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Urinary cytokine and chemokine profiles across the menstrual cycle in healthy reproductive age women

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Abstract

Objective—To assess utility of urinary cytokines for monitoring reproductive function by considering detection, variation across the menstrual cycle, and relations with hormones.

Design—Longitudinal cohort study

Setting—Academic institution.

Participants—Healthy, reproductive aged women with self-reported regular menstrual cycles and at least one observed ovulatory cycle (n=248).

Intervention—None

Main outcome measure—Urinary cytokines measured by 30-plex immunoassays in 3550 biospecimens. Nested random-effects ANOVA and marginal structural models used to evaluate variability and relations with hormones.

Results—For 24 of 30 evaluated factors, detectable levels were observed in at least 50% of urine samples. Interleukin (IL)-6, IL-8, IL-10, IL-15, granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), interferon (IFN)- α , and RANTES levels varied significantly across the menstrual cycle. The pro-inflammatory factors IL-1 β , IL-6, IL-8, and HGF were 1.5 to 3 times higher during menses than the late follicular phase. In marginal structural models, IL-1 β , IL-6, IL-8 were associated with lower estradiol and progesterone.

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CONFLICT OF INTEREST

None to declare

Conclusions—Variability during the menstrual cycle and correlations with reproductive hormone levels support a role of cytokines in the menstrual cycle; however, because of the limited variability for most cytokines considered, the utility of urine as a matrix for assessment of inflammation in menstrual cycle function appears limited for clinical purposes.

Keywords

Cytokines; chemokines; inflammation; menstrual cycle; immunology

INTRODUCTION

Normal menstrual cycle function suggests a link between the endocrine and immune systems. Decidualization involves recruitment of a specialized population of leukocytes including macrophages and natural killer cells (1, 2). Leukocytes are also present in the ovary, and the type and count vary across the menstrual cycle (3, 4). Further, the rupture of the follicle and release of the ovum that occurs in ovulation has been described as an inflammatory process (5, 6), as has been menstruation (7, 8). Regulation of inflammation during these processes may be critical for embryo implantation and successful pregnancy.

Cytokines and chemokines play important roles in inflammation, angiogenesis, and tissue turnover. *In vitro* experiments and epidemiologic studies suggest involvement of cytokines regulating menstrual cycle function and throughout reproduction (4, 6, 9–12). Production of cytokines including interleukin (IL)-1 β , IL6, tumor necrosis factor (TNF)- α , and colony stimulating factors (CSF) is observed in ovarian tissue and/or follicular cells (13, 14). Leukemia inhibitory factor 1 – structurally similar to granulocyte (G)-CSF and related hematopoietic cytokines – appears critical for embryo implantation (15). Chemokines, including IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP) 1 α and 1 β , and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) are thought to be involved in decidualization, targeting specialized leukocyte populations to the endometrium (16). Cytokines appear to be active in a range of pregnancy-related processes (for review, see 17).

Cytokines have been previously evaluated for possible roles in endometrial receptivity to embryo implantation (18). Cytokine levels during the menstrual cycle in women of reproductive age correlate with treatment outcomes among women undergoing assisted reproduction (19). Additionally, if cytokine-regulated inflammation mediates ovarian function, cytokine profiles during ovulation and the window of implantation may help explain failures of embryo implantation and early pregnancy loss (20), or indicate underlying gynecologic disorders (2).

Research of menstrual cycle variability of cytokines has been conflicting (1, 20–22). Assessment of menstrual cycle function and within-person variation of inflammatory factor levels requires a longitudinal design, along with large sample sizes and multiple, well-timed biospecimens per person, representing significant practical challenges for investigators and potential burdens for research subjects. Prior research has utilized tissue samples and vaginal wash, or assessed systemic levels via venous blood draw (23). These samples require invasive approaches for collection, limiting their utility for longitudinal assessments.

Urine samples are commonly utilized for longitudinal studies of reproductive function. These samples are easily obtainable and may be self-collected by participants at home, and thus may be more feasible for longitudinal studies requiring repeated sample collection than other matrices with more invasive collection procedures. Urinary cytokine levels have been explored for assessment of inflammation in congestive heart failure (24), HIV-associated wasting (25), and non-Hodkins lymphoma (26), as well as response to acute stress (27). However, urinary cytokine levels have not been utilized to assess menstrual cycle function in asymptomatic women, where inflammation is expected to be low. In this study we considered urinary levels of cytokines and chemokines in a longitudinal study of menstrual cycle function. Specifically, our aims were: to assess the degree to which cytokine and chemokine levels are detectable in urine; to evaluate variability of urinary cytokine levels and determine whether changes in levels at menses and ovulation observed in blood and vaginal wash are also seen in urine; and to estimate relations with those of reproductive hormones.

