**Distribution Agreement**

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Renee Donahue Carlson Date

Microbiome Composition and Female Genital Tract Compartmental Antiretroviral Drug Exposure in HIV-Infected Women

By

Renee Donahue Carlson

Doctor of Medicine

Clinical Research

_____

Igho Ofotokun, M.D., M.Sc.

Advisor

_____

Mitch Klein, Ph.D.

Committee Member

_____

Muna Qayed, M.D., M.Sc.

Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.

Dean of the James T. Laney School of Graduate Studies

_____

Date

# Microbiome Composition and Female Genital Tract Compartmental Antiretroviral Drug Exposure in HIV-Infected Women

By

Renee Donahue Carlson

M.D., University of Minnesota, 2010

B.A., Gustavus Adolphus College, 2005

Advisor: Igho Ofotokun, M.D., M.Sc.

An abstract of

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

in Clinical Research

2016

ABSTRACT

Microbiome Composition and Female Genital Tract Compartmental Antiretroviral Drug Exposure in HIV-Infected Women
By Renee Donahue Carlson, M.D.

***Background:***
Optimal female genital tract (FGT) antiretroviral exposure is essential for genital virologic suppression and relevant to HIV prevention methods utilizing antiretroviral therapy in women. Changes in FGT microbiota are common and occur with bacterial vaginosis (BV), sexually transmitted infections (STIs), and certain hygienic practices, and may alter genital pH and other factors that influence compartmental drug penetration. We therefore characterized the FGT microbiome serially over a menstrual cycle in HIV-infected women and examined the relationship between FGT microbiota and antiretroviral concentrations.

***Methods:***
Virologically suppressed HIV-infected women on tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) and atazanavir/ritonavir (ATV/RTV) without clinical BV/STIs were prospectively enrolled. Twenty participants underwent 6 twice-weekly visits (N=117) over one menstrual cycle where paired samples for plasma and cervicovaginal trough antiretroviral concentrations, and cervicovaginal lavage (CVL) were collected. Antiretroviral concentrations were measured using high-performance liquid chromatography-tandem mass spectroscopy. Illumina MiSeq 16S rRNA gene sequencing of CVL samples, with analysis using Dirichlet Multinomial Mixtures, clustered each participant-visit into a unique microbiome community type (CT) based on similar bacterial taxa abundances. Generalized mixed models were used to evaluate predictors of microbiome CT and to estimate the association between CT and FGT antiretroviral concentrations controlling for significant predictors.

***Results:***
Participants were 95% African American with median age 38 (range, 24-48) years. High-quality sequencing data (N=109) lead to 3 unique microbiome CTs: a low-diversity CT dominated by *Lactobacillus* (N=40), and intermediate- (N=28) and high-diversity (N=41) CTs with increased abundance of multiple anaerobic taxa. In multivariable models, controlling for plasma antiretroviral concentrations, body mass index, recent sexual activity and CVL blood contamination, low- and high-diversity CTs were associated with 52% (95% Confidence Interval (CI), 31-88%) and 46% (CI, 23-92%) of the ATV concentrations (P=0.03), and 51% (CI, 28-91%) and 54% (CI, 23-127%) of the TDF concentrations (P=0.06) in the FGT, respectively, compared to intermediate-diversity CTs. FTC concentrations were not significantly associated with CT (P=0.27).

***Conclusions:***
We demonstrate in this proof-of-concept study that certain microbiome CTs are associated with decreased FGT antiretroviral drug concentrations. Validation of these findings in larger studies and with additional antiretrovirals could influence antiretroviral drug choice for biomedical HIV prevention in women.

# Microbiome Composition and Female Genital Tract Compartmental Antiretroviral Drug Exposure in HIV-Infected Women

By

Renee Donahue Carlson

M.D., University of Minnesota, 2010

B.A., Gustavus Adolphus College, 2005

Advisor: Igho Ofotokun, M.D., M.Sc.

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

in Clinical Research

2016

ACKNOWLEDGEMENTS

I thank my mentor, Dr. Igho Ofotokun for his extraordinary dedication to teaching and mentoring me during the course of my research. I also thank my co-mentor, Dr. Anandi Sheth, for providing additional mentorship and expertise, as well as access to clinical samples. I additionally thank collaborators, Dr. Timothy Read, Michael Frisch, Dr. Christina Mehta and Dr. Colleen Kraft for their contributions to this project. I also thank the additional members of my Infectious Disease Fellowship Mentorship Committee, Dr. Wendy Armstrong, Dr. Elizabeth Corwin, Dr. Lisa Haddad and Dr. Jeffrey Lennox, for their feedback on this work. Thank you to the Emory University Department of Medicine, Division of Infectious Diseases for supporting this work. Furthermore, this work was supported in part by the Bristol-Myers Squibb 2014-2015 Virology Fellows Program, the Emory Center for AIDS Research (NIAID P30AI050409), the Atlanta Clinical Research Site of the Women's Interagency HIV Study (NIAID U01A1103408-01) and the Atlanta Clinical and Translational Science Institute (NCCR KL2TR000455 and UL1TR000454).

TABLE OF CONTENTS

# INTRODUCTION

Women comprise 16 million of the 35 million HIV-infected individuals worldwide (1), and efforts directed at understanding HIV transmission among women could have a major impact on the global HIV/AIDS epidemic. Antiretroviral therapy decreases mother-to-child and sexual transmission of HIV and is associated with suppression of HIV-1 viral shedding in blood and genital secretions (2-4). Since genital tract HIV-1 RNA levels predict HIV transmission (5), optimizing antiretroviral therapy to achieve adequate female genital tract (FGT) concentrations resulting in complete viral suppression is paramount for multiple prevention strategies that use ART. However, differences in FGT antiretroviral concentrations relative to plasma concentrations have been reported for several antiretroviral drugs (6-9). Compartmental drug penetration is generally known to be influenced by a number of factors including a drug's physicochemical properties such as its affinity for membrane transporters, degree of protein binding, lipophilicity, and its acid dissociation constant (pKa), though, the specific etiology for variability in FGT compartmental exposure of antiretroviral drugs has not been fully elucidated.

The FGT microenvironment can be highly dynamic between- and within- women, owing to alterations in the distribution and types of bacterial species colonizing the FGT, termed the microbiome. *Lactobacillus* species are typically thought to dominate the FGT microbiome in normal women, secreting lactic acid and maintaining an acidic pH. However, nearly 30% of asymptomatic, healthy U.S. women (10, 11), and over 50% of non-Hispanic African American women(11) have increased microbiome community diversity, increased pH and gram stain characteristics consistent with the condition, bacterial vaginosis (BV). BV is associated with an increased risk of both HIV acquisition (12, 13) and FGT HIV shedding (14-16), which in turn has been associated with HIV transmission (5). Fluctuations in the FGT microbiome, such as toward BV, have the potential to impact local pH and other characteristics in the vaginal microenvironment, which theoretically could impact compartmental penetration of antiretroviral or other drugs.

Though the specific impact of vaginal microbiome fluctuations on FGT compartmental antiretroviral drug penetration has not been described, because of the potential for compartmental microbiome composition to influence the local pH and other factors that may affect drug disposition across membranes, we hypothesize that antiretroviral drug concentrations in the FGT vary by FGT microbiome community type. Thus, the aims of this study are to 1) describe the FGT microbiome composition in HIV-infected women on antiretroviral therapy over a menstrual cycle, evaluating for predictive factors including demographic and behavioral factors, menstrual cycle phase and female sex hormones, and 2) to determine if the female genital tract microbiome composition is associated with FGT antiretroviral drug concentrations for the antiretroviral drugs tenofovir disoproxil fumarate (TDF), emtricitabine (FTC) and ritonavir (RTV)-boosted atazanavir (ATV).

To assess this hypothesis, we carried out a prospective, longitudinal cohort study of 20 HIV-infected adult women with regular menstrual cycles and plasma HIV viral suppression who were taking the antiretroviral regimen TDF, FTC and RTV-boosted ATV. After a screening visit to assess entry criteria and rule-out clinical evidence of genital tract infections, participants underwent six visits over a menstrual cycle, each with collection of samples for the FGT microbiome and genital and plasma trough antiretroviral drug concentrations. The distribution and types of bacteria colonizing the FGT were characterized using 16S rRNA gene sequencing with clustering of similar microbiome community types (CTs) using Dirichlet Multinomial Mixtures (17). Microbiome CTs were described and demographic, behavioral and other clinical factors were assessed as potential clinical predictors of CT using generalized mixed models with a random intercept for each individual to account for repeated measures. The association between the primary predictor, microbiome CT, and the outcome, log-transformed FGT antiretroviral drug concentrations, was assessed using univariate mixed linear models with a random intercept for the individual for each of the antiretroviral drugs. Multivariable models additionally controlled for the covariates: plasma antiretroviral drug concentrations and significant demographic, behavioral

or clinical univariate predictors of either microbiome CT or FGT antiretroviral drug concentrations.

## BACKGROUND

Globally, there are an estimated 35 million people living with HIV, 16 million of whom are women aged 15 years and older (1). HIV antiretroviral therapy is a lifesaving treatment, as well as a key component of modern HIV prevention efforts. Antiretroviral therapy has averted an estimated 7.6 million deaths globally since 1995, however there continue to be 2.1 million new infections annually (1). Additional efforts directed at mitigating HIV transmission among women could have a major impact on the epidemic.

Antiretroviral therapy suppresses HIV viral shedding in blood and genital secretions and leads to prevention of mother-to-child transmission (PMTCT) and decreased sexual transmission of HIV (2-4). Antiretroviral concentrations achieved in the female genital tract (FGT), therefore, have the potential to impact prevention strategies that rely on antiretroviral therapy, including pre-exposure prophylaxis (PrEP), PMTCT, and treatment for the prevention of HIV transmission. Furthermore, the FGT as well as in the gut, peripheral blood mononuclear cells and other sites may be reservoirs of HIV viral persistence (18-28). Efforts to eradicate the virus are theorized by some to have been unsuccessful owing to insufficient tissue antiretroviral concentrations to fully suppress viral replication in these reservoirs (20, 28). Therefore, optimizing antiretroviral therapy to achieve concentrations in the genital tract that result in complete suppression of viral replication is paramount for HIV prevention efforts and the ongoing HIV eradication and cure strategies.

The normal FGT, the primary site of HIV transmission and acquisition in women, is colonized by diverse communities of bacteria, which impact the vaginal microenvironment (10). Bacteria of the *Lactobacillus* genus are traditionally thought to be the predominant colonizers of the healthy vagina, secreting lactic acid and thereby maintaining an acidic pH below 4.5. However a variety of diverse bacteria may play important roles in some women's bacterial communities. The clinical condition bacterial vaginosis (BV) occurs when altered FGT microbiota occur, with an increased abundance of anaerobic organisms and a loss of an acidic

vaginal pH. BV can be diagnosed by clinical criteria, using factors including pH, vaginal discharge, amine odor, vaginal wet mount findings (29), as well as by vaginal gram stain. While BV can cause symptomatic vaginitis, it has also been linked to multiple negative health outcomes including increased rates of pre-term labor and spontaneous abortions (30), and risk for pelvic inflammatory disease (31) and sexually transmitted infections (STIs) (32, 33). Furthermore, BV is frequently asymptomatic, though still associated with altered microbiota, increased pH and local inflammation (34). Gram staining of vaginal secretions to visually characterize the distribution of bacterial morphotypes using a Nugent score allows BV diagnosis in asymptomatic women (35). Using the vaginal gram stain, BV prevalence was estimated, among over 3,700 U.S. women aged 14-49 years, to be 29% overall, though there is significant variability in prevalence based on race, with rates above 50% among Black, non-Hispanic women compared with the lowest rates among white women and more intermediate rates among Mexican American and other Hispanic women(11).

To enhance our understanding of the vaginal microbiota including during episodes of BV, recent advancements in genetic sequencing techniques have allowed improved characterization of the wealth of bacterial species in the FGT, termed the microbiome. These sequencing techniques allow detection of many bacterial organisms that historically have been unable to be cultured or were not previously identified using standard microbiologic techniques (36, 37). High-throughput sequencing of the marker gene encoding the16S ribosomal RNA (rRNA), which is common to all bacteria, combined with specialized bioinformatics techniques allowing storage and mining of the vast amount of generated data, have allowed precise and simultaneous characterization of the multiple types of bacteria living in an environment of interest. Using this technology, HIV infection has been reported to alter the oral, respiratory, gut and vaginal microbiomes (37-42), the significance of which is only recently being elucidated.

