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Correlation of Estradiol, Parathyroid Hormone, Interleukin-6, and Soluble Interleukin-6 Receptor During the Normal Menstrual Cycle

K. M. CHIU,[1,2] C. D. ARNAUD,[2] J. JU,[3] D. MAYES,[4] P. BACCHETTI,[5] S. WEITZ,[3] and E. T. KELLER[1]

[1]*Unit of Laboratory Animal Medicine and Department of Pathology, School of Medicine and the Institute of Gerontology, University of Michigan, Ann Arbor, MI, USA*
[2]*Program in Osteoporosis and Bone Biology and* [5]*Department of Epidemiology & Biostatistics, University of California, San Francisco, CA, USA*
[3]*Metra Biosystems, Inc., Mountain View, CA, USA*
[4]*Endocrine Sciences, Inc., Agoura Hills, CA, USA*

Rodent models suggest that estradiol deficiency promotes bone loss through increasing interleukin-6 (IL-6) activity. However, it is controversial as to whether these findings are applicable to humans. To evaluate estradiol-mediated modulation of IL-6 activity in relation to bone metabolism in humans, we measured serum IL-6, soluble interleukin-6 receptor (sIL-6R), estradiol (E_2), progesterone, luteinizing hormone, follicle-stimulating hormone, intact parathyroid hormone (PTH), serum and urine Ca, and bone biochemical markers (serum bone-specific alkaline phosphatase, osteocalcin, and serum and urine deoxypyridinoline [Dpd]) across one menstrual cycle for 20 women. Neither IL-6 nor sIL-6R levels differed between the follicular phase (FP) and the luteal phases (LP). However, IL-6 was negatively correlated with E_2 during the FP ($p = 0.003$). Furthermore, IL-6 correlated positively with serum Ca over the entire cycle ($p = 0.009$). Serum Ca correlated positively with serum ($p = 0.040$) and urine ($p = 0.006$) Dpd. PTH was significantly higher during the FP than in the LP ($p = 0.004$). PTH was negatively related to E_2 ($p = 0.002$), serum Ca ($p < 0.001$), and urine Ca ($p = 0.036$), whereas it was positively correlated with IL-6 ($p = 0.027$). These data demonstrate that IL-6 and PTH fluctuate with E_2, and serum IL-6 is associated with PTH levels during the menstrual cycle. However, the role of IL-6 in bone remodeling during the normal menstrual cycle remains to be determined. (Bone 26:79–85; 2000)

Key Words: Estradiol (E_2); Parathyroid hormone (PTH); Interleukin-6 (IL-6); Deoxypyridinoline (Dpd); Menstrual cycle.

Introduction

In vitro and ex vivo rodent models have implicated a large number of potential mediators of estrogen's (E_2) action on bone. One of these factors, interleukin-6 (IL-6), has been postulated to play a role in induction of E_2-deprivation bone loss (reviewed in Manolagas[43]). Specifically, E_2 inhibits IL-6 expression in murine bone marrow stromal cells.[38,53,54,61] Thus, E_2 deprivation promotes IL-6 expression, which induces osteoclastogenesis.[22] However, the role of IL-6 in human postmenopausal bone loss is controversial. Specifically, E_2 has been shown both to have no effect[5,8,25,33,44,55,56] and to inhibit[4,9,24,34] IL-6 secretion from human osteoblasts. Studies in both pre- and postmenopausal women show that circulating IL-6 levels did not correlate with either serum E_2 levels or bone remodeling markers.[29,46] Thus, the role of IL-6 in E_2-depletion-mediated osteopenia in women is not clear.

One approach to defining the interactions between IL-6, E_2, and bone remodeling (bone biochemical markers) is by examining relationships among the serum concentrations of these indices that occur during the menstrual cycle. For example, serum estrogen levels change cyclically during the menstrual cycle. It follows that IL-6 should also fluctuate. However, several investigators have reported that serum IL-6 concentrations do not change during the menstrual cycle.[23,40,41] By contrast, plasma[2] and endometrial[63] IL-6 levels were reported as menstrual-cycle-dependent by others.

