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The influence of sex hormones on proinflammatory cytokines in gingiva of periodontally healthy premenopausal women

E. Markou[1], E. Boura[2], L. Tsalikis[1], A. Deligianidis[3], A. Konstantinidis[1]

[1]Department of Preventive Dentistry, Periodontology and Implant Biology, School of Dentistry, Aristotle University of Thessaloniki, Thessaloniki, Greece, [2]Private Practice, Agathoupoleos street 19, 54636 Thessaloniki, Greece and [3]Immunology Department and Regional Tissue Typing Laboratory of Hippocrates General Hospital, Thessaloniki, Greece

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Background and Objective: The aim of this work was to investigate any correlation between the fluctuation of levels of specific proinflammatory cytokines in gingival crevicular fluid and the fluctuation of sex hormones in peripheral blood at ovulation and progesterone peak.

Material and Methods: Eighteen premenopausal women with normal and consistent menstrual cycles and healthy periodontium were included in this study. The exclusion criteria were as follows: (i) pregnancy; (ii) use of oral contraceptives; (iii) metabolic or systemic disease that might affect the periodontium; (iv) use of antimicrobial or nonsteroidal anti-inflammatory drugs during the past 6 mo; and (v) smoking. The measurements were performed at two specific time points for each participant [(i) on the day of ovulation; and (ii) on the day of the progesterone peak) and included the following: (i) plaque index; (ii) bleeding on probing; and (iii) the gingival crevicular fluid levels of interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α).

Results: During the menstrual cycle, plaque index values remained unchanged (0.71 ± 0.07 at ovulation; 0.73 ± 0.08 at progesterone peak; $p > 0.05$), as did bleeding on probing (0.35 ± 0.07 at ovulation; 0.41 ± 0.07 at progesterone peak; $p > 0.05$). At ovulation, mean gingival crevicular fluid levels were as follows: IL-1β, 13.3 pg/sample; IL-6, 5.9 pg/sample; IL-8, 18.7 pg/sample; and TNF-α, 25.9 pg/sample. The corresponding values at progesterone peak were as follows: 14.1, 10.1, 19.5 and 26.3 pg/sample. Only IL-6 gingival crevicular fluid levels were significantly different between ovulation and progesterone peak ($p < 0.05$). This could reflect sensitivity to subclinical amounts of plaque and biofilm constituents.

Conclusion: The subclinical increase of IL-6 at progesterone peak is not accompanied by clinical changes in the periodontium.

Lazaros Tsalikis, DDS, Dr MedDent, Department of Preventive Dentistry, Periodontology and Implant Biology, School of Dentistry, Aristotle University of Thessaloniki, 30 Agias Sofias street, 54622 Thessaloniki, Greece
Tel: +30 2310 999603
Fax: +30 2310 999613
e-mail: tsalikis @dent.auth.gr

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The menstrual cycle of women is a period of 25–30 d controlled by the secretion of sex hormones. The cycle is divided into two phases: the proliferative (phase A) and the secretory phase (phase B). The proliferative phase is characterized by a gradual increase in the production of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol and, to a lesser degree, progesterone. Around the 14th day of the menstrual cycle, there is a sudden and marked increase in production of LH, leading to ovulation. In the subsequent secretory phase, the production of LH and FSH decline, while there is an increase in the production of progesterone (1,2).

Sex steroid hormones can significantly influence the periodontal tissues (3,4). For example, estrogens may influence the proliferation, differentiation and growth of epithelial cells and fibroblasts, resulting in a reduced epithelial barrier to bacterial insult and altered collagen metabolism (3,5,6). In addition, estrogen receptors in osteoblast-like cells mediate the direct impact on bone (4). In contrast, progesterone affects the local vascularity, increasing vascular permeability and proliferation, and stimulates the production of interleukin-6 (IL-6) by human gingival fibroblasts (7–9). Additionaly, female hormones may influence oral microbiota, as an increased number of *Porphyromonas gingivalis*, *Prevotela intermedia* or *Capnocytophaga species* has been reported during puberty and pregnancy (3,10–12).

