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Impact of Standard Bacterial Vaginosis Treatment on the Genital Microbiota, Immune Milieu, and Ex Vivo Human Immunodeficiency Virus Susceptibility

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Background. Genital immunology is a key determinant of human immunodeficiency virus (HIV) susceptibility. Both factors are modulated by bacterial vaginosis (BV) and, to some extent, by *Lactobacillus iners*, the genital *Lactobacillus* spp. that predominates in African, Caribbean, and other Black (ACB) women. We conducted a clinical trial to assess the impact of oral metronidazole treatment on the genital immune parameters of HIV acquisition risks in Kenyan women with BV.

Methods. The primary endpoint was *ex vivo* cervical CD4+ T-cell HIV susceptibility after 1 month; secondary endpoints included genital cytokine/chemokine levels, cervical immune cell populations, and the composition of the cervico-vaginal microbiota by 16S ribosomal RNA gene amplicon sequencing.

Results. BV resolved (Nugent score ≤ 3) at 1 month in 20/45 participants, and cervical CD4+ T-cell HIV entry was moderately reduced in all participants, regardless of treatment outcome. Resolution of BV and reduced abundances of BV-associated gram-negative taxa correlated with reduced genital interleukin (IL)-1 α/β . However, BV resolution and the concomitant colonization by *Lactobacillus iners* substantially increased several genital chemokines associated with HIV acquisition, including interferon- γ inducible protein (IP)-10, macrophage inflammatory protein (MIP)-3 α , and monokine induced by gamma interferon (MIG). In an independent cohort of ACB women, most of whom were BV-free, vaginal chemokines were again closely linked with *L. iners* abundance, though not other *Lactobacillus* spp.

Conclusions. BV treatment reduced genital CD4+ T-cell HIV susceptibility and IL-1 levels, but dramatically increased the genital chemokines that may enhance HIV susceptibility; the latter effect was related to the restoration of an *Lactobacillus iners*-dominated microbiota. Further studies are needed before treatment of asymptomatic BV can be recommended for HIV prevention in ACB communities.

Keywords. bacterial vaginosis; HIV susceptibility; *Lactobacillus iners*; chemokines; metronidazole.

Bacterial vaginosis (BV) is the most frequent cause of vaginal discharge, and has been associated with adverse reproductive health outcomes and an increased incidence of several sexually transmitted infections, including the human immunodeficiency virus (HIV) [1, 2]. In research settings, the microbiological diagnosis of BV uses the Nugent score, which is the semi-quantitative scoring of a Gram's stained vaginal smear based on the presence of gram-positive rods (*Lactobacillus* spp.), Gram-negative rods

(*Gardnerella* and *Bacteroides* spp.), and curved, Gram-variable rods (*Mobiluncus*) [3]. This scoring system assigns women to 1 of 3 categories: normal vaginal microbiota (Nugent score 0–3), intermediate vaginal flora (Nugent score 4–6), or BV (Nugent score 7–10). The latter 2 categories (collectively defined here as altered vaginal flora [AVF], Nugent score = 4–10) are associated with a >50% increased risk of HIV acquisition [1]. Furthermore, the presence of BV in an HIV-infected woman results in a 3-fold increase in the risk of HIV transmission to her male partner [4], independent of the genital HIV viral load. As a result, BV may be responsible for up to a fifth of heterosexual HIV transmission in sub-Saharan Africa [4, 5].

More sophisticated methods to assess the vaginal microbiota involve sequencing 16S rribosomal RNA (rRNA) gene amplicon, which afford a higher resolution of the composition of the microbiota and the assignment to 1 of 5 community state types (CSTs) [6]. CSTs from women with BV are characterized by

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high bacterial diversity, with an abundance of facultative and strict anaerobes, such as *Gardnerella*, *Atopobium*, *Prevotella*, *Sneathia*, BV associated bacterium (BVAB) 1–3, and *Mobiluncus* spp. [6]. In keeping with the epidemiological links between BV and HIV transmission, African women with high-diversity bacterial CSTs and/or those dominated by *Lactobacillus iners* are at a higher risk of acquiring HIV [7–9].