An alternative mechanism to control of IL-6 activity is through regulation of soluble IL-6 receptor (sIL-6R) expression levels. IL-6 binds the sIL-6R and circulates in the blood as an active IL-6/sIL-6R complex.[11] The presence of both IL-6 and sIL-6R is required to induce osteoclast formation in some,[64] but not all,[12] model systems. Moreover, significant variations of serum sIL-6R levels during the menstrual cycle have been reported and sIL-6R was correlated with urinary type I collagen C-telopeptide breakdown products and serum bone-specific alkaline phosphatase.[17] Thus, estrogen may promote osteoclastogenesis through modulation of sIL-6R expression.

In addition to its direct effects on IL-6 activity, estrogen may modulate IL-6 activity indirectly through parathyroid hormone (PTH). Specifically, estrogen blocks the action of PTH on bone resorption.[28,57] Several studies have shown that serum PTH is positively associated with bone resorption marker (type I collagen cross-linked N-telopeptide, pyridinoline, deoxypyridinoline) excretion in both young (24–35 years) and postmenopausal (71–78 years) women.[37,47] Furthermore, PTH induces IL-6 expression[16,18,48,52,59] in osteoblastic cells. Taken together, these

Address for correspondence and reprints: Evan T. Keller, Ph.D., Box 0940 CCGCB Room 5304, 1500 East Medical Center Drive, University of Michigan, Ann Arbor, MI 48109-0940. E-mail: etkeller@umich.edu

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reports suggest that PTH modulates IL-6-induced bone resorption during changes in estrogen status.

We hypothesized that cycling of E_2 during the menstrual cycle modulates bone metabolism through regulation of PTH and IL-6 activity. In the current study, our objective was to determine if an association exists with menstrual-cycle-associated changes in E_2, PTH, and IL-6 in the context of bone remodeling. Accordingly, we measured serum IL-6, sIL-6R, E_2, PTH, bone formation markers (serum bone-specific alkaline phosphatase [BAP] and osteocalcin [OC]), and bone resorption markers (serum and urine deoxypyridinoline [Dpd]) during the menstrual cycle in 20 healthy premenopausal women.

Subjects and Methods

Subjects and Study Design

Approval for the present study was obtained from the University of California, San Francisco (UCSF) Committee on Human Research. Subjects were recruited from populations of hospital employees, students, and the local community surrounding the UCSF and Mount Zion Medical Centers. Informed consent was obtained from all subjects.

Inclusion criteria required that the subjects were women between the ages of 20 and 40 years, had a history of spontaneous, regular (24–34-day) menstrual cycles, were ambulatory, and engaged in their usual activities. Exclusion criteria included pregnancy or a history of endocrine, renal, or metabolic disease and the use of oral contraceptives during the 3 months prior to the study or any medication known to influence calcium metabolism such as glucocorticoids, thiazide diuretics, and anticonvulsants. Dietary calcium was determined using a validated focused food-frequency questionnaire.[10]

Blood samples were collected between 7:00 A.M. and 10:00 A.M. three times a week (Monday, Wednesday, and Friday), beginning within 4 days of the onset of menses and ending after the onset of the next menses. Blood was allowed to clot for 2 h at 4°C and serum was prepared by centrifugation at 4°C. Urine samples were first morning voids (overnight fasting) and were collected on the same days as blood draws for one complete menstrual cycle. All serum and urine samples were stored at −80°C until analyzed. Frozen samples were transported on dry ice. All samples were from ovulatory cycles.

Analytic Methods

All analyses described were performed on each serum or urine sample. All samples from one individual were analyzed in the same analytic run.

