

## Impact of the Ovulatory Cycle on Virologic and Immunologic Markers in HIV-Infected Women

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An individual's sex influences plasma human immunodeficiency virus type 1 (HIV-1) RNA level and rate of CD4 cell decline, but the mechanism for this effect is currently unknown. To determine the effect of the ovulatory cycle on HIV-1 RNA level and lymphocyte subsets in HIV-infected women, blood specimens were obtained weekly from 14 women infected with HIV. Participants reported regular menses and were not using hormonal medications or narcotics. The occurrence of ovulation was verified by use of endocrine criteria. Ovulation occurred in 10 of the 14 women. Among women who ovulated, median HIV-1 RNA level fell by a median of 0.16 log<sub>10</sub> from the early follicular phase to the midluteal phase ( $P = .03$ , Wilcoxon signed-rank test). When women who did not ovulate were included in the analysis, no significant fluctuation in plasma HIV RNA level was identified. Thus, the ovulatory cycle influenced circulating HIV-1 RNA levels, a finding that is plausible because of the known effect of sex hormones on lymphocyte function and cytokine production.

Despite reports to the contrary [1, 2], recent findings of sex-based differences in circulating human immunodeficiency virus type 1 (HIV-1) RNA levels (virus load) and lymphocyte subsets have generated renewed interest in the effects of sex on the course of HIV infection [3–6]. The recent studies found that, for a given CD4 cell count, women had plasma HIV-1 RNA levels ~30%–50% lower than those in men. The physiological bases for the findings are unclear, but, because the participants in these studies had not yet received potent antiretroviral therapy combinations, treatment and access to it do not appear to explain the sex-based differences in RNA level. Since current treatment guidelines are based on the prognostic value of HIV-1 RNA quantitation, several authors have discussed the potential impact of sex differences in viral RNA level on antiretroviral treatment guidelines [4, 5].

One possible source of the effect of sex is the fluctuation of

reproductive hormone levels, which, in premenopausal women, do not exist in a steady state [7]. The menstrual or ovulatory cycle has not previously been thought to influence HIV-1 RNA levels. Goulston and colleagues reported that, among 6 women with normal menstrual cycles, plasma HIV-1 RNA did not appear to vary with stage of cycle [8]. The participants in this study reported regular menses and were sampled weekly. However, anovulatory cycles occur in up to 5%–31% of healthy women with regular menstruation [9, 10] and may be influenced by age, exercise, chronic illnesses, extremes of body mass, and alcohol or narcotic use [11–13]. Confirmation of ovulation is particularly important in studies of HIV-infected women, who may be at increased risk for cycle irregularities.

Gonadotropins and/or ovarian hormones, which vary with stage of the ovulatory cycle and in pregnancy, might influence key virologic and immunologic parameters. In 1993 Henin and coworkers reported cervicovaginal shedding of greater quantities of HIV among pregnant women, although this group of women had less-advanced HIV disease, than among a group of nonpregnant women [14]. However, human chorionic gonadotropin (hCG), which is present during pregnancy, has been reported to inhibit HIV replication [15–17] and, in an animal model, to provide some clinical benefit [18]. Other investigators have identified varied effects of specific hormonal agents, such as the antiprogesterin RU-486 [19] and antiestrogens [20].

Similarly, gonadotropin and hormone levels can influence lymphocyte responses and overall immunologic function. Maini and colleagues found that women had significantly higher CD4 cell counts and percentages than men, even when smoking, which increases CD4 cell percentage, was controlled for [21]. Luteinizing hormone (LH), which peaks midcycle, and its homologue hCG, which is expressed during pregnancy, have been

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Informed consent was obtained from the participants of this study in accordance with procedures and consent materials that were reviewed and approved by the Committee on Human Experimentation at the University of California, San Francisco, and each of the collaborating institutions.

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shown to increase the percentage of circulating CD4 cells. By contrast, follicle-stimulating hormone (FSH) decreased the percentage of CD4 cells, while increasing CD8<sup>+</sup>CD28<sup>+</sup> subsets [22]. Postmenopausal women were found to have lower CD4 cell percentages, which correlated inversely with FSH level, whereas premenopausal women had higher percentages of CD4 cells, which correlated with LH level [23].

