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Anar S. Patel Date

The association between female genital tract microbiome composition and female genital tract antiretroviral drug penetration in HIV-infected women: a pilot study

By

Anar S. Patel

Master of Science
Clinical Research

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

_____
Christine L. Kempton, M.D., M.Sc.
Committee Member

_____
Amita Manatunga, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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Anar S. Patel

B.A., University of Cincinnati, 2008

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An abstract of

A thesis submitted to the Faculty of the

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in partial fulfillment of the requirements for the degree of

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2018

ABSTRACT

The association between female genital tract microbiome composition and female genital tract antiretroviral drug penetration in HIV-infected women: a pilot study

By Anar S. Patel

Background: The impact of the vaginal microenvironment on genital tract antiretroviral drug penetration is unknown. We sought to characterize the vaginal microbiome of virally-suppressed HIV-infected women and examine the association between the vaginal microbiome and FGT ARV drug concentration.

Methods: We enrolled 58 virally-suppressed HIV-infected women in a single urban HIV clinic in Atlanta, GA between 2015-2016 who were on three ARV regimens with one of the following anchor drugs: ATV, DRV, and RAL. Participants underwent two study visits each for microbiome sampling and cervicovaginal and plasma ARV concentration collection. Microbiome analysis was performed utilizing 16s rRNA sequencing and each sample was characterized by diversity and abundance of *Lactobacillus* species. We used mixed linear models for bivariate and multivariable models to evaluate the association between microbial community type and FGT ARV drug concentration.

Results: Microbial community types were characterized as *Lactobacillus* dominant (low diversity) and non-*Lactobacillus*-dominant (high diversity) in 67% and 33% of the women, respectively. Significant clinical characteristics associated with percent change in FGT ARV concentration for any drug type in bivariate analyses included recent sexual activity, cigarette smoking, vaginal pH level, and log plasma ARV concentration. In multivariable analyses controlling for these factors, there was no significant association between microbial community type and FGT ARV concentration.

Conclusion: In this single center study of 58 virally-suppressed HIV-infected women on combination ART including either ATV, DRV, and RAL, female genital tract microbiome composition was not found to be significantly associated with change in FGT ARV concentration.

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ACKNOWLEDGEMENTS

This work was supported by: the National Center for Advancing Translational Sciences of the National Institutes of Health award number UL1TR000454 (PI: Dr. David S. Stephens), the National Institute of Allergy and Infectious Diseases award number 5U01AI103408-02 (PI: Dr. Igho Ofotokun), the National Institute of Allergy and Infectious Diseases award number 1K23AI114407 (PI: Anandi N. Sheth), Emory CFAR (NIAID P30AI050409), Atlanta WIHS (NIAID U01A1103408-01), ACTSI (UL1TR000454), and the Bristol-Myers Squibb Virology Fellows Program (A1424-536). Assay services were provided by the Biomarkers Core Laboratory at the Yerkes National Primate Research Center which is supported by the Yerkes National Primate Research Center Base (Grant 2P51RR000165-51). Ribosomal 16S sequencing and STI sample analysis was supported in part by the Center for AIDS Research (P30 AI050409).

I would like to thank my mentors, Drs. Igho Ofotokun and Anandi Sheth, my thesis committee members Drs. Amita Manatunga and Christine Kempton, and Dr. Mitch Klein for their teaching and insightful comments. I thank Dr. Renee Donahue Carlson for her valuable input and contributions to this research project. I would also like to thank my co-authors and collaborators including Dr. Tim Read, Michael Frisch, Dr. Christina Mehta, Dr. Caitlin Moran, Dr. Lisa Haddad and Dr. Colleen Kraft. I also thank my collaborators at the University of Alabama, Dr. Ed Acosta and Kevin Ryan, for their assistance. I would also like to thank the research participants and staff of the Women's Interagency HIV Study and the Grady Hospital Ponce de Leon Center.

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INTRODUCTION

Women comprise more than half of the nearly 37 million people living with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) worldwide. (1) Sexual transmission of HIV is the principle mode of spread of the virus throughout the world. (2) Due to a complex array of social, behavioral, and biological factors, women are particularly vulnerable to HIV infection. (1) Worldwide, women experience sexual violence at higher rates than men and are often unable to negotiate condom use in their sexual relationships. (3) Additionally, women are biologically twice as susceptible to HIV acquisition during unprotected vaginal sexual intercourse compared to men and this risk is increased if either partner has a sexually transmitted infection (STI), if the partner has a high viral load (VL) or in the setting of mucosal inflammation or vaginal dysbiosis such as the presence of bacterial vaginosis (BV). (1)

The use of combination antiretroviral treatment (ART) to prevent disease progression and development of effective regimens to prevent mother-to-child transmission have achieved substantial success in slowing the spread of HIV. The advancement of effective antiretroviral (ARV) drugs in HIV-infected individuals over the past decade has also led to the conception of biomedical prevention tools in the form of oral and topical ARVs to decrease HIV susceptibility in uninfected individuals; this concept is known as pre-exposure prophylaxis (PrEP). (4) PrEP is an HIV prevention strategy that is recommended for individuals at substantial risk of HIV as part of combination HIV prevention package. (1) Currently the only drug approved for use for PrEP is called Truvada, which is a combination of two ARVs: emtricitabine and tenofovir. Truvada can be more than 96% effective in preventing HIV acquisition in HIV-uninfected adults when taken with optimal adherence. The exact mechanism by which Truvada works to protect against HIV acquisition is unknown but it is hypothesized that it plays a role in suppressing the virus

from taking hold in sites of HIV acquisition, such as in the genital tract. (5,6) While Truvada is an effective medication used for PrEP, it requires long-term renal function and bone density monitoring for those who take it. Therefore, additional studies are underway to find other target medications that can potentially serve as agents for PrEP. (4,7)

Reduction of HIV viral load not only in blood, but in sites of HIV acquisition such as mucosal tissues potentially have an influence on the efficacy of ARVs when used for the purposes of PrEP. (2) Prior investigation on the penetration of ARVs into the female genital tract (FGT) has shown that achievable drug concentrations in that compartment are highly drug specific and have high intra- and inter-individual variability (8). It has been hypothesized that the degree of compartmentalization between the genital tract and peripheral circulation is a result of differences in viral load response to ART or local responses to mucosal inflammation or infection. (2) Antiretrovirals that quickly and effectively accumulate into the female genital tract represent the best candidates for PrEP in HIV-uninfected women (9) and, furthermore, enhance prevention of mother-to-child transmission, control of local viral replication, and decrease viral burden in HIV sanctuary sites in women who are HIV-infected. (9)

The penetration of ARVs into the FGT is regulated by a number of complex variables that include both drug-related factors and those factors related to the genital tract mucosal environment. Drug factors include drug lipophilicity, degree of protein binding, expression of efflux and uptake transporters in cervical and vaginal cells, drug pH dissociation constant (K_d), and the effect of variability of endogenous hormones during phases of the menstrual cycle. (10,11) The vaginal microbiome is a dynamic bacterial community with many functions and influences. It is known to be composed of many different species of bacterial that collaborate to enhance the health of the vaginal mucosa, and also influence its susceptibility to HIV infection

and other STI's. (12) The vaginal microbiota is considered optimal when *Lactobacillus* is the predominant bacterial morphotype (13); its function is to maintain an acidic environment by secretion of lactic acid. (11) Conversely, highly diverse vaginal bacterial communities including the presence of *Gardnerella vaginalis* alone or codominant with other anaerobic bacteria are known to increase HIV susceptibility and lead to negative reproductive outcomes. Bacterial vaginosis (BV) occurs after there has been a shift from a *Lactobacillus* dominated microbial community to one characterized by increased heterogeneity and higher vaginal pH. (14) Various factors contribute to the distribution and diversity of the female genital tract microbiome including race and ethnicity, inflammation and infection, sexual and hygiene practices, cigarette smoking, (15) endogenous and exogenous sex hormones (16), and antimicrobial use. (11)

