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The effect of periodontal scaling and root polishing on serum IL-17E concentrations and the IL-17A:IL-17E ratio.

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Abstract

OBJECTIVES:
The serum IL-17A:IL-17E ratio has previously been demonstrated to be a clinical marker of periodontitis. The aim of this study was to determine the effects of non-surgical periodontal treatment on the serum IL-17A:IL-17E ratio.

MATERIALS AND METHODS:
Forty chronic periodontitis patients completed this study and received periodontal treatment comprising scaling and root planing plus ultrasonic debridement. Clinical data were recorded at baseline, six weeks (R1) after treatment completion (full-mouth or quadrant-scaling and root planing) and 25 weeks after baseline (R2). Serum samples were taken at each time point and cytokines concentrations determined by ELISA.

RESULTS:
Following treatment, statistically significant reductions were noted in clinical parameters. However, IL-17A and IL-17E concentrations were significantly greater than baseline values before- and after- adjusting for smoking. The IL-17A:IL-17E ratio was lower at R1 and R2. Serum IL-6 and TNF levels were significantly lower at R1 only. Also exclusively at R1, serum IL-17A and IL-17E correlated positively with clinical parameters, while the IL-17A:IL-17E ratio correlated negatively with probing pocket depth and clinical attachment.

CONCLUSION:
Increased serum IL-17E and a reduced IL-17A:IL-17E ratio may be indicative and/or a consequence of periodontal therapy. Therefore the role of IL-17E in periodontal disease progression and the healing process is worthy of further investigation.

CLINICAL RELEVANCE:
IL-17E may be a valuable biomarker to monitor the healing process following periodontal treatment as increased IL-17E levels and a reduced IL-17A:IL-17E ratio could reflect clinical improvements post-therapy. Therefore, monitoring serum IL-17E might be useful to identify individuals who require additional periodontal treatment.
**Introduction**

Interleukin-17A (IL-17A) has been implicated to play a role in the pathogenesis of periodontal disease. *In vivo* studies have implicated a protective role for IL-17A as Interleukin-17 receptor subunit A (IL-17RA) deficient mice showed exacerbated bone loss in a *Porphyromonas gingivalis* (*P. gingivalis*) induced disease model [1]. However, an excessive IL-17A response has been demonstrated to drive inflammatory alveolar bone loss in an ageing mouse model of periodontitis [2]. Therefore an effective, but balanced, IL-17A response is required for the protection of the oral mucosa against periodontal pathogens.

In humans, polymorphisms in the *IL-17A* gene have been associated with incidence of chronic periodontal diseases [3-5]. Furthermore, elevated levels of IL-17A have been detected in serum [6-8] saliva [8,9] and gingival crevicular fluid (GCF) [8,10,11] of periodontitis patients. Serum and GCF levels of IL-17A have also been demonstrated to correlate with clinical attachment loss in patients with chronic periodontitis [7, 8,11] and aggressive periodontitis [7].

In comparison to IL-17A, the role of interleukin-17E (IL-17E) in periodontitis is less understood. IL-17E has been described as being a ‘double edged sword’ as it is known to promote type 2 T helper cell (Th2)immunity as well as play a role as a negative regulator of inflammatory responses and attenuator of destructive inflammation [12]. Indeed, IL-17E may have opposing roles to IL-17A in the pathogenesis of periodontal disease [8]. IL-17E has been found to inhibit both the *P. gingivalis* and IL-17A induced expression of neutrophil chemotactic chemokines by oral keratinocytes [8]. In addition, the serum IL-17A:IL-17E ratio was suggested to be a predictive marker of disease state [8].