We now report the results of a study of HIV seropositive women, who reported regular menstrual cycles, for whom we performed weekly measurement of plasma HIV-1 RNA levels and lymphocyte subsets, in addition to verifying the occurrence of ovulation via measurement of gonadotropin and ovarian steroid hormone levels.

## Methods

The present research is a cross-sectional study of 14 HIV seropositive women who reported regular menstrual cycles during the preceding 6 months.

**Participants.** Participants were enrolled between February and July 1996. The 14 ovulatory study participants were recruited during a routine, semiannual visit in the Women's Interagency HIV Study (WIHS), a longitudinal cohort study of the natural history of HIV. Women were recruited for participation at WIHS study sites located at the University of California, San Francisco (UCSF), General Clinical Research Center and the Alameda County Medical Center in Oakland, California. Women 18–45 years of age were offered enrollment if they reported molimina and regular menses during the preceding 6 months (menstruation occurred at 25–32-day intervals, cycles did not vary >3 days in length, and missed menses or intermenstrual vaginal bleeding had not occurred). In addition, women were excluded if they had received a vaccination (within 2 months), narcotics, estrogens, progestagens, or antiretroviral medications; had undergone hysterectomy; or were pregnant or postpartum within the past 6 months.

**Study visits.** Historical and physical examination data were obtained by trained interviewers and research clinicians using standardized data collection instruments [24]. Participants were asked to contact project staff within 3 days of the onset of menstrual bleeding and were then scheduled for a series of 4 weekly study visits. Weight, height, body mass index, medications, menstrual history, smoking status, and confirmation of the presence of serum HIV antibody were gleaned from WIHS interview data. Medical history was updated (dates of onset of menstruation, new medications, use of illicit drugs, acute illnesses) and phlebotomy performed at each visit.

**Laboratory testing.** Because serial ovarian ultrasonography and daily serum sampling were not performed, the ovulatory status of study participants was determined by careful review of menstrual history and endocrine analyses of weekly blood samples. We established endocrine criteria to ensure normal ovulatory function, using radioimmunoassays standardized in the UCSF Reproductive Endocrinology Center research laboratory specifically for the purpose of assessing the hypothalamic-pituitary-ovarian axis [25]. Serum was separated and frozen for batch testing. Serum concentrations of FSH, LH, estradiol, progesterone, and prolactin were

quantified in duplicate by use of established radioimmunoassays with inter- and intraassay coefficients of variation of <15% and <10%, respectively [25]. The criteria for inclusion in the ovulatory group included a follicular phase FSH <20 mIU/mL, an increase in estradiol between the follicular and periovulatory phases, a midluteal progesterone >5 ng/mL, and a prolactin level <20 ng/mL. In this way, we were able to classify 3 of each 4 weekly visits as during the early follicular, periovulatory, or midluteal phase of the cycle (referring to days 4–7, 12–16, or 19–24, respectively, after onset of menses).

Plasma was recovered from citrate-anticoagulated blood and was frozen at –70°C within 6 h of collection. Plasma HIV-1 RNA quantity was determined by use of the branched chain DNA assay version 2, which has a lower limit of detection of 500 copies/mL (Chiron HIV-1 RNA v2.0 Assay, Chiron Corporation, Emeryville, CA). The minimum virus load measured in this study was 507 copies/mL, so retesting of specimens with more-sensitive assays was not required. Lymphocyte subsets (CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>) were determined by flow cytometry using EDTA-treated blood at a laboratory certified by the AIDS Clinical Trials Group quality assurance program.

**Data analysis.** Data were entered into a relational database (Microsoft Access) and analyzed in SAS (SAS Institute, Cary, NC). HIV-1 RNA data were reported as log<sub>10</sub> copies/mL. Binomial comparisons were made by use of Fisher's exact and  $\chi^2$  tests. Means were compared by use of Student's *t* test. The significance of changes over time in lymphocyte subsets and HIV-1 RNA quantities, which were not normally distributed, were tested by Wilcoxon signed-rank tests. Random-effects regression models were constructed of HIV-1 RNA levels and lymphocyte subset by phase of cycle.