It has been hypothesized that alterations of the FGT microbiota towards a community type that is more diverse has the potential to influence drug concentrations in the FGT. The CAPRISA 004 study evaluated the efficacy of topical tenofovir gel for HIV prevention in 688 sub-Saharan African women. The study overall demonstrated that topical tenofovir gel had only moderate efficacy (39%) in preventing HIV acquisition, but importantly, that the genital tract microbiome community type played an important role in modulating the level of efficacy. In study participants who had a vaginal microbiome with increased dysbiosis and low levels of *Lactobacillus* species, efficacy of topical tenofovir gel decreased to 18%; further in vitro studies demonstrated that tenofovir was depleted secondary to metabolism by *Gardnerella vaginalis* species. (17) Another post-hoc analysis of the Partners-PrEP study demonstrated that the efficacy of Truvada, which is only formulated for oral administration, for PrEP did not differ in women with bacterial vaginosis (13). Further studies are needed to characterize the intersection between the vaginal microenvironment and antiretroviral drug levels, and to provide evidence about the

importance of the vaginal microbiome on prevention efficacy to improve this HIV-prevention strategy for women.

The primary aim of this study is to estimate the association between the female genital tract microbiome composition and female genital tract antiretroviral drug concentration in HIV-infected women in three different antiretroviral drug types: atazanavir (ATV), darunavir (DRV), and raltegravir (RAL). We hypothesize that FGT ARV concentration will differ in microbial community types that are low in *Lactobacillus* species compared to those that are dominated by *Lactobacillus* species.

To assess this hypothesis, we performed a prospective, longitudinal cohort study of 58 HIV-infected adult women with plasma HIV viral suppression who were on three different antiretroviral drug regimens anchored by darunavir, atazanavir, and raltegravir. Each participant underwent two study visits with collection of plasma/serum and genital tract samples. The genital tract microbiome samples were analyzed utilizing 16s rRNA gene sequencing with clustering of similar cervicovaginal microbial community types (MCT). Demographic, behavioral, and clinical factors were assessed as covariates in a model which assessed the association between the causal factor: the microbial community type and the outcome: percent change in FGT ARV drug concentration. The association was assessed using bivariate mixed linear models with a random intercept for the individual and multivariable models controlling for significant covariates for change in FGT ARV concentration.

BACKGROUND

Antiretroviral therapy has averted approximately 7.6 million deaths globally and is now a key component of modern HIV prevention efforts. (11) Because the incidence of HIV remains alarmingly high in many parts of the world, the use of antiretroviral drugs for pre-exposure prophylaxis is important in reducing the risk of infection after unprotected sexual exposure. (18) ARV drug penetration into genital mucosal tissues is thought to play a critical role in both preventing HIV infection at time of exposure (19) and HIV cervical viral shedding in HIV-infected women. (8) The extent to which systemically administered drugs distributes into various compartments, including the FGT, depends on several host-specific and drug-specific factors (11), though studies assessing the impact of these influences on ARV drug penetration in the genital tract are currently limited.

The human vagina and the bacterial communities that reside within it have a balanced mutualistic association. These indigenous bacterial communities play a protective role in preventing colonization by potentially pathogenic organisms including those that cause bacterial vaginosis. (20) In particular, cumulative evidence shows that a group of vaginotropic *Lactobacillus* species (21) are the keystone in creating a healthy vaginal microenvironment. (20) These species, *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*, among others, produce lactic acid as a fermentation product to create a low protective pH between 3.5 - 4.0. (22) In addition to lactic acid, many *Lactobacillus* species are also known to produce other antimicrobial compounds, such as bacteriocins and hydrogen peroxide. Bacteriocins are proteinaceous, bactericidal substances synthesized by bacteria that play a major role in combating growth of pathogenic organisms. Hydrogen peroxide, produced in vitro under aerobic conditions, also inhibit colonization of potential pathogenic bacteria. (20) Overall, evidence suggests that there

are a variety of compositions of the vaginal microbiota but those dominated by *Lactobacillus* species are considered to be "healthy."

Vaginal bacterial communities that lack *Lactobacillu*s species and are comprised of a diverse array of facultative or strictly anaerobic bacteria can result in a clinical diagnosis of bacterial vaginosis. BV is the most prevalent vaginal disorder in reproductive age women and can be characterized by vaginal discharge, elevated vaginal pH, and the presence of "clue" cells on microscopic examination, though many women are asymptomatic. (20) BV can be diagnosed by a variety of methods including gram stain and the use of Amsel criteria which utilize symptomatic and clinical criteria. The Nugent score (23), primarily used in research settings, is optimal for diagnosing asymptomatic women with BV using a scored gram stained vaginal smear to assess the relative number of bacteria and presence of polymorphonuclear leukocytes. (23,24)

There is evidence for an association between BV and acquisition of other STI's, such as HSV-2 infection, *Trichomonas vaginalis*, human papilloma virus infection, and HIV (25,26). Other longitudinal studies demonstrate an increased risk of gonorrhea and chlamydia infection when BV is present. (21) Prevalence of BV also varies in populations of women across the world and among subsets of women in the United States. Highest prevalence of BV in the United States is found in African-American women, which also the group most heavily affected by the HIV epidemic. (21) These racial difference are not fully explained by known risk factors for BV which include sexual behavior, presence of other STI's, smoking, contraceptive use, douching and socioeconomic status (15,27–29). In summary, African-American women more often have a vaginal microbiota that is not dominated by *Lactobacillus* species compared to those in Caucasian women. (21)

There are a variety of clinical, behavioral, and sociodemographic factors that influence microbiome composition in each individual. Sexual behaviors are shown to be associated with concentration of specific vaginal microbiota (30). Prostate specific antigen (PSA) located in semen, which is a validated marker of recent unprotected intercourse within the prior 72 hours, was strongly associated with decreased overall prevalence of *Lactobacillus* species (31,32). BV and increased presence of *Gardnerella vaginalis* and *L. iners* is associated with exposure to a new partner and with more than one sexual partner within three months. It has been postulated that a new partner could possible introduce a new strain of *G. vaginalis* that leads to microbial instability. (33) Studies also show that intravaginal cleansing are a risk factor for the development of intermediate vaginal flora and bacterial vaginosis in women with normal vaginal flora at baseline (28). A meta-analysis provided compelling evidence that women using hormone contraceptives have a decreased risk of BV through several mechanisms. It is hypothesized that estrogen-containing contraceptives increase the glycogen-content of vaginal epithelial cells which in turn becomes metabolized to lactic acid and thus elicits a cytokine response in the genital tract that inhibits BV. (16,29) Both progesterone- and estrogen-containing contraceptives regulate numerous immune mechanisms in the genital epithelial tract, such as immunoglobulins, leukocyte protease inhibitor, and recruitment of functional immune cells (such as lymphocytes and macrophages) and production of cytokines. (16,29,34) These hormonal actions are complex and concentration-dependent. Another mechanism by which progesterone-containing hormone contraceptives are thought to protect against BV is by reducing the frequency of menstruation. A number of studies have additionally reported that BV is detected more commonly at the beginning of the menstrual cycle when estradiol levels are lowest. (29)

Orally and topically administered antiretroviral drugs have been explored for use as pre-exposure prophylaxis (PrEP) in HIV-negative individuals at risk for HIV acquisition. Rapid penetration, high accumulation, and long half-life within sites of HIV transmission, such as the female genital tract, are important features for PrEP interventions. (7,35) Drug distribution into the female genital tract is influenced by multiple factors such as hormonal changes (16), inflammation (36), and concomitant sexually transmitted infections (37). Drug-related factors of the antiretrovirals such as protein binding, lipophilicity, and pK_a are also hypothesized to impact the drug's ability to penetrate mucosal tissue in the female genital tract. (18)

