Study Title: Accelerated Genital Tract Aging in HIV: Estradiol Clinical Trial NCT04079218
IRB Number: 2019-10529

Accelerated genital tract aging in HIV: estradiol clinical trial

1. Background

HIV is associated with accelerated and accentuated aging as evidenced by *earlier onset* and *increased prevalence* of several age-related conditions including cardiovascular, neurocognitive and bone disease[1-4]. Chronic HIV infection and aging may be linked by a common mechanism: inflammation[5], which is fueled by ongoing low-level viral replication, microbial translocation, immunosenescence, immune activation and ART use[6]. However, the link between HIV infection and genital tract aging in women is understudied and has important clinical implications. The phenotype of female genital tract (FGT) aging is characterized by vaginal dysbiosis (increase in diverse anaerobes and decrease in acid-producing lactobacilli) and mucosal inflammation. These changes are associated with an increased risk for urinary tract and sexually transmitted infections (STI), vaginal atrophy, HIV acquisition and shedding[7,8,9]. Among incident US HIV infections in 2015, 17% occurred in persons >50 years of age. Approximately 45% of people living with HIV in the US are aged ≥50[10,11], and by 2030 an estimated 70% will be over age 50, underscoring the importance of studying the effects of aging on the FGT and genital health as well as HIV acquisition and shedding.

This proposal will test the hypothesis that HIV is associated with earlier onset and more pronounced changes in the FGT characterized by vaginal dysbiosis and mucosal inflammation. I propose a cross-sectional study to compare a phenotype of FGT aging in HIV-infected (HIV+) and HIV-uninfected (HIV-) menopausal women aged 45-70 years and predict that younger HIV+ menopausal women (45-55 years) will have a phenotype similar to older (56-70 years) HIV- menopausal women. I will also explore whether topical estradiol therapy in HIV+ women with vaginal atrophy will improve symptoms and the age-related phenotype of dysbiosis and inflammation. Results of these studies have important clinical implications and could lead to recommendations for earlier and/or more aggressive treatment of vaginal atrophy in HIV+ women.

Menopause is associated with vaginal dysbiosis, inflammation, and atrophy: hydrogen peroxide, lactic acid-producing lactobacilli (*L. crispatus, L. jensenii and L. gasseri*) are protective by maintaining an acidic vaginal pH <4.7 and producing antimicrobial molecules such as bacteriocins. In contrast, dysbiosis, defined as a decrease in protective lactobacilli and increase in diverse anaerobes, is associated with a greater risk for HIV and other infections. Among the clinical conditions associated with dysbiosis are the estrogen deficient states of menopause and associated vaginal atrophy. These conditions are associated with increases in proinflammatory molecules and disruption of epithelial integrity[12,13]. A study comparing the vaginal microbiome of HIV- pre- and postmenopausal women showed that premenopausal women were more often in community state type (CST)-I (predominance of *L. crispatus*), whereas postmenopausal women were most often in CST-IV (dominated by diverse anaerobes). Limited data suggest that symptoms of vaginal atrophy may be at least partly mediated by vaginal dysbiosis. Postmenopausal participants with symptoms of vaginal atrophy had 25X greater odds of having CST-IV vs. CST-I[13]. Moreover, postmenopausal women reporting moderate to severe dryness had more *Prevotella* and other anaerobes, whereas women with little or no dryness had a predominance of lactobacilli[12]. Further, women with atrophy and dryness had increased expression of proinflammatory genes and decreased expression of genes associated with epithelial integrity. The findings of dysbiosis and inflammation may be linked mechanistically as studies have shown that bacteria commonly found in CST-IV (e.g. *Prevotella*, *Mobiluncus* and *Sneathia*) induce higher levels of proinflammatory cytokines including interleukin (IL)-1α, IL-1β and IL-8 compared to *L. crispatus* (CST-I)[14,15]. Similar shifts in the vaginal microbiome and

concomitant inflammation are associated with increased risk of HIV shedding and transmission[16-20]. However, there are no studies that specifically address changes in the FGT in postmenopausal HIV+ women. I propose to address this knowledge gap and to study whether HIV+ menopausal women develop an earlier and greater degree of dysbiosis and mucosal inflammation compared to HIV- menopausal women and to evaluate the impact of vaginal estradiol treatment in HIV+ women with vaginal atrophy.

Vaginal atrophy is common but undertreated especially in women living with HIV (WLWH). Vaginal atrophy affects up to 45% of menopausal women[21] and presents with symptoms of vaginal dryness and dyspareunia, as well as urinary symptoms. Although the systemic vasomotor symptoms of menopause (e.g. hot flashes) often improve with time, atrophy symptoms persist. Intravaginal estrogen is effective for the treatment of vaginal atrophy with a favorable safety profile[22-25,26-29] compared to systemic preparations. However, it is estimated that only 25% of symptomatic women seek medical attention[21] and even in these women it is undertreated. With the increased availability of antiretroviral therapy (ART) and associated aging of the HIV population[30,31], large numbers of WLWH will enter menopause, placing them at risk for vaginal atrophy. Topical estrogen therapy has not been studied in WLWH, despite the findings that they experience and report menopausal symptoms at an earlier age than HIV- women[32-35]. Moreover, severe menopausal symptoms have been associated with reduced adherence to ART placing menopausal WLWH at risk for possible drug resistance and adverse HIV related health outcomes[36]. Low dose intravaginal estrogen (Vagifem 10 μg) is safe, effective and FDA approved for the treatment of symptomatic vaginal atrophy. Because of low systemic absorption (3-11 pg/ml)[37,38], there is little risk of complications nor need for progesterone supplementation[22,23,39]. The boxed warning for risk for endometrial cancer and cardiovascular disease (CVD) is based on extrapolations of data from clinical trials of systemic hormonal therapy, with substantially higher levels of exposure. There is no randomized trial or consistent observational evidence linking low-dose vaginal estrogen to cancer, CVD, dementia, or thromboembolism. A Working Group on Women's Health and Well-Being in Menopause has proposed to the FDA a labeling change[40].