## Results

The 14 participants were 19–44 years of age. Initial CD4 cell counts were 184–1177 cells/mL, and initial HIV RNA levels were 2.7–4.8 log<sub>10</sub> copies/mL. Of the 14 subjects studied, 10 met the required criteria for normal ovulatory function. Table 1 provides a summary of descriptive findings of the study participants, including those who met criteria for ovulation and those who did not. The mean age of women who ovulated was 31 years, versus 38 years among those who did not. Mean body mass index among women who ovulated was 23.9, versus 25.8 among those who did not. Initial mean absolute CD4 cell count was 392 cells/mL (2.6 log<sub>10</sub>) among women who ovulated, versus 632 cells/mL (2.7 log<sub>10</sub>) among those who did not. Sixty-three percent of the women who ovulated, versus all the women who did not ovulate, were smokers at the time of study participation.

Table 2 summarizes differences in median HIV-1 RNA levels between cycle phases. When the analysis included data from all participants, HIV-1 RNA did not vary significantly from the early to the later postmenstrual visits. Median HIV RNA level was 3.06 log<sub>10</sub> copies/mL at the early postmenstrual visit and fell by a median value of 0.08 log<sub>10</sub> copies/mL, a value not significantly different from changes in RNA levels at other times

**Table 1.** Age, body mass, and endocrine measurements among study participants.

Measurement	All women (n = 14)	Women who ovulated (n = 10)	Women who did not ovulate (n = 4)
Mean age (years)	33.10 ± 8.00	31.00	38.00
Mean body mass index (kg/m <sup>2</sup> )	24.50 ± 2.50	23.90	25.80
Mean follicular phase CD4 cell count (log <sub>10</sub> cells/mL)	2.61 ± 0.22	2.57	2.73
Mean follicular phase plasma HIV-1 RNA level (log <sub>10</sub> copies/mL)	3.35 ± 0.68	3.53	2.92
Mean follicular phase FSH (mIU/mL)	15.80 ± 13.30	11.40	27.50
Mean follicular phase estradiol (pg/mL)	57.90 ± 37.70	70.20	29.10
Mean periovulatory phase LH (mIU/mL)	31.60 ± 24.80	30.80	NA
Mean periovulatory phase estradiol (pg/mL)	112.90 ± 97.60	122.40	NA
Mean midluteal phase progesterone (ng/mL)	9.40 ± 8.00	12.70	1.20
Mean midluteal phase prolactin (ng/mL)	9.60 ± 5.00	10.50	7.50

NOTE. Follicular phase, days 4–7 of menstrual cycle or first postmenstrual visit among women who did not ovulate; periovulatory phase, days 12–16 of cycle or second postmenstrual visit among women who did not ovulate; midluteal phase, days 19–24 of cycle or third postmenstrual visit among women who did not ovulate. HIV-1, human immunodeficiency virus type 1; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NA, not applicable.

in the cycle ( $P = .31$ , Wilcoxon signed-rank test). By contrast, when only women who ovulated were included in the analysis, a decrease in HIV RNA level over the cycle occurred. Median HIV-1 RNA was 3.41 log<sub>10</sub> copies/mL in the early follicular phase and fell by a median of 0.16 log<sub>10</sub> copies/mL in the midluteal phase, a decrease that was statistically significant ( $P = .03$ , Wilcoxon signed-rank test). The range of HIV-1 RNA levels varied by individual participant. Figure 1 summarizes HIV-1 RNA levels and lymphocyte subsets from the early follicular phase to the midluteal phase of the women who ovulated. Figure 2 summarizes HIV-1 RNA levels and lymphocyte subsets over 3 visits among women who did not ovulate.

Among women who ovulated, absolute CD4 cell counts also decreased over the cycle, but this trend did not reach statistical significance by Wilcoxon signed-rank test ( $P = .15$ ) or random-effects regression ( $P = .27$ ). No pattern of change over the cycle was seen for absolute CD8 cell counts ( $P = .84$  by Wilcoxon signed-rank test and  $P = .91$  by random-effects regression model). No pattern of variation in lymphocyte subsets was discernible among the small number of women who did not ovulate.

**Table 2.** Differences in plasma RNA level (log<sub>10</sub>) between cycle phases among women who ovulated.

Ovulatory cycle phases	Median difference (range), log <sub>10</sub> plasma RNA	$P^a$
All participants		
Initial median at early follicular phase	3.06 (2.69–4.81)	
Early follicular minus periovulatory	0.02 (−0.67–0.49)	.58
Early follicular minus midluteal	0.08 (−0.25–0.75)	.31
Periovulatory minus midluteal	0.07 (−0.24–0.87)	.10
Women who ovulated		
Initial median at early follicular phase	3.41 (2.70–4.81)	
Early follicular minus periovulatory	<0.01 (−0.13–0.50)	.156
Early follicular minus midluteal	0.16 (0–0.75)	.031
Periovulatory minus midluteal	0.02 (−0.24–0.28)	.375

NOTE. Follicular phase, days 4–7 of menstrual cycle or first postmenstrual visit among women who did not ovulate; periovulatory phase, days 12–16 of cycle or second postmenstrual visit among women who did not ovulate; midluteal phase, days 19–24 of cycle or third postmenstrual visit among women who did not ovulate.