Serum E_2 and progesterone (P) levels were measured by radioimmunoassay (Endocrine Sciences). Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by immunochemiluminometric assay (Endocrine Sciences). Serum bone-specific alkaline phosphatase (BAP), osteocalcin (OC), and urine deoxypyridinoline (Dpd) were measured by enzyme-linked immunosorbent assay (ELISA; Metra Biosystems). Intact parathyroid hormone was measured by a two-site immunoradiometric assay (IRMA; Nichols Institute Diagnostics). Serum and urinary calcium levels were measured by standard laboratory tests (Clinical Laboratory, Veterans Affairs Medical Center, San Francisco, CA). Urine Dpd and urinary calcium values were corrected for changes in urine concentration by normalizing them to urine creatinine. Urine creatinine was measured by automated analysis. Serum IL-6, sIL-6R, and IL-1 were measured by high-sensitivity ELISA kits (#HS600, DR600, and HSLB50; R&D Systems, Minneapolis, MN). The ELISA kits measure total cytokine complexed to specific and nonspecific proteins.

Table 1. The intra- and interassay coefficients of variation and sensitivities of the assays

	Coefficients of variations (%)		
	Intraassay	Interassay	Sensitivities
E_2	8.3	9.9	5 pg/mL
P	4.5	6.7	0.1 ng/mL
LH	4.3	6.0	0.02 mIU/mL
FSH	7.8	7.8	0.05 mIU/mL
PTH	3.4	5.6	1 pg/mL
IL-6	5.9	6.7	0.094 pg/mL
sIL-6R	2.6	4.2	3.5 pg/mL
IL-1	6.9	7.7	0.1 pg/mL
BAP	3.9	7.6	0.7 U/L
OC	4.8	4.8	0.45 U/L
Serum Dpd	7.5	11.5	100 pmol/L
Urinary Dpd	4.3	4.6	1.1 nmol/L

KEY: BAP, bone-specific alkaline phosphatase; Dpd, deoxypyridinoline; E_2, estradiol; FSH, follicle-stimulating hormone; IL, interleukin; LH, leutinizing hormone; OC, osteocalcin; P, progesterone; PTH, parathyroid hormone.

The intra- and interassay coefficients of variation and sensitivities of the assays are shown in **Table 1**.

Statistical Analysis

Each menstrual cycle was normalized to 29 days. The day of the LH surge was defined as day 0, the first day of menses was defined as day −14, and the last day before the start of the next menses was defined as day +14. Visits falling between these points were assigned a time based on how far their actual date was from the ends of the interval that they fell into (either days −14 and 0, or days 0 and +14). For purposes of graphing or examining summary values by day, the visits were assigned to 2 day bins, so that a visit on day −14 or −13 was counted as day −14, a visit on day −12 or −11 was counted as day −12, etc. Cycles were divided into FP (days −14 to −1) and LP (days 1 to 14). The significance of the difference of variables between menstrual phases (FP vs. LP) was assessed by the Wilcoxon signed-rank test.

It is a well-established statistical principle that ignoring within-person dependence can severely distort the results of statistical analyses,[14] but nonnormality of many of the outcome measures precluded the use of standard repeated measures models. Therefore, we calculated Spearman rank correlations between measures separately for each woman over her cycle, rather than simply using all data points from all women as if they were all independent. For a given pair of measures, we summarized the rank correlations of the 20 women by median score (denoted r_m in the text) and tested them for a tendency to be more positive than negative, or vice versa, by the Wilcoxon signed-rank test (also called the one-sample Wilcoxon test).

All data are expressed as median (95% confidence interval of the median). Data analyses were performed using SAS software (SAS Institute, Cary, NC) and STATVIEW software, version 4.02 (Abacus Concepts, Berkeley, CA).