MATERIALS and METHODS

The BioCycle Study

BioCycle is a prospective study of menstrual cycle function previously described in detail (28). Female participants ($n=259$) of reproductive age (mean=27.3 years, range=18–44) were followed for one ($n=9$) or two ($n=250$) menstrual cycles (total observed cycles = 509). The mean body mass index (BMI) in the cohort was 23.9 kg/m², 60% were Caucasian, 20% were Black/African American, 16% Asian and 4% were of other race/ethnicities. Participants reported high levels of physical activity and included few current smokers (4%). The University at Buffalo Health Sciences Institutional Review Board (IRB) approved the study and served as the IRB designated by the NIH for this study under a reliance agreement. All participants provided written informed consent.

Participants completed questionnaires at baseline and subsequent clinic visits, and maintained daily diaries as well. Blood and urine specimens were collected at eight clinic visits during each menstrual cycle, per study protocol. Participants came to the clinic after fasting overnight and visits were scheduled for approximately the same time of the morning. All participants completed at least five of the eight scheduled visits, and 94% completed at least seven. Fertility monitors measuring urinary estrone-3-glucuronide (E3G) and luteinizing hormone (LH) (Clearblue® Easy Fertility Monitor, Inverness Medication Innovations, Inc., Waltham, MA) were used to assist timing clinic visits. Use of fertility monitors has been shown to improve upon other methods of timed sample collection during the menstrual cycle to capture phases of the menstrual cycle with the most hormonal variability (29).

Hormone analysis

Estradiol, progesterone, LH, and follicle stimulating hormone (FSH) were measured in fasting serum samples collected at each clinic visit at the Kaleida Health Center for Laboratory Medicine in Buffalo, NY. Estradiol, progesterone, LH, and FSH were measured using solid phase competitive chemiluminescent enzymatic immunoassay by Specialty

Laboratories, Inc. (Valencia, CA) on the DPC Immulite®2000 analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL). Across the study period the analytical coefficients of variation for these tests were <10% for estradiol, <5% for LH and FSH, and <14% for progesterone. In order to allow assessment of cytokine variability in ovulatory cycles, ovulatory status was determined based on observed peak progesterone and luteinizing hormone levels (28). For the current study, 469 cycles classified as ovulatory were contributed by 248 women, were included in analyses. A minimum of five samples were collected during a cycle, and at least seven urine samples were collected in 95% of ovulatory cycles, yielding a total of 3550 samples available for analysis.

Cytokine assessment in urine samples

Fasting urine samples were collected in 50mL sterile cups. Specimen cups were placed in a cooler and delivered to the processing laboratory within 30 min of collection. Urine was transferred to 50mL conical tubes and centrifuged ($1500 \times g$) for 10 min at 4°C, decanted into 5mL polypropylene storage tubes and remained in storage at -80°C until being shipped on dry ice to the University of Florida.

Cytokines and chemokines were measured using BioSource 30-plex human cytokine assays (Invitrogen Corporation, Carlsbad, CA). This standard panel included a large number of cytokines with suspected involvement in menstrual cycle function and implantation. Specifically, analytes included: epithelial growth factor (EGF), eotaxin, fibroblast growth factor-basic (FGF-b), G-CSF, GM-CSF, hepatocyte growth factor (HGF), IFN- α , IFN- γ , IL-1 β , IL-1 receptor antagonist (RA), IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, interferon γ inducible protein (IP)-10, MCP-1, monokine induced by interferon γ (MIG), MIP-1 α , MIP-1 β , RANTES, TNF- α , and vascular endothelial growth factor (VEGF). All assays were performed following manufacturer recommendations. Concentrations were measured using the Luminex 100 IS system (Luminex Corp, Austin, TX).

Cytokine/chemokine levels were measured in 93 batches. Each batch included a blank, 7 standards, and 40 unknowns, all run in duplicates. Batches were random with regard to cycle phase to avoid imbalance.

