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ORIGINAL ARTICLE

Insertive vaginal sex is associated with altered penile immunology and enrichment of *Gardnerella vaginalis* in uncircumcised Ugandan men

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Abstract

Problem: HIV susceptibility is linked to the penile immune milieu (particularly IL-8 levels) and microbiome. The effects of insertive vaginal sex itself on penile immunology and microbiota are not well described.

Method of study: We compared the immune milieu and microbiology of the coronal sulcus (CS) and distal urethra in 47 uncircumcised Ugandan men reporting ever ($n = 42$) or never ($n = 5$) having had vaginal intercourse. Soluble immune factors were assayed by multiplex ELISA, and penile bacteria abundance by 16S rRNA qPCR and sequencing. Co-primary endpoints were penile levels of IL-8 and soluble E-cadherin.

Results: Independent of classical STIs, men reporting prior vaginal sex demonstrated elevated IL-8 levels in both the coronal sulcus (1.78 vs. 0.81 log10 pg/mL, $p = .021$) and urethra (2.93 vs. 2.30 log10 pg/mL; $p = .003$), with a strong inverse relationship between urethral IL-8 levels and the time from last vaginal sex ($r = -0.436$; $p = .004$). Vaginal sex was also associated with elevated penile IL-1α/β and soluble E-cadherin (sEcad), a marker of epithelial disruption. *Gardnerella vaginalis* (*Gv*) was only present in the penile microbiome of men reporting prior vaginal sex, and urethral *Gv* absolute abundance was strongly associated with urethral inflammation ($r = 0.556$; $p < .001$); corynebacteria were enriched in the CS of men reporting no prior vaginal sex and were associated with reduced CS inflammation.

Conclusions: Sexual intercourse was associated with sustained changes in penile immunology, potentially mediated through microbial alterations, in particular the urethral abundance of *G. vaginalis*. Future studies should further characterize the effects of sexual debut on penile bacteria and immunology.

Erin Day and Ronald M. Galiwango contributed equally to the manuscript.

KEYWORDS
HIV susceptibility, microbiome, penile immunology, penile-vaginal sex

1 | INTRODUCTION

Sub-Saharan Africa remains the epicenter of the global Human Immunodeficiency Virus type 1 (HIV) pandemic, and in this region, most transmission occurs during penile-vaginal sex.[1] Pre-exposure prophylaxis (PrEP) is an effective way to prevent transmission, but PrEP roll-out is far short of UNAIDS targets in resource-limited settings and HIV incidence remains high.[2] Understanding the biological factors that contribute to HIV transmission may lead to novel prevention strategies. The genital immune milieu at the site of virus exposure strongly influences HIV transmission risk in both men and women.[3–6] Penile acquisition is thought to occur primarily through two tissue sites, the inner foreskin[7] and the distal urethra,[8] and acquisition is linked to inner foreskin levels of the chemoattractant cytokine IL-8.[6,9] In addition, in uncircumcised men the coronal sulcus (CS) level of soluble E-cadherin (sEcad), a tight junction protein, has been correlated with both increased coronal sulcus levels of IL-8[10] and with genital epithelial disruption[11] that may enhance virus access to sub-epithelial HIV target cells.

Sexually transmitted infections are known to alter penile immunology,[12] and recently it has been demonstrated that the coronal sulcus microbiome is also an important determinant of the local immune milieu, including both IL-8 and sEcad levels.[9,10] Specifically, six bacterial species have been defined as Bacteria Associated with HIV Seroconversion and Immune Cells (BASIC).[9] These include *Peptostreptococcus anaerobius*, *Prevotella bivia* and *disiens*, and three species of *Dialister*. There is only partial overlap between these BASIC species and bacteria found in the vagina. For instance, *Prevotella* spp. are key BASIC species and are also important contributors to bacterial vaginosis (BV), a condition which causes vaginal inflammation and enhances HIV transmission risk.[13–16] Other BV-associated bacteria such as *Gardnerella vaginalis* are less frequent on the penis, although they are found in a minority of men and have been linked to the vaginal microbiome of a man's sexual partner,[17–20] but to our knowledge they have not been linked with penile immune changes or increased penile HIV susceptibility.[21,22] Not all genital microbes enhance HIV susceptibility, and some may play a protective role. Specifically, both genital inflammation and HIV acquisition risk are decreased in the context of an increased *Lactobacillus* spp. density in the vagina, and of an increased *Corynebacterium* spp. density in the penile coronal sulcus.[23] Several prior studies have demonstrated that there may be sharing of genital bacteria between female and male partners after penile-vaginal sex,[17–20,24,25] but the impact of these shared bacteria on penile immunology and HIV susceptibility is unclear. Here we explore associations between insertive penile-vaginal sex and the immune milieu and microbiome of the coronal sulcus and distal urethra in a cohort of uncircumcised Ugandan men.

