CLINICAL STUDY

Serum leptin and C-reactive protein levels in the physiological spontaneous menstrual cycle in reproductive age women

D M Wunder¹, M Yared¹, N A Bersinger¹, D Widmer¹, R Kretschmer² and M H Birkhäuser¹¹Department of Obstetrics and Gynaecology, University of Berne, Switzerland and ²Central Laboratory, Inselspital, Switzerland

(Correspondence should be addressed to D M Wunder; Universitäts-Frauenklinik, Inselspital, Effingerstrasse 102, Berne, CH-3010, Switzerland; Email: dorothea.wunder@insel.ch)

Abstract

Objective: Only a few studies have investigated variations of different markers for inflammatory processes during the physiological menstrual cycle. The results are conflicting, particularly concerning the correlation between the marker leptin and steroid hormones. The aim of the study was to investigate the inflammatory markers C-reactive protein (CRP) and leptin in the serum of healthy, normally ovulating women and to correlate these with each other and with the hormones of the gonadal axis. A cycle-dependence of the markers studied would imply an exact timing of the blood sampling for clinical needs.

Design: Observational study investigating the two inflammatory markers CRP and leptin in relation to the hormonal pattern of the gonadal axis during the normal cycle.

Methods: Ovulatory cycles of 36 healthy, young, normo-androgenic women, having a normal body mass index were evaluated. Serum concentrations of leptin and CRP, as well as of follicle-stimulating hormone, luteinising hormone, 17 β -oestradiol, progesterone, prolactin (PRL) and free testosterone were measured every 1–2 days during one full cycle.

Results: Serum levels of leptin and CRP behaved differently during ovulatory cycles, with higher concentrations for leptin only during certain phases. Significant correlations were found in the follicular phase between leptin and PRL and leptin and free testosterone.

Conclusions: Leptin levels change during the menstrual cycle. Leptin levels are more stable on cycle days 1–5 than later in the cycle. For precise cycle-independent measurements, these fluctuations have to be taken into account. There is no similar cyclic pattern for CRP.

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Introduction

Leptin is a hormone encoded by the obesity (*ob*) gene. Initially, it was found to have anti-obesity effects, but then it has been shown to have also immunoregulatory (1), pro-inflammatory (2) and angiogenic (1, 3) properties. Influences on reproduction (4) and involvement in the pathogenesis of endometriosis (5) and infertility (6, 7) have also been demonstrated. Concentrations of circulating leptin are increased by inflammatory mediators (8). Results published on serum leptin levels during the physiological menstrual cycle vary considerably. Some studies showed significant increase in the late follicular phase (9, 10), and some did so on the day of the onset of the luteinising hormone (LH) surge (11, 12). Other investigations described significant increase in serum leptin levels in the late luteal phase (13–17), and again others reported only small, not statistically significant variations during the menstrual cycle (18–21). The acute phase C-reactive protein (CRP) is produced by the liver. A strong correlation has been found between the body mass index (BMI) and both

leptin and CRP levels (22–25). Shamsuzzaman *et al.* (26) showed an association between plasma CRP and leptin in men and non-pregnant women, and Bullo *et al.* (27) even demonstrated that leptin could induce the production of CRP. Maruna *et al.* (28) confirmed the correlation between CRP and leptin, and identified the latter as an acute phase reactant with potential haematopoietic, immunomodulatory, and hepatocyte-stimulating activity. To our knowledge only two studies have been published to date investigating CRP levels during the physiological menstrual cycle: in a small sample of patients, Jilma *et al.* (29) found increased CRP concentrations at midcycle and in the luteal phase, with a significant correlation with progesterone levels. Blum *et al.* (30) also reported varying CRP concentrations during the menstrual cycle. As ovulation is an inflammatory process, the interactions and correlations between leptin, CRP and the gonadal hormones in the physiological menstrual cycle are of great interest in this context. To the best of our knowledge, no longitudinal studies comparing these two acute phase reactants in the normal menstrual cycle have been performed

yet; and generally only a small body of information on CRP in reproduction is currently available. The aim of the present study was to examine, in the physiological menstrual cycle, whether leptin and CRP, which are both involved in inflammatory processes, are correlated with each other and with the other hormones of the pituitary–gonadal axis. If there would be a correlation with cycle-dependent hormones such as oestradiol (E2), progesterone or androgens, clinical recommendations would be needed concerning the timing of the blood sampling of inflammatory factors within the cycle. Otherwise, a misinterpretation of cycle-dependent values would be possible, with potentially serious clinical consequences.

