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Sex Differences in CMV Replication and HIV Persistence During Suppressive ART

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Background. The association between subclinical cytomegalovirus (CMV) replication and HIV persistence has not been investigated in cis-gender women with HIV.

Methods. Fifty virologically suppressed female participants with HIV were prospectively enrolled and provided oral, vaginal, and urine samples and peripheral blood mononuclear cells at 1 cross-sectional time point. CMV DNA was quantified in each specimen by real-time polymerase chain reaction (PCR). Cellular HIV DNA and HIV RNA transcripts (unspliced and multiply spliced [ms] encoding tat-rev) were quantified by droplet digital (dd) PCR in peripheral blood cells. Forty-nine male individuals with HIV and CMV (historical data) were used as controls.

Results. Levels of cellular HIV DNA and unspliced HIV RNA were not different between sexes, but female participants had less detectable msHIV RNA and CMV DNA compared with males (both $P < .01$). Unlike previously described for males, the presence of CMV DNA was not associated with increased HIV DNA in females. Among female participants, premenopausal status was independently associated with lower HIV DNA compared with postmenopause, after adjusting for nadir CD4 count ($P < .01$).

Conclusions. Female participants with HIV had reduced cellular HIV RNA and less subclinical CMV DNA compared with males but overall similar HIV DNA levels in our study. Postmenopausal status was independently associated with higher HIV DNA levels among female participants.

Keywords. HIV; CMV; sex differences.

Antiretroviral therapy (ART) improves health, prolongs survival, and reduces HIV transmission [1]. Nevertheless, HIV infection remains associated with morbidity and mortality due to non-AIDS-defining events [1–3]. Increased morbidity and mortality are associated with inflammation and immune dysfunction, which persist in some people with HIV on suppressive ART [2]. Potential contributors to inflammation include the direct effect of low-level HIV replication, translocation of bacterial/fungal products from the gut into the systemic circulation, and viral co-infections such as cytomegalovirus (CMV) [1, 2]. Further, CMV-driven inflammation might modulate and maintain the HIV reservoir through various mechanisms in the setting of suppressive ART [3].

Our group has investigated extensively the connections between CMV replication, systemic immune activation, and levels of HIV DNA in men who have sex with men (MSM) with HIV in Southern California. We reported that subclinical CMV reactivation in seminal plasma and detectable CMV DNA in peripheral blood cells were associated with T-cell dysfunction, and also with higher levels of HIV DNA [9–11]. These findings support clinical trials of anti-CMV drugs or vaccines to decrease systemic immune activation in order to prevent end-organ diseases and reduce the latent HIV DNA reservoir. However, female participants cannot be included in these trials without optimizing detection of CMV reactivation and investigating if CMV reactivation in females might induce the same immunologic and virologic outcomes as observed in males. Only sparse data exist regarding asymptomatic CMV shedding in females with HIV, including the following: 2 studies in the United States including cis-gender women with HIV with partial uptake of ART found that CMV DNA was detected infrequently in cervicovaginal lavage (CVL), with 55/764 (7%) and 10/312 (3%) positive samples [12, 13]. Two studies in Sub-Saharan Africa reported significantly higher levels of CMV detectability between 59% and 78% of provider-collected cervical swab samples [14, 15]. These data demonstrate the variability of CMV shedding and regional differences but also the sparse data available about CMV shedding from mucosal sites in females on suppressive ART. Here, we prospectively enrolled 50 virologically suppressed cis-gender women with HIV in New York City to collect oral, vaginal, and

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urine samples and peripheral blood mononuclear cells (PBMCs) at 1 time point. We quantified CMV DNA in each specimen, as well as cellular HIV DNA and HIV RNA transcripts in PBMCs. Forty-nine MSM (historical data) were used as controls. Our primary goals were (i) to describe the burden of CMV among women with HIV in the United States and (ii) to investigate if presence of CMV DNA (among other factors) was associated with levels of HIV DNA in female participants.