Data on drug penetration in the female genital tract have lagged behind studies on men due to practical difficulties in sample acquisition. In the past few years, advances in sampling strategies and microbial analytic techniques have allowed for significant progress to be made on detailing the pharmacokinetics of ART in the female genital tract. (2) Repeated cervicovaginal sampling at regular intervals after drug dosing have allowed the creation of concentration-time curves (AUC) of the matrix of genital tract relative to plasma. Cervicovaginal fluid (CVF) sampling is the primary method by which an AUC can be calculated for the female genital tract due to its non-invasive approach and ease of sample collection. (18) However, even with using AUC, results obtained from the same drug between and within studies can be disparate; this is largely because individual time-specific genital tract:blood plasma ratios can vary considerably depending on time of drug intake and sample collection. (2)

Recent pharmacokinetic studies have confirmed that all nucleoside/nucleotide analogue-type antiretrovirals (NRTI/NnRTI) reach concentrations in cervicovaginal fluid that exceed corresponding plasma levels. In contrast, protease inhibitor (PI) disposition into the genital tract varies significantly by drug with protein binding being the most important factor determining the

achievable drug concentration. There is limited data on integrase inhibitor pharmacokinetics in the genital tract though a few limited studies have evaluated single and steady-state raltegravir pharmacokinetics in the FGT. Raltegravir concentrations in cervicovaginal fluid was equivalent or higher than blood levels and its half-life was noted to be twice as long in CVF than in the blood. Since raltegravir blocks the entry of HIV into host cell DNA, it is thought to be particularly useful for preventive use. (2)

Mucosal tissues represent a particularly dynamic microenvironment that contributes significantly to pharmacological factors that determine drug distribution into those compartments. Some of these factors include protein binding, membrane drug transporter activity, and endogenous hormones. Protein binding is important to consider because only the unbound fraction or free drug is available for pharmacological activity. The two main proteins responsible for binding ARVs include albumin and alpha-acid glycoprotein but the extent of drug binding is variable among ARV classes. (38,39) Additionally, transporter proteins on the cell membranes of genital tract mucosal cells are involved in the uptake and efflux of drugs. Drug transport affinity for ARVs have been found to correlate with genital tract tissue concentrations. (2,38,40) The expression of efflux transporters is also influenced by inflammatory cytokines released in response to infection or injury. Certain efflux transporters (MDR1, BCRP) are decreased by inflammatory mediators such as IL-6, IL-1β, ad INF-γ. It can thus be inferred that genital tract infection, inflammation, or injury may affect ARV drug distribution due to cytokine-mediated variations in drug transporter expression. (10) Physiological and hormonal variations of endogenous hormones alter protein binding though the data is very limited. Two studies by Sheth, et al.(8) and Clavel, et al. (41) demonstrated high inter-individual variability in genital tract ARV concentrations throughout the menstrual cycle

though the impact of menstrual cycle phase on drug distribution in the female genital tract compartment has not yet been clearly delineated.

ART distribution into the FGT is regulated by a number of complex variables including drug lipophilicity, degree of protein binding, expression of efflux and uptake transporters in cervical and vaginal cells, drug's pH-dependent dissociation constant (K_d) and the effect of variability of endogenous hormones during phases of the menstrual cycle. (10,42) It has been hypothesized that transformation of the microbiome composition, particularly to one that has decreased protective *Lactobacillus* species or increased dysbiosis, can greatly impact drug penetration into the FGT. (42) Previous studies (43–45), including a study by our group in a cohort of 20 HIV-infected women receiving the same ART regimen (8), reported highly variable FGT antiretroviral concentrations, suggesting that, in addition to drug-specific properties, the vaginal environment may affects compartmental drug distribution.

In this prospective cohort study, we will characterize the vaginal microbiome of 58 virally-suppressed HIV-infected women on three different antiretroviral anchor drugs: darunavir (DRV), atazanavir (ATV), and raltegravir (RAL) and examine its mediating factors, such as age, race, menstrual cycle phase, and hormonal contraception with the ultimate goal to explore the interaction between FGT ART exposure and vaginal microbiome. We speculate that the variability in FGT antiretroviral concentration is related, in part, to perturbation in the vaginal microbiota and that these mechanisms may be related to factors that relate to both the drug and the mucosal environment.

METHODS

Hypothesis and specific aims

This analysis was conducted with the following specific aim:

1) Primary aim: To estimate the association between FGT microbiome composition and FGT ARV concentration in HIV-infected women in three different antiretroviral drug types: atazanavir, darunavir, and raltegravir.

Our hypothesis was that the female genital tract ARV drug concentration would differ in microbial community types that were not low in *Lactobacillus* species compared to those that were dominated by *Lactobacillus* species.

Patient selection and data collection

This was a single-center, prospective, observational cohort study of 58 virologically suppressed HIV-infected women who were adherent to one of three common antiretroviral regimens. The women were enrolled and recruited from the Atlanta Clinical Research Site (CRS) of the Women's Interagency HIV Study (WIHS), a multicenter prospective cohort of HIV-infected women in the U.S., and the Grady Infectious Diseases Program (Atlanta, GA).

Inclusion criteria were: engagement in HIV care within 6 months prior to enrollment, biological female ≥ 18 years of age, HIV-1 infection documented by WIHS enrollment or by any currently approved diagnostic test, HIV viral suppression as defined by HIV-1 RNA <200 cells/mL within the 6 months prior to study enrollment, taking combination ART regimen containing either atazanavir (ATV), darunavir (DRV), or raltegravir (RAL) with at least two additional HIV antiretroviral drugs for ≥ 4 weeks prior to study enrollment, and ability and willingness to undergo serial pelvic examination, cervicovaginal sample collection, and venipuncture. Exclusion criteria were: ART non-adherence within 1 month of screening for

enrollment (as defined by less than 90% ART adherence in prior month and missed ART doses in 4 days prior to study visit 1), pregnancy as documented by clinical history or positive urine pregnancy test, pelvic examination already performed by another provider on the day of study visit 1, or cervicovaginal lavage or pap smear within 14 days or colposcopy with biopsy within 1 month of study visit 1, contraindication to specified ART-regimens, or known history of any medical condition that would interfere with conduct of the study, in the opinion of the study investigator. The protocol and protocol consent form was approved by the Emory Institutional Review Board and the Grady Health System Research Oversight Committee. All participants provided informed consent.

Study visits

Detailed demographic, medical, sexual, and reproductive histories were collected and physical examination including pelvic examinations were performed on all participants. Participants underwent two study visits during follicular and luteal phases of a single menstrual cycle timed to correspond with plasma trough concentrations (~12 hours for RAL and ~24 hours for ATV and DRV) for the collection of antiretroviral trough plasma samples and cervicovaginal lavage (CVL) (TearFlo wicks, HUB Pharmaceuticals, Rancho Cucamonga, CA) separated within 2 hours of sample collection into supernatants and cellular pellets via centrifugation. All samples were stored at -80^{o}C until analyses. Study visit 1 was scheduled at least 14 days following a WIHS core research visit or any other clinical or research visit that included a pap smear or use of a cervicovaginal lavage and at least 1 month following any colposcopy with biopsy to allow healing from any trauma secondary to these procedures. A medical history was obtained at study visit 1 with interim history at the subsequent study visit. A general medical history included

questions about genitourinary symptoms, HIV ART treatment and adherence, menstrual history, sexual history, and behavioral history. A general physical exam including genitourinary/pelvic exam was performed at each study visit and any genitourinary lesions, ulcers, or abnormal vaginal discharge was documented. Complete blood count, chemistries, liver function tests, CD4, and HIV-1 RNA at baseline was abstracted from the participants most recent WIHS core visit records or Grady clinic records. HIV-1 infection was verified by review of serologic test, western blot, or plasma HIV-1 RNA documentation within the participants WIHS study or clinical chart.

