WOMEN'S INTERAGENCY HIV STUDY

SECTION 36: MALT/GALT SUBSTUDY PROTOCOL

A. STUDY PURPOSE

The purpose of this study is to evaluate the effects of HIV infection in gut-associated lymphoid tissue (GALT) and endometrial mucosal-associated lymphoid tissue (MALT) in HIV-negative and HIV-positive women at selected stages of HIV disease progression.

Our aims are to compare endometrial, ileal and sigmoid colon tissue samples in selected WIHS participants in order to determine if:

1. The perturbations in GALT, HIV-specific immune responses, apoptosis, and collagen deposition that have been reported in HIV-positive men are also found in HIV-positive women.
2. The patterns of these alterations differ among HIV disease state groups.
3. These alterations are similarly present in endometrial tissue.
4. HIV infection will be associated with reduced numbers of CD4 memory cells, increased lymphocyte activation, apoptosis, cellular turnover, T regulatory cells and collagen deposition in both gut and endometrium; the extent of the abnormalities will be directly correlated with intracellular levels of HIV RNA.
5. The extent of immune abnormality in MALT tissues will be correlated with the extent of HIV disease progression, and with expression of cellular activation markers and level of circulating soluble biologic mediators (in a pattern consistent with inflammation).
6. It is feasible to use endometrial tissue to evaluate overall mucosal lymphoid immunity in future HIV research.

B. BACKGROUND, SIGNIFICANCE, AND PRELIMINARY STUDIES

1. GALT IN HIV DISEASE

While peripheral blood T-cell (CD3+CD4+ cell) counts are an accessible measure of immunologic status in HIV infection, they are an incomplete measure of immune function in general, and are particularly poor indicators of mucosal CD4 cell populations, which are the largest in the human body. Persistence of perturbations of mucosal lymphoid tissues after HIV infection and HAART indicate that even optimal host and treatment responses do not insure recovery of normal immunologic functions.

The recent recognition of the extent of mucosal lymphoid abnormalities after HIV infection and their persistence during HAART has led to major revisions in concepts of HIV pathogenesis. Current hypotheses of immune and viral events over the course of untreated HIV infection hold that CD4 cells from GALT are severely depleted by day 14 of HIV or Simian Immunodeficiency Infection (SIV) infection, particularly CD4+ memory T cells in the lamina propria. These changes precede peripheral CD4 cell depletion.

This persistent cellular depletion in GALT occurs in humans despite near complete recovery of CD4 T cells in peripheral blood after initiation of HAART. Long-term

nonprogressors (natural controllers) are spared the loss of mucosal T cells and the chronic expression of inflammatory response genes in gut mucosa seen in patients with a more typical HIV course. Since mucosal cells can be easily replaced by cells that traffic in from other sites, depletion of CD4 memory cells within mucosal tissues does not explain the subsequent progression of HIV disease. Chronic immunologic activation and inflammatory responses are closely linked with the long-term outcomes of HIV infection. Lymphocyte activation and changes in blood inflammatory mediators often accompany mucosal abnormalities in both untreated and treated HIV patients. Immune activation is also associated with the extent of CD4 cell recovery after HAART, as well as functional abnormalities. Since activation is not limited to HIV-specific cells, persistent activation is not driven solely by lymphocyte responses to HIV itself. However, repeated episodes of HIV-induced cellular proliferation, differentiation and death may over time lead to increased cellular activation and turnover. Persistent activation could also result from CD4-depleted gut mucosa that permit leakage of bacterial antigens into systemic circulation, a concept that is supported by a recent study by Douek, *et al.,* who found that increased blood bacterial lipopolysaccharide (LPS) levels were associated with HIV infection, increased plasma IFN-γ and frequency of CD38+ HLADR+ CD8 T cells. However, HAART treatment is associated with improvements in systemic immune activation even though mucosal lymphocyte populations remain depleted. Because immune activation is to some extent responsive to HAART, viral replication thus appears to contribute directly to persistent activation, perhaps via stimulation of bystander cells by HIV-triggered responders. Immune activation plays a vital role in maintaining viral replication in mucosal tissues by providing newly susceptible cells, while direct and indirect viral effects result in depletion of susceptible substrate. Pro-inflammatory products of activated cells within the mucosa likely cause injury and apoptosis within the mucosal epithelium.

2. THE ENDOMETRIUM

The female upper genital tract is a site of mucosal-associated lymphoid tissue (MALT). While their functions obviously differ, endometrium shares several characteristics with GALT. (*See Appendix A: Comparison of Endometrium to GALT.)* Endometrium is distinguished by the presence of a unique immune cell component — uterine natural killer (NK) cells, the ability to receive and tolerate an embryo and monthly cycles of proliferation, differentiation and tissue breakdown in response to endocrine and paracrine factors. Leukocytes comprise a substantial portion of endometrial cell constituents, ranging from 7% to 30% depending on ovulatory cycle phase. Polymorphonuclear leukocytes are common during menses, but T cells and macrophages, combined with the uterine NK cells, comprise 90% of endometrial leukocytes. T cells comprise approximately 50% of leukocytes; of these one half to two thirds are CD8+, and a smaller but cycle-constant proportion express CD4. In contrast, the number and proportion of NK cells increases as the ovulatory cycle progresses. CD8 + T cells occur as stromal cells, intraepithelial lymphocytes and in lymphoid aggregates. Lymphoid aggregates or follicles are present in the basal layer, formed during the proliferative phase, and include a core of B-cells surrounded by CD8+CD45RO+CD45RA-, T cells with an outer halo of macrophages. Aggregates arise primarily from trafficking of cells to nucleation sites; a small number of CD8+ cells show evidence of division at the site. Macrophages account

for 5% to 15% of stromal cells. Phenotypically unique uterine NK cells are present in deep tissue layers.