BV and/or a diversity-associated vaginal microbiota are thought to mediate increased host susceptibility to HIV through their effects on host mucosal immunology. HIV is acquired across the cervico-vaginal mucosa during penile-vaginal sex, and the immune parameters that increase susceptibility at this site include a reduced integrity of the cervico-vaginal mucus and the epithelial barrier, the presence of activated mucosal HIV target cells (CD4+ T cell and dendritic cell subsets), and elevated pro-inflammatory cytokines/chemokines [10]. Importantly, BV-associated vaginal microbiota increase mucosal levels of interleukin (IL)-1 α and IL-1 β , and the resulting mucosal inflammation can recruit activated CD4+ T cells [8, 11], as well as disrupt epithelial integrity [11, 12], impair mucosal repair [13], and reduce the ability of cervical mucus to bind HIV [14]. While it is unclear which mechanisms are most important, these immune alterations are thought to underpin the consistent association of genital inflammation and, more recently, are thought to impact the genital microbiota in cases of HIV acquisition in both women [8, 15, 16] and men [17, 18].

More sophisticated techniques to assess the genital microbiota have permitted a more nuanced assessment of women with a *Lactobacillus*-predominant vaginal microbiota. While *Lactobacillus crispatus* has been found to predominate in Caucasian women [6], *L. iners* is much more common in women from ACB communities [6, 19, 20]. This is important, since an *L. iners*-predominant microbiota (CST-III) frequently transitions into a diversity-type microbiota [21], is not associated with the same mucosal immune quiescence that appears to be provided by *L. crispatus* [19, 20], and tends to increase HIV acquisition risks [8]. Therefore, despite calls for BV screenings and/or treatment to be implemented at the community level as a means to reduce HIV incidence in at-risk populations [22], it is important to first understand the impact of existing BV treatment on the genital immune parameters that increase HIV susceptibility. To achieve this, we assessed the impact of BV treatment on cervico-vaginal immunology, *ex vivo* HIV susceptibility, and the genital microbiota in a longitudinal clinical study.

RESULTS

Participant Characteristics

Screening, enrollment, and study participants' demographic data are shown in Figure 1 and Table 1 and described in the Supplementary Materials.

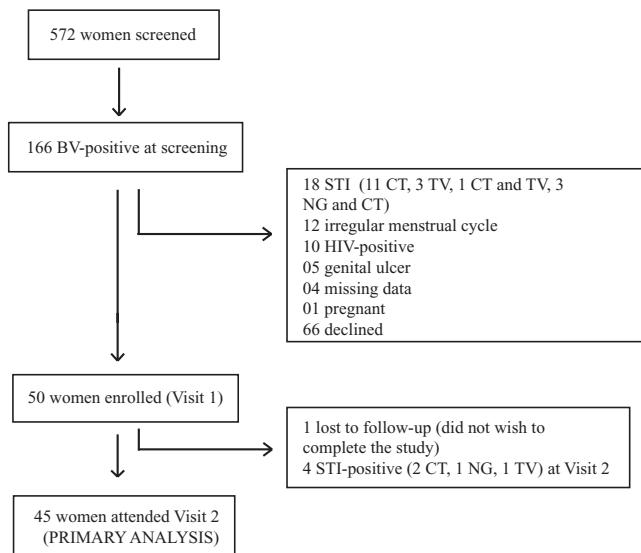


Figure 1. Participant screening and enrollment. BV-positive was defined as Nugent scores 7–10 on Gram's stain. Primary analysis included an assessment of the frequency and number of *ex vivo* HIV-infected endocervical CD4+ T cells. Abbreviations: BV, bacterial vaginosis; CT, *Chlamydia trachomatis*; HIV, human immunodeficiency virus; NG, *Neisseria gonorrhoeae*; STI, sexually transmitted infection; TV, *Trichomonas vaginalis*.

Clinical Impact of Metronidazole Treatment

All 45 study participants had BV at screening (Nugent score ≥ 7), and the median time between screening and enrollment was 6 days (interquartile range [IQR] 4–11 days). At enrollment (day of metronidazole administration), 33/45 (73.3%) participants had BV, 9/45 (20%) had an intermediate flora, and 3/45 (6.7%) were BV-free. All participants reported completing 1 week of oral metronidazole without serious adverse effects. Enrollment

Table 1. Participant Characteristics at Study Enrollment

Characteristic (N = 45 Participants)	Median (%)
Age, y, median (range)	26 (19–46)
Currently married	18 (40%)
Sexually active (past 12 months)	45 (100%)
Vaginal sex within past 3 days	9 (20%)
>1 sexual partner in the past year	9 (20%)
HSV-2 infection	23 (51%)
Regular menstrual cycle ^a	24 (53%)
Long-acting hormonal contraceptive use ^b	21 (47%)
Clinician diagnosed current vaginal discharge, odor or irritation	3 (7%)
Intra-vaginal practices, ^c ever	25 (56%)
Intra-vaginal practices, daily	14 (31%)

Data are number (%) of participants, unless indicated otherwise.