Subjects were asked on screening if they had regular menstrual periods, defined as periods occurring within a 22 to 35 day interval for the preceding three cycles. Women not meeting these criteria due to post-menopausal status, having menstrual cycle irregularity, or using long-acting hormonal contraception were documented as such.

The scheduling of study visit 1 was irrespective of current phase of the menstrual cycle and the visit was rescheduled if the woman was actively menstruating. Study visit 2 was scheduled in the next phase of the menstrual cycle after study visit 1 (typically within the following 14 days but no sooner than 5 days) for pre-menopausal women with regular menstrual cycles. For post-menopausal women or women whose menstrual cycles were not regular, study visit 2 was scheduled 14 days after study visit 1 (with acceptable range 10-21 days after study visit 1). (**Table 1**)

Female genital tract sample collection occurred after placement of a speculum. It was preferable for the speculum to be inserted without lubricant or water to avoid specimen contamination but was utilized if requested by the patient or if extreme discomfort was observed during examination. The Catch-ALL^{TM} swab was collected first to minimize specimen contamination for microbiome analysis.

Measurements and laboratory testing

Sexually transmitted infections

Serum rapid plasma regain (RPR) to assess for syphilis infection was collected as part of study visit 1 on all participants. A positive RPR reflexed to a treponemal confirmatory test. A participant's result was recorded as positive if they had a positive RPR and treponemal test and if the RPR was elevated ≥ 4-fold from their prior RPR titer or, if unavailable, participant self report of no prior treatment or testing for syphilis. Serology for herpes simplex virus 2 (HSV-2) infection was performed using HerpeSelect 2 (Focus Diagnostics, Cypress, CA). Clinically collected cervicovaginal swabs were tested for *Neisseria gonorrhea*, *Chlamydia trachomatis,* and *Trichomonas vaginalis* by multiplex PCR (Gen-Probe APTIMA, San Diego, CA) at each study visit.

Bacterial vaginosis

Gram stain for Nugent score was performed on whole CVL fluid or CVL cell pellet depending on availability of specimens. Nugent score was characterized as positive for bacterial vaginosis (BV) if ≥ 7 and negative if < 7. Nugent scoring was performed by two individuals trained with Nugent score methodology: medical study Connor Blackwell and laboratory technician Aswani Vunnava.

Semen contamination

The presence of semen in whole CVL was tested using the ABACard p30 antigen detection kit (Abascus Diagnostics, West Hill, CA). The ABACard p30 tests for the presence of prostate specific antigen in semen which has high sensitivity and specificity for determining if sexual activity took place within 72 hours of the test and up to 5 days. Women were considered

to have recent sexual activity (within 7 days of study visit) if this was self-reported and/or if semen contamination was present at the study visit.

Hormone levels

Plasma estradiol and progesterone concentrations were measured using immunochemistry analyzer (DRG Hybrid XL, DRG International, Springfield, NJ) as well as liquid chromatography-mass spectrometry.

Vaginal pH

Vaginal pH was measured utilizing microelectrode pH meter (Oakton Model pH Spear) which was applied to the vaginal mucosa at the highest point in the posterior fornix to obtain the pH reading. Two measurements were taken during each pelvic examination and the average of both was utilized in analysis. To assess the accuracy of pH electrode measurements, pH paper was also used to document pH levels by applying it to gram stain swab of the posterior fornix.

Vaginal microbiome composition

Catch-ALL^{TM} swabs were collected from each study participant at each study visit for microbiome analysis. Prior to storage, one swab was immediately swirled in MoBio buffer in a MoBIo tube and the other was replaced in its sterile packaging.

ARV drug concentrations

Antiretroviral drug concentrations were measured at each study visit in the plasma and cervicovaginal wick sample (Weck-Cel) collected from the ectocervix and/or posterior fornix of the vagina. Each sample was analyzed by the Department of Pharmacology at the University of Alabama (Dr. Ed Acosta) using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS).

16S rRNA gene sequencing

DNA was extracted from CVL pellets using the Qiagen EZ1 DNA Tissue kit (Qiagen, Germantown, MD) with the Qiagen bacterial card on the Qiagen EZ1 Advanced XL instrument according to manufacturer's instructions.16S rRNA gene microbial census sequencing library preparation was carried out using the Illumina MiSeq procedures. Sequence processing was performed using Mothur software. After generating contigs from reads, sequences with ≥ 1 ambiguous bases and a length ≥ 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 for removal of chimeric sequences and classification with a Bayesian classifier and the GreenGenes database. Operational taxonomic units (OTU) clustering was performed using 95% sequence homology and taxonomic assignments were made using the GreenGenes database. Further analysis of sequencing data utilized R Studio and Phyloseq package. Each participant visit was clustered into a microbial community type (mCT) compromised of similar abundance and type of bacterial taxa using Dirichlet Multinomial Mixtures with the Dirichlet Multinomial R package. Alpha-diversity was measured for each sample using the Shannon Index and results averaged across mCT. Principle coordinates analysis utilized unifrac distances.

Statistical analysis

Sample size calculations

A previous study by our research group of 20 HIV-infected women on ART demonstrated a geometric mean cervicovaginal wick trough ATV concentration of 1440 ng/mL (standard deviation ~300 ng/mL). Using this estimate, a sample size of 60 enrolled subjects for Aim 1 analysis allows a 90% power to detect a 255 ng/mL (>15%) difference or larger in FGT

drug concentration between two vaginal bacterial communities (using a two-sample t-test on change, $\alpha = 0.05$). Assuming a high standard error of 500 ng/mL in the coefficient describing the impact of bacterial community on FGT drug concentration, a sample size of 20 subjects in each group allows 80% power to detect a 455 ng/mL difference or larger between ART regimen groups. However, it is noted that no threshold concentration for either treatment or protective efficacy has been reported in the literature for ARV drug concentration in either the plasma or genital tract.

Analyses

For our primary aim, the primary exposure was the predominant vaginal bacterial community or microbial community type (MCT). The primary outcome was percent change in FGT plasma antiretroviral drug concentration. All antiretroviral drug concentrations were log-transformed prior to analysis to normalize its distribution. Separate linear regression models for each independent variable was performed to estimate its association with drug concentration.

Clinical and behavioral factors that served as independent variables included: age, body mass index (BMI), antibiotic use within 7 days of either study visit, sexual activity within 7 days of either study visit as defined by participant self report or positive PSA testing, menstrual cycle phase (follicular, luteal), ovulatory phase (ovulatory, non-ovulatory), presence of bacterial vaginosis, any frequency of cigarette smoking within 6 months of study visit, presence of diabetes, intravenous or oral antibiotic use within 7 days of either study visit, vaginal douching within 7 days of either study visit, presence of STI (including gonorrhea, chlamydia, HSV-2, syphilis or trichomonas), use of hormonal contraception within 30 days of screening visit (including oral contraceptive, intrauterine device, implant or injectable), vaginal pH, plasma sex hormone levels (estradiol and progesterone), and plasma ARV concentration. To evaluate

associations between these variables and the outcome, mixed linear models were carried out using the covariates as expressed above as the exposure and the percent change in log-transformed FGT antiretroviral drug concentration as the outcome.

The association between MCT (non-*Lactobacillus*-dominant versus *Lactobacillus*-dominant) and the percent change in FGT drug concentration were also assessed in bivariate mixed linear models. Multivariable models were subsequently conducted to assess for percent change in log-transformed FGT drug concentration which included those covariates which were found to be significantly associated with change in FGT antiretroviral concentration for any drug type. SAS v. 9.4 (Cary, NC) was used for statistical analysis.

