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Project Title: Association of sex hormones and DMPA use with HIV-resistance and acquisition: An analysis of the Pumwani Sex Worker Cohort and the CAPRISA-004 Trial

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SUMMARY: (no more than 250 words single spaced)

Recent studies have associated endogenous sex hormones (estradiol and progesterone) and exogenous sex hormones (e.g. depot medroxyprogesterone acetate) with HIV resistance/ susceptibility and mucosal immune factors expressed in the female reproductive tract (FRT). To better understand the association between endogenous and synthethic sex hormones with HIV status and mucosal immunity, we performed an analysis of hormone data on plasma samples and proteomic data on cervicovaginal (CVL) samples collected from participants in the Pumwani Sex Worker Cohort (n = 112 plasma samples, 40 CVL samples) and CAPRISA-004 Trial (n = 40 plasma and CVL samples). Estradiol and progesterone levels from the Pumwani cohort were analyzed against HIV status group (HIV-resistant, HIV-infected and HIV-uninfected) and immunolgical proteomic biomarkers expressed in CVL fluid. Depot medroxyprogesterone acetate (DMPA) plasma levels were measured in CAPRISA-004 participants and were also analyzed against immune factors expressed in CVL. Endogenous sex hormone (estradiol and progesterone) levels did not significantly differ between HIV-resistant, HIV-uninfected and HIV-infected women in the Pumwani Cohort. However, CVL analysis showed that estradiol positively correlates with FRT mucosal proteins involved in maintaining epithelial integrity of the FRT. This showcases a potential mechanism in which estradiol may play a role in creating a robust physical FRT mucosa that is less penetrable by pathogens such as HIV. Moreover, progesterone was shown to have an inverse association with acute phase response proteins expressed in the CVL. This finding provides a correlative link between progesterone and an immunosuppressed FRT environment, which may lead to an increased HIV risk. This study is the first to directly measure and characterize the association between plasma sex hormone levels, HIV status and immune factors expressed in the FRT mucosa.

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Introduction & Background

The female reproductive tract (FRT) plays an important role in HIV acquisition and transmission.[1] It is the first line of defense against heterosexual HIV transmission.[1,2] The epithelial lining of the FRT serves as a physical barrier against HIV virions while its mucosal fluid secretions are composed of various antimicrobials, many of which are protective against HIV.[1,3] The FRT environment is influenced by the sex hormones estradiol and progesterone.[4] These hormones heavily determine the FRT's permeability to various pathogens by regulating the differentiation and proliferation of the FRT's epithelial layers.[4] Moreover, estradiol and progesterone are found to have significant roles in the innate and adaptive immune responses in the FRT. [5,6]

Many anti-microbial peptides (AMPs) expressed in the FRT have been linked with sex hormones.[5] Examples of these AMPs include defensins and protease inhibitors which possess anti-bacterial, anti-fungal and anti-viral properties.[6, 7,8] In a study conducted by Burgener et al., which characterized the cervicovaginal proteome of women from the Pumwani Sex Worker Cohort, it was shown that protease inhibitors, specifically serpins and cystatins, were differentially abundant in HIV-1-resistant women.[9] Serpins (serine protease inhibitors) have known roles in regulating inflammation, apoptosis and host defense in the FRT. [10-12] They have also been implicated in decreased HIV-1 infectivity due to their ability to inhibit several cytokine-mediated pathways involved in excessive immune activation and HIV-1 target cell migration.[9,13-15] In a follow-up study on HIV-resistant women from the same cohort, it has been shown that serpins vary with the menstrual cycle and are affected by hormonal contraceptive use.[16] They are elevated in the estradiol-abundant/progesterone-deficient proliferative phase and are decreased during hormonal contraceptive use. Thus, estradiol and progesterone, including their synthetic counterparts (e.g. depot medroxyprogesterone acetate) may have a role in HIV resistance. This study aims to further characterize the links between sex hormones and HIV resistance/susceptibility.

Estradiol and progesterone levels vary along a woman's reproductive cycle. Estradiol increases in the follicular (proliferative) phase, peaks towards ovulation, drops at early luteal (secretory) phase and increases at mid-luteal phase, whereas progesterone only starts to rise at early luteal phase.[17] Studies have suggested that women in low-estrogen states (menopause, hormonal contraceptive use) or high-progesterone states (luteal phase, depot medroxyprogesterone acetate use, pregnancy) are more likely to become infected with HIV.[18-22] Of importance are the several studies that analyze the link between depot medroxyprogesterone acetate (DMPA) and HIV acquisition and transmission.[25] DMPA (Depo-Provera ®) is a progestin-based injectable contraceptive that therapeutically simulates the high-progesterone luteal phase by maintaining increased serum progestin levels.[24] It is the cheapest hormonal contraceptive and is widely used in sub-saharan Africa, where HIV is prevalent.[14] DMPA has been linked to increased rates of HIV (with a study suggesting a hazard risk ratio as high as 2.0), although this topic remains controversial, with mixed results from various cohort studies.[21,23,25] Previous evidence have demonstrated DMPA's immunosuppressive effects and potential role in increasing HIV susceptibility.[26] It has been shown to inhibit cytokine production, diminish T-cell response and prevent down-regulation of HIV-1 coreceptors (CXCR4 and CCR5).[26,27] Moreover, cervicovaginal secretions of women on DMPA were found to have increased pro-inflammatory cytokines and decreased secretory leukocyte protease inhibitors (SLPI), both of which are associated with increased HIV seroconversion rates.[28] Access to the CAPRISA-004 clinical trial