2. Innovation

This is the first study specifically designed to test an intervention to restore local estrogen in HIV+ menopausal women with the goal of improving symptoms of atrophy, promoting an optimal vaginal microbiome and reducing GT inflammation. Previous studies have demonstrated a reduction in symptoms of vaginal atrophy and increased lactobacilli after *oral* estrogen replacement in HIV- menopausal women[41]. However, the effect of *topical* estradiol on the vaginal microbiome has not been studied nor has local estradiol treatment been systematically assessed in *HIV+* women with vaginal atrophy. We will fill this research gap in this pilot randomized clinical trial. In addition to assessing improvement in clinical symptoms, we will explore the effects of topical estradiol on the vaginal microbiome, markers of mucosal inflammation, and HIV shedding.

Despite numerous studies documenting the composition of the vaginal microbiome in various disease states, our understanding of the functional relationship between the vaginal microbiome and mucosal health is limited. In order to fill this knowledge gap, we will evaluate the vaginal microbiome with Illumina MiSEq and quantitative PCR for select bacteria**,** and also propose to apply a **more novel technique of Ig-SEQ, which combines flow cytometry-based bacterial cell sorting and 16S sequencing to identify subpopulations of IgA and/or IgG coated bacteria, with the goal of understanding the link between bacterial species,**

mucosal inflammation and genital tract aging. We hypothesize that functional consequences of differences in the microbiome may link to immunoglobulin (Ig) coating.

3. Approach

We propose to test the hypothesis that menopausal women living with HIV (WLWH) develop genital tract aging earlier than HIV- menopausal women and that the magnitude of changes in microbiome and mucosal inflammation will be greater in HIV+ versus HIV- women. We will also test whether vaginal estradiol treatment in menopausal WLWH with vaginal atrophy will result in improvement of symptoms and an increase in Ig-coated, lactobacillus-dominant vaginal microbiota and reduction in mucosal inflammation. If the hypothesis is supported by this pilot interventional study, results would provide the foundation for a future study to evaluate the efficacy of earlier use of topical estradiol and generate new testable hypotheses to link aging, the vaginal microbiome and host mucosal immunity to HIV care.

4. Objectives and Study Design

A. To determine if HIV is associated with earlier and more significant age-associated changes in FGT by comparing the vaginal microbiome and mucosal inflammation in menopausal WLWH and HIV- menopausal women.

B. To conduct a pilot randomized clinical trial in menopausal WLWH with symptomatic vaginal atrophy to evaluate the impact of vaginal estradiol treatment on symptoms and biomarkers of FGT aging.

We propose a study nested in the Bronx Women's Interagency HIV Study (WIHS) to compare a phenotype of FGT aging between menopausal WLWH and HIV- women. We will use the following biomarkers of aging: lower levels of lactobacilli (primary outcome), increased dysbiosis, less Ig bacterial coating, and higher levels of mucosal inflammatory mediators. We will also assess bacterial and host cell gene expression using metatranscriptomics (vaginal swabs) and RNAseq (vaginal biopsies). These exploratory "omic" studies may provide insights into the functional significance of observed differences in the composition and Ig coating of vaginal microbiota and their link to vaginal health.

In the first part of the study (Aim 1), 50 HIV+ and 50 HIV- participants aged 45-70 will have 2 study visits one month apart to assess reproducibility of findings. Participants will be frequency matched by age, race and ethnicity into 4 groups: (i) HIV+ aged 45-55; (ii) HIV+ aged 56-70; (iii) HIV- aged 45-55; and (iv) HIV- aged 56-70 years. We have chosen to dichotomize into these age groups based on differences in the taxonomic composition of the microbiome in a recently published study[42] and potential eligible participants in WIHS. **Figure 1** illustrates the overall study design. Sampling at both study visits will include vaginal swabs for pH, microbiome assessment and evaluation for vaginal infections (candida, bacterial vaginosis [BV], trichomonas), cervicovaginal lavage (CVL) for soluble immune mediators, glycogen, and HIV CVL RNA (shedding), blood for HIV RNA (plasma viral load), CD4 count, estradiol and follicular stimulating hormone (FSH), and vaginal biopsies for exploratory RNAseq (Visit 2 only).