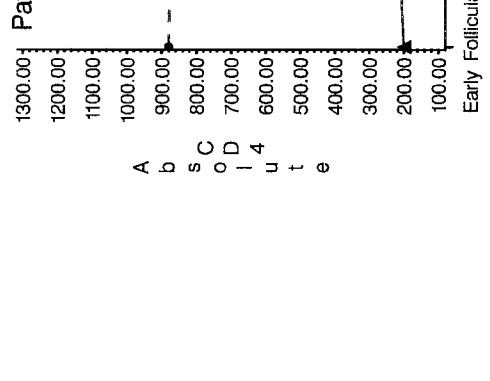
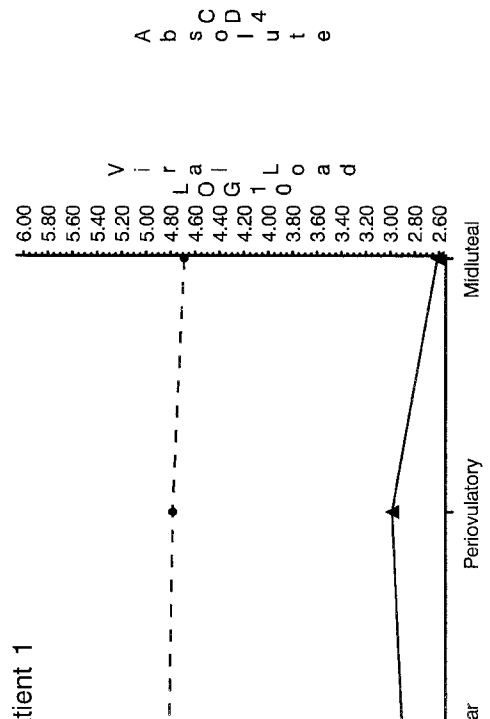
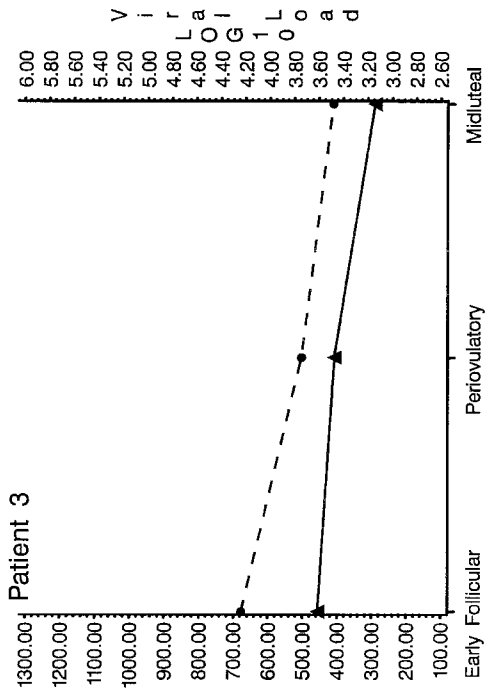
<sup>a</sup> Wilcoxon signed-rank test.