RESULTS

Fifty eight women were enrolled and completed a total of 116 study visits. Participants had median age of 48 years (IQR 39 – 54) and the majority identified as African American (95%). The median CD4 count was 586 cells/mcl (IQR 394 – 792) and 56 women had HIV-1 viral suppression as demonstrated by viral load <40 copies/mL prior to study enrollment (two women had clinically insignificant low level viremia). Nearly one fifth (12%) of the women reported diabetes as a medical comorbidity and 48% endorsed cigarette smoking with any frequency within the prior 6 months. Only 3% of women reported using hormonal contraception (implant method only) in the prior month while no women reported use of probiotics, chemotherapy or steroids in the week prior to their screening study visit. More than half (52%) of the women were not sexually active in the prior 6 months, while 38% of women had 1 sexual partner in the same time period. There was low incidence of vaginal infection on screening visit (study visit 1): 8 (14%) trichomonas, 3 (5%) syphilis, 4 (7%) HSV-2 IgG positivity, 5 (9%) bacterial vaginosis and no positive testing of gonorrhea or chlamydia. (**Table 2**)

A total of 113 samples had adequate CVL pellet specimen available for 16S rRNA gene sequencing; 3 samples were removed in quality processing steps due to lack of high quality specimen. Unsupervised clustering of similar microbial communities by identity and distribution of bacterial taxa yielded two distinct microbial community types based on abundance of *Lactobacillus* species. Approximately two-thirds of the study visits (66%) were characterized as *Lactobacillus*-dominant community type while one-third had non-*Lactobacillus*-dominant community type (34%). (**Figure 1**) The microbial community types dominated by *Lactobacillus* species were comprised primarily of *Lactobacillus iners* (65% mean abundance) followed by *Lactobacillus crispatus* (11% mean abundance). The taxa most common in the non-*Lactobacillus*

dominant microbial community type included *Lactobacillus iners* (24% mean abundance), *Atopobium vaginae* (15% mean abundance), *Prevotella amnii* (10% mean abundance), *Mycoplasma hominis* (9% mean abundance), and *Prevotella timonensis* (8% mean abundance). (**Table 3**)

The distribution of clinical factors across study visits by microbial community type is presented in **Table 4**. The FGT and plasma antiretroviral concentrations were lower for all drug types in non-*Lactobacillus*-dominant MCT compared to *Lactobacillus* dominant MCT except for FGT DRV concentration. There was higher incidence of bacterial vaginosis and higher median pH level in microbial community types that were characterized as non-*Lactobacillus*-dominant.

Clinical factors associated with percent change in FGT ARV concentration were assessed utilizing bivariate analyses using mixed linear regression models with a random intercept (**Table 5**). Log plasma antiretroviral drug concentration was found to be significantly associated with change in FGT ATV concentration ($p < 0.005$) and FGT DRV concentration ($p = 0.01$). Recent sexual activity ($p = 0.02$), cigarette smoking ($p = 0.05$), and vaginal pH level ($p = 0.01$) was found to be significantly associated with percent change in FGT DRV concentration. No clinical factors were found to be significantly associated with change in FGT RAL concentration. The percent change in FGT drug concentration was also evaluated between microbial community types (non-*Lactobacillus*-dominant MCT versus *Lactobacillus*-dominant MCT) in bivariate analyses. There was no significant change found in FGT ATV concentration ($p = 0.48$), DRV ($p = 0.32$) or RAL ($p = 0.38$) concentration between microbial community types with non-*Lactobacillus*-dominant MCT versus *Lactobacillus*-dominant MCT. Multivariable analyses of the association between percent change in FGT concentration for each drug type and microbial community type controlled for any significant factor found in bivariate analysis for any drug

type: plasma antiretroviral drug concentration, recent sexual activity, cigarette smoking, and vaginal pH level. In multivariable analysis, there was no significant change found in FGT ATV concentration ($p = 0.29$), DRV ($p = 0.50$) or RAL ($p = 0.67$) concentration between microbial community types with non-*Lactobacillus*-dominance versus those with *Lactobacillus*-dominance. (**Table 6**)

DISCUSSION

The interaction between the microbiome and human drug metabolism is complex and evolving. (46) Changes in the vaginal microbiome, including presence of bacterial taxa associated with bacterial vaginosis (47) and chronic STIs such as HSV-2 (48), are associated with increased risk of HIV acquisition. (37, 38) There is growing research on the importance of achieving antiretroviral concentrations that inhibit viral replication at sites of HIV acquisition, such as in the genital tract. (49) The CAPRISA 004 trial showed that increased levels of topical tenofovir in the female genital tract was associated with high levels of protection against HIV. (50) However, there is limited knowledge about the interaction between female genital tract microbiome and antiretroviral pharmacokinetics. This hypothesis-generating study, which sought to estimate the association between female genital tract microbiome composition and genital tract antiretroviral drug penetration, contributes to this growing body of literature.

In this pilot study, we found that vaginal microbial community type was not associated with change in antiretroviral drug concentration in the female genital tract of HIV-infected women taking atazanavir, darunavir, and raltegravir. Using an unsupervised clustering method, two distinct microbial community types were identified among 116 study visits by 58 women followed longitudinally. MCT's with low diversity and high abundance of *Lactobacillus* species were characterized as *Lactobacillus*-dominant while MCTs with high diversity and low abundance of Lactobacillus species were characterized as non-*Lactobacillus*-dominant.

From a multitude of clinical and behavioral factors, we found plasma ARV concentration, recent sexual activity, vaginal pH level, and cigarette smoking within 6 months to be significantly associated with change in FGT ARV concentration for either ATV or DRV, though none of the clinical or behavioral factors we assessed were significantly associated with change

in FGT RAL concentration. In multivariable analysis for each drug type, when we controlled for those previously mentioned clinical factors, we found that there was no change in FGT ARV concentration for ATV, DRV, or RAL between non-*Lactobacillus*-dominant MCT versus *Lactobacillus*-dominant MCT. This pilot study demonstrates that the FGT microbiome composition did not have an association with change in FGT antiretroviral drug concentration for any of the three ARV's tested.

The lack of association between FGT microbiome composition and genital tract ARV drug levels may be due to several factors related to drug pharmacokinetics. Atazanavir and darunavir are potent drugs with widespread use that have the ability to rapidly suppress viral replication in the blood in HIV-infected individuals. However, existing evidence suggests that protease inhibitors exhibit limited penetration into the CVF with a mean CVF to plasma trough concentration ratio ranging from 0.03 to 0.8. (18) This trend was also found in our study. For protease inhibitors, such as atazanavir and darunavir, protein binding plays an important role in the availability of free drug in various compartments (2). As a result, significant variability exists between the drug types and between individuals. In addition to protein binding, efflux transporters such as MRB1 and MRP2 serve as substrates for P-glycoprotein. The combined effect of these transporters may minimize the ability of protease inhibitors to concentrate within cells in the FGT (18). No studies have investigated free drug concentration of protease inhibitors in the FGT (18).

We found that the mean CVF to plasma ratio of raltegravir was significantly lower in our study than has been previously reported in the literature. Historically, raltegravir has shown to have good penetration in the genital tract due to its low molecular weight and its fraction not bound to plasma proteins; the DIVA 01 study (41) showed that, in addition to raltegravir

accumulation in the genital tract due to active transport and low clearance, patients with BV had higher CVF/BP concentration ratios, suggesting that local inflammation may have increased the accumulation of raltegravir in the genital tract. (18,41) This effect was found in our study as well with 184% increase in RAL concentrations in the setting of bacterial vaginosis though this change was not found to be statistically significant. Raltegravir blocks entry of HIV into host cell DNA so some experts believe it may be particularly well suited for use in HIV prevention efforts for pre-exposure prophylaxis (18). Therefore, raltegravir may demonstrate to be optimal for use as an agent for PrEP given its extensive FGT penetration and minimal change in concentration in the setting of dysbiotic genital tract microbial communities.

