**ORAL MICROBIOLOGY
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Cytokines in gingival crevicular fluid of adolescents and young adults

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Background/aim: The purpose of this study was to compare the levels of the cytokines interleukin-1β (IL-1β), IL-4, and IL-8 in the gingival crevicular fluid (GCF) of adolescents and young adults.
Methods: Twenty-five adolescents aged between 14 and 16 years (Group A) and 20 periodontally healthy young adults aged between 25 and 35 years (Group B) were selected from two private dental clinics limited to pedodontics and periodontics respectively in Piraeus Greece. All subjects were systemically healthy. Clinical examination included probing pocket depth (PPD), presence or absence of plaque, and bleeding on probing (BOP). GCF was collected from four sites per subject. IL-1β, IL-4, and IL-8, measured as total amounts (pg/30 s), were evaluated in 180 samples using a commercially available sandwich enzyme-linked immunosorbent assay.
Results: IL-1β mean levels of Groups A and B were adjusted for BOP and PPD. Differences of IL-1β mean levels between the two age groups were statistically significant ($F = 50.245$, $P < 0.001$) in favour of Group A. Adolescents showed statistically significantly lower mean levels of IL-4 than young adults in the presence of BOP ($F = 10.690$, $P = 0.001$). There was no statistically significant difference between adolescents and adults for the means of IL-8 adjusted for BOP and plaque presence ($F = 2.032$, $P = 0.161$).
Conclusions: Within the limits of this study the differences reported in mean levels of IL-1β and IL-4 may be attributed to the different age status.

Cytokines have been defined as regulatory proteins produced by immune cells and other cells of the body. Their pleiotropic action includes numerous effects on the cells of the immune system and modulation of inflammatory responses. The immune system, through cytokine- and interleukin-mediated pathways can regulate the reproductive system by inducing the release of gonadotropins, such as luteinizing hormone and follicle-stimulating hormone. It seems that communication between the endocrine and immune systems determines the expression of interleukins in puberty (19). In an effort to elucidate possible mechanisms of age-associated changes in immunity, the impact of age on cytokine production has been examined in humans. However, studies examining alterations in proinflammatory cytokine production with age are contradictory: both an increase and/or no change have been reported between different age groups (9, 21, 25, 26).

The analysis of cytokine production levels has been also used as a tool for studying the local host response to a bacterial challenge. In particular, a large number of cytokines present in the gingival crevicular fluid (GCF) have been proposed as potentially useful diagnostic or prognostic markers of periodontal destruction (3, 11). Among these, interleukin-1β (IL- 1β), IL-4, and IL-8 have been shown to function in concert with other members of the cytokine network to regulate the cellular inflammatory response in the periodontium. In all these studies, it is important, however, to acknowledge the variation of cytokines with age.

The present cross-sectional study was designed to determine the levels of IL-1β, IL-4, and IL-8, in the GCF of healthy adolescents and young adults.

Materials and methods
Experimental design

This study was a cross-sectional single-blinded randomized clinical trial.

Sample size

Twenty-one subjects per age group would achieve 90% power to detect a difference of IL-1β, IL-4, and IL-8 means equal to 5 pg/30 s between adolescents and young adults with a standard deviation of 5 pg/30 s, an assumed correlation between four repeated measurements within the mouth equal to 0.5 and a level of significance equal to α = 0.05 (8).

Study population

A total of 45 healthy adolescents and young adults participated in the study. They were selected from two private dental clinics in Piraeus, Greece limited to pedodontics and periodontics, respectively.

Inclusion criteria for both groups

No subjects had a history of systemic diseases (e.g. diabetes) or pregnancy; they had not received antibiotics or non-steroidal antiinflammatory drugs in the 6 months prior to entering the study. They were non-smokers. They all had a healthy periodontium without radiographic evidence of bone loss and a probing pocket depth less than 3 mm.