Materials and methods

Evaluation of volunteers

The present study was conducted amongst 36 Caucasian women. They were recruited through advertising at the University campus and most of them were students. They were aged between 20 and 32 years, reported regular menstrual cycles and had not been taking oral contraceptives or other medicines for at least 3 months. Other inclusion criteria were a good health status, a normal ($18.5\text{--}25\text{ kg/m}^2$) BMI and normal pretreatment hormonal values for follicle-stimulating hormone (FSH), LH, prolactin (PRL), $17\beta\text{-E}_2$, free testosterone and dehydro epiandrosterone sulphate. In addition, haematological and chemical laboratory analyses, a physical examination, and the results from a gynaecological ultrasound and a Papanicolou (PAP) cervical smear had to be normal. Therefore, women with polycystic ovary syndrome have been excluded. Finally, a biphasic pattern of the basal body temperature (BBT) was required. All examinations were performed prior to the collection of blood from the subjects for the study cycle. Primary criteria for exclusion were pregnancy, dysovulation with a monophasic BBT chart and a short luteal phase, use of medicines, or inaccessibility of follow-up. The investigation was approved by the ethical committee of the University of Berne, and written informed consent was obtained from each volunteer prior to involvement in the project.

Collection of sera

Blood samples were obtained by venipuncture, using 21-gauge needles and 9 ml serum monovettes. All blood samples were drawn, after an overnight fast, between 06 00 and 10 00 h every second day from the onset of the cycle and during the whole follicular phase, until 2–5 days before ovulation. From then, samples were drawn on each day until 4 days after ovulation had taken place. Ovulation was monitored by performing five to eight urinary LH detection tests (Today-Ovulation Test; Ultimate Products Gabmed, Ahrensburg, Germany) from

the late follicular phase. During the luteal phase and until the end of the cycle (onset of menstruation), blood samples were again taken every second day. Thus, in one ovulatory cycle, 14–19 (mean 16) samples per volunteer could be collected depending on the length of the cycle. Every participant registered her own morning BBT 1–4 months prior to the beginning as well as during the study cycle. The collected blood was allowed to clot and centrifuged within 60 min after venipuncture, and the obtained serum was divided into 1 ml aliquots and stored at -80°C .

Measurement of leptin, CRP, gonadotrophins, and steroid hormones in serum

Leptin was quantified with a microplate enzyme immunoanalytical method (ELISA) developed in our laboratory (32) using a matched pair of MABs (R&D Systems Europe; Abingdon, England). The samples were diluted 1:50 in PBS containing 0.5% non-fat milk proteins (Blotto; Pierce, USA; obtained from Socochim; Lausanne, Switzerland). Subsequent incubations were as described (32). The determination of leptin after frozen storage has been validated (32). Intra- and inter-assay coefficients of variance for leptin were 2.7 and 8.9%, respectively. CRP was also measured manually, according to our recently published immunoanalytic method (33). The initial sample dilution was 1:250, which sometimes had to be readjusted to a higher or lower factor. With 0.5 ng/ml, the sensitivity of the assay was higher than the one of currently used nephelometric methods; the lowest CRP concentration measured in this study was 66 ng/ml. Intra- and inter-assay coefficients of variance for CRP were 3.1 and 5.5%, respectively. All other hormones (FSH, LH, E2, progesterone, free testosterone, PRL) were determined in the central routine laboratory of the University hospital, by means of the automated Elecsys 2010 method (Roche).

Statistical methods

Comparisons between the medians of marker levels at the early follicular, ovulatory, or midluteal stage were made non-parametrically using the Mann–Whitney *U*-test. Associations between these concentrations were investigated non-parametrically by Spearman-rank correlation analysis, and those between leptin with the BMI by linear regression.

Results

Clinical data

Clinical parameters of the volunteers are given in Table 1. The population was very homogeneous in terms of age, BMI, basal FSH levels, and length of the menstrual cycle.

Table 1 Clinical parameters of the volunteers. Values given are means $\pm$ S.D.

Clinical parameter	Values
Age (yrs)	25.5 $\pm$ 5.5
Body mass index (kg/m ²)	21.9 $\pm$ 3.1
Basal FSH (mIU/ml)	7.1 $\pm$ 1.9
Cycle length (days)	28.7 $\pm$ 6.7

Steroid hormone, leptin and CRP concentrations in the sera

Hormonal values at the different phases of the spontaneous cycle Cross-sectional results are given in Table 2 for the early follicular phase, the day of ovulation, and the midluteal phase. On the day of ovulation, sera from all subjects were available; for the other two time points, they were combined for the days -12 and -11 , or for the days $+7$ and $+8$, respectively, thus yielding a value for each subject (see Methods). Median leptin levels were significantly higher in the midluteal than in the early follicular phase ($P=0.042$). Leptin levels strongly correlated with the BMI independent of the cycle stage ($r>0.63$, $P<0.0001$ in all three groups). No significant differences in CRP concentrations were observed between the three time points in the cycle. This did not change after correction of the CRP concentrations for BMI (data not shown).