Though the vaginal microbiome has not been well characterized across all populations, significant diversity in bacterial communities can occur, with variation depending upon

race/ethnicity, geographic area, hormonal factors, menses, and sexual and vaginal health practices (10, 43, 44). In a population of asymptomatic healthy women from 4 racial/ethnic groups in the United States, the vaginal microbiota clustered into 5 unique bacterial community state types (CSTs) based on similar types and relative abundances of bacterial taxa (10). Over a quarter of women's vaginal microbiota in this study clustered into a CST that was not dominated by *Lactobacillus*, but instead had increased community diversity and prevalent anaerobic bacteria. This CST was in turn, strongly associated with the diagnosis of BV by Nugent score (10). Among these healthy women, the BV-associated community group was present in up to 40% of Hispanic and black women, compared to only 10% of white women and nearly 20% of Asian women (10). In addition to racial and ethnic difference in BV prevalence, HIV-infected women have been reported by some investigators to have even higher vaginal microbial diversity than HIV-uninfected women (41).

Though BV is common, it also may negatively impact multiple important HIV-related outcomes and thus it is highly relevant to ongoing HIV prevention efforts. BV is associated with increased risks of both HIV acquisition (12, 13) and FGT HIV viral shedding, which in turn has been associated with HIV transmission (5, 14-16). Furthermore, while the vaginal microbiome can vary markedly between women (10, 44), vaginal microbiota have also been identified, in HIV-negative women, to be highly dynamic in many individuals, changing significantly over the course of a month (43, 45, 46). These changes could lead to periods of increased HIV acquisition risk or transmission potential. Given the importance of vaginal microbiota to HIV transmission, further characterization of the temporal dynamics of the FGT microbiome in HIV-infected women and potential interactions between antiretroviral therapy and vaginal microbiota are imperative for continued HIV prevention efforts.

There are an abundance of identified risk factors for alterations of FGT microbiota. Multiple behavioral and sexual risk factors have been associated with increased rates of BV including new or multiple sexual partners, vaginal sexual activity, sexual activity without condom

use, receptive oral sex, and vaginal douching (44, 47, 48). Additionally, certain STIs, such as herpes simplex virus-2 (HSV-2), may also increase BV risk (49). While previous data on BV as diagnosed by clinical factors or gram stain with Nugent score have identified general associations with altered microbiota, microbiome studies utilizing molecular methods have the ability to reveal associations with more subtle changes in the vaginal microenvironment.

Molecular methods of characterization of vaginal microbiota reveal relationships between specific species that cannot be detected with traditional diagnostics for BV. A large review identified consensus across multiple studies that the species *Lactobacillus crisipatus* is not typically associated with BV, while *L. iners* is commonly associated with BV or intermediate vaginal flora (50). The specific *Lactobacillus* species may also impact stability of FGT microbiome communities; while *L. crisipatus* and *L. gasseri*-dominated communities tend to be associated with increased community stability, *L. iners*-dominant and non-*Lactobacillus*-dominated community types that have increased diversity are associated with increased fluctuation in microbiome communities (46). Identifying specific bacterial taxa can help to explain traditional BV risk factors such as sexual activity; for example, vaginal exposure to semen, as assessed by detection of the marker prostate specific antigen (PSA) in vaginal samples, is quantitatively associated with decreased abundance of *L. crisipatus* along with increased abundance of *L. iners* and *L. gasseri* (51).

The menstrual cycle has been identified as an additional determinant of fluctuations in FGT microbiota, and increasing data suggest that female sex hormones play an important role. Menses are a major disturbing factor in the FGT microbiota (52), and the rate of change of microbiome community type is associated with the time in the menstrual cycle phase. The highest frequency of community change was found to occur during menses while the lowest rates of change occurred during the peaks of plasma estradiol and progesterone in pre-menopausal women (46). Alterations in FGT microbiota among women using hormone contraceptives or hormone replacement therapy or among post-menopausal women add additional evidence for the

importance of female sex hormones on the FGT microbiome. For example, various types of hormone contraceptives have been found to be protective for BV (47, 53-55). Post-menopausal women are significantly more likely than pre-menopausal women to have community types dominated by *L. gasseri*, or *L. jensenii* or to have low-*Lactobacillus* and increased diversity community types compared to those dominated by *L. crisipatus* (56). Furthermore BV prevalence may be decreased with hormone replacement therapy in post-menopausal women (57).

The changing FGT microenvironment, through fluctuations in the microbiota, is subject to conditions that affect both its tissue drug penetration and HIV transmissibility. Abnormal FGT microbiota are associated with increased risk for genital HIV shedding (14-16) and at least one study has demonstrated that the association between vaginal microbiota and HIV shedding varies depending upon antiretroviral therapy use (58). Compartmental drug penetration is generally impacted by each drug's physicochemical properties, affinity for efflux pump transporters, degree of protein binding, lipophilicity, and the acid dissociation constant (pKa) (59). However, it is not known if alterations of the FGT microbiome could affect FGT drug penetration, though the kinetics of FGT drug distribution are known to vary by specific antiretroviral drug (6-8). Additionally, highly variable FGT antiretroviral concentrations between different women as well as longitudinally among women have been identified by our group (9) and others (6-8). Among 20 HIV-infected women regularly taking the antiretroviral drugs tenofovir, emtricitabine and ritonavir-boosted atazanavir, the within-person coefficients of variation (CV) for these drugs ranged from 66-82% and the between-person CVs were 64-93%, in excess of plasma variability of the same drugs (9). Though the etiology for such variability in genital tract drug penetration has not been fully elucidated, this finding suggests that in addition to drug-specific properties, other changes in the vaginal microenvironment could impact compartmental drug distribution.

The composition of the FGT microbiome has the potential to impact compartmental drug disposition by way of multiple potential mechanisms. Though research to date on this type of relationship, termed 'pharmacomicrobiomics,' has focused on the gut microbiome (60), similar

theoretic mechanisms may apply in the FGT. In the gut, a number of antimicrobial compounds and other types of drugs have been found to have altered pharmacokinetic and pharmacodynamic effects mediated by gut microbiota. Previously documented mechanisms include pH alteration, selective activation or inactivation of a target drug or it's metabolites, or by selective uptake or active efflux of a drug by microbial membrane proteins (60).

Pharmacomicrobiomic interactions have not been previously explored in the FGT, however similar mechanisms may apply and have the potential to differentially impact the concentrations of common antiretrovirals that have diverse physicochemical properties. Varying pH of the vaginal microenvironment, secondary to fluctuations in the microbiota, may dissimilarly affect compartmental penetration of drugs with different acid dissociation constants (pKa) since drugs that are ionized are less likely to cross membranes than those that are not ionized. Antiretrovirals such as the protease-inhibitor, atazanavir (ATV), with a high pKa of 4.7 (61) is more likely to be ionized in a moderately acidic environment compared to the reverse transcriptase inhibitors, tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC), which have lower pKa values (3.75 and 2.65, respectively) (62). These drugs have additional diverse physiochemical properties which may impact compartmental penetration. While atazanavir is a large, highly-protein bound molecule (molecular weight 704.9 and 86% plasma protein binding), tenofovir and emtricitabine are comparatively smaller molecules (molecular weights 287.2 and 247.2) with low plasma protein binding (<0.7% and <4%), respectively (63-66). These along with other differences across antiretroviral drugs, may lead to distintive interactions with fluctuating microbiota, which could explain some of the variability of FGT antiretroviral drug concentrations.

Since a major goal of HIV antiretroviral therapy is to reduce HIV transmission to partners and newborns, ensuring adequate and consistent drug penetration in the genital tract to suppress viral shedding is paramount. To further explore the etiology of variability in FGT antiretroviral drug penetration, this study aims to characterize the FGT microbiome and significant predictors,

among virologically-suppressed HIV-infected women on antiretroviral therapy and to assess the relationship between the composition of the FGT microbiome and antiretroviral drug disposition in the FGT for the common antiretroviral drugs: TDF, FTC and ritonavir (RTV)-boosted ATV.

# METHODS

## *Aims and Hypotheses*

The aims of this study are to 1) describe the female genital tract (FGT) microbiome composition in HIV-infected women on antiretroviral therapy over a menstrual cycle, evaluating for predictive factors including demographic and behavioral factors, menstrual cycle phase, and female sex hormones, and 2) to determine if the FGT microbiome composition is associated with FGT antiretroviral drug concentrations for the antiretroviral drugs tenofovir disoproxil fumarate, emtricitabine and ritonavir-boosted atazanavir. It is hypothesized that FGT antiretroviral drug concentrations vary by FGT microbiome community type for each of the antiretroviral drugs.

## *Study Design:*

This study was a single-center, prospective, longitudinal cohort study which included 6 study visits over the course of a menstrual cycle among 20 HIV-infected women who had plasma HIV viral suppression while taking an antiretroviral drug regimen consisting of TDF, FTC and RTV-boosted ATV. This cohort was originally designed to assess the effects of genital antiretroviral drug concentrations on the frequency of genital HIV-1 RNA and DNA shedding throughout the menstrual cycle among women highly adherent to antiretroviral therapy without baseline bacterial vaginosis (BV) by clinical criteria (Amsel's criteria) (9). Sample collection occurred between April and December, 2010, though subsequent analysis using 16S rRNA gene sequencing of cervicovaginal lavage specimens that had been consistently stored at -80°C since sample collection was undertaken in 2015 for the purposes of this analysis.

## *Characteristics of Study Population*

HIV-1 infected women included in this study were ≥18 years of age with reported regular menses (occurring within 22-35 day intervals for the previous 3 cycles) and with undetectable plasma HIV-1 RNA viral loads (<75 copies/ml) within 90 days of enrollment, and were taking the combination antiretroviral therapy regimen TDF/FTC (300/200 mg) and ATV/RTV (300/100 mg) each once daily for ≥ 30 days and had taken any combination antiretroviral therapy regimen

for ≥ 6 months. Exclusion criteria were: BV by Amsel's criteria (29) at baseline visit, *Trichomonas* infection (by wet mount examination), vaginal candidiasis (by wet mount and potassium hydroxide staining of wet mount examination) or genital ulcers or purulent vaginal discharge on examination at the baseline visit, non-adherence to antiretroviral therapy (participant reported missed antiretroviral doses in the 3 days before study enrollment), and current pregnancy or post-menopausal status (absence of menses ≥ 12 months). Participants were receiving routine HIV care at the Grady Hospital Infectious Disease Program (IDP) Clinic (Atlanta, GA) where they were recruited using flyers or direct referrals from medical providers. Twenty women fitting inclusion and exclusion criteria after the screening visit were scheduled for the first study visit after completion of the next menses. Written informed consent was obtained from all participants prior to study entry and the study was approved by the Emory University Institutional Review Board (IRB) and the Grady Research Oversight Committee (GROC).

***Study Visits***

All study visits took place at the Grady IDP Clinic. At the initial screening visit a detailed baseline interview with medical, menstrual, reproductive and sexual histories were collected in addition to a pelvic exam to evaluate for vaginal infections that were a part of the study's exclusion criteria. Baseline demographic and clinical data were collected by interview with study staff. Chart review was performed to evaluate the most recent CD4 count and to confirm relevant clinical history including use of other medications such as hormone contraceptives, recent antimicrobial use and most recent Pap smear results. Participants were screened for pregnancy, syphilis (by rapid plasma reagin (RPR) with reflex *Treponema pallidum* particle agglutination assay testing for positive RPR results), HSV-2 (by IgG antibody) and vaginal *Neisseria gonorrhea* and *Chlamydia trachomatis* (by nucleic acid testing), *Trichomonas* (by wet mount), BV (by wet mount and gram stain) and *Candida* (by potassium hydroxide staining of wet mount and by gram stain) as previously described (9). Chart review was also performed after the first

study visit to document the most recently recorded height and weight prior to the first study visit, for calculation of the body mass index (BMI).

During six twice-weekly study visits after completion of menses and over the course of each participant's menstrual cycle, paired plasma and cervicovaginal specimens were collected for antiretroviral drug concentrations with additional cervicovaginal specimens collected for microbiome analysis and characterization of bacterial vaginosis (BV) status by Nugent criteria. Women with onset of menses before completing their final study visit were asked to complete remaining study visits after cessation of menstruation. Study visits were scheduled to occur at the time of trough antiretroviral drug concentrations (approximately 24 hours since the last doses of TDF, FTC and RTV-boosted ATV). Women were instructed to abstain from sexual intercourse or use of intravaginal products or douching for ≥ 24 hours before study visits.

