C-reactive protein (CRP) is one of the most commonly used markers of acute phase reaction in clinical settings and predictors of cardiovascular risk in healthy women; however, data on its physiologic regulation in premenopausal women are sparse. The objective of this study was to evaluate the association between endogenous reproductive hormones and CRP in the BioCycle Study (2005–2007). Women aged 18–44 years from western New York were followed prospectively for up to 2 menstrual cycles (n = 259). Serum levels of CRP, estradiol, progesterone, luteinizing hormone, and follicle-stimulating hormone were measured up to 8 times per cycle, timed by fertility monitors. CRP levels varied significantly across the cycle (P < 0.001). More women were classified as being at elevated risk of cardiovascular disease (CRP, >3 mg/L) during menses compared with other phases (12.3% vs. 7.4%; P < 0.001).

A 10-fold increase in estradiol was associated with a 24.3% decrease in CRP (95% confidence interval: 19.3, 29.0). A 10-fold increase in luteal progesterone was associated with a 19.4% increase in CRP (95% confidence interval: 8.4, 31.5). These results support the hypothesis that endogenous estradiol might have antiinflammatory effects and highlight the need for standardization of CRP measurement to menstrual cycle phase in reproductive-aged women.

Abbreviations: CRP, C-reactive protein; hs-CRP, high-sensitivity C-reactive protein.
assay, in multiple well-timed measurements across the menstrual cycle in premenopausal women.

MATERIALS AND METHODS

The BioCycle Study was a prospective cohort study among 259 healthy, regularly menstruating women aged 18–44 years recruited between 2005 and 2007 from western New York. Exclusion criteria included use of oral contraceptives during the last 3 months, current use of vitamin and mineral supplements or prescription medications, pregnancy or breastfeeding in the past 6 months, diagnosis of polycystic ovary syndrome, recent history of infections or diagnosis of chronic medical conditions, and self-reported body mass index at screening of <18 or >35 kg/m². Details of the study have been published elsewhere (18). Of the 449 women screened, 318 (70.8%) were eligible, and a total of 276 women enrolled (9 women completed 1 cycle as part of a pilot study, 250 women completed 2 cycles, and 17 women withdrew before completing 1 cycle). Although not markedly different, those eligible were slightly younger and more likely to report being of the white race. The most common reasons for ineligibility were a positive Chlamydia screen, outside the body mass index range, history of sexually transmitted disease, current use of an excluded medication, and self-reported fibroids. The University at Buffalo Health Sciences Institutional Review Board approved the study, and all participants provided written, informed consent. Under a reliance agreement, the National Institutes of Health depend on the designated University at Buffalo Health Sciences Institutional Review Board for review, approval, and continuing oversight of their human subject research for the BioCycle Study.

Study visits were scheduled on approximately the second day of menstruation, mid- and late-follicular phase, 2 days around expected ovulation, and early, mid-, and late-luteal phase in each cycle. Fertility monitors (Clearblue Easy Fertility Monitor; Inverness Medical, Waltham, Massachusetts) were used to assist in the timing of specimen collection and to capture critical windows of hormonal variation, including ovulation (19). The date of the observed serum luteinizing hormone surge was used to further classify visits into correct cycle phase categories. For ovulatory cycles (defined as cycles with luteal progesterone concentrations of >5 ng/mL and observed serum luteinizing hormone peaks before the luteal phase (20)), the visit with the peak serum luteinizing hormone level was classified as the luteinizing hormone surge, and data for other cycle visits were reclassified accordingly (21). Visits from ovulatory cycles that lacked evidence of a luteinizing hormone surge (due to missed clinic visits) were classified on the basis of data from the fertility monitors, calendar dates of cycle visits, hormone levels at each visit, and menses information. Visits from anovulatory cycles were not reclassified and were included in the current analysis.