has provided us with an opportunity to determine the relationship between DMPA levels in Depo-Provera®-using participants, its effect on mucosal immune factor expression, and HIV acquisition..

Using previous evidence, we hypothesize that HIV-resistant women from the Pumwani Sex Worker Cohort (Study Population #1 under Methods section) have higher estradiol plasma levels and/or lower progesterone plasma levels compared to their HIV-uninfected and HIV-infected counterparts. In addition, we hypothesize that HIV-protective proteomic biomarkers found in CVL fluid will be positively correlated with estradiol plasma levels and/or negatively correlated with progesterone plasma levels. Moreover, we hypothesize that participants enrolled in the CAPRISA-004 trial, who recently acquired HIV, (Study Population # 2 under Methods section) have elevated plasma DMPA levels that negatively correlate with HIV-protective immune factors expressed in the female genital tract.

This study will be the first to measure sex hormone levels and correlate them with HIV status groups (HIV-resistant, HIV-uninfected and HIV-infected). Moreover, this will be the first to directly measure and correlate estradiol, progesterone and DMPA plasma levels with immunological protein expression in the cervicovaginal fluid. Results of this study may have implications for hormone-mediated mechanisms of HIV susceptibility, HIV preventative strategies and/or family planning methods, especially in HIV-prevalent areas.

Materials & Methods

Ethics Statement

This study was performed with prior approval from the University of Manitoba, Nairobi Human Research Ethics Board, University of KwaZulu-Natal's Bio-medical Research Ethics Committee, Family Health International Protection of Human Subjects Committee, and South African Medicines Control Council.[16,34]

Study Population

Depending on which steroids (endogenous estradiol and progesterone or synthetic DMPA) were analyzed, participants were taken either from the Pumwani Sex Worker Cohort or the CAPRISA-004 Trial. The need to study two different populations is based on the fact that hormonal contraceptive usage alters estradiol and progesterone levels in plasma.[17] Consequently, the CAPRISA-004 population, which is composed of 85% women on Depo-Provera ®, was used to study DMPA while the Pumwani Sex Worker Cohort, with more women that are not in any hormonal contraceptives, was used in the estradiol/progesterone study.

Study Population #1

The Pumwani Sex Worker Cohort in Nairobi, Kenya was established for the study of immunobiology and epidemiology of sexually transmitted infections. It is a HIV high-risk cohort, with 60% of enrollees being HIV-positive upon enrolment.[16] All participants used in our study were engaged in active sex work during the time of plasma and cervicovaginal lavage (CVL) collection. Current study population: 112 women that provided blood plasma samples, with 40 also providing CVL on the same date of plasma collection (Table 1). The plasma samples were grouped into three HIV status groups: those that came from HIV-resistant (n=31), HIV-uninfected (n=52) or HIV-infected (n=29) women. HIV-resistant women were defined as having been enrolled in the cohort for more than or equal to 7 years and have been HIV-negative (PCR and antibody-negative) for more than or equal to 7 years. Within each HIV status group, plasma samples were also categorized based on menstrual phase (follicular or luteal). To determine menstrual phase, backtracking from the date of sample collection to the first day of menses (as

determined by self-reported last menstrual period LMP date) was done (menses: days 1-7, proliferative: 8-14, secretory: 15-28). Participants in transition days (7/8, 14/15) were excluded from the analysis. Moreover, participants who tested positive for concurrent genital tract infections such as *Chlamydia trachomatis, Neisseria gonorrhea*, *Trichomonas vaginitis* or bacterial vaginosis were excluded from the study. In addition, women in menses, in menopause or who are pregnant were not included in the analysis. Cervicovaginal lavage (n=40) samples used in this study were all taken from HIV-uninfected women.

Study Population #2

The CAPRISA-004 trial is a two-arm, double-blind randomized placebo-controlled trial used to assess the effectiveness of tenofovir (TFV) gel for HIV prevention. It was conducted from 2007 to 2010 in women enrolled at an urban or a rural STI clinic in KwaZulu-Natal, South Africa. Women who were HIV-negative, from 18 to 40 years of age, sexually active, not pregnant and are using nonbarrier contraceptive methods were eligible for enrollment.[34] Consequently, a majority (85%) of this cohort is comprised of women on hormonal contraception, particularly the injectable progestin-based contraceptive, Depo-Provera ®. A pilot of CAPRISA-004 cases (n=20, with plasma and CVL from their latest uninfected visit prior to HIV seroconversion) and controls (n=18, HIV-uninfected) were used in this study. Cases and controls had no significant differences in age, contraceptive use, HSV status and TFV/placebo use.