METHODS

Cohort and Samples

Fifty virologically suppressed cis-gender women with HIV were prospectively enrolled in New York City at Weill Cornell Medicine between July 2014 and September 2016. Participants provided genital samples (self-collected vaginal swabs n = 50, provider collected cervical swabs n = 14, and cervicovaginal lavage [CVL] n = 14), oral samples (rinses n = 50, swabs n = 42), peripheral blood mononuclear cells (n = 50), and urine (n = 50) at 1 cross-sectional time point.

As controls, we used historical data from cis-gender MSM with HIV and CMV enrolled in the California Collaborative Treatment Group (CCTG) 592 [16]. Briefly, CCTG 592 is a study of an Internet-based behavioral intervention of sexually active MSM that included baseline collection of semen and longitudinal follow-up on a total of 180 MSM with HIV who were either on or off ART. For this study, we included baseline data from a subset of 49 cis-gender men who were receiving effective ART with blood plasma HIV RNA <50 copies/mL within 3 months before the seminal sample collection.

The studies were conducted with appropriate written subject consent and were approved by the Human Research Protections Program at the University of California, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, the University of Southern California, and Weill Cornell Medicine.

CMV Quantification in Genital Secretion, Oral Secretion, and Urine

For oral rinse and CVL, the volumes of supernatants were measured (20–30 mL mouthwash, 8–11 mL CVL) and ultra-centrifuged at 23 500 × g, 4°C for 1 hour; 200 μL of supernatant was used to isolate DNA following the QIAamp DNA Mini Kit (Qiagen, Carlsbad, CA, USA) protocol. Cellular pellets (obtained from CVL and oral rinse) directly underwent DNA extraction using the same QIAamp DNA Mini Kit. Further, 500 μL of eluted DNA per sample was concentrated via standard ethanol DNA precipitation protocol.

Urine was pelleted by high-speed centrifugation at 23 500 × g 4°C for 1 hour; 140 μL of concentrate was used to isolate DNA using the QIAamp Viral RNA Mini Kit (Qiagen, Carlsbad, CA, USA), per the manufacturer's protocol.

For dry oral, vaginal, and cervical swabs, DNA was extracted by re-suspending the swab in 400 μL of 1X Dulbecco's Phosphate-Buffered Saline (DPBS). The entire 400-μL suspension of each swab sample was collected using autoclave-able assemblies of spin-baskets (Kerafast, Boston, MA, USA) and 1.5 mL of Eppendorf snap-cap microcentrifuge safe-lock tubes (Fisher Scientific, Waltham, MA, USA) after centrifugation at >10 000 × g 23°C for 1 minute [9]. DNA was extracted from the total 400-μL suspension with the QIAamp DNA Mini Kit. Ethanol-based DNA precipitation was performed to concentrate the DNA. For semen, DNA was extracted as previously described [9, 11].

CMV was measured from all extracted and precipitated DNA by real-time PCR using known CMV plasmid concentrations as the standard curve on Applied Biosystems' 7500 Real Time PCR Systems (Thermo Fisher Scientific, Kerafast, Boston, MA, USA) [17].

Cellular HIV DNA and CMV DNA in PBMCs

DNA was extracted from 5 million PBMCs for each time point using AllPrep DNA/RNA Mini Kit (Qiagen, CA). Total HIV DNA (pol) and 2-LTR junction were quantified by droplet digital PCR (ddPCR) from extracted DNA as previously described [18]. Copy numbers were calculated as the mean of replicate PCR measurements and normalized to 1 million CD4+ T cells as determined by RPP30 (total cell count) and flow cytometry (percentage of CD4+ T cells) [4].