At Visit 2, 50 HIV+ participants with vaginal atrophy will be offered immediate enrollment into a 1:1 randomized trial of vaginal estradiol (n=25) versus no treatment (n=25) to assess whether

estradiol will improve vaginal symptoms and modify the genital tract aging phenotype. HIV+ women who agree to participate in the trial will have 3 additional study visits: randomization (Visit 3), 6 weeks after randomization (Visit 4) and 12 weeks after randomization (Visit 5). HIV- women can only participate in Aim 1 and will have 2 study visits. HIV+ women can elect to participate in Aim 1 and/or Aim 2 (2 study visits if only Aim 1 is completed; 4 study visits if only Aim 2 is completed; 5 visits if both Aims 1 and 2 are completed).

Inclusion criteria for Aim 1 (first 2 study visits):

- HIV infection or HIV negative
- Females aged 45-70
- Menopause defined by having no menstrual periods for 12 consecutive months, confirmed with serum follicle-stimulating hormone (FSH) level >40 IU/ml and serum estradiol level <20 pg/ml

In the second part of the study (Aim 2), we propose a pilot randomized clinical trial nested in the Bronx WIHS in 50 menopausal WLWH with symptomatic vaginal atrophy to assess the impact of 12 weeks of a topical low-dose estradiol vaginal tablet (Vagifem® 10 µg) on symptoms of atrophy and measures of the vaginal microbiome. Participants will be randomized 1:1 to estradiol (n=25) or no therapy (n=25) and will be followed for 12 weeks. The dosing of Vagifem® will be 1 tablet inserted using a plastic applicator (similar to a tampon) in the vagina daily for 2 weeks followed by 1 tablet inserted twice weekly (i.e., Tuesday and Friday). If we find benefit among women assigned to the estradiol arm, participants randomized to the no therapy arm will be offered vaginal estradiol treatment at the end of the study by their primary care doctor. If participants assigned to Vagifem are symptomatic beyond the 12-week study period and wish to continue Vagifem®, it can be prescribed by their primary care physician. Women will have 3 visits before (Visits 1, 2 and 3) and 2 visits following Vagifem® treatment (Visits 4 and 5). Adherence to Vagifem will be assessed with the use of participant diaries of Vagifem use and counting of used Vagifem applicators.

Inclusion criteria for Aim 2 (randomized clinical trial for HIV+ participants only):

- HIV infection
- Females aged 45-70
- Menopause defined by having no menstrual periods for 12 consecutive months, confirmed with serum follicle-stimulating hormone (FSH) level >40 IU/ml and serum estradiol level <20 pg/ml
- Symptomatic vaginal atrophy defined as reporting at least once per week in the past 30 days, 1 or more of the following symptoms of moderate or severe intensity:
  -Dryness
  -Itching
  -Irritation
  -Soreness or pain

  OR
  -Pain associated with sexual activity at least once
- Evidence of atrophy on exam, including thin, pale and dry vaginal and vulvar surfaces
- Agrees not to use vaginal products other than Vagifem during the clinical trial

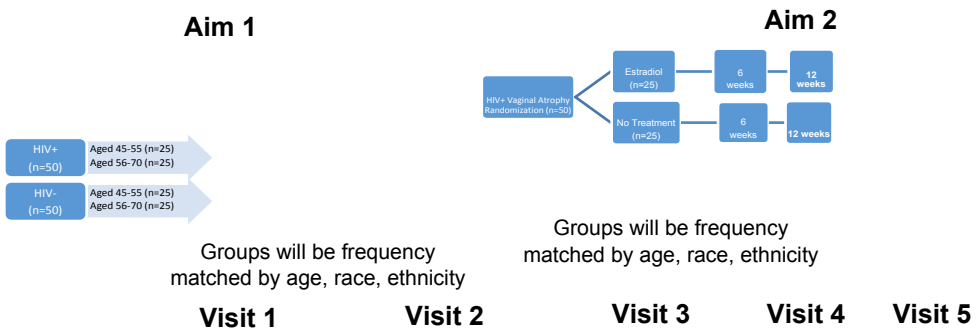
Exclusion criteria for Aims 1 & 2:

- Current unexplained or unevaluated abnormal genital bleeding
- Current or suspected pregnancy
- If < age 55, hysterectomy and has at least one ovary
- Pelvic or vaginal surgery in the 60 days prior to enrollment
- Use of systemic reproductive hormones in the 2 months prior to study enrollment
- Antibiotic use in the past 30 days
- Use of immunosuppressive medications in the prior 60 days including biologics, chemotherapeutics or post-transplant immunosuppressive medications.
- Use of any vaginal or vulvar preparations 1 month prior to enrollment
- Current acute vaginal infection (diagnosed by wet mount at Visit 1 or 2)
- Any serious disease or chronic condition that might interfere with study compliance
- Participants who are unwilling to agree to the provisions of the protocol

Additional Exclusion Criteria for Aim 2 only:

- Current or previous history of breast cancer or estrogen dependent neoplasia
- Known current or past thromboembolic disease (deep vein thrombosis or pulmonary embolism, not including thrombophlebitis), myocardial infarction or stroke
- Known blood clotting disorders including Protein C, Protein S and antithrombin deficiency, Factor V Leiden or prothrombin mutations
- Known severe liver disease including cirrhosis or active Hepatitis B
- History of adverse reaction to vaginal estradiol

Figure 1. Study Design



5. Recruitment

The study is nested in Bronx WIHS, which is an NIH-funded prospective observational cohort study of HIV+ and at-risk HIV- women. We have identified 90 HIV- and 248 HIV+ Bronx and Brooklyn WIHS participants who are potentially eligible for this study. Potential participants will be invited to discuss this study at semiannual Core WIHS visits. We will recruit participants from the Bronx and Brooklyn WIHS for this study. Bronx WIHS staff will call potentially eligible participants and schedule them to come in to the Clinical Research Center at the Albert Einstein College of Medicine or the Bronx WIHS site for a screening visit. As we have done for prior studies, Brooklyn WIHS participants who are interested in participating in the study, will be contacted by the Bronx WIHS staff and seen at the Bronx WIHS site for all visits. Additional participants may be recruited

from Montefiore Medical center, and may be identified using the Center for AIDS Research Clinical Database.