Statistical methods

Analyses included: 1. Evaluation of the proportion of samples with detectable levels by visit; 2. Assessment of variability (analytical, inter-individual, and intra-individual, i.e., day variability); 3. Evaluation of mean cytokine levels at up to 8 times during the menstrual cycle; and, 4. Assessment of relations among cytokines and reproductive hormones.

As an initial step in assessing measurement of cytokines and chemokines during the menstrual cycle, we determined the total number of specimens with measurable values. In order to separate between factors that were consistently undetectable and those that varied between detectable and non-detectable levels during the cycle, we also assessed detection by visit, and the number of specimens with detectable levels by woman. Additionally, the

median, 25th and 75th percentiles of lab-measured levels were determined for each of the evaluated cytokines at each phase (Supplemental Table 1).

For analyses of variability, levels were log transformed to meet distributional assumptions. Analytical coefficients of variation (CV_A) were calculated for each measured factor in all 93 batches using the replicates for the lab standards used to generate calibration curves; an average CV_A from all batches was calculated as well. Two-way nested random effects ANOVA models were used to partition total variability into between- (σ_B^2), and within-women over time (σ_W^2). In turn, these were used to calculate CVs for each component, the acceptability index (CV_A/CV_W), and the intraclass correlation coefficient (ICC) as, $\sigma_B^2/(\sigma_B^2 + \sigma_W^2)$ to reflect reliability of the repeated measures across the menstrual cycle (31). For these models, the potential influence of missing data related to lower limits of detection/quantification was addressed by use of multiple imputation (32,33). Use of multiple imputation is suggested to be effective in this circumstance to address potential issues of bias even when a large proportion of values (e.g., 50%) are below detection limits and values imputed (34).

Mean log transformed cytokine levels by phase were estimated using repeated measures analysis of variance. A potential batch effect was addressed through inclusion of batch as indicator variables considered as nuisance parameters. Models were used to compare mean levels of each log-transformed cytokine by menstrual cycle phase and test equivalency of means. These adjusted log averages were exponentiated to yield geometric means to evaluate cytokine levels across the menstrual cycle (Figure 1). As previously described, multiple imputation was used to address non-detects and derive estimates (32,33).

Linear mixed models were utilized to evaluate the associations among log transformed cytokines (dependent variable) and log transformed reproductive hormones. For these models, a realignment algorithm was applied to hormone and cytokine data in order to address potential misclassification related to issues of timing of sample collection (35). For each cytokine considered, two models were evaluated: 1. Unadjusted models with each reproductive hormone as the independent variable; 2. Marginal structural models that included estradiol, progesterone, FSH and LH simultaneously, as well as BMI and age, as well as physical activity as a time-varying factor. Stabilized inverse probability weights were used for multivariable models to address the relations among hormone levels across the cycle; these models make use of longitudinal data to address the potential biases due to factors that are simultaneously intermediates and time-varying confounders (36, 37). These models also included a random effect to address variability in cytokine levels due to batch effects.

RESULTS

1. Cytokines/chemokines are largely present at detectable levels in urine samples

Table 1 displays the percent of biospecimens with quantifiable levels (*i.e.*, within the standard curve) for each measured factor. Of 30 factors considered, six were detected in few biospecimens. Eotaxin, FGF-b, IL-2, IL-5, and IP-10 were detected in fewer than 11% of samples. EGF was detected in most samples but was unquantifiable due to optical densities

above that of the highest standard in the calibrator range and is thus not considered in subsequent analyses.

Detection was greater than 90% for most factors. Quantifiable levels of IL-1RA, IL-12, and MCP-1 were observed in greater than 99% of biospecimens. For most factors, detection varied across the menstrual cycle. For example, IL-4 was detected in 79% of biospecimens, though for 23.5% of cycles had measurable levels throughout. The cytokines IL-1 β and IL-6 were detected in 55% and 50% respectively of samples overall and for both of these cytokines, detection varied across the menstrual cycle. In at least 94% of all cycles, measureable levels were observed in biospecimens collected at some point in the cycle.

2. Levels of cytokines and chemokine in urine have moderate within-woman variability relative to between-woman variability

Table 2 displays the analytical CV (CV_A) as a marker of consistency between replicates, as well as an average CV_A across 93 batches for each factor. Overall, the average CV_A from cytokine/chemokine analyses was 13%. Higher CV_A values were observed for IL-2, IL-5, IL-7, IL-13, MIP-1 β and VEGF, each having CVs above 15%. For most factors, CV_A was low relative to CV_W , with values of the acceptability index within the desirable region less than 0.5 (Fraser and Harris, 1989).