2 | METHODS

2.1 | Study enrollment and sexual behavior data collection

The study protocol was reviewed and approved by the Research and Ethics Board (REB) of the Uganda Virus Research Institute in Entebbe (Uganda) and the Institutional Review Board at the University of Toronto (Canada), and all study participants gave written informed consent. As previously described,[10] study participants consisted of uncircumcised Ugandan men over 18 years of age, without genital symptoms, presenting to the Rakai Health Sciences Program for voluntary medical male circumcision to reduce HIV risk. Molecular diagnostics for *N. gonorrhoeae* and *C. trachomatis* were performed using first-void urine samples, with directed treatment provided as needed according to Uganda National Guidelines. A social-behavioral questionnaire was administered that included a detailed sexual history. In addition to other socio-behavioral questions, participants indicated whether they had ever engaged in penile-vaginal, oral or anal sex, and if so, how many days ago (if within the last week), how many weeks ago (if within the last month), how many months ago (if within the last year) or how many years ago (if longer than 1 year). For analysis purposes, these answers were coded into a single variable indicating days since last insertive penile-vaginal sex.

2.2 | Sample collection and processing

Penile samples were collected from all participants, as follows. Sterile, polyester-tipped swabs (Puritan Medical Products, ME, USA) pre-moistened in phosphate-buffered saline (PBS) were used by the study clinician to swab the inner foreskin. Nylon-flocked urethral swabs (Hardy Diagnostics, CA, USA) were used to swab the distal urethra. All swabs were collected by the same clinician, using a standardized operating procedure. Swabs were placed into 500 μL of PBS and all transferred to the laboratory on ice. Swabs were vigorously vortexed for 60 s in the laboratory, prior to inverting the swab within the tube and performing a quick spin to dry out the swab. Swabs were then discarded and the eluants were pulse-vortexed before splitting into two aliquots, each containing 250 μL, and frozen at −80°C.

2.3 | Multiplex chemiluminescent ELISA

Using a multiplex electro-chemiluminescence ELISA platform (Meso Scale Discovery, Rockville, MD), levels of nine soluble immune biomarkers were assayed, as previously described.[10,13] Prototypic

proinflammatory cytokines interleukin-1 alpha (IL-1α), and IL-1 beta (IL-1β) were included in the biomarker panel, as well as chemoattractant chemokines IL-8 and macrophage inflammatory protein 1 beta (MIP-1β), and finally, a biomarker of epithelial integrity/breakdown (soluble E-cadherin; sEcad). The panel also included four other exploratory analytes; resistin, an atypical proinflammatory biomarker, the novel biomarkers tissue inhibitor of metalloproteases 1 (TIMP-1), vascular endothelial growth factor (VEGF), and matrix metalloproteinase 9 (MMP-9). A standard curve was generated using serial dilutions of stock analyte from the manufacturer and was used to calculate analyte concentrations within each sample. To limit any potential impact of plate-plate variation, all samples belonging to a given individual were run on the same plate. A frozen control media aliquot was included on each plate to monitor inter-plate/run variability.

2.4 | Bacterial community analysis

Using a combination of enzymatic and chemical lysis, DNA was extracted from 80 μL of diluted swab eluent. At 37°C and for 1 h, each sample was treated with an enzymatic cocktail containing 122 μL Tris-EDTA, 50 μL 10 mg/mL lysozyme (L6876-1G, Sigma-Aldrich), 4 μL 25 KU/mL mutanolysin (M4782-5KU, Sigma-Aldrich), and 3 μL 4 U/μL lysostaphin (SAE0091-2MG, Sigma-Aldrich) followed by extraction using MagMax DNA Multi-Sample Ultra 2.0 Kit (including Proteinase K treatment) with 80 μL final elution volume. Penile bacterial communities were assessed using 16S rRNA gene-based broad-range real-time PCR and sequencing. A modified protocol from Fadrosh[26] with forward (341F) and reverse (786R) primers from Liu[27] was used to perform the sequencing analysis. Using MiSeq Reagent Kit v3 (600 cycle), sequencing was performed on MiSeq platform. Using cutadapt v2.4, primer sequences were removed during processing, and Trimmomatic v0.39 was used to quality trim the resultant sequences. For reads-filtering, chimera check, and inferred error models to identify amplicon sequence variants (ASVs), DADA2 v1.10 modules were used. The Naïve Bayesian Classifier (v.2.12) was used to classify the ASVs at each taxonomic level at 80% bootstrap confidence level. To generate an abundance matrix for analysis, classification results for each sample were enumerated. Additional details can be found at https://github.com/araclab/mb_analysis. Absolute abundances were calculated as: Taxon absolute abundance/swab = Total bacterial load/swab (total 16S rRNA copies per swab) × proportional taxon abundance (number of sequences assigned to that taxon, divided by the total 16S rRNA sequences for the sample). SRA project number PRJNA738496 can be used to access sequence data for this study. Each amplicon PCR plate included no template control (NTC) and positive template controls (PTC) which were analyzed to assess cross-contamination during PCR and verify PCR performance.