Abbreviation: HSV, herpes simplex virus.

^aMenstrual cycle reported to be 21–35 days.

^bLong-active hormonal contraceptive use included depot medroxyprogesterone acetate (DMPA) use within the past 3 months or a sub-dermal implant within the past 5 years.

^cIntra-vaginal practices were defined as the use of soap, a cloth, bleach, a drying agent, a herbal product, or detergent.

and follow-up visits occurred 28 days apart (IQR 27–29). At follow-up, 20/45 participants (44%) were BV-free, while the remainder had AVF: 7/45 (16%) had a Nugent score 4–6, and 18/45 (40%) participants had persistent or recurrent BV. Treatment reduced the vaginal pH from pH 5.1 (IQR 4.7–5.5) to 4.7 (IQR 4.3–5.5; $P = .006$, $n = 41$, Supplementary Figure 1); pH readings were uninterpretable in 4/45 participants. The reduction in pH was limited to participants with Nugent scores 0–3 at follow-up (1.2-fold reduction, $P = .0005$, $n = 16$, Supplementary Figure 2).

Impact of Metronidazole Treatment on the Vaginal Microbiota

The 16S rRNA gene amplicon sequencing was successful at both enrollment and follow-up for 41/45 participants (91%); 4 failed to either amplify or sequence. At enrollment, vaginal microbiota were clustered into 4 distinct CSTs: 33/41 (80%) of participants fell within CST-IV, characterized by a paucity of *Lactobacillus* spp. and a wide array of strict and facultative anaerobes, while 8/41 (20%) had a *Lactobacillus*-dominated vaginal microbiota ($n = 6$ that were *L. iners*-dominated [CST-III]; $n = 1$ that were *L. crispatus*-dominated [CST-I]; and $n = 1$ that were *Lactobacillus gasseri*-dominated [CST-II]). Most participants with BV at enrollment carried CST-IV vaginal microbiota (29/31 participants), 1 participant carried CST-III microbiota, and 1 carried CST-I microbiota. At follow-up, the proportion of participants with a *Lactobacillus*-dominated vaginal microbiota had increased to 20/41 (49%): these were dominated by *L. iners* in 15/20 (CST-III, 75%) participants and by non-*iners* *Lactobacillus* spp. in 5/20 (25%) participants ($n = 2$ CST-I, $n = 2$ CST-II, and $n = 1$ CST-V). The remaining 21/41 participants had a CST-IV vaginal microbiota. The overall vaginal microbiota diversity was significantly reduced after metronidazole treatment (median Jensen-Shannon score 2.7, IQR 1.0–3.5, vs. 3.2 at follow-up, IQR 2.8–3.6; $P = .02$).

Impact of Metronidazole Treatment on Human Immunodeficiency Virus Entry Into Cervical CD4+ T-Cells

Metronidazole treatment significantly reduced the *ex vivo* susceptibility of endocervical CD4+ T cells to HIV entry, the pre-defined primary study endpoint, from 9.1% (IQR 4.9–15.1%) to 6.7% (IQR 3.4–9.9%; $P = .02$; $n = 45$; Figure 2 and Supplementary Figure 3), though there was no impact on the total number of infected cervical CD4+ T cells per cytobrush (Figure 2). The reduction in percent HIV entry was independent of treatment outcome: HIV entry was reduced in 13/18 (72%) participants, with a Nugent score ≤ 3 at follow-up; in 5/7 (71%) participants with intermediate flora; and in 12/20 (60%) participants with persistent/recurrent BV (likelihood ratio = 1.1; $P = .6$; $n = 45$). The reduction in virus entry was independent of post-treatment CST, of changes in the total cervico-vaginal bacterial load, and of the baseline abundance, follow-up abundance, or change in abundance of the specific BV-associated bacterial species or genera previously linked to HIV acquisition, including *Gardnerella*, *Prevotella*, *Atopobium*, *Mobiluncus*, *Sneathia*, or *Lactobacillus* spp. (data not shown). Co-factors that may modify the risk of HIV acquisition, including herpes simplex virus (HSV)-2 infection, hormonal contraception use, and the phase of the menstrual cycle, were not associated with HIV entry at baseline ($P > .05$ for all). In summary, BV treatment reduced the HIV susceptibility of endocervical CD4+ T cells at 1 month, regardless of the treatment outcome as defined by Nugent score or genital CST.