6. Study procedures

Study visits will occur at the Einstein Clinical Research Center or the Bronx WIHS clinical study site. The study clinicians or study coordinator will screen participants for eligibility, discuss the study, answer any questions, and obtain written informed consent. At each scheduled research visit, a pelvic examination will be performed for the collection of the following specimens: vaginal swabs for pH, and microbiome studies; CVL; vaginal swabs to assess for infection (Visits 1 and 2 only) and vaginal biopsies and a vaginal scraping (VMI) to assess for vaginal aging at (Visits 2 and 5 only) (**Table 1**). If candida, BV, or trichomonas is diagnosed, participants will be notified and referred to their primary doctor for treatment prior to enrollment in the study. After treatment, participants may return for repeat testing and if the infection has resolved, they may be enrolled in the study.

At Visit 2 blood will be collected for estradiol and follicular stimulating hormone (FSH) levels, aging biomarkers and future studies and for HIV positive participants, CD4 and HIV-1 RNA. At Visit 5, blood will be collected for aging biomarkers and future studies. Participants will be asked at Visits 2-5 to complete a questionnaire about the presence and severity of the most bothersome symptom of atrophy, defined as vulvovaginal itching, pain, dryness, irritation or pain with penetration (dyspareunia)[43].

Participants will rate the severity of symptoms based on a four-point scale (0=none, 1=mild, 2=moderate, 3=severe). Presence or absence of atrophy will also be assessed via clinical exam at Visits 2-5 and at Visits 2 and 5 with the Vaginal Maturation Index (VMI) which is done by lightly scraping the upper 1/3rd of the vaginal wall with a saline dipped vaginal spatula to examine cells under the microscope to assess for vaginal aging[43]. Following placement of a speculum into the vaginal vault, swabs will be collected from the lateral vaginal walls. CVL is collected using a small syringe to place 10 cc of normal saline in the vaginal area. The fluid is immediately removed and sent to the Herold lab for storage and analysis. Vaginal biopsies will be collected at Visits 2 and 5 only. Women will be counseled to abstain from vaginal product use and sexual intercourse for one week after biopsy collection to allow adequate time for healing. In our experience, re-epithelialization occurs within one to two days, and the biopsy sites are completely healed within seven days. Adult women who decline or state that they are unlikely to abstain from sexual activity for one week after biopsy visits will not undergo biopsy collection. Tissue samples are typically ~3 x 5 mm. Biopsies are obtained with a Tischler or other biopsy forceps. All participants will receive a follow-up phone call the day after the vaginal biopsies from the study clinician to ensure they are feeling well. The specimens will be obtained strictly for research purposes and used only for the specific objectives outlined in the study. At all scheduled return visits or at any visit initiated by the participant due to genitourinary symptoms, an appropriate examination for the evaluation of specific symptoms and adverse events will be performed (for example, performance of a pelvic examination in women with complaints of abnormal vaginal discharge or bleeding). If any examination findings warrant further diagnostic evaluation for clinical management, participants will be referred to their medical provider for appropriate diagnostic studies and treatment.

Table 1. Timeline of Study Visits, Procedures, & Sample Collection

	Aim 1 Study of Menopausal HIV- and WLWH		Aim 2 Pilot Randomized Clinical Trial of Topical Estradiol in Menopausal WLWH			
	Visit 1 Screening	**Visit 2 2nd visit and/or Screening for Trial**	**Visit 3 Randomized Estradiol vs. No therapy**	**Visit 4 6 weeks Estradiol vs. No therapy**	**Visit 5 12 weeks Estradiol vs. No therapy**	**Safety Call 2, 8, 13 weeks**
Eligibility/Informed consent	X	X	X			
Randomization to Estradiol vs. No treatment			X			
WIHS database extraction Demographics, CD4 count, plasma HIV-1 RNA, ART regimen and reported adherence, etc.	X	X				
Vaginal atrophy symptom questionnaire		X	X	X	X	X
Adverse event assessment	X	X	X	X	X	X
Vaginal swabs (n=4-5 per visit) pH, microbiome studies	X	X	X	X	X	
Cervicovaginal lavage (CVL) supernatant Immune mediators, HIV-1 RNA, RSID, saline/KOH, glycogen	X	X	X	X	X	
CVL cell pellet Nugent score, additional microbiome	X	X	X	X	X	
Wet mount (candida, trichomonas, bacterial vaginosis)	X	X				
Vaginal biopsies (2) RNA seq, future immunohistochemistry studies		X			X	
Blood collection Estradiol/FSH levels, HIV-1 RNA, CD4, plasma for future aging biomarkers		X			X	
Clinical Exam to assess for Vaginal Atrophy (VMI)		X			X	