2.5 | Statistical analysis

Analysis was performed using SPSS version 28.0.0.0 (Armonk, New York, USA). Immune parameters and bacterial density were compared between groups using an unpaired *t*-test where variances were equal, and a Welch's *t*-test where equal variances could not be assumed. Immune and microbial correlations were assessed by Pearson regression, using log10 transformed cytokine and bacterial density data. Categorical variables were compared between groups using Chi-squared test with calculation of the Likelihood Ratio (LR). Analyses were visualized using GraphPad Prism version 9 (La Jolla, CA, US). Since coronal sulcus levels of IL-8 were the strongest immune predictor of HIV acquisition in a prospective cohort uncircumcised Ugandan men,[6] our co-primary immune endpoints were predefined to be the level of IL-8 in each of the coronal sulcus and urethra. Levels of the 8 other soluble immune factors were considered as exploratory secondary endpoints.

3 | RESULTS

3.1 | Participant demographics

The study enrolled $n = 47$ uncircumcised male participants with penile swabs available for microbiome and immune analysis. The mean age of participants was 22 years (range 18—36 years). Five participants (10.6%) self-reported never having had vaginal intercourse; the remainder ($n = 42$; 89.4%) reported prior insertive vaginal sex with a wide range of lifetime partner numbers (1–40, mean = 7). Information was also collected regarding prior oral and anal sex, which were much less common. Overall 3/47 (6%) participants reported prior oral sex, and 1/47 (2%) reported prior anal sex; no participant who had not had vaginal sex reported either prior oral or anal sex. The median time from the last vaginal sex was 60 days (range, 1—4380 days). Most participants (94%) reported regular retraction of the foreskin during washing, and a small minority (4%) reported antibiotic exposure during the preceding 3 months. Two participants (4%) tested positive for *C. trachomatis* and were provided with treatment according to Uganda National Guidelines.

3.2 | Impact of prior vaginal sex on penile immunology

We first assessed the impact of prior vaginal sex on penile immune parameters. In the coronal sulcus, males who reported prior insertive vaginal sex demonstrated higher levels of our primary endpoint IL-8 (1.78 vs. 0.81 log10 pg/mL in those reporting no prior sex; $p = .021$); in addition, there were increased coronal sulcus levels of secondary analytes IL-1β (1.16 vs. 0.317 log10 pg/mL; $p = .011$), sEcad (4.22 vs. 3.32 log10 pg/mL; $p = .008$) (Figure 1A–C), and resistin (2.25 vs. 1.24 log10 pg/mL; $p = .017$). Similarly, in the urethra of men reporting prior insertive vaginal sex there were higher levels of our primary endpoint IL-8 (2.93 vs. 2.30 log10 pg/mL; $p = .003$); in addition there were higher levels of secondary analytes IL-1β (0.77 vs. −0.24 log10 pg/mL; $p < .001$), IL-1α (1.74 vs. 1.02 log10 pg/mL; $p < .001$), MIP-1β (1.16 vs. 0.40 log10 pg/mL; $p = .002$), MMP-9 (4.40 vs. 3.79 log10 pg/mL; $p = .036$) and resistin (3.27 vs. 3.63 log10 pg/mL; $p = .011$), although there were no differences in urethral sEcad levels (Figure 1D–F).

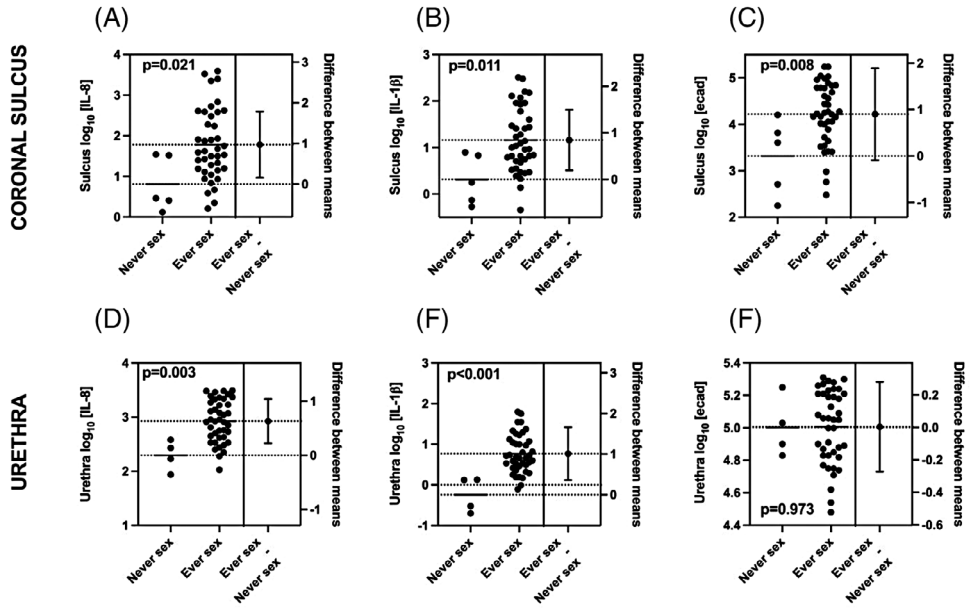

FIGURE 1 Prior vaginal sex and penile immune parameters in the coronal sulcus and urethra. Log_{10} transformed levels of IL-8, IL-1β, and soluble E-cadherin in the coronal sulcus (A–C) and urethra (D–F) were compared between study participants reporting ever or never having had vaginal intercourse.

