suggest that subgroups of patients, as yet unidentified, are susceptible to develop more severe forms of periodontal disease and the distinctive patterns of tissue destruction observed between gingivitis, chronic and aggressive periodontitis would suggest that differences in cellular and molecular pathogenic mechanisms exist between the disease classifications.

Within all forms of the disease, a dysbiosis in the natural microflora leading to the release of virulence factors derived from bacterial pathogens in the gingival sulcus, results in activation of immuno-inflammatory host responses within the surrounding periodontal tissues [4–6]. The inflammatory response is characterised by the infiltration of neutrophils, macrophages and leukocytes which are intended to be protective by aiding in the resolution of inflammation and halting disease progression. Within this scenario T-helper cells (Th1, Th2 and Th17) and regulatory T-cells (Treg) are recognised to play a key roles. These T-cell subpopulations derive from the activation of naïve CD4+ T-cells with the cognate bacterial antigen. In addition, a complex interplay of various pro- and anti-inflammatory cytokines and co-stimulatory signals act either to induce or to inhibit differentiation to the respective subpopulations, defining the final phenotypic nature of the developing T-cell. Subpopulations produced are described to exhibit either beneficial or detrimental effects and thus the balance between Th1/Th2/Th17/Treg cells is important in directing tissue destruction or in preventing it (reviewed by [7–9]).

Numerous studies have shown some success towards identifying clear involvement for Th1, Th2, Th17 and Treg cells, although the precise contribution for how each cell subset stimulates periodontal tissue destruction is unclear. Studies in mice, involving the induction of periodontal tissue destruction by known periodontal pathogens, have demonstrated a prominent role for CD4+ cells in mediating alveolar bone destruction (reviewed by [7]). However, it has been difficult to clarify which subset plays the greater pathogenic role. A plethora of animal studies have proposed that Th1 cells are associated with a stable lesion, whereas Th2 cells are associated with the progressive lesion [6]. Conversely, clinical studies have also suggested that raised levels of Th1 cells occur in diseased tissues, whereas other studies identify as yet undefined roles for both Th1 and Th2 cells in periodontal disease. Mechanistic understanding is further complexed when considering the additional involvement for Th17 and Treg cells [6]. Mice deficient for interleukin (IL) 17 A, a pro-inflammatory cytokine produced by Th17 cells, were more susceptible to periodontally-induced alveolar bone resorption, suggesting a protective role for this T-cell subset [10]. This contradicts *in vitro* studies suggesting osteoclastogenic activity for Th17 cells [11]. Likewise, human gingival biopsies from patients with gingivitis and periodontitis have identified the co-existence of both Th1 and Th2 [12,13]. However, the subset that is responsible for disease progression to more advanced forms of periodontitis is still controversial with contradictory results published [14–16]. Cytokines characteristic for Th17 cells (IL-17 A, IL-17, IL-21, IL-22, IL-26) have been shown to be elevated in both the periodontal tissues and gingival crevicular fluid of patients exhibiting either periodontitis or gingivitis [17–20]. Several reports have also described increased infiltration of Treg cells in human gingival tissue associated with periodontal lesions [18,21]. It is also recognised that the balance of T-cell subgroups formed also sits within a wider reciprocal signalling environment involving other cells involved in the immune response, including neutrophils, macrophages and B cells. The activity of such cells is equally critical in dictating either resolution of inflammation leading to tissue repair or progressive tissue destruction [7–9].

Against this background of limited evidence, the aims of the present study were to identify a multi-array profile for a number of T-cell-related cytokines present within an individual sample of gingival crevicular fluid. Samples were taken from patients stratified as presenting with either gingival inflammation, chronic or aggressive forms of periodontitis. Results were compared to GCF samples from relatively healthy controls demonstrating minimal inflammation.