ART=antiretroviral therapy; RSID=rapid semen identification; FSH=follicle stimulating hormone; VMI=vaginal maturation index

7. Potential Risks:

There is a small risk of bruising, blood clots or infection from blood draws. Sometimes the area of needle insertion becomes sore or red. A woman may experience mild discomfort at the time of speculum placement and sample collection. There is a very small risk of injury to the lining of the vagina from the speculum or from the vaginal scraping. There is no discomfort or risk to placement or removal of sterile saline into the vaginal area. There is no risk to CVL or vaginal swab collection. Women may experience a sharp pinch or have pain when vaginal biopsies are performed. In order to minimize the pain and discomfort of the biopsy, we will apply a topical numbing medication (benzocaine gel) to the tissue. There may be stinging, burning, redness, or itching associated with local anesthesia (topical benzocaine gel) which is rare. Biopsies may cause light bleeding, Rarely, a biopsy can cause infection and/or prolonged bleeding. Sometimes a medication is needed to stop the bleeding, which would be applied directly to the

site. This medication can cause a temporary dark-colored discharge from the vagina. In rare cases a stitch might be needed or a machine (cautery) may be used to stop the bleeding. Potential risks of vaginal estrogen tablets (Vagifem® 10 µg) include back pain, diarrhea, vulvovaginal mycotic infection, and vulvovaginal pruritus (treatment emergent adverse reactions reported at a frequency of ≥5 percent in a 12-month randomized, double-blind, placebo-controlled study in which 309 postmenopausal women received either placebo or Vagifem® 10 µg). Vagifem applicator may cause vaginal abrasion. The Division of AIDS (DAIDS) Female Genital Grading Table for Use in Microbicide Studies will be the primary tool for grading adverse events available at https://rsc.niaid.nih.gov/sites/default/files/addendum-1-female-genital-grading-table-v1-nov-2007.pdf. AEs will be reported to the IRB.

Women who use systemic forms of unopposed estradiol who have an intact uterus may have an increased risk of endometrial hyperplasia, which in some women may be a precursor to endometrial cancer after prolonged use. Women in this study will be using a low-dose topical estradiol vaginal tablet, which does not increase serum levels of estradiol beyond the baseline menopausal levels and has been proven to be safe for the treatment of vaginal atrophy without adverse effects on the endometrium, including endometrial hyperplasia. Further, low-dose vaginal estrogen has not been shown to increase risk for coronary heart disease, stroke, venous thromboembolism or dementia. The proposed study will test the effect of 6 and 12 weeks of vaginal estrogen on clinical symptoms, the vaginal microbiome, and soluble markers of inflammation. Participants will be followed closely by the study team and will be instructed to report immediately any vaginal bleeding or spotting while using Vagifem®. Women may remain symptomatic beyond the 3-month study period and may elect to continue Vagifem® with close medical follow-up by their primary clinician. If benefit is determined in the estradiol arm of the study, participants randomized to the no treatment group, will be offered Vagifem® by their primary care physicians. The study team will contact participants' medical provider if the decision is made to continue treatment or initiate Vagifem®.

8. Adequacy of Protection Against Risks:

The consent will contain the following: investigators' statement identifying the study; purpose and benefits; procedures; risk, benefits, stress, and discomfort associated with the study; request for permission for future use of samples (as a separate checkbox, so that participants may be in the study and not agree to storage of samples for future use) and other information deemed necessary. The consent will also identify the investigators and provide contact information. The participant must verbally assent that they understand the nature of the study, its inherent risks and benefits, other treatment alternatives, their right to terminate participation in the study without affecting their health care or involvement in the core WIHS cohort study, and that they have freely given informed consent to participate in the study. A signed copy of the informed consent form will be given to the participant. The participants are informed that all medical records are kept confidential. Participants will be compensated for time and travel. Participants will receive a total of $125 ($50 for Visit 1 and $75 for Visit 2) if they complete only the first part of the study (Aim 1, 2 visits). Participants with HIV who complete both parts of the study (Aims 1 and 2, 5 visits), will receive a total of $300 ($50 at Visits 1, 3 and 4 and $75 at Visits 2 and 5). HIV+ participants who enroll only in the second part of the study (Aim 2, randomized trial) will receive a total of $250 ($50 at Visits 3 and 4 and $75 at Visits 2 and 5).

All specimens and study forms are labeled with a unique study identifier that has no relationship to participant's identifying information (such as name, initials, or date of birth). All records and

data are kept in locked offices and a secure computer database. The Bronx site of the WIHS has a long history of participation in many sub-studies and the staff is very familiar with the need to maintain participant confidentiality and security of study data. Samples will be stored in Dr. Betsy Herold's lab and will not be destroyed at the end of the study, unless participants do not agree to use of remaining samples for related studies. A secure, web-based application, REDCap (Research Electronic Data Capture), will be used to collect and store data from questionnaires obtained in this study. REDCap is designed exclusively to support data capture for research studies. REDCap servers are housed in a data center at Einstein and all web-based information transmission is encrypted. REDCap was developed specifically around HIPAA-security guidelines and it includes full audit trail.