Biologic specimens

Fasting blood specimens were generally collected between 7:00 and 8:30 AM at each clinic visit. Blood collection and handling protocols were designed to minimize variability in preanalytical factors (22). All samples were processed and frozen at −80°C within 90 minutes of phlebotomy and shipped on dry ice to analytical laboratories as a complete cycle (up to 8 samples) from each participant. Ninety-four percent of participants completed at least 7 clinic visits per cycle, and 100% completed 5 visits or more per cycle.

Reproductive hormones. Estradiol, progesterone, luteinizing hormone, and follicle-stimulating hormone were measured in fasting serum samples collected at each visit at the Kalieda Health Center for Laboratory Medicine (Buffalo, New York). Estradiol was measured by radioimmunoassay (<10% total coefficient of variation). Progesterone, luteinizing hormone, and follicle-stimulating hormone were measured by solid-phase competitive chemiluminescent enzymatic immunoassay by Specialty Laboratories, Inc. (Valencia, California) on the Immulite 2000 analyzer (Diagnostic Products Corporation, now part of Siemens Medical Solutions Diagnostics, Deerfield, Illinois) (<14% total coefficient of variation for progesterone, <4% total coefficient of variation for luteinizing hormone and follicle-stimulating hormone).

C-reactive protein. To assess coronary heart disease risk, investigators must measure CRP by high-sensitivity methods (hs-CRP) capable of determining concentrations within the healthy reference interval (23). For purposes of sample economy, hs-CRP was measured simultaneously with progesterone and gonadotropins by using the IMMULITE 2000 platform as opposed to the conventional nephelometric high-sensitivity method. This hs-CRP chemiluminescent immunoassay is sensitive to 0.3 mg per liter and is cleared by the Food and Drug Administration to meet guidelines from the American Heart Association and the Centers for Disease Control that recommend hs-CRP as a more sensitive assay for the prediction of vascular disease, compared with traditional assays for circulating CRP levels (24, 25). Levels of serum total cholesterol, high density lipoprotein cholesterol, and triglycerides were determined on a Beckman LX20 automated chemistry analyzer (Beckman, Brea, California) (<5% total coefficient of variation for all assays). Low density lipoprotein cholesterol was determined by using the formula of Friedewald et al. (26).

Covariate assessment

At baseline, participants completed questionnaires regarding lifestyle, family and reproductive health history, and physical activity (International Physical Activity Questionnaire) (27). High, moderate, and low physical activity categories were created on the basis of standard International Physical Activity Questionnaire cutpoints. Height and weight were measured in the clinic by standardized protocols and used to calculate body mass index (kg/m²). At each clinic visit, women reported whether they had been ill (cold, flu, or other) in the past 7 days. In daily diaries, women recorded the type, frequency, and dosage of nonsteroidal antiinflammatory drug use. All covariates had less than 5% total missing data.

Statistical analysis

Descriptive statistics were computed for all study variables according to tertile of baseline CRP levels and American Heart Association cardiovascular disease risk cutpoints: CRP <1 mg/L, low; CRP 1–3 mg/L, moderate; or CRP >3 mg/L, elevated (24). Baseline CRP levels were

measured on the second day of menses during the first cycle. CRP measurements of $>10$ mg/L ($<1\%$ of the data), generally indicative of viral or bacterial infection, were excluded from baseline comparisons and analyses of American Heart Association classifications across the cycle (28). Exact chi-square tests and analysis of variance were used to test for associations between demographic variables and CRP levels.

CRP and hormone levels were log transformed for normality. Six percent of the CRP measurements were below the limit of detection (0.1 mg/L) and were accounted for by using multiple imputation (29). Median, interquartile range, and variance of CRP, as well as the percentage of women with CRP levels above desirable ranges as identified by the American Heart Association, were calculated for each cycle phase visit. Linear mixed models were used to test for associations between cycle phase and CRP level and a difference in American Heart Association classifications by cycle phase while taking multiple cycles per woman into account (30).