The penile immune changes associated with prior penile-vaginal sex remained essentially unchanged if the two participants testing positive for urethral *C. trachmatis* infection were excluded. Specifically, males who reported prior insertive vaginal sex demonstrated higher levels of coronal sulcus IL-8, IL-1β, sEcad and resistin ($p = .034$, .023, .041, and .17, respectively), and higher urethral levels of IL-8, IL-1α, IL-1β, MIP-1β, MMP-9 and resistin ($p = .003$, <.001, <.001, .002, <.001, and .011, respectively). All immune differences associated with prior vaginal sex were also significant (Mann-Whitney $p < .05$) when a nonparametric analysis approach was taken, both with and without inclusion of the two participants with *C. trachomatis* infection (data not shown).

3.3 | Impact of prior vaginal sex on penile microbiology

Prior analysis in this cohort had demonstrated strong associations between penile immune parameters and genital bacteria,[10] and therefore we assessed the association of prior vaginal sex with penile bacteria. In the coronal sulcus, lower levels of *Corynebacterium* were found in men who reported prior insertive vaginal sex (4.50 vs. 6.35 log10 bacteria/swab in those reporting no prior sex; $p < .001$; Figure 2) and there were higher levels of *Gardnerella vaginalis* (0.94 vs. 0.00 log10 bacteria/swab respectively; $p = .005$). *Gardnerella vaginalis* was only detected in the coronal sulcus of men who were also colonized in the urethra (8/18 men with urethral colonization vs. 0/29 without; $p < .001$). In the urethra, higher levels of *Gardnerella vaginalis* were found in men who reported prior insertive vaginal sex (2.31 vs. 0.00 log10 bacteria/swab; $p < .001$; Figure 2), and no *Gardnerella vaginalis* was detected in the urethra of men who reported never having had vaginal sex (0/5 men reporting no prior sex vs. 18/42 reporting prior sex; LR = 5.2, $p = .023$). The bacterial species *Prevotella bivia*, which has been previously associated with penile inflammation and HIV acquisition, was common in the coronal sulcus and urethra of all men, with no differences based on prior vaginal sex. Prior vaginal sex was not associated with differences in the total bacterial load (calculated based on 16S qPCR) in either the coronal sulcus (7.35 vs. 7.37 log10 16S copies/swab; $p = .966$) or urethra (6.29 vs. 6.04 log10 16S copies/swab; $p = .718$).

Associations of IL-8 and sEcad levels with several bacteria have been previously reported in this cohort, in both the urethra and coronal sulcus,[10] but those analyses were specifically focused on the genera *Prevotella*, *Dialister*, *Corynebacterium*, and *Staphylococcus* that have been linked with differences in HIV acquisition risk. Given the association of prior vaginal sex with *Gardnerella*, penile immune associations with this genus were now examined (Figure 2). In the urethra there were strong positive correlations between the absolute abundance of *Gardnerella vaginalis* and concentration of the cytokines IL-8 ($r = 0.556$; $p < .001$), IL-1α ($r = 0.573$; $p < .001$), and IL-1β ($r = 0.520$; $p < .001$); these urethral immune associations all remained independently and strongly significant ($p \leq .003$) in logistic regression models that incorporated the density of *Prevotella*, *Dialister*, *Corynebacterium*, and *Staphylococcus* (Table 1). No associations were seen with urethral sEcad levels. In the coronal sulcus, *Gardnerella* density was positively correlated with levels of IL-1α ($r = 0.36$, $p = .014$); this association was lost in multivariable analysis, and no associations were seen with other coronal sulcus immune parameters (Table 1).