Estimates of the ICC are shown as a reflection of consistency of repeated measures across the menstrual cycle, or temporal variation. Observed ICCs indicate minimal temporal variability for most factors; for 15 of the 24 cytokines and chemokines evaluated, less than 20% of all variability was due to within-person variation. Several factors had ICCs close to 1, indicating very little within-subject variation as a proportion of all biological variability. These included GM-CSF, IFN- γ , MIP-1 β , RANTES, IL-1RA, and IL-12. A slightly greater proportion of total variability due to within-subject variation was observed for HGF (ICC=0.61), IFN- α (ICC=0.71), TNF- α (ICC=0.70), IL-1 β (ICC=0.73), and IL-10 (ICC=0.71).

3. Mean levels of cytokines and chemokines measured in urine vary across the menstrual cycle

P-values from repeated measures ANOVA models with adjustment for batch effects are shown (Table 2). Of 24 factors consistently quantified in biospecimens, statistically significant differences in mean levels by phase of biospecimen collection (*i.e.*, $P < 0.05$ for the phase effect) were observed for 10 cytokines: IL-1 β , IL-1RA, IL-6, IL-8, IL-10, IL-15, G-CSF, HGF, IFN- α , and RANTES.

Figure 1 illustrates plots of geometric mean cytokines by menstrual cycle phase. Factors shown are those with levels varying significantly by menstrual cycle phase. Greater than two-fold higher levels of HGF, IL-6, IL-8 were observed at menses compared to the rest of the cycle. Roughly 1.5-fold higher levels of IL-1 β and RANTES, and 1.5 fold lower levels of IL-1RA, were observed at menses compared to the rest of the menstrual cycle. A small rise was observed around the time of ovulation for IL-6. Only small differences in geometric

mean levels were observed for G-CSF, IFN- α , IL-10 and IL-15, and these factors were dropped from consideration for assessment of associations with hormone levels.

4. Urinary levels of cytokines/chemokines are associated with reproductive hormone levels

Table 3 displays results of linear mixed regression models of cytokines/chemokines (as dependent variables) and their associations with reproductive hormones (as independent variables; both log transformed). Regression coefficients, which take the interpretation of the proportional change in cytokine levels per one unit log transformed hormone, and *P*-values for reproductive hormones from unadjusted models and inverse probability weighted marginal structural models are shown (36, 37).

In both unadjusted and adjusted models, estradiol was significantly inversely associated (*i.e.*, higher levels of estradiol related to lower levels of cytokines) with IL-1 β ($P<0.001$), IL-6 ($P<0.001$), IL-8 ($P<0.001$) and RANTES ($P<0.001$). Estradiol was positively associated with IL-1RA ($P=0.002$). Progesterone was significantly inversely associated with IL-1 β ($P=0.001$), IL-6 ($P<0.001$), IL-8 ($P=0.001$), and RANTES ($P=0.002$); and was positively associated with IL-1RA ($P=0.001$). LH was significantly inversely associated with IL-6 ($P=0.03$), IL-8 ($P=0.02$) and RANTES ($P=0.02$) in adjusted models. Significant positive associations with FSH were observed in adjusted models for HGF ($P=0.01$), IL-1 β ($P=0.007$), and IL-6 ($P=0.003$); inverse associations with FSH were seen in both unadjusted and unadjusted models for IL-1RA ($P<0.001$).

DISCUSSION

We evaluated cytokines and chemokines measured in urine samples collected throughout the menstrual cycle from women in the BioCycle Study with consideration of detection, variability and profiles. In our data, most factors were present at measurable levels and varied only minimally over the menstrual cycle. Urinary levels of pro-inflammatory factors including IL-1 β , IL-8 and RANTES were observed to vary significantly over the menstrual cycle, with peak levels occurring at menses. Elevated levels of pro-inflammatory cytokines have been observed in some studies using blood samples (42,44,45). With a total of 3550 biospecimens evaluated in ovulatory cycles from 248 women at up to 8 times per menstrual cycle, this is the largest study to date evaluating cytokines and chemokines during the menstrual cycle, and among very few to evaluate their levels in urine.