Results

Study Population

Twenty-seven healthy women, ages 23–40 years, were enrolled in the study. Twenty women completed the study (median age

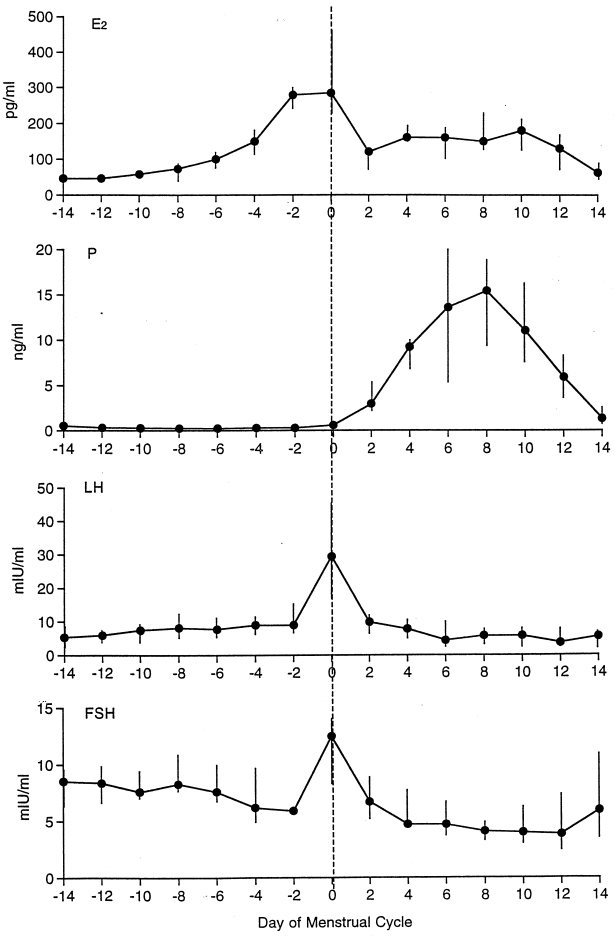

Figure 1. Serum levels of estradiol (E_2), progesterone (P), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) during the menstrual cycle. Each cycle was divided into follicular phase (FP) and luteal phase (LP) using the LH peak. Day −14: onset of menses. Results are reported as median (±upper and lower 95% confidence intervals) from 20 women.

34.5 years, 95% confidence interval 31–36 years). Five women dropped out of the study after signing the consent form and two women withdrew from the study after 1 week of blood draws (specimens were not used for analyses).

The median calcium intake of our subjects was 523 mg/day (362–686 mg/day). The median hip and spine bone mineral densities of the subjects (0.89 g/cm^2 [0.83–0.91 g/cm^2] and 1.04 g/cm^2 [0.98–1.13 g/cm^2], respectively) were within the normal range for young women, except for one subject who had low bone density (T score of hip and spine −1.9% and −2.5%, respectively).

Menstrual Cycle

The median length of the menstrual cycles was 28 days (28–30 days) in the 20 women studied (**Figure 1**). The follicular phase was 14 days (11–16 days) and the luteal phase was 14 days (13–14 days), separated by the LH peak (20.5 mIU/mL [17.0–45.0 mIU/mL]). All examined cycles were ovulatory, as judged from the rise in serum P during the luteal phase (day 8) (15.45 ng/mL [9.46–18.86 ng/mL]).