Cellular HIV RNA in PBMCs

Cellular HIV RNA was extracted from PBMCs using the AllPrep DNA/RNA Mini Kit (Qiagen, Carlsbad, CA, USA) following the manufacturer's protocol with the addition of a DNAse step to avoid DNA contamination (RNase-free DNase Set, Qiagen, Carlsbad, CA, USA). Extracted RNA (500 ng) was reverse-transcribed into 20 μL of cDNA (iScript Advanced cDNA Synthesis Kit for RT-qPCR, Bio-Rad) using the manufacturer's protocol. The cDNA product (~300 ng) was added to the ddPCR reaction. Unspliced (us) HIV RNA (Gag) and multiply-spliced (ms) HIV RNA (TatRev) PCRs were performed as a duplex with HEX (usGag) and FAM (msTatRev) probes, respectively, using primers and probes as previously described [5, 6]. Copy numbers were calculated as the mean of replicate PCR measurements and normalized to total RNA as determined by A260/A280 absorptivity ratio using a NanoDrop 2000 spectrophotometer (Thermo Scientific) [9].

Anti-CMV IgG Antibody Levels

Anti-CMV IgG antibody levels were measured in blood plasma. Units per mL (U/mL) were determined by interpolation from a standard curve of a known anti-CMV IgG solution, as previously described [7, 8].

Sex Hormone Levels

Levels of estradiol, progesterone, and follicle-stimulating hormone (FSH) were measured using the estradiol enzyme-linked immunosorbent assay (ELISA) kit (Ref EIA-2693, DRG International, Inc.), progesterone ELISA kit (Ref EIA-1561, DRG

International, Inc.), and FSH ELISA kit (Ref EIA-1288, DRG International, Inc.) following the manufacturers' protocols.

Statistical Analysis

We used descriptive statistics to characterize the study populations. We used the Fisher exact test to compare dichotomous variables between groups and the Kruskal-Wallis test to compare continuous variables between groups. We examined predictors of HIV DNA using generalized linear models. To build the multivariable models, we included all variables with a P value <.15 in the univariable analysis. Then, we removed variables with a P value >.15 in a stepwise fashion to build the final model. Continuous variables were log-transformed as needed to normalize the distribution. All statistical analyses were performed using SAS, version 9.4.

RESULTS

Cohort and Samples

For female participants, the median age was 53 years; 26 (52%) were postmenopausal (self-reported), and 43 (86%) acquired HIV through heterosexual contact. The median time on suppressive ART (interquartile range [IQR]) was 7.8 (4.2–13.1) years. The median current $CD4^+$ T-cell count (IQR) was 721 (490–930) cells/μL, and the nadir $CD4^+$ was 172 (56–265) cells/μL. For clinical details and comparison with the male participants, see Table 1. Importantly, the 2 groups were significantly different by ethnicity/race, mode of HIV transmission, age, and current CD4 T-cell count. For hormonal levels in women by menopausal status, see Supplementary Table 1.

Differences in CMV Replication and HIV Persistence Among Women and Men

One woman was CMV IgG negative. Of the 49 female participants with both HIV and CMV, 15/49 (29%) had detectable CMV DNA in at least 1 specimen type (Figure 1), most frequently self-collected vaginal swabs (8/49, 16%) and PBMCs (7/48, 15%), followed by oral rinse (combined supernatant and pellet; 4/49, 8%), oral swabs (1/39, 2.6%), CVL, and urine (both 0%). Detectability was often inconsistent across sampled locations. As a comparison, of the 49 cis-gender men with HIV and CMV, 26/49 (53%) had detectable CMV DNA in at least 1 specimen type, most frequently in semen (40%) and in PBMCs (21%). Overall, male participants had significantly more detectable CMV replication than females (Fisher $P < .01$). We also obtained quantitative CMV DNA levels. Specifically, CMV DNA in PBMCs ranged from 3 to 18 copies/10^6 cells in females and 2–59 in males ($P > .2$), CMV DNA in vaginal swabs ranged from 7 to 40 copies/mL elution, seminal CMV DNA ranged from 220 to 26 671 copies/mL seminal plasma ($P < .001$), and CMV in oral mucosa ranged from 6 to 12 744 copies/mL elution for females. Data on oral mucosa were not available for men.

Levels of cellular HIV DNA and unspliced HIV RNA were not different between sexes, but female participants were less likely to have detectable msHIV RNA (54% vs 100% in male participants; Fisher $P < .001$).