## Discussion

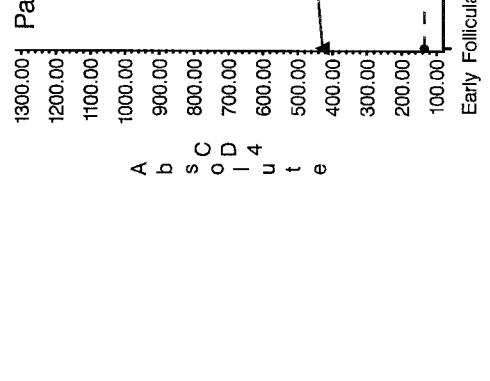
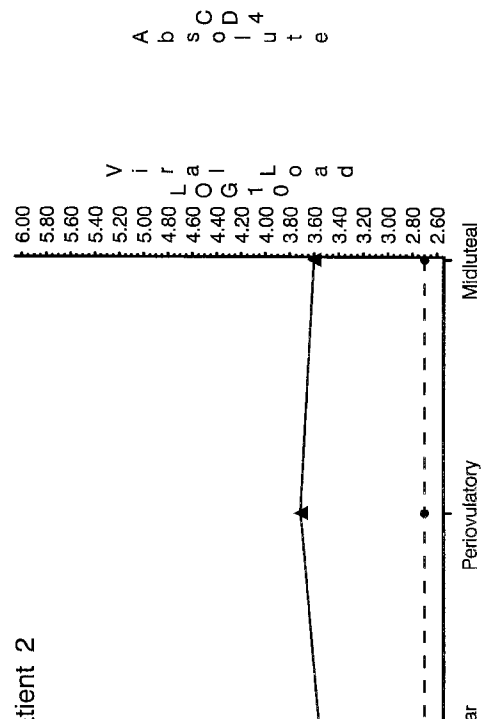
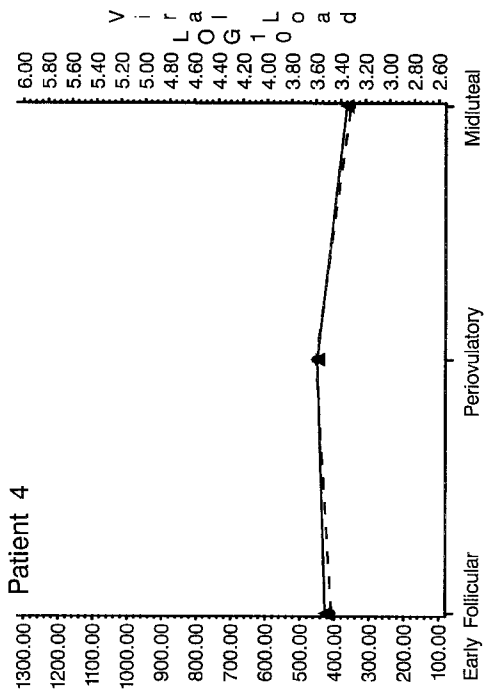
The findings of this study show that key prognostic indicators in HIV infection may vary during the ovulatory cycle. The extent of the variation in HIV-1 RNA level and CD4 cell counts, although small, could contribute to sex-based differences recently reported in HIV-1 RNA level and lymphocyte subsets [4–6]. No consistent pattern of variation in HIV-1 RNA level was discerned among women who did not ovulate. Age differences between women who ovulated and those who did not also could have influenced HIV RNA levels in this study. Although this study is too small to directly compare cyclic effects in ovulatory and anovulatory women, the cyclic effect among women who ovulate would have been missed by an analysis that included both groups. Since unrecognized anovulatory cycles are relatively common [9, 10], inclusion of data from women during anovulatory cycles could explain the failure of previous studies to associate cycle phase and HIV-1 RNA level [8]. Serial endocrinologic evaluations or vaginal ultrasound monitoring of ovarian follicles and endometrial thickness should be used to verify ovulation, in studies of the influence of the menstrual cycle on virologic and immunologic parameters.

