

WOMEN'S INTERAGENCY HIV STUDY

SECTION 27: SEX STEROID PROTOCOL

A. STUDY PURPOSE

Specific Aim: To evaluate the effects of HIV infection on the age of onset of diminished ovarian reserve (increased serum estradiol and FSH and low inhibin B levels at days two through four of the menstrual cycle), on peri-menopausal changes in menstrual cycle characteristics, and on the age at menopause.

- **Hypothesis 1.** HIV-infected women will demonstrate younger age at onset of evidence of diminished ovarian reserve, and irregular menstrual cycles, than HIV-uninfected women.
- **Hypothesis 2.** HIV-infected women will transition to menopause at an earlier age than HIV-uninfected women.
- **Hypothesis 3.** In the absence of hormone replacement therapy, treatment with protease inhibitor regimens will be associated with earlier perimenopausal symptoms and increased FSH levels.
- **Hypothesis 4.** Increased FSH and diminished ovarian function will be associated with reduced fertility and increased risk of miscarriage when controlled for age.

B. BACKGROUND AND SIGNIFICANCE

1. SEX DIFFERENCES IN HIV BIOLOGY

Sexual dimorphism in immune responses has long been recognized in animal and human models and is clinically appreciable in patterns of autoimmune illnesses. An accumulating body of evidence indicates that sex influences the amount HIV-1 RNA in plasma, CD4+ cell counts, and rates of change in these parameters. Several reports have demonstrated lower HIV-1 RNA levels in women than in men with similar CD4+ cell counts. These differences are greatest shortly after HIV infection, and tend to diminish with disease progression. Other studies have identified additional sex-based differences in disease progression. Clinical AIDS-defining events and AIDS-related deaths may occur at higher CD4+ cell counts in women than in men. There is a relative dearth of data on the effect of sex on response to antiretroviral therapy (ART), but such differences are plausible since the effects of sex involve immunologic and pharmacokinetic functions. When ART and access to it are well controlled, sex itself does not appear to influence the outcome of therapy, though most of the studies of sex and treatment outcome are quite small and do not consider ovulatory function and hormonal treatments.

2. SEX STEROIDS AND HIV

Research is clearly needed on the mechanisms by which sex influences the course of HIV infection and treatment responses. Elucidation of the mechanisms for such differences can contribute to our understanding of immunologic recovery during HAART, and could lead to new treatment modalities. One possible mechanism for sex effects in HIV infection is fluctuations of reproductive hormone levels, which, in premenopausal women, do not exist in a steady state. In a pilot study of 14 HIV-1 infected WIHS participants in San Francisco, we found that among women with ovulatory cycles, the plasma HIV-1 RNA levels were 0.16 log₁₀ lower in the midluteal than the early follicular phase of the menstrual cycle, indicating that sex steroids may influence virologic or immunologic parameters. We also noted a high rate of anovulation (29%) in these women, an observation that has been reported by others.

3. EFFECT OF HIV ON OVULATORY FUNCTION AND MENOPAUSE

Many anecdotal reports from women living with HIV suggest changes in the character of their menstrual periods and symptoms associated with menstruation. The WIHS National Community Advisory Board has long considered the effects of HIV on menstruation as a priority for WIHS research. Several studies have been conducted on the effects of HIV infection on ovulatory function and menstruation; however, in general, HIV-infection was not associated with alterations of the prevalence of menstrual abnormalities, regardless of CD4 cell count and CDC stage of HIV infection. Chirgwin and colleagues studied 248 HIV-infected women and 82 negative controls and found that the HIV-infected women were more likely to experience irregular periods (>6 week intervals without menstrual bleeding) and amenorrhea (>3 month interval). Accordingly, several premenstrual symptoms were less common among the HIV-infected women. A past history of substance abuse was associated with this difference, but not current drug use or level of HIV infirmity. Chirgwin concluded that socioeconomic factors might underlie their findings. Menstrual calendars were used for a joint HERS/WIHS study of menstrual cycle length. 75-83% of enrolled participants submitted three monthly calendars, and trends toward irregular cycles (<18 days or >90 days) were independent of age, race, body mass and substance use, but were associated with higher plasma HIV RNA level and lower CD4 cell count.