Participants will receive telephone calls at 2 and 8 weeks after initiation of estradiol and 1 week after completion of therapy to assess for safety. Participants will also receive telephone calls a day after vaginal biopsies are performed. If symptoms are reported, participants will be instructed to return to the study site for evaluation. Participants will receive local anesthesia with topical benzocaine gel prior to each biopsy procedure and will be advised that they may take a mild pain reliever (Tylenol) before and after each biopsy procedure. Women who have any history of estrogen responsive cancer including breast cancer, endometrial cancer or vaginal bleeding will be excluded from this study to minimize risk for adverse events. In the event of an injury resulting from participation in this study, participants will be instructed to seek immediate appropriate medical care and inform the study doctor. In the event of an emergency they will be instructed to go to the emergency room. Participants will be provided with a 24-hour telephone number to call to contact a study clinician at any time. Appropriate blood and secretion precautions will be employed by all personnel in the drawing of blood and handling of all specimens, as currently recommended by the US Centers for Disease Control and Prevention (CDC). Universal precautions will be observed at all times.

9. Potential Benefits:

Women with HIV infection experience menopausal symptoms at an earlier age and are more likely to report symptoms compared to women who do not have HIV. Participants may benefit from study participation because the use of low dose topical estradiol, which has been FDA approved since 2009 for the treatment of vaginal atrophy in menopausal women, may relieve symptoms of vaginal dryness, itching, soreness, pain and urinary symptoms in women with HIV. Participants and others may benefit in the future from information learned from this study. Specifically, if we find that HIV accelerates genital tract aging, this may prompt recommendations for earlier treatment of vaginal atrophy in menopausal women with HIV.

10. Data analysis and sample size:

Aim 1: The primary exposure variables are HIV infection and age. The primary outcome is the vaginal microbiome, specifically the relative abundance and quantities of lactobacillus species as measured by Illumina MiSeq and qPCR respectively. Secondary outcomes are relative abundance and quantities of specific BV associated bacteria and quantities of CVL cytokine/chemokines. Exploratory outcomes are quantities and composition of Ig coated bacteria and levels of HIV-1 in the genital tract. Multivariate linear regression will be applied for our analysis. General form of the linear regression model will be: $y_i = \mu + \beta age_i + \gamma HIV_i + \theta age_i \times HIV_i + \delta_1 Covariates_i + \ldots + e_j$, in which y_i is the outcome variables subject to necessary transformation as necessary. Age will be analyzed as a continuous variable to examine the slope or rate of change in lactobacillus and as

a dichotomous variable (e.g. age 45-55 vs. 56-70) to examine the magnitude of difference between younger and older HIV+ and HIV- menopausal participants. We have chosen the age cut offs above given the significant differences observed in the taxonomic composition of the microbiome in pilot studies when comparing HIV+ menopausal participants in these age ranges and the distribution of eligible participants in WIHS. We will adjust the regression analysis for important covariates including years since menopause, time since HIV diagnosis, HIV plasma viral load, CD4 count, ART use, and HSV-2 serostatus. Both raw *p* values and the false discovery rate (FDR) according to Benjamini and Hochberg will be examined. To limit the false positive rate in our analysis of secondary outcomes, an arbitrary cutoff of 0.05 for FDR will used.

Power Considerations: We estimated the power of the proposed study using software PASS (Power and Sample Size Analysis, version 11). Given the sample size of 50 HIV- and 50 HIV+ menopausal participants, we will have >80% power for the minimal detectable difference of 0.6SD and 0.9SD between HIV (or age) group with the significance level of 0.05 and 0.0005 (a conservative Bonferroni correction for 100 independent tests). Data from preliminary studies in our lab investigating the impact of menopause and HIV status on the vaginal microbiome indicated 51 HIV+ menopausal participants had a significantly lower relative abundance of lactobacillus species of 7.3% compared to 23% for 19 HIV- menopausal participants, equivalent to about 0.8SD change. Further when comparing upper and lower age ranges of HIV+ menopausal participants, HIV+ participants age 56-70 (n=28) had significantly lower relative proportions of *L. iners* 7.4% compared to 44.7% in HIV+ menopausal participants aged 47-55.9 (n=23), equivalent to about 1.2SD change. Based on the preliminary data, we are confident that this study will have excellent power to detect effects of age and HIV.

Aim 2: The primary outcomes are clinical symptoms measured by most bothersome symptom of vaginal atrophy and changes in the vaginal microbiome, specifically the relative abundance of protective lactobacillus species *L. crispatus* as measured by Illumina MiSeq and quantities of protective lactobacilli species (*L. crispatus, L. jensenii* and *L. gasseri*) as measured by qPCR between baseline and 6 and 12 weeks within individual participants and between participants randomized to estradiol vs. no drug at baseline, 6 and 12 weeks. The 12-week time frame was chosen as this was the endpoint utilized in several clinical trials demonstrating Vagifem efficacy and is recommended in FDA guidelines[22,43]. The secondary outcomes are changes relative abundance and quantities of specific BV associated bacteria and levels CVL cytokine/chemokines between baseline, 6 and 12 weeks. Exploratory outcomes are the quantities and composition of Ig coated bacteria and levels of HIV-1 in the genital tract. Spearman's correlation coefficients (SCC) will be calculated to evaluate associations between pairs of quantitative outcome variables and covariates, such as levels of protective lactobacilli species and MBS of atrophy, quantity of Ig coated bacteria, inflammatory mediators, CVL HIV-1 RNA, levels of estradiol in the blood. Linear mixed effects models or generalized linear mixed models will be used to examine effects of time and estradiol use on outcomes (bacterial communities, vaginal bacterial quantities, symptoms) after adjustment for factors such as ART, duration of HIV, plasma viral load, and CD4+ T cell count. We will also address the variability of the microbiome and inflammatory mediators over time in the absence of an intervention by comparing these measures at the screening (Visit 2) and enrollment visits (Visit 3).