Associations Between CMV DNA, HIV Reservoir Measures, and Other Variables in Women

Unlike our previous findings in males [11, 19], CMV DNA (overall and at any sampled location) had no effect on any measure of HIV persistence in female participants ($P > .2$).

Table 1.   Sex Differences in Demographics

Demographics		Women (n = 50)	Men (n = 49)	P Values
Ethnicity	Hispanic	7 (14)	19 (39)	<.001
	Non-Hispanic	43 (86)	30 (61)	
Race	Asian	1 (2)	2 (4)	<.001
	Black	37 (74)	11 (22)	
	Native American	1 (2)	0	
	Other	3 (6)	0	
	White	8 (16)	36 (74)	
HIV transmission	IDU	3 (6)	0	<.001
	Sex with men	43 (86)	48 (98)	
	Both	2 (4)	0	
	Other	2 (4)	1 (2)	
Menopausal status	Premenopausal	24 (48)	NA	
	Postmenopausal	26 (52)	NA	
Age, y		53 [47–57]	46 [38–51]	<.01
Current $CD4^+$ cells, cells/mm^3		721 [490–930]	625 [538–774]	.04
Nadir $CD4^+$ cells, cells/mm^3		172 [56–265]	215 [68–350]	.12

Data are presented as No. (%) or median [IQR].

Abbreviations: IDU, injection drug users; IQR, interquartile range; NA, not available.

Obs	Vag swab	Oral swab	Oral Pellet	Oral Sup	PBMC
1	■				■
2	■				■
3	■				
4	■				
5	■				
6	■				
7	■				
8	■				■
9			■	■	
10			■		■
11				■	■
12					■
13					■
14					■
15			■		

Figure 1. CMV shedding pattern among 15 women with detectable CMV DNA in at least 1 sample site. Abbreviations: CMV, cytomegalovirus; PBMC, peripheral blood mononuclear cell.

There was also no difference in cellular HIV RNA transcription and CMV shedding between pre- and postmenopausal women.

Among female participants, premenopausal status was associated with significantly lower HIV DNA compared with postmenopause (42 vs 150 HIV DNA copies/10^6 cells; $P < .01$; males: 90 copies/10^6 cells) (Figure 2). Overall among female participants, higher HIV DNA concentrations were associated with lower nadir CD4 counts, higher FSH concentrations, older age, and postmenopausal status. In the multivariable model, lower nadir CD4 counts and postmenopausal status remained independently associated with higher HIV DNA concentrations. When evaluating concentrations of sex hormones, we did not find any effect of estradiol or progesterone on HIV RNA transcription or HIV DNA levels. However, we did observe a positive correlation between FSH and HIV DNA ($P < .01$) (Figure 3). In the multivariable model, FSH was not independently correlated with HIV DNA when accounting for menopausal status.

DISCUSSION

If we are to cure HIV, we must understand the mechanisms that allow the HIV DNA reservoir to persist despite ART. We have previously shown that asymptomatic CMV reactivation in MSM is associated with higher levels of HIV DNA and with increased systemic immune activation and proliferation of CD4$^+$ T cells. This was observed in male participants both receiving and not receiving antiretroviral therapy [9, 11]. It remains unclear, however, if CMV reactivation in females induces the same immunologic and virologic consequences. Additionally, while CMV is most reliably detected in the male genital tract, the optimal location or strategy for detecting CMV replication in women is unclear.

Overall, our study found that female participants have significantly less detectable CMV DNA compared with males (Fisher $P < .01$), even if sampled in more mucosal sites (ie, genital, urine, oral, and PBMCs for females compared with seminal plasma and PBMCs for males). For females, CMV DNA was most frequently detected in self-collected vaginal swabs (16%) and PBMCs (15%), followed by oral rinse (8%), oral swabs (3%), and CVL and urine (both 0%). These data are similar to previous reports in the United States [12, 13] but lower than data reported from Sub-Saharan Africa [14, 15]. When comparing the genital compartment, females had significantly lower levels of CMV DNA (average, 15 copies/mL vs 7600 copies/mL; $P < .001$) compared with males. On the other hand, the detection of CMV DNA in PBMCs was similar between males and females (21% and 15%, respectively). The presence of CMV DNA in blood cells (usually monocytes) may reflect low-level viral replication but may also simply reflect latent CMV infection and not necessarily CMV replication.