It is generally accepted that increased sex hormones during the menstrual cycle modulate the development of gingival inflammation, although this is not fully confirmed experimentally (8,13–15). This may be partly explained by the fact that longitudinal studies of any minor changes in the quality of the gingiva are seriously hampered by the limitations of the clinical and laboratory methods of investigation. Additionally, subclinical inflammatory changes could become overt, modulated by the host response and sensitivity to small amounts of plaque, depending on the composition of the plaque biofilm. More specifically, Holm-Pedersen and Loe (16) showed that there is no correlation between the condition of the gingiva and the different phases of the menstrual cycle in clinically healthy gingiva, whereas a significant deterioration in pre-existing gingivitis was observed during the first day of menstruation. In contrast, gingival crevicular fluid volume increased when FSH and estrogen levels were higher, whereas gingival crevicular fluid volume decreased when progesterone levels were higher during the menstrual cycle, according to Lindhe and Attstrom (17). More recently, Machtei *et al*. (15), in a longitudinal study of 18 premenopausal women, reported no statistically significant difference in plaque index, but an increase in gingival index in the ovulatory and premenstrual periods.

In gingival inflammation, proinflammatory cytokines play a major role in the progression of the disease. Specifically, interleukin-1β (IL-1β) mediates the production of prostaglandins, leukotrienes and platelet-activating factor and promotes bone resorption. Tumor necrosis factor-α (TNF-α) activates inflammatory leukocytes and stimulates the production of cytokines as well as antibodies. Interleukin-6 is responsible for the production of the acute-phase proteins (such as fibrinogen) by the liver, and is the basic growth factor for activated B-cells, whereas interleukin-8 (IL-8) is not only the main chemotactic factor for neutrophils but also for eosinophils, basophils and lymphocytes (18–21).

The purpose of this study was to examine the fluctuation of specific immune factors (IL-1β, IL-6, IL-8 and TNF-α) in the gingival crevicular fluid of periodontally healthy women at ovulation and progesterone peak, in order to investigate possible mechanisms for the hormone–tissue interaction in the periodontium.

Material and methods

Eighteen premenopausal women, 19–25 years old, exhibiting a stable menstrual cycle, volunteered to be included in this longitudinal study, which was held from September 2008 to October 2009. These patients were recruited from a pool of students of Aristotle University of Thessaloniki. Written informed consent was provided by all participants. The study design and protocol were approved by the Ethics Committee of Aristotle University of Thessaloniki and were found to conform to the guidelines issued in the Declaration of Helsinki. Inclusion criteria were as follows: (i) premenopausal women; (ii) normal and consistent menstrual cycle, 25–35 d long; and (iii) healthy periodontium, with no radiographic evidence of bone loss, and plaque index $< 10\%$ and bleeding on probing $< 10\%$ at initial examination. Exclusion criteria were as follows: (i) pregnancy; (ii) use of oral contraceptives; (iii) metabolic or systemic condition that might affect the periodontium; (iv) antibiotic therapy or use of nonsteroidal anti-inflammatory drugs within the past 6 mo; and (v) smoking. An ovulation test (which measures LH in urine) was given to each patient in order to determine each woman's exact day of ovulation. The LH surge is very brief, and in order to avoid any mistakes the subjects started taking the test 3 d before the middle of their cycle, at the same time point every day. This test works by detecting the LH surge, and so a timetable was set, consisting of the following two time points during the menstrual cycle: (i) the day of ovulation; and (ii) the day of the progesterone peak (7 d after the day of ovulation). A plaque control program, including professional tooth cleaning, was undertaken prior to treatment to ensure gingival health at baseline.

The measurements performed for all patients at each of the two time points included the following: (i) plaque index (22); (ii) bleeding on probing; and (iii) gingival crevicular fluid collection for determination of IL-1β, IL-6, IL-8 and TNF-α. For the determination of clinical parameters, full-mouth records were obtained. The collection of the gingival crevicular fluid samples has been previously described by Tsalikis *et al*. (23), and it was always performed prior to the clinical assessments. The samples were collected from the Ramfjord teeth as suggested by Rams *et al*. (24). Each site under study was isolated

with cotton rolls and air dried. Paper strips (Periopaper; Harco Electronics Ltd., Winnipeg, MB, Canada) were gently inserted into the gingival crevice of the mesial-buccal site of each Ramfjord tooth and left in place for 30 s. The paper strips were then placed in one test tube for each subject (pooled sample), which contained 150 μL of phosphate-buffered saline, sealed and immediately sent to the laboratory, where they were centrifuged (5 min, 3000*g*) at room temperature with the vortex unit. Then the strips were removed and the tubes immediately frozen and kept at −70°C until transferred to Hippocrates General Hospital of Thessaloniki for further processing.