At each study visit interval genitourinary history was collected and paired cervicovaginal and plasma samples were obtained. A speculum pelvic exam was performed and cervicovaginal fluid was collected on 3 TearFlo wicks (Hub Pharmaceuticals, Rancho Cucamonga, CA) applied to the ectocervix until saturated prior to storage at -80°C. A cervicovaginal lavage (CVL) was subsequently performed by repeatedly instilling and aspirating 10ml of sterile phosphate-buffered saline (PBS) aimed toward the ectocervix and vaginal walls, allowing it to pool in the posterior fornix prior to final sample collection. CVLs were stored on ice prior to processing within 4 hours including qualitative evaluations for leukocytes and blood with a urine dipstick test, evaluation for semen contamination by PSA detection (67, 68) and Gram stain. CVL samples were subsequently centrifuged to separate the supernatant and pellet components prior to storage at -80°C. Blood was collected into sodium-citrate-containing CPT vacutainer tubes, centrifuged and plasma removed for storage at -80°C with subsequent antiretroviral concentration testing. Women with symptoms suggestive of cervicovaginal infection during the study were tested and treated as per standard of care at the IDP clinic.

***Laboratory Techniques and Definition of Variables***

To obtain 16S rRNA gene sequencing of cervicovaginal fluid, DNA from the CVL pellet specimens was first extracted using the Qiagen EZ1 DNA Tissue kit (Qiagen, Germantown MD Cat#953034) with the Qiagen bacterial card on the Qiagen EZ1 Advanced XL instrument. 16S rRNA gene metagenomic sequencing library preparation was carried out using the Illumina MiSeq procedures (69) with the following modifications. Universal PCR primers for the V1-V2 hypervariable regions of the 16S rRNA gene (underlined) with Nextera tags (bold) were used for sequencing: 8F:AGAGTTTGATCCTGGCTCAG, 338R:TGCTGCCTCCCGTAGGAGT and with Nextera tags,

8F:**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGTTTGATCCTGGCTCAG and

338R:**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGCTGCCTCCCGTAGGAGT. Nextera-tagged primers were used in the initial stage of PCR and amplicons were subsequently purified using Agencourt AMPure XP beads (Beckman Coulter, Brea CA Cat#A63880). Dual indices and Illumina sequencing adapters were attached to the purified product during the Index PCR using the Nextera XT Index Kit, the products of which were purified using the Ampure XP beads. The final product was quantified using the Qubit dsDNA high sensitivity Assay kit (Life Technologies Cat#Q32851). Amplicon libraries were diluted to 4 nM, and 5 uL of each were pooled, denatured and diluted to 4 pM along with a PhiX control sample. The denatured and diluted PhiX/amplicon library sample was added to the Illumina Miseq v2 500 cycle Flow Cell and targeted resequencing was performed using paired-end, 2 index reads.

Further sequence processing was performed using Mothur software (70). After generating contigs from reads, sequences with ≥ 1 ambiguous bases and a length > 385 bases were removed. Sequences starting at position 1046 and ending at position 6424 with maximum homopolymer length of 8 bases were screened and unique sequences underwent a pre-clustering

step using UCHIME (71) for removal of chimeric sequences and classification with a Bayesian classifier and the GreenGenes database (72). Operational taxonomic unit (OTU) clustering was performed using 95% sequence homology and taxonomic assignments were made using the GreenGenes database (72).

Microbiome data was further analyzed using RStudio (73) and Phyloseq software (74). Each participant-visit was clustered into a microbiome community type (CT) comprised of similar abundance and type of bacterial taxa using Dirichlet Multinomial Mixtures with the DirichletMultinomial package (17). Further classification of *Lactobacillus* sequences at the species-level, as has been reported previously using SpeciateIT (speciateIT.sourceforge.net), was attempted, however we were unfortunately unable to carry out this analysis given software incompatibility. Alpha-diversity was measured using the Shannon Index (75) calculated for individual microbiome samples and subsequently averaged across microbiome CT.

High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was used to characterize concentrations of plasma and TearFlo genital wick TDF, FTC and ATV concentrations as described previously (9, 76) with dynamic ranges for both plasma and cervicovaginal wicks for all three drugs of 10–2000 ng/mL, with 15% inter- and intra- day coefficients of variation. Plasma estradiol and progesterone concentrations were assessed using a radioimmunoassay (Siemens Healthcare) with lower limits of detection of 5 pg/mL and 0.1 ng/mL, respectively. Progesterone concentrations were used to characterize each person-visit into follicular (if study visit occurred before the onset of progesterone rise) or luteal phase of the menstrual cycle (if the study visit occurred at or after the onset of progesterone rise). Participants who did not have a rise in progesterone during the course of the study were classified as having a non-ovulatory menstrual cycle (vs. a normal ovulatory cycle).

Semen contamination, a qualitative variable, was determined to be positive based on a positive result from the ABACard p30 antigen detection kit (Abascus Diagnostics, West Hill, CA) for detection of PSA, which typically returns to a negative result within 48 hours of vaginal

semen exposure (67, 68). The variable, reported sexual activity within 7 days of a study visit, was corrected to a 'positive' result from a 'negative' reported result if semen contamination was positive in the sample for the same study visit. Additionally, since FGT leukocytes and blood results were both markedly skewed, for the purposes of univariate and multivariable analyses, these variables were dichotomized to be either > vs. ≤ the median value for each of these variables.

Nugent score for identification of BV was defined by gram stain per standard protocol whereby a score (range 0-10) ≥ 7 was characterized as positive for BV and a score <7 was characterized as negative for BV (35). Gram stain for Nugent score was performed on specimens from whole CVL fluid (prior to centrifugation) for available specimens (n=53). For some visits, whole CVL was unavailable, thus CVL cell pellet was used for the gram stain (n=55). A subset of 15 specimens had available gram stain specimens with Nugent scores for both whole CVL and CVL cell pellet; for these samples a correlation analysis of Nugent score was performed, identifying a Pearson correlation coefficient of 0.84267 ($p<0.0001$). This suggested that the source of gram stained CVL material (whole vs. cell pellet) did not markedly alter the resulting Nugent score.

***Analytic Methods***

Descriptive statistics were performed to describe baseline data and the distribution of clinical and demographic variables evaluated in this study by microbiome CT. Trough antiretroviral drug concentrations were natural log-transformed to normalize the distribution prior to univariate and multivariate analyses; they were back-log-transformed (exponentiated) for the purposes of presentation of results.

For the AIM 1 analysis, to evaluate predictors of microbiome CT, univariate models evaluated each potential clinical predictor as the exposure in a model with the outcome being the pairwise-comparison of two microbiome CTs. Generalized mixed models were used for univariate associations with a random intercept for the individual to account for repeated

measures among participants. Clinical predictors evaluated in AIM 1 included age, BMI, antibiotic use for vaginal infection in the prior 30 days, sexual activity in the past 7 days, vaginal semen contamination present, FGT leukocytes or blood greater than the median value for each of these variables, plasma estradiol and progesterone concentrations, as well as follicular vs. luteal phase for those with ovulatory cycles, and ovulatory vs. non-ovulatory cycles for all participants.

For the AIM 2 analyses, the same clinical predictors evaluated in AIM 1 analyses were evaluated, with the exception of recent antibiotic use for vaginal infection which is not expected to influence FGT antiretroviral drug concentrations, and Nugent score, which is an alternative marker for microbiota community composition by 16S rRNA gene sequencing. Additionally, semen contamination was not considered for inclusion in multivariable models given the small number of positive results identified as well as potential for collinearity with the variable, recent sexual activity within 7 days, which was included as a predictor in final multivariable models using the described criteria.

Relevant clinical predictors of FGT antiretroviral concentrations were evaluated for the first portion of AIM 2. Each clinical predictor was evaluated in a univariate model with the outcome of log-transformed antiretroviral concentration (ATV, TDF or FTC) using mixed linear models with a random intercept for individual. Subsequently, in the second portion of AIM 2, univariate mixed linear models with a random intercept for individual were performed for each antiretroviral drug with the outcome log-transformed FGT antiretroviral concentration and the predictor being microbiome community type (a categorical variable with differing levels for each unique microbiome CTs). Multivariable models with the same structure additionally included clinical predictors which were found to be significantly associated ($P<0.05$) with either any of the three FGT antiretroviral concentrations (from the first portion of AIM 2) or of microbiome CT (from AIM 1 univariate analyses). Clinical predictors deemed “significant” for any comparison of microbiome CT, or for any of the FGT antiretroviral concentrations were included in all

multivariable models. Additionally, multivariable models each, *a priori*, included the plasma antiretroviral concentration of the same drug evaluated as the outcome in the model.

Because this study was an analysis of stored specimens from a previously sampled cohort, a power analysis to determine optimal sample size could not be carried out prior to sample collection. SAS v. 9.4 (Cary, NC) was used for statistical analyses and a P-value of $<0.05$ was considered statistically significant. Missing data were overall rare, so complete case analysis was performed.

# RESULTS

Twenty women were enrolled and completed 119 study visits in the initial cohort (9). Participants had median age 38 years, were 95% African American and approximately half were obese (median BMI 30 kg/m$^2$, range 21-51 kg/m$^2$). Most participants had CD4 ≥ 200 cells/mcl, were taking their current antiretroviral therapy regimen for a median of 14 (range 3-41) months, were not using hormone contraception and were sexually active with 1 partner in the past 6 months. The majority of participants had prior exposure to HSV2 (IgG positive in 19/20), and at screening 5 had asymptomatic BV by Nugent score and 5 had asymptomatic identification of Candida on gram stain (**Table 1**).

A total of 117 samples had adequate CVL pellet specimen available for 16S rRNA gene sequencing, which yielded high-quality sequences used for microbiome analyses from 109 participant-visits; 7 samples were removed in quality processing steps and one was removed post-processing because it was comprised of only rare OTUs (those below the top 1000 most frequent OTUs).

Unsupervised clustering of similar microbiome communities by identity and distribution of bacterial taxa using Dirichlet Multinomial Mixtures yielded three distinct microbiome community groups characterized by low- (N=40), intermediate- (N=28), and high-diversity (N=41) using Shannon Index (**Figure 1**). Of classified bacterial taxa, the low-diversity community type (CT) was comprised of primarily *Lactobacillus*-species (95.9% relative abundance) followed by *Aerococcus* (2.0%) and *Gardenerella* (0.8%), whereas the intermediate-diversity CT had a lower proportion of *Lactobacillus* (49.7%) followed by *Prevotella* (11.5%), unclassified genus (9.0%), and *Megasphaera* (8.6%), respectively. The high-diversity CT had *Megasphaera* (23.3%), *Prevotella* (23.0%) and *Shuttleworthia* (16.1%), respectively, comprising the top three identified taxa with a minority of *Lactobacillus* (1.0%) overall (**Figure 2**).

Among 109 participant-visits over the menstrual cycle, 5 (4.6%) study visits among 4 participants were completed outside the study window due to menses onset prior to study

completion (**Figure 3**). Trough FGT antiretroviral concentrations were obtained, with the time from last antiretroviral drug dose to FGT sampling of median 24 (Quantile (Q)1, Q3: 23, 25) hours, though 9 (8.3%) samples were collected >4 hours from a true 24-hour trough. Plasma sampling occurred a median of 24 (Q1, Q3: 22, 24) hours from last antiretroviral therapy dose and occurred within an hour of FGT sampling for 82 (75.2%) of visits. Plasma sampling occured not more than 1 hour from genital sampling in the remaining visits (27 visits, 24.8%).

The majority of participants had stable microbiome CTs during the course of the study; 6 study participants experienced a CT change 9 times during the course of the study. The majority of CT changes occurred from the low-diversity group to another group (6 times, 66.7% of CT changes), and from one CT to the intermediate-diversity CT (7 times, 77.8%). Among the three CTs, the high-diversity group was most stable longitudinally, with 1 CT change from the high-diversity CT to another CT, and zero CTs changes from one CT to the high-diversity group (**Figure 3**).

The distribution of clinical factors across study visits by microbiome CT are presented in **Table 2**. The low-diversity CT was associated with low Nugent scores and only 9.1% of visits were characterized as BV (Nugent score ≥ 7), while BV was identified among 67.9% and 91.9% of visits with intermediate- and high-diversity CTs, respectively. Univariate associations between clinical predictors and pairwise microbiome CTs revealed that study visits characterized by the intermediate-diversity CT were associated with increased BMI; a 1 unit increase in BMI was associated with a 34% reduction in odds of high- compared to intermediate-diversity BMI (P=0.0191) (**Table 3**). Intermediate-diversity CTs were less commonly identified (10.7%) following use of an antibiotic for a vaginal infection within 30 days of the screening visit compared to low- (35%) and high-diversity (29.3%) CTs, however univariate analyses did not identify significant pairwise associations (both P>0.05). FGT semen contamination, leukocytes >125 cells/mL, and red blood cells (RBCs) > 25 cells/mL occurred more commonly in intermediate- compared to low- and high-diversity CTs but statistically significant univariate

associations were not identified (P>0.05 for all). Menstrual cycle phase or presence of an ovulatory cycle compared to a non-ovulatory cycle, in addition to plasma estradiol and progesterone concentrations, did not significantly vary by microbiome CT (P>0.05 for all).

