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Female Genital Tract Host Factors and Tenofovir and Lamivudine Active Metabolites

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Background. We previously reported the effect of contraception on cervical tenofovir concentrations in Ugandan women with human immunodeficiency virus (HIV). Here we explored the role of cervicovaginal cytokines and drug metabolizing enzymes and transporters (DMETs) to elucidate female genital tract (FGT) drug disposition in a Ugandan cohort.

Methods. Cervicovaginal fluid and cervical biopsies were collected from Ugandan women with HIV receiving tenofovir/lamivudine-based therapy and intramuscular depot medroxyprogesterone acetate (n = 25), copper intrauterine device (cuIUD; n = 12), or condoms (n = 13) as contraception. Cytokines were measured in cervicovaginal fluid (CVF). Ectocervical tenofovir diphosphate (TFVdp), lamivudine triphosphate (3TCtp), and deoxyadenosine triphosphate (dATP)/deoxycytidine triphosphate (dCTP) concentrations and immune marker/DMET gene expression were measured in cervical biopsies.

Results. Cervical 3TCtp was not correlated with any CVF cytokines. Cervical TFVdp was correlated with IL-10, IL-7, and IL-17 in CVF. CCR5 mRNA expression in cervical biopsies was higher in cuIUD users versus condom users. Using multivariable linear regression, CVF IL-17, tissue dATP, plasma estradiol, and plasma tenofovir were all significant predictors of cervical TFVdp. Tissue dCTP and plasma lamivudine were significant predictors of cervical 3TCtp.

Conclusions. TFVdp concentrations in cervix appear to be influenced by local inflammation. In contrast, 3TCtp FGT exposure was not affected by genital inflammation or DMETs. CuIUD users have more immune cells present, which may in turn influence local TFVdp disposition.

Main Finding. We investigated changes in tenofovir diphosphate and lamivudine triphosphate due to the microbiome and inflammation. While lamivudine triphosphate was not affected by either, tenofovir diphosphate appeared to be affected by local inflammation. Specifically, Th17 cells may influence tenofovir disposition.

Keywords. tenofovir; lamivudine; contraception; inflammation; female genital tract.

Cisgender women make up half of people with human immunodeficiency virus (HIV) globally and yet the factors that regulate drug efficacy in the female genital tract (FGT) are understudied, which has implications for antiretrovirals used as preexposure prophylaxis (PrEP) and treatment (ie, reduce viral shedding in women with HIV). Modeling studies have shown minimal pharmacologic forgiveness in the FGT with 6–7 doses a week required to reach protective concentrations [1]. It is critical to understand factors affecting antiretroviral disposition in the FGT to optimize dosing strategies and advance development of new prevention options.

Mucosal antiretroviral pharmacology is complex and involves many components of the FGT microenvironment, which may play a role in efficacy of PrEP. Some of these components include vaginal microbiota, inflammation, and drug metabolizing enzymes and transporters (DMETs). In addition, both endogenous and exogenous hormones regulate the FGT microenvironment and may play a role in efficacy.

A healthy vaginal microbiome is mainly comprised of *Lactobacillus* species that release lactic acid to keep the pH low [2]. Vaginal dysbiosis, clinically known as bacterial vaginosis, is when there is an increase in microbiota diversity. Bacterial vaginosis in women of reproductive age is associated with the presence of different genera including *Prevotella*, *Sneathia*, *Dialister*, *Megasphaera*, and *Gardnerella* [3]. Vaginal dysbiosis has been associated with a decrease in efficacy of topical tenofovir. In a retrospective analysis of Centre for the AIDS Programme of Research in South Africa 004, women with a *Lactobacillus*-dominated vaginal microbiota had 3-fold higher protection from HIV acquisition using 1% tenofovir gel than women with non-*Lactobacillus*-dominated vaginal microbiota [4]. There is less known how the vaginal microbiome

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affects antiretroviral exposure in the FGT. Carlson et al showed that microbiome community types with intermediate diversity had the highest concentration of tenofovir in cervicovaginal fluid, but tenofovir concentrations were similar between high-diversity community types and low-diversity community types [5]. One potential mechanism for decreased tenofovir diphosphate (TFVdp) is that when the diversity of the vaginal microbiome increases, there is a probability of increased abundance of proinflammatory taxa, which leads to an increase in genital inflammation. Indeed, McKinnon et al found the efficacy of 1% tenofovir gel was significantly reduced in the presence of genital inflammation, even in participants with high adherence (>50%) [6].