Weighted linear mixed-effects models with random intercepts were used to evaluate the association between log hormone levels and log CRP concentrations (30). Random intercepts were included to account for variation in baseline CRP among women. Because hormone levels change over the cycle in response to complex feedback mechanisms with other hormones, stabilized inverse probability weights were used to appropriately adjust for time-dependent confounding by the other reproductive hormones (31). To estimate the stabilized weights, the conditional density of estradiol levels at each cycle visit while adjusting for other factors was obtained by ordinary least-squares regression and estimated by the normal distribution (31, 32).

Choice of the covariates considered in the weighted models was determined by a review of prior literature and included age, body mass index, race, serum total cholesterol, and other reproductive hormone levels. Measures of triglycerides, acute illness, nonsteroidal antiinflammatory drug use, physical activity, and smoking status were considered as potential covariates but did not appreciably alter the estimates. For estradiol and follicle-stimulating hormone, models included up to 8 measurements per cycle as separate observations. For progesterone, only measurements during the luteal phase were included and, for luteinizing hormone, only measurements immediately preceding and during ovulation were included because of minimal biologic variation during other phases. Because the exposure and outcome variables were log-transformed, results of the models are presented as a percent change in the nontransformed values per 10-fold increase in the nontransformed exposure using the following formula: $[(10^{\beta} - 1) \times 100 \%]$, where $\beta$ is the regression coefficient. All statistical analyses took multiple cycles per woman and the multiple imputation into account by the American Heart Association classifications across the cycle (28). Exact chi-square tests and analysis of variance were used to test for associations between demographic variables and CRP levels.

C-reactive protein variation over the cycle

Levels of CRP varied significantly across the menstrual cycle ($P < 0.001$). CRP was highest during menses (median, 0.74 mg/L), decreased during the follicular phase, was lowest on the expected day of ovulation (median, 0.45 mg/L), and increased in the luteal phase (Figure 1). The variance of CRP was 50% higher during menses as compared with the rest of the cycle (0.82 vs. 1.23 (mg/L)$^2$).

The distribution of CRP in cardiovascular disease risk categories by menstrual cycle phase is illustrated in Figure 2. The largest percentage of women were classified as moderate-to-elevated risk of cardiovascular disease (hs-CRP, $>1$ mg/L) when measured during menses (40.0%), while the fewest number of women were classified as such on the predicted day of ovulation (28.9%). Every clinic visit, except for the midluteal phase visit ($P = 0.2$), had significantly fewer women classified as high-to-moderate risk of cardiovascular disease when compared with the menses visit ($P < 0.05$). Of note, only 2% ($n = 6$) of women had CRP levels consistently above 3 mg/L at all visits. However, 83 women (32.1%) had levels above 3 mg/L on at least 1 cycle visit. Of cycles where only 1 visit was $>3$ mg/L, 41% of the time this occurred during menses. To simulate the current American Heart Association recommendation for CRP testing (24), we compared the mean of 2 tests taken approximately 2 weeks apart. When one of the 2 tests was taken during menses, the mean CRP was significantly higher than when neither of the 2 tests was taken during menses (1.75 vs. 1.35 mg/L; $P = 0.004$).

Reproductive hormones

Estradiol was inversely associated with CRP levels across the menstrual cycle after controlling for age, race, body mass index, serum total cholesterol, luteinizing hormone, follicle-stimulating hormone, and progesterone levels ($P < 0.001$) (Table 2). Specifically, a 10-fold increase in estradiol (as is characteristic of typical menstrual cycle fluctuation) was associated with a 24.3% decrease in CRP levels. In the fully adjusted model, a 10-fold increase in progesterone was associated with a 19.4% increase in CRP in the luteal phase. In the unadjusted model, follicle-stimulating hormone was inversely associated with CRP across the cycle (3.6% decrease in CRP per 10-fold increase in follicle-stimulating hormone); however, after adjustment, this association was no longer statistically significant ($P = 0.41$). Luteinizing hormone was not associated with CRP levels.