Only eight reports have been published on the endometrium and HIV infection. Active HIV replication occurs in monocytes and macrophages in endometrium. Very limited information is available on the effects of HIV infection on endometrial tissue. Johnstone reported increases in CD45+ cells and CD3+ expression among HIV-positive women at the time of menses, compared with HIV-negative women. In a study of three HIV-infected women, White, *et al,.* found that despite the presence of HIV-specific cytotoxic T lymphocytes (CTL) in peripheral blood mononuclear cells (PBMCs), CTL activity was absent in the endometrium of the two antiretroviral therapy (ART) recipients, both of whom lacked endometrial CD8+ cells. By contrast, the one long-term nonprogressor (LTNP) studied demonstrated CD8+ cells and HIV-specific CTL in endometrium.

Clearly, this study can make significant contributions to the understanding of how the endometrial MALT site is affected by HIV, as well as investigating the potential value of endometrial tissue for future studies of MALT and HIV.

C. STUDY DESIGN AND METHODS

1. STUDY POPULATION AND SAMPLE SIZE

A total of 50 WIHS participants, 10 to 15 participants each from five phenotype groups, will be enrolled. Once 15 women from any of the categories have completed the study, that category will be closed to enrollment. The phenotype groups are as follows: *(See Appendix B for complete definitions.)*

- HIV-uninfected women
- Natural controllers
- Good CD4 responders to highly active antiretroviral therapy (HAART)
- Poor CD4 responders to HAART
- Off-treatment progressors

2. STUDY PROCEDURES

a. *Pre-eligibility:*

WIHS sites will be provided with lists of potentially eligible participants based on the Inclusion and Exclusion Criteria listed below. Study staff will then approach potentially eligible participants at their next regularly scheduled Core Visit. Participants who are interested will have a Pre-Eligibility Screening Interview at the end of the Core Visit. Those who appear eligible will be scheduled for a Screening Visit. (*See Appendix C: Time and Events Schedule*.)

b. *Screening Visit*

The Screening Visit will be scheduled as soon as possible after the WIHS Core Visit. Informed consent will be obtained at this visit. All participants will answer questions about medical history, current medications, and use of intravaginal products. Urine testing for pregnancy will be performed. Participants with a positive pregnancy test will not continue in the study. Blood will be drawn for prothrombin and partial

thromboplastin tests (PT and PTT). HIV-positive participants will have blood drawn for HIV viral load, since it is anticipated that results from the prior WIHS Core Visit will not yet be available. HIV-positive participants will also be asked to report any acute medical illnesses occurring during the study period and to avoid vaccinations during the study if this can be done without compromising their health. These measures are requested because of the potential for HIV viral load changes associated with acute medical illness and vaccination.

Participants will be instructed how to perform at-home urine testing to detect luteinizing hormone (LH). LH peaks just before ovulation, and is detectable in the urine in the early luteal phase.

Study staff will dispense Clearblue brand LH surge kits (Proctor & Gamble, Cincinnati, OH) and assist the participant in determining when to start testing. (*See Appendix D: When to Start LH Surge Testing.*) The participant will notify site staff when LH is detected in the urine, so that the Endometrial Biopsy Visit can be scheduled within 7 to 9 days, at which time the participant will be in the mid-to-late luteal phase. Participants will be encouraged to contact site staff should *any* questions or concerns arise during urine testing. Study staff may also contact participants.

Staff will also dispense Golytely laxative (polyethylene glycol) or other bowel cleansing medication, at the discretion of the site colonoscopist. You may need to get a prescription from the colonoscopist for this, your PI may write the prescription, or you may order it from your pharmacy. Participants will be given written instructions for Bowel Prep. Participants will be instructed to have a clear liquid diet the day before the Biopsy Visit and to refrain from any food or liquids at all for 8 hours before the Colonoscopy Visit. Participants will receive personal cleansing wipes for use during Bowel Prep, which will cause frequent liquid stools.

Participants who do not feel they will be able to refrain from vaginal intercourse for 72 hours before the biopsy will receive a supply of non-lubricated condoms.

c. *At Home Phase:*

Participants deemed ineligible because of screening PT/PTT or HIV viral load results will be notified by study staff. All others will be in contact with staff regarding at-home urine testing for LH surge.

Participants will receive calls from staff to assist in urine testing as needed. The Endometrial Biopsy Visit will be scheduled 7 to 9 days after LH surge. The Colonoscopy Visit will be scheduled for the same day, or within 7 days of the Endometrial Biopsy.