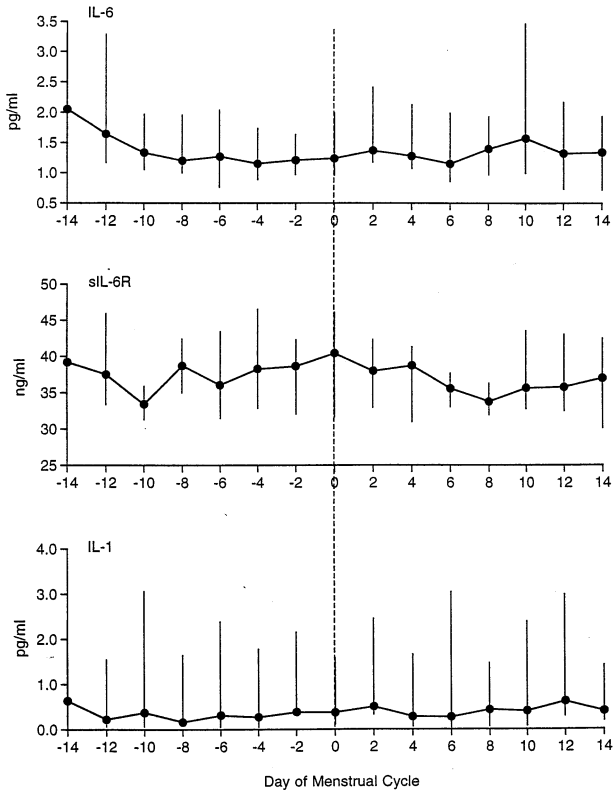

Figure 2. Interleukin-6 (IL-6), soluble interleukin-6 receptor (sIL-6R), and interleukin-1 (IL-1) levels during the menstrual cycle. Each cycle was divided into follicular phase (FP) and luteal phase (LP) using the LH peak. Day −14: onset of menses. Results are reported as median (± upper and lower 95% confidence intervals) from 20 women.

Menstrual Cycle and IL-6

There were no differences between IL-6 or sIL-6R levels between the FP and LP (**Figure 2** and **Table 2**). IL-6 correlated negatively with E_2 during the FP (**Table 3**). Both IL-6 and sIL-6R were positively correlated with serum Ca throughout the entire cycle (Table 3). IL-6 was positively correlated with PTH (Table 3). In contrast, sIL-6R was negatively correlated with PTH (Table 3).

Menstrual Cycle and PTH

Serum PTH was increased during the FP (**Figure 3** and Table 2). Serum and urine Ca, E_2, and P were negatively correlated with PTH (Table 3). As mentioned earlier, PTH was positively correlated with IL-6, but negatively correlated with sIL-6R (Table 3).

Calcium Metabolism and Bone Remodeling Markers

Serum DpD was increased during the FP (**Figure 4** and Table 2). Serum Ca was positively correlated with serum and urine Dpd (Table 3). There were no other significant correlations between Ca and bone remodeling markers.

The serum concentrations of IL-1β were not analyzed for correlation because 25% of the concentrations were below detectable levels.

Table 2. The levels of bone biochemical markers at the follicular phase (FP) and luteal phase (LP) during the menstrual cycle in 20 premenopausal women (values expressed as median with range in parentheses)

	FP	LP	FP − LP	*p* value
PTH (pg/mL)	29.89 (16.66–57.80)	27.46 (13.30–54.33)	3.45 (−3.35–16.67)	0.004[a]
Serum Ca (mg/dL)	9.3 (9.02–9.83)	9.31 (8.99–9.90)	−0.005 (−0.17–0.28)	0.794
Urine Ca (mg/dL/mmol/L creatinine)	0.97 (0.08–1.95)	1.01 (0.23–1.96)	−0.145 (−0.6–0.65)	0.38
IL-6 (pg/mL)	1.66 (0.68–12.17)	1.49 (0.61–4.08)	−0.01 (−1.16–8.09)	0.601
Serum IL-6R (ng/mL)	35.7 (29.90–47.06)	35.92 (24.43–44.98)	0.50 (−5.73–11.29)	0.502
IL-1β (pg/mL)[b]	0.62 (0.05–3.84)	0.43 (0.05–3.76)	0.05 (−0.68–0.63)	0.444
BAP (U/L)	14.52 (10.69–22.89)	14.48 (11.00–22.95)	0.07 (−0.70–2.60)	0.370
OC (U/L)	8.63 (5.41–16.28)	8.89 (5.90–15.30)	0.32 (−1.97–1.09)	0.445
Serum Dpd (pmol/L)	268.04 (195.83–392.5)	259.59 (116.71–385.71)	15.21 (−26.46–79.12)	0.028[a]
Urine Dpd (nmol/L/mmol/L creatinine)	6.25 (3.48–9.87)	5.78 (3.62–10.2)	0.05 (−0.98–2.66)	0.380

See Table 1 for abbreviations.
[a]Significant difference, $p \leq 0.05$, by Wilcoxon signed-rank test.
[b]Twenty-five percent of the IL-1β levels were below assay sensitivity (0.1 pg/mL); 0.05 pg/mL was substituted for the levels that were below 0.1 pg/mL.