Oral hygiene performance was monitored and tooth polishing was performed for all subjects twice a month during the 2 months before the study started. The day of the examination they presented no non-treated carious lesions. Subjects were divided into two groups:

Group A comprised 25 adolescents with permanent dentition (15 male, 10 female, mean age 15.3 ± 0.9 years) and Group B comprised 20 young adults (10 male, 10 female, mean age 28.6 ± 1.3 years).

The qualified subjects were consecutively entered in the study. All participants (parents of adolescents) gave their consent to take part in the study. The participants were recruited from May 2002 to July 2005. The study was conducted according to the principles outlined in the Helsinki declaration of 1975 as revised in 1983, on experimentation involving human subjects.

Periodontal examination

The clinical evaluation was performed by one periodontist (J.K.) and included the presence or absence of plaque (24), assessment of probing pocket depth (PPD) and bleeding upon probing (BOP) (1) at four sites around each tooth, excluding the third molars. Measurements of PPD were carried out to the nearest mm using a Goldman/Fox Williams periodontal probe.

Intra-examiner reproducibility

A total of 10 periodontally healthy adolescents and young adults who were not included in the study group were used for the calibration evaluation. The single designated examiner (J.K.) performed full mouth PPD measurements for all 10 subjects on two separate occasions, 15-min apart. The intra-examiner standard deviation for repeated measures was 0.1 mm. Examiner's reproducibility was 99.8%.

Gingival crevicular fluid sampling

The GCF was collected from four sites in each adolescent/young adult using Durapore filter membranes (pore size = 0.22 mm; Millipore Corp., Bedford, MA). Four experimental sites were designed comprising one crevice in each quadrant. All teeth within a quadrant were numbered from one to seven. One tooth and the respective mesial or distal site from each quadrant were randomly sampled using the random number generator of SPSS 13.0 (SPSS, Chicago, IL).

After isolation of the test sites from saliva, a first Durapore strip was inserted 1 mm into the sulcus and left in place for 15 s. Three minutes after removal of the first strip, a second Durapore strip was similarly inserted in the same site for 15 s. The two strips were then placed into a microcentrifuge tube and immediately frozen at −70°C until the day of the analysis. In case of visible contamination with blood, the strips were discarded.

Analysis of cytokine production

The samples were transferred to the Department of Periodontology at the University of Geneva and analyzed blindly by C.G.

The content of IL-1β, IL-4, and IL-8 was measured in each of the four preselected sites from each patient. A total of 180 samples in 45 subjects were analyzed. On the day of the analysis, 350 μl phosphate-buffered saline (pH 7.2) was added to the tubes containing the strips. The strips were gently shaken for 1 min and then centrifuged at 2000 *g* for 5 min, with the strips kept at the collar of the tube to elute GCF components completely. After strip removal, the supernatant was divided into three aliquots for the determination of each biochemical compound. The amount of IL-1β, IL-4, and IL-8, was determined by enzyme-linked immunosorbent assays (ELISA) (31) specific for each compound (Ruwag Diagnostics, Zurich, Switzerland). The assays were carried out in accordance with the manufacturer's instructions and the levels of the biochemical compounds were reported as total amount (pg) per 30 s sample.

Total cytokine amounts per 30 s samples were calculated, based on ELISA concentration values. Sites with cytokine levels below the limits of the assay's detectability were scored as 0 pg.

Statistical analysis

Statistical analyses were carried out on the full set of patients who participated in the study. Baseline demographic and clinical characteristics were summarized by the mean and standard deviation (± SD) for continuous measures and the number and per cent for categorical variables. Differences of demographic data between genders and differences between BOP levels were tested using the *t*-test or the Mann–Whitney test in the case of PPD values. By design, each subject had four repeated measurements on four quadrants. These observations are typically correlated. Therefore, a mixed effects model with a random intercept was fitted for each interleukin to take into account this correlation. Since BOP was considered as a confounding variable, all differences between the two groups were adjusted for its presence. In addition, PPD and the presence of plaque were also considered for possible additional adjustment. Comparisons between mean levels for all interleukins have been made using the Bonferroni adjustment (4) to compensate for the multiple testing. All computations were made using SPSS 13.0.