Power Considerations: We have increased our sample size and added randomization for this clinical trial based on feedback from the NIH reviewers. If we assume an attrition rate of 15%, a sample of 50 HIV+ women randomized to estradiol (Vagifem) vs. no treatment will have >80%

power for the minimal detectable difference in lactobacilli of 0.85 SD and 1.4 SD with the significance level of 0.05 and 0.0005 (a conservative Bonferroni correction for 100 independent tests), respectively, at 12 weeks post treatment. Although data is lacking in HIV+ women treated with Vagifem, a previous study with a smaller sample size (30 HIV-) investigating the use of low dose (0.3mg) oral conjugated estrogens for treatment of vaginal atrophy in demonstrated a significant increase in the relative abundance of Lactobacillus from 11.2% to 71% ($p<0.0001$) comparing baseline to 4 weeks estrogen treatment. Further the vaginal symptom score decreased from 3.93 vs. 1.00 ($p<0.0001$) at the same time points[41], suggesting this proposed study may have adequate power for detecting a realistic difference of candidate Lactobacillus and symptom score. It should be noted that this is a pilot study and as such we acknowledge that it may be underpowered for testing interaction effects of time and estradiol use. However, the goal of the study is to determine an effect size of topical estradiol in planning for a future large-scale randomized controlled trial.

References

1. Sabin CA, Reiss P. Epidemiology of ageing with HIV: what can we learn from cohorts? *AIDS (London, England).* 2017;31 Suppl 2:S121-s128.
2. Van Epps P, Kalayjian RC. Human Immunodeficiency Virus and Aging in the Era of Effective Antiretroviral Therapy. *Infectious disease clinics of North America.* 2017;31(4):791-810.
3. De Francesco D, Wit FW, Burkle A, et al. Do people living with HIV experience greater age advancement than their HIV-negative counterparts? *AIDS (London, England).* 2019;33(2):259-268.
4. Wing EJ. HIV and aging. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases.* 2016;53:61-68.
5. Kennedy BK, Berger SL, Brunet A, et al. Geroscience: linking aging to chronic disease. *Cell.* 2014;159(4):709-713.
6. Pathai S, Bajillan H, Landay AL, High KP. Is HIV a model of accelerated or accentuated aging? *The journals of gerontology Series A, Biological sciences and medical sciences.* 2014;69(7):833-842.
7. Hawes SE, Hillier SL, Benedetti J, et al. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. *The Journal of infectious diseases.* 1996;174(5):1058-1063.
8. Martin HL, Richardson BA, Nyange PM, et al. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *The Journal of infectious diseases.* 1999;180(6):1863-1868.
9. Borgdorff H, Tsivtsivadze E, Verhelst R, et al. Lactobacillus-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *The ISME journal.* 2014;8(9):1781-1793.
10. Centers for Disease Control and Prevention. *HIV Surveillance Report.* 2015.
11. New York City Department of Health and Mental Hygeine. *New York City HIV/AIDS Surveillance Slide Sets.* New York2017.
12. Hummelen R, Macklaim JM, Bisanz JE, et al. Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness. *PloS one.* 2011;6(11):e26602.
13. Brotman RM, Shardell MD, Gajer P, et al. Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause (New York, NY).* 2014;21(5):450-458.
14. Anahtar MN, Byrne EH, Doherty KE, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity.* 2015;42(5):965-976.