Discussion

Many factors, including IL-1α, IL-1β, IL-1 receptor antagonist,[32,49] tumor necrosis factor,[30,31] transforming growth factor-β,[21] prostaglandin E_2,[26,27] macrophage colony-stimulating factor,[36,60] and osteoprotegerin,[20] have been implicated as modulators of E_2-mediated bone metabolism. In the present study, we focused on one cytokine, IL-6, and its receptor, to gain a better appreciation for its role in estrogen-modulated bone remodeling. We found that serum IL-6 levels declined during the rise in estradiol levels that accompanies the FP, although IL-6 levels did not change between the FP and LP. Furthermore, we detected that increased IL-6 and sIL-6R were associated with increased serum Ca, although we could not detect any significant correlation between IL-6 or sIL-6R and specific bone remodeling markers. IL-6 and sIL-6R were also correlated with PTH during the cycle. These results are consistent with the hypothesis that IL-6 expression is regulated by estradiol and PTH, but do not support a role for IL-6 in bone remodeling during the menstrual cycle.

Consistent with several studies,[23,40,41] we did not observe any significant change of serum IL-6 levels across the menstrual cycle. However, we did find a negative correlation between serum IL-6 and estrogen levels during the follicular phase. Disparate results have been reported regarding the regulation of IL-6 expression by estradiol. For example, it has been documented that estradiol has both no effect on serum IL-6 levels[25,55,56] and decreases IL-6 production[9,34,35,53] in vitro. Furthermore, it has been reported that estrogen deficit (either due to oophorectomy or natural menopause) can increase[3,7,68] or have no effect on IL-6 levels[58,66,67] or IL-6 production by mononuclear cells.[51] Our findings are consistent with studies documenting how estrogen inhibits IL-6 gene promoter activity in vitro.[53,54,62]

Even if IL-6 is increased due to estradiol deficiency, the presence of IL-6 itself may not be sufficient to induce bone remodeling. Several studies have established a precedent for

Table 3. Summaries of within-woman rank correlations from 20 women

Variables	Rank correlation: Positive	Rank correlation: Negative	Median rank correlation (r_m)	Wilcoxon signed-rank *p* value
PTH vs. E_2	3	17	−0.292	0.002
PTH vs. P	3	17	−0.379	<0.001
PTH vs. sCa	2	18	−0.442	<0.001
PTH vs. uCa	7	13	−0.271	0.036
PTH vs. IL-6	15	5	0.063	0.027
PTH vs. sIL-6 receptor	5	15	−0.147	0.007
IL-6 vs. sCa	11	9	0.225	0.009
sIL-6R vs. sCa	18	2	0.138	0.001
sDpd vs. sCa	13	7	0.209	0.04
uDpd vs. sCa	17	3	0.164	0.006
E_2 vs. IL-6 (in FP only)	3	17	−0.393	0.003

KEY: FP, follicular phase; s, serum; u, urinary. See Table 1 for other abbreviations.