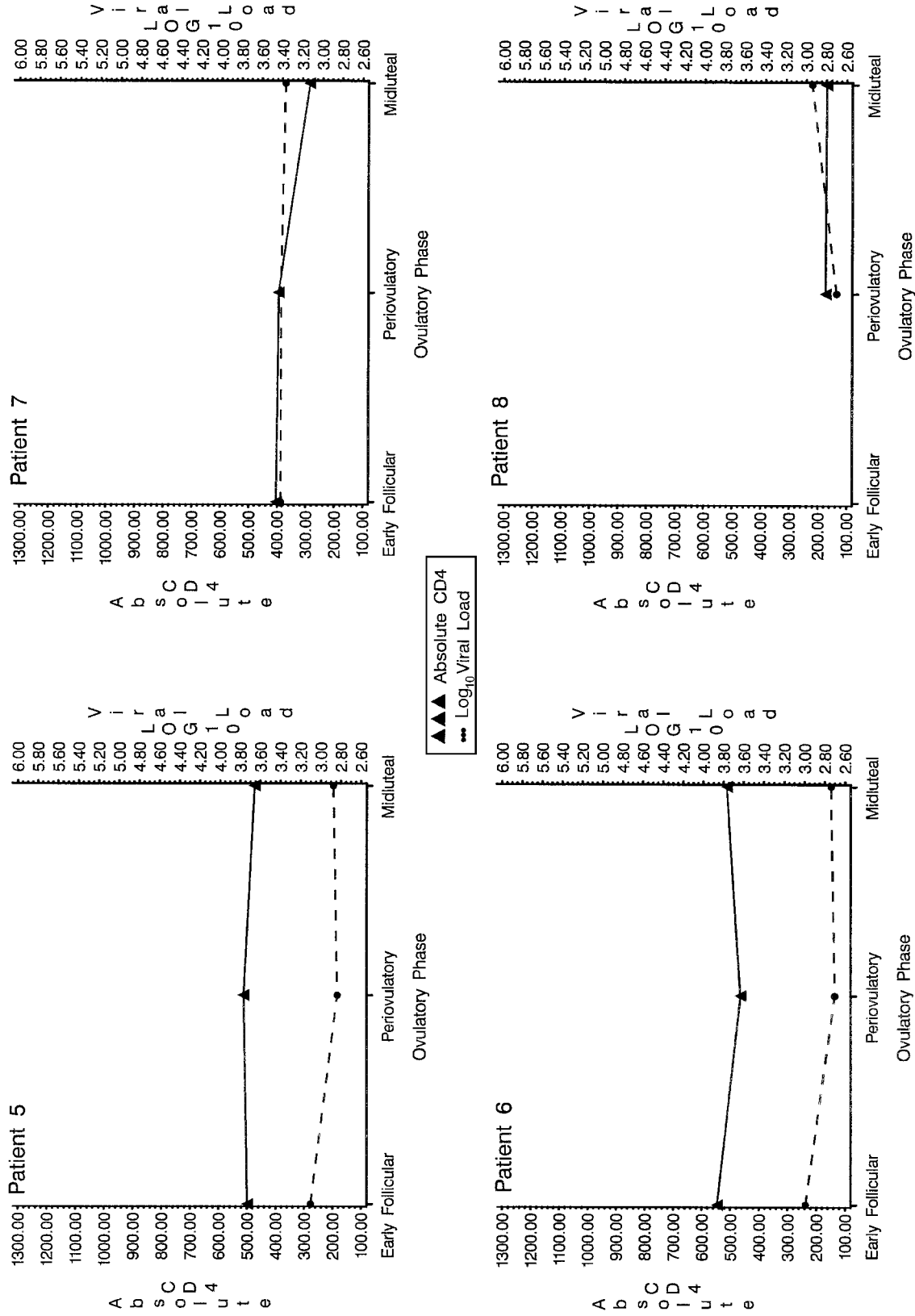
It is biologically plausible that gonadotropins and ovarian hormones influence the rate of viral replication and circulating lymphocyte subsets via their known effects as modulators of cytokine gene expression or via direct effects on HIV replication. However, with few studies examining the effects of sex steroids on HIV and immunity to it, the potential effect of these substances on cytokine expression and HIV infection must be inferred from studies of osteoporosis and autoimmune and cardiovascular diseases [26].

The effects of estrogen on the immune system are complex and not fully characterized. Estrogens have long been thought to contribute to the tendency for premenopausal women to develop autoimmune diseases such as systemic lupus erythematosus, which tends to be exacerbated during pregnancy and after administration of exogenous estrogen [27]. However, beneficial effects of estrogens on some autoimmune processes are