Clinical predictors of FGT antiretroviral concentrations were evaluated using univariate analyses (**Table 4**). Sexual activity within 7 days of a study visit was associated with 48% increased TDF concentrations (P=0.0492) in the FGT compared to study visits without recent sexual activity. FGT RBCs > 25 cells/mL compared to ≤ 25 cells/mL were associated with 46% increased FGT ATV concentrations (P=0.0356). Increased BMI was associated with borderline increased FGT TDF concentrations (4.5% increase per unit ($kg/m^2$) increase in BMI; P=0.0897). Evaluation of these predictors for the other antiretrovirals as well as additional predictors (age, FGT leukocytes>125 cells/mL, estradiol, progesterone and menstrual cycle phase, or ovulatory status) were not significantly associated with FGT antiretroviral concentrations (P>0.05 for others). Plasma concentrations of ATV were positively associated with FGT ATV concentrations (Estimate=1.56, 95% CI 1.28-1.89; P<0.0001) though plasma TDF and FTC concentrations were not significantly associated with their respective FGT concentrations in this analysis (P>0.05 for both).

The proportional change in ATV, TDF and FTC concentrations in the FGT across microbiome CTs were assessed with univariate and multivariable mixed linear models (**Table 5**). In univariate models, TDF concentration was reduced 49% (Estimate=0.51, 95% CI 0.29-0.91) in low- compared to intermediate- diversity CTs, and reduced 54% (Estimate=0.46, 95% CI 0.21-1.03) in high-compared to intermediate-diversity CTs. For ATV and FTC there were similar but non-significant trends toward reduced FGT antiretroviral concentrations in low- and high-diversity CTs compared to the intermediate-diversity CT.

Multivariable analyses of the association between FGT antiretroviral concentrations and microbiome CT controlled for plasma antiretroviral concentrations and significant predictors of either microbiome CT or FGT antiretroviral concentrations: BMI, FGT RBCs > 25 cells/mL and

sexual activity within the past 7 days. In a multivariable analysis, ATV was associated with 48% and 54% reduced FGT concentrations in low- and high-diversity CTs, compared to the intermediate-diversity CT (Estimate=0.52, 95% CI 0.31-0.88 and 0.4, 95% CI 0.23-0.92; P=0.0260), respectively. Similarly, TDF was 49% and 46% reduced in low- and high-diversity CTs, compared to the intermediate-diversity CT, though this was of borderline statistical significance (Estimate=0.51, 95% CI 0.28-0.91 and 0.54, 95% CI 0.23-1.27; P=0.06). FTC concentrations in the FGT were not significantly associated with microbiome CTs in the multivariable model (P=0.2716) (**Figure 4**).

## DISCUSSION/CONCLUSIONS

In this study, the FGT microbiome was measured in HIV-infected women taking TDF/FTC and RTV-boosted ATV antiretroviral therapy and was demonstrated to be associated with altered antiretroviral drug concentrations in the FGT. Using an unsupervised clustering method, three distinct microbiome CTs were identified among 109 study visits by 20 women followed longitudinally across the menstrual cycle. CTs were of low-diversity and >95% relative abundance of *Lactobacillus* species, or of intermediate- and high-levels of diversity CTs, both with increased abundance of anaerobic taxa and reduced *Lactobacillus*. While age, recent antibiotic use for vaginal infection, menstrual cycle, and FGT detection of semen or increased leukocytes or blood were not identified as predictors of microbiome CT, increased BMI was significantly associated with increased odds of the intermediate- compared to high-diversity microbiome CTs. Recent sexual activity, increased FGT blood and plasma antiretroviral concentrations were identified as significant predictors of some antiretroviral drug concentrations and thus were included with BMI in multivariable analyses. Interestingly, controlling for these predictors, both the low- and high-diversity microbiome CT's concentrations of ATV were found to be decreased approximately 50% compared to the intermediate-diversity CT. Similar reductions were identified for TDF by microbiome CT, however the reductions were not statistically significant. FTC concentrations were not significantly associated with FGT microbiome composition in the multivariable model. This proof-of-concept study demonstrates the hypothesis that FGT microbiota have the potential to influence compartmental concentrations of some drugs, however further studies are needed to confirm these findings and to evaluate the direction, as well as mechanisms, of causation.

The FGT microbiome has not been previously well characterized in HIV-infected women taking antiretroviral therapy. During up to six time points over a menstrual cycle, the majority of the study population's FGT microbiome were characterized by diverse CTs with an abundance of

anaerobic taxa and high frequencies of BV by Nugent score. A minority of time points were identified as having a *Lactobacillus* dominated, low-diversity CT which is typically characterized as the "normal" vaginal microbiome. Nevertheless, the microbiome communities in this population were relatively stable during the one month follow up period, with occurrence of a change in microbiome CT over the course of the study among only 30% of participants.

While this study identified three primary FGT CTs among the study population of HIV-infected women on antiretroviral therapy, varying numbers of community groups have been reported in the literature previously among differing populations. Given there are multiple previously identified demographic, sexual, hygiene and clinical factors associated with changes in FGT microbiota, it is not surprising that the number of community groups may vary across the literature with additional differences due to variation in study populations and sample sizes. In their landmark cross-sectional study characterizing the FGT microbiome among 396 healthy U.S. women, Ravel and colleagues identified 5 main community-subtypes, four of which were *Lactobacillus*-dominated and one that was associated with increased microbial diversity and BV (10). Notably, only 26.3% of participants in the study were African American, though 40% of African American women, compared to 10.3% of Caucasian women fell into the high-diversity/non-*Lactobacillus*-dominated FGT community group.

Other studies have also reported differences in numbers and types of microbiome community groups. In Gaejer and colleagues' longitudinal cohort of healthy women in Baltimore, MD, three *Lactobacillus*-dominant CTs were identified, but two additional CTs, with increased diversity and a minority of *Lactobacillus* were distinguished, though these two groups were distinct, with different species compositions identified (46). In our population, composed of HIV-infected women who were primarily African American with prior HSV-2 exposure, both known risk-factors for BV, it is not unexpected that two of three major community groups comprising the majority of the visits had increased diversity compared to the low-diversity, highly-*Lactobacillus* dominated and non-BV associated group. Similarly, in a longitudinal study

of the vaginal microbiome in HIV-infected women in Tanzania, 8 major community clusters were identified, only two of which were associated with *Lactobacillus iners* or *crisipatus*-dominated flora not associated with BV while multiple other community groups were strongly associated with BV and with increased proportions of various anaerobic taxa such as *Prevotella* and taxa of genus *Lachnospiraceae* (77). Furthermore, among a longitudinal cohort over 8-10 years of at-risk and HIV-infected women, 6 primary microbiome CTs were identified, four of which comprised over half of the 581 visits and had increased diversity and low *Lactobacillus*-abundance (78) similar to intermediate- and high-diversity CTs identified in the current analysis. Importantly, HIV status was not associated with a particular CT in the longitudinal cohort of at-risk and HIV-infected women (78), suggesting that clinical, demographic or other factors may account for differences across studies. Our study adds to the body of literature in this area, in particular defining the microbiota among HIV-infected but virologically suppressed U.S. women on antiretroviral therapy, a majority of whom were African American, thus reflecting the general population of new and prevalent HIV infected women in the U.S. (79).

An additional strength of the methodology used in this study was that unlike some authors' method of community classification using a complete linkage clustering methodology which requires specification by the investigators of the number of unique CTs to be reported (10), the current analysis made use of Dirichlet Multinomial Mixtures. This unsupervised approach utilizes the data to determine the best-fit for the models, thus identifying the optimal number of community groups in a population (17). Therefore, microbiome community classification using Dirichlet Multinomial Mixtures may be a preferred method, and thus has been increasingly employed by additional authors (80-82).

Previous longitudinal studies in healthy women suggest that FGT microbiome communities can be stable among some women, and highly variable among others over the menstrual cycle (43, 46). Overall among the current study's population of virologically suppressed HIV-infected women on antiretroviral therapy, microbiome communities were

relatively stable during the menstrual cycle, with no change in CT between visits nearly 90% of the time and with stable CTs across all study visits among 70% of participants. However, of CT changes, microbiome communities were most likely to leave the low-diversity CT and to enter the intermediate-diversity CT, while the high-diversity CT was the most stable longitudinally. Given the small numbers of CT changes, this study is limited in it's ability to characterize factors associated with CT change, however menstrual cycle phase, sex-hormone levels and comparisons of women with typical ovulatory- compared to non-ovulatory cycles did not identify these factors as significant predictors of microbiome CT in this population. Gajer and colleagues modeled community constancy using beta-diversity and the Jensen-Shannon index. Menses were associated with the least constancy in microbiome CT while the peaks of estradiol in the follicular phase, followed by the peak of progesterone, in the luteal phase, were associated with the most constancy, respectively, across the menstrual cycle (46). In the current analysis, limited sample size and the fact that 6 of 20 of women had non-ovulatory cycles without a progesterone peak, may have limited the ability to detect relationships between the microbiome, the menstrual cycle and hormone concentrations. Furthermore, more subtle changes, such as occurring in the relative abundance of certain taxa, though with differences still defined within a specified microbiome CT, would not be detected within the current analysis.

Importantly, this study supports the hypothesis that the FGT microbiome is associated with, and may influence, variability of compartmental penetration of some drugs into the FGT. 'Pharmacomicrobiomic' effects have been previously identified in the gut (60), however this is the first demonstration, to our knowledge, of an important pharmacomicrobiomic effect of vaginal microbiota on FGT drug distribution. In certain women, concentrations of ATV, and potentially tenofovir, may vary by up to 50%, compared to other women due to differences in FGT microbiome communities. The large degree of these fluctuations in compartmental drug exposure is clinically relevant and deserving of further characterization. This finding, if validated, as well as if demonstrated for additional antiretroviral drugs, may influence antiretroviral drug

choice for future prevention efforts. This may particularly be the case for PrEP where suboptimal adherence may occur frequently, and for which increased dosing intervals have been considered in some clinical studies. Thus, in these settings, clinically significant reductions in FGT concentrations of antiretrovirals could be even more likely to occur in some women due to fluctuations in the FGT microbiome. Furthermore, this study's findings may have broader clinical implications for other treatments which require adequate genital drug penetration, such as drugs for the treatment of STIs, where increasing prevalence of resistance of some organisms, including *Neisseria gonorrhea,* could be attributed to suboptimal genital penetration of certain treatments due to pharmacomicrobiomic effects.

Further studies are needed to determine the mechanisms of the association between microbiome CT and FGT drug distribution. Differences in drug physiochemical properties, in the setting of varying microbiome CTs, are one potential etiology for differences across drug types. Increased ionization of a drug, which reduces transport across membranes, is expected to occur more frequently for drugs with pKa values closer to the physiologic range in the FGT such as ATV or TDF (pKa 4.7 and 3.4, respectively) (61, 62) compared to FTC which has a pKa below the lowest expected pH concentration in the FGT (pKa 2.7)(62) and would be therefore less likely to become ionized in fluctuating FGT microenvironments. However, further characterization of differences in the intermediate-diversity CT compared to the low- and high-diversity CTs are necessary, and pH not be the primary implicated mechanism, given this study's findings. The high-diversity CT, is associated with the highest Nugent scores, and is expected to have the highest vaginal pH, compared to the lowest pH in the low-diversity CT. This study identified marked antiretroviral drug concentration differences in the intermediate-diversity compared to both low- and high-diversity groups, thus if pH impacts the pharmacomicrobiomic effect, there may be a non-linear association across the range of physiologic pH, or there may be additional implicated factors. Other potential mechanisms of differential compartmental drug exposure which require further investigation include variability secondary to metabolism or selective

activation/inactivation of certain drugs by microbiota or by changes in membrane transport proteins in certain microbiome CTs.

This study has several important limitations. Because this study was a secondary analysis, in which the FGT microbiome was characterized from previously collected stored specimens, a power analysis to determine optimal sample size could not be performed prior to sample collection. Thus, results must be considered hypothesis-generating and must be validated in future studies. The relatively small sample size, with as few as 28 participant-visits in the smallest microbiome CT, may have limited the ability to detect meaningful associations with clinical predictors and with FGT antiretroviral concentrations; larger studies are needed to further investigate these associations.