2. Materials and methods

2.1. Recruitment of patients

Ethical approval was provided by South East Wales Local Research Ethics Committee (reference number 08/WSE03/3), following Good Clinical Practice regulations and with informed consent obtained from each patient. A total of 40 patients, representing 10 patients with chronic periodontitis, 10 patients with generalised aggressive periodontitis, 10 patients with gingivitis, and 10 healthy subjects, were recruited from patients referred to the Restorative Clinic at Cardiff University Dental School. Exclusion criteria included pregnancy, medical conditions or medications that may influence periodontal health, intake of any antibiotic or anti-inflammatory drugs in the previous six months or a history of periodontal therapy in the previous six months. Patient recruitment was based on the clinical and radiographic criteria proposed at the 1999 International World workshop for classification of periodontal disease conditions [22]. Thus for the study presented herein, the aggressive periodontitis group presented with attachment loss of greater than 6 mm affecting a minimum of six teeth; all subjects in this group were younger individuals below the age of 40. The chronic periodontitis group presented with attachment loss in the range of 6–10 mm in 2–5 teeth, which was typically associated with substantial amounts of plaque and calculus and a radiographic indication of gradual horizontal bone loss; patients in this group represented a broader age range. Gingivitis was associated with plaque-induced inflammation and demonstrated no evidence of attachment loss either clinically or radiographically. The control group were essentially free of large deposits of plaque and inflammation of the gingival tissue.

2.2. Sample collection

GCF was collected from two sites per patient. Considering that periodontal inflammation and tissue destruction is site specific [4] samples represented independent collections. Contaminating saliva was isolated from the collection site, supragingival plaque was gently removed and the tooth was air dried. GCF was collected by capillary action into 2 µl capillary tubes (Drummond Microcaps, Drummond Scientific Co, Pennsylvania, USA), placed at the gingival margin for a period of 10 min and then immediately stored at −80 °C until further analysis. During collection, capillary tubes were renewed when full or blocked by plaque debris. GCF samples were visually examined in the capillary tube and those tubes that did not contain a relatively translucent fluid expected of GCF, due to contamination with excessive blood were discarded. GCF volume was determined by measuring the proportion of the tube filled with fluid.

2.3. Measurement of clinical parameters

GCF was collected prior to clinical measurements, which may have increased exudate production. Clinical parameters were recorded by a single operator, using the Florida Probe with a computerised, pressure-controlled PASHA probe, that was calibrated for each patient assessment (Florida Probe Corporation, Gainesville, FL, USA). Probing depths and recession were recorded at six points per tooth.

2.4. Cytokine analysis of GCF fluid

Cytokine levels in each GCF sample was analysed using a BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA.) according to the manufacturer's protocol. Each GCF sample was expelled from the capillary tube, mixed with 15 µL of assay diluent and centrifuged at 1000*g* for 5 min to remove debris. The recovered supernatant was adjusted to a total volume of 35 µL with assay diluent and mixed with 35 µL of bead mix containing bead populations coated with capture antibodies specific for IL-

2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)α, interferon (IFN)γ and IL-17A (prepared immediately beforehand) and 35 µL of phycoerythrin (PE) detection reagent. Samples were incubated at 4 °C overnight in the dark and then re-equilibrated to room temperature for 1 h. 700 µL of wash buffer was added to each sample, centrifuged at 200*g*, for 5 min, and pelleted beads were re-suspended in 200 µL of wash buffer prior to flow cytometry. Sample data was acquired using a BD FACS Calibur flow cytometer (BD Biosciences). FCAP ArrayTM software was used to generate results. Within this software standard curves were generated using standards supplied with the assay kit and unknown values were calculated from the linear region with a 20–5000 pg/ml detection limit for each cytokine analysed.

2.5. Statistical analysis

Descriptive statistics and graphical methods were used to explore the data using the SPSS V20 statistical software package. Medians, means and standard deviations were calculated. STATA V13 was used to produce dot plots of cytokine levels (pg per ml) as a function of periodontal status group (i.e. control, aggressive periodontitis, chronic periodontitis and gingivitis), and line plots of the mean and median values for each group were superimposed on these charts. Normality of data distribution for each cytokine was assessed via normal plots and the Kolgorov-Smirnov and Shapiro-Wilk tests of normality. Due to non-normality of the data and / or the presence of outliers for all cytokines, differences between periodontal status groups were examined using non-parametric ANOVA (i.e., the Kruskal-Wallis test) for each cytokine separately. Dunn's *post-hoc* tests were employed after non-parameter one-way ANOVA in order to detect differences between specific pairs of groups. Statistical tests were carried out using GraphPad Instat V3.10.