The ACTG determined progesterone and follicle stimulating hormone levels and reviewed reported menstrual history among a small group of women aged 20-42 years. No statistical differences in CD4 cell count existed between anovulatory and menopausal women, but women who ovulated and had regular cycles had higher CD4 cell counts than those who did not. Cu-Uvin and colleagues determined that progesterone and estradiol levels during the luteal and follicular phases (determined by day of cycle) among HIV-infected women who reported normal menstrual cycles did not differ from uninfected women. The ACTG also examined self-reported age at menopause among 55 female participants of clinical trials and found this group of HIV-infected women reported menopause at an average of 47 years, a value lower than the average for the U.S. population (51 years), but this comparison may have been affected by errors of recollection.

4. DIMINISHED OVARIAN RESERVE

The transition to menopause occurs over years, and women may vary with respect to the age at which it occurs and the point in the transition when perimenopausal symptoms begin to occur. The number of primordial ovarian follicles falls progressively in women from menarche to the late 30s. Subsequently, a much steeper decline in the number of follicles occurs until menopause, when few follicles remain. Early in ovarian aging, the relative lack of responsive follicles is associated with sentinel endocrine changes, which constitute diminished ovarian reserve (DOR). As DOR is associated with reduced fertility and recurrent pregnancy loss, a number of clinical studies have focused on the definition and detection of this state. DOR is defined as elevations of serum follicle stimulating hormone (FSH) and estradiol levels on day three of the menstrual cycle (FSH \geq 10mIU/mL and estradiol \geq 50pg/mL). These findings are associated with lower numbers of ovarian follicles and ovarian volume as visualized by ultrasound, and are predictive of reduced fecundity and perimenopausal transition. Luteinizing hormone and serum inhibin-B are also elevated on day three. Hormonal changes do not occur with consistency during other phases of the ovulatory cycle. Because of inhibitory effects of nicotine on aromatase activity, elevations of FSH can also occur among heavy smokers. Since measurement of FSH and estradiol on day three of the ovulatory cycle is a feasible and accurate indicator of ovarian reserve, this method would be an excellent means to determine whether the transition to menopause is influenced by HIV infection and its treatment in a large cohort.

C. PRELIMINARY STUDIES

Several WIHS substudies have investigated sex steroid influences on HIV viremia, genital tract shedding and the effect of HIV infection on self-reported menstrual cycle characteristics. These studies demonstrate our ability to perform ovulatory cycle based methods and to gain the cooperation of WIHS participants in this complex field of research. The following publications demonstrate our experience in this type of research:

1. Minkoff H, Ahdieh L, Watts H, Greenblatt RM, Schmidt J, Schneider M, Stek A. The relationship of pregnancy to the use of highly active retroviral therapy. *Am J Obstet Gynecol.* 184:1221-1227, 2001.
2. Harlow SD, Schuman P, Cohen M, Ohmit DE, Cu-Uvin S, Lix X, Anastos K, Burns D, Greenblatt R, Minkoff H, Muderspach L, Rompalo A, Warren D, Young MA, Klein RS. Effect of HIV infection on menstrual cycle length. *J Acquir Immune Defic Syndrome.* 24:68-75, 2000.
3. Greenblatt RM, Ameli N, Grant RM, Bacchetti P, Taylor RN. Impact of the ovulatory cycle on virologic and immunologic markers in HIV-infected women. *J Infect Dis.* 181:82-90, 2000.
4. Kovacs A, Wasserman SS, Burns D, Wright DJ, Cohn J, Landay A, Weber K, Cohen M, Levine A, Minkoff H, Miotti P, Palefsky J, Young M, Reichelderfer P. Determinants of HIV-1 shedding in the genital tract of women. *Lancet.* 358:1593-1601, 2001.
5. Cu-Uvin S, Wright DJ, Anderson D, Kovacs A, Watts DH, Cohn J, Landay A, Reichelderfer PS. Hormonal levels among HIV-1-seropositive women compared with high-risk HIV-negative women during the menstrual cycle. Women's Health Study (WHS) 001 and WHS 001a Study Team. *J Womens Health Gend Based Med.* 9:857-863.
6. Reichelderfer PS, Coombs RW, Wright DJ, Cohn J, Burns DN, Cu-Uvin S, Baron PA, Cohen MH, Landay AL, Beckner SK, Lewis SR, Kovacs AA. Effect of menstrual cycle on HIV-1 levels in the peripheral blood and genital tract. *AIDS* 2000;14:2101-7.