RESULTS

Demographics

BioCycle Study participants were predominately young (mean age, 27.3 years), normal weight (mean body mass index, 23.9), Caucasian (59.7%), nonsmokers (81.8% never smoked), and physically active (90.3% reported moderate to high physical activity) (Table 1). Women in the second and third tertiles of baseline CRP level tended to be older, of higher body mass index, and to have slightly higher lipoprotein cholesterol and significantly higher triglyceride levels than those in the first tertile. Additionally, a greater proportion of Caucasians, past or current smokers, and past oral contraceptive users were observed among women in the second and third tertiles of baseline CRP compared with the first tertile.
CRP was inversely associated with estradiol across the menstrual cycle and positively associated with luteal progesterone levels. These associations persisted after controlling for demographic (i.e., age, race, and body mass index) and time-dependent (i.e., total cholesterol and other reproductive hormones) confounders. These results support the hypothesis that endogenous estrogens may act as antiinflammatory agents. Additionally, these findings have important implications for research and clinical practice because of CRP's predictive value for cardiovascular disease in premenopausal women. In our study, the cardiovascular disease risk categorization of women by CRP level varied significantly across the menstrual cycle, suggesting the importance of consideration of menstrual cycle variability in measurement of CRP in women of reproductive age.

The cyclic changes in CRP and association with estradiol are supported biologically. Generally speaking, the events of the menstrual cycle involve inflammatory-like mechanisms (33). Thus, observing cyclic changes in an inflammatory biomarker, such as CRP, across the menstrual cycle is not unexpected. The antiinflammatory effect of estradiol on CRP is consistent with estradiol’s effect on inflammation in many tissue types, as estrogens have negative effects on inflammatory cell migration and inflammatory marker production in diverse nonreproductive, nonimmune tissues (8). Estrogen receptors are highly expressed in endothelial and vascular smooth muscle cells throughout the human body (9). One of estrogen’s main antiinflammatory roles is through generating...
nitric oxide, an important vasoprotective molecule (34). Apart from being a potent vasodilator, nitric oxide exerts an antiinflammatory role on the endothelium, as manifested by decreased leukocyte recruitment and scavenging of reactive oxygen species (35). Estrogen has also been shown to reduce levels of tumor necrosis factor-alpha, a major proinflammatory cytokine (36). This in turn reduces the synthesis and release of chemokines like interleukin-8 and platelet activating factor, as well as down regulating adhesion molecules such as intercellular adhesion molecule 1 and E-selectin, which leads to recruitment of leukocytes (the hallmark of inflammation) (37). Finally, estrogen can act as an antiapoptotic agent on various cell types including endothelial cells by preventing the release of cytochrome c from the mitochondria, thus reducing subsequent vascular inflammation (38).

Our finding that progesterone is positively associated with CRP is difficult to reconcile with many observations of progesterone exerting overall antiinflammatory effects on the immune system. Although progesterone can promote the chemotaxis of neutrophils and increase production of some inflammatory mediators (interleukin-6 and leukemia inhibiting factor) by monocytes (39), progesterone also decreases natural killer cell activity, macrophage tumor necrosis factor, and nitric oxide synthase production and inhibits T-cell