While the Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial showed no increased HIV risk with 3 methods of contraception, a substudy showed an association between copper intrauterine device (IUD), increased bacterial diversity, and increased inflammation after 6 months of use [7, 8]. We previously reported, in a observational pharmacokinetic study in 50 Ugandan women with HIV on an antiretroviral regimen containing tenofovir, lamivudine, and efavirenz, that copper IUD users had lower TFVdp concentrations compared to intramuscular depo-medroxyprogesterone acetate (DMPA-IM) or condom users [9]. We hypothesize that copper IUDs may alter the microbiome and thus create an inflammatory environment in the FGT, causing low TFVdp concentrations. Therefore, we sought to further explore how different components in the FGT influence mucosal pharmacology of TFVdp, lamivudine triphosphate (3TCtp), and the endogenous nucleotides deoxyadenosine triphosphate (dATP) and deoxycytidine triphosphate (dCTP). We conducted an analysis on secondary endpoints, examining the effect of the vaginal microbiome and genital inflammation on the active metabolites in cervical tissues by investigating the presence and quantity of cytokines, drug metabolizing enzymes, transporters, and vaginal microbiota in the FGT.

METHODS

Study Population

The study procedures have been previously described and primary results published [9]. The Uganda Virus Research Institute, the Human Research Ethics Committee of the University of the Witwatersrand, and the Uganda National Council of Science and Technology reviewed and approved the procedures for the study (NCT03377608). The study population was a subset of women who participated in the BONE: Contraception and Anti-Retroviral Effects (BONE- CARE) study [10]. All women were on an antiretroviral therapy regimen that consisted of tenofovir disoproxil fumarate (TDF), lamivudine, and efavirenz. Women who were enrolled in the BONE-CARE study, stable on their TDF-containing regimen, and virally suppressed (plasma HIV RNA <50 copies/mL) for ≥6 months were offered participation in this substudy. Exclusion criteria included pregnancy, breastfeeding, symptomatic vaginal infection, abnormal vaginal bleeding, history of genital dysplasia or human papillomavirus, or use of oral/vaginal antibiotics or antifungals within 30 days (exception made for sulfamethoxazole-trimethoprim). Written informed consent was obtained prior to study participation [9].

Sample Collection

All samples were collected at a single visit and are detailed previously [9]. Relevant to this analysis, 1 vaginal swab for microbiome sequencing and 1 cervical swab for cytokine quantification were collected using polyester swabs. Two cervical biopsies were collected using Baby Tischler Biopsy forceps (McKesson) and blood was collected in ethylenediaminetetraacetic acid tubes.

Cytokine Quantification

Cytokine extraction from cervicovaginal swabs was performed similar to methods previously described [11]. Cervical swabs were centrifuged in 500 µL of phosphate-buffered saline (PBS) and then scraped on the side of the tube. Concentrations of 27 cytokines were measured using the 27-plex Human Cytokine Assay (Bio-Rad) and the MAGPIX Assay Reader. The 27-plex Human Cytokine Assay was chosen based previous literature and the targets being relevant to HIV [8, 12, 13].

Antiretroviral Quantification

A detailed description of antiretroviral quantification was previously published [9]. In brief, tenofovir and lamivudine were quantified in blood using a validated assay that had a lower limit of 1 ng/mL. TFVdp, 3TCtp, dATP, and dCTP were quantified in cervical tissue with a dynamic range of 0.02–20 ng/mL and peripheral blood mononuclear cells (PBMCs), with a dynamic range of 0.02–100 ng/mL for dATP and TFV-DP, 0.2–600 ng/mL for 3TCtp, and 0.06–100 ng/mL for dCTP.

Microbiome Sequencing

The vaginal microbiome was analyzed using 16S sequencing as described previously [9]. In brief, DNA was extracted from vaginal swabs using the DNeasy Powersoil Kit. The V4 hypervariable region of the 16S ribosomal RNA gene was used for amplification.

Western Blot Analysis

Nine of the 50 snap-frozen cervical biopsies were used for protein expression with Western blot, while the remaining 41 were used for gene expression. Cervical biopsies for Western blot were chosen based on TFVdp concentrations, selecting samples with low, medium, and high cervical TFVdp. Human liver was collected postmortem as previously described [14] and was used as a positive control. Total proteins from snap-frozen cervical tissue biopsies and liver were lysed using radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific). A Bradford assay (Sigma) was used to quantify total protein concentration. Forty micrograms of protein was separated using

10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. Membranes were incubated overnight with RAR-related orphan receptor gamma (ROR-γ) (Bioss, 1:500, BS-6217R), CD4 (Sigma-Aldrich, 1:500, 104R-1), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Bioss, 1:5000, BS-10900R) antibodies. Membranes were then incubated with horseradish peroxidase–conjugated secondary antibody (Prometheus, 1:4000) for 1 hour. Protein bands were visualized using Western Lighting Plus-ECL reagents and imaged on iBright imaging system (Thermo Fisher Scientific).