Participants will receive reminder calls when it is time to stop using intravaginal products, as well as when to stop using prohibited medications (those which increase risk of bleeding), when it is time to begin clear liquid diet and to begin Bowel Prep. They will also be contacted to determine if they have had any acute medical illnesses (for example, cold or flu-like illnesses, fever, diarrhea, urinary tract infections, genital herpes outbreaks, vaginitis) that would necessitate rescheduling of the Biopsy Visit.

Study staff will be available via pager to assist the participant in adherence with Bowel Prep. For participants who may find it very difficult to do the Bowel Prep at

home (for example, those marginally housed or living in places where bathroom access is limited), one option is to offer hospitalization at a local research center overnight. Sites should also consider this option for participants with very difficult peripheral venous access, for whom placement of a peripherally inserted central catheter (PICC) line is necessary. Sites with access to CTSA-funded CRCs may consider applying for overnight admission visits for participants who have difficulty traveling to study visits, or who are anticipated to have difficulty with the Bowel Prep.

d. *Biopsy Visit:*

FOR SOME SITES, IT WILL NOT BE POSSIBLE TO SCHEDULE THE COLONOSCOPY AND ENDOMETRIAL BIOPSY ON THE SAME DAY. IN THAT CASE, THE ENDOMETRIAL BIOPSY SHOULD STILL BE SCHEDULED BASED ON THE LH SURGE. THE COLONOSCOPY CAN BE SCHEDULED WITHIN 7 DAYS ON EITHER SIDE OF THE ENDOMETRIAL BIOPSY.

SITES PERFORMING GYNECOLOGICAL SPECIMEN COLLECTION WITHOUT SEDATION MAY WANT TO CONSIDER OFFERING PARTICIPANTS IBUPROFEN AND/OR PARACERVICAL BLOCK.

This visit will occur 7 to 9 days after the LH surge is detected, during the mid-to-late luteal phase of the menstrual cycle, when the depth of endometrial tissue is greatest. Participants will undergo a colonoscopy and endometrial biopsy (EMB). Immediately prior to these procedures, we will conduct a brief interview to collect health history and information on medication use and recent intravaginal product use. A urine sample will be collected to rule out pregnancy. Participants with a positive pregnancy test or recent intravaginal product use will not continue in the study.

Continuing participants will be assessed by the site colonoscopist who will determine the appropriate intravenous (IV) sedation to administer. Participants will have an IV line placed by Endoscopy Nursing staff. Before sedation is administered, blood for serum progesterone will be drawn through the IV line. Serum progesterone levels will be run locally to confirm menstrual cycle phase in case the histologic dating of the endometrium is equivocal. If colonoscopy and endometrial biopsy are being performed at separate visits, serum progesterone should be drawn at the EMB visit.

For HIV-positive participants, one 5-6 ml lavender top EDTA tube should be drawn at the same time as the serum progesterone. This will be used to compare HIV viral load in plasma to that in cervical secretions.

After venipuncture, gently invert the tube 8 to 10 times and store upright at room temperature until centrifuging. Blood should be centrifuged within 6 hours. Centrifuge at room temperature at $\leq$1300 x g for 10 minutes. Aliquot at least 1.1 ml into a labeled Wheaton cryovial and refrigerate for no more than six hours before freezing at -80 for shipping.

The SF site only will also draw two 10-cc lavender top EDTA tubes for peripheral blood monocytes (PBMCs) to be shipped to the UC Davis Lab.

Sedation will be administered and blood drawn through the PICC line for those participants who required PICC placement; all peripheral and PICC lines will be

removed before the participant is discharged from the study. After sedation, the participant will be placed in a right side-lying position and a colonoscope will be passed through the large intestine and through the ileo-cecal valve. At least 8 to 10 biopsy samples each of the ileum and sigmoid colon (total 16 to 20 samples) will be obtained with cold forceps using standard techniques. In the event the colonoscopist identifies any lesions, additional biopsies of the lesion(s) will be obtained as clinically indicated, and sent for routine clinical pathology at the site.

Four to five samples will be placed in Wheaton cryovials supplied by the San Francisco site and flash frozen; four to five samples will be placed in a container of 10% formalin, and one sample will be placed in another container of 10% formalin. Sites will supply their own formalin containers. All will be shipped to San Francisco.

Endoscopy nurses are used to collecting biopsy specimens; if you give her/him the tubes, s/he will probably use a small needle to tease the tissue out of the forceps into your cryovial or container of formalin. Please collect the frozen sample first so that there is no chance of contaminating the frozen specimen with formalin. The study specimens will be used to measure inflammatory cells, cytokines and other immune markers, and collagen deposition. Routine histology will be performed on the second formalin container.

(San Francisco site only will also send fresh endometrial specimens in antibiotic medium to the Shacklett Lab at UC Davis.)

- If the colonoscopist determines that the Bowel Prep is inadequate to allow visualization of the colon, s/he will withdraw the endoscope; the participant will remain in the unit to recover, but no further study procedures (i.e., endometrial biopsy) will be done. Blood, if drawn for serum progesterone level or HIV viral load, will be discarded.
- After colonoscopy is completed, all gynecological specimens will be collected. The participant will be placed in the lithotomy position. A pelvic examination will be performed to rule out signs of infection; if any are present, no further procedures will be performed.