Bacterial Vaginosis Therapy Dramatically Alters Genital Cytokine and Chemokine Levels

Metronidazole treatment reduced the cervico-vaginal level of cytokine IL-1 α (1.9-fold; $P = .004$) and increased levels of the chemokine interferon- γ inducible protein (IP)-10 (6.0-fold,

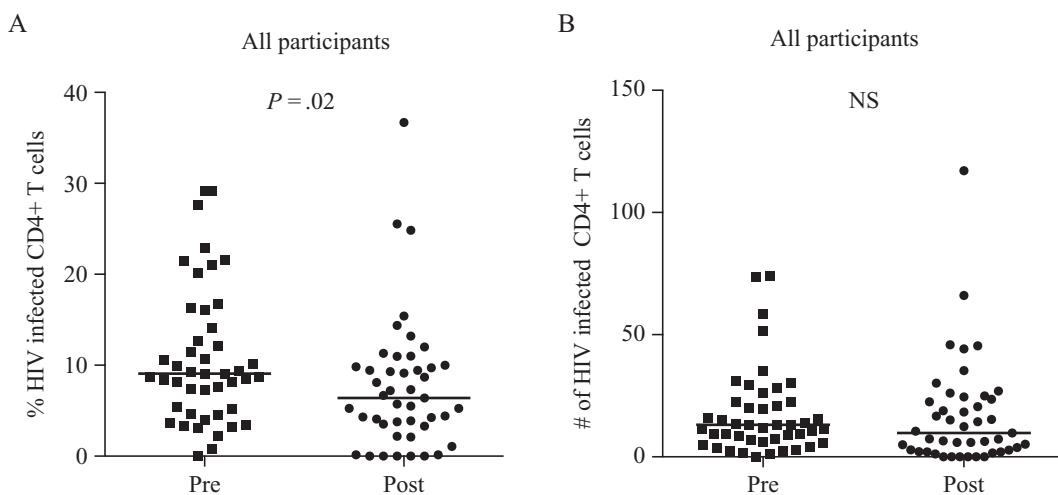


Figure 2. Metronidazole treatment reduces the frequency of HIV entry into endocervical CD4+ T cells. Panels show the (A) frequency and (B) number of endocervical cytobrush-derived CD4+ T cells per cytobrush infected by a clade A HIV pseudovirus *ex vivo* prior to (Pre) and after (Post) metronidazole treatment in Kenyan women ($n = 45$) with bacterial vaginosis. P values represent the results of Wilcoxon matched-pairs signed rank tests. Abbreviations: BV, bacterial vaginosis; HIV, human immunodeficiency virus; NS, not significant.

$P = .03$), as well as increased monokine induced by gamma interferon (MIG), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-3 α , IL-17, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (all increased >1.4 -fold, $P < .05$; **Figures 3** and **4**, left panels). In contrast to cellular changes, cytokine/chemokine changes were highly dependent on the treatment outcome. Specifically, participants who were BV-free after treatment (Nugent score 0–3) demonstrated a 3.6- and 5.2-fold reduction in IL-1 α and IL-1 β , respectively ($P \leq .006$ for both; $n = 18$ and 20, respectively; **Figure 3**), and substantial increases in the chemokines/cytokines IP-10, MIG, MCP-1 (median fold changes = 21-, 6.5-, and 8.0-fold respectively, $P < .0001$), MIP-3 α and MIP-1 α (6.2- and 1.5-fold, respectively; $P \leq .01$), and interferon (IFN)- γ and GM-CSF (1.7- and 1.9-fold, respectively; $P < .05$; $n = 20$; **Figure 4**, right panels). In contrast, these chemokines/cytokines were unchanged in participants with AVF at follow-up ($P > .05$ for all; $n = 25$; **Figures 3** and **4**, right panels). Furthermore, the degree

of treatment efficacy (visit 2 Nugent scores 7–10, 4–7, and 0–3, indicative of increasing treatment efficacy) was associated with stepwise increases in the genital levels of chemokines IP-10, GM-CSF, MCP-1, MIG, and MIP-3 α ($P < .05$ for all; $n = 45$), and with a stepwise decrease in the level of IL-1 β ($P = .02$; $n = 45$; **Table 2** and **Supplementary Table 1**).

In summary, metronidazole treatment-induced reductions in the Nugent score were associated with concomitant reductions in genital IL-1 α/β , but with increased chemokines, most notably IP-10, but also MIG, MIP-3 α , and MCP-1.