Cytokine and chemokine levels appear to be largely measurable in urine, though the detection varies in part due to temporal variability of levels. For 17 of the 30 factors considered, more than 90% of biospecimens had measurable levels; for another 7 factors, levels were measurable in at least 75% of biospecimens. For those factors with measurable levels, reliability as reflected by the analytical coefficient of variation was moderate, ranging from 9% to 23%. For most factors, CV_A was low relative to CV_W , as reflected by the acceptability index. ICCs were calculated to reflect temporal variability over time, and were observed to range from 0.61 to 0.96. ICCs were above 0.80 for most cytokines and chemokines evaluated, reflecting a minimal within-subject variation relative to between-subject variation over the menstrual cycle for these factors. Due to the large number of

measurements available for analysis, we observed statistically significant variation even for some factors with minimal fluctuation.

We observed the largest changes in geometric mean levels across the cycle for several pro-inflammatory related factors, including IL-1 β , IL-6, IL-8, HGF and RANTES. Although temporal fluctuations of cytokines/chemokines in this study may result from random biological sources, results of the mixed models with reproductive hormones suggest an association with the menstrual cycle for many of these factors. The largest changes in cytokine/chemokines levels were seen early in the menstrual cycle, with high levels at menses observed for IL-1 β , IL-6, IL-8, HGF and RANTES that were up to 3 fold greater than levels in the mid-follicular phase. Menstruation is described as a pro-inflammatory event (7, 8). The observation of elevated pro-inflammatory factor levels in urine samples collected three days post-onset of menses is consistent with studies evaluating levels in blood samples; however, prior studies of variability of pro-inflammatory cytokines during the menstrual cycle have yielded mixed results.

IL-1 β is a widely expressed pro-inflammatory cytokine with suspected involvement in implantation (38). IL-1 β and its inhibitor, IL-1RA, are produced in the endometrium (39), and the IL-1 system is suggested to be involved in regulation matrix metalloproteinase enzymes (MMP) (40, 41). In prior research, levels of IL-1 β in cervical-vaginal secretions have been observed to be increased at menses or in the follicular phase (2, 42), though studies of these were small ($n < 10$). Prior studies of menstrual cycle variability of IL-6 have presented conflicting findings. No variation was observed among 18 normal cycling fertile women (43) or among eight women undergoing infertility treatment (44), though elevated levels were observed during the follicular phase in plasma samples from five volunteers followed for up to four menstrual cycles (45). High follicular phase IL-6 levels and an inverse relation with progesterone have been seen in cervico-vaginal secretions as well (42). We observed two-fold higher geometric mean levels of IL-6 at menses than throughout the rest of the menstrual cycle; however, these levels had limited variability after menses. We also observed similar changes with IL-8 and RANTES levels during menses. A previous investigation compared serum levels of RANTES between follicular and luteal phases in five women and observed non-significant differences, while IL-8 levels were significantly higher during the follicular phase (42). Levels of endometrial IL-8 mRNA expression have been shown to be highest around menses, consistent with our observations of higher urinary levels (21). We observed levels of HGF to be two-fold higher at their peak than in menses. HGF has been shown to be hormonally regulated *in vitro* (46). HGF promotes angiogenesis in wound repair, embryonic development, and tumorigenesis (46, 47). The variability of HGF across the menstrual cycle has not been evaluated previously in human studies, and further consideration is warranted.

The BioCycle Study provided a unique opportunity to evaluate urine cytokine levels during the menstrual cycle in healthy, reproductive aged women. The sampling strategy and relatively large sample size provide an excellent resource to describe temporal patterns in healthy women, though some limitations are notable. In our data, levels were below detection limits in some biospecimens for all cytokines and chemokines we considered; we did not consider six factors with quantifiable levels in fewer than 20% of all biospecimens

(i.e., bFGF, eotaxin, IL-2, IL-5, IP-10); levels of EGF were detectable, but above the linear range of the standard curve. To address missingness related to detection limits we utilized multiple imputation, which has been shown to yield unbiased estimates with as much as 50% of values below detection limits (34). Using marginal structural models, we modeled time-varying effects of hormones, physical activity, which can influence inflammation and vary over the menstrual cycle, as well as age and body mass index which also are related to endocrine function and reproductive hormone levels. Many modifiable factors are postulated to impact inflammation. Future analyses may consider additional factors in relation to urine cytokines/chemokines levels over the menstrual cycle.