1. <u>Endocervical wick/sponge sampling:</u>

   Endocervical mucus and secretions for analysis of cytokines will be collected with Merocel ophthalmic sponges (Medtronic Xomed, Jacksonville, FL). Using a ring forceps, two sponges will be inserted sequentially into the cervical os; each will be held in place to absorb sample for 60 to 90 seconds. The sponges are inserted gently directly into the os. The depth of insertion is based on the size of the os; in some women it is possible to insert just the tip of the sponge, while in others, the entire sponge may be inserted. The sponges should never be forced, as this may cause bleeding which will contaminate the mucus collected. They should not be twisted or turned; simply allow the sponge to rest and absorb secretions. It should be possible to see the sponge expanding as it absorbs secretions.

   After removal, the sponges will be placed in separate cryovials, supplied by the San Francisco site. Place the sponge spearhead down in the vial and cut off the part of the handle that protrudes from the vial so that the sponge will fit into the

tube. Then place this cut-off handle in the vial with the sponge section and close the vial. This is necessary because the lab must weigh the whole collection device in order to ascertain the volume of fluid collected. The cryovials will be placed in liquid nitrogen and the samples will be shipped frozen.

For HIV-positive participants ONLY, endocervical mucus and secretions for HIV viral load will be collected with TearFlo ophthalmic strips. Using a ring forceps to hold three strips on the squared end of the strips, gently insert them simultaneously into the cervical os and hold in place for approximately 60 to 90 seconds to adsorb sample. Only the TearFlo strips and **not** the forceps should be placed in the cervical os. If a woman has a small or pinpoint os, gently place the tip of the TearFlo strips to the opening of the os and get as much secretion as possible. The strips may also be folded vertically to make them thinner, and thus, able to pass through the os. After removal, all three strips will be placed in one Corning cryovial with lysis buffer. Hold the round end of the three strips over and slightly inside one labeled vial. Cut the round head off the strips with sterile scissors, allowing the heads to fall into the vial. Cap the vial and gently invert five times. The cryovials will be placed in liquid nitrogen and the samples will be shipped frozen.

CAUTION: Buffer is caustic! Always wear gloves and eye protection!

2. Endocervical cytobrush sampling: (*SAN FRANCISCO SITE ONLY*!)

   Two cytobrushes will be inserted into the cervix sequentially. Each will be rotated five times to collect cells. Brushes will be placed in antibiotic medium and shipped fresh to the Shacklett Lab at UC Davis.

3. Endometrial biopsy:

   We will use the Pipelle aspirator, a flexible polypropylene sheath with an inner plunger that is attached to a syringe; the entire device is disposable. The procedure requires a total of 10 to 15 min, with 5 to 15 seconds for tissue sampling. With the participant in the lithotomy position, a bimanual exam is performed to determine uterine size and position and to determine whether marked uterocervical angulation exists. A sterile speculum is inserted and the cervix visualized. An antiseptic solution is applied to the cervix, and if it is not readily visualized, a tenaculum is used to position the cervix. The Pipelle is introduced through the cervical os, endocervical canal, internal os, lower uterine segment and into the uterine fundus. The depth of the uterus is measured (note the scale markings on the Pipelle catheter) and documented. The syringe piston is retracted, and the device is gently rotated with a gentle in-and-out motion at least four times within the upper endometrial cavity, maintaining a vacuum. The device is withdrawn when the Pipelle lumen has been filled with tissue. The sample will be dispelled with care not to contaminate the catheter so that it can be re-inserted for additional specimen collection if needed. After final withdrawal, the tip of the device is cut off and discarded. Half the specimen is discharged into a Wheaton cryovial by pushing the syringe piston. This specimen is flash frozen by placing the cryovial in liquid nitrogen. The other half is discharged into a container of

10% formalin. Again, it is important not to contaminate the frozen specimen with formalin.

After specimen collection is complete, the speculum and tenaculum will be gently removed, and swab pressure applied to any sites of bleeding.

(San Francisco site only will collect more endometrium and send fresh endometrial specimens in antibiotic medium to the Shacklett Lab at UC Davis.)

4. Cervical Transformation Zone Sampling:

   The active cervical transformation zone is usually visible without colposcopy in younger women as a circular zone surrounding the os where the pale pink squamous epithelium (distally) meets the dark red epithelium of the columnar epithelium (proximally); this region includes the squamocolumnar junction. The active transformation zone, which includes the squamocolumnar junction at its proximal or leading edge can be more precisely delineated using Lugol's (iodine) solution. Lugol's stains mature squamous epithelium (dark brown); the active transformation zone and the columnar epithelium are non-staining. The cervix will be swabbed with Lugol's solution for disinfection and to visualize the transformation zone. Biopsies will be obtained with Tischler biopsy forceps; the longer jaw placed in the os on the columnar epithelium and the short jaw of the forceps placed on the squamous epithelium, spanning a portion of the transformation zone for each biopsy. Two adjacent cervical biopsies from the transformation zone at 12:00 and 1:00 will be obtained. The average size of a typical biopsy is approximately 2 to 3 mm^3. One biopsy will be placed into a cryovial, snap frozen in liquid nitrogen, and shipped for storage in a -80 degree freezer until RNA extraction can be performed. The other biopsy will be placed in 10% formalin for histology and immunohistochemistry analyses. If the cervical transformation zone is not visible due to recession into the cervical canal (i.e., the entire ectocervix stains with Lugol's solution), biopsies will be performed at the cervical os with the long jaw of the forceps placed as deeply as possible into the endocervical canal and the short blade on the ectocervix. Silver nitrate will be applied for hemostasis.