The micro-ELISA method was used for measuring cytokine levels. All samples and standards were assayed in duplicate. The levels of IL-1β, IL-6, IL-8 and TNF-α were determined using commercial ELISA kits, according to the manufacturer's instructions. The amounts for each cytokine were examined using a microplate reader at 450 nm as the primary wavelength and at 620 nm as the reference wavelength. Positive control samples confirmed the validity of each assay procedure, and standard curves were linearized by using point/point paper and regression analysis applied to the point transformation.

Statistical analysis

For the concise presentation of results, indicators of central tendency (means and medians) and variance (standard deviations, minimum and maximum values) as well as coefficient of correlations (Spearman's rho) were calculated. The nonparametric Wilcoxon test was used for the various comparisons of distributions and mean values. Nonparametric statistical processes were used, because sample size was relatively small and it was not possible to support and test normality of parameters' values. In all nonparametrical statistical tests, the observed level of significance (*p*-value) was calculated with the 'exact' method. This approach leads to valid inferential conclusions even in cases when the presuppositions of statistical testing procedures (random samples, normal distribution, independent observations and large sample sizes) are not satisfied (25). Statistical analyses were performed using the statistical software SPSS v.15 (IBM, Chicago, IL, USA) with the module Exact Tests installed. In order to increase the power of the correlations' significance testing procedures, the significance level was predetermined at $p < 0.10$.

Results

All subjects completed the trial. The changes in the clinical parameters were not significant, whereas the results of statistical analysis for the immunological parameters are presented in Table 1. During the menstrual cycle of each participant, the plaque index and bleeding on probing values did not reveal any significant difference ($p > 0.05$), indicating that periodontal health was successfully maintained. There was no statistically significant difference between the two measurements for IL-1β, IL-8 and TNF-α ($p > 0.05$), whereas a statistically significant difference was observed between ovulation and progesterone peak for IL-6. Specifically, the IL-6 mean value at ovulation was 5.9 pg/sample and at progesterone peak it reached 10.1 pg/sample ($p < 0.05$). Finally, at each time point, the correlation between two cytokines was checked for both measurements, and statistical significance was detected only between TNF-α and IL-1β (Spearman's $\rho = -0.463$, $p = 0.082$ at ovulation; Spearmans $\rho = -0.493$, $p = 0.062$ at progesterone peak). In addition, for the second measurement the correlation is stronger for the window of TNF-α values >27.5 pg/sample.

Discussion

This prospective, longitudinal study examined the periodontal condition of 18 periodontally healthy premenopausal women exhibiting stable menstrual cycles, on the day of ovulation and on the day of the progesterone peak. Machtei *et al.* (15) also examined the periodontal condition of 18 women at two time points during the menstrual cycle and found a statistically significant increase in the gingival index. In the present study, it was observed that on the day of progesterone peak the levels of TNF-α, IL-1β and IL-8 remained stable. On the contrary, IL-6 values were significantly increased.

The cellular pathway for this hormone–tissue interaction during menstruation has been studied widely in peripheral blood. Brannstrom *et al.* (13) reported that TNF-α showed a significant fluctuation during the menstrual cycle, with surges just before ovulation and in the premenstrual period, whereas no significant fluctuations in the levels of IL-2 and IL-6 were observed. In contrast, Lapp *et al.* (9), Gornstein *et al.* (14) and Konecna

Table 1. Values of central tendency and variance for the four parameters at ovulation and at progesterone peak

	Mean	Median	SD	Minimum	Maximum	Exact *p*-value
Tumor necrosis factor-α						
Ovulation	25.85	28.00	7.46	12	36	0.551
Progesterone peak	26.33	31.00	8.66	11	40	
Interleukin-1β						
Ovulation	13.34	11.00	8.57	6	31	0.901
Progesterone peak	14.10	11.00	8.28	6	31	
Interleukin-6						
Ovulation	5.88	6.40	1.84	2.7	9.4	0.034*
Progesterone peak	10.13	6.40	8.41	4	34	
Interleukin-8						
Ovulation	18.67	20.00	3.24	11	23	0.531
Progesterone peak	19.47	21.00	3.23	11	22	