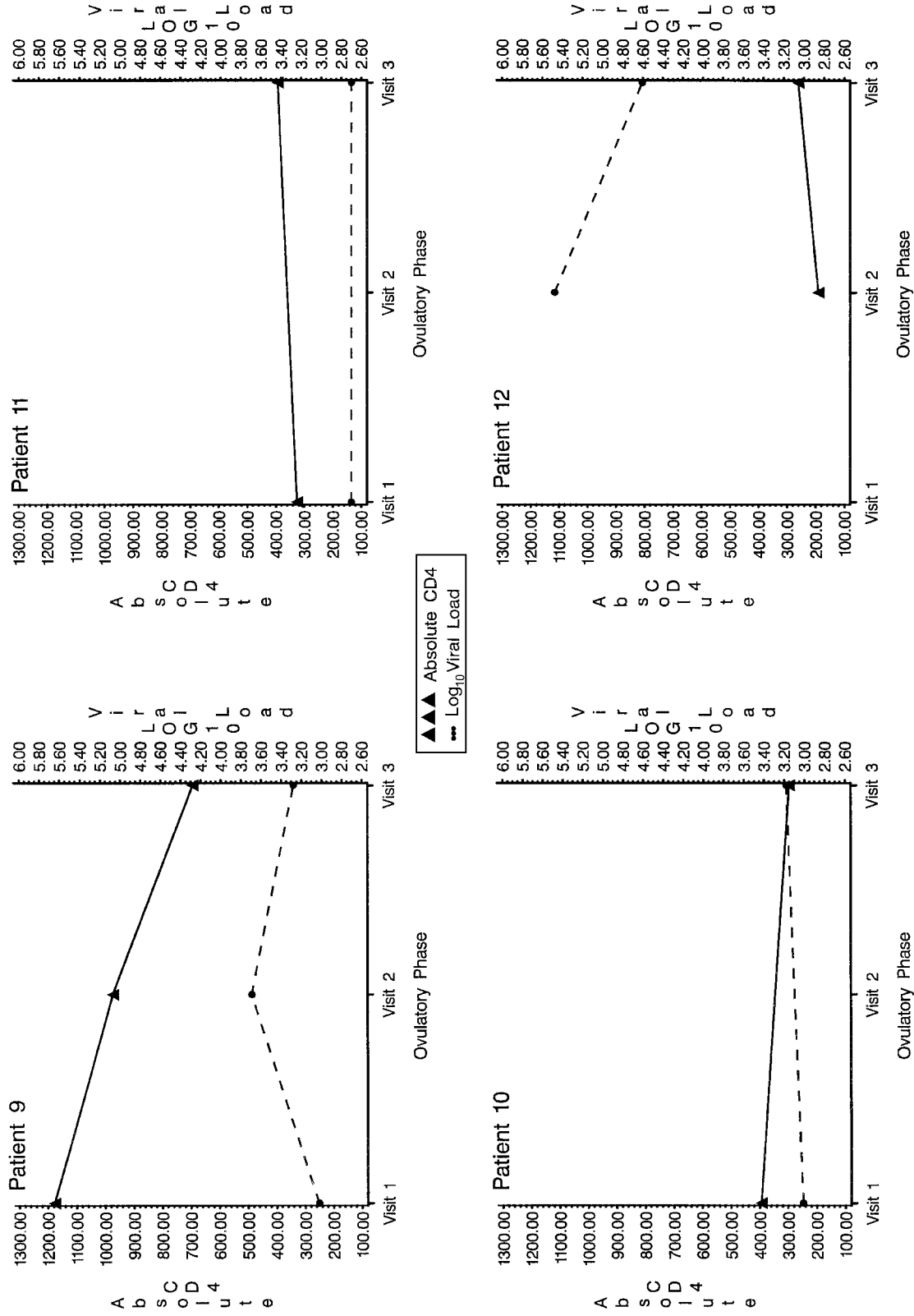


▲▲▲ Absolute CD4  
 --- Log<sub>10</sub> Viral Load





**Figure 1.** Changes in CD4 cell count/mL and plasma human immunodeficiency virus RNA level over ovulatory cycle among women who ovulated, patients 1–8. Follicular phase, days 1–8; Perioviulatory phase, days 19–24; Midluteal phase, days 4–7 of the cycle.



**Figure 2.** Changes in CD4 cell count/mL and plasma human immunodeficiency virus RNA level over 3 visits among women (patients 9–12) who did not ovulate. Visit 1, first postmenstrual visit; visit 2, second postmenstrual visit; visit 3, third postmenstrual visit.

offset by exacerbation of other autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and psoriasis with estrogen “deficiency” [27, 28]. These conditions frequently remit during pregnancy and relapse in the postpartum period [29].

Estrogens and progestins appear to have complex roles in regulating the balance between pro- and anti-inflammatory signals, primarily via the production of immunoregulatory cytokines [30, 31]. The effects of estrogen, as either a suppressor or an enhancer of cytokine expression, are tissue and concentration dependent [27, 30, 32]. For example, menopausal women have elevated levels of interleukin (IL)-1 and tumor necrosis factor (TNF), which are reversed by hormone replacement therapy [26]. Estrogen decreases apoptosis and reduces TNF- $\alpha$  in peripheral blood mononuclear cells, but it also has been associated with increased production of TNF- $\alpha$  in an animal model of endotoxemia [33]. Overall, estrogens appear to have a biphasic effect on the secretion of TNF- $\alpha$ , with low levels being stimulatory and higher levels inhibitory [30].