   (San Francisco site only will do an additional biopsy and send fresh cervical specimens in antibiotic medium to the Shacklett Lab at UC Davis.)

The participant will be sent to the recovery area, where she will be monitored for approximately 2 to 3 hours, until sedation wears off. Because of safety concerns after sedation, the participant will be advised not to drive or operate heavy machinery for 24 hours after the procedures. It is conventional for Endoscopy staff to refuse to discharge a patient who has received sedation without a responsible adult to accompany her home. The SC will arrange transportation via a medical cab service for participants who do not have a responsible adult to accompany them. Regardless of participant's transportation home, SC will be present at discharge. Furthermore, some Endoscopy staff may also require that the provider who performed the EMB clear the participant for discharge (i.e., confirm that there are no complications from the EMB), in addition to the clearance given by the colonoscopist.

The participant will be given post-procedure instructions. The SC will also contact the participant that evening, the next day, and again one and two weeks later to be sure she is not experiencing any adverse events.

3. **INCLUSION CRITERIA:**

1. WIHS participants between 18 and 44 years of age.
2. Regular menstrual cycles (21 to 35 days).
3. HIV-negative women *OR* women who meet the criteria for the HIV disease progression groups listed above. All positive participants must have documented HIV positivity for at least three years. *(See Appendix B for detailed definitions of the eligibility groups*.)
4. Participant must be willing and able to perform urine testing at home and to report for an Endometrial Biopsy Visit 7 to 9 days after testing confirms that LH surge has occurred, with Colonoscopy Visit occurring the same day or within seven days.
5. Participant must be willing and able to refrain from using vaginal products for 10 days prior to the Endometrial Biopsy Visit.
6. Participant must be willing and able to refrain from vaginal intercourse or to use nonlubricated condoms for 72 hours prior to the Endometrial Biopsy Visit.
7. Participant must be willing and able to perform Bowel Prep.

4. **EXCLUSION CRITERIA:**

1. History of hysterectomy.
2. Allergies to latex or iodine.
3. History of bleeding dyscrasias; current anticoagulant therapy or abnormal screening coagulation studies.
4. History of inflammatory bowel disease.
5. Currently breastfeeding, pregnant, or planning a pregnancy in the near future. Has breastfed in the last six months.
6. Current or recent abnormal vaginal discharge and/or abnormal vaginal bleeding.
7. Current genital herpes outbreak, and/or greater than four outbreaks/year. (Suppressive therapy for genital herpes is **NOT** exclusionary.)
8. Any active uterine or gut infection.
9. Recent (within 14 days of Biopsy Visit) upper respiratory infection, urinary tract infection, vaginitis, systemic illness with fever or diarrhea, or vaccination.
10. Abnormal Pap smear in the last 12 months.
11. Use of intrauterine device in the last 12 months.
12. Use of hormonal contraceptives or sex hormones within the last 12 months.

13. Use of supraphysiologic levels of glucocorticoid therapy or other potential immunomodulatory therapy within the past 30 days. (Use of inhaled steroids for asthma is **NOT** exclusionary.)

14. Other conditions that, in the opinion of the investigator, would compromise the participant's ability to comply with the study protocol, such as a major psychiatric disorder, or inebriation or cognitive impairment sufficient to interfere with participant's ability to give informed consent.

D. COMPENSATION

Participants will be reimbursed for the Screening Visit and for the Biopsy Visit(s) at the discretion of each site. For example, SF is reimbursing $25 for screening and $125 for the Biopsy Visit. Participants whose Bowel Prep is deemed inadequate to perform colonoscopy will be reimbursed $20. The participant will be offered re-enrollment with her next menstrual cycle at the discretion of the site's Principal Investigator and the Study Coordinator.

E. SPECIMEN DISPOSITION

1. ***LABELING SPECIMENS:***

San Francisco will supply Merocel sponges, Wheaton cryovials and cryo-labels to all the sites. They will also supply preprinted labels for the formalin containers. Sites will supply their own formalin containers. A layer of blank packing tape should placed over the formalin container label to prevent running of the ink should containers come in contact with liquids.

San Francisco will supply cryo-labels and Corning cryovials with lysis buffer (orange cap) for collection of cervical secretions in HIV-positive participants, and cryo-labels and cryovials for collection of plasma for HIV viral load. Sites will supply their own EDTA blood collection tubes.

All biopsy specimens should be labeled with the date of collection and the unique identifier (called the Lab ID#), which the site will assign to each participant. We are not labeling the actual specimens with the WIHS ID because we are using such small cryovials and the receiving lab here in San Francisco requested we use a system that allows all the information to be on the tube directly.