The median concentration (pg per ml) for each of the cytokines analysed within each of the respective periodontal status groups were graphically depicted as a heat map created by using the statistical software package R. Hierarchical cluster analysis was performed to arrange the heat maps to indicate clustering of cytokine concentrations with respect to both cytokine type and periodontal status group. Dendograms generated were incorporated into the resulting heat maps to indicate the degree of similarity in cytokine median concentration between data sample groups.

3. Results

3.1. Clinical data

Patient and sample clinical data is presented in Table 1. Two sites were analysed per patient, with 10 patients per group recruited. Considering that periodontal disease is site specific [4], samples were treated independently. Although flow cytometry was attempted, cytokine levels could not be measured with any confidence for 11 samples in the control group because the sample volume was too small and the cytokine concentration was below the detection limit identified within the standard curve. For samples collected from patients with gingivitis, chronic periodontitis or aggressive periodontitis, 3–4 samples were not analysed due to excess blood contamination which would have greatly influenced the calculation of cytokine concentration within the GCF exudate. The GCF volume is thus provided in Table 1 only for samples analysed, which demonstrated a small increase in flow rates for samples collected from patients within the chronic and aggressive periodontitis clinical group (median 2.4 µl over a 10 min collection time) compared to patients with gingivitis or relative healthy gingival tissue (median 1.6 µl / 10 min and 2 µl / 10 min respectively). For all clinical groups the age range was similar and a gender balance between male and female patients was obtained. Attachment loss was much greater for the two periodontitis groups, indicating a broad range of values.

Table 1
Patient and sample characteristics following collection of clinical parameters.

	Control	Gingivitis	Chronic periodontitis	Aggressive periodontitis
Number of samples analysed	9	17	16	16
Age (years)				
Range	28-40	28-56	33-54	28-40
Median	33.5	42.5	48.0	34.5
Male / Female	5/5	5/5	6/4	4/6
AL (mm)				
Range	0	0-1	6-10	6-10
Median	0	0	7	8
GCF volume (µl)				
Range	1.25 - 3.3	0.8-2.6	1.25 - 4	2-4.7
Median	2	1.6	2.4	2.4

3.2. Measures of central tendency, spread of data and analysis of variance

Individual results obtained from the cytokine array analysis were reported as pg per ml of GCF; making the assumption that the concentration of cytokine bathing the underlying periodontal tissues at the time of analysis was the same as the GFC released into the gingival crevice. Mean, standard deviation, and median cytokine concentrations were calculated for the control group, the aggressive periodontitis, chronic periodontitis and gingivitis groups (Table 2). The lowest concentrations were observed for IL-2, IL-4, and IL-17A, whereas the highest concentrations were for IL-6 and IL-10. Median and mean values also showed that the cytokine concentrations for the gingivitis group were highest for all cytokines analysed compared to concentrations for the control, chronic periodontitis and aggressive periodontitis. Cytokine concentrations for the aggressive periodontitis group are lowest for all cytokines compared to the concentrations for the other periodontal groups. A scatterplot of the data obtained for each periodontal groups is shown in Fig. 1, with respective mean and median values overlaid. This graphical representation shows the non-normal distribution in a high number of the data sets within each periodontal group, particularly for the gingivitis and periodontal groups, with high value outliers evident. Significant differences were identified between the gingivitis group and the aggressive periodontitis groups for cytokines IL-2 (P =0.004), IL-4 (P = 0.004), IL-6 (P = 0.035), IL-10 (P = 0.048), TNFα (P = 0.037), IFNγ (P = 0.001), and IL-17A (P = 0.001). No other significant differences between groups were demonstrated.

3.3. Heat maps and cluster analysis

Due to the skew in the data evident for many of the data sets, median values for each cytokine concentrations within each periodontal group were used for hierarchical cluster analysis. Heat maps and tree-based dendrograms for hierarchical clustering are shown in Fig. 2. The colour scales progress from blue that indicate low median cytokine concentrations, through green, yellow and to a beige colour indicating the highest concentrations. The tree-based dendrograms indicate the data groups connected by lines; those groups connected at the bottom of the tree (drawn close to the heat map grid) demonstrate similar cytokine concentrations, whilst data groups at the top of the tree indicate the greatest difference. Hierarchical clustering clearly identifies that the concentration of IL-6 is much higher compared to all other cytokines analysed. Data presented in Table 2 confirms that IL-6 concentration was 10.5 fold higher for gingivitis samples compared with concentrations for aggressive periodontitis samples. Lowest concentrations of IL-2, IL-4, and IFNγ were identified within the heat map and cluster together with greatest similarity for cytokine concentrations when compared to each other. Analyses also suggest that there is little difference in the concentration of IL-2, IL-4, and IFNγ cytokines between chronic

Table 2
Mean, median, and standard deviation (SD) of cytokine levels (pg per ml) for the control group, the aggressive periodontitis, chronic periodontitis, and gingivitis groups.