The effect of periodontal therapy on serum levels of cytokines has been investigated previously. In particular the effects of therapies on serum interleukin-6 (IL-6) and Tumor necrosis factor (TNF) are well documented [7, 13-17]. The effect of periodontal therapy on levels of IL-17 family cytokines in biological fluids is less well known. Previous studies have suggested that GCF levels of IL-17A in patients with chronic periodontitis [11] and serum levels of IL-17A in patients with aggressive periodontitis [7] are reduced after non-surgical therapies. To date, however, no studies have investigated the effect of therapies on serum levels of IL-17E or the serum IL-17A:IL-17E ratio. Therefore, the purpose of this study was to establish the effect of periodontal therapy on serum levels of IL-17E and the IL-17A:IL-17E ratio and compare full-mouth against quadrant-scaling and root
planing. A lesser aim was to investigate the effect of smoking on these outcomes. IL-6 and TNF were also measured to allow for comparison with published studies.
Materials and Methods

Study participants

Forty patients (24 non-smokers and 16 smokers) completed the study. All had been referred to the Unit of Periodontics at Glasgow Dental Hospital for non-surgical treatment of previously untreated chronic periodontitis. This study was performed in studies involving human participants and was reviewed and approved by The Glasgow Dental Hospital and School Ethics Committee and written informed consent was obtained from all participants.

Inclusion and exclusion criteria

Patient recruitment started in January 2000 and clinical intervention concluded in July-2002. All participants were systemically healthy. Apart from periodontitis, the participants had no other known disease and had received no antibiotic or anti-inflammatory therapy in the previous 3 months or during the course of the study. ‘Smokers’ smoked a median of 15 cigarettes per day (range: 5-30) with at least 10 pack years of cigarette consumption. ‘Non Smokers’ reported no cigarette use in the previous 10 years; or ever. ‘Periodontitis’ was assigned to patients with a minimum of 18 teeth and at least two non-adjacent sites on different teeth in each quadrant with a probing pocket depth (PPD) and clinical attachment levels (CAL) of ≥5 mm and radiographic evidence of bone loss (American Academy of Periodontology) [18]. Non-surgical periodontal treatment included detailed oral hygiene instruction in conjunction with scaling and root planing and ultrasonic debridement. Treatment was carried out by one calibrated clinician (DAA) and was completed during a single or multiple consecutive visits (FM-SRP) or quadrant-by-quadrant at 2-weekly intervals (Q-SRP).

Clinical examination and sample collection

Full-mouth periodontal assessments were carried out by the same examiner (DAA) at baseline, 6-weeks (R1) after treatment and six months (R2) from baseline assessment [19,20]. For the FM-SRP group R1 was on week 7 and for the Q-SRP group on week 13. For both groups R2 was 25 weeks post therapy. Clinical parameters recorded PPD, CAL using a manual periodontal probe (UNC-15, Hu-Friedy, Chicago, IL, USA), bleeding on probing (BOP), plaque index (PI) and the number of pockets ≥5 mm in depth [19].
Peripheral venous blood was collected from all participants at baseline, R1 and R2. Blood was collected in a glass vacutainer between 9:00 AM and 11:00 AM (to minimise diurnal variations in biochemical parameters). After coagulation, blood was centrifuged (200 x g) then serum stored in aliquots at -80 °C prior to analysis.

**Enzyme-linked immunosorbent assays:**

IL-17A and IL-17E were assayed using commercially available ELISA kits (Peprotech, London, UK) as described previously [8]. According to the manufacturer there is no reactivity between IL-17A antibodies or IL-17E antibodies with the other IL-17 cytokines. TNF and IL-6 levels were also assayed using commercially available ELISA kits (R & D systems, Abingdon, UK). The minimum detection limits of each assay were as follows: IL-17A & IL-17E = 1.9 pg/ml; TNF = 1.5 pg/ml and IL-6 = 0.8 pg/ml.

**Statistical analyses:**

Statistical analyses of clinical and biochemical data were carried out using SPSS (Version 21, IBM Chicago USA). Statistical power calculations were based on the assumption that the biochemical data were normally distributed data. With alpha set at 0.05 in a statistical analysis, and where the standard deviation did not exceed the mean difference, 16 subjects on average per treatment-group were sufficient to give greater than 80% power. However the number present in each of the two treatment groups was increased to 20 on the likelihood that the data did not conform to the normal distribution and that non-parametric statistical analyses would be required.

Initial investigations were performed on the FM-SRP and Q-SRP data separately. However, as both treatment regimens were shown to equally effective regarding the improvement in the clinical indices at R2 it was decided to perform further analysis of all 40 patients at baseline and at R2 as if they were a single treatment group. This meant that the statistical power in this analysis was increased to greater than 95% with an effect size =1 and that smaller effect sizes would be accommodated in the analysis and still have sufficient statistical power.