CCR5, CD4, AK2, and MRP4 Expression

Total RNA was extracted from snap-frozen cervical biopsies using the RNeasy Fibrous Tissue Kit (Qiagen). Tissues were homogenized in lysis buffer and underwent a 10-minute digestion in Proteinase K prior to RNA isolation and purification. RNA was quantified and tested for purity using a NanoDrop. First-strand cDNA synthesis was performed from total RNA using Superscript1 Vilo IV cDNA Synthesis kit (Life Technologies, Grand Island, New York) and messenger RNA (mRNA) expression was quantified using quantitative polymerase chain reaction (qPCR) with TaqMan Gene Expression Assays. Genes were normalized to GAPDH and relative expression was calculated as $2^{-\Delta Ct}$. CCR5 and CD4 genes were chosen because $CD4^+$ T cells are the target cells of HIV and CCR5 is a coreceptor that facilitates entry into the cell for R5 tropic variants, the most common variant to be transmitted through sexual contact [15]. AK2 is a kinase that phosphorylates TFVdp, and we wanted to explore if the copper IUD decreased AK2 expression, thus lowering TFVdp concentrations compared to DMPA-IM and condom users. MRP4 is a transporter that has been implicated in tenofovir disposition [16, 17].

Statistical Analysis

Kruskal-Wallis tests were performed to test differences in gene expression between DMPA-IM users, copper IUD users, and condom users. Differences in cytokine concentrations between DMPA-IM users, copper IUD users, and condom users were tested by Dunn test. All statistical comparisons were corrected for multiple comparisons using the Bonferroni method. Correlations with drug active metabolites, endogenous nucleotides, and the ratio of metabolite to related nucleotide were assessed using Spearman correlations, specifically looking at correlations with cytokines, bacterial relative abundance, and gene expression. Bacteria taxa with >1% relative abundance in vaginal swabs, which includes *Lactobacillus*, *Prevotella*, *Dialister*, *Sneathia*, *Gardnerella*, and *Megasphaera* were included in the analysis. Associations between antiretrovirals and the microbiome have already been reported [9]. Here, linear regression was used to identify interactions between cytokines and the microbiome when predicting antiretroviral concentrations.

Western blot results for ROR-γ and CD4 were quantified using Image J software. To further investigate our hypothesis of the involvement of Th17 cells, we compared ROR-γ and CD4 expression in tissue by TFVdp cervical concentrations separated into tertiles: T1, 1544–10 825 fmol/g; T2, 10 286–18 175 fmol/g; and T3, ≥18 176 fmol/g. Spearman correlations were used to compare interleukin (IL) 17 and CD4/ROR-γ.

Stepwise regression in both directions was performed to identify predictors of TFVdp and 3TCtp in cervical tissue. Covariates tested in the models were age, CD4 cervical expression, cervicovaginal IL-17, CCR5 cervical expression, progesterone, estradiol, respective parent drug in plasma, and respective nucleotide in tissue. Since all cytokines measured were correlated, only 1 cytokine was included in the regression model, which was determined by the best fit in single linear regression model with cervical TFVdp. All models were tested using model diagnostics and the final model was the best fit. All statistical analyses were performed in R Studio.

RESULTS

Participant Characteristics

Supplementary Table 1 describes the participant characteristics for the exploratory analysis. Fifty women with HIV were enrolled for this study. Twenty-five women were on DMPA-IM, 12 had a copper IUD, and 13 were using condoms for primary contraceptive methods. The mean age was 26.2 years (range, 23.9–30.7 years). Two women using nonhormonal contraception and 2 women using DMPA-IM tested positive for gonorrhea. Three women using nonhormonal contraception tested positive for syphilis.

Cervical Cytokine Concentrations and Relative Bacterial Abundance by Contraception Method

Condom users had 65% higher granulocyte macrophage–colony-stimulating factor than DMPA-IM ($P = .023$) and it was 47% higher than copper IUD users ($P = .033$). RANTES (CCR5-binding chemokine) was 113% lower in copper IUD users compared to DMPA-IM users but was not significant ($P = .06$). While RANTES was 62% lower in copper IUD users compared to condom users, it was not significant ($P = .33$). The rest of the cytokines measured were not significantly different between contraceptive methods.

Associations Between Cytokines, Antiretrovirals, and the Microbiome

3TCtp was not correlated with any cytokines measured. TFVdp was positively correlated with 3 of the 27 cytokines. Specifically, TFVdp was positively correlated with IL-7 ($R = 0.35$, $P = .016$), IL-10 ($R = 0.36$, $P = .01$), and IL-17 ($R = 0.48$, $P = .00075$) (Figure1*A*–*C*).

Dialister, *Megasphaera*, *and Prevotella* were negatively correlated with TFVdp:dATP ratio (Table 1). *Prevotella* was positively correlated with dATP in tissue, but not correlated with