*$p < 0.05$, statistical significance.

et al. (26) found that serum IL-6 showed a negative correlation with estradiol and progesterone. These cytokines are considered to play an important role in the initiation, progression and the host modulation of periodontal disease, and even a minimal imbalance of cytokine production may affect induction of bone and collagen destruction in periodontal disease (27,28). High levels of IL-1β in gingival crevicular fluid and in gingival tissue have been associated with gingival inflammation, while inhibition of IL-1β reduced tissue destruction and the progression of inflammation in experimental periodontitis (29–35). Tumor necrosis factor-α stimulates collagenase production and bone resorption and impairs the repair capacity of the periodontium (36). Baser *et al*. (37) reported that there is a correlation between local inflammatory markers, such as IL-1β, and systemic hormonal changes, but failed to present any significant change of TNF-α values during the menstrual cycle. Interleukin-6 is a pleiotropic cytokine secreted in response to bacterial challenges, such as lipopolysacharide, as well as IL-1 and is involved in leukocyte recruitment, apoptosis and T-cell activation, as well as in bone resorption (36–38). Becerik *et al*. (38) evaluated the effect of hormonal changes occurring in the menstrual cycle on gingival inflammation and the gingival crevicular fluid levels of IL-6, prostaglandin E_2, tissue plasminogen activator and plasminogen activator inhibitor-2 and concluded that changes in the sex steroid hormones might have a limited effect on gingival inflammation but that levels of gingival crevicular fluid cytokines are not affected. Finally, IL-8 is a chemotactic cytokine involved in the recruitment and activation of neutrophils, as well as in angiogenesis, and plays a key role in the host defence mechanism in inflammatory diseases, including periodontitis (20,21).

The detection of proinflammatory cytokines in clinically healthy gingival, indicating sensitivity to a subclinical amount of plaque and biofilm constituents, has also been reported. Faizuddin *et al*. (39) detected the presence of IL-1β in gingival crevicular fluid in 12 of 20 periodontally healthy patients. Interleukin-8 was found in gingival crevicular fluid of 20 periodontally healthy young adults aged between 25 and 35 years old, while high levels of IL-6 in gingival crevicular fluid have also been associated with increased stress (40,41).

Another possible mechanism for the interaction between hormones and periodontal tissues was proposed by Jensen *et al*. (10), who reported a dramatic increase in *Bacteroides* species in women taking oral contraceptives and in pregnant women. This influence can be explained by the fact that female hormones are substitutes for the requirements of certain periodontal pathogens. Raber-Durlacher *et al*. (42) found that the proportion of *Prevotella intermedia* increased during pregnancy, and Klinger *et al*. (43) detected the presence of *P. intermedia* in 22 of 29 samples of subgingival plaque from women taking oral contraceptives, although no changes in the clinical parameters were observed. In contrast, Fischer *et al*. (44) examined the fluctuation in subgingival pathogens during the menstrual cycle of 20 periodontally healthy premenopausal women and found no cyclic variation of bacteria under the influence of hormones.

Hormonal changes aggravate gingivitis in the presence of plaque. This study illustrates the fact that when good plaque control is maintained with minimal gingival inflammation, hormones would have limited influence. The observed increase in IL-6 may represent subclinical changes, which should be considered when analyzing data from epidemiological and treatment studies in premenopausal periodontally healthy women, where IL-6 levels are used as primary or secondary outcome variables. The detection of proinflammatory cytokines in healthy gingiva indicates subclinical inflammation, which could become overt with increased plaque/inflammatory burden. The strict inclusion criteria of plaque index and bleeding on probing that were applied in the present study might have excluded subjects who have average oral hygiene habits and are also more sensitive to hormonal changes and left only those with minimal disease who may not show differences unless a very large group is examined. Therefore, no general conclusions can be drawn from this study. Further studies will be required to explore the mechanism by which this phenomenon occurs and to examine whether these subclinical changes have any lasting negative effect on either healthy or inflamed periodontium.