Additionally, this study has the following limitations: 1) to further characterize microbiome CTs, it would be beneficial to characterize the specific *Lactobacillus* species prevalent in each microbiome CT, given the potential for varied species to have different effects on the microenvironment. For example, *L. crisipatus* tends to be associated with a more acidic pH compared to *L. iners* (10, 77). Other potential differential effects on the microenvironment by certain *Lactobacillus* species include varied hydrogen peroxide production, differential inhibitory effects on other organisms, as well as differing associations between certain species and stability of the community composition, vs. propensity for CT fluctuation and risk for developing BV (83-86). In this study, both the low- and intermediate- diversity CTs have *Lactobacillus* as the most predominant taxa (with 96%, vs. nearly 50% relative abundance, respectively). However, the primary *Lactobacillus* species predominating in these two CTs, could differ, further accounting for disparate effects on FGT antiretroviral drug penetration, though this study was not able to identify *Lactobacillus* at the species-level. 2) The effect of repeated CVL sampling on microbiome composition has not been previously well defined, though this method of sample collection has been frequently used in the literature. However, only two CT changes occurred between a participant's first and second visit, suggesting performing the CVL may not be a major

determinant of CT change. 3) Additional clinical factors are known to influence microbiome community composition, such as smoking status, but were not measured in this study. If certain factors additionally influence FGT antiretroviral drug distribution, confounding could occur; however relatively little is known about other etiologies of FGT antiretroviral drug exposure variability, and this study uniquely identified and controlled for, several important predictors. Further studies are needed to evaluate additional demographic, clinical, sexual, hygiene and other factors' influence on microbiome composition in HIV-infected women taking antiretroviral therapy. 4) A minority of study visits were collected outside the study window due to early menses and 5) not all study visits occurred at the time of the true antiretroviral trough, with 9 of 109 visits occurring 4 or more hours outside of the true trough. All drugs were at steady state and multivariable models adjusted for plasma concentrations, likely partially mitigating the magnitude of this factor's influence on antiretroviral concentration variability. Nevertheless, given differences in drug distribution kinetics between FGT and plasma (6, 8), this may have contributed to some of the variability in FGT antiretroviral concentrations, though it is not suspected to have induced substantial bias in the current analysis. Finally, 6) though pharmacomicrobiomic associations were identified in this analysis, the direction of causation is unproven. The first approved HIV antiretroviral drug, a nucleoside analogue reverse transcriptase inhibitor, zidovudine, has been previously shown to have anti-bacterial effects against some organisms of the family *Enterobacteriacea* which commonly colonize the gut (such as *E. coli)* (87). Though there is not published data reporting similar testing for the majority of other antiretrovirals, including those investigated in this study, the non-nucleoside reverse transcriptase inhibitor, neviripine, was evaluated similarly and *in vitro* anti-bacterial effects were not identified (88). Thus, we cannot rule-out the potential that some antiretrovirals may alter microbiome community composition, which could lead to variability in microbiome CTs in the setting of varied FGT penetration due to other factors. However, the potential that fluctuations in the FGT microbiome, by way of altering the pH or other changes to the microenvironment such as

influence on membrane proteins or selective activation/inactivation of certain drugs by bacterial species, is suspected to be more biologically plausible.

In summary, this study identified three major microbiome CTs in HIV-infected women on antiretroviral therapy which are characterized by differences in the diversity and distribution of bacterial taxa including varied relative abundances of *Lactobacillus*. We identified a significant association between the FGT microbiome community composition and the concentration of the antiretroviral drug ATV, with a trend toward a similar effect for TDF, but no significant effect for FTC. Multivariable models adjusted for a number of important predictors and potential confounders, and are a strength of this analysis. Limited selection bias, due to differing clinical, demographic or other factors among participants, or confounding by indication is suspected in the current analyses given all three drugs evaluated in this study were measured concurrently among the same group of participants at steady state and all participants were highly adherent to their three-drug antiretroviral therapy regimen. Furthermore, this study described the FGT microbiome composition in a population of primarily African American women receiving regular HIV medical care in urban Atlanta. Though the study population was a convenience sample, results from this study are suspected to be generalizable to the U.S. population of prevalent and newly infected HIV-positive women. Future studies will serve to validate this study's findings, evaluate mechanisms of the association between microbiome community composition and FGT antiretroviral drug distribution, and evaluate additional antiretroviral and non-antiretroviral drugs for which optimized FGT penetration are essential.

REFERENCES

1. UNAIDS. The Gap Report. http://www.unaids.org/sites/default/files/media_asset/UNAIDS_Gap_report_en.pdf, 2014.
2. Cu Uvin S, Caliendo AM, Reinert SE, et al. HIV-1 in the female genital tract and the effect of antiretroviral therapy. *AIDS* 1998;12(7):826-7.
3. Cohen MS, Chen YQ, McCauley M, et al. Prevention of HIV-1 Infection with Early Antiretroviral Therapy. *N Engl J Med* 2011; 365(6):493-505.
4. Siegfried N, van der Merwe L, Brocklehurst P, et al. Antiretrovirals for reducing the risk of mother-to-child transmission of HIV infection. *Cochrane Database Syst Rev* 2011(7):CD003510.
5. Baeten JM, Kahle E, Lingappa JR, et al. Genital HIV-1 RNA predicts risk of heterosexual HIV-1 transmission. *Sci Transl Med* 2011;3(77):77ra29.
6. Dumond JB, Yeh RF, Patterson KB, et al. Antiretroviral drug exposure in the female genital tract: implications for oral pre- and post-exposure prophylaxis. *AIDS* 2007;21(14):1899-907.
7. Kwara A, Delong A, Rezk N, et al. Antiretroviral drug concentrations and HIV RNA in the genital tract of HIV-infected women receiving long-term highly active antiretroviral therapy. *Clin Infect Dis* 2008;46(5):719-25.
8. Dumond JB, Nicol MR, Kendrick RN, et al. Pharmacokinetic modelling of efavirenz, atazanavir, lamivudine and tenofovir in the female genital tract of HIV-infected pre-menopausal women. *Clin Pharmacokinet* 2012;51(12):809-22.
9. Sheth AN, Evans-Strickfaden T, Haaland R, et al. HIV-1 genital shedding is suppressed in the setting of high genital antiretroviral drug concentrations throughout the menstrual cycle. *J Infect Dis* 2014;210(5):736-44.

10. Ravel J, Gajer P, Abdo Z, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4680-7.

11. Allsworth JE, Peipert JF. Prevalence of bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. *Obstetrics and gynecology* 2007;109(1):114-20.

12. 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. *J Infect Dis* 1999;180(6):1863-8.

13. Taha TE, Hoover DR, Dallabetta GA, et al. Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. *AIDS* 1998;12(13):1699-706.

14. Cu-Uvin S, Hogan JW, Caliendo AM, et al. Association between bacterial vaginosis and expression of human immunodeficiency virus type 1 RNA in the female genital tract. *Clin Infect Dis* 2001;33(6):894-6.

15. 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. *J Infect Dis* 2005;191(1):25-32.

16. Coleman JS, Hitti J, Bukusi EA, et al. Infectious correlates of HIV-1 shedding in the female upper and lower genital tracts. *AIDS* 2007;21(6):755-9.

17. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. *PLoS One* 2012;7(2):e30126.

18. Zalar A, Figueroa MI, Ruibal-Ares B, et al. Macrophage HIV-1 infection in duodenal tissue of patients on long term HAART. *Antiviral research* 2010;87(2):269-71.

19. Wong JK, Hezareh M, Gunthard HF, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* 1997;278(5341):1291-5.

20. Poles MA, Boscardin WJ, Elliott J, et al. Lack of decay of HIV-1 in gut-associated lymphoid tissue reservoirs in maximally suppressed individuals. *J Acquir Immune Defic Syndr* 2006;43(1):65-8.

21. Palmer S, Josefsson L, Coffin JM. HIV reservoirs and the possibility of a cure for HIV infection. *Journal of internal medicine* 2011;270(6):550-60.

22. Lampinen TM, Critchlow CW, Kuypers JM, et al. Association of antiretroviral therapy with detection of HIV-1 RNA and DNA in the anorectal mucosa of homosexual men. *AIDS* 2000;14(5):F69-75.

23. Imamichi H, Degray G, Dewar RL, et al. Lack of compartmentalization of HIV-1 quasispecies between the gut and peripheral blood compartments. *J Infect Dis* 2011;204(2):309-14.

24. Finzi D, Hermankova M, Pierson T, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 1997;278(5341):1295-300.

25. Di Stefano M, Favia A, Monno L, et al. Intracellular and cell-free (infectious) HIV-1 in rectal mucosa. *Journal of medical virology* 2001;65(4):637-43.

26. Chun TW, Stuyver L, Mizell SB, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* 1997;94(24):13193-7.

27. Chun TW, Nickle DC, Justement JS, et al. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. *J Infect Dis* 2008;197(5):714-20.

28. Chun TW, Carruth L, Finzi D, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* 1997;387(6629):183-8.

29. Amsel R, Totten PA, Spiegel CA, et al. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *The American journal of medicine* 1983;74(1):14-22.

30. Leitich H, Bodner-Adler B, Brunbauer M, et al. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *American journal of obstetrics and gynecology* 2003;189(1):139-47.

31. Ness RB, Kip KE, Hillier SL, et al. A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *American journal of epidemiology* 2005;162(6):585-90.

32. Wiesenfeld HC, Hillier SL, Krohn MA, et al. Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. *Clin Infect Dis* 2003;36(5):663-8.

33. Cherpes TL, Meyn LA, Krohn MA, et al. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clin Infect Dis* 2003;37(3):319-25.

34. Mitchell C, Marrazzo J. Bacterial vaginosis and the cervicovaginal immune response. *Am J Reprod Immunol* 2014;71(6):555-63.

35. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology* 1991;29(2):297-301.

36. Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. *Science* 2006;312(5778):1355-9.

37. Saxena D, Li Y, Yang L, et al. Human microbiome and HIV/AIDS. *Current HIV/AIDS reports* 2012;9(1):44-51.

38. Iwai S, Fei M, Huang D, et al. Oral and airway microbiota in HIV-infected pneumonia patients. *Journal of clinical microbiology* 2012;50(9):2995-3002.

39. Vujkovic-Cvijin I, Dunham RM, Iwai S, et al. Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci Transl Med* 2013;5(193):193ra91.

40. Gori A, Tincati C, Rizzardini G, et al. Early impairment of gut function and gut flora supporting a role for alteration of gastrointestinal mucosa in human immunodeficiency virus pathogenesis. *Journal of clinical microbiology* 2008;46(2):757-8.

41. Spear GT, Sikaroodi M, Zariffard MR, et al. Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. *J Infect Dis* 2008;198(8):1131-40.

42. Spear GT, Gilbert D, Landay AL, et al. Pyrosequencing of the genital microbiotas of HIV-seropositive and -seronegative women reveals Lactobacillus iners as the predominant Lactobacillus Species. *Applied and environmental microbiology* 2011;77(1):378-81.

43. Srinivasan S, Liu C, Mitchell CM, et al. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *Plos One* 2010;5(4):e10197.

44. Brotman RM. Vaginal microbiome and sexually transmitted infections: an epidemiologic perspective. *J Clin Invest* 2011;121(12):4610-7.

45. Brotman RM, Ravel J, Cone RA, et al. Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. *Sex Transm Infect* 2010;86(4):297-302.

46. Gajer P, Brotman RM, Bai G, et al. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;4(132):132ra52.

47. McClelland RS, Richardson BA, Graham SM, et al. A prospective study of risk factors for bacterial vaginosis in HIV-1-seronegative African women. *Sex Transm Dis* 2008;35(6):617-23.

48. Fethers KA, Fairley CK, Hocking JS, et al. Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clin Infect Dis* 2008;47(11):1426-35.

49. Esber A, Vicetti Miguel RD, Cherpes TL, et al. Risk of Bacterial Vaginosis Among Women With Herpes Simplex Virus Type 2 Infection: A Systematic Review and Meta-analysis. *J Infect Dis* 2015;212(1):8-17.

50. van de Wijgert JH, Borgdorff H, Verhelst R, et al. The vaginal microbiota: what have we learned after a decade of molecular characterization? *PLoS One* 2014;9(8):e105998.

51. Jespers V, Menten J, Smet H, et al. Quantification of bacterial species of the vaginal microbiome in different groups of women, using nucleic acid amplification tests. *BMC microbiology* 2012;12:83.

52. Hickey RJ, Abdo Z, Zhou X, et al. Effects of tampons and menses on the composition and diversity of vaginal microbial communities over time. *Bjog* 2013;120(6):695-704; discussion -6.