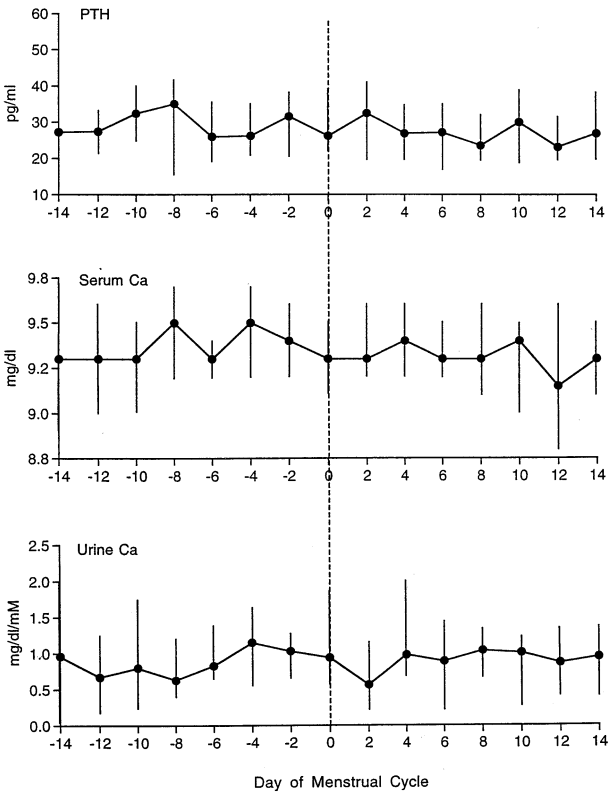

Figure 3. Parathyroid hormone (PTH), serum calcium (Ca), and urine Ca during the menstrual cycle. Each cycle was divided into follicular phase (FP) and luteal phase (LP) using the LH peak. Day −14: onset of menses. Results are reported as median (±upper and lower 95% confidence intervals) from 20 women.

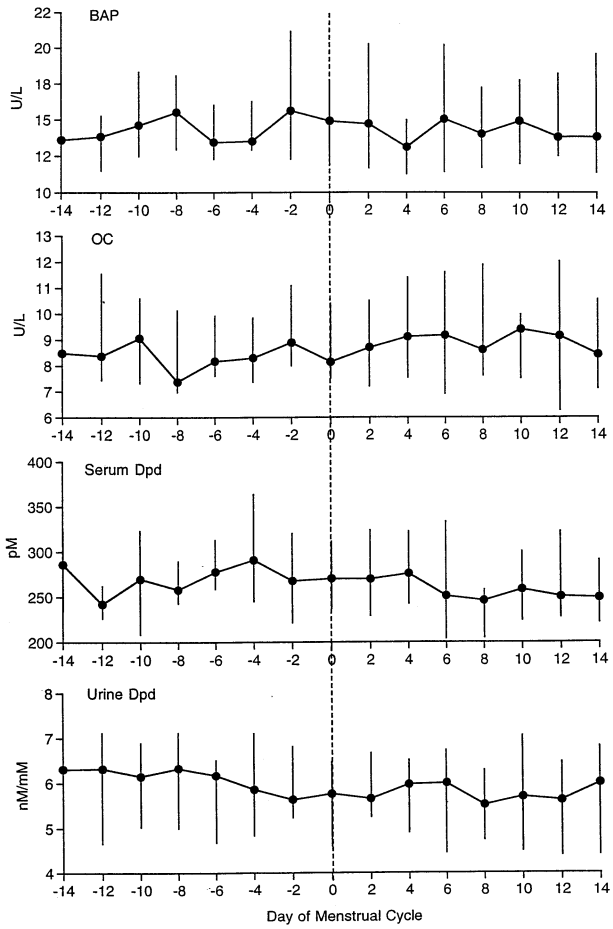

Figure 4. Bone remodeling parameters during the menstrual cycle. Each cycle was divided into follicular phase (FP) and luteal phase (LP) using the LH peak. Day −14: onset of menses. Results are reported as median (±upper and lower 95% confidence intervals) from 20 women.