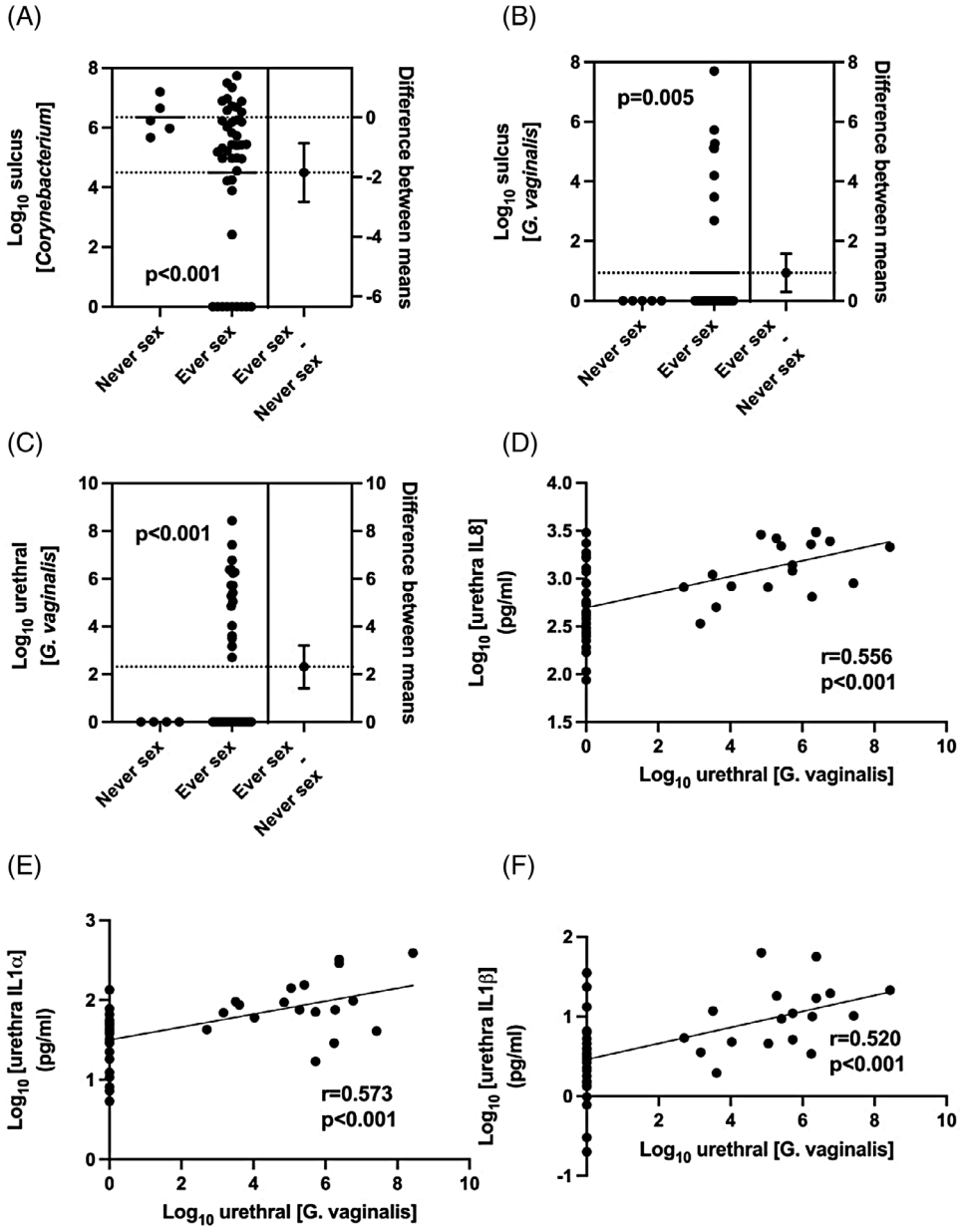

FIGURE 2 Penile *Gardnerella vaginalis* abundance is strongly associated with prior vaginal sex and immune parameters in the penile urethra. Log_{10} transformed levels of *Corynebacterium* (A) and *Gardnerella vaginalis* (B,C) were compared between study participants reporting ever or never having had vaginal intercourse. Associations are also shown between log_{10} transformed levels of urethral IL-8, IL-1α, and IL-1β and log_{10} transformed levels of urethral *Gardnerella vaginalis* (D–F).

3.4 | Time from sex and penile immune parameters

Given the penile immune and bacterial associations of ever having had vaginal sex, we next examined whether these parameters were associated with the time from last vaginal sex, limiting analysis to the 42 participants who reported prior sexual debut (Figure 3). A longer time since last vaginal sex was associated with reduced urethral levels of IL-8 ($r = -0.436$; $p = .004$) and IL-1α ($r = -0.402$; $p = .009$). No associations were seen with urethral levels of sEcad ($p > .05$), and immune parameters in the coronal sulcus were not correlated with time from the last sex (all $p > .05$).

4 | DISCUSSION

The penis is the tissue site of most HIV acquisition among uncircumcised men in sub-Saharan Africa, with virus acquisition occurring during/after penile-vaginal sex.[7] Since the composition of bacterial communities on the penis is a critical determinant of local immunology and HIV susceptibility, our goal in this cross-sectional study was to assess the relationship between prior penile-vaginal sex and the penile immune/microbial correlates of HIV susceptibility. Our findings suggest that insertive penile-vaginal sex may cause sustained changes in the penile microbiome. Specifically, we demonstrate that a history

TABLE 1 Association of *Gardnerella vaginalis* absolute abundance with immune parameters in the urethra and coronal sulcus.