53. Rifkin SB, Smith MR, Brotman RM, et al. Hormonal contraception and risk of bacterial vaginosis diagnosis in an observational study of women attending STD clinics in Baltimore, MD. *Contraception* 2009;80(1):63-7.

54. Riggs M, Klebanoff M, Nansel T, et al. Longitudinal association between hormonal contraceptives and bacterial vaginosis in women of reproductive age. *Sex Transm Dis* 2007;34(12):954-9.

55. Bradshaw CS, Walker J, Fairley CK, et al. Prevalent and incident bacterial vaginosis are associated with sexual and contraceptive behaviours in young Australian women. *PLoS One* 2013;8(3):e57688.

56. Brotman RM, Shardell MD, Gajer P, et al. Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause* 2014;21(5):450-8.

57. Heinemann C, Reid G. Vaginal microbial diversity among postmenopausal women with and without hormone replacement therapy. *Canadian journal of microbiology* 2005;51(9):777-81.

58. 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):13-9.

59. Thompson CG, Cohen MS, Kashuba AD. Antiretroviral pharmacology in mucosal tissues. *J Acquir Immune Defic Syndr* 2013;63 Suppl 2:S240-7.

60. Saad R, Rizkallah MR, Aziz RK. Gut Pharmacomicrobiomics: the tip of an iceberg of complex interactions between drugs and gut-associated microbes. *Gut pathogens* 2012;4(1):16.

61. Evotaz [package insert]. Bristol Myers Squibb Australia Pty Ltd. Victoria AS. (http://www.medicines.org.au/files/bqpevota.pdf). (Accessed December 7 2015).

62. Truvada [package insert]. Gilead Sciences, Inc. Foster City, CA; Jan. 2016. (http://www.gilead.com/~/media/Files/pdfs/medicines/hiv/truvada/truvada_pi.PDF). (Accessed February 6, 2016).

63. Reyataz [package insert]. Bristol-Myers Squibb Co. Princeton, NJ; Sept. 2015. (http://packageinserts.bms.com/pi/pi_reyataz.pdf). (Accessed December 6, 2015).

64. Viread [package insert]. Gilead Sciences, Inc. Foster City, CA; May 2015. (http://www.gilead.com/~/media/Files/pdfs/medicines/hiv/viread/viread_pi.pdf). (Accessed).

65. Emtriva [package insert]. Gilead Sciences, Inc. Foster City, CA; Nov. 2015. (http://www.gilead.com/~/media/Files/pdfs/medicines/hiv/emtriva/emtriva_pi.pdf). (Accessed December 5, 2015).

66. National Center for Biotechnology Information. PubChem Compound Database; CID=464205. (https://pubchem.ncbi.nlm.nih.gov/compound/464205). (Accessed February 8 2016).

67. Macaluso M, Lawson L, Akers R, et al. Prostate-specific antigen in vaginal fluid as a biologic marker of condom failure. *Contraception* 1999;59(3):195-201.

68. Hobbs MM, Steiner MJ, Rich KD, et al. Good performance of rapid prostate-specific antigen test for detection of semen exposure in women: implications for qualitative research. *Sex Transm Dis* 2009;36(8):501-6.

69. Preparing Libraries for Sequencing on the MiSeq®. Illumina Inc.; 2013. (https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/preparing-libraries-for-sequencing-on-miseq-15039740-d.pdf). (Accessed Nov. 25, 2015).

70. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology* 2009;75(23):7537-41.

71. Edgar RC, Haas BJ, Clemente JC, et al. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics (Oxford, England)* 2011;27(16):2194-200.

72. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology* 2006;72(7):5069-72.

73. RStudio Team. RStudio: Integrated Development for R. Boston, MA: RStudio, Inc., 2015.

74. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;8(4):e61217.

75. Shannon CE. A mathematical theory of communication. *Bell System Technical Journal* 1948;27:379–423, 623–56.

76. Kuklenyik Z, Martin A, Pau CP, et al. Effect of mobile phase pH and organic content on LC-MS analysis of nucleoside and nucleotide HIV reverse transcriptase inhibitors. *Journal of chromatographic science* 2009;47(5):365-72.

77. Hummelen R, Fernandes AD, Macklaim JM, et al. Deep sequencing of the vaginal microbiota of women with HIV. *PLoS One* 2010;5(8):e12078.

78. Mehta SD, Donovan B, Weber KM, et al. The vaginal microbiota over an 8- to 10-year period in a cohort of HIV-infected and HIV-uninfected women. *PLoS One* 2015;10(2):e0116894.

79. Centers for Disease Control and Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD and TB Prevention, Div. of HIV/AIDS Prevention. HIV Surveillance by race/ethnicity (through 2014). (http://www.cdc.gov/hiv/pdf/library/slidesets/cdc-hiv-surveillance-race-ethnicity.pdf). (Accessed February 21 2016).

80. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. *Nature* 2014;509(7500):357-60.

81. Srinivasan S, Morgan MT, Liu C, et al. More than meets the eye: associations of vaginal bacteria with gram stain morphotypes using molecular phylogenetic analysis. *PLoS One* 2013;8(10):e78633.

82. Nakatsu G, Li X, Zhou H, et al. Gut mucosal microbiome across stages of colorectal carcinogenesis. *Nature communications* 2015;6:8727.

83. Ghartey JP, Smith BC, Chen Z, et al. Lactobacillus crispatus dominant vaginal microbiome is associated with inhibitory activity of female genital tract secretions against Escherichia coli. *PLoS One* 2014;9(5):e96659.

84. Martinez RC, Franceschini SA, Patta MC, et al. Analysis of vaginal lactobacilli from healthy and infected Brazilian women. *Applied and environmental microbiology* 2008;74(14):4539-42.

85. Hillier SL, Krohn MA, Rabe LK, et al. The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. *Clin Infect Dis* 1993;16 Suppl 4:S273-81.

86. Verstraelen H, Verhelst R, Claeys G, et al. Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC microbiology* 2009;9:116.

87. Elwell LP, Ferone R, Freeman GA, et al. Antibacterial activity and mechanism of action of 3'-azido-3'-deoxythymidine (BW A509U). *Antimicrobial agents and chemotherapy* 1987;31(2):274-80.

88. Jackson JB, Dick J, Tekle T, et al. Lack of antimicrobial activity by the antiretroviral drug nevirapine against common bacterial pathogens. *Antimicrobial agents and chemotherapy* 2009;53(8):3606-7.

TABLES AND FIGURES

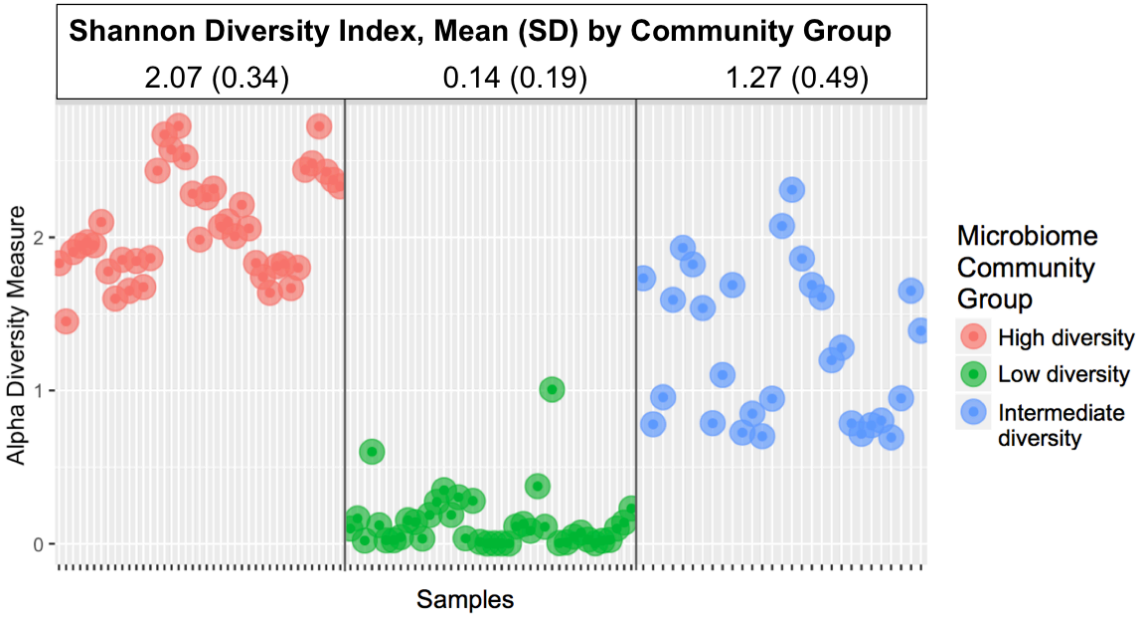
**Table 1.** Baseline demographic and clinical characteristics of study participants (N=20 participants)

| Variable | N (%), or median (range) |
|---|---|
| Age (years) | 38 (24-48) |
| Weight (lbs) | 184 (123-270) |
| BMI (kg/m$^2$) | 30 (21-51) |
| Race | |
| African American | 19 (95) |
| White | 1 (5) |
| Years since HIV diagnosis | 9 (1-17) |
| Nadir CD4 cell count (cells/mcl) | 110 (2-320) |
| Most recent CD4 cell count (cells/mcl) | 383 (71-1189) |
| < 200 | 2 (10) |
| 200 - 500 | 12 (60) |
| > 500 | 6 (30) |
| Antiretroviral therapy history | |
| Months since first ART regimen | 90 (9-115) |
| Months on current ART regimen | 14 (3-41) |
| Current hormone contraceptive use[a] | 1 (5) |
| Treatment of vaginal infection within 30 days | 6 (30) |
| Antibacterial agent[b] | 5 (20) |
| Antifungal agent[c] | 3 (15) |
| Vaginal product use or douching reported within 7 days of any study visits | 0 |
| Sexually active in the past 6 months | 17 (85) |
| 1 sexual partner | 16 (94) |
| 2 sexual partners | 1 (6) |
| Dysplasia on most recent pap smear | 5 (25) |
| Genital infections at screening visit[d] | |
| Gonorrhea | 0 |
| Chlamydia | 0 |
| Syphilis | 2 (10) |
| HSV2 IgG positive | 19 (95) |
| Candida on gram stain | 5 (25) |
| Bacterial vaginosis from vaginal Gram stain[e] | 5 (25) |

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; FGT, female genital tract; HSV2, herpes simplex virus type 2, IgG, immunoglobulin G

[a] Depot medroxyprogesterone
[b] Metronidazole alone or with additional oral antibacterial agents within 30 days of screening
[c] Fluconazole single oral dose or topical vaginal antifungal agent prescribed within 30 days of screening
[d] Women were excluded if bacterial vaginosis by Amsel's criteria, *Trichomonas* or vaginal candidiasis by wet mount or potassium-hydroxide staining of wet mount, or if abnormal vaginal discharge or genital ulcers at screening visit.
[e] Bacterial vaginosis diagnosed on vaginal gram stain by Nugent score ≥ 7

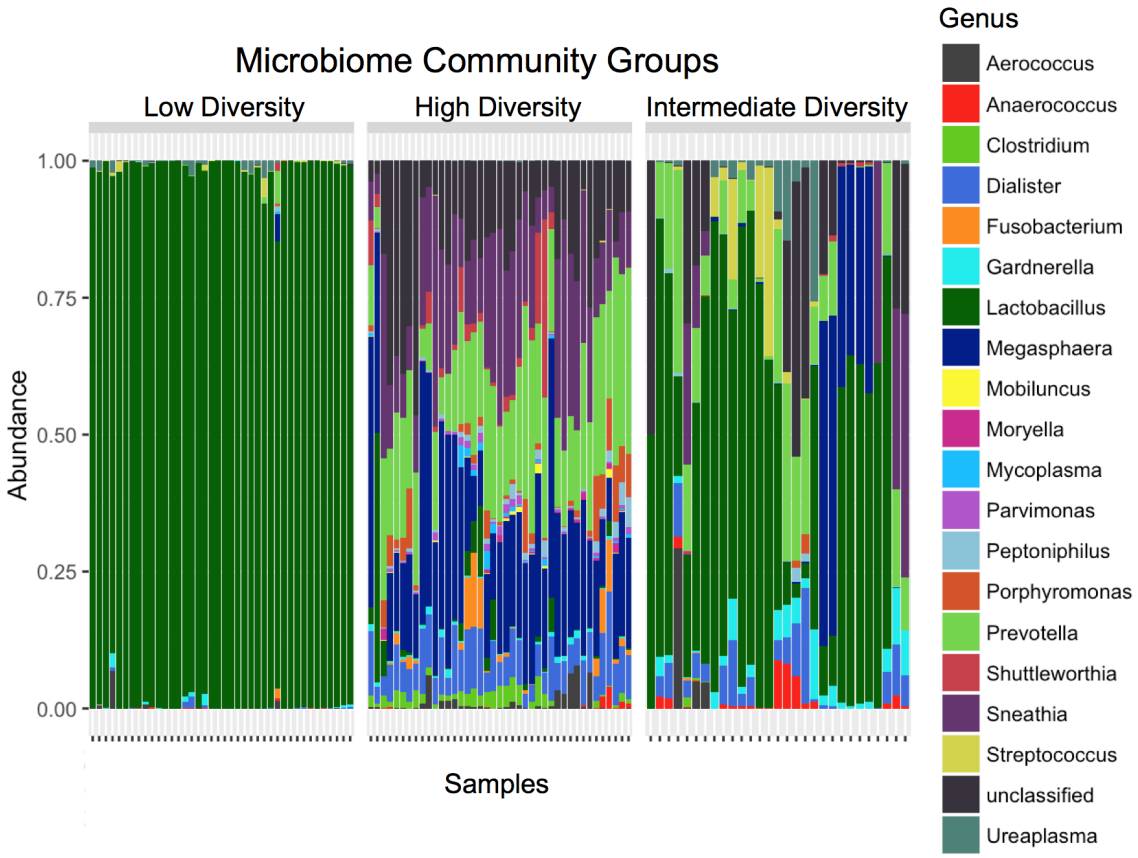
Figure 1. Alpha-diversity by Shannon Diversity Index of microbiome community groups[a]