Every specimen shipping form will have both the WIHSID # and the Lab ID#. The Lab code always starts with W (for WIHS), then a number from 1-6, denoting the WIHS Site (1=Bronx; 2=Brooklyn; 3=Washington, D.C.; 4=Los Angeles; 5=San Francisco; 6=Chicago). The last number is assigned sequentially as participants are enrolled. First participant at your site =1, second =2, etc.

Each specimen will be labeled with a 5-digit alphanumeric code that contains the Lab ID# and a code for the type of specimen. The code is explained below. For example, W51-EP would be assigned for endometrium preserved in formalin for the first participant enrolled at the San Francisco WIHS site. W52-IF would be assigned for a frozen ileal specimen from the second San Francisco WIHS participant enrolled.

W = WIHS
[S] = indicates which WIHS Site nationally (will be a number from 1-6)
[N] = Unique to participant, assigned in the order in which participants at each site are enrolled. First participant = 1, second = 2, etc.
Next letter = Specimen type
S = Sigmoid colon
I = Ileum
P = Cervical sPonge (Merocel spear)
W=Cervical Wick for HIV VL (TearFlo strip)
E = Endometrium
Y = Endocervical cYtobrush (San Francisco only)
C = Cervical transformation zone
B = Blood

Last Letter = How specimen received in Lab
F = Frozen
P = Formalin (Preserved)
C = Fresh (Cells) (San Francisco only)

SPECIMENS IN ORDER OF COLLECTION	Frozen Specimens	Fresh Specimens (SAN FRANCISCO ONLY)	Specimens in Formalin
Ileal biopsy	W _ _ -IF	W _ _ -IC	W _ _ -IP (2 containers: one with 4-5 pieces, one with 1 piece)
Sigmoid colon biopsy	W _ _ SF	W _ _ -SC	W _ _ -SP (2 containers: one with 4-5 pieces, one with 1 piece)
Cervical sponge for cytokines	W _ _ -PF	W _ _ -PC	N/A
Cervical wick in lysis buffer for HIV VL (HIV + participants only)	W _ _-WF	N/A	N/A
Endocervical cytobrush *	N/A	W _ _ -YC	N/A
Endometrial biopsy	W _ _ -EF	W _ _ -EC	W _ _ -EP
Cervical transformation zone biopsy	W _ _ -CF	W _ _ -CC	W _ _ -CP
Blood for PBMCs*	N/A	W _ _ -BC	N/A
Plasma for HIV VL (HIV + participants only)	W _ _-BF	N/A	N/A

2. ***SHIPPING SPECIMENS:***

a. ***ALL SITES EXCEPT SAN FRANCISCO:***

Both Fixed and Frozen tissues should be shipped to:

McCune Lab
Cancer Center, Mt. Zion
2340 Sutter Street, Room S-371
San Francisco, CA. 94115
Attention: Margaret Takeda

In order that the formalin not freeze, the frozen specimens and the formalin specimens should be shipped in separate containers, **OR** all the specimens can be shipped in one container, as long as the frozen specimens are in a separate Styrofoam container, ie., a box within in a box.

Before shipping specimens, please email Jane Pannell at jane.pannell@ucsf.edu to inform her of the pending shipment. Please include WIHSID, Lab ID#, and date of specimen collection.

When the results of the serum progesterone are available, please mark clearly with participant's WIHSID and Lab ID#, obliterate other identifying information, and fax to Jane Pannell at 415-353-9792.

Please also fax a copy of the colonoscopist's report and any pathology reports (if lesions are seen) to Jane Pannell. Again, mark clearly with participant's WIHSID and Lab ID#, and obliterate other identifying information. Thank you.

b. ***SAN FRANCISCO SITE ONLY:***

As above, plus:

Courier all fresh specimens in antibiotic medium at ambient temperature to:

Barbara L. Shacklett, PhD
Department of Medical Microbiology and Immunology
School of Medicine
3146 Tupper Hall
1 Shields Avenue
Davis, CA 95616
Phone: Dr. Shacklett: 530-752-6785
FAX: 530-752-8692

Prior to sending specimens to UC Davis, please e-mail blshacklett@ucdavis.edu to inform the lab of the pending shipment.

APPENDIX A: COMPARISON OF GALT TO ENDOMETRIUM

Comparator	***Endometrium***	***GALT***
Barrier function	Frequent entry of lower genital tract constituents (Barnhart 2001; Dayal 2003)	Intense bacterial colonization
Induction of immune tolerance	Distinguishes foreign embryo from pathogens (Doukouhaki 2006)	Distinguishes desirable flora from pathogens
Variation in cell types	Cellular constituents vary over ovulatory cycle	Cellular constituents vary with anatomic location
Lymphoid aggregates	Cycle variable B cells surrounded by CD8/45 RO+ T cells, and an outer halo of CD14+ monocytes/macrophages present at the stratum basalis, and NK aggregates at endothelial layers (Sentman 2004)	Peyers patches, M cells, isolated follicles
Microvillus epithelium	Post-luteal epithelium	Small bowel
Antigen presentation	Epithelial cells (Fahey 2006)	Epithelial cells
Origin of lymphoid cells	Most traffic in (Yeaman 2001)	Most traffic in
T cell type	CD8+ T cells predominate	CD8+ T cells predominate
γδ-T cells	γδ-T cells represent approximately 10% of intraepithelial T cells	γδ-T cells represent approximately 10% of intraepithelial T cells
Defensins	α– and β–defensins are present depending on inflammation, infection, and cycle phase	α– and β–-defensins are present depending on inflammation and infection
Sex steroid responsive	Significant modulation of cell types, apoptosis, tissue anatomy	Estrogen receptor ligands affect IBD, estrogen inhibit gut NK-κB transcription (Kayisli)