The Friedman test was used to investigate the differences between baseline, R1 and R2 within each treatment group. A Bonferroni corrected Wilcoxon test was used for post hoc analysis of the paired data (within group analyses). The Mann–Whitney U-test was employed for analyses of differences between the FM-SRP and Q-SRP groups at baseline and R2. The Spearman rho was used to investigate correlations between the clinical and biochemical parameters. To allow a correction for potential confounding factors such as age, PI, tobacco use and
gender a weighted least squares regression analysis on log transformations of the cytokine data was performed. A partial correlation analysis of the clinical and biochemical parameters was also performed to correct for age, PI and cigarette consumption.

Cross tabulation and Chi squared analysis was performed to determine whether there were differences in the proportion of smokers or the gender of the treatment groups.
Results

Demographic and Clinical data

Table 1 shows the demographic data for the study participants allocated to the two treatment groups. A significant difference in the gender makeup of the Q-SRP and FM-SRP treatment groups was observed (p<0.001) (Table 1).

Table 2 shows the clinical data for the study participants in the FM-SRP and Q-SRP groups at baseline, R1 and R2. Significant reductions in PPD, CAL, PI and BOP were observed at both R1 and R2 following FM-SRP and Q-SRP (all p<0.01) (Table 2). There was a significantly greater clinical improvement between baseline and R1 following both FM-SRP and Q-SRP than between R1 and R2 (p<0.001). No significant differences in clinical outcomes were seen between the FM-SRP and Q-SRP treatment groups at R1 or R2, therefore both treatment regimens appear equally effective.

Serum IL-17A and IL-17E concentrations and the IL-17A:IL-17E ratio following treatment:

To investigate the temporal relationships between the cytokines following treatment both treatment groups, FM-SRP & Q-SRP, were investigated separately at baseline, R1 and R2 in the first instance. All IL-17A and IL-17E measurements were greater than the assay background. In addition, comparisons between the two regimes were made at baseline and at R2. Between the two study groups the following significant differences were noted following non-parametric analysis. For the FM-SRP group, serum levels of IL-17A (Figure 1A) were significantly greater than baseline levels at R1 (p=0.040) and R2 (p=0.006). Also in this group serum levels of IL-17E (Fig 1B) were significantly greater than baseline levels at R1 (p=0.001) and R2 (p<0.001). However, in comparison with baseline, the IL-17A:IL-17E ratio showed a significant reduction at R2 (p=0.036) for the FM-SRP group (Fig 1C).

For the Q-SRP group, serum levels of IL-17A were significantly greater than baseline levels at R1 and R2 (Figure 1A). The IL-17E concentrations (Figure 1B) were also greater than baseline at both R1 and R2 (both p<0.001). In addition, IL-17E levels at R2 were significantly greater than those at R1 (p=0.007). In comparison with baseline, the IL-17A:IL-17E ratio showed a significant reduction at R2 (p=0.040) for the Q-SRP group (Fig 1C).
No significant differences were seen in the serum IL-17A:IL-17E ratio between the two treatment groups. However, after a least squares regression weighted analysis correcting for age, smoking and gender the IL-17A:IL-17E ratio was significantly higher at R2 in the Q-SRP group compared to the FM-SRP group (p=0.003).

IL-6 (Fig 2A) and TNF (Fig 2B) levels were lower at R1 and R2 than at baseline for both the FM-SRP and Q-SRP treatment groups (all p<0.05). However, it was noted that IL-6 (Fig 2A) and TNF (Fig 2B) levels rose again in both groups at R2 and in the case of TNF were significantly higher than R1 levels (p=0.013). At baseline all IL-6 and TNF levels were greater than the assay background for all 40 serum samples. At R1 IL-6 and TNF concentrations were lower than the assay baseline in three and five out of 40 sera, respectively and at R2, the IL-6 and TNF concentrations were below the assay background in one sample and two samples out of 40 sera, respectively.