Abbreviation: SD, standard deviation

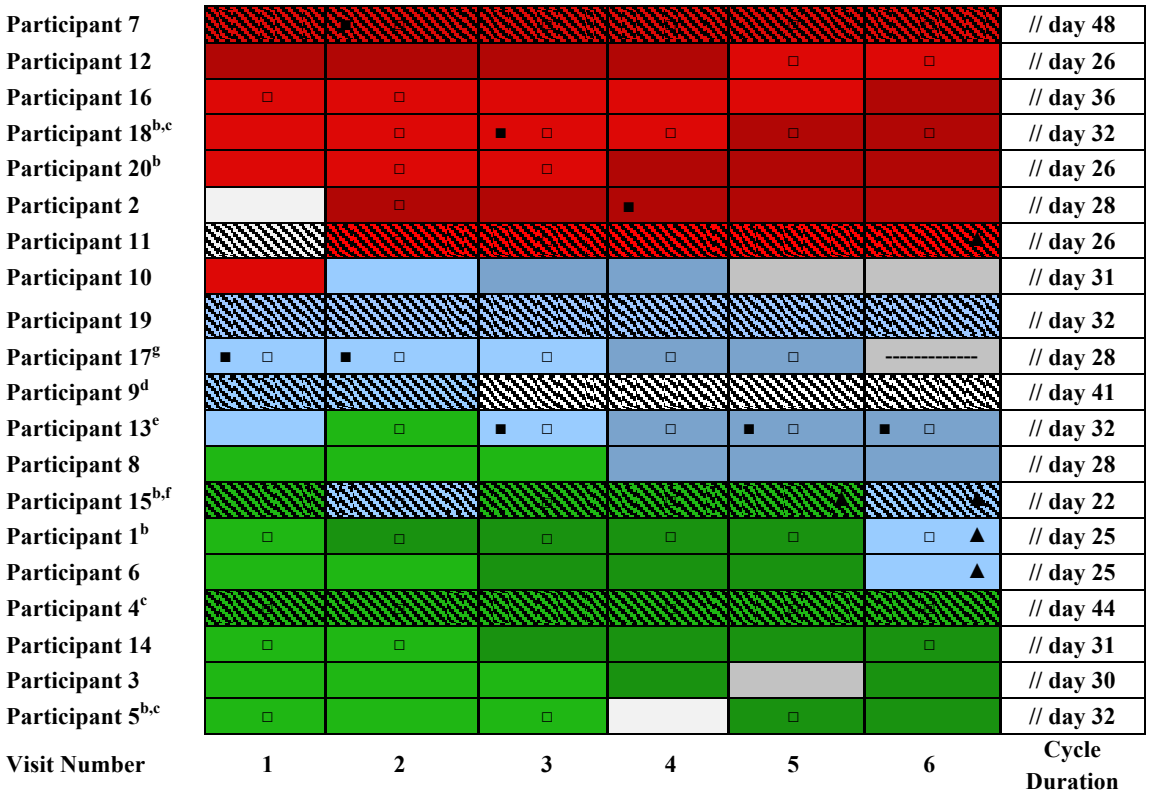
[a] Individual samples from each study visit (horizontal axis) underwent 16S rRNA gene sequencing and individual participant-visits were classified into microbiome community groups (colored red, green, or blue) using Dirichlet Multinomial Mixtures. Microbiome community groups were defined as high-, low-, or intermediate-diversity based on mean Shannon Diversity Index (as depicted in the box at the top of the figure).

**Figure 2.** Relative abundance of bacterial taxa by microbiome community group[a]




[a] Individual samples from each study visit (horizontal axis) underwent 16S rRNA gene sequencing and individual participant-visits were classified into microbiome community groups using Dirichlet Multinomial Mixtures. The relative abundance of the top-20 most abundant bacterial taxa by genus for each participant-visit are depicted by colored boxes.

**Figure 3.** Timeline of female genital tract microbiome community types by menstrual cycle phase over a single menstrual cycle for 20 HIV-infected women on antiretroviral therapy[a]




[a] Visits occurred twice weekly for three weeks following the week of menses and are numbered sequentially by order of time since preceding menses (visits 1-6). Microbiome community type for each visit is colored by box to correspond with high-diversity (red), intermediate-diversity (blue) and low-diversity (green) community types. Cycle duration was defined as the duration from onset of menses until the onset of the next menses according to self-report. Follicular phase (box not shaded) was determined as visits occurring between the end of menses and the start of the rise in serum progesterone. Luteal phase (light shading [ ]) was determined as any remaining days of the cycle until the onset of the next menses. A non-ovulatory phase (diagonal line shading [▨]) was defined as a cycle without a rise in serum progesterone during the study period. Reported vaginal sex within 7 days of a study visit [□] and vaginal semen contamination at the study visit [■] are shown. Five study visits were completed outside the study window due to menses onset prior to completion of study visits [▲].

[b] Oral antibiotic (metronidazole with or without additional oral antibiotic medications) received within 30 days of screening visit

[c] Oral or topical vaginal antifungal used within 30 days of screening visit

[d] Treated for symptomatic *Trichomonas* infection on visit 5

[e] Treated for symptomatic vaginal yeast infection on visit 3

[f] Was receiving hormonal contraception (depo medroxyprogesterone).

[g] Completed only 5 study visits (missed visit 6 marked [----])

**Table 2.** Distribution of clinical factors and $C_{24}$ antiretroviral drug concentrations by FGT microbiome community type over the menstrual cycle in 20 HIV-infected women[a]

| Variable | Total cohort, (N=109 Visits)[a] | Microbiome Community Type (CT) | | |
|---|---|---|---|---|
| | | Low-Diversity (N= 40 Visits) | High-Diversity (N= 41 Visits) | Intermediate-Diversity (N= 28 Visits) |
| Nugent score, median (Q1, Q3)[b] | 7 (4, 9) | 3 (1, 5) | 9 (8, 10) | 7 (5, 8) |
| Bacterial vaginosis, Nugent score ≥ 7, N (%)[c] | 56 (57) | 3 (9.1) | 34 (91.9) | 19 (67.9) |
| Age, years, median (Q1, Q3) | 38 (33, 41) | 37 ( 36, 43) | 40 (30, 41) | 35 (39, 36) |
| BMI, kg/m$^2$ median (Q1, Q3) | 27 (25, 37) | 26 (26, 37) | 27 (23, 32) | 37 (35, 41) |
| Antibiotic for vaginal infection within 30 days of screening, N (%)[c] | 29 (26.6) | 14 (35) | 12 (29.3) | 3 (10.7) |
| Sexually active within 7 days of study visit, N study visits (% )[d] | 47 (43.1) | 17 (42.5) | 20 (48.8) | 11 (39.3) |
| FGT semen contamination, visits (%)[e] | 8 (7.3) | 0 (0) | 3 (7.3) | 5 (17.9) |
| FGT Leukocytes, median cells/mcl (Q1, Q3) | 125 (15, 150) | 70 (70, 412) | 70 (15, 125) | 125 (42, 700) |
| FGT leukocytes > 125 cells/mcl (%) | 30 (27.5)) | 10 (25) | 9 (22) | 11 (39.3) |
| FGT RBCs, median cells/mcl (Q1, Q3) (N=108) | 25 (10, 100) | 25 (25, 100) | 25 (10, 165) | 80 (10, 525) |
| FGT RBCs > 25 cells/mcl (%) | 62 (57.4) | 18 (45) | 13 (32.5) | 15 (53.6) |
| Menstrual cycle characteristics, N study visits (%) | | | | |
| Non-ovulatory phase | 31 (28.4) | 10 (25) | 11 (26.8) | 10 (35.7) |
| Ovulatory phase | 78 (71.6) | 30 (75) | 30 (73.2) | 18 (64.3) |
| Follicular (N(%) of Ovulatory) | 39 (50) | 15 (50) | 16 (53.3) | 8 (44.4) |
| Luteal (N(%) of Ovulatory) | 39 (50) | 15 (50) | 14 (46.7) | 10 (55.6) |
| Plasma hormone concentrations, pg/ml (Q1, Q3) | | | | |
| Estradiol (N=108) | 27.01 (6.24, 58.5) | 27.14 (3.58, 61.15) | 24.88 (0, 51.10) | 35.96 (12.98, 63.15) |
| Progesterone (N=109) | 0.58 (0.31, 4.64) | 0.59 (0.29, 4.04) | 0.7 (0.38, 5.03) | 0.54 (0.3, 4.68) |
| FGT Antiretroviral concentrations, median $C_{24}$ ng/mL (Q1, Q3)[f] | | | | |
| ATV | 1435 (705, 2655) | 1200 (705, 2510 | 1435 (541, 2330) | 1818 (1048, 47778) |
| TDF | 215 (117, 473) | 157 (100, 251) | 202 (90, 343) | 560 (141, 1272) |
| FTC | 1252 (495, 1840) | 1252 (431, 1780) | 1284 (455, 1670) | 1133 (640, 2678) |
| Plasma Antiretroviral concentrations, median $C_{24}$ ng/mL (Q1, Q3) | | | | |
| ATV | 620 (380, 957) | 612 (400, 880) | 666 (346, 1390) | 612 (407, 915) |
| TDF | 74 (44, 104) | 89 (63, 120) | 76 (35, 101) | 55 (40, 88) |
| FTC | 69 (46, 131) | 98 (54, 152) | 64 (43, 135) | 55 (40, 88) |

Abbreviations: ATV, atazanavir; $C_{24}$, antiretroviral drug concentration measured 24 hours after last dose; CT, microbiome community type; FGT, female genital tract; FTC, emtricitabine; RBCs, red blood cells; SD, standard deviation; TDF, tenofovir; Q1, quartile 1; Q3, quartile 3

[a] Samples collected from N=20 participants during N=109 study visits unless N for the individual variable is otherwise specified. Study visits were completed in a single menstrual cycle for 16 participants or were completed outside the cycle window because of early menses prior to completion of study visits (4 participants, N= 5 (4.6 %) study visits).
[b] Participant visits with Gram stain and Nugent scores available, N=98 (low-diversity CT, N= 33; intermediate-diversity CT, N= 28 ; high-diversity CT, N= 37).
[c] Metronidazole alone or in combination with another oral antibiotic(s). Among the 5 participants with antibiotic use within 30 days of screening, the microbiome CT at the time of the first study visit was low-diversity (N=3), intermediate-diversity (N=0) and high-diversity (N=2).
[d] Includes participants with reported sexual activity in the past 7 days or participants with positive semen contamination of vaginal secretions but without reported sexual activity in the past 7 days (N=1).
[e] Semen contamination of cervicovaginal lavage fluid was assessed by detection of prostate specific antigen using the ABACard p30 antigen detection test.
[f] FGT antiretroviral concentrations available for N=107 visits (N=2 missing from 2 study visits by 1 participant, both visits with concurrent high-diversity microbiome CT identified).