Regarding measures of HIV persistence, levels of cellular HIV DNA and unspliced HIV RNA were not different between
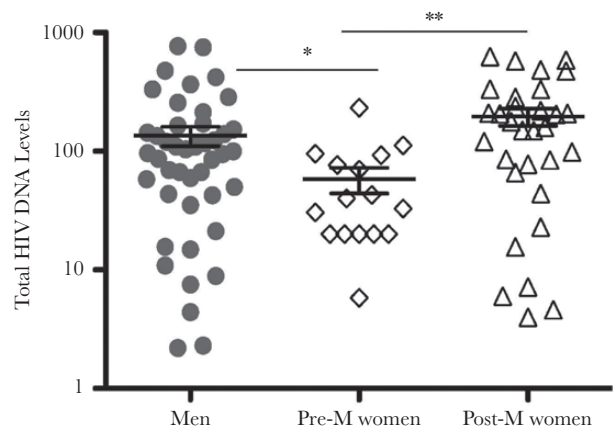
Figure 2. Levels of total HIV DNA in men, premenopausal (pre-M) women, and postmenopausal (post-M) women. $^{**}P < .001$; $^*P < .05$.
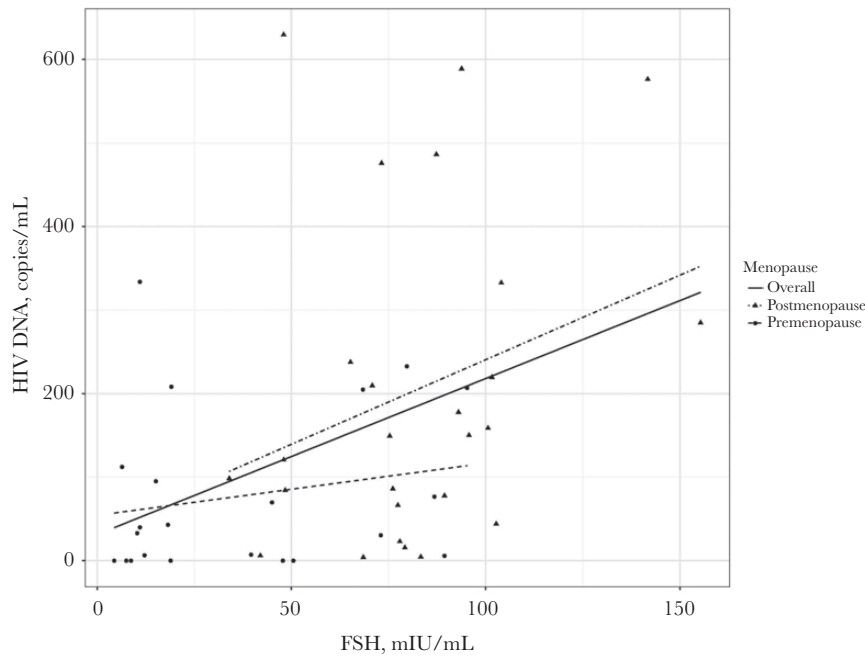
Figure 3. Regression analysis between FSH and total HIV DNA in the pre- and postmenopausal groups (continuous and dotted lines, respectively). Abbreviation: FSH, follicle-stimulating hormone.

sexes, but female participants were less likely to have detectable msHIV RNA (54% vs 100% in cis-gender men; Fisher $P < 0.01$). This is in line with a previous study from Scully et al. [20] that showed higher cell-associated HIV RNA, higher plasma HIV-1 (single copy assay), and higher T-cell activation and PD-1 expression in females compared with males, but this previous study did not evaluate CMV persistence.