APPENDIX B: DEFINITIONS OF PHENOTYPE GROUPS FOR POSITIVE WOMEN

Women in all categories must have been HIV positive for at least three years, plus:

Natural Controllers	• HIV viral load consistently below the WIHS level of detectability. • Never used HAART ***or*** HAART use during pregnancy only.
Good CD4 Responders to HAART	• CD4 count <350 before initiating HAART. • HAART use for at least 2.5 years. • CD4 count increase ≥ 200 cells above pretreatment count. • Consistent increases in CD4 cell count since starting treatment. • HIV viral load consistently below WIHS level of detectability since starting HAART.
Poor CD4 Responders to HAART	• CD4 count <350 before initiating HAART. • HAART use for at least 2.5 years. • CD4 cell increase ≤100 cells over pretreatment count. • HIV viral load consistently below WIHS level of detectability since starting HAART.
Off Treatment Progressors	• HIV viral load consistently detectable for at least three years. • Never used HAART ***or*** HAART use during pregnancy only.

APPENDIX C: TIME AND EVENTS SCHEDULE

Requirement	*Screening Visit*	*At home Phase*	*4 to5 Days before Biopsy Visit*	*1-2 Days before Biopsy Visit*	*Biopsy Visit*	*Evening of & Day after Biopsy Visit*	*1 wk and 2 wks post Biopsy Visit*
Consent.	X						
Urine pregnancy test.	X				X		
Blood draw for PT and PTT.	X						
Ascertainment of medical history, medication use, intravaginal product use.	X				X		
Condoms dispensed as needed.	X						
Golytely dispensed with instructions. Personal wipes dispensed.	X						
SC* and Ppt** in contact. Ppt to perform home urine testing and to refrain from use of vaginal products once LH surge detected. Biopsy Visit to be scheduled 7 to 9 days after LH surge.		X					
SC makes reminder call to Ppt: Stop all prohibited medications. SC makes arrangements for medical taxi as needed.			X				
SC makes reminder call to Ppt: Clear liquid diet to begin day before (Colonoscopy) Biopsy Visit. Begin Golytely Bowel Prep at 6 pm same day *(or* admit to CRC for Bowel Prep).				X			
SC available via pager to assist with adherence to Bowel Prep.				X			
Colonoscopy with biopsies, EMB performed. Serum progesterone drawn at EMB Visit.					X		
SC contacts Ppt to assess for adverse events.						X	X

*SC=Study Coordinator ** Ppt=Participant

APPENDIX D: HOW TO USE CLEARBLUE URINE TEST KITS TO DETECT LH SURGE

When Luteinizing Hormone (LH) rises in the body, it causes ovulation, which occurs in the middle of the menstrual cycle. This increase is called the "LH surge." We will schedule the Biopsy Visit for 7to 9 days after the LH surge is detected, when the depth of the endometrial tissue is at its greatest, so it is important that participants are aware how to test properly and to call as soon as the LH surge is detected.

DETERMINE WHEN TO START TESTING

To determine when to start testing, you must first determine the length of the participant's menstrual cycle. The length of the menstrual cycle is the number of days from the first day of bleeding to the day before bleeding begins on the next period.

Determine the usual length of the menstrual cycle over the last six months. Then, refer to the Cycle Chart below to decide on which day of the menstrual cycle to begin testing.

Cycle Length	Day to Begin Testing
21 days	Day 5
22 days	Day 5
23 days	Day 6
24 days	Day 7
25 days	Day 8
26 days	Day 9
27 days	Day 10
28 days	Day 11
29 days	Day 12
30 days	Day 13
31 days	Day 14
32 days	Day 15
33 days	Day 16
34 days	Day 17
35 days	Day 18

TEST PROCEDURE

Instruct the participant:

1. You may test your urine at any time of the day; however, you should try to do it the same time of the day every day. Most people find it easiest to do first thing in the morning.

Also, reduce your liquid intake and try not to urinate for about four hours before testing, since a diluted urine sample can hinder LH detection.

2. To begin testing, open the sealed pouch and remove the test stick from the pouch. Remove the white cap. This will expose the absorbent strip.
3. Point the absorbent strip downward. Place it in your urine stream for about five seconds so that it gets thoroughly wet. Try not to get any urine on the results window.
4. When you take the stick out of your urine stream, keep holding it with the absorbent strip down. You do not have to recap the stick, but can if you want to.
5. Lay the stick flat on a table, and wait for colored lines to appear. You can read the results anytime between three and ten minutes after using the test.

INTERPRETATION OF RESULTS

Negative - No LH Surge: Only one colored line appears **OR** the surge line (closest to the arrow) band is much lighter than the reference line. There is no LH surge. Test again tomorrow.