**Table 3.** Univariate pairwise associations between FGT microbiome community types and clinical predictors among 20 HIV-infected women over the menstrual cycle (N=109 visits)[a]

| **Variable**[b] | **ORs for Microbiome CT** | | | | | |
|---|---|---|---|---|---|---|
| | Low- vs. Int.-Diversity (Ref=Int.) (95% CI) | P-value (Low- vs. Int-) | Low- vs. High-Diversity (Ref = High) (95% CI) | P-value (Low- vs. High-) | High- vs. Int-Diversity (Ref = Int.) (95% CI) | P-value (High- vs. Int.-) |
| Age (years) | 1.24 (0.95, 1.61) | 0.1049 | 1.01 (0.73, 1.40) | 0.9712 | 1.19 (0.88, 1.62) | 0.2593 |
| BMI (kg/m$^2$) | 0.87 (0.72, 1.04) | 0.1145 | 1.16 (0.85, 1.58) | 0.3370 | 0.68 (0.50, 0.94) | 0.0191 |
| Antibiotic for vaginal infection within 30 days of screening (Ref=None)[c] | 7.44 (0.22, 256.28) | 0.2607 | 2.15 (0.02, 213.34) | 0.7412 | 2.56 (0.03, 245.38) | 0.6815 |
| Sexual activity within 7 days of study visit (Ref=None)[d] | 1.38 (0.19, 10.11) | 0.7502 | 0.94 (0.08, 10.83) | 0.9594 | 1.38 (0.10, 18.36) | 0.8017 |
| FGT semen contamination present[e] | - | - | - | - | 1.38 (0.10, 18.36) | 0.7978 |
| FGT Leukocytes > 125 cells/mcl (95% CI) | 0.58 (0.11, 3.06) | 0.5162 | 1.12 (0.06, 22.83) | 0.9387 | 0.35 (0.03, 4.15) | 0.3991 |
| FGT RBCs > 25 cells/mcl (95% CI) | 0.83 (0.19, 3.56) | 0.8006 | 1.32 (0.12, 14.19) | 0.8139 | 0.72 (0.08, 6.79) | 0.7736 |
| Menstrual cycle phase | | | | | | |
| Follicular | 1.73 (0.33, 9.09) | 0.5070 | 0.98 (0.07, 14.84) | 0.9885 | 2.06 (0.16, 26.57) | 0.5719 |
| Luteal | Ref | Ref | Ref | Ref | Ref | Ref |
| Ovulation status | | | | | | |
| Non-ovulatory cycle | 0.36 (0.01, 11.22) | 0.5541 | 0.74 (0.01, 101.92) | 0.9048 | 0.485 (0.01, 34.65) | 0.7353 |
| Ovulatory cycle | Ref | Ref | Ref | Ref | Ref | Ref |
| Plasma hormone concentrations, pg/ml | | | | | | |
| Estradiol | 1.00 (0.98, 1.02) | 0.9003 | 1.01 (0.96, 1.05) | 0.8283 | 0.99 (0.96, 1.03) | 0.6713 |
| Progesterone | 0.97 (0.76, 1.24) | 0.0802 | 0.99 (0.70, 1.40) | 0.9528 | 0.94 (0.69 1.30) | 0.7090 |

Abbreviations: CT, Microbiome community type; FGT, female genital tract; Int., Intermediate; RBCs, red blood cells; OR, odds ratio; Ref, Reference group

[a] Generalized mixed models including a random intercept for the individual modeled the comparison between two CTs for each predictor.
[b] N=109 visits for each variable unless N is otherwise specified for the variable as per Table 2
[c] Metronidazole alone or in combination with other oral antibiotic(s)

[d] Includes participants with reported sexual activity in the past 7 days or participants with positive semen contamination of vaginal secretions but without reported sexual activity in the past 7 days (N=1).
[e] Unable to estimate the association between semen contamination and low-diversity CT as there were not any low-diversity CT study visits with semen contamination identified.

**Table 4.** Univariate associations between clinical predictors and genital antiretroviral drug concentrations among 20 HIV-infected women over the menstrual cycle (N=109 visits)

| | **Univariate associations with geometric mean FGT antiretroviral drug concentrations (ng/ml) (95% CI)[a]** | | | | | |
|---|---|---|---|---|---|---|
| **Variable Name** | **ATV** | **ATV P-value** | **TDF** | **TDF P-value** | **FTC** | **FTC P-value** |
| Age (years) | 0.99 (1.04-0.95) | 0.7810 | 0.981 (1.05-0.92) | 0.5873 | 0.98 (1.04-0.92) | 0.4814 |
| BMI ($kg/m^2$) | 1.01 (1.04-0.97) | 0.7563 | 1.045 (1.10-0.99) | 0.0897 | 1.03 (1.07-0.98) | 0.2758 |
| Sexual-activity within 7 days of study visit (Ref=None)[b] | 1.24 (1.85-0.83) | 0.2833 | 1.48 (1.001-1.48) | 0.0492 | 1.22 (1.68-0.89) | 0.2197 |
| FGT Leukocytes > 125 cells/mcl (95% CI) | 1.30 (1.97-0.89) | 0.2158 | 1.008 (1.51-0.67) | 0.9680 | 1.000 (1.39-0.72) | 0.9994 |
| FGT RBCs > 25 cells/mcl (95% CI) | 1.46 (2.09-1.03) | 0.0356 | 1.022 (1.44-0.73) | 0.8987 | 0.968 (1.28-0.73) | 0.8147 |
| Menstrual cycle phase | | | | | | |
| Follicular (N=39) | 1.02 (1.57-0.66) | 0.9355 | 1.165 (1.67-0.81) | 0.3972 | 0.94 (1.26-0.70) | 0.6677 |
| Luteal (N=39) | Ref | | Ref | | Ref | |
| Ovulation status | | | | | | |
| Non-ovulatory cycle (N=29) | 0.96 (1.84-0.50) | 0.8991 | 0.590 (1.48-0.24) | 0.2576 | 0.51 (1.10-0.24) | 0.0864 |
| Ovulatory cycle (N=78) | Ref | | Ref | | | |
| Plasma hormone concentrations, pg/ml | | | | | | |
| Estradiol | 1.00 (1.00-0.99) | 0.5936 | 1.003 (1.01-1.00) | 0.2635 | 1.001 (1.01-0.98) | 0.4888 |
| Progesterone | 0.99 (1.04-0.94) | 0.6911 | 0.987 (1.04-0.94) | 0.6177 | 0.997 (1.04-0.96) | 0.8707 |
| Plasma antiretroviral concentration (ng/ml)[c] | 1.5565 (1.89-1.28) | < 0.0001 | 1.021 (1.40-0.75) | 0.8946 | 1.210 (1.52-0.96) | 0.1019 |

Abbreviations: ATV, atazanavir; FGT, female genital tract; FTC, emtricitabine; RBCs, red blood cells; Ref, Reference group; TDF, tenofovir

[a] Univariate associations of log-transformed FGT drug concentrations with each clinical predictor was performed using univariate mixed linear models accounting for repeated measures with a random intercept for each participant. The proportional (multiplicative) change in geometric mean FGT ARV concentration is presented for a 1 unit increase in a continuous predictor or for the comparison to the reference outcome of a dichotomous predictor variable.

[b] Includes participants with reported sexual activity in the past 7 days or participants with positive semen contamination of vaginal secretions but with no reported sexual activity in the past 7 days (N=1).
[c] Plasma antiretroviral drug concentrations were log-transformed prior to analyses. The association between the concentration of the same FGT and plasma drug was assessed in each univariate model.

**Table 5.** Association between the female genital tract microbiome and the female genital tract concentrations of TDF, FTC and RTV-boosted ATV among 20 HIV-infected women

| **Microbiome CT** | Proportional change in FGT antiretroviral concentrations by univariate models (95% CI)(N=107 study visits)[a, b] | | | Proportional change in FGT antiretroviral concentrations by multivariable models (95% CI) (N=106 study visits)[b, c] | | |
|---|---|---|---|---|---|---|
| | **ATV** | **TDF** | **FTC** | **ATV** | **TDF** | **FTC** |
| **Low-Diversity** | 0.65 (0.37-1.12) | 0.51 (0.29-0.91)* | 0.71 (0.44-1.14) | 0.52 (0.31-0.88)* | 0.51 (0.28-0.91)* | 0.68 (0.42-1.10) |
| **High-Diversity** | 0.59 (0.31-1.12) | 0.46 (0.21-1.03) | 0.78 (0.38-1.62) | 0.46 (0.23-0.92)* | 0.54 (0.23-1.27) | 0.90 (0.43-1.91) |
| **Intermediate-Diversity** | Ref | Ref | Ref | Ref | Ref | Ref |
| P-value (column-wise comparison) | 0.1749 | 0.0384 | 0.3509 | 0.0260 | 0.0644 | 0.2716 |

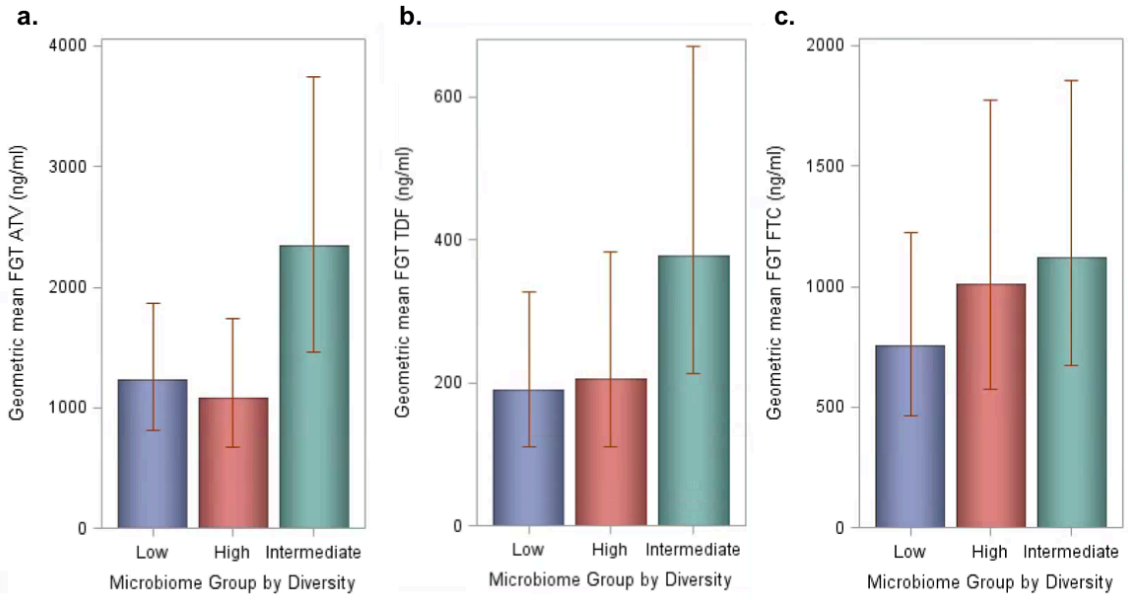
Abbreviations: ATV, atazanavir; BV, bacterial vaginosis; TDF, tenofovir; FTC, emtricitabine; CT, microbiome community type; Ref, reference group;
* denotes P<0.05

[a] Univariate mixed linear models accounted for repeated measures and modeled the outcome of log-transformed FGT antiretroviral concentration for each antiretroviral drug

[b] The proportional geometric mean (ng/ml) change in the estimate of the antiretrovirals TDF, FTC and RTV-boosted ATV for each CT compared to the reference CT is presented.

[c] Multivariable mixed linear models (one for each antiretroviral drug) accounted for repeated measures and modeled the outcome of change in log-transformed FGT ARV concentration. Covariates included in each model in addition to microbiome CT were: log-transformed plasma antiretroviral concentrations (same antiretroviral as evaluated for the model's outcome), body mass index, FGT red blood cells > 25 cells/mcl and reported sexual activity within the past 7 days (corrected to a positive response if vaginal semen contamination present (N=1)).

**Figure 4.** Estimated geometric mean female genital tract antiretroviral drug concentrations by microbiome community type from multivariable models[a]




Abbreviations: ARV, antiretroviral; conc., concentration; ATV, atazanavir; BV, bacterial vaginosis; CT, microbiome community type; TDF, tenofovir; FTC, emtricitabine

[a] Antiretroviral estimates depicted for ritonavir-boosted ATV (a), TDF (b) and FTC (c). Estimates of geometric mean antiretroviral drug concentrations were obtained using multivariable mixed linear models accounted for repeated measures. Covariates included in each model in addition to microbiome CT were: log-transformed plasma antiretroviral drug concentrations (same antiretroviral as evaluated for the model's outcome), body mass index, FGT red blood cells > 25 cells/mcl and reported sexual activity within the past 7 days (corrected to a positive response if vaginal semen contamination present (N=1)).