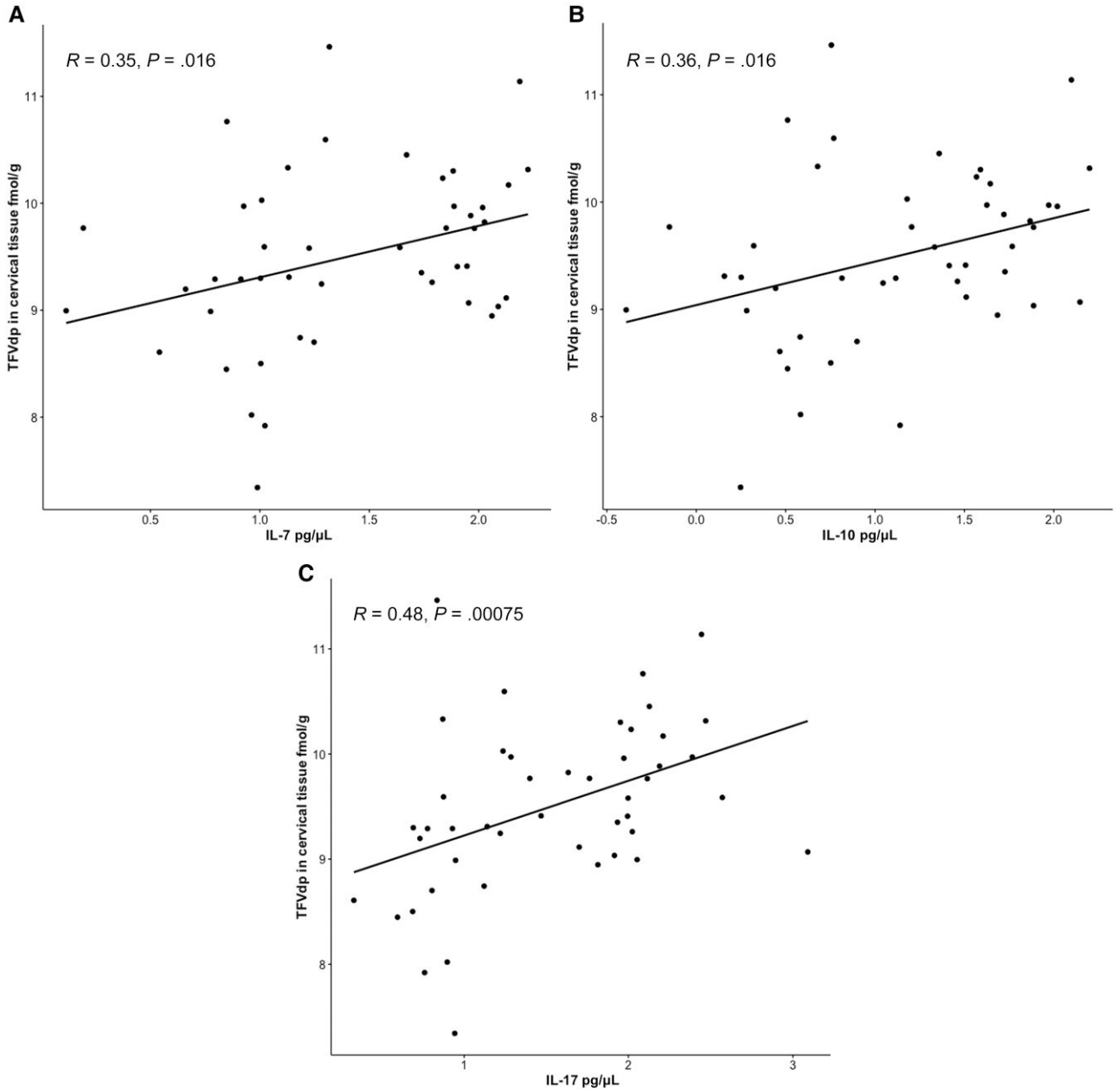

Figure 1. Cytokines were measured in cervicovaginal fluid and tenofovir diphosphate (TFVdp) was measured in cervical biopsies. Correlations between cervical TFVdp concentrations (fmol/g) and all 27 cytokines measured were analyzed using Spearman correlations. Of the 27 measured, only interleukin 7 (IL-7; *A*), interleukin 10 (IL-10; *B*), and interleukin 17 (IL-17; *C*) were significantly correlated with TFVdp.

TFVdp. *Dialister* trended to have a positive correlation with dATP in cervical tissue, but it was not significant ($R = 0.25$, $P = .092$). *Mycoplasma* was negatively correlated with 3TCtp: dCTP ratio ($R = 0.35$; $P = .017$). Both cervical concentrations of 3TCtp and dCTP separately were positively correlated with *Lactobacillus* ($P = .032$, $P = .044$, respectively; Table 2).

Two of the 6 most prevalent bacteria were correlated with proinflammatory cytokines (Table 3). *Sneathia* was positively correlated with 4 proinflammatory cytokines: IL-1β, tumor necrosis factor alpha (TNF-α), IL-8, and IL-17. *Lactobacillus* was negatively correlated with IL-1β and TNF-α. *Shuttleworthia* was positively correlated with IL-1β. *Gardnerella*, *Prevotella*, and *Megasphaera* were not correlated with any of the 27 cytokines measured.