		IL-2	IL-4	IL-6	IL-10	TNFα	IFNγ	IL-17A
Control (n = 9)	Mean	1181.6	1178.9	5003.1	2482.5	1743.2	944.1	1720.0
	Median	925.3	917.5	2602.2	1913.0	1553.4	710.4	1507.1
	SD	727.7	757.2	5938.6	1913.1	843.8	626.8	1051.4
Aggressive periodontitis (n = 16)	Mean	1407.5	1327.2	8735.9	2768.3	2090.8	969.0	1968.6
	Median	729.9	723.0	1637.7	1428.9	1434.8	565.8	1089.6
	SD	2723.9	2437.0	11173.5	3496.9	1763.4	1507.2	3558.9
Chronic periodontitis (n = 16)	Mean	1093.1	1098.0	11263.6	1827.2	2452.3	887.6	1709.1
	Median	854.7	861.7	3950.5	1419.2	1563.3	688.5	1376.1
	SD	843.9	830.8	16257.7	1197.2	2484.2	639.2	1246.2
Gingivitis (n = 17)	Mean	1398.5	1401.8	27184.6	4118.6	3816.9	1133.7	2173.9
	Median	1232.1	1191.4	17289.2	2759.6	3248.3	1017.3	2082.4
	SD	570.7	574.1	37736.8	4815.3	2780.1	456.3	834.3

periodontitis, aggressive periodontitis and the control group. Again, slightly higher concentrations for these cytokines are detected in the gingivitis group. IL-10, IL-17A and TNFα form an additional cluster group for demonstrating a similar concentration to one another. Concentrations of these cytokines are again notably 2–2.2 fold higher in the gingivitis group, with IL-10 concentration also high in the control group. Clustering is also found with respect to the different periodontal groups, shown in the dendrogram on the left-hand side of the heat map. Gingivitis demonstrates the most dissimilar cytokine profile compared with other periodontal status groups. Cytokine concentrations for the chronic periodontitis and the control group clustered together, where the cytokine profiles were broadly similar, although subtle differences were observed in the cytokine concentrations for IL-6 and IL-10. Cytokine levels are again found to be lowest for aggressive periodontitis which demonstrates the greatest dissimilarity compared with the gingivitis group.

4. Discussion

Using a commercial multi-array assay system, this study was successful in providing an analysis of seven T-cell relevant cytokines within individual GCF samples collected from patients with strong clinical evidence of gingivitis, chronic periodontitis or aggressive periodontitis or little inflammation (“healthy” control). Representing an exploratory analysis, the data collected provides new information to demonstrate descriptive trends in cytokine concentrations that are of value to develop and support hypotheses relating to the role of these cytokines in the propagation or arrest of periodontal disease. For all cytokines analysed in this study, the data indicates that GCF samples from sites with aggressive periodontitis had the lowest concentrations of cytokines, whereas gingivitis samples had the highest concentrations. Significantly, our results indicated raised concentrations of IL-6, IL-17 A, IL-10 and TNFα in gingivitis samples compared with more advanced forms of periodontitis, suggesting potential roles for these cytokines in preventing disease progression and resolution of inflammation.