Table 1. Continued

<table>
<thead>
<tr>
<th>AHA Classification</th>
<th>&lt;1 mg/L</th>
<th>1–2.99 mg/L</th>
<th>≥3 mg/L</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%)</td>
<td>Mean (SD)</td>
<td>No. (%)</td>
<td>Mean (SD)</td>
<td>No. (%)</td>
</tr>
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<td>144</td>
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<td>29.4 (8.4)</td>
<td>28.7 (8.5)</td>
<td>0.003</td>
</tr>
<tr>
<td>13</td>
<td>22.5 (2.8)</td>
<td>25.6 (3.9)</td>
<td>26.8 (4.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>58</td>
<td>50.7</td>
<td>52.0 (7.6)</td>
<td>58.4 (8.4)</td>
<td>0.60</td>
</tr>
<tr>
<td>73</td>
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<td>75.8 (8.4)</td>
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</tr>
<tr>
<td>12</td>
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<td>12.3 (3.9)</td>
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</tr>
<tr>
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<td>20.3 (3.9)</td>
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</tr>
<tr>
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<td>67.2 (8.4)</td>
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</tr>
<tr>
<td>95</td>
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<td>12.7 (1.6)</td>
<td>0.03</td>
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<tr>
<td>22</td>
<td>15.3</td>
<td>18.9 (7.6)</td>
<td>18.9 (8.4)</td>
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<tr>
<td>8</td>
<td>5.6</td>
<td>6.9 (1.2)</td>
<td>7.0 (1.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>12</td>
<td>29.0</td>
<td>29.0 (4.8)</td>
<td>29.0 (4.4)</td>
<td>0.99</td>
</tr>
<tr>
<td>100</td>
<td>0.4 (2.8)</td>
<td>1.8 (3.1)</td>
<td>4.9 (4.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>162.5</td>
<td>100.5</td>
<td>160.8 (31.0)</td>
<td>171.0 (25.8)</td>
<td>0.26</td>
</tr>
<tr>
<td>100.5</td>
<td>160.8</td>
<td>161.0 (28.6)</td>
<td>171.0 (25.8)</td>
<td>0.26</td>
</tr>
<tr>
<td>51.1</td>
<td>48.6</td>
<td>48.6 (11.4)</td>
<td>49.6 (13.4)</td>
<td>0.31</td>
</tr>
<tr>
<td>54.6</td>
<td>62.9</td>
<td>62.9 (21.9)</td>
<td>63.6 (27.9)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Abbreviations: AHA, American Heart Association; CRP, C-reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein; NSAID, nonsteroidal antiinflammatory drug; OC, oral contraceptive; SD, standard deviation.

* Of 11 missing values, 2 were missed serum collections and 9 were set to missing because of concentrations >10 mg/L.

* Body mass index: weight (kg)/height (m)².

* Serum biomarkers were measured on day 2 of the first menstrual cycle.
development and activity (40). Therefore, our results suggesting that increases in progesterone typical of normally cycling women have an overall inflammatory effect require further study.

The observed fluctuation in CRP across the menstrual cycle and its association with endogenous reproductive hormones are consistent with results from several studies (13–15) and at odds with 2 studies (16, 17). In general, previous studies were limited by small sample size (<37 women) (13–17), restricted follow-up time (1 cycle) (14–17), and issues with serum collection timing (13). In general, studies with several CRP measurements were able to detect cyclic changes in CRP.
levels across the menstrual cycle. In particular, 2 studies with 7–12 measurements across the cycle observed fluctuations in CRP across the cycle, with maximum values occurring during the menses and the early follicular phase (13, 14). Similar to our findings with estradiol, those of Wander et al. (13) and Blum et al. (14) observed that a 10-fold increase in estrogen across the cycle resulted in a 29% and a 41% decrease in CRP, respectively. Wander et al. (13) further observed a 29% decrease in CRP per 10-fold increase in progesterone. Most studies that compared only 3 phases per cycle did not observe the same cyclic changes in CRP (16, 17). Although 1 study did find significant differences in CRP when comparing 3 phases (follicular, midcycle, and luteal), they were not in the same pattern that we observed (15).