Plasma Collection

Plasma was isolated from 10 mL of blood collected in heparin, placed in transport containers at room temperature and stored in a -80C freezer.

Extraction and Hormone Measurement

Estradiol & progesterone: Plasma samples were extracted using acetonitrile and TFA. They were then transferred on a 96-well plate and run on a steroid/thyroid hormone magnetic bead panel (Cat.# STTHMAG-21K, Milliplex®) with magnetic anti-estradiol and anti-progesterone beads. The plate was read using the Bio-Plex Mgr 5.0 software.

Depot medroxyprogesterone acetate: Plasma samples were extracted using 80%ethyl acetate/20% hexane and ammonium formate and then run on LC-MS/MS. Readings and interpretation of data are made using Analyst 1.52 Software.

Cervicovaginal Lavage Collection, Protein Digestion and Proteomic Analysis

A phosphate-buffered saline lavage of the ectocervix and vagina was collected from each participant. CVL protein content was measured by standard BCA protein assay, and equal amount of protein from each participant was individually trypsin digested, cleaned and analyzed via label-free mass spectrometry. Mass-spectrometer data output was analyzed using Progenesis (Nonlinear Dynamics) software.

Statistical Analysis

Plasma sex hormones (estradiol and progesterone) and HIV Status: Plasma hormone data were analyzed against HIV status using GraphPad Prism 6 ($p < 0.05$ = significant). Across group variations in hormone, age and other epidemiological factors were measured by non-parametric Kruskal-Wallis testing (1-way ANOVA). Intergroup/two-group variations were calculated using Mann-Whitney testing (non-parametric, unpaired, two-tailed). Multivariate linear regression analysis (SPSS Statistics Software) was used to analyze multiple variables (HIV status, menstrual phase, age, contraceptive use) against plasma hormone levels.

Plasma sex hormones (estradiol, progesterone and DMPA) and CVL proteomic biomarkers: Perseus (v1.3.0.4, Max Planck Institute of Biochemistry) was used to normalize all protein abundance data via log 2 transformation. Pearson correlation (GraphPad Prism 6, $p < 0.05$ = significant) tests were run comparing all protein factors to estradiol, progesterone and DMPA levels.

Results

Estradiol and Progesterone

Plasma Hormone Levels of HIV-Resistant Women are Not Significantly Different from HIV-Uninfected and HIV-Infected Women

Estradiol and progesterone levels of women from the same menstrual phase were compared. As seen in Figure 1A, there is no significant difference (all p values > 0.05) between sex hormone levels of HIV-resistant, HIV-uninfected and HIV-infected women, regardless of menstrual phase. Upon measurement of hormone levels, it was noted that a few of the samples' hormone levels are at the minimum threshold of detection for the magnetic bead panel (estradiol = 0.01 ng/mL, progesterone = 0.045 ng/mL). As a result, these samples were removed from the analysis (Figure 1B).

Plasma Sex Hormone Levels Do Not Vary Significantly with Menstrual Phase

As seen on Table 1, plasma samples within the same HIV-status group were also classified into follicular phase or luteal phase. Only women within the same menstrual phase are compared as literature states that women from different menstrual phases should have significantly different sex hormone levels.[17] To confirm this, a two-tailed Mann Whitney test was done to compare estradiol and progesterone levels between women in follicular phase and women in luteal phase (Figure 2A, 2B). No significant difference ($p > 0.05$) was found between these women, demonstrating that determining participants' menstrual phase based on self-reported history of LMP dates may be an inaccurate method.

Ratios of Plasma Sex Hormone Levels in HIV-Resistant Women are Not Significantly Different from HIV-Infected and HIV-Uninfected Women

Since using self-reported LMP dates to determine women's menstrual phase may be inaccurate in this cohort, the ratio of estradiol to progesterone is instead used to compare women from the three different HIV status groups. This eliminates the need to group women based on menstrual phase and also allows the experimenters to look at the interaction between estradiol and progesterone. Upon analysis, it is shown that sex hormone ratios do not vary significantly between HIV statuses (Figure 3).

Age Vary Significantly Between HIV-Status Groups

As seen on Figure 4, there is a significant difference in age between HIV groups. HIV-resistant women are found to be older than the two other HIV groups. This is an inevitable outcome of our selection criteria for HIV-resistance where women need to be enrolled in the cohort and engaged in active sex work for at least 7 years while being consistently HIV-negative. Conversely, HIV-uninfected women are youngest since they are the newest enrollees in the cohort. Subsequent analysis showed no association between estradiol plasma levels and age (Figure 5A), and age, therefore was not a confounding factor. This was not the case for progesterone, which was found to be negatively associated with age (Figure 5A).