Whilst cytokine regulatory networks are complex and involve secretions from other immune and stromal cells, a number of cytokine activities relevant to T-cell activity have been proposed (reviewed by [7–9]); from which commercial cytokine array assays have been developed. Considering only those cytokines analysed within this study, Th1 differentiation is known to be accelerated by IL-2, leading to the secretion of interferon (IFN)-γ which promotes cell-mediated immunity involving activation of macrophages, CD8 + T (cytotoxic) cells and NK cells; thus providing a defence against intracellular bacteria. IL-2 and IL-4 promote Th2 differentiation. Th2 secretes IL-4, IL-6 and IL-10 to mediate humoral immunity, via activation of B cell proliferation and antibody production, and mast cell activation, the presence of which has been suggested as characteristic of a progressive lesion [23]. Moreover, Th17 cells form in response to cytokines IL-1β and IL-6 and characteristically produce IL-17 A, IL-17. Th17 cells are proposed to protect against extracellular bacteria and fungi, which is not effective via Th1-mediated immunity [24]. Inflammation is further controlled by Foxp3+ Tregs which suppress the activation, proliferation and effector function for a wide range of immune cells [25]. Differentiation of naïve T-cells into Tregs is supported by IL-2 and they characteristically produce IL-10 [8]. Within the assay used in this study TNFα represents a generic pro-inflammatory cytokine produced by many cell types. Although the cytometric bead array assay kit used in this study was developed commercially to study cytokines associated with regulating activity of T helper cells, this is placing a too simplistic viewpoint in the interpretation of the data we have obtained. It is also important to note that many of these cytokines analysed are also produced by other host cells within an inflammatory environment. A more expansive summary of cells producing these cytokines is reviewed by [26], the relevance of which is also indicated in the discussion below.

Within this study, we have reported cytokine levels as a concentration (pg/ml), which we regard as equivalent to the concentration bathing the underlying periodontal connective tissues and thus a highly appropriate unit of measure to analyse the data. In addition, it is recognised that periodontal disease is associated with variable periods of active tissue destruction, quiescence or repair, where the duration and intensity of active periods of tissue destruction will vary. Active tissue destruction is thus very difficult to predict, especially since defining disease activity relies on historical evidence [27]. Consequently, data of this type invariably produces a skewed distribution, with outliers evident and this was observed within the data presented herein. For this reason both means and medians have been provided. However, medians provide a more robust “measure of centre” in the presence of outliers and so heat maps and cluster analysis where created using median rather than mean concentration values.

Probably the most notable and striking observation identified within this study, is the differences in IL-6 concentration, which descriptive analysis and the heat maps / dendrograms indicated was highest in the gingivitis group, potentially suggesting roles in prevention of disease progression and resolution of inflammation. Compared to gingivitis samples, we observed a 10.5-fold decrease in IL-6 concentration in GCF of patients with aggressive periodontitis, where rapid tissue destruction is clinically evident. Again, when compared to median concentrations in gingivitis samples, 6.6- and 4.3- fold decreases in IL-6 concentrations were observed within the control and chronic periodontitis group respectively. IL-6 is described as a pleiotrophic mediator, assigned roles both in mediating inflammation, bone tissue destruction, and also in regenerative processes [28]. In rheumatoid arthritis IL-6 is ascribed prominent roles in promoting tissue destruction and disease progression [29]. IL-6 is invariably associated in a local periodontal lesion during the initial stages of gingival inflammation, which is produced by Th2, along with a variety of other