Subsequently, we assessed the effect of CMV and other clinical variables on the HIV reservoir among female participants. Unlike our previous findings in males [11, 19], CMV had no effect on any measure of HIV persistence in females. Among the other variables, premenopausal status was associated with significantly lower HIV DNA compared with postmenopause, even after adjusting for age and duration of suppression on ART ($P < .01$). In line with these findings, higher levels of FSH were associated with higher HIV DNA. One possible mechanism is

in vivo exposure to estrogen, which has been shown to repress HIV transcription in latency models and patient cells [21] as well as in in vitro infection systems [22], indicating a direct role for hormones in mediating sex differences.

This study had several limitations. First, the limited sample size and the cross-sectional study design likely limit our capacity to observe some associations. Specifically, a single time point does not adequately capture participants with CMV DNA because of the intermittent nature of the viral shedding. This results in the potential for misclassification, which would affect the ability to detect an effect. Also, the study populations were different and recruited at different sites (New York vs Southern California), which might have introduced some selection bias. There was a notable difference in race/ethnicity between the 2 cohorts, with a majority of black females compared with white males. It is important to note that the seroprevalence of CMV

Table 2. Sex Differences in CMV and HIV Reservoir Data

CMV Data	Women (n = 50)	Men (n = 49)	P Values
CMV IgG seropositive	49 (98)	48 (98)	>.2
CMV IgG levels, UI/mL	37 [33–41]	34 [26–43]	>.2
Detectable CMV DNA (any samples)	15 (29)	26 (53)	<.01
HIV reservoir data			
HIV DNA levels, copies/10^6 cells	89 [19–209]	90 [18–148]	>.2
Unspliced cellular HIV RNA levels (per 200 ng DNA input)	7 [3–15]	7 [4–15]	>.2
Detectable multiply spliced cellular HIV RNA	27 (54)	41 (100)[a]	<.001

Data are presented as No. (%) or median [IQR].

Abbreviations: CMV, cytomegalovirus; IQR, interquartile range.

[a]Cellular HIV RNA data for men only available on a subset of n = 41.

Table 3. Predictors of HIV DNA (Copies/10^6 PBMC) in Women

Factor	Univariable, β, PValue	Multivariable, β, PValue
Current CD4, per 100 cells/mm^3	−8, .43	
Current CD4%, per %	−0.97, .71	
Nadir CD4, per 100 cells/mm^3	**−36, .02**	**−43, <.01**
Time on ART, per 10 y	−55, .26	
Low level viremia (yes vs no)	−15, .84	
CMV detected (yes vs no)	54, .31	
Menopause (postmenopausal vs premenopausal)	**123, <.01**	**144, <.01**
Age, per 10 y	77, <.01	

Bold indicates significant results.
Abbreviations: ART, antiretroviral therapy; CMV, cytomegalovirus.

was similar in both cohorts, and a difference in CMV shedding by race/ethnicity is currently not known. Our data from the United States are likely not generalizable to the population of females with HIV worldwide.

Further, while we were not able to observe a direct effect of estradiol on any marker of HIV persistence at single time points, we did not measure expression of estrogen receptor as part of our study. Also, the age range of the women was relatively narrow, which might explain the lack of association between estradiol and any marker of HIV persistence. A longitudinal approach should better address the impact of the contemporaneous hormone levels on virus activity.

Nevertheless, this study provides key information regarding the pattern of CMV shedding in females, as compared with males. Specifically, the male genital tract constitutes a concentrated reservoir of CMV DNA replication, which likely alters the local and systemic immunologic and viral dynamics [3, 23, 24]. For females in our US study, CMV shedding is much less frequent, with inconsistent detection at various locations with significantly lower viral loads. While we were not able to observe an effect of CMV shedding on HIV persistence in our study, the systemic immunologic effect of CMV shedding in females needs further elucidation. Also, the optimal sampling strategy to measure CMV shedding in females likely requires a combination of oral and genital swabs.

Our study also provides some interesting insights into the association between menopausal status and HIV persistence. Overall, our observations provide further evidence that virologic outcomes after curative interventions may differ by sex and menopausal status, requiring careful attention to enrolling an adequate number of women of all ages in cure studies and to reporting sex-delineated outcomes.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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