Results
Demographic and periodontal variables

The demographic data including gender and age are summarized in Table 1. The periodontal status of each group, including mean PPD of the four selected sites in each patient, is also presented. Mean PPD was significantly higher in young adults (Group B) than adolescents (Group A), Mann–Whitney ($z = -11.589$, $P < 0.01$), but was within normal acceptable values

Table 1. Demographic and periodontal variables

	Group A Adolescents (N = 25)	Group B Adults (N = 20)
Age (years)	14–16	25–35
Mean age (years)	15.3 ± 0.9	28.6 ± 1.3
Men/women	15/10	10/10
Probing depth (mm) at sampling sites (0/1/2/3)[1]	$n = 100$ 96/4/0/0	$n = 80$ 17/20/19/24
Plaque (no/yes)[2]	36/64	36/44
Bleeding on probing (no/yes)[3]	66/34	66/14

N = number of subjects; n = number of sites.
[1]Mann–Whitney ($z = -11.589$, $P < 0.01$); [2]$\chi^2 = 1.5$, $P = 0.221$; [3]$\chi^2 = 6.188$, $P = 0.013$.

(0–3 mm). Furthermore, Table 1 shows the percentage of sites with plaque accumulation and BOP. The difference in the plaque percentage between the two groups (64/100 = 64% and 44/80 = 55% for the adolescents and young adults, respectively) was not statistically significant ($\chi^2 = 1.5$, $P = 0.221$). However, BOP percentages between adolescents and adults (34/100 = 34% and 14/80 = 17.5% respectively) were statistically different ($\chi^2 = 6.188$, $P = 0.013$).

Comparisons of the mean IL-1β, IL-4, and IL-8 between Group A and Group B

Table 2 shows mean total amounts of IL-1β for adolescents and young adults adjusted for BOP and PPD. Differences of IL-1β mean total amounts between the two age groups were statistically significant ($F = 50.245$, $P < 0.001$). The two groups were found to interact with presence of BOP for IL-4 ($F = 10.690$, $P = 0.001$). There was no significant difference between adolescents' and young adults' mean IL-4 total amounts for subjects with absence of BOP ($P = 0.793$). In contrast, adolescents showed statistically significantly lower mean total amounts of IL-4 than young adults in the presence of BOP ($P = 0.001$). There was no statistically significant difference between adolescents and adults for the mean total amounts of IL-8 adjusted for BOP and

presence of plaque ($F = 2.032$, $P = 0.161$).

Discussion

The total amounts of IL-1β, IL-4, and IL-8 in the GCF of periodontally healthy adolescents and young adults were defined as diagnostic markers in relation to the periodontal status and age.

We were unable to measure the extremely small quantities of GCF available from healthy sites, so the levels of the biochemical compounds have been reported as total amounts per 30-s sample, as an alternative to concentrations. This is in accordance with the findings of several authors (12, 22, 28), suggesting that total amounts, rather than concentrations of GCF components, should be used when estimating periodontal disease activity.

The mean PPD is higher in the group of young adults; although < 3 mm, and a PPD up to 3 mm was found in the absence of detectable attachment loss in 24 out of 80 sites examined in the group of young adults, which sites were not bleeding upon probing.

It is known that IL-1β plays a crucial role in the regulation of destruction and repair in the periodontium (14) while IL-4 is a potent downregulator of macrophage function, and its localized absence has been associated with periodontal disease activity and progression (15).

Table 2. Adjusted means of interleukin-1β (IL-1β), IL-4, and IL-8 total amounts for Groups A and B with corresponding *F*-tests and *P*-values for comparison

	Group A ($n = 100$)	Group B ($n = 80$)	*F* value	*P*-value
IL-1β (adjusted for BOP)	15.1	9.6	50.245	<0.001
IL-4 for BOP (+)[1]	9.6	15.4	10.690	0.001
IL-4 for BOP (−)[1]	10.8	11.2		
IL-8 (adjusted for BOP and PI)	28.4	25.3	2.032	0.161

[1]Comparison of mean IL-4 total amounts between the two age groups for bleeding on probing (BOP) presence ($P = 0.002$) and absence ($P = 0.793$).
BOP (+), BOP presence; BOP (−), BOP absence; PI, presence of plaque; IL, interleukin.