Since both treatment approaches were equally effective regarding clinical outcome (Table 2) the treatment groups (FM-SRP and Q-SRP) were combined to investigate differences between baseline and R2. Serum levels of IL-17A (Fig 3A) and IL-17E (Fig 3B) were significantly greater at R2 than baseline (p<0.001 & p<0.001). However, the elevation in serum levels was greater for IL-17E than IL-17A at R2. Therefore, the serum IL-17A:IL-17E ratio (Fig 3C) was significantly reduced, compared to baseline, at R2 (p<0.05).

Serum levels of IL-6 (Fig 3D) and TNF (Fig 3E) were significantly lower than baseline at R2 (TNF, p<0.001, & IL-6, p=0.016).

The influence of cigarette smoking on serum IL-17A and IL-17E concentrations and the IL-17A:IL-17E ratio following treatment

Serum levels of IL-17A (Fig 4A) and IL-17E (Fig 4B) were significantly elevated in both smokers and non-smokers at R2 compared to baseline (p<0.043 & p=0.001, respectively). However, IL-17E levels were significantly lower (p=0.001) in the smokers than in the non-smokers at R2 (Fig 4B). The IL-17A:IL-17E ratio at R2 was significantly higher (p<0.01) in smokers than non-smokers (Fig 4C).
Serum levels of IL-6 were significantly lower only in the non-smokers at R2 than at baseline (p=0.010). At R2, serum IL-6 levels were significantly higher in smokers than in the non-smokers (p=0.009) (Fig 4D). No differences in serum TNF levels were observed between baseline and R2 in non-smokers and smokers (Fig 4E).

**Correlations between biochemical and clinical parameters**

At baseline, there were no significant correlations between IL-17A, IL-17E or the IL-17A:IL-17E ratio with the clinical data (data not shown). At baseline, the serum TNF levels correlated with PPD (r=0.357, p=0.039) and IL-6 levels had a significant Spearman correlation with the PI (ρ=0.367, p=0.028).

At R1, serum IL-17E levels correlated positively with PPD and CAL (ρ=0.364, p=0.029 & ρ=0.444, p=0.007, respectively) and the serum IL-17A:IL-17E ratio correlated negatively with PPD (ρ=-0.441, p=0.007) and CAL (ρ=-0.493; p=0.002). The serum IL-17A:IL-17E ratio also correlated negatively with the number of sites >5mm (ρ=-0.402, p=0.019). However, after adjusting for cigarette usage this finding was no longer significant. In contrast there were no significant correlations between biochemical and clinical parameters at R2.

There was no correlation between IL-6 levels and changes in the clinical parameters at either R1 or at R2 but at R1 serum TNF levels correlated with the reduction in PPD and CAL (r=-0.431, p=0.018 and r=-0.379, p=0.030).
Discussion

This study reports for the first time a significant decrease in the serum IL-17A:IL-17E ratio 25 weeks post baseline assessment (R2) compared to baseline (pre-therapy) assessment. This decreased ratio is due to significant elevations in serum IL-17E levels to an extent greater than the increases observed for IL-17A at R2. The increased serum IL-17E and reduced serum IL-17A:IL-17E ratio appear to be associated with clinical improvements following the mechanical removal of plaque and calculus. Despite this fact, we failed to see significant correlations between IL-17A and IL-17E with the clinical parameters such as PPD and CAL at baseline in this investigation compared with our recent cross-sectional study [8] where serum IL-17A correlated positively with PPD and CAL and IL-17E correlated negatively with these parameters. There is plausible reason for this discrepancy. The earlier investigation involved a greater number of participants with a greater variation in the level of periodontitis, whereas in this study the number was far fewer and the variation in clinical condition of the participants was much narrower. Thus small biological variations could have masked undying relationships between the clinical and biochemical parameters of the participants in this study. The detection of significant correlations at R1 following treatment might be explained by larger variations in differences in individual responses to the SRP reflected in parallel at a clinical and biochemical level. The failure to see significant correlation between the clinical and biochemical measures at R2 was possibly because the variation in the measured responses under comparison was more restricted.