	Immune parameter	Crude correlation (Pearson *r*)	*p*-value	Multivariable correlation[a] (*t*-value)	*p*-value
Urethral [*G. vaginalis*]	IL-8	0.556	<.001	3.650	<.001
	IL-1α	0.573	<.001	3.697	<.001
	IL-1β	0.520	<.001	3.65	.003
	sE cadherin	0.006	.968	−0.374	.711
Coronal sulcus [*G. vaginalis*]	IL-8	0.169	.266	0.729	.471
	IL-1α	0.321	.032	1.620	.113
	IL-1β	0.139	.363	0.923	.362
	sE cadherin	0.084	.581	0.551	.584

[a]Linear regression model incorporated the absolute abundance of *Gardnerella vaginalis*, *Prevotella* spp, *Dialister* spp, corynebacteria and staphylococci.

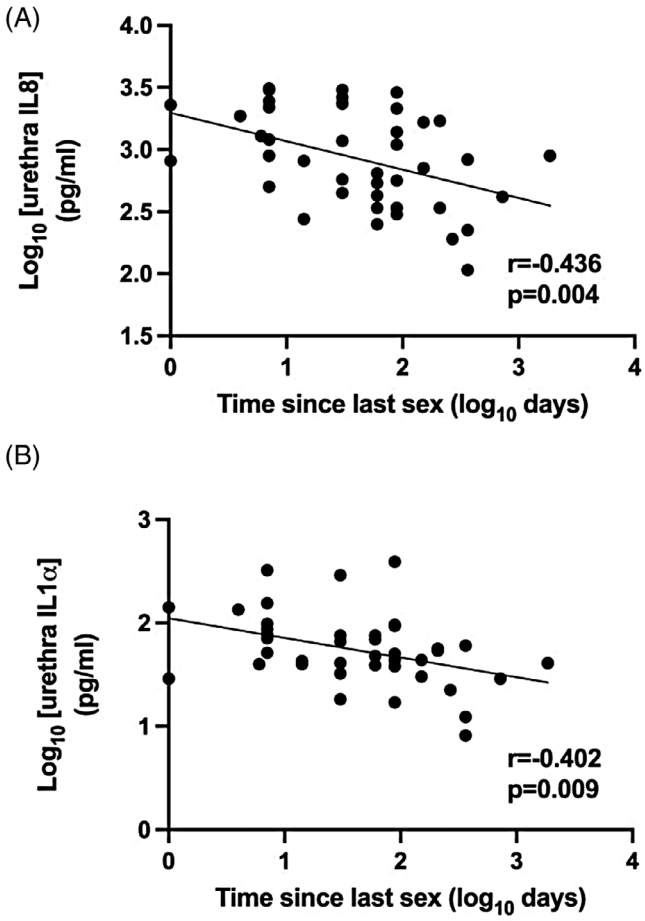

FIGURE 3 The impact of time from vaginal sex on penile immune parameters. Associations show between time from sex (in participants who reported prior sexual debut) and $\log_{10}$ transformed levels of urethral IL-8 and IL-1α (A,B).

of prior vaginal sex was associated with increased pro-inflammatory cytokines/chemokines in both the urethra and coronal sulcus, as well as with enrichment of *G. vaginalis* and a reduced abundance of *Corynebacterium* spp. In addition, a shorter time from sex was associated with increased urethral (but not CS) levels of inflammatory cytokines. These preliminary findings strongly suggest that penile-vaginal sex causes sustained changes in the penile microbiome that induce inflammation, and that would be expected to enhance penile HIV susceptibility.

We observed a higher abundance of *Corynebacterium* spp. on the CS of males who reported never having had penile-vaginal sex. An increased abundance of penile *Corynebacterium* has been correlated with reduced levels of IL-8 in the coronal sulcus,[10] and men with the highest abundance of *Corynebacterium* in the coronal sulcus were less likely to acquire HIV.[23] Corynebacteria were common in men who were sexually active, despite the reduced absolute abundance of this genus. The reason for this reduced abundance is unclear, although it is possible that *Corynebacterium* may be displaced by new, competing bacteria.

Interestingly, the BV-associated bacterial species *Gardnerella vaginalis* was only found in the urethra (and less commonly the CS) of men who reported prior insertive vaginal sex, and we demonstrate for the first time that the absolute abundance of *Gardnerella vaginalis* in the penile urethra was strongly and independently correlated with elevated levels of IL-8, IL-1α and IL-1β. This suggests that *Gardnerella vaginalis* may not be native to the penile microbiome but that acquisition occurs from a female partner after vaginal sex debut and causes urethral inflammation, and is in keeping with previous studies that have linked the coronal sulcus microbiome with the cervico-vaginal microbiome of a man's female partner,[17,18] although these studies did not assess the urethral microbiome. While, to our knowledge, a link between urethral inflammation and HIV acquisition risk has not yet been explored in observational studies, if inflammation at this site were to enhance penile HIV susceptibility then this would provide a rationale for the previously described association between BV in women living with HIV and viral transmission to their male partners.[28]