D. RESEARCH DESIGN AND METHODS

1. SUMMARY

We plan a longitudinal study of the association between HIV infection and the age of onset of diminished ovarian reserve and menopause among WIHS participants. A second analysis will focus on fertility and miscarriage rates between HIV-infected and -uninfected women. A third analysis will focus on the possible association between protease inhibitor use and hypogonadism.

2. INCLUSION CRITERIA

Inclusion criteria for the Sex Steroid Substudy will include:

- HIV-infected and -uninfected women.
- Complete WIHS enrollment, including HIV RNA level, CD4 cell count and demographic data.
- Complete antiretroviral treatment data and full ovulatory and hormone treatment histories.
- Completion of study phlebotomy on one of days two through four of the menstrual cycle among women who have ovulated in the past six months.
- No history of unilateral ovariectomy.
- No breast-feeding within the past six months.

- No use of exogenous hormones within the past 12 months. Hormonal sources include therapy. (**NOTE:** See SSSCR Question-by-Question Specifications for a complete list of exclusionary medications.)

To best match the hormone level data with the core WIHS interview data, the Sex Steroid visit should be scheduled within one month of the core visit, if possible.

3. MEASUREMENT OF HORMONE LEVELS

Serum collected at the baseline Sex Steroid visit will be utilized for determination of FSH, estradiol and inhibin B levels. Many women will also have baseline DHEA, DHEA-s and SHBG testing completed. The Bayer Centaur and DPC RIA methods will be used to determine FSH and estradiol concentrations, respectively. Inhibin B levels will be determined using a double antibody ELISA such as the Serotech assay. Intra- and interassay coefficients of variation will be determined for each assay. The FSH and estradiol assays will be performed centrally by Quest Diagnostics (Baltimore, Maryland); the Inhibin B assays will be performed centrally by Women's and Infants' Hospital (Providence, Rhode Island).

Testing will be done longitudinally for DHEA, DHEA-s, SHBG, MIS and testosterone, beginning with specimens collected during visit 24. For follow-up, specimens collected during the core visit will be resposited, then withdrawn in batches for central testing at the University of Michigan.

4. PREDICTOR VARIABLES

Predictor variables will include: HIV infection status, age, body mass index, smoking (pack years and recent use), history of irregular cycles, desire for pregnancy, and barrier and intrauterine IUD use.

5. OUTCOME VARIABLES

Outcome variables will include: day two through four FSH, inhibin B and estradiol levels; age at menopause; pregnancy history; and spontaneous miscarriage history.

E. PARTICIPANT ELIGIBILITY AND ENROLLMENT

HIV-infected and -uninfected participants from all six WIHS sites who meet the inclusion criteria in Section D2 will be eligible to participate in the Sex Steroid Substudy. Women will be screened for eligibility during visits 18 through 23 at their WIHS core visit. Sites should use SSSCR (Sex Steroid Screening Form) to screen participants. This form is not entered into Apollo. Women who are determined to be eligible will be invited to participate in the substudy.

F. SCHEDULING THE SEX STEROID VISIT

1. BASELINE

For those women who consent to participate in the substudy, the Sex Steroid visit should ideally be scheduled to take place during their next menstrual period; however, if necessary, it can be scheduled for a later menstrual period as long as it is within three months of the core visit. The visit will be scheduled by asking the participant to call her WIHS clinical site on the first day of her next menstrual period. The participant's LMP (collected in the screening form) can also be used to track eligible participants. Reminder calls can be made a few days before she gets her next period. The participant will be asked to come in for her sex steroid visit on day two, three or four of her period.

NOTE: If the participant is on day two, three or four of her menstrual period at the time of her core WIHS visit, and is willing to participant in the Sex Steroid Substudy, she may have her Sex Steroid visit on the same day as her core WIHS visit.

The Sex Steroid visit, to give consent and have blood drawn, will last approximately 20 minutes.