The circulating levels of IL-17A (range 2 pg/ml - 360 pg/ml), IL-17E (4 pg/ml - 96 pg/ml), TNF (range = 0 pg/ml - 42 pg/ml) and IL-6 (range = 0 pg/ml – 34 pg/ml) reported in this study are similar to levels reported in other studies [7, 8, 13, 14]. In addition the trends in these mediators post-therapy are generally in agreement with other studies [7, 8, 13, 14]. While there is no exact agreement on the timing of reassessments between studies, some comparisons can be made. Indeed, at the 6 week post-treatment reassessment (R1) findings for TNF and IL-6 were similar to results reported in other studies [7, 13-17]. However, unlike some other studies, an increase in the levels of IL-6 and TNF, was observed after R1 with higher levels being recorded at R2; although these generally remained below baseline values. Indeed, in the case of TNF, levels had returned to near baseline values by 25 weeks post FM-SRP and Q-SRP. Earlier studies on IL-6 showed that serum levels of this cytokine were similar at 2 months and 6 months post-treatment [13,16]. In the case of TNF, however, Duarte et al. [7] reported no significant difference
between serum TNF levels at baseline and at 6 months post-treatment in periodontitis patients suggesting that the similar observation in our study was not an artefact.

Comparison of treatment regimens revealed that for the Q-SRP group the serum IL-17A levels were higher at R2 than at R1 and significantly greater than in the FM-SRP group where the levels appeared to be lower at R2 than at R1. Interestingly, Duarte et al. [7] has also reported a small but not statistically significant increase in IL-17A levels at 6 months following a Q-SRP regime in chronic periodontitis patients. However, despite the differences in serum IL-17A levels between the FM-SRP and Q-SRP groups at R2, overall no differences in the IL-17A:IL-17E ratio were observed between the two treatment regimens. Although the small group sizes may provide an explanation for this result, it is probably not surprising given that there were no significant differences in clinical improvements between these two treatment groups.

The increase in serum levels of IL-17A and IL-17E post-therapy is in contrast to findings reported in similar studies investigating the effects of therapies on serum levels of key inflammatory cytokines; such as TNF, IL-6 and IL-1β; where decreases 3 and 6 months after treatment are generally seen [13-17]. Interestingly, despite increases in serum levels of both IL-17A and IL-17E post-therapy, the actual IL-17A:IL-17E ratio was reduced. We also saw that before and after correcting for age, smoking and gender the IL-17A:IL-17E ratio was significantly higher at R2 in the Q-SRP group compared to the FM-SRP group. This appears to have been brought about partly by a smaller increase in IL-17A levels at R2 for the FM-SRP group than was seen for the Q-SRP group. In addition, while there were no statistically significant differences in IL-17E at R2 between both treatment groups, higher median levels were seen in the Q-SRP group at R2 and this also contributed to the reduced IL-17A:IL-17E ratio.

IL-17E has been shown recently to reduce both IL-17A and P. gingivalis biofilm-stimulated secretion of chemokines by oral keratinocytes and reduce the level of phosphorylation of the p65 subunit of NF-κB [8]. This therefore suggests that IL-17E can act to regulate IL-17A and periodontal pathogen mediated immune responses. This was consummate with its reported role as a negative regulator of inflammatory responses and attenuator of destructive inflammation [12]. In addition, IL-17E has been reported to promote healing and repair through the activation of alternatively activated macrophages (M2) [21]. Therefore, the elevated IL-17E levels observed in serum post-therapy could be hypothesised to be due to localised increases in expression of IL-17E which occurs to
promote healing and repair of periodontal tissues. The fact that higher IL-17E and lower IL-17A:IL-17E ratios associated with greater PPD and CAL at R1 is interesting and may reflect ongoing attempts to down-regulate the inflammatory response and initiate repair mechanisms.

A three way interaction involving smoking treatment and the visit was shown previously to influence clinical measurements at selected sites [20] at R1 but not at R2 when the clinical improvements and healing time were seen to be similar [19,20]. Although we saw no difference in baseline values between smokers and non-smokers, smoking appears to have influenced the results observed post-treatment. In the non-smokers, the median IL-17A levels at R2 were not significantly different from baseline, whereas in the smokers the levels were increased. There are conflicting reports on the effect of smoking on IL-17 levels in serum, with evidence for a decrease [22] and increase [11, 23]. In addition, smokers have greater numbers of CD4+ IL-17+ T cells in the lung tissue than non-smokers [24]. If a similar phenomenon also occurred in the periodontium it could conceivably influence local and circulating levels of IL-17A and IL-17E.