Changes in the FGT MCT characterized by increased microbial diversity and bacteria associated with bacterial vaginosis i.e. *Gardnerella vaginalis* and other anaerobic species are associated with increased vaginal pH and, thus, are hypothesized to alter drug disposition into the vaginal mucosa through a variety of mechanisms. (19) Two major vaginal microbial communities were identified in our study: one dominated with *Lactobacillus* species and the other dominated by non-*Lactobacillus* species. While the CAPRISA 004 demonstrated decreased protective efficacy of tenofovir in women with non-*Lactobacillus* dominant microbial types with a topical formulation of the drug (17), Truvada had no change in PrEP efficacy when taken as an oral formulation in the setting of bacterial vaginosis in a post-hoc analysis of the Partners PrEP study (13). It is thought that the pathways that oral and topical drugs take to reach HIV target cells and prevent HIV acquisition are distinct and therefore, modulation of efficacy by a local mediator, such as BV, was much less likely in an orally administered drug. Adherence to daily oral PrEP was also very high in the Partners PrEP study. However, what is known is that adherence to the PrEP regimen is needed by women at levels much higher than men and there is

less forgiveness with missed doses. (13) Therefore, research on the genital microbiome and its modification of drug metabolism in sites of HIV acquisition can be beneficial to the understanding of the optimal regimen for PrEP as well as necessary levels of adherence needed for adequate HIV protection.

This study has several important limitations. The study was not randomized which can result in unmeasured confounders. The participants in this study were a relatively homogenous population and we had a small sample size with twenty patients or less in each group taking each type of antiretroviral drug. Some data collection was based on participant self report which can be subject to recall error, misrepresentation, and social desirability bias. Additionally, we studied the association of the vaginal microbiome on only two classes of antiretroviral drugs – protease inhibitors (ATV and DRV) and integrase inhibitors (RAL). Studies do report significant intra-class variability in mucosal tissue drug levels (8,41,43) and thus, it could also be considered an advantage to capture potential changes among drugs within the same drug class. Through unsupervised bacterial clustering methods, the majority of the participants' microbial community states were categorized into two community types – either dominated by *Lactobacillus* or low *Lactobacillus* spp.; this finding may be in part to the homogeneity of our study population. Other studies (51–53), including one performed by our group in HIV-infected women in different phases of the menstrual cycle (42), have characterized microbial communities in more than two states; therefore, it is understood that microbial community type classification is very dependent on the methodology used for clustering of community types based on bacterial diversity and species abundance.

Additional clinical factors are known to influence microbiome composition, such as cervical dysplasia, parity, use of intravaginal products, and use of proton-pump inhibitors, but

were not incorporated as covariates in our study. Additionally, it can be extrapolated by data demonstrating long-term effects of antibiotics on changes in the gastrointestinal tract microbiome (53) that this effect may also apply to the vaginal microbiome. While we only measured antibiotic effect within 7 days of microbiome sample collection on either study visit, it may be beneficial to assess the effect of antibiotic use up to 6 months prior to sample collection.

Further research is needed to fully understand how the microbiome interacts with topical and systemically delivered antiretroviral drugs. Future steps include longitudinal pharmacokinetic studies with frequent vaginal sampling as well as in-depth time-varying pharmacokinetic and pharmacodynamics studies in small study samples to determine threshold concentrations for each antiretroviral drug that is necessary to both achieve viral suppression in various compartments (blood, genital tract, etc.) as well as provide optimal protection against HIV acquisition. The success of ARV drugs for HIV treatment and prevention has provided hope that further transmission of HIV can be eliminated. The strengths of our analysis include evaluation of HIV-infected women, a group in which pharmacokinetic and microbiome research has been limited, longitudinal collection of clinical and laboratory measurements, and the ability to utilize novel methodologies to characterize the cervicovaginal microbiome. Multivariable models adjusted for a number of important covariates and potential confounders, and are another strength of this study.

In conclusion, the genital tract microbiome composition in HIV-infected women was not found to be associated with change in genital tract antiretroviral drug penetration for three different antiretroviral drug types including: atazanavir, darunavir, and raltegravir. Future research is needed to validate this study's findings and to determine mechanisms and influences

on FGT antiretroviral drug penetration to optimize efforts to limit HIV transmission and enhance HIV prevention.

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TABLES AND FIGURES

Table 1. Study schedule of events for 58 virologically-suppressed HIV-infected women on three antiretroviral drug regimens

Event	Study Visit	
	1	2
Informed consent for study participation	X	
Demographic Data	X	
Clinical, medication, menstrual, sexual histories	X	
Interval clinical, menstrual, sexual histories		X
Speculum pelvic examination		
Vaginal Catch-All™ swabs for microbiome analysis	X	X
Collect pH via microelectrode pH meter	X	X
Collect Weck-Cel for ARV drug trough concentrations	X	X
Vaginal swab for gram stain/Nugent score	X	X
Vaginal swab for STIs via Multiplex PCR testing for *N. gonorrhea, C. trachomatis, T. vaginalis* and HSV-2 serology	X	X
Vaginal swab for semen contamination by ABAcard p30 PSA analysis	X	X
Blood Collection		
RPR	X	
Plasma ARV drug concentration	X	X
Serum estradiol/progesterone levels	X	X
Other Testing		
Urine pregnancy test	X	

Abbreviations: RPR, rapid plasma reagin

Table 2. Demographic and clinical characteristics of 58 HIV-infected women on three antiretroviral drug regimens

Characteristic	Total, N (%) or median (QR), n = 58	ATV, N (%) or median (IQR), n = 20	DRV, N (%) or median (IQR), n = 20	RAL, N (%) or median (IQR), n = 18
Age (years)	48 (39, 54)	46 (41, 53)	45 (33, 52)	53 (44, 56)
BMI (kg/m^2)	30 (26, 35)	29 (26, 35)	31 (26, 39)	30 (27, 35)
Race				
African American	55 (95%)	19 (95%)	19 (95%)	17 (94%)
White	1 (2%)	1 (5%)	0 (0%)	0 (0%)
Years since HIV diagnosis	14 (10, 21)	14 (11, 21)	12 (7, 16)	18 (11, 28)
CD4 cell count (cells/mcl)	586 (394, 792)	518 (394, 784)	660 (396, 767)	640 (394, 861)
HIV viral load <40 copies/mL[a]	56 (97%)	19 (95%)	19 (95%)	18 (100%)
Diabetes[b]	7 (12%)	0 (0%)	2 (10%)	5 (28%)
Cigarette smoking within 6 months	28 (48%)	11 (55%)	9 (45%)	8 (44%)
Menstrual cycle phase[c]				
Non-ovulatory	24 (45%)	8 (40%)	6 (40%)	10 (56%)
Follicular	11 (21%)	7 (35%)	8 (53%)	3 (17%)
Luteal	18 (34%)	5 (25%)	1 (7%)	5 (28%)
Hormonal contraceptive use[d]	2 (3%)	0 (0%)	2 (10%)	0 (0%)
Chemotherapy or steroid use within 7 days of study visit	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Antibiotic use within 7 days of study visit[e]	7 (6%)	2 (5%)	4 (11%)	1 (3%)
Probiotic use within 7 days of study visit	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Vaginal douching within 7 days of study visit	6 (5%)	1 (3%)	1 (3%)	4 (12%)
Sexual activity in the prior 6 months				
0 sexual partners	30 (52%)	9 (45%)	13 (65%)	8 (44%)
1 sexual partner	22 (38%)	7 (35%)	7 (35%)	8 (44%)
2 sexual partners	5 (8%)	3 (15%)	0 0%)	2 (11%)
Genital infection at screening visit				
Gonorrhea	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chlamydia	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Trichomonas	8 (14%)	5 (25%)	2 (10%)	1 (2%)
Syphilis	3 (5%)	2 (10%)	1 (5%)	0 (0%)
HSV-2 IgG positive	4 (7%)	1 (5%)	0 (0%)	3 (17%)
Bacterial vaginosis[f]	5 (9%)	3 (16%)	2 (11%)	0 (0%)