Gene Expression and ROR-γ in Cervical Tissues

Women on copper IUDs had significantly higher gene expression of CCR5 (Figure 2*B*) and AK2 (Figure 2*C*) compared to

Table 1. Correlations Between Cervical Tenofovir Diphosphate, Deoxyadenosine Triphosphate, Their Ratio, and Bacteria Genera Measured Using 16S Microbiome Sequencing

Bacteria	*R*	*P* Value
TFVdp:dATP ratio in ectocervix		
Dialister	−0.4	.0063**
Prevotella	−0.34	.021*
Megasphaera	−0.39	.0076**
Sneathia	−0.13	.38
Mycoplasma	0.15	.39
Gardnerella	−0.069	.65
Lactobacillus	0.16	.27
dATP in ectocervix		
Dialister	0.22	.13
Prevotella	0.3	.04*
Megasphaera	0.075	.62
Sneathia	0.027	.86
Mycoplasma	0.15	.32
Gardnerella	0.023	.88
Lactobacillus	0.073	.63
TFVdp in ectocervix		
Dialister	−0.16	.28
Prevotella	−0.1	.5
Megasphaera	−0.28	.056
Sneathia	−0.02	.89
Mycoplasma	0.0033	.83
Gardnerella	0.011	.94
Lactobacillus	0.11	.45

Abbreviations: dATP, endogenous deoxyadenosine triphosphate; TFVdp, tenofovir diphosphate.

*$P < .05$.

**$P < .01$.

Table 2. Correlations Between Cervical Lamivudine Triphosphate, Deoxycytidine Triphosphate, Their Ratio, and Bacteria Genera Measured Through 16S Microbiome Sequencing

Bacteria	*R*	*P* Value
3TCtp:dCTP ratio in ectocervix		
Dialister	−0.25	.09
Prevotella	−0.25	.09
Megasphaera	−0.12	.42
Sneathia	−0.28	.06
Mycoplasma	−0.35	.017*
Gardnerella	−0.12	.44
Lactobacillus	0.25	.098
dCTP in ectocervix		
Dialister	−0.065	.67
Prevotella	−0.004	.98
Megasphaera	−0.11	.45
Sneathia	−0.1	.5
Mycoplasma	0.0058	.97
Gardnerella	−0.021	.89
Lactobacillus	0.3	.044*
3TCtp in ectocervix		
Dialister	−0.13	.41
Prevotella	−0.12	.41
Megasphaera	−0.059	.7
Sneathia	−0.13	.39
Mycoplasma	−0.11	.45
Gardnerella	0.005	.97
Lactobacillus	0.32	.032*

Abbreviations: 3TCtp, lamivudine triphosphate; dCTP, endogenous deoxycytidine triphosphate.

*$P < .05$.

condom users, with CD4 trending to be higher although not significant (Figure 2*A*). There was no differences in relative mRNA for CD4, CCR5, AK2, or MRP4 between women on DMPA-IM and copper IUD users. There were no correlations between the 4 genes measured and TFVdp, 3TCtp, dATP, dCTP, and any cytokines measured.

ROR-γ and CD4 were quantified in 9 cervical biopsies using Western blot to confirm Th17 cell presence in cervical tissue. Neither CD4 nor ROR-γ was correlated with IL-17 ($R = -0.37$, $P = .34$ and $R = -0.067$, $P = .067$, respectively). Both ROR-γ and CD4 had the highest mean expression in TFVdp tertile 1, which contrasts with our hypothesis that ROR-γ and CD4 would be higher in tertile 3 (Figure 3*A* and 3*B*).

Stepwise Regression

Table 4 includes the data from stepwise regression for both TFVdp and 3TCtp. Cervical TFVdp was significantly predicted by IL-17 ($P < .01$), dATP in cervical tissue ($P < .05$), tenofovir in plasma ($P < .05$), and estradiol ($P < .05$). Cervical 3TCtp was predicted by dCTP in cervical tissue ($P < .01$) and lamivudine in plasma ($P < .01$). Other covariates tested were age, CD4 expression, MRP4 expression, and progesterone.

Table 3. Significant Spearman Correlations Between Genera of Vaginal Microbiome and Cytokines Measured in Cervicovaginal Fluid

Cytokine	*R*	*P* Value
Sneathia		
IL-1β	0.31	.037
TNF-α	0.35	.018
IL-8	0.38	.0088
IL-17	0.33	.024
Lactobacillus		
IL-1β	−0.39	.0078
TNF-α	−0.31	.036

Abbreviations: IL-1β, interleukin 1-beta; IL-8, interleukin 8; IL-17, interleukin 17; TNF-α, tumor necrosis factor alpha.

DISCUSSION

Understanding how the genital tract environment affects antiretroviral concentrations in mucosal tissues is critical for improving antiretroviral efficacy in the FGT. One objective of this study was to further explore the observation that copper IUD users had the highest concentration of cervical TFVdp, which could be due to alterations in the inflammatory milieu or the vaginal microbiome. We did not find an increase in