In conclusion, this investigation for the first time reports the measurement of serum levels of IL-17E and the determination of the IL-17A:IL-17E ratio in patients prior to, and following, periodontal treatment. In addition, it compares serum levels of IL-17A, IL-17E and the IL-17A:IL-17E ratio in smokers and non-smokers post-treatment. The data from this study supports the notion that IL-17 cytokines may be valuable biomarkers to monitor the healing process following periodontal therapy. Indeed, the results suggest that increased IL-17E (IL-25) levels, which result in a reduced IL-17A:IL-17E ratio could reflect clinical improvements post-therapy. If this is true, monitoring of IL-17E levels in serum might be useful for identify individuals in need of additional periodontal treatment in the long term. Within the limitations of this study smokers had a lower reduction of the IL-17A:IL-17E ratio compared to the non-smokers, which might argue for an negative effect on the healing process.
Compliance with Ethical Standards:

Ethical approval: This study was performed in studies involving human participants and was reviewed and approved by The Glasgow Dental Hospital and School Ethics Committee and was conducted in accordance with the ethical principles described in the WORLD MEDICAL ASSOCIATION’S DECLARATION OF HELSINKI 1964 and its amendments [25,26].

Informed consent:
Written informed consent was obtained from all individual participants included in the study.

Conflict of interest:
We have no conflict of interest in publishing the results presented in this manuscript.

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25 WORLD MEDICAL ASSOCIATION Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects 52nd WMA General Assembly, Edinburgh, Scotland, October 2000.

Legends

Figure 1: Median and semi-inner quartile ranges depicting serum levels of IL-17A (A), IL-17E (B) and the IL-17A:IL-17E (C) ratio in periodontitis patients undergoing FM-SRP (N=20, dark grey diamonds) and Q-SRP (N=20, light grey squares). The measurements are recorded prior to SRP and for both treatment groups at 7 weeks (6 weeks after FM-SRP was completed = R1: FM-SRP), for both treatment groups at 13 weeks (6 weeks after Q-SRP was completed = R1: Q-SRP) and at 25 weeks (R2) following the commencement of SRP.

* Significantly different from FM-SRP baseline.

‡ Significantly different from Q-SRP baseline.

† Significant differences between FM-SRP and Q-SRP at R2.

Figure 2: Median and semi-inner quartile ranges depicting serum levels of IL-6 (A) and TNF (B) in periodontitis patients undergoing FM-SRP (N=20, dark grey diamonds) and Q-SRP (N=20, light grey squares) prior to SRP and at 7 weeks (6 weeks after FM-SRP was completed = R1: FM-SRP R1), at 13 weeks (6 weeks after Q-SRP was completed = R1: Q-SRP) and at 25 weeks (R2) following the commencement of SRP.

* Significantly different from FM-SRP baseline.

‡ Significantly different from Q-SRP baseline.

† Significant differences between FM-SRP and Q-SRP at R2.

§ Significant differences between R1 & R2.

Figure 3: Box and whisker plots depicting the median and quartile ranges (with outliers indicated by solid circles) of serum levels of IL-17A (A), IL-17E (B), the serum IL-17 A:IL-17E ratio (C), serum levels of IL-6 (D) and TNF (E) in periodontitis patients prior to SRP (N=40, Empty boxes) and at 25 weeks following the commencement of SRP (R2; grey boxes).

* Significant differences between baseline and R2.
Figure 4: Box and whisker plots depicting the median and quartile ranges (with outliers indicated by solid circles) of serum levels of IL-17A (A), IL-17E (B), the serum IL-17 A:IL-17E ratio (C), serum levels of IL-6 (D) and TNF (E) in non-smoker (N=24, Empty boxes) and smoker (N=16, grey boxes) periodontitis patients prior to SRP and at 25 weeks following the commencement of SRP (R2).

* Significant differences in non-smoker between baseline and R2.

† Significant differences in smokers between baseline and R2.

‡ Significant differences between smokers and non-smokers.
Figure 3

A. IL-17A

B. IL-17E

C. IL-17A:IL-17E

D. IL-6

E. TNF
Figure 4

(A) IL-17A
(B) IL-17E
(C) IL-17A:IL-17E
(D) IL-6
(E) TNF