Abbreviations: ATV, atazanavir; BMI, body mass index; DRV, darunavir; HSV2, herpes simplex virus type 2; IgG, immunoglobulin G; RAL, raltegravir

[a] Two patients with viral load 43 copies/mL and 120 copies/mL in ATV and DRV groups, respectively

[b] Based on patient self report

[c] Determined based on menopausal stage and self report of last menstrual period

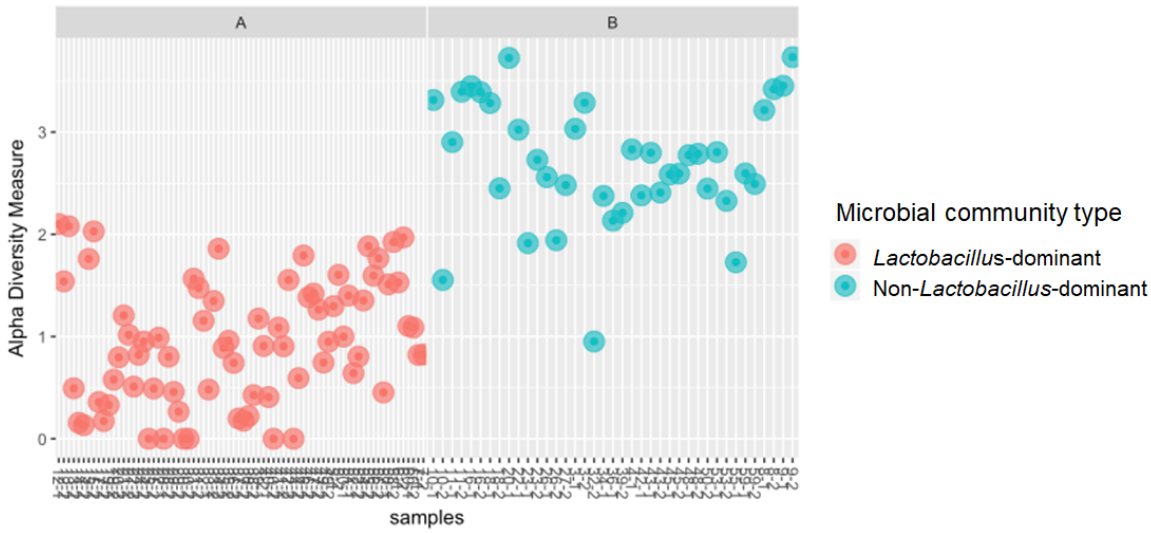
[d] Use of hormonal patch, oral contraceptive pill, intrauterine device, implant, or injectable within 1 month of screening visit

[e] Oral or intravenous antibiotic use for any reason

[f] Bacterial vaginosis diagnosed on vaginal gram stain by Nugent score ≥ 7

Figure 1. A. Alpha-diversity by Shannon diversity index of microbial community type (MCT). Individual cervicovaginal lavage pellet samples from each study visit (horizontal axes) underwent 16S ribosomal RNA gene sequencing, and individual participant visits were classified into MCTs using Dirichlet multinomial mixtures. The MCTs are defined as *Lactobacillus*-dominant (pink) and non-*Lactobacillus*-dominant (blue) based on mean (standard deviation) Shannon diversity index. B. Relative abundance of the top 20 most abundant bacterial taxa by genus and species for each participant visit depicted in the colored boxes.

A.



B.

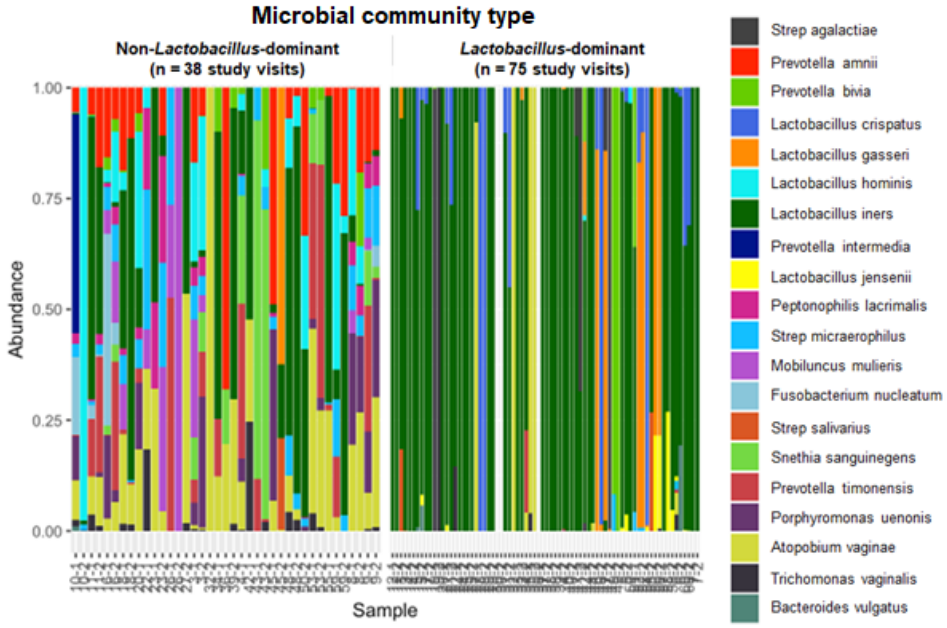


Table 3. A) Most abundant bacterial taxa in *Lactobacillus*-dominant (A) and non-L*actobacillus*-dominant (B) microbial community types of 58 HIV-infected women on three antiretroviral drug regimens

A

***Lactobacillus*-dominant MCT (n = 75)**	**Mean % abundance**
Lactobacillus iners	65
Lactobacillus crispatus	11
Atopobium vaginae	5
Lactobacillus gasseri	4
Prevotella bivia	4

B

Non-*Lactobacillus* dominant MCT (n = 38)	**Mean % abundance**
Lactobacillus iners	24
Atopobium vaginae	15
Prevotella amnii	10
Mycoplasma hominis	9
Prevotella timonensis	8

Table 4. Distribution of clinical factors and C_{24} antiretroviral drug concentrations by FGT microbial community type in 58 HIV-infected women on three antiretroviral drug regimens (n = 116 study visits)

Variable	Total cohort (n = 116 study visits)	Microbial community type	
		Lactobacillus-dominant (n = 75 study visits)	Non-*Lactobacillus*-dominant (n = 38 study visits)
Age, years, median (Q1, Q3)	48 (39 - 54)	50 (41, 56)	44 (37, 52)
BMI, kg/m^2, median (Q1, Q3)	30 (26 – 35)	28 (26, 34)	34 (27, 36)
Antibiotic use within 7 days of study visit, N study visits (%)	7 (6%)	5 (7%)	2 (5%)
Bacterial vaginosis at study visit[a], N study visits (%)	14 (13%)	4 (6%)	10 (29%)
Sexual activity within 7 days of study visit[b], N study visits (%)	25 (23%)	11 (16%)	14 (38%)
Sexually transmitted infection at study visit[c], N study visits (%)	24 (21%)	13 (10%)	14 (38%)
Vaginal douching within 7 days of study visit, N study visits (%)	6 (5%)	3 (4%)	3 (8%)
Smoking within 6 month, N study visits (%)	56 (48%)	30 (40%)	25 (66%)
Diabetes, N study visits (%)	14 (12%)	10 (13%)	4 (11%)
Menstrual cycle characteristics, N study visits (%)			
Non-ovulatory phase Ovulatory phase Follicular Luteal	48 (46%) 28 (2%) 28 (26%)	39 (57%) 12 (18%) 17 (25%)	9 (26%) 15 (43%) 11 (31%)
Vaginal pH level (Q1, Q3)	4.9 (4.3, 5.5)	4.7 (4.2, 5.4)	5.1 (4.6, 5.5)
Hormonal contraceptive use[d]	4 (3%)	3 (4%)	1 (3%)
Plasma hormone concentrations			
Estradiol pg/mL (Q1, Q3)	55.9 (26.8, 112.0)	45.9 (23.3, 111.3)	62.0 (31.8, 141.5)
Progesterone, ng/mL (Q1, Q3)	0 (0, 0.3)	0 (0, 0.3)	0 (0, 0.4)
FGT antiretroviral concentrations, median C_{24} (Q1, Q3)			
ATV	110.0 (71.1, 160.0)	123.0 (70.3, 165.0)	81.2 (71.8, 145.0)
DRV	133.0 (80.5, 259.0)	130.5 (52.8, 220.0)	143.0 (98.2, 301.0)
RAL	18.4 (8.8, 37.0)	20.6 (10.4, 39.3)	9.9 (7.5, 26.6)
Plasma antiretroviral concentrations, median C_{24} ng/mL (Q1, Q3)			
ATV	720.7 (330.2, 1254.9)	720.7 (392.2, 1414.0)	688.5 (242.0, 877.0)
DRV	1939.5 (1265.8, 3616.9)	3142.4 (1372.0, 3988.2)	1348.8 (1072.9, 2443.5)
RAL	236.9 (134.0, 506.1)	340.3 (136.7, 776.3)	185.7 (40.4, 257.2)