To determine whether any other measured variables were confounding the relationship between age and progesterone, a multivariate linear regression was fitted using HIV status group, contraceptive use, and menstrual phase as co-variates (Table 2A). A significant inverse

relationship between age and progesterone was observed, as seen in univariate analysis. Being in the HIV-resistant group also had an inverse correlation with progesterone levels that is approaching significance ($p = 0.057$, Table 2B). However, analyzed with age, this association is lost ($p = 0.912$) and age ($p = 0.001$) becomes the significant determinant of progesterone levels (Table 2C, 2D). This supports the idea that progesterone levels do not vary between HIV statuses. Rather, it is age that brings about the apparent difference in progesterone levels between HIV-resistant women and the two other HIV status groups, given that HIV-resistant women are relatively older.

Estradiol Plasma Levels are Significantly Positively Correlated with CVL Host Proteins Involved in Maintaining Epithelial Integrity while Progesterone Plasma Levels are Significantly Negatively Correlated with CVL Proteins Associated with the Acute Phase Response

Plasma estradiol levels positively correlated with 7 host proteins (Table 3), including 3 that have known roles in maintaining epithelial integrity: Annexin 2, sciellin and calmodulin-like protein 5. Annexin is involved in proper F-actin arrangement within cells, sciellin plays an important role in the cornification of stratified squamous epithelia and calmodulin-like protein 5 is a key enzyme in keratinocyte differentiation.

Plasma progesterone levels positively correlated (Pearson correlation, $p<0.05$) with 4 host proteins and negatively correlated with 17 host proteins (Table 4) found in the cervicovaginal lavage samples from 40 HIV-uninfected women from the Pumwani cohort. Many of these progesterone correlates are factors that are involved in the acute phase response ($n = 13$). All of these acute phase response proteins had an inverse association with plasma progesterone levels.

Depot Medroxyprogesterone Acetate

Results will be presented during BSc Med Dissertation. CAPRISA-004 samples are still running on LC-MS/MS at the time this report is written.

Discussion

Recent studies suggest a potential link between sex hormones and HIV acquisition and transmission.[4-6,29] Several experiments on macaques, non-human primates with histologically similar reproductive tracts as humans, have shown that estradiol and progesterone impact HIV infection rates.[30,31] Estradiol is known promote vaginal epithelial thickening and endometrial gland proliferation.[18,29-31] Macaques treated with intravaginal estriol prior to a vaginal challenge with SIV (Simian Immunodeficiency Virus, a virus similar to HIV) had significantly reduced rates of infection.[18,29,30] Moreover, histological samples of estradiol-treated macaques showed thicker vaginal epithelium.[18] Consequently, estradiol was proposed to be protective against HIV due to its epithelium-proliferating effect that keeps host target cells (e.g. T cells and dendritic cells) at a longer distance from HIV virions.[18,30] On the other hand, progesterone is known to promote vaginal thinning and changes in the vaginal pH and mucosal viscosity.[17] In another study on macaques, subcutaneous implantation of progesterone led to vaginal atrophy and a potentiated risk for SIV infection. Moreover, progesterone-treated macaques that acquired SIV had increased rates of progression to AIDS.[31] Consequently, progesterone-abundant states have been implicated in increased HIV susceptibility. However, despite previous evidence that support endogenous sex hormones' roles in HIV resistance/susceptibility, we did not find any significant difference in estradiol and progesterone levels between HIV-resistant, HIV-uninfected

and HIV infected women. However, we did find that sex hormones do associate with CVL immune factors that may serve an important role in HIV protection/susceptibility.

Proteomic analysis on CVL collected at the same date of plasma collection showed that plasma progesterone levels had a significant inverse relationship with several acute phase response proteins. The acute phase response is a non-specific innate immune reaction against immunological stress and provides immediate host protection against damage that may occur during the acute stages of an infection.[17] Acute phase proteins have been implicated in the host antiviral response during the earliest stages of HIV infection.[33] Thus, these preliminary findings suggest progesterone's potential immunosuppressive role in the FRT, which can possibly increase a woman's risk to HIV. Moreover, as the acute phase response pathway involve several proteins, including serpins which are upregulated in HIV-resistant women,[7,10-13] our findings provide a potential mechanism by which progesterone can regulate some processes important for HIV resistance, even if its levels are not significantly higher or lower in HIV-resistant women themselves.