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References

1. Laufer N, Navot D, Schenker JG. The pattern of luteal phase plasma progesterone and estradiol in fertile cycles. *Am J Obstet Gynecol* 1982;**143:**808–813.
2. Ganong WF, Ganong W. *Review of Medical Physiology*. Norwalk, CT: Appleton & Lange, 1989:379–387.
3. Mariotti A. Sex steroid hormones and cell dynamics in the periodontium. *Crit Rev Oral Biol Med* 1994;**5:**27–53.
4. Mascarenhas P, Gapski R, Al-Shammari K, Wang HL. Influence of sex hormones on the periodontium. *J Clin Periodontol* 2003;**30:**671–681.
5. Amar S, Chung KM. Influence of hormonal variation on the periodontium in women. *Periodontol 2000* 1994;**6:**79–87.
6. Mealey BL, Moritz AJ. Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones on the periodontium. *Periodontol 2000* 2003;**32:**59–81.
7. Lindhe J, Branemark PI. Changes in vascular permeability after local application of sex hormones. *J Periodontal Res* 1967;**2:**259–265.
8. Lindhe J, Brånemark PI, Lundskcg J. Changes in vascular proliferation after local application of sex hormones2. *J Periodont Res* 1967;**2:**266–272.
9. Lapp CA, Thomas ME, Lewis JB. Modulation by progesterone of interleukin-6 production by gingival fibroblasts. *J Periodontol* 1995;**66:**279–284.
10. Jensen J, Liljemark W, Bloomquist C. The effect of female sex hormones on subgingival plaque. *J Periodontol* 1981;**52:**599–602.

11. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. *Infect Immun* 1982;**35:**256–263.
12. Mombelli A, Lang NP, Burgin WB, Gusberti FA. Microbial changes associated with the development of puberty gingivitis. *J Periodontal Res* 1990;**25:**331–338.
13. Brannstrom M, Friden BE, Jasper M, Norman RJ. Variations in peripheral blood levels of immunoreactive tumor necrosis factor alpha (TNFalpha) throughout the menstrual cycle and secretion of TNFalpha from the human corpus luteum. *Eur J Obstet Gynecol Reprod Biol* 1999;**83:**213–217.
14. Gornstein RA, Lapp CA, Bustos-Valdes SM, Zamorano P. Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *J Periodontol* 1999;**70:** 604–609.
15. Machtei EE, Mahler D, Sanduri H, Peled M. The effect of menstrual cycle on periodontal health. *J Periodontol* 2004;**75:**408–412.
16. Holm-Pedersen P, Loe H. Flow of gingival exudate as related to menstruation and pregnancy. *J Periodontal Res* 1967;**2:**13–20.
17. Lindhe J, Attsfrom R. Gingival exudation during the menstrual cycle. *J Periodontal Res* 1967;**2:**194–198.
18. Gowen M, Mundy GR. Actions of recombinant interleukin 1, interleukin 2, and interferon-gamma on bone resorption in vitro. *J Immunol* 1986;**136:**2478–2482.
19. Billingham MEJ. Cytokines as inflammatory mediators. *Br Med Bull* 1987;**43:**350.
20. Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *Int J Hematol* 2000;**72:** 391–398.
21. Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Characterization of cellular infiltrate, detection of chemokine receptor CCR5 and interleukin-8 and RANTES chemokines in adult periodontitis. *J Periodontal Res* 2001;**36:**194–203.
22. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 1963;**21:**533–551.
23. Tsalikis L, Malaka E, Pavlitou E, Konstantinidis A. Aspartate aminotransferase levels in gingival crevicular fluid before and after initial periodontal treatment. *J Int Acad Periodontol* 2001;**3:**68–74.
24. Rams TE, Oler J, Listgarten MA, Slots J. Utility of Ramfjord index teeth to assess periodontal disease progression in longitudinal studies. *J Clin Periodontol* 1993;**20:** 147–150.
25. Mehta CR, Patel NR. *SPSS Exact Tests 7.0 for Windows*. Chicago: SPSS Inc, 1996.
26. Konecna L, Yan MS, Miller LE, Scholmerich J, Falk W, Straub RH. Modulation of IL-6 production during the menstrual cycle in vivo and in vitro. *Brain Behav Immun* 2000;**14:**49–61.
27. Salvi GE, Lang NP. Host response modulation in the management of periodontal diseases. *J Clin Periodontol* 2005;**32**(suppl 6)**:**108–129.
28. Seymour GJ, Gemmell E. Cytokines in periodontal disease: where to from here? *Acta Odontol Scand* 2001;**59:**167–173.
29. Masada MP, Persson R, Kenney JS, Lee SW, Page RC, Allison AC. Measurement of interleukin-1alpha and -1beta in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *J Periodontal Res* 1990;**25:**156–163.
30. Stashenko P, Jandinski JJ, Fujiyoshi P, Rynar J, Socransky SS. Tissue levels of bone resorptive cytokines in periodontal disease. *J Periodontol* 1991;**62:**504–509.
31. Engebretson SP, Lamster IB, Herrera-Abreu M *et al*. The influence of interleukin gene polymorphism on expression of interleukin-1b and tumor necrosis factor-a in periodontal tissue and gingival crevicular fluid. *J Periodontol* 1999;**70:**567–573.
32. Tsalikis L, Parapanisiou E, Bata-Kyrkou A, Polymenides Z, Konstantinidis A. Crevicular fluid levels of interleukin-1alpha and interleukin-1beta during experimental gingivitis in young and old adults. *J Int Acad Periodontol* 2002;**4:**5–11.
33. Graves DT, Delima AJ, Assuma R, Amar S, Oates T, Cochran D. Interleukin-1 and tumor necrosis factor antagonists inhibit the progression of inflammatory cell infiltration toward alveolar bone in experimental periodontitis. *J Periodontol* 1998;**69:**1419–1425.
34. Ataoglu H, Alptekin NO, Haliloglu S *et al*. Interleukin-1β, tumor necrosis factor-α levels and neutrophil elastase activity in peri-implant crevicular fluid. *Clin Oral Implants Res* 2002;**13:**470–476.
35. Delaleu N, Bickel M. Interleukin-1β and interleukin-18: regulation and activity in local inflammation. *Periodontol 2000* 2004;**35:**42–52.
36. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. *Nature* 1986;**319:** 516–518.
37. Baser U, Cekici A, Tanrikulu-Kucuk S, Kantarci A, Ademoglu E, Yalcin F. Gingival inflammation and interleukin-1 beta and tumor necrosis factor-alpha levels in gingival crevicular fluid during the menstrual cycle. *J Periodontol* 2009;**80:**1983–1990.
38. Becerik S, Ozcaka O, Nalbantsoy A *et al*. Effects of menstrual cycle on periodontal health and gingival crevicular fluid markers. *J Periodontol* 2010;**81:**673–681.
39. Faizuddin M, Bharathi SH, Rohini NV. Estimation of interleukin-1beta levels in the gingival crevicular fluid in health and in inflammatory periodontal disease. *J Periodontal Res* 2003;**38:**111–114.
40. Kamma J, Mombelli A, Tsinidou K, Vasdekis V, Giannopoulou C. Cytokines in gingival crevicular fluid of adolescents and young adults. *Oral Microbiol Immunol* 2009;**24:**7–10.
41. Johannsen A, Rylander G, Soder B, Asberg M. Dental plaque, gingival inflammation, and elevated levels of interleukin-6 and cortisol in gingival crevicular fluid from women with stress-related depression and exhaustion. *J Periodontol* 2006;**77:** 1403–1409.
42. Raber-Durlacher JE, van Steenbergen TJ, Van der Velden U, de Graaff J, Abraham-Inpijn L. Experimental gingivitis during pregnancy and post-partum: clinical, endocrinological, and microbiological aspects. *J Clin Periodontol* 1994;**21:**549–558.
43. Klinger G, Eick S, Klinger G *et al*. Influence of hormonal contraceptives on microbial flora of gingival sulcus. *Contraception* 1998;**57:**381–384.
44. Fischer CC, Persson RE, Persson GR. Influence of the menstrual cycle on the oral microbial flora in women: a case-control study including men as control subjects. *J Periodontol* 2008;**79:**1966–1973.