15. Masson L, Mlisana K, Little F, et al. Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. *Sexually transmitted infections.* 2014;90(8):580-587.
16. Mauck C, Chen PL, Morrison CS, et al. Biomarkers of Cervical Inflammation and Immunity Associated with Cervical Shedding of HIV-1. *AIDS research and human retroviruses.* 2016;32(5):443-451.
17. Sha BE, Zariffard MR, Wang QJ, et al. Female genital-tract HIV load correlates inversely with Lactobacillus species but positively with bacterial vaginosis and Mycoplasma hominis. *The Journal of infectious diseases.* 2005;191(1):25-32.
18. Mitchell C, Balkus JE, Fredricks D, et al. Interaction between lactobacilli, bacterial vaginosis-associated bacteria, and HIV Type 1 RNA and DNA Genital shedding in U.S. and Kenyan women. *AIDS research and human retroviruses.* 2013;29(1):13-19.
19. Mitchell C, Hitti J, Paul K, et al. Cervicovaginal shedding of HIV type 1 is related to genital tract inflammation independent of changes in vaginal microbiota. *AIDS research and human retroviruses.* 2011;27(1):35-39.
20. Baeten JM, Kahle E, Lingappa JR, et al. Genital HIV-1 RNA predicts risk of heterosexual HIV-1 transmission. *Science translational medicine.* 2011;3(77):77ra29.
21. Lindahl SH. Reviewing the options for local estrogen treatment of vaginal atrophy. *International journal of women's health.* 2014;6:307-312.
22. Simon J, Nachtigall L, Gut R, Lang E, Archer DF, Utian W. Effective treatment of vaginal atrophy with an ultra-low-dose estradiol vaginal tablet. *Obstetrics and gynecology.* 2008;112(5):1053-1060.
23. Ulrich LS, Naessen T, Elia D, Goldstein JA, Eugster-Hausmann M. Endometrial safety of ultra-low-dose Vagifem 10 microg in postmenopausal women with vaginal atrophy. *Climacteric : the journal of the International Menopause Society.* 2010;13(3):228-237.
24. Rahn DD, Carberry C, Sanses TV, et al. Vaginal estrogen for genitourinary syndrome of menopause: a systematic review. *Obstetrics and gynecology.* 2014;124(6):1147-1156.
25. Santoro N, Fan M, Maslow B, Schoenbaum E. Women and HIV infection: the makings of a midlife crisis. *Maturitas.* 2009;64(3):160-164.
26. Bachmann G, Lobo RA, Gut R, Nachtigall L, Notelovitz M. Efficacy of low-dose estradiol vaginal tablets in the treatment of atrophic vaginitis: a randomized controlled trial. *Obstetrics and gynecology.* 2008;111(1):67-76.
27. Eriksen PS, Rasmussen H. Low-dose 17 beta-estradiol vaginal tablets in the treatment of atrophic vaginitis: a double-blind placebo controlled study. *European journal of obstetrics, gynecology, and reproductive biology.* 1992;44(2):137-144.
28. Simunic V, Banovic I, Ciglar S, Jeren L, Pavicic Baldani D, Sprem M. Local estrogen treatment in patients with urogenital symptoms. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics.* 2003;82(2):187-197.
29. Lethaby A, Ayeleke RO, Roberts H. Local oestrogen for vaginal atrophy in postmenopausal women. *The Cochrane database of systematic reviews.* 2016(8):Cd001500.
30. Nakagawa F, Lodwick RK, Smith CJ, et al. Projected life expectancy of people with HIV according to timing of diagnosis. *AIDS (London, England).* 2012;26(3):335-343.
31. Nakagawa F, May M, Phillips A. Life expectancy living with HIV: recent estimates and future implications. *Current opinion in infectious diseases.* 2013;26(1):17-25.
32. Miller SA, Santoro N, Lo Y, et al. Menopause symptoms in HIV-infected and drug-using women. *Menopause (New York, NY).* 2005;12(3):348-356.

33. Schoenbaum EE, Hartel D, Lo Y, et al. HIV infection, drug use, and onset of natural menopause. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2005;41(10):1517-1524.
34. Ferreira CE, Pinto-Neto AM, Conde DM, Costa-Paiva L, Morais SS, Magalhaes J. Menopause symptoms in women infected with HIV: prevalence and associated factors. *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology.* 2007;23(4):198-205.
35. Clark RA, Cohn SE, Jarek C, et al. Perimenopausal symptomatology among HIV-infected women at least 40 years of age. *Journal of acquired immune deficiency syndromes (1999).* 2000;23(1):99-100.
36. Duff PK, Money DM, Ogilvie GS, et al. Severe menopausal symptoms associated with reduced adherence to antiretroviral therapy among perimenopausal and menopausal women living with HIV in Metro Vancouver. *Menopause (New York, NY).* 2017.
37. Santen RJ, Pinkerton JV, Conaway M, et al. Treatment of urogenital atrophy with low-dose estradiol: preliminary results. *Menopause (New York, NY).* 2002;9(3):179-187.
38. Eugster-Hausmann M, Waitzinger J, Lehnick D. Minimized estradiol absorption with ultra-low-dose 10 microg 17beta-estradiol vaginal tablets. *Climacteric : the journal of the International Menopause Society.* 2010;13(3):219-227.
39. Weisberg E, Ayton R, Darling G, et al. Endometrial and vaginal effects of low-dose estradiol delivered by vaginal ring or vaginal tablet. *Climacteric : the journal of the International Menopause Society.* 2005;8(1):83-92.
40. Manson JE, Goldstein SR, Kagan R, et al. Why the product labeling for low-dose vaginal estrogen should be changed. *Menopause (New York, NY).* 2014;21(9):911-916.
41. Shen J, Song N, Williams CJ, et al. Effects of low dose estrogen therapy on the vaginal microbiomes of women with atrophic vaginitis. *Scientific reports.* 2016;6:24380.
42. Murphy K, Keller MJ, Anastos K, et al. Impact of reproductive aging on the vaginal microbiome and soluble immune mediators in women living with and at-risk for HIV infection. *PloS one.* 2019;14(4):e0216049.
43. US Food and Drug Administration. Guidance for industry: estrogen and estrogen/progestin drug products to treat vasomotor symptoms and vulvar and vaginal atrophy symptoms—recommendationsfor clinical evaluation. Rockville, MD: US Food and Drug Administration; 2003.