Our study on plasma hormone levels and HIV status groups has several potential limitations. First, plasma samples were classified into two menstrual phase groups (Follicular: Days 8-14 and Luteal: Days 15-28) based on last menstrual period (LMP) dates. Only women within the same menstrual group are compared since literature states that sex hormone levels vary significantly between follicular phase and luteal phase, with progesterone peaking only at luteal phase.[17] Thus, only comparing women within the same menstrual phase group theoretically eliminates menstrual status as a confounding factor in the study. However, the menstrual phasing method (based on LMP) used, assumes that women accurately self-report their LMP and have regular 28-day menstrual cycles. This is not the case as shown by our non-parametric Mann-Whitney Test (Figure 2A and 2B). This implies that women may not have been grouped and compared correctly. To address this problem, the ratios of estrogen to progesterone in the three groups were compared instead. Hormone ratios also did not vary significantly between HIV status groups, thus supporting our previous findings that sex hormones do not significantly differ between HIV status groups. Second, age vary significantly between women from different HIV status groups. As expected, HIV-resistant and HIV uninfected women were older and younger, respectively. Age-matching or grouping women in different age brackets (e.g. 20-24 y.o., 25-29 and so forth) cannot be done since most of the HIV-resistant women are in their forties and the power of our study will significantly decrease, respectively. Univariate correlation analysis showed that progesterone had an inverse relationship with age. Multivariate linear regression analysis confirmed the same observation and showed that it is age that solely significantly determines progesterone levels in our study population (Table 2A-D). This highlights the lack of association between HIV status group and hormone levels but also emphasizes the importance of having age-matched HIV status groups in subsequent studies to eliminate age from confounding the results.

Our cross-sectional study on CVL and hormone levels suggests an inverse relationship between progesterone and acute phase response proteins important for inhibition of HIV infection. Moreover, estradiol correlated with markers important in epithelial integrity, thus supporting previous evidence of estradiol's HIV-protective role through epithelial thickening.[18,30] However, a causal relationship between sex hormones and the above factors can only be established by examining the rate of change in the expression of significantly correlated CVL proteins in relation to controlled variations in sex hormones levels in the plasma. This requires not only a longitudinal study but also a design similar to studies on non-human primates, where procedures such as ovariectomies are used to control hormone levels present in the plasma. This is obviously not feasible in our study population and even in almost all other studies

involving human participants. At best, it is important to at least establish a temporal relationship between sex hormone levels and CVL protein expression in future studies.

Overall, our preliminary findings suggest that, contrary to what previous studies suggest, there is no association between sex hormone levels and HIV-resistance. However, an inverse association between progesterone levels and acute phase response proteins have been observed, suggesting a correlative link between high-progesterone states and an immunosuppressed, HIV-susceptible FGT environment. Moreover, estradiol positively correlated with proteins involved in epithelial integrity and can thus be potentially linked to a physically robust FRT that is less penetrable by pathogens such as HIV. Future studies are needed to establish a causal relationship between hormone levels and innate immune factor expression in the genital tract. Findings of this study demonstrate a possible mechanism in which a person's susceptibility to HIV can be altered due to fluctuations in hormone levels, particularly progesterone's. This relationship between progesterone and the acute phase response, if proven causal, could have a profound impact on HIV prevention strategies and family planning methods, particularly those that involve the use of hormonal contraceptives (e.g. DMPA).

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Figures, Figure Legends and Tables

Table 1: Final study population (plasma and CVL samples for estradiol/progesterone study) after application of exclusion criteria. Participants were also classified based on menstrual phase (Follicular: Days 8-14, Luteal: Days 15-28) with women in transition periods (7/8, 14/15) removed from the study. Out of the 52 HIV-uninfected women, 40 provided CVL samples for proteomic analysis.

	Plasma Samples			CVL Samples
Menstrual Phase	**HIV-Resistant**	**HIV-Uninfected**	**HIV-Infected**	**HIV-Uninfected**
Follicular Phase	9	21	13	40
Luteal Phase	22	31	16	
Total: Phases Combined	31	52	29	
Total	112			40

Table 2A: Multivariate linear regression analysis with menstrual phase (luteal), age, HIV status group (resistant) and contraceptive method as covariates. Only age (p=0.001) has a significant inverse (t=-3.3357) association with the dependent variable, progesterone. **Table 2B**: HIV-resistant status has an inverse association with progesterone levels that is approaching significance (t=-1.928, p =0.057). **Table 2C:** When both covariates (HIV-resistance and age) are analyzed together, only age has a significant inverse relationship (p=0.001) with progesterone levels. **Table 2D**: Age has a significant inverse association with plasma progesterone levels (p=0.002).