Prior studies of inflammation during the menstrual cycle have evaluated protein levels in matrices other than urine, including blood (2, 6, 42–45), follicular fluid (4) and vaginal wash (40), which may be differentially regulated. In research including diseased individuals, various investigators have observed measurable levels of urinary cytokines related to outcomes (24–26). A limited body of work suggests that urinary cytokine levels are correlated with those in cellular secretions (48) and plasma (24), though assessments have been limited to IL-1, IL-6 and TNF- α . Urine is easily, inexpensively, and non-invasively collected; measurement of inflammation in urine may hold potential for research, though clinical utility is limited related to the minimal variability we observed for most factors.

In conclusion, urinary levels of cytokines and chemokines are detectable during the menstrual cycle, and elevated levels of pro-inflammatory cytokines during menses suggest a role of cytokines in menstrual cycle function. However, for most evaluated factors, minimal biological variation was observed, which we were able to evaluate in the BioCycle Study due to its sample size and longitudinal design. Thus the utility of urine for assessment of menstrual cycle function is unclear. Future research may consider what information urinary cytokine levels may add to levels measured in other matrices as well as the relation of cytokines with menstrual cycle characteristics and reproductive health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 1 panels A–J shows geometric mean cytokine and chemokine levels (ng/mL) and 95% confidence intervals by cycle phase for timed clinic visits. Phases are identified as: M, menses; F1, mid-follicular; F2, late-follicular; F3, LH surge; Ov, ovulation; L1, early luteal; L2, mid-luteal, and; L3, late luteal. Cytokines shown were observed to have statistically significant menstrual cycle temporal variability from repeated measures ANOVA models of log transformed factors. Figures are scaled to the interquartile range of each factor shown.

Table 1

Detection/non-detection^a of cytokines and chemokines as proportion of all urine samples ($n=3550$), and proportion of all clinic visits from ovulatory cycles ($n=463$) with levels outside of the detectable range.

	Biospecimens (total $n=3550$)		Cycles (total $n=463$)			
	Detectable levels		Non-detectable levels at every clinic visit		Detectable levels at every clinic visit	
	%	(n)	%	(n)	%	(n)
bFGF	5.8	(205)	84.4	(396)	0.0	(0)
EGF^b	~		~		~	
EOTAXIN	10.8	(382)	79.6	(372)	1.3	(6)
GCSF	91.3	(3241)	0.2	(1)	70.0	(328)
GMCSF	92.7	(3291)	0.0	(0)	67.6	(317)
HGF	80.5	(2857)	1.7	(8)	38.4	(180)
IFNα	93.0	(3301)	0.2	(1)	73.7	(345)
IFNγ	93.7	(3325)	0.0	(0)	73.4	(344)
IL1β	54.8	(1944)	2.0	(9)	1.9	(9)
IL1RA	99.8	(3544)	0.0	(0)	98.3	(460)
IL2	1.9	(66)	95.2	(445)	0.0	(0)
IL2R	97.9	(3474)	0.0	(0)	86.4	(405)
IL4	79.0	(2804)	0.0	(0)	23.5	(110)
IL5	3.3	(118)	87.6	(409)	0.0	(0)
IL6	50.3	(1785)	3.9	(18)	4.3	(20)
IL7	91.1	(3235)	0.0	(0)	53.6	(251)
IL8	77.9	(2765)	2.0	(9)	27.6	(130)
IL10	92.3	(3278)	0.0	(0)	67.2	(315)
IL12	99.2	(3522)	0.0	(0)	95.9	(450)
IL13	94.0	(3337)	0.2	(1)	74.5	(349)
IL15	92.0	(3266)	0.0	(0)	66.7	(313)
IL17	95.0	(3371)	0.0	(0)	78.2	(367)
IP10	1.2	(44)	98.0	(457)	0.2	(1)
MCPI	99.1	(3519)	0.0	(0)	94.6	(443)

Biospecimens (total n=3550)		Cycles (total n=463)			
Detectable levels		Non-detectable levels at every clinic visit		Detectable levels at every clinic visit	
%	(n)	%	(n)	%	(n)
MIG	97.9 (3474)	0.0	(0)	87.7	(411)
MIP1A	86.8 (3083)	0.0	(0)	44.5	(208)
MIP1B	93.6 (3323)	0.0	(0)	72.6	(340)
RANTES	97.4 (3458)	0.0	(0)	90.9	(426)
TNFα	84.5 (2998)	2.6	(12)	58.3	(274)
VEGF	97.1 (3448)	0.0	(0)	87.9	(412)

Abbreviations: EGF, epithelial growth factor; bFGF, basic fibroblast growth factor; G(M)CSF, granulocyte (macrophage) colony stimulating factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IP, interferon γ inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by interferon γ ; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

^a Detection calculated as the percent of samples with quantifiable levels of cytokine.