Significant fluctuation in circulating IL-6 levels and variation in its physiologic effects have been documented to occur in vivo during normal ovulatory cycling [34]. Cyclic fluctuations in the manifestations of autoimmune diseases in premenopausal women also are well described. Estrogens are reported to increase production of granulocyte-macrophage colony-stimulating factor in uterine epithelium [35] and increase production of IL-1 and IL-6 in blood mononuclear cells [32, 36, 37] in vitro; however, estrogen is associated with decreased cytokine production (including IL-6) from lymph node lymphocytes in a mouse rheumatoid arthritis model [27]. Estrogens also decrease IL-6 production by bone mononuclear cells at sites of inflammation [32]. Estrogens attenuate IL-6 production in bone marrow [38] and increase bone loss at sites of local inflammation, in part via enhanced IL-1 $\beta$  promoter activity in the presence of bacterial lipopolysaccharide [39]. By contrast, IL-6 is constitutively secreted in marrow of postmenopausal women [38] and is associated with elevated levels of IL-6 in blood. Both IL-1 and IL-6 are known to activate NF- $\kappa$ B and induce proteins of the long terminal repeat region of HIV-1, and thus they could act as mediators of estrogen-induced effects on HIV replication [40]. However, estradiol also results in small but statistically significant increases in human endometrial IL-6 production [41]. Estrogens have minimal effects on lymphocyte proliferation, but even small changes in concentration influence antibody and TNF production and activate the interferon (IFN)- $\gamma$  promoter [42, 43]. Cytokine concentrations in cervical mucus also have a cyclic pattern in women that follows peaks in circulating estradiol levels [44].

Progesterone, which is generally considered anti-inflammatory, often opposes estrogen effects [31], for example by inhibiting the production of IL-6 by endometrial cells [45]. Whereas estrogens have been demonstrated to induce a Th-1 type of immune response, progesterone induces a Th-2 shift in vitro [28, 46]. Inhibition of progesterone increases expression of IFN-

$\gamma$  and decreases IL-10 production in splenic cells of pregnant mice [46]. Both IL-6 and IL-6-receptor levels in the blood fluctuate during the normal menstrual cycle, with the peak levels occurring in the preovulatory phase and the lowest levels occurring during the luteal phase, in an inverse correlation with serum progesterone [34, 47]. Progesterone also inhibits synthesis of the  $\beta$ -chemokine MCP-1 (monocyte chemoattractant protein-1), an effect that is reversed by the antiprogestin RU-486 [48]. Progesterone may also have direct inhibitory effects on HIV replication, perhaps via increased phosphorylation (and activation) of nucleoside antiviral agents [49].

There are few available data that address the impact of sex steroids on the course of HIV infection, although some studies have raised the possibility. Clark and Bessinger studied a small group of older women with HIV infection and found that hormone replacement therapy was marginally associated with a reduced risk of death, in a multivariate analysis [50]. These authors noted that hormone replacement could produce this finding via direct biologic effects or if it were a marker of better access to health care or of health-seeking behaviors. The antiestrogen tamoxifen has been demonstrated to inhibit HIV replication in lymphocytes, although the mechanism of this effect is unclear [20]. However, since sex steroids influence establishment of Th-1 versus Th-2 immunological responses, a recognized factor associated with outcome of HIV infection [51], as well as production of cytokines, which also influence HIV replication rates, they may well result in sex-based differences in HIV-1 levels.

These findings indicate that, by supporting sophisticated modeling of disease course, the identification of sensitive prognostic markers (such as current HIV-1 RNA assays) will lead to recognition of the effects of more-subtle processes, such as an effect that may be produced by reproductive hormones. The effect of the ovulatory cycle should be further investigated to determine the mechanisms that cause fluctuation in HIV RNA levels and to determine whether this process influences the large differences in RNA level by sex. Although this kind of research, which requires repeated sampling and concurrent measurement of hormone levels, is cumbersome, it could lead to a better understanding of basic mechanisms of viral pathogenesis and to new treatment modalities.

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