Our study was not appropriately powered to perform a detailed analysis of the microbiome associations of prior vaginal sex, and instead focused on the presence/abundance of *Gardnerella vaginalis*, the prototypic bacterial species associated with the development of

bacterial vaginosis (BV).[29] However, the recent study from Toh and colleagues analyzed the penile urethra microbiome in relation to the time from vaginal intercourse, and found that several bacterial species associated with BV were only present only in the urethra of men reporting recent vaginal intercourse.[19] Likewise, earlier work from Zozoya et al. demonstrated that in heterosexual couples where the female partner has BV, there is greater similarity between the vaginal microbiota and that of the male partner's penile skin and urethra, suggesting the frequent sexual exchange of BV-associated bacteria,[20] although others have also found BV-associated bacteria in the urethra of adolescents reporting no prior sexual experience.[30] Our findings build on this important prior work, demonstrating that the exchange of specific BV-associated bacteria (*Gardnerella vaginalis*) may cause significant inflammation, which is in keeping prior observations that *G. vaginalis* in a female partner was associated with more frequent leukocytospermia in the semen of her male partner,[24] and that other BV-associated bacteria in the male urethra may cause clinical urethritis.[25] We opted to focus our immune analyses on the absolute (rather than relative) abundance of *G. vaginalis* since this has recently been demonstrated to be more strongly predictive of changes in vaginal immunology after the treatment of BV.[13]

There are a number of limitations to this pilot study. Our sample size was limited, with only five men reporting no prior vaginal, oral or anal sex. However, the significant associations demonstrated with penile immunology suggest a strong biologic effect that was apparent not just for our co-primary endpoints of IL-8 levels in the coronal sulcus and urethra, but was also seen across 3/8 exploratory immune endpoints in the coronal sulcus and 5/8 exploratory immune endpoints in the urethra. Other factors beyond vaginal sex may alter the penile microbiome, including antibiotics and genital hygiene practices.[7] Only two men reported recent antibiotics in our study, and all but two reported regularly retracting the foreskin to wash the coronal sulcus. While our conclusions remained broadly unchanged if these participants were excluded (data not shown), it is possible that our results were affected by unmeasured behavioral or biological confounders. A longitudinal study with repeated penile sampling from the same participant before/after sexual debut, or before/after an episode of sex, would enable a formal assessment of their impact, with subsequent statistical controlling as needed. Furthermore, as this was a relatively small cross-sectional study, we were not able to demonstrate the direction of causality in the associations demonstrated between the penile microbiome and immunology, or to assess their association with actual HIV acquisition. However, we found significant and sustained differences in penile microbial and immune parameters based on the self-report of prior vaginal sex; these have been linked with HIV acquisition in prospective human studies, strongly suggesting that the exchange of microbiome components during vaginal-penile sex can be sustained and can alter HIV susceptibility. Finally, most men did not report very recent penile-vaginal sex, and so whether insertive vaginal sex leads to even more dramatic short-term microbiome and/or immune changes on the penis, as has been demonstrated in recent longitudinal studies,[31] could not be assessed but would be an interesting area for future research.

In summary, we demonstrate that prior insertive vaginal sex is strongly associated with both the immunology and microbiome of the penis, with differences most pronounced in the urethra and particularly correlated with the presence and absolute abundance of *G. vaginalis*. It will be important to confirm these cross-sectional findings in prospective studies, which would be better able to characterize the impact of sexual debut and sexual intercourse itself on the penile microbiome and immunology.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Joint United Nations Programme on HIV/AIDS (UNAIDS). UNAIDS data 2021. 2021. Accessed November 28, 2023. https://www.unaids.org/en/resources/documents/2021/2021_unaids_data
2. Joint United Nations Programme on HIV/AIDS (UNAIDS). Confronting inequalities: lessons for pandemic responses from 40 years of AIDS. 2021. Accessed November 28, 2023. https://www.unaids.org/sites/default/files/media_asset/2021-global-aids-update_en.pdf
3. Kaul R, Prodger J, Joag V, et al. Inflammation and HIV transmission in sub-Saharan Africa. *Curr HIV/AIDS Rep*. 2015;12(2):216-222.
4. Levinson P, Kaul R, Kimani J, et al. Levels of innate immune factors in genital fluids: association of alpha defensins and LL-37 with genital infections and increased HIV acquisition. *AIDS*. 2009;23(3):309-317.
5. Masson L, Passmore JA, Liebenberg LJ, et al. Genital inflammation and the risk of HIV acquisition in women. *Clin Infect Dis*. 2015;61(2):260-269.
6. Prodger JL, Gray RH, Shannon B, et al. Chemokine levels in the penile coronal sulcus correlate with HIV-1 acquisition and are reduced by male circumcision in Rakai, Uganda. *PLoS Pathog*. 2016;12(11):e1006025.
7. Prodger JL, Kaul R. The biology of how circumcision reduces HIV susceptibility: broader implications for the prevention field. *AIDS Res Ther*. 2017;14(1):49.
8. Ganor Y, Zhou Z, Bodo J, et al. The adult penile urethra is a novel entry site for HIV-1 that preferentially targets resident urethral macrophages. *Mucosal Immunol*. 2013;6(4):776-786.
9. Prodger JL, Abraham AG, Tobian AA, et al. Penile bacteria associated with HIV seroconversion, inflammation, and immune cells. *JCI Insight*. 2021;6(8):e147363.
10. Galiwango RM, Park DE, Huibner S, et al. Immune milieu and microbiome of the distal urethra in Ugandan men: impact of penile