Time course of leptin and CRP concentrations during the spontaneous cycle Leptin levels were lowest on the first and the last day of the cycle and reached their maximum in the midluteal phase (Day $+7$, Fig. 1). CRP level patterns showed no significant change during the cycle (Fig. 2). The mean values ranged between 200 and 4000 ng/ml. Three subjects showed isolated extremely high and clinically unexplained spiking levels of CRP from one sampling to the

next reaching 60 000 ng/ml and dropping again immediately to normal levels. These erratic patterns were excluded from further analysis. However, it has to be stressed that no significant difference could be found between the cycle pattern of the 36 women before exclusion of the spiking CRP levels (data not shown) and the remaining 33 women.

Correlations between leptin and CRP levels, steroid hormones, FSH, and PRL Significant correlations were observed in the follicular phase between leptin and PRL ($P=0.028$), leptin and free testosterone ($P=0.005$), and between CRP and free testosterone ($P<0.001$). Spearman's rank-correlation analysis showed no correlations between CRP and leptin, either in the follicular (day -16 to -3) (243 sera), periovulatory (day -2 to $+2$) (159 sera, $P>0.1$ for both), or luteal phase (day $+3$ to $+15$) (199 sera, $P=0.079$). The ovarian steroid hormones (E2, progesterone) did not show any correlations with leptin or CRP. In the luteal phase, no correlations between any pair of markers were found.

Discussion

Endocrine diagnosis in women depends on the exact knowledge of cyclic fluctuations of the hormones measured. The aim of the present study was to explore the possibility of cycle-dependent fluctuations of leptin and of the inflammatory marker CRP. In daily routine, this knowledge is particularly important for CRP, a frequently used clinical marker. The results published to date on leptin levels in the menstrual cycle differ remarkably. Some studies showed significant increase of leptin in the late follicular phase (9, 10), some on the day of the LH surge onset (11, 12), while others in the late luteal phase (13–17). Further studies reported small, non-significant variations during the menstrual cycle (18–21). Regarding the correlations between leptin and other hormones, similar inconsistencies

Table 2 Leptin, CRP, gonadotrophins, PRL and ovarian steroid hormone concentrations in the serum of 36 subjects during a normal ovulatory cycle. Values (medians and range) are given for the early follicular (day LH $-12/-11$), midcycle (day LH), and the midluteal phase (day LH $+7/+8$).

Marker or hormone	Cycle phase		
	Early follicular	Day LH	Midluteal
Leptin (ng/ml)	12.9 (3.9–37.5)	13.8 (5.4–57.0)	17.6 (5.8–53.2) [†]
Leptin/BMI	0.64 (0.22–1.54)	0.62 (0.26–2.34)	0.84 (0.25–2.25) [†]
CRP (ng/ml)	2313 (254–8265)	1817 (232–7078)	1883 (245–5921)
FSH (mIU/ml)	7.30 (3.40–12.00)	13.10 (3.53–25.30)	3.16 (1.19–6.04)
LH (mIU/ml)	5.06 (2.46–15.80)	40.3 (13.8–89.7)	5.54 (0.16–20.80)
Prolactin (ng/ml)	27.3 (5.64–79.8)	31.0 (10.8–70.3)	28.7 (6.9–78.8)
Oestradiol (pmol/l)	135 (12–409)	1095 (266–2075)	752 (126–1484)
Progesterone (ng/ml)	0.77 (0.34–2.53)	1.12 (0.76–2.15)	13.25 (2.02–28.8)
Testosterone (ng/ml)	0.47 (0.17–1.48)	0.63 (0.23–1.33)	0.50 (0.10–1.44)

[†] $P=0.04$ compared to the early follicular phase (Mann–Whitney U-test).