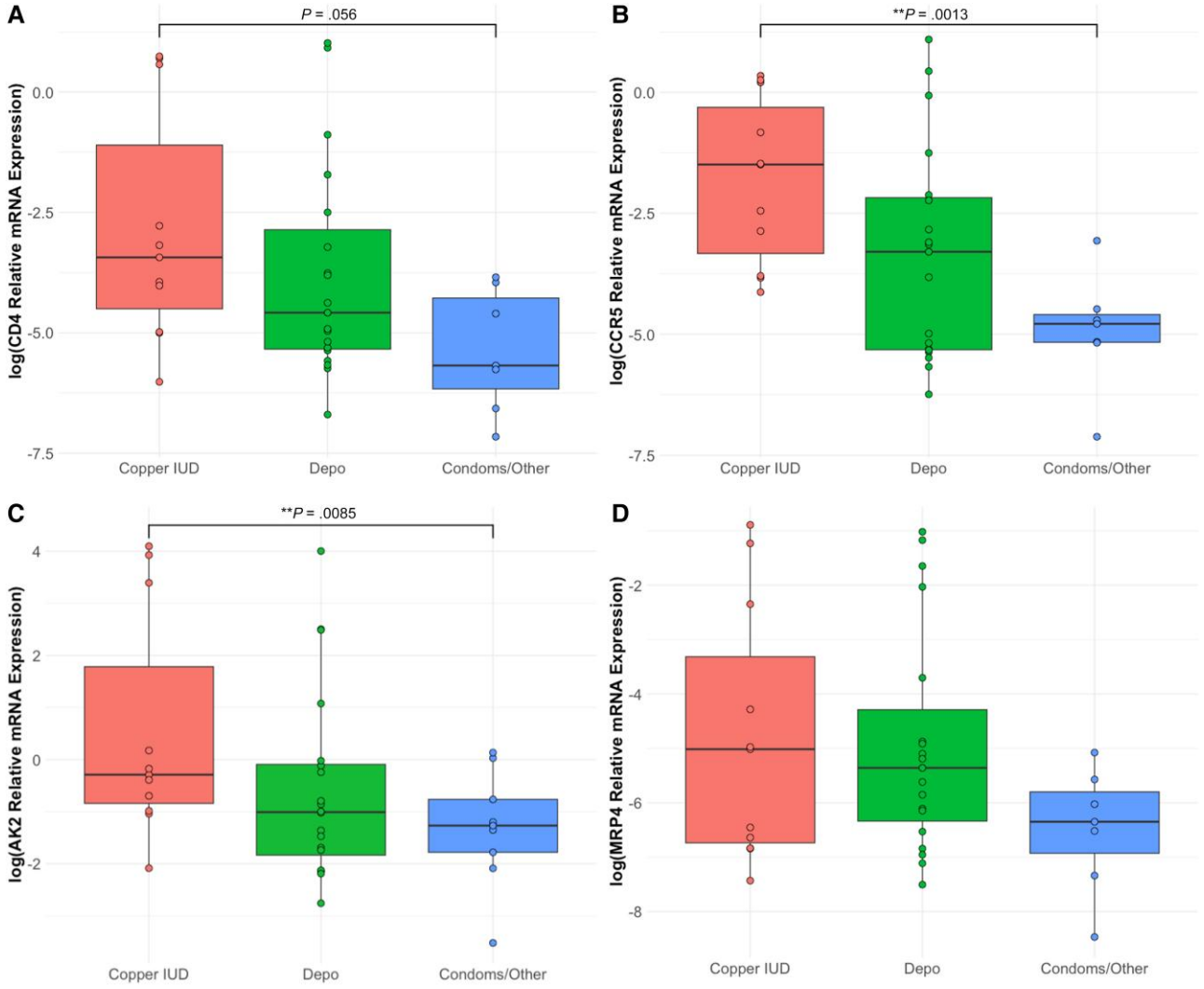

Figure 2. RNA was extracted from cervical biopsies and gene expression was quantified using quantitative polymerase chain reaction. CD4 (*A*), CCR5 (*B*), AK2 (*C*), and MRP4 (*D*) were normalized to glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene, and Kruskal-Wallis tests were run to compare expression between 3 contraception methods: copper intrauterine device (IUD; most left plot), intramuscular depot medroxyprogesterone acetate (Depo; middle plot), and condoms (most right plot). $^{**}P < .01$.

cytokine concentrations for copper IUD users compared to DMPA-IM or condom users, which contrasts with findings from the ECHO trial [8], where the copper IUD was associated with increased cytokine concentrations, including IL-1β, IL-2, IL-17, and TNF-α. However, mean duration with copper IUD in our study was 29.6 months (range, 2–84 months), which is longer than the 6 months in the ECHO trial substudy [7]. It is possible that the difference in cytokine concentrations seen in this study is due to having more time to heal from epithelial damage from IUD insertion, thus decreasing the immune response to the IUD. Furthermore, this was not a longitudinal study, so we were unable to see changes over time in cytokine concentrations. It is also possible that a change in pH because of the copper IUD alters tenofovir disposition as it is known that tenofovir uptake into cells is decreased as pH increases [18]. Further research into the mechanisms related to lower TFVdp in women with copper IUD is needed.

We saw higher AK2 expression in women on copper IUDs compared to women using condoms. This was unexpected given copper IUD users had lower TFVdp compared to women on DMPA-IM or using condoms. Several kinases, including AK2, are critical for tenofovir to become pharmacologically active in PBMCs and vaginal tissue [19]. It is possible that other kinases including pyruvate kinase isozymes (muscle or liver and red blood cell) are decreased instead of AK2. Additionally, we only measured gene expression, not protein, so it is possible that there is lower AK2 protein.