A

	Unstandardized Coefficients		**Standardized Coefficients**	**t**	**Sig. (p-value)**	**95.0% Confidence Interval for B**	
Constant	**B**	**Std. Error**	**Beta**			**Lower Bound**	**Upper Bound**
Luteal	.095	.248	.040	.382	.703	-.399	.589
Age	-.062	.018	-.397	-3.357	.001	-.098	-.025
Resistant	-.042	.280	-.018	-.149	.882	-.600	.516
Contraceptive	-.394	.558	-.073	-.705	.483	-1.505	.717

B

	Unstandardized Coefficients		**Standardized Coefficients**	**t**	**Sig. (p-value)**	**95.0% Confidence Interval for B**	
Constant	B	Std. Error	Beta			Lower Bound	Upper Bound
Resistant	-.498	.259	-.209	-1.928	.057	-1.013	.016

C

	Unstandardized Coefficients		**Standardized Coefficients**	**t**	**Sig. (p-value)**	**95.0% Confidence Interval for B**	
Constant	B	Std. Error	Beta			Lower Bound	Upper Bound
Resistant	-.031	.277	-.013	-.111	.912	-.581	.520
Age	-.063	.018	-.407	-3.495	.001	-.099	-.027

D

	Unstandardized Coefficients		**Standardized Coefficients**	**t**	**Sig. (p-value)**	**95.0% Confidence Interval for B**	
Constant	B	Std. Error	Beta			Lower Bound	Upper Bound
Age	-.057	.018	-.284	-3.110	.002	-.094	-.021

Dependent Variable = progesterone

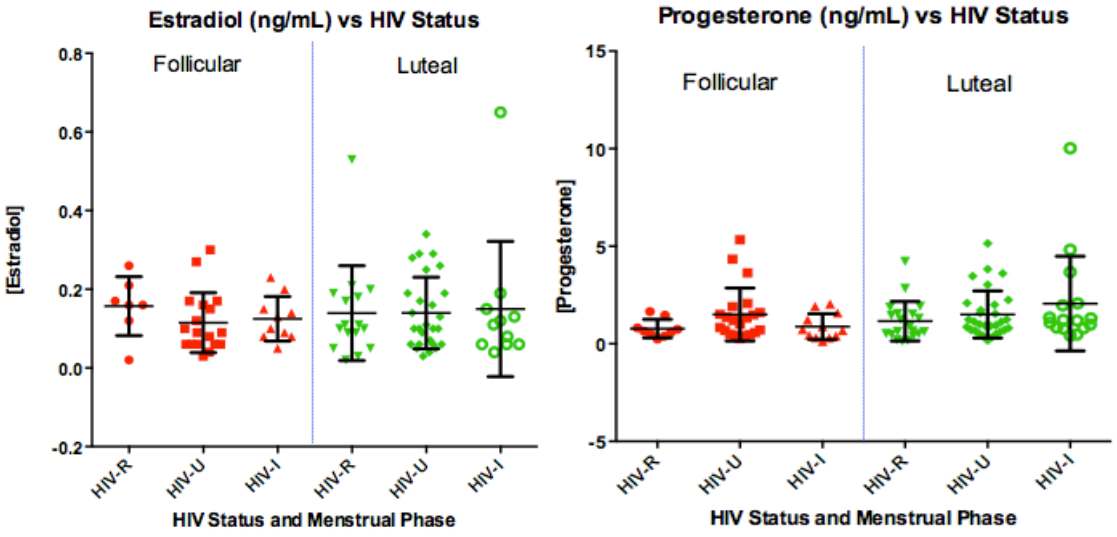
Table 3: Protein factors that had significant positive (n=7) and negative (n=4) correlations with estradiol, along with their functions (based on UniProt).

Gene Name	Protein	Function (UniProt)	Pearson Correlation	Trend
SCEL	Sciellin	Assembly and regulation of cornified envelope	0.456	Positive
rpsP	30S ribosomal protein S16	Ribosomal protein	-0.4742	Negative

ldhA	D-lactate dehydrogenase	Mitochondrial protein	0.4646	Positive
AHNAK	Neuroblast differentiation-associated protein	Protein oligomerization RNA splicing regulation	0.4424	Positive
RNASET2	Ribonuclease T2	Lysosomal degradation of ribosomal RNA	0.4425	Positive
ARF1	ADP-ribosylation factor 1	GTP-binding protein	-0.4276	Negative
CDA	Cytidine deaminase	Cytidine scavenging for UMP synthesis	0.3866	Positive
TMPRSS11E	Transmembrane protease serine 11E	Serine protease with gelatinolytic and caseinolytic activity	0.3742	Positive
CALML5	Calmodulin-like protein 5	Differentiation of keratinocytes	-0.3726	Negative
ANXA2	Annexin A2	Membrane-binding protein	0.36	Positive
LRG1	Leucine-rich alpha-2-glycoprotein	Brown fat cell differentiation	-0.3561	Negative

Table 4: Protein factors that had significant positive (n=4) and negative (n=17) correlations with progesterone, along with their functions (based on UniProt). 13 out of the 17 negative correlates are involved in the acute phase response.