regulation of IL-6-mediated activity through the sIL-6R. For example, Tamura et al. reported that IL-6 required sIL-6R to induce osteoclast formation.[64] In addition, Lin et al. demonstrated that ovariectomy induces IL-6 receptor and IL-6 mRNA expression in ex vivo murine bone marrow cell cultures,[38] suggesting that both IL-6 and IL-6 receptor are important for skeletal homeostasis in the sex-steroid-deficient state. Although we did not observe any direct correlation between IL-6 or sIL-6R and bone biochemical markers, we found that IL-6 and sIL-6R correlated positively with serum Ca. Our study did not evaluate if IL-6 increases Ca or if Ca increases IL-6. However, oral calcium intake has been associated with increased IL-6 secretion from mononuclear blood cells in humans.[6] Furthermore, increasing intracellular Ca in monocytes induces IL-6 mRNA expression.[45] These data suggest that increased Ca may lead to increased IL-6. One possible reason we observed a correlation of IL-6 with Ca, but not with Dpd, might be due to the possibility that measurements of calcium in biologic fluids (blood and urine) are more accurate and precise, albeit less specific, than measurements of urine and serum Dpd. It is also possible that IL-6 does not play a role in bone resorption during the normal menstrual cycle.

PTH is another well-known factor that enhances osteoclastic bone resorption. Generally, estrogen and PTH seem to have opposite actions on bone resorption. Our findings of an inverse correlation between PTH and E_2 during the menstrual cycle is consistent with the observation that E_2 replacement therapy decreased PTH secretion in early postmenopausal women.[19] We also found that both PTH and serum Dpd levels were higher during the follicular phase when E_2 levels were relatively low. This observation further strengthens the inverse association between E_2 and bone resorption.

Progesterone (P) is another ovarian steroid hormone that plays an important role in regulating bone metabolism. We observed a negative correlation between P and PTH in our study. It is possible that there is a direct physiologic interaction between P and PTH production. Progestogens have been demonstrated both in vivo and in vitro to increase PTH gene expression and also that progesterone receptor is present in the parathyroid gland.[15] Progestogens have been shown to prevent bone loss,[39] increase periosteal diameter, and cause less loss of cortical width when compared with control subjects.[13] Their positive effect on bone metabolism is manifested by the reduction of urinary calcium/creatinine, urine hydroxyproline/creatinine,[1,42] serum calcium, and alkaline phosphatase in postmenopausal women.[50]

Our observation that PTH correlated positively with IL-6 during the entire cycle is consistent with previous reports that PTH stimulates IL-6 expression by cells of the osteoblastic lineage.[16,18,48,52,59] PTH may therefore serve as a tropic agent for IL-6 during the low estrogen phase (follicular phase) of the normal menstrual cycle. Because IL-6 has been reported to inhibit the expression of the sIL-6R gene both in vivo and in vitro, it is plausible that PTH-induced IL-6 repressed sIL-6R gene expression, thus accounting for the negative correlation between PTH and sIL-6R. However, our data did not show an inverse relationship between IL-6 and sIL-6R. This might be due to insufficient power to detect this correlation. The number of subjects in the study was determined by attempting to detect a 15% difference in urine Dpd levels with 80% power. However, this power may be insufficient to study the correlation between IL-6 and sIL-6R.

Based on the average dietary calcium intake (600 mg/day)[65] and median hip and spine bone mineral densities of our population, results from the current study should be representative of young adult women in the United States. However, these data may not be representative of the bone loss observed in postmenopausal women. Specifically, the estradiol levels during the menstrual cycle fluctuated between 25 and 350 pg/mL (95% confidence intervals of median), whereas in postmenopausal women serum estradiol levels declined to below 20 pg/mL. Thus, the full effect of estrogen deficiency was not realized in our population.

A limitation of this study is that IL-6 is derived from other tissues besides bone, and therefore sampling peripheral blood for IL-6 levels may not necessarily reflect bone IL-6 levels. Furthermore, IL-6 circulates in both a free form and bound to sIL-6R. However, our assays measured total levels of IL-6 and sIL-6R. The IL-6:sIL-6R complex is active and the free IL-6 itself is active on membrane-bound IL-6 receptor.[11] Thus, it is difficult to confidently determine IL-6 bioactivity in vivo based on measurement of IL-6 and sIL-6R levels.

We conclude that physiologic changes in serum estradiol during the menstrual cycle are associated with changes in IL-6 and PTH levels. However, the role of IL-6 in bone remodeling during the menstrual cycle remains to be determined.

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