Cervical TFVdp was positively correlated with IL-7, IL-10, and IL-17. One previous study found that incubation of IL-7 with cervical explants followed by HIV challenge had a higher

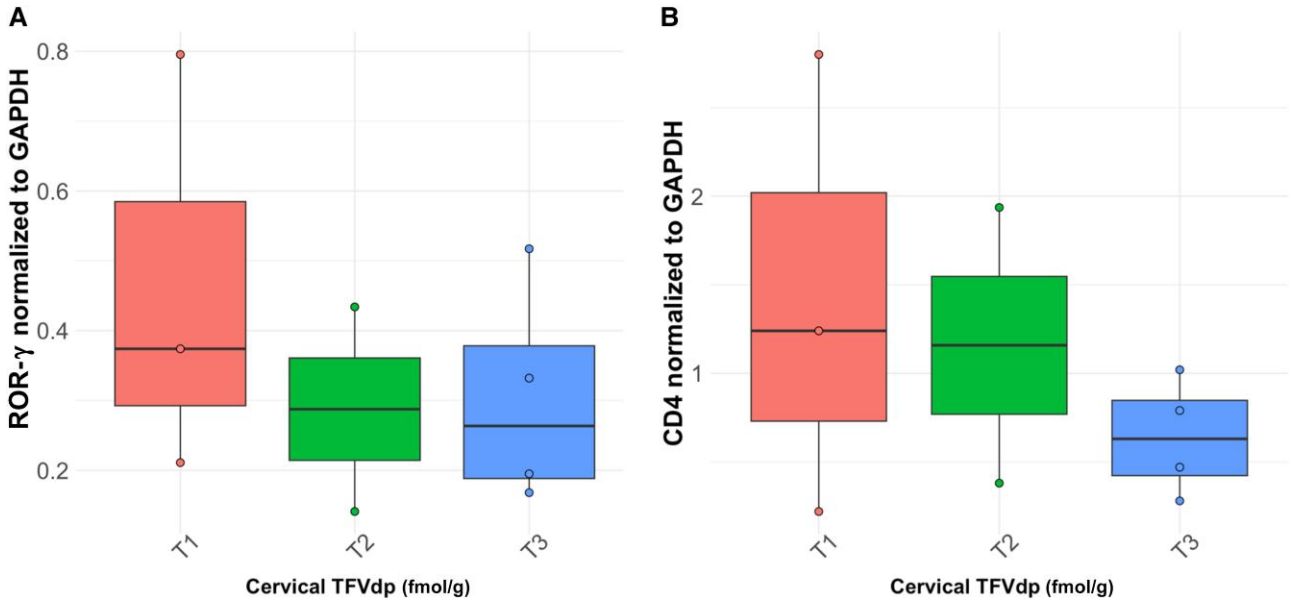

Figure 3. Nine of 50 cervical biopsies were homogenized in radioimmunoprecipitation assay buffer, and expression of RAR-related orphan receptor gamma (*A*) and CD4 (*B*) was measured using Western blot. Expression was graphed by tenofovir diphosphate concentration tertiles. The 3 tertiles were divided as follows: T1 (1544–10 285 fmol/g), T2 (10 286–18 175 fmol/g), and T3 (≥18 176 fmol/g). No statistical analyses were done due to the small sample size. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ROR-γ, RAR-related orphan receptor gamma; TFVdp, tenofovir diphosphate.

Table 4. Final Stepwise Regression Model for Predicting Tenofovir Diphosphate and Lamivudine Triphosphate in Cervical Tissue

Covariate	Estimate	Standard Error
TFVdp		
Log(dATP in cervical tissue)*	0.417	0.158
Log(IL-17)**	0.262	0.061
Estradiol*	−0.002	0.001
Age	−0.017	0.01
Log(TFV in plasma)*	0.36	0.136
3TCtp		
Log(dCTP in cervical tissue)**	0.799	0.1
Log(3TC in plasma)**	0.34	0.141

Significant: *$P < .05$, **$P < .01$.

Abbreviations: 3TC, lamivudine; 3TCtp, lamivudine triphosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; IL-17, interleukin 17; TFV, tenofovir; TFVdp, tenofovir diphosphate.

number of CD4 cells in the tissue than without IL-7 present, suggesting that IL-7 prevents CD4 apoptosis and induced CD4 proliferation [20]. One possible mechanism for IL-7 being correlated with TFVdp is an increased number of CD4 cells in cervical tissue that phosphorylate tenofovir. A higher number of CD4 T cells present in cervix could lead to a higher production of TFVdp.