The present data indicate that adolescents had significantly higher levels of IL-1β and lower IL-4 levels compared to young adults after adjusting for BOP without any sign of periodontal destruction.

The different response of the local immune system without any significant difference in plaque levels of Group A and Group B leads to the assumption that these variations in the expression of cytokines might be attributable to different age status.

Gingival inflammation is described in children of all ages. Gingivitis with similar levels of plaque accumulation increased gradually in prevalence and severity from early childhood to the early teenage years, thereafter subsiding slightly and levelling off for the remainder of the second decade of life (2, 13, 32).

A cross-sectional survey of 909 Zurich schoolchildren reported that gingivitis was present in 90% of children at all age levels between 8 and 17 years (6). The increase of gingivitis during puberty compared to infancy, has been attributed not only to local factors such as the higher number of sites, to plaque accumulation, and the inflammatory changes associated with tooth eruption and exfoliation, but also to systemic factors, such as the hormonal changes that take place during puberty.

An increase in the sex steroid hormones during puberty is believed to have a transient effect on the inflammatory status of the gingiva (20). Human gingiva is capable of metabolizing sex hormones. Vittek et al. (30) have shown a positive correlation between progesterone levels and gingival inflammation, more specifically increasing inflammation has been reported in pre-existing gingivitis (23). These effects could account for many of the changes seen in puberty where there are increased circulating sex hormones.

So far only a few studies have considered age status as a modifying factor for the variations in intracellular cytokine production that can be observed by comparing younger and aging adults or children and adolescents (5, 17). Contradictory results have, however, been reported. Studies have shown both an increase and no change between different age groups examining alterations in proinflammatory systemic cytokine production.

No age-dependent changes were found in the levels of cytokines (IL-1β, IL-6, and IL-8) in the normal clinically non-inflamed gingiva in groups: 6–14 years old, 18–35 years old (young adults), 36–54 years old (mature adults), and more than 55 years old (old adults) (32). Increasing age had

either no effect on the IL-4 production or the effect was not very robust because variations in experimental design result in very different outcomes (10). It is known that IL-4 stimulates T helper type 2 cell activity, which in turn stimulates antibody production by B cells. Yakovlev et al. (32) found that serum levels of antibodies to oral microorganisms involved in periodontal disease increased gradually with both age and severity of gingivitis.

The assessment of immune function is easily influenced by numerous parameters, such as health status of the subjects and genetic variability, as well as behavioural factors such as diet, stress, cognitive status, and level of physical activity (27).

In the present study, inflammation was reflected by higher GCF levels of IL-1β and lower levels of IL-4, which were more pronounced in adolescents than in young adults. Although it has been proposed that the severity of gingivitis in childhood is linked to several factors, including puberty (20, 29) no evidence of a direct link between gingivitis and puberty has been established for three reasons: chronological age is a poor indicator of puberty, the measurement of gingivitis is subjective, and the hormone levels are not measured.

Sex hormones are known to affect several cell types in the body (30), the gingival vasculature (18) and the oral biofilm. Kamma et al. (16) showed that children at a young age with mixed dentition harbour a multiform oral biofilm consisting of gram-negative anaerobic bacteria, some of which are suspected periodontal pathogens. This suggested that children colonized early with periodontal pathogenic species may be at greater risk of the outbreak of periodontal disease either in childhood or later in life and may have a more robust local host response of IL-1β from lipopolysaccharide-stimulated mononuclear cells while a transient increase of black-pigmented gram-negative anaerobic rods has been reported in children during puberty (7).

Within the limits of this cross sectional study it might be a possible association of the local expression of cytokines with the age-related biological activity in the periodontium of adolescents, despite the evident difficulty of studying the direct effect of age on immune function. Therefore, further longitudinal studies are essential to a more complete definition of the potential modifying effects of age on the local expression of cytokines in the periodontium.

Conflict of interest and source of funding statement

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