2. FOLLOW-UP

Follow-up Sex Steroid visits began during visit 24, and then were discontinued by the EC in August 2006, mid-way through the visit. As of August 1, 2006, separate follow-up Sex Steroid visits (on day 2 through 4 of the menstrual period) will no longer be required. Future follow-up testing will use specimens collected at the core visit as part of the standard phlebotomy protocol. These specimens will be reposit, and then withdrawn at a later date for the specified testing.

G. OVERVIEW OF VISIT

1. BASELINE

An *SSNOTI* (Sex Steroid Participant Notification/Specimen Collection Form) should be completed and data entered for all women who come in for a Sex Steroid visit (for a total of 1,000 women). After the participant consents to enroll in the study, the date and time of her blood draw, as well as the first date of the participant's current menstrual period, will be recorded on this form. If the enrollment date is not on the second, third or fourth day of the participant's current menstrual period, the participant will not be eligible for enrollment into the substudy.

Serum will be collected from the participant for measuring FSH, estradiol and inhibin B levels. Two to three SSTs (one or two for FSH and estradiol, one for inhibin B) will be collected at the visit. Each tube should contain 8 ml of blood.

2. FOLLOW-UP

No separate Sex Steroid visit is necessary for follow-up data collection, as specimens collected during the standard core visit phlebotomy will be used for all future testing.

H. BASELINE SAMPLE COLLECTION, PROCESSING AND SHIPMENT

1. FSH and ESTRADIOL

a. FSH and estradiol assays will be performed centrally at Quest Diagnostics (Baltimore, MD) at a cost of \$12 per sample for FSH and \$10 per sample for estradiol.

b. Procedure and Supplies:

- Refer to Section 10, Part II, for Blood Drawing Protocol
- Refer to Section 10, Part IX, for Phlebotomy supply list
- One or two (8ml minimum) serum separator tube(s) (SST)
- One (8ml) transport tube

c. Blood for FSH and estradiol testing should be collected aseptically in one SST (8 ml). A total of 2 ml of serum are needed to complete both assays. If a minimum of 4 ml of blood cannot be obtained from a participant in one SST tube, phlebotomists can attempt a second draw using another SST tube. (Therefore, IRB submissions should note that, in the event of a difficult blood draw, this protocol could require three SST tubes total.) After collection, gently invert the tube approximately five times to activate the clotting process. Let blood clot 30 minutes in a vertical position.

d. Ideal serum processing:

Within one hour of blood collection, spin SST for 10 minutes in a swinging-bucket centrifuge or for 15 minutes in a fixed-angle centrifuge at 1100 to 1300 x g. Refrigerate separated serum prior to freezing. Freeze serum in 1 ml aliquots within eight hours of blood collection.

e. Alternative acceptable serum processing:

Within eight hours of blood collection, spin SST for 10 minutes in a swinging-bucket centrifuge or for 15 minutes in a fixed-angle centrifuge at 1100 to 1300 x g. Refrigerate separated serum prior to freezing. Freeze serum in 1 ml aliquots within 24 hours of blood collection.

f. Frozen specimens should be batched and shipped monthly to Quest Diagnostics-Baltimore for testing. Specimens should be shipped overnight on dry ice.

Quest Diagnostics, Inc.
ATTN: Department of Virology
1901 Sulphur Spring Road
Baltimore, MD 21227
Phone: (410) 536-1713
Fax: (410) 536-1474
Contacts: William Meyer, Larry Hirsch, Denise Bopst

The following instructions must be followed when shipping specimens to Quest Diagnostics-Baltimore:

- 1) The submitting WIHS site must complete a custom Quest Diagnostics-Baltimore test requisition for each submitted sample and provide this form to Quest along with the specimen aliquots.
- 2) Specimen aliquots will be packaged by the submitting WIHS site in a sequential, organized fashion within appropriate cyrovial primary shipping boxes.
- 3) The WIHS site will package and ship specimens (with test requisitions) according to applicable governmental packaging requirements (see Communications Memo #344, New ICAO Regulations for the Transport of Diagnostic Specimens).
- 4) The WIHS site will acquire the packaging materials necessary to ship samples for the Sex Steroid Substudy from a non-Quest Diagnostics-Baltimore source.
- 5) The WIHS site will cover the costs to ship the sample aliquots to Quest Diagnostics-Baltimore.