In contrast, cervical 3TCtp was not correlated with any of the 27 cytokines measured, nor was it correlated with any of the genes measured, although AK2 and MRP4 were not expected to be associated with 3TC disposition. Cervical 3TCtp was positively correlated with *Lactobacillus*, but not correlated with any of the other bacterial genera measured. *Lactobacillus* dominates a "normal," less diverse microbiome and is associated with a healthy microbiome environment [3]. This suggests that lamivudine distribution is not negatively affected by genital inflammation, nonoptimal vaginal microbiota, or DMETs in the FGT.

We also showed that IL-17 was a significant predictor of cervical TFVdp in a stepwise regression model. One of the major T-cell subsets in the FGT is T-helper type 17 (Th17) cells. These cells are produced for an immune response against extracellular bacteria/fungus and help maintain mucosal barrier integrity [21, 22]. ROR-γ is a main transcription factor in this cell population, and we hypothesized that Th17 cells were the reason for high TFVdp [23]. However, in our samples, expression of ROR-γ was highest when TFVdp concentrations were in T1 and ROR-γ was not correlated with IL-17. This suggests that Th17 cells are not playing a role in the distribution of TFVdp and that other immune cells may be involved.

Th17 cells activation and expression are influenced by many factors though, including DMPA-IM initiation and the microbiome [24, 25]. DMPA-IM has been shown to increase Th17 cell frequency in cervical tissue, but this has only been looked at 1 month postinitiation. In addition, we showed that the *Sneathia* genus was positively correlated with IL-17 and *Lactobacillus* negatively correlated with IL-1β and TNF-α, suggesting an increase in an immune response to increased diversity and proinflammatory bacteria. We did not see a correlation between Th17 and AK2 and we did not measure ROR-γ and AK2 in the same participants as separate biopsies were selected

for protein versus expression. Other innate immune cells can also produce IL-17, including innate lymphoid cells and natural killer cells [26]. It is possible that other cells are responsible for elevated IL-17 in our population. These data suggest that immune cells may be involved either in tissue distribution of TFVdp or conversion of tenofovir to its active metabolite due to an increased immune response to proinflammatory bacteria, but future confirmatory studies are needed to identify cell type(s) that are involved.

Estradiol was also a significant predictor of cervical tenofovir in plasma. This contradicts with our previous findings [9]. However, it was a weak association (coefficient = −0.002) and while it was statistically significant, it may not be clinically relevant. Moreover, the addition of cytokines, specifically IL-17, as a covariate in the stepwise regression could also alter the results of other covariates. Finally, the *Prevotella* genus was negatively correlated with TFVdp:dATP ratio and positively correlated with dATP cervical tissue concentrations (Table 1). This suggests that *Prevotella* genus may be increasing endogenous dATP, thus decreasing tenofovir efficacy. Both 3TCtp and dCTP were positively correlated with *Lactobacillus* genus, but *Lactobacillus* was not correlated with the 3TCtp:dCTP ratio (Table 2). This may suggest that while dCTP is increasing, it does not affect lamivudine efficacy.

The results of this study are limited by a small sample size, especially for subanalyses. This study only had 1 point in time, so further investigations of how these findings translate over time are needed. We only measured ROR-γ and CD4 protein expression in a small subset of our participants (9/50) to elucidate if the correlation between TFVdp and IL-17 was due to the presence of Th17 cells. In addition, the ROR-γ antibody does not distinguish between isoforms.

The study population was women with HIV. Differences in vaginal microbiota between women with and without HIV may limit the translatability for prevention in the HIV-negative population. The community state type of the vaginal microbiome that has anaerobic bacteria associated with bacterial vaginosis is believed to be more prevalent in women with HIV but does not seem to be statistically different [27–29]. In addition, it is possible that TFVdp tissue pharmacokinetics in the FGT is different between women with and those without HIV. However, the median peak tenofovir concentrations in plasma after 15 days in women with and women without HIV were 306 and 357 ng/mL, respectively [30, 31]. This suggests that there seems to be no difference by HIV status, but further work to evaluate the hypotheses generated from this study in women without HIV is needed.

In conclusion, 3TCtp is likely not affected by the local microenvironment of the FGT. TFVdp tissue concentrations, on the other hand, seem to be regulated by multiple components of the FGT. Most importantly, immune cells may play a role in tenofovir tissue distribution and phosphorylation, although we did not find a correlation between Th17 cells and AK2. These data will ultimately help develop hypotheses to improve antiretroviral therapies targeted to FGT mucosa. In addition, these data highlight the importance of the microenvironment in tenofovir distribution. This highlights the importance of involving the whole system in further studies of tenofovir for PrEP, either in a preclinical animal model or an ex vivo tissue model. This will further our understanding of the complex mucosal pharmacology in the FGT and accelerate the development of future prevention options.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Author contributions. Sequence processing and analysis was done using the resources of the Minnesota Supercomputing Institute. Drug concentrations were measured at the University of North Carolina Center for AIDS Research Clinical Pharmacology and Analytical Chemistry Laboratory. F. K. M. and M. R. N. designed research. A. L., R. J., E. I., R. N., S. K., and M. E. B. performed research. A. L. did data analysis and wrote the manuscript. All authors participated in editing of the manuscript.

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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