Our study included intensive monitoring of a large number of young, ethnically diverse women throughout 2 menstrual cycles. To our knowledge, this is the largest, most comprehensive study of CRP across the menstrual cycle to date. Having 8 clinic visits per cycle timed with fertility monitors significantly decreased the probability of misclassifying menstrual cycle phase. The use of the hs-CRP assay as opposed to the standard CRP assay allowed us to measure very small amounts of CRP in the blood (range, from 0.5 to 10 mg/L) that are not quantifiable with the standard methods (range, from 10 to 1,000 mg/L). Although the regular CRP test is more common for patients at risk for infections or chronic inflammatory diseases, the hs-CRP assay is required to assess potential risk for heart problems among seemingly healthy people. Thus, given the range of levels observed in this study, regular CRP methods would have been insufficient. We had a wide variety of information on participants’ characteristics that increased our ability to adjust for confounding.

Finally, the prospective design and exclusion criteria of the study strengthen the ability to draw inference, having reduced the potential for bias from known risk factors for inflammation and hormonal abnormalities.

Although hs-CRP is currently considered a reliable biomarker of chronic inflammation (24), caution should be taken to infer an antiinflammatory mechanism based solely on this biomarker, because we did not have direct measurements of other serum inflammatory markers to confirm our results (i.e., serum interleukin-6, tumor necrosis factor-alpha, and other cytokines). While our study included the use of fertility monitors to time visits and visits were realigned on the basis of post-hoc evaluation of serum luteinizing hormone peaks, misclassification could have been introduced through mistimed sample collection; however, various indicators of successfully timed visits were found to be unrelated to hormone or CRP concentrations, and thus any misclassification is likely to be nondifferential. We restricted our study sample to healthy, regularly menstruating women in order to exclude potential confounders by design, but such restrictions could also limit the generalizability of our findings to other populations.

In conclusion, we observed that CRP concentrations varied across the menstrual cycle, attaining the highest and most variable levels during menses. Furthermore, CRP was significantly inversely associated with endogenous estradiol and positively associated with luteal progesterone. Given that CRP is one of the most commonly used markers of acute-phase reaction in clinical settings and the strongest predictor of cardiovascular disease in young women, measurement of CRP in clinical settings and future research studies should be standardized to menstrual cycle phase. To date, this study

### Table 2. Weighted Linear Mixed-Effect Models of the Association Between Reproductive Hormones and C-reactive Protein in the BioCycle Study, Buffalo, New York, 2005–2007

<table>
<thead>
<tr>
<th>Hormones and Model</th>
<th>β (SE)</th>
<th>% Change Per 10-Fold Increase in Exposure</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (all phases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−0.10 (0.01)</td>
<td>20.57</td>
<td>−25.53, −15.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−0.12 (0.01)</td>
<td>24.32</td>
<td>−29.04, −19.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Progesterone (luteal phase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.08 (0.02)</td>
<td>19.95</td>
<td>9.40, 31.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>0.08 (0.02)</td>
<td>19.40</td>
<td>8.39, 31.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH (ovulation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>−0.02 (0.02)</td>
<td>−3.84</td>
<td>−11.49, 4.71</td>
<td>0.37</td>
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<tr>
<td>2</td>
<td>−0.01 (0.02)</td>
<td>−2.73</td>
<td>−11.28, 6.66</td>
<td>0.57</td>
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<tr>
<td>FSH (all phases)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>−0.05 (0.03)</td>
<td>−3.62</td>
<td>−19.46, −2.95</td>
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</tr>
<tr>
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<td>−0.02 (0.02)</td>
<td>−4.06</td>
<td>−12.70, 5.68</td>
<td>0.41</td>
</tr>
</tbody>
</table>

**Abbreviations:** β, effect estimate; CI, confidence interval; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SE, standard error.

<sup>a</sup> Unadjusted.

<sup>b</sup> Weighted for age (continuous), race (white, black, other), body mass index (continuous), total cholesterol (continuous, log transformed), and other reproductive hormones (continuous, log transformed).
provides the most comprehensive assessment of the interplay between endogenous hormones and CRP among women of reproductive age. More research is warranted to further clarify the role of endogenous hormones on other biomarkers of inflammation in premenopausal women.

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REFERENCES