circumcision and implications for HIV susceptibility. *Microbiome*. 2022; 10(1):7.
11. Liu R, Armstrong E, Constable S, et al. Soluble E-cadherin: a marker of genital epithelial disruption. *Am J Reprod Immunol*. 2023;89(3):e13674.
12. Gray CM, O'Hagan KL, Lorenzo-Redondo R, et al. Impact of chemokine C-C ligand 27, foreskin anatomy and sexually transmitted infections on HIV-1 target cell availability in adolescent South African males. *Mucosal Immunol*. 2020;13(1):118-127.
13. Armstrong E, Hemmerling A, Miller S, et al. Metronidazole treatment rapidly reduces genital inflammation through effects on bacterial vaginosis-associated bacteria rather than lactobacilli. *J Clin Invest*. 2022;132(6):e152930.
14. Armstrong E, Kaul R. Beyond bacterial vaginosis: vaginal lactobacilli and HIV risk. *Microbiome*. 2021;9(1):239.
15. Gosmann C, Anahtar MN, Handley SA, et al. Lactobacillus-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in young South African women. *Immunity*. 2017;46(1):29-37.
16. McClelland RS, Lingappa JR, Srinivasan S, et al. Evaluation of the association between the concentrations of key vaginal bacteria and the increased risk of HIV acquisition in African women from five cohorts: a nested case-control study. *Lancet Infect Dis*. 2018;18(5):554-564.
17. Mehta SD, Nandi D, Agingu W, et al. Longitudinal changes in the composition of the penile microbiome are associated with circumcision status, HIV and HSV-2 status, sexual practices, and female partner microbiome composition. *Front Cell Infect Microbiol*. 2022;12:916437.
18. Onywera H, Williamson AL, Ponomarenko J, Meiring TL. The penile microbiota in uncircumcised and circumcised men: relationships with HIV and human papillomavirus infections and cervicovaginal microbiota. *Front Med (Lausanne)*. 2020;7:383.
19. Toh E, Xing Y, Gao X, et al. Sexual behavior shapes male genitourinary microbiome composition. *Cell Rep Med*. 2023;4(3):100981.
20. Zozaya M, Ferris MJ, Siren JD, et al. Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome*. 2016;4:16.
21. Liu CM, Hungate BA, Tobian AA, et al. Penile microbiota and female partner bacterial vaginosis in Rakai, Uganda. *mBio*. 2015;6(3):e00589.
22. Liu CM, Prodger JL, Tobian AAR, et al. Penile anaerobic dysbiosis as a risk factor for HIV infection. *MBio*. 2017;8(4):e00996-17.
23. Kaul R, Liu CM, Park DE, Galiwango RM, Tobian AAR, Prodger JL. The penis, the vagina and HIV risk: key differences (aside from the obvious). *Viruses*. 2022;14(6):114.
24. Mandar R, Punab M, Borovkova N, et al. Complementary seminovaginal microbiome in couples. *Res Microbiol*. 2015;166(5):440-447.
25. Manhart LE, Khosropour CM, Liu C, et al. Bacterial vaginosis-associated bacteria in men: association of Leptotrichia/Sneathia spp. with nongonococcal urethritis. *Sex Transm Dis*. 2013;40(12):944-949.
26. Fadrosh DW, Ma B, Gajer P, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome*. 2014;2(1):6.
27. Liu CM, Aziz M, Kachur S, et al. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiol*. 2012;12:56.
28. Cohen CR, Lingappa JR, Baeten JM, et al. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS Med*. 2012;9(6):e1001251.
29. Muzny CA, Blanchard E, Taylor CM, et al. Identification of key bacteria involved in the induction of incident bacterial vaginosis: a prospective study. *J Infect Dis*. 2018;218(6):966-978.
30. Nelson DE, Dong Q, Van der Pol B, et al. Bacterial communities of the coronal sulcus and distal urethra of adolescent males. *PLoS One*. 2012;7(5):e36298.
31. Mohammadi A, Bagherichimeh S, Choi Y, et al. Insertive condom-protected and condomless vaginal sex both have a profound impact on the penile immune correlates of HIV susceptibility. *PLoS Pathog*. 2022;18(1):e1009948.

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