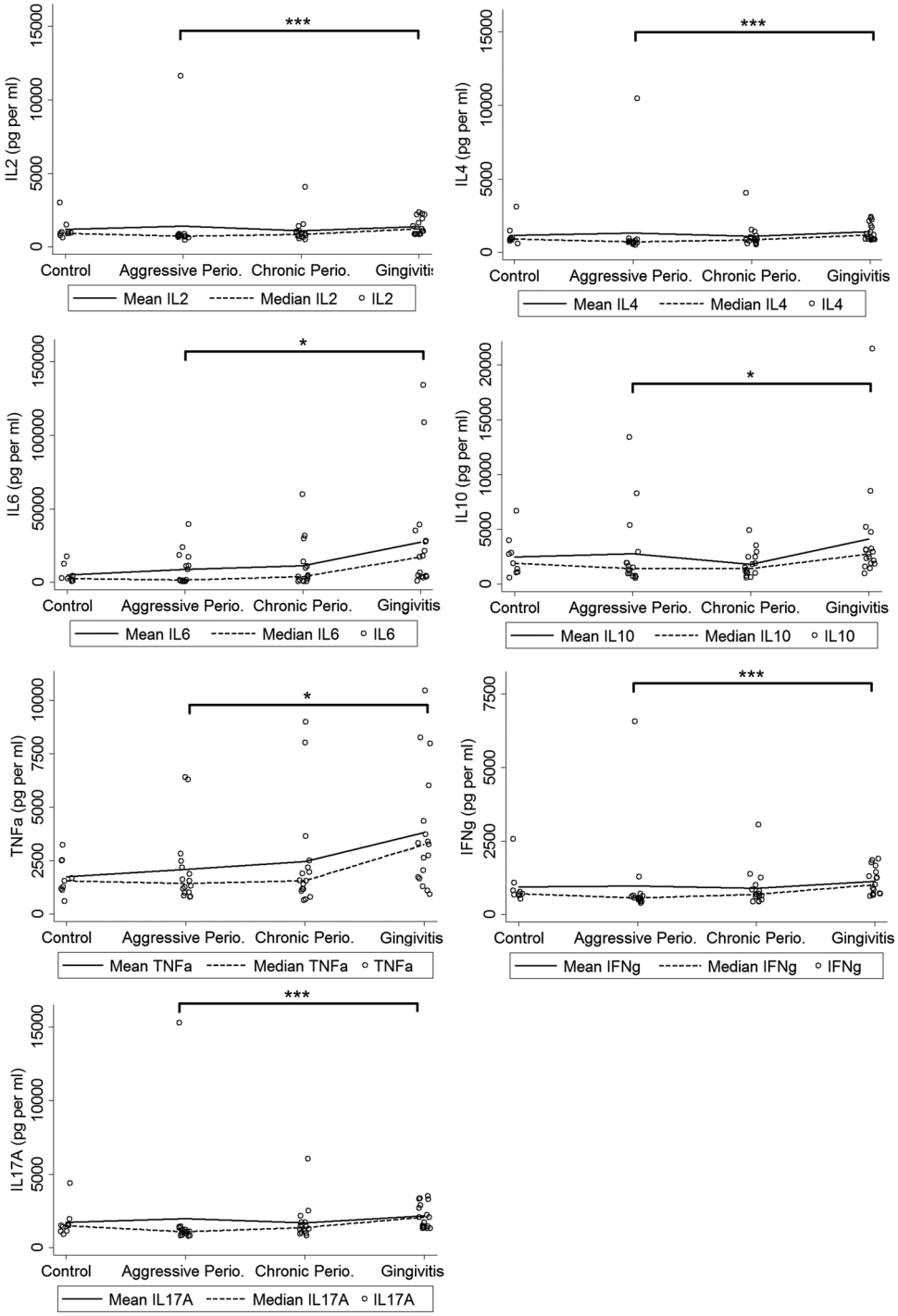

Fig. 1. Cytokine concentrations within each patient group. Results are presented as actual values depicted as scatter plots (data for individual GCF samples represented as open circle) with the mean (solid line) and median (broken line) values overlaid. Results indicate the continued trend for higher cytokine concentrations within the gingivitis group, which was significantly different for IL-17 A, IFNɣ, IL-2 and IL-4 when compared to aggressive periodontitis samples. *** $p < 0.001$ * $p < 0.05$.

cell types including B cells, endothelial cells, dentritic cells, macrophages, stromal cells, osteoblasts, adipocytes, fibroblasts and keratinocytes following activation by microbial products, IL-1β and TNFα [26,30,31]. Within this clinical scenario, it is noted that IL-6 has also been proposed roles that aid resolution of inflammation during acute inflammation and aids in the transition from innate to acquired immunity [28]. Scheller *et al* [28] have put forward a hypothesis that IL-6 acts, along with a plethora of chemokines, to recruit neutrophils to the local site. On entering the site of inflammation, the neutrophils then produce soluble IL-6 receptor (sIL6R) as a consequence of proteolytic cleave of the membrane bound precursor. This brings about a switch between *classic* to *trans* IL-6 signalling route within the endothelial cells; changing endothelial phenotype from one that recruits neutrophils to one that facilitates recruitment and survival of monocytes and T-cells [32,33]. This change to IL-6 *trans*-signalling within neutrophils has also been proposed to induce neutrophil apoptosis, leading to their clearance [28]. Although neutrophils have been recognised as important in bacteria clearance, over activity of neutrophils can cause