Abbreviations: BMI, body mass index; FGT, female genital tract; ATV, atazanavir; DRV, darunavir; RAL, raltegravir; OCP, oral contraceptive pill; IUD, intrauterine device
intrauterine device

[a] Bacterial vaginosis diagnosed on vaginal gram stain by Nugent score ≥ 7

[b] Included participants with self-reported sexual activity and/or positive semen contamination of vaginal secretions

[C] Included participants who tested positive for gonorrhea, chlamydia, trichomonas, syphilis (screening visit only), or HSV IgG positivity

[d] Use of hormonal patch, OCP, IUD, implant, or vaginal ring within 1 month of screening visit

Table 5. Bivariate associations between clinical covariates and FGT antiretroviral drug concentration in 58 HIV-infected women on three antiretroviral drug regimens (n = 116 study visits)

	Percent change[b] in FGT antiretroviral drug concentration, 95% CI					
Variable	**ATV**	**ATV P-value**	**DRV**	**DRV P-value**	**RAL**	**RAL P-value**
Age (increase in 1 year)	-1.87 (-4.76, 1.11)	0.20	-0.64 (-4.35, 3.21)	0.73	-1.75 (-8.19, 5.14)	0.58
BMI (increase in 1 kg/m^2)	1.71 (-1.37, 4.89)	0.26	-0.56 (-5.16, 4.27)	0.81	2.24(-5.01, 10.03)	0.52
Recent sexual activity vs None[c]	-4.40 (-41.87, 57.22)	0.85	267.23 (26.87, 963.04)	0.02*	73.95 (-42.25, 423.92)	0.29
Antibiotic use vs None	-27.63 (-68.73, 67.49)	0.43	-40.71 (-76.42, 49.03)	0.25	80.05 (-85.59, 2149.71)	0.62
Bacterial vaginosis vs None[d]	45.91 (-11.35, 140.17)	0.13	-31.94 (-78.63, 116.75)	0.49	184.95 (-74.34, 3063.94)	0.36
Sexually transmitted infection vs None[e]	3.22 (-36.30, 67.24)	0.89	12.06 (-61.32, 224.67)	0.82	12.50 (-66.06, 272.83)	0.83
Vaginal douching vs None	-64.73 (-88.47, 7.83)	0.07	-51.33 (-95.40, 414.64)	0.53	-6.89 (-80.01, 333.84)	0.92
Cigarette smoking vs None	-14.61 (-49.42, 44.13)	0.54	136.59 (1.51, 451.40)	0.05*	-53.35 (-86.87, 65.72)	0.22
Diabetes vs None	-	-	-29.31(-84.99, 233.00)	0.64	196.44 (-24.19, 1059.22)	0.11
Hormonal contraceptive use vs None[f]	-	-	81.47 (-60.88, 741.73)	0.42	-	-
Vaginal pH level	-9.56 (-27.77, 13.24)	0.36	-45.45 (-64.14, -17.03)	0.01*	-48.69 (-75.03, 5.29)	0.07
Log plasma antiretroviral concentration (ng/ml)[g]	50.40 (22.89, 84.05)	<0.005*	37.45 (7.91, 75.09)	0.01*	22.28 (-31.23, 117.45)	0.46
Menstrual cycle phase						
Follicular vs Luteal	43.25 (-10.89, 130.29)	0.12	60.08 (-46.77, 381.50)	0.34	61.57 (-51.90, 442.66)	0.37
Ovulatory status						
Non-ovulatory vs Ovulatory phase	1.48 (-40.71, 73.69)	0.96	91.54 (-21.63, 368.10)	0.14	2.61(-72.54, 283.47)	0.97
Plasma hormone concentrations						
Estradiol, pg/mL	0.018 (-0.12, 0.15)	0.78	-0.05 (-0.23, 0.13)	0.57	0.08 (-0.42, 0.59)	0.73
Progesterone, ng/mL	3.16 (-3.17, 9.92)	0.31	-4.00 (-14.85, 8.23)	0.48	19.21(-6.85, 52.55)	0.15
Microbial community type						
Non-*Lactobacillus*-dominant vs *Lactobacillus*-dominant	-15.19 (-47.63, 37.33)	0.48	43.30 (-32.22, 202.96)	0.32	-39.06 (-81.46, 100.29)	0.38

Abbreviations: ATV, atazanavir; CI, confidence interval; FGT, female genital tract; DRV, darunavir; RAL, raltegravir; OCP, oral contraceptive pill; IUD, intrauterine device

[b] 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 bivariate model.

[c] Included participants with self-reported sexual activity and/or participants with positive semen contamination of vaginal secretions

[d] Bacterial vaginosis diagnosed on vaginal gram stain by Nugent score ≥ 7

[e] Included participants who tested positive for gonorrhea, chlamydia, trichomonas, syphilis (screening visit only), or HSV IgG positivity

[f] Use of hormonal patch, OCP, IUD, implant, or vaginal ring within 1 month of screening visit

[g] Percent change in FGT antiretroviral concentration is presented for a 1 unit increase in a continuous predictor or for the comparison to the reference outcome of dichotomous predictor variables with 95% confidence interval.

- None in these categories

* Denotes statistical significance ($p < 0.05$)

Table 6. Bivariate and multivariable associations of clinical covariates and FGT antiretroviral drug concentration between microbial community types (non-*Lactobacillus*-dominant versus *Lactobacillus*-dominant) in 58 HIV-infected women on three antiretroviral drug regimens (n = 116 study visits)

Antiretroviral drug	Percent change in FGT concentration (95% CI)	P value
Bivariate		
ATV (n=20)	-15.19 (-47.63, 37.33)	0.48
DRV (n=20)	43.30 (-32.22, 202.96)	0.32
RAL (n=18)	-39.06 (-81.46, 100.29)	0.38
Multivariable[a]		
ATV	25.77 (-19.47, 96.41)	0.29
DRV	18.13 (-30.28, 100.18)	0.50
RAL	-25.02 (-84.08, 253.17)	0.67

Abbreviations: ATV, atazanavir; CI, confidence interval; DRV, darunavir; RAL, raltegravir

[a] Multivariable associations between clinical covariates and percent change in FGT drug concentrations were performed by each drug type holding constant the variables found to be significantly associated with FGT antiretroviral drug concentration for any drug type including recent sexual activity, cigarette smoking, vaginal pH level, and log plasma ARV concentration.