NOTE: All specimens will be tested at Quest Diagnostics in Baltimore, Maryland. The Los Angeles WIHS consortium will pay for all testing.

2. INHIBIN B

a. Procedure and Supplies:

- Refer to Section 10, Part II, for Blood Drawing Protocol
- Refer to Section 10, Part IX, for Phlebotomy supply list
- One (8ml) SST
- -20° C frozen cold packs

b. Blood for the inhibin B assay should be collected aseptically in a second SST (8 ml). 1 ml of serum are needed for this assay. After collection, gently invert the tube approximately five times to activate the clotting process. Place the tube in a vertical position for 30 minutes to allow for the clotting process to conclude.

c. Filled SSTs should be kept at room temperature and centrifuged within six hours of collection in horizontal rotor at 1100 x g for 15 minutes. If a site uses a specimen bank for specimen processing, they should send the tubes there to be spun down and frozen at -70° C. If the

ambient temperature is above 75° F when samples are being transported from the clinic to the bank, they should be placed in a cooler bag with –20° C frozen cold packs. When using cold packs, make sure that the blood does not come into direct contact with the cold pack.

If a site processes their own specimens, they should spin down and freeze the specimens at –70° C. Aliquot serum and ship only 1 mL to the W&I Hospital. Any excess serum should be sent to BBI with s-code 1.

- d. After specimens are frozen, they should be batched and then shipped overnight on dry ice to the central lab once a month for testing. Results will be returned to the site within one week for a shipment of approximately 30 samples.

Women's and Infants' Hospital, 2nd floor
Division of Prenatal and Special Testing
70 Elm St.
Providence, RI 02903
Phone: (401) 453-7650
Contact: Diane or Kristin

3. SEX HORMONE BINDING GLOBULIN (SHBG), DHEA, DHEA-SULFATE (DHEA-S)

- a. SHBG, DHEA and DHEA-s assays will be performed centrally at Quest Diagnostics (Baltimore, MD) using specimens left-over after FSH and estradiol testing has been completed.
- b. Procedure and Supplies:
 - See above for procedure and supplies for FSH and estradiol. SHBG, DHEA and DHEA-s will be run on specimens remaining subsequent to FSH and estradiol testing.
- c. Frozen specimens should be batched and shipped to Quest Diagnostics-Baltimore for testing. Specimens should be shipped overnight on dry ice.

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- 2) Specimen aliquots will be packaged by the submitting WIHS site in a sequential, organized fashion within appropriate cyrovial primary shipping boxes.
- 3) The WIHS site will package and ship specimens (with test requisitions) according to applicable governmental packaging requirements (see Communications Memo #344, New ICAO Regulations for the Transport of Diagnostic Specimens).
- 4) The WIHS site will acquire the packaging materials necessary to ship samples for the Sex Steroid Substudy from a non-Quest Diagnostics-Baltimore source.

- 5) The WIHS site will cover the costs to ship the sample aliquots to Quest Diagnostics-Baltimore.

NOTE: All specimens will be tested at Quest Diagnostics in Baltimore, Maryland.

I. FOLLOW-UP SAMPLE COLLECTION, PROCESSING AND SHIPMENT

Specimens for follow-up Sex Steroid testing will be collected as part of the standard core phlebotomy. No extra specimens need be collected. Future Sex Steroid testing (for DHEA, DHEA-s, SHBG, MIS and testosterone) will be performed using repositied specimens. Testing is proposed to take place at two-year intervals. The criteria for selecting women for testing have not yet been determined.

J. DATA REPORTING

1. BASELINE

Test results for FSH and estradiol (from Quest Diagnostics-Baltimore) and inhibin B (from Women's and Infants' Hospital) will be transmitted back to the clinical sites. Sites should transcribe results onto *SSOI* (Lab Report Form) and data enter the form into Apollo. Test results for SHBG, DHEA and DHEA-s will be transmitted electronically from Question Diagnostics-Baltimore to WDMAC.

2. FOLLOW-UP

Test results for DHEA, DHEA-s, SHBG, MIS and testosterone will be transmitted electronically from the University of Michigan lab to WDMAC after each back of specimens is tested.