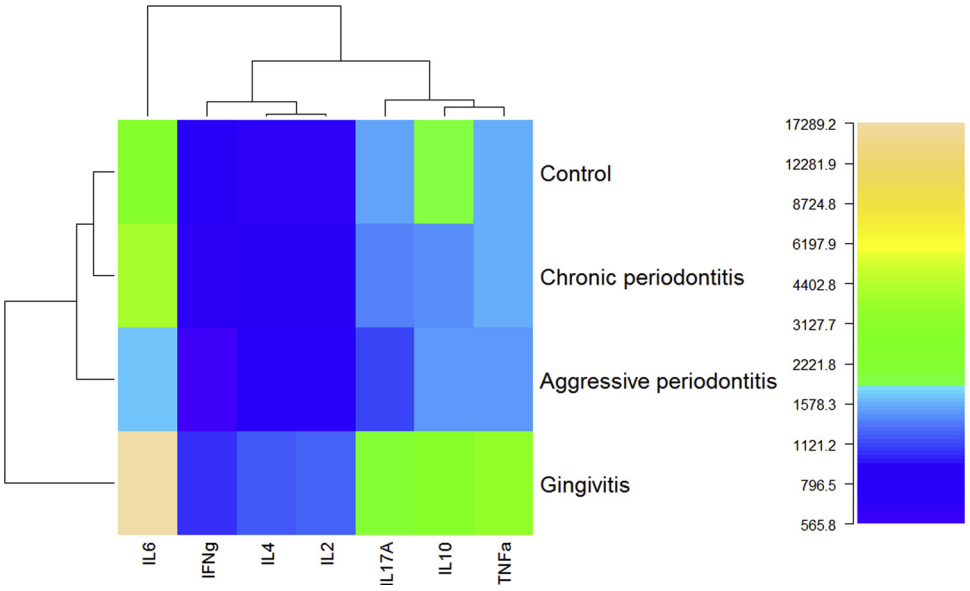

Fig. 2. Heat map for relative comparison of median cytokine concentrations within each patient group. Cluster analysis provides hierarchical ordering based on these cytokine median values. Descriptive analysis indicates that cytokines IL-6, IL-17 A, IL-10 and TNFα are present in much higher concentrations in samples collected from sites associated with gingivitis. Conversely, the analysis demonstrates low cytokine concentrations apparent in GCF collected from patient with aggressive periodontitis.

tissue damage and it can prolong the duration and severity of inflammatory periodontal diseases [34].

Within this study, a raised concentration of IL-17A was also detected in GCF samples of the gingivitis group compared with other groups. IL-17A is produced by Th17 cells, suggesting elevation in Th17 cells during acute phases of inflammation. This also correlates with the raised concentrations of IL-6 in the gingivitis group, which, in combination with TGFβ directs differentiation of naïve T-cells towards a Th17 phenotype [35]. IL-17A is also produced by macrophages, dendritic cells, mast cells and natural killer cells and elevations of all these cells have been associated with acute phases of gingival inflammation; cells which also all produce IL-6. Recent studies have also renewed interest in γδT-cells which are also producers of IL-17A and IL-6 along with IFNγ and TNFα [36,37]. Whilst representing a small proportion of total number of circulatory leukocytes within the body, γδT-cells are proposed to represent as much as 30% of leukocytes in inflamed ginigiva [37] where they are described a sentinel cells in mucosal tissue where bacterial challenge is prevalent [36]. However, Th17 has also been show to produce Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) that promotes osteoclastogenesis and thus promote bone resorption [38]. IL-17 produced by Th17 cells has also been described to lead to increases in TNFα, IL-1 and IL-6 within the local environment, which stimulates further RANKL expression by the resident stromal cells [39]. These proposed roles for IL-17 and IL-6 do not concur with the lack of bone loss within our gingivitis group of patients and thus would suggest that these two factors are unable to mediate osteoclast formation within the signalling environment created during gingivitis.

This is not to say that IL-17A and IL-6 do not play a role in promoting bone loss within the cytokine environment associated with chronic and aggressive forms of periodontitis. Triggered by a dysbiotic microbiome, an increase in Th17 cells and neutrophils has been reported associated with inflammatory periodontal tissue destruction and defects in these cells resulted in reduced bone loss [40]. However, it is also noted that mice deficient for the receptor for IL-17A were susceptible to bacterial induced alveolar bone loss, due to deficient neutrophil migration [41]. Some authors have reported elevated neutrophil counts in patients with generalized aggressive periodontitis [42], although others have reported that neutrophil numbers in serum are similar in aggressive and chronic forms of periodontitis [43]. Studies have also reported that deleterious superoxide production by neutrophils was raised in both resting and stimulated neutrophils collected from patients diagnosed with aggressive periodotitis [44]. Additionally, incubation of control neutrophils with P. gingivalis lipopolysaccharide and serum from patients with aggressive forms of periodontitis generated a higher deleterious response, compared with incubation with control serum [45]. These studies would support a prime role for impaired neutrophil function in propagating aggressive forms of periodontitis [42]. High or impaired neutrophil levels associated with aggressive periodontitis correlates well with the observation in this study for low concentrations of IL-6 within this patient group, assuming the hypothesis that high IL-6 levels effect neutrophil apoptosis (as proposed above). It is thus tempting to speculate that reduced concentrations of IL-6 and IL-17 associated with aggressive periodontitis may partially be responsible for persistent and enhanced neutrophil activity.