Positive - LH Surge: If two colored lines are visible and the surge line (the line closest to the arrow) is equal to or darker than the reference line, you are about to ovulate. ***Call us to schedule your Biopsy Visit!***

Invalid: No visible line at all. The reference line will not appear if not enough urine hit the absorbent tip. Repeat with a new test kit. Please reread the instructions and follow them precisely. The most common reason for an invalid test result is that urine has splashed into the interpretation window. This prevents liquid from properly "wicking" up the test and through the band of dye. Please note that invalid results can nearly always be avoided by placing your thumb over the interpretation window while holding the test in the urine stream.

FREQUENTLY ASKED QUESTIONS

1. *Should I restrict my diet before taking the test?*
   No, diet will not affect the test results.
2. *Today's reference line is a different shade of blue than yesterdays. Is this a concern?*
   No. Variations in the color of the reference line will not affect the test result. Always compare the color of the surge line to that of the reference line of the same device on the day the test is performed. Do not compare bands from different devices.

STORAGE AND STABILITY

Store the test kit below 28ºC; do not freeze.

APPENDIX E: JANE'S MALT/GALT CHEAT SHEET

Jane's MALT/GALT Cheat Sheet
NOT FOR DATA ENTRY. SHRED AFTER PARTICIPANT COMPLETES STUDY.

LMP? Menstrual hx? How many days usually b/w periods? (should be 21-35)
Pregnant? Breastfeeding? Birth control?
Any medical problems? Meds? +/-?
Allergies?
Schedule screen visit—Send consent to read prn □ Screening visit scheduled for _____ Confirmed □

SCREENING VISIT: Consent - preg test - blood draw (with HIV VL as needed) □
Dispense Golytely and "care pkg" □ *OR* Plan admission for Bowel Prep □
Dispense Clearblue kits, instructions, wipes, condoms □
Contact info given □
Special needs? IV access?

AT HOME PHASE: Screening labs OK? □ Notify ppt
Check in with ppt-- menses start date? _____LH surge date ? _____
Any vaccine, fever, illness, UTI, genital HSV, vaginits, etc?

SCHEDULE EMB VISIT 7-9 DAYS AFTER LH SURGE.
SCHEDULE COLONOSCOPY VISIT 7 DAYS BEFORE OR AFTER EMB VISIT.

EMB VISIT scheduled for: _____	**COLONOSCOPY VISIT** scheduled for: _____
□ _____ Remind ppt no vag products or vag intercourse for al least 3 days before visit. □ _____ Day of visit: Check with ppt re: any illnesses/vaccine, vag product use. Urine preg test before any procedures. □ _____ Night of visit: check in with ppt	□ Offer assist with meds. Usually ppts should not take any meds before procedure. Some exceptions-- BP meds. You may have to check with ppt's provider or the endoscopist. □ Check transport home/ arrange medical cab □ _____ Remind ppt to stop NSAIDS, other prohibited meds 5 days before Visit □ _____ Remind ppt to begin clear liquid diet day before visit, and start Golytely solution at 6pm. NPO for 8 hours before colonoscopy. □ _____ Day of visit: Check with ppt re: any illnesses or vaccines. Urine preg test before any procedures. □ Evening of colonoscopy—Check in with ppt □ _____ 1 week after colonoscopy—Check in with ppt □ _____ 1 week after colonoscopy—Check in with ppt

BRING TO EMB VISIT:	**BRING TO COLONOSCOPY VISIT:**
▪ Supplies to draw serum progesterone ▪ Supplies to draw plasma HIV VL for + participants ONLY- will be shipped frozen ▪ Preg test ▪ Specula ▪ Ring forceps ▪ Tenaculum ▪ Kevorkian/Tischlers ▪ Merocel eye spears (sponges for cytokines) ▪ TearFlo eye strips (wicks for cervical VL in HIV+ participants ONLY) ▪ Endometrial biopsy catheter (Pipelle) ▪ Sterile scissors ▪ Betadine, cotton balls, swabs, Lugols and/or Monsel's, etc. – clinician's preference ▪ Wheaton cryovials x 2—1 for endometrium, 1 for cervical transformation zone ▪ Individual containers of 10% formalin ▪ Liquid nitrogen ▪ Ibuprofen and/or supplies for cervical block—clinician's preference ▪ Mini pad for participant ▪ Extra formalin containers in case clinician sees any lesions ▪ Appropriate transport/shipping containers and/or lab tracking from for shipping to UCSF	▪ Liquid nitrogen ▪ Wheaton cryovials x 2 ▪ Individual containers of 10% formalin x 4 (1 sigmoid and 1 ileum for study specimens to be shipped to UCSF, plus 1 sigmoid and 1 ileum for local routine pathology) ▪ Extra formalin containers in case clinician sees any lesions ▪ Appropriate transport/shipping containers and/or lab tracking from for shipping to UCSF

□ **Email Jane Pannell re: impending lab shipments: jane.pannell@ucsf.edu**

FAX to Jane Pannell at 415-353-9792:

□ NOTI Form
□ Serum Progesterone Results
□ Colonoscopy Report
□ Any Pathology Reports