^b EGF was non-quantifiable due to levels above the upper limit of the calibration curve

Table 2

Measures of variability in cytokine levels during ovulatory menstrual cycles ($n=469$) from participants^a in the BioCycle Study with at least one ovulatory cycle ($n=248$).

	Analytical CV ^b	A _i ^c	ICC ^d	P-value for day-effect ^e
G-CSF	0.131	0.273	0.822	0.001
GM-CSF	0.152	0.115	0.934	0.257
HGF	0.115	0.081	0.612	0.012
IFN- α	0.119	0.307	0.712	0.001
IFN- γ	0.122	0.169	0.925	0.314
MCP-1	0.117	0.230	0.889	0.149
MIG	0.130	0.233	0.859	0.271
MIP-1 α	0.165	0.231	0.955	0.186
MIP-1 β	0.104	0.229	0.811	0.087
RANTES	0.124	0.101	0.950	0.050
TNF- α	0.102	0.132	0.695	0.551
VEGF	0.229	0.550	0.769	0.133
IL-1 β	0.128	0.115	0.735	0.021
IL-1RA	0.118	0.368	0.958	<0.001
IL-2R	0.130	0.285	0.815	0.087
IL-4	0.131	0.162	0.860	0.477
IL-6	0.137	0.018	0.752	0.015
IL-7	0.184	0.283	0.949	0.189
IL-8	0.116	0.055	0.809	0.013
IL-10	0.109	0.214	0.714	0.005
IL-12	0.137	0.182	0.948	0.119
IL-13	0.177	0.361	0.883	0.421
IL-15	0.094	0.123	0.869	0.002
IL-17	0.103	0.283	0.775	0.465

^aParticipants with at least one ovulatory cycle considered for analysis ($n=248$)

^bAnalytical variability determined from replicates of the standards across all 93 batches

^cA_i (Acceptability index), ratio of CV_A (replicate reliability) to CV_W (within-subject variability)

^dICC as calculated from two-way nested ANOVA models

^eP-value for the day effect in repeated measures ANOVA using log transformed cytokines

Table 3

Regression coefficients and p-values from mixed linear models of cytokine/chemokine levels (ng/mL) and reproductive hormones in biospecimens ($n=3550$) from the members of BioCycle Study cohort with at least one observed ovulatory cycle ($n=248$).

Cytokine	Model ^a	Estradiol (pg/mL)		Progesterone (ng/mL)		LH (ng/mL)		FSH (mIU/mL)	
		Coeff.	P-value	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value
HGF	Unadj.	0.014	0.522	-0.016	0.217	-0.023	0.269	0.003	0.911
	Adj.	0.020	0.384	-0.011	0.396	-0.045	0.077	0.063	0.052
IL-1 β	Unadj.	-0.046	0.001	-0.018	0.021	-0.028	0.024	0.017	0.348
	Adj.	-0.071	<0.001	-0.025	0.001	-0.009	0.523	0.048	0.010
IL-1RA	Unadj.	0.049	0.001	0.036	<0.001	0.000	0.997	-0.071	<0.001
	Adj.	0.052	0.001	0.032	<0.001	-0.034	0.054	-0.151	<0.001
IL-6	Unadj.	-0.186	<0.001	-0.083	<0.001	-0.061	0.009	0.099	0.003
	Adj.	-0.204	<0.001	-0.076	<0.001	-0.056	0.041	0.120	<0.001
IL-8	Unadj.	-0.166	<0.001	-0.061	0.001	-0.063	0.020	0.070	0.093
	Adj.	-0.150	<0.001	-0.063	0.000	-0.141	<0.001	-0.108	0.014
RANTES	Unadj.	-0.039	<0.001	-0.014	0.001	-0.016	0.017	0.013	0.181
	Adj.	-0.042	<0.001	-0.019	<0.001	-0.020	0.010	0.011	0.289

^aUnadjusted model includes each log transformed hormone as the sole independent variable. Adjusted model is a marginal structural model that includes all log transformed hormones, age and body mass index as independent variables with inverse probability of exposure weights to address time-varying confounding.

Bold indicates $P<0.05$

Abbreviations: HGF, hepatocyte growth factor; IL, interleukin; RA, receptor antagonist; RANTES, regulated upon activation normal T-cell expressed and secreted