Gene Name	Protein	Function (UniProt)	Pearson Correlation	Trend
ORM1	Alpha-1-acid glycoprotein 1	Modulation of the immune system during the acute-phase reaction	-0.4498	Negative
HPX	Hemopexin	Heme transport Acute phase response protein	-0.4376	Negative
ORM2	Alpha-1-acid glycoprotein 2	Modulation of the immune system during the acute-phase reaction	-0.4234	Negative
ALB	Serum albumin	Plasma transport protein Regulation of colloidal osmotic pressure Acute phase response protein	-0.397	Negative
AHSG	Alpha-2-HS-glycoprotein	Promotion of endocytosis Influences mineral phase of bone Acute phase response protein	-0.3923	Negative
gap	Glyceraldehyde-3-phosphate dehydrogenase	Key enzyme in glycolysis	0.3833	Positive
TTR	Transthyretin	Thyroid hormone-binding protein Acute phase response protein	-0.382	Negative
A1BG	Alpha-1B-glycoprotein	Blood microparticle Acute phase response protein	-0.3792	Negative
GSN	Gelsolin	Role in ciliogenesis	-0.3754	Negative
LRG1	Leucine-rich alpha-2-glycoprotein	Brown fat cell differentiation	-0.3755	Negative
rplL	50S ribosomal protein L7/L12	Ribosomal protein	-0.4694	Negative
C3	Complement C3	Activation of the complement system Acute phase response protein	-0.353	Negative
KRT19	Keratin, type I cytoskeletal 19	Myofiber organization	-0.3523	Negative
CP	Ceruloplasmin	Iron transport and ferroxidase activity Acute phase response protein	-0.352	Negative
TF	Serotransferrin	Iron binding transport proteins Acute phase response protein	-0.3521	Negative
groL	60 kDa chaperonin		0.3481	Positive
QSOX1	Sulfhydryl oxidase 1	Oxidation of sulfhydryl groups in proteins	0.3469	Positive
APOA1	Apolipoprotein A-I	Reverse transport of cholesterol Acute phase response protein	-0.3415	Negative
SPRR2F	Small proline-rich protein 2F	Keratinocyte protein	0.3363	Positive
FGA	Fibrinogen alpha chain	Fibrin polymerization Cofactor in platelet aggregation Acute phase response protein	-0.3329	Negative
C4B	Complement C4-B	Propagation of the classical component pathway Acute phase response protein	-0.3275	Negative



A

Menstrual Phase	HIV-Resistant			HIV-Uninfected			HIV-Infected		
	Initial	Used in Estradiol Analysis	Used in Progest. Analysis	Initial	Used in Estradiol Analysis	Used in Progest. Analysis	Initial	Used in Estradiol Analysis	Used in Progest. Analysis
Follicular	9	**7**	**9**	21	**18**	**21**	13	**10**	**12**
Luteal	22	**16**	**20**	31	**27**	**30**	16	**11**	**16**
Total: All Phases	31	**23**	**29**	52	**45**	**51**	29	**21**	**28**
Total	Initial: 112 Used In Estradiol Analysis: **89** Used In Progesterone Analysis: **102**								

B

Figure 1A: Analysis of estradiol and progesterone levels from the 3 HIV-status groups (R-resistant, U-uninfected, I-infected), respectively. Samples were classified into follicular phase and luteal phase. Two-group comparison (Mann-Whitney test) and intergroup variation analysis (Kruskal-Wallis test) was done in samples that are within the same menstrual phase. There are no significant differences (all p-values >0.05) in hormone levels between the 3 HIV groups within the same menstrual phase. **Figure 1B:** Number of plasma samples used in estradiol and progesterone analysis after samples on the minimum threshold of detection for the magnetic bead panel (estradiol = 0.01 ng/mL, progesterone = 0.045 ng/mL) were removed.

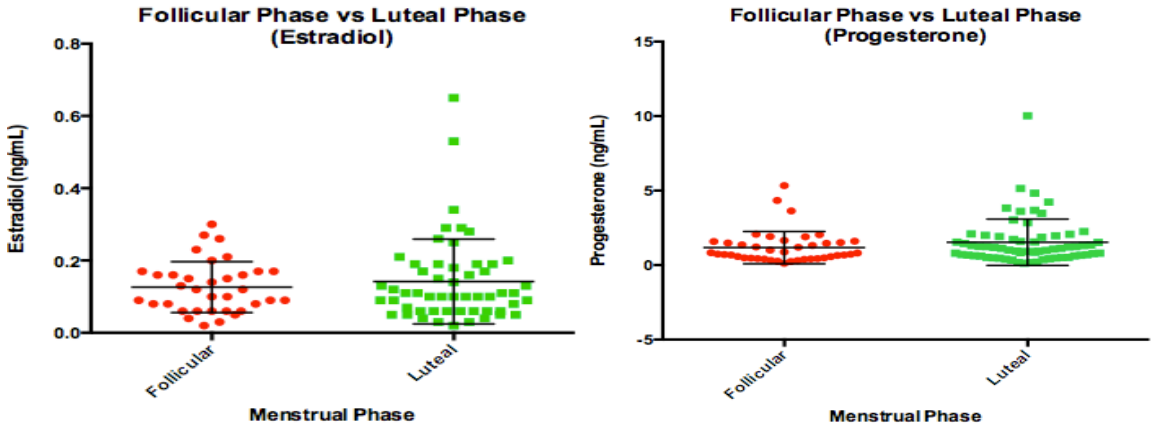


Figure 2A: A comparison of estradiol levels between women in follicular phase and women in luteal phase. No significant differences between these groups are found (p = 0.9850) after a non-parametric, two-tailed Mann-Whitney test (t-test). **Figure 2B:** A comparison of estradiol