Figure 1 Concentrations of circulating leptin (●) and oestradiol (○) during the normal ovulatory menstrual cycle in 36 healthy women. Values shown are means $\pm$ S.E.M. The curve shown was smoothed using the cubic spline (4-value) algorithm.

predominate in the literature. Some publications and some authors described correlations between leptin and oestrogens (11) or progesterone (15), whereas a majority of reports find no correlations at all (9, 10, 14, 16, 18, 21). One publication reported an association between leptin and LH (19), but in that study (19) only six normal-weight women at only

three-defined time points have been evaluated. In contrast, in our study, blood samples were taken at least every other day during a full menstrual cycle in 36 women. Therefore, the considerable differences in the number of patients included have to be taken into consideration. There are also differences in the assay methodologies; while in many older studies CRP was

Figure 2 Concentrations of circulating CRP during the normal ovulatory menstrual cycle in 33 healthy women. Values shown are means $\pm$ S.E.M. The curve shown was smoothed using the cubic spline (4-value) algorithm.

determined by a nephelometric method, we used the more recently validated and highly sensitive ELISA method (33). In our study presented here, precisely timed measurements were performed every day or every second day of the cycle with 36 Caucasian women. In the follicular phase, we could find a parallel of leptin, PRL ($P=0.028$) and free testosterone ($P=0.005$). Although it is well known that free testosterone and PRL rise during follicular maturation, there is no causative explanation for the correlation between leptin and follicular maturation. No correlations between sex steroids, PRL and leptin were found in the luteal phase. Concerning the inflammatory mediator CRP, only a few trials with low number of subjects have been studying its concentration pattern during the normal spontaneous cycle. Blum *et al.* (30) recently investigated eight young, normal-weight women with normal ovarian function and found that CRP concentrations changed significantly during the menstrual cycle. Concentrations were highest in the early follicular phase and inversely correlated to E2 concentrations. In the present study, we could not confirm cycle-dependent CRP variations in a clearly larger patient group and we did not find any correlation with E2. The differences between the results of Blum *et al.* (30) and the present data can most likely be explained by the nearly four times larger sample size and the slightly shorter (and thus more sensitive) intervals between samplings (2 days maximum) in our study. In another study on CRP levels in the menstrual cycle with 18 patients (29), a CRP increase of 44% at midcycle and 31% in the luteal phase in comparison to follicular phase levels were found. There was a significant correlation between the relative increase in CRP at midcycle and the relative increase in progesterone levels during midcycle and the luteal phase. This finding is also in contrast with our results; we could not find significant correlations between either the absolute concentrations of CRP and progesterone, or between their increments in the luteal phase. Knowing that CRP is an acute phase protein and that it is produced only a few hours after the onset of an inflammatory process, one could expect a cycle peak of CRP production around ovulation. On the other hand, the isolated, cycle-independent spikes in the three individuals in our sample could be explained by variables supposed to influence the CRP levels, such as physical activity (34, 35), consumption of coffee, stress (35) or genetic polymorphisms (36). The fact is intriguing that the pattern of CRP in the spontaneous cycle is completely different from the one observed in gonadotrophin-stimulated *in-vitro* fertilization (IVF) cycles. As we have shown, there is a significant increase in CRP levels on the day of ovarian pick-up (OPU), compared to the day of ovulation induction or 1 day before (33). The timing of the blood sampling, taken immediately before the OPU procedure, corresponds to the period just before ovulation. The rise in CRP seems quite logical given that

the ovulation is an inflammatory process. But these findings from IVF cycles do not concur with our present results from spontaneous menstrual cycles: in spontaneous, normal cycles there is no increase of CRP levels neither around the time of ovulation nor around the time of menstruation. This discrepancy might be explained by the supra-physiological E2 concentrations reached in assisted reproductive therapies. These high oestrogen levels may stimulate the production of CRP in the liver, resulting in a CRP pattern strongly different from the one seen in spontaneous, non-stimulated cycles. Similarly, elevated CRP levels have been found in women using oral contraceptives (37, 38) and also in women with tubal factor subfertility due to persistent *Chlamydia trachomatis* infection (39). Another theoretical explanation could be the fact that in contrast to physiological cycles, 5000–10 000 IU of human chorionic gonadotrophin (hCG) is administered in IVF treatment cycles. hCG could possibly have an impact on inflammatory reactions; however, precise data in the literature on this topic are missing. No association between CRP and leptin could be found neither in the present study (spontaneous menstrual cycles) nor in gonadotrophin-stimulated cycles (33). Considering that both these mediators are involved in inflammatory processes, the different concentration patterns indicate the presence of complex mechanisms. We conclude that in the menstrual cycle, CRP as an inflammatory marker and leptin in its role as an inflammatory mediator follow different concentration patterns. The inflammatory-mediating effects of leptin and CRP are unrelated to each other. Cycle-dependent fluctuations of leptin might be important in the diagnosis of metabolic dysfunctions, whereas there is no cycle-dependence for CRP. Therefore, CRP levels of fertile women in daily routine do not have to be evaluated in function of the menstrual cycle.

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