Notably, our data also suggests that the anti-inflammatory cytokine IL-10 is also elevated within the gingivitis group compared to the periodontitis group. The major source of IL-10 is considered to be Tregs, but it is also produced by other T-cells, macrophages, dendritic cells and B cells [26]. IL-10 has been ascribed a wide range of functions in general immuno-suppression, by inhibiting proliferation and pro-inflammatory cytokine production of a wide range of immune cells [8] including neutrophils [46]. Most notably, IL-10 has been reported to inhibit IFNγ production, suppress Th1 and promote Th2 cell activity [23]. Within this study, although elevated concentrations of IL-10 were reported in both control and gingivitis samples there was not a reduced concentration for IFNγ, although this may be because levels were generally low across all periodontal sample groups. Treg cells are also proposed to inhibit osteoclastogenesis, mediated by cytokines TGFβ, IL10 and IL-4 [47].

A further striking observation was that the lowest concentrations of IL-6, IL-17A and IL-10 were detected in the aggressive periodontitis group. Indeed for all cytokines analysed, lowest concentrations were observed within the aggressive periodontitis GCF samples. It is unlikely that this would infer that the producer cells for these cytokines are also low. It is possible that the cytokines produced by immune cells within this periodontal tissue are either quickly consumed or cells became toleranced to bacterial stimulation, preventing the release of beneficial cytokines, changing the signalling environment to one promoting tissue destruction.

Within our analysis there is little discernible change in the concentrations of IL-2 and IFNγ, (secreted by Th1 cells) and IL-4 (secreted by Th2 cells) in GCF samples collected from advanced or chronic periodontitis compared with the control, suggesting little change in the ratio of Th1 / Th2 cells. Considering the number of other cells producing these cytokines, this is a dangerous assumption. Small but detectable rises in the concentration of these cytokines are noted within

gingivitis. Unfortunately, our data is unable to add to the extensive debate within the area [6].

5. Conclusion

The results presented within this study serve to highlight the complex nature of cytokine signalling activity in dictating periodontal disease progression or resolution of inflammation. It is important to note that the data presented represents a cross-sectional study, measuring cytokine levels from an individual where progression of the disease to advanced forms or resolution of gingival inflammation is unknown. Defining markers for identifying susceptible patients has thus not been possible, which is better achieved through longitudinal studies. Notwithstanding this limitation, this study has been successful in identifying statistically significant changes in the concentrations of IL-6, IL-10 and IL-17 A, which were all found to be highest in GCF samples collected from patients with inflammatory gingivitis, but were lowest in GCF associated with aggressive forms of periodontitis. In considering the immuno-pathology of periodontitis, these observations would add support to those studies in the literature that suggest IL-6, IL-10 and IL-17A can collectively act to suppress and prevent extensive tissue destruction. It is likely that the protective roles these cytokines provide in the gingivitis lesion are due to the network of other cytokine with which they interact. Our discussion above did not dismiss the ability of these cytokines to also promote periodontal tissue loss, particularly that of bone, as indicated within the literature. The lack of detection of these cytokines in association with aggressive periodontitis may be because the cytokines are quickly consumed by the cells they are regulating. However, as a pilot study, the analysis of seven cytokines in a single sample has allowed the growth of hypotheses for further functional studies to identify the potential multiple roles for each cytokine within a defined extracellular signalling networks. This data is thus pertinent for supporting future studies in the field that aim to further elucidate the mechanism of periodontal disease or for supporting the development of therapies and in elucidating differing and possibly contradictory metabolic information content available when using cytokines as diagnostic biomarkers.

Declarations of interest

None.

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