levels between women in follicular phase and women in luteal phase. No significant difference between these groups are found (p = 0.1573) after a non-parametric, two-tailed Mann-Whitney test (t-test).

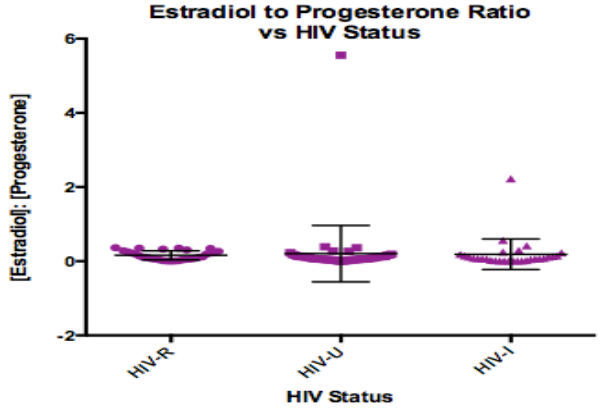


Figure 3: The ratios between estradiol and progesterone plasma levels between the 3 HIV status groups are compared. No significant intergroup variation (p =0.2225) or two-group differences (all p-values >0.05) are found after Kruskal-Wallis testing and Mann-Whitney testing, respectively.

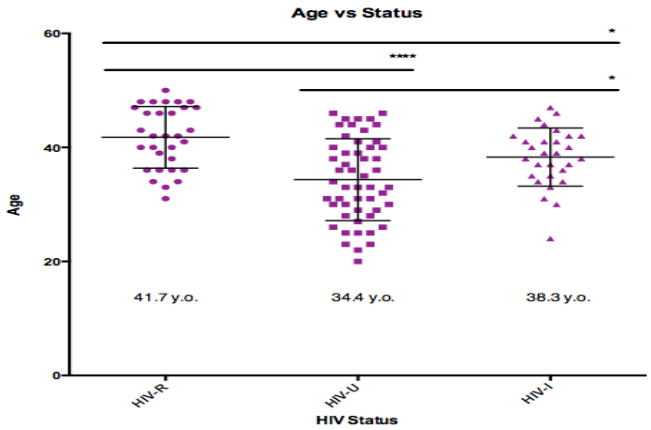


Figure 4: Ages of women from each HIV-status group are compared using a non-parametric, two-tailed Mann-Whitney test. HIV-resistant women, with an average age of 41.7 years old, are significantly older than HIV-uninfected (p <0.0001) and HIV-Infected women (p = 0.0190). HIV-uninfected women are significantly younger than HIV-R and HIV-I women (p = 0.0414).

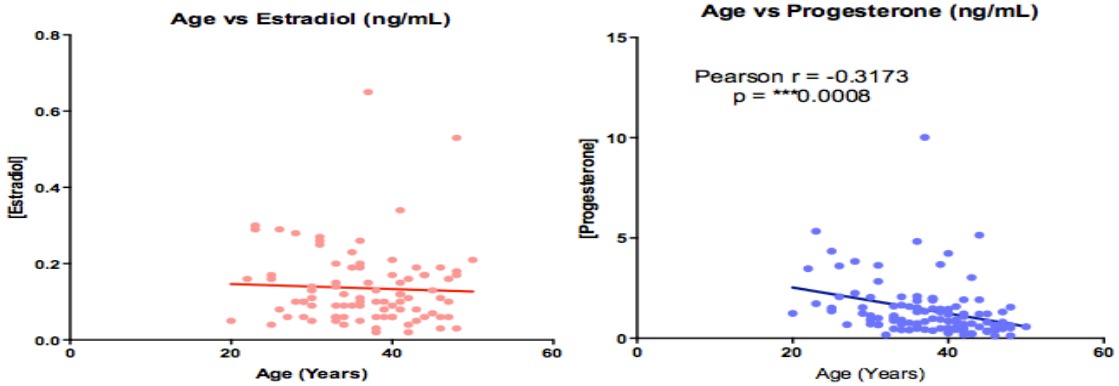


Figure 5A: No significant correlation (p = 0.6668) is found between plasma estradiol levels and age of participants after Spearman correlation testing. **Figure 5B:** A significant negative correlation (p = 0.0008) is found between plasma progesterone levels and age of participants after Spearman correlation testing.