Associations of Bacterial Taxa With Genital Cytokines and Chemokines

The impact of more nuanced microbiota alterations, as defined by 16S rRNA gene amplicon sequencing, on genital chemokines/cytokines was then explored descriptively using a principal component analysis. Bacterial taxa were selected if their relative abundance changed significantly ($>0.1\%$) after metronidazole treatment, and non-iners *Lactobacillus* spp. (0.03% increased

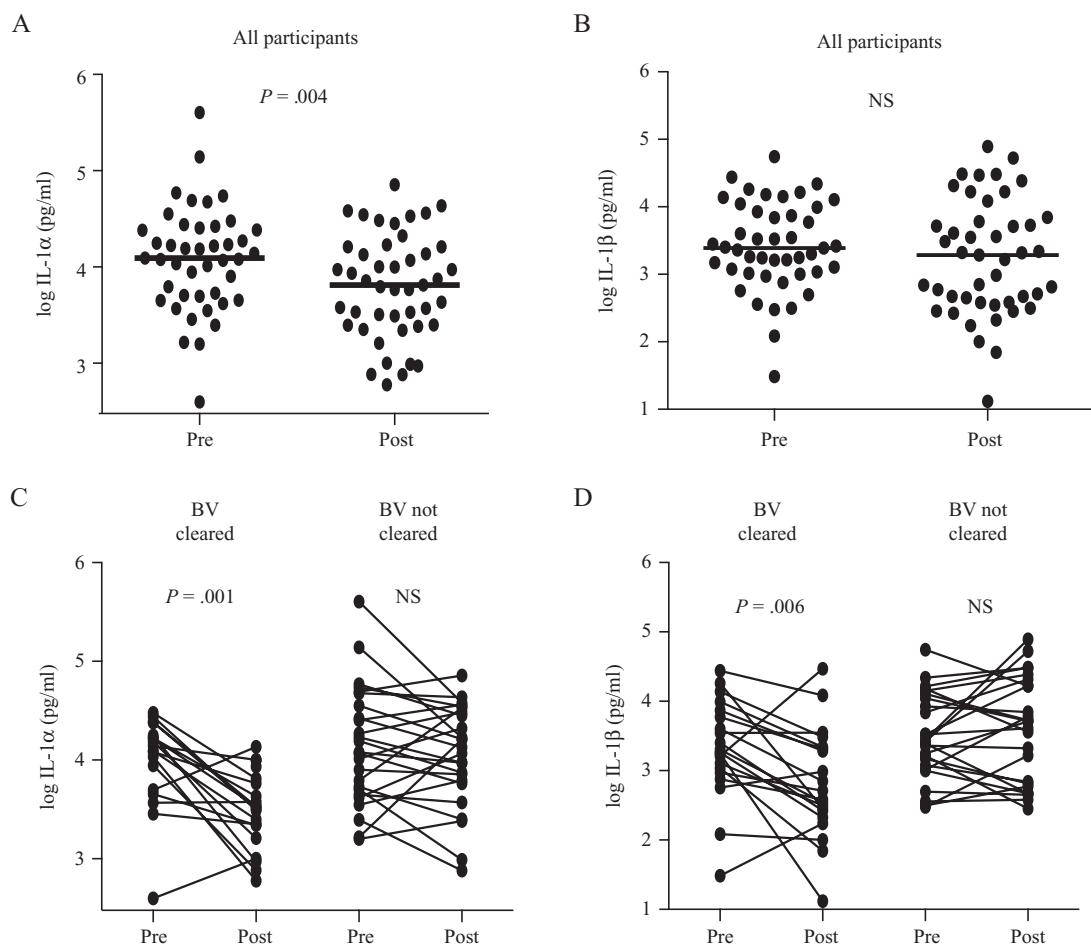


Figure 3. BV resolution causes a reduction in genital IL-1 α and IL-1 β . Panels show \log_{10} -transformed values of IL-1 α (A and C) and IL-1 β (B and D) prior to (Pre) and after (Post) metronidazole treatment in Kenyan women with BV. In A and B, all BV-positive participants that completed the study are included. In C and D, participants are stratified according to Nugent scores after BV treatment. $n = 20$ in BV cleared (Nugent score ≤ 3); $n = 25$ in BV not cleared (Nugent score 4–10) groups. IL-1 α levels could not be measured by enzyme-linked immunosorbent assay in 2/45 participants. All paired comparisons were performed using the Wilcoxon matched-pairs signed rank test. Abbreviations: BV, bacterial vaginosis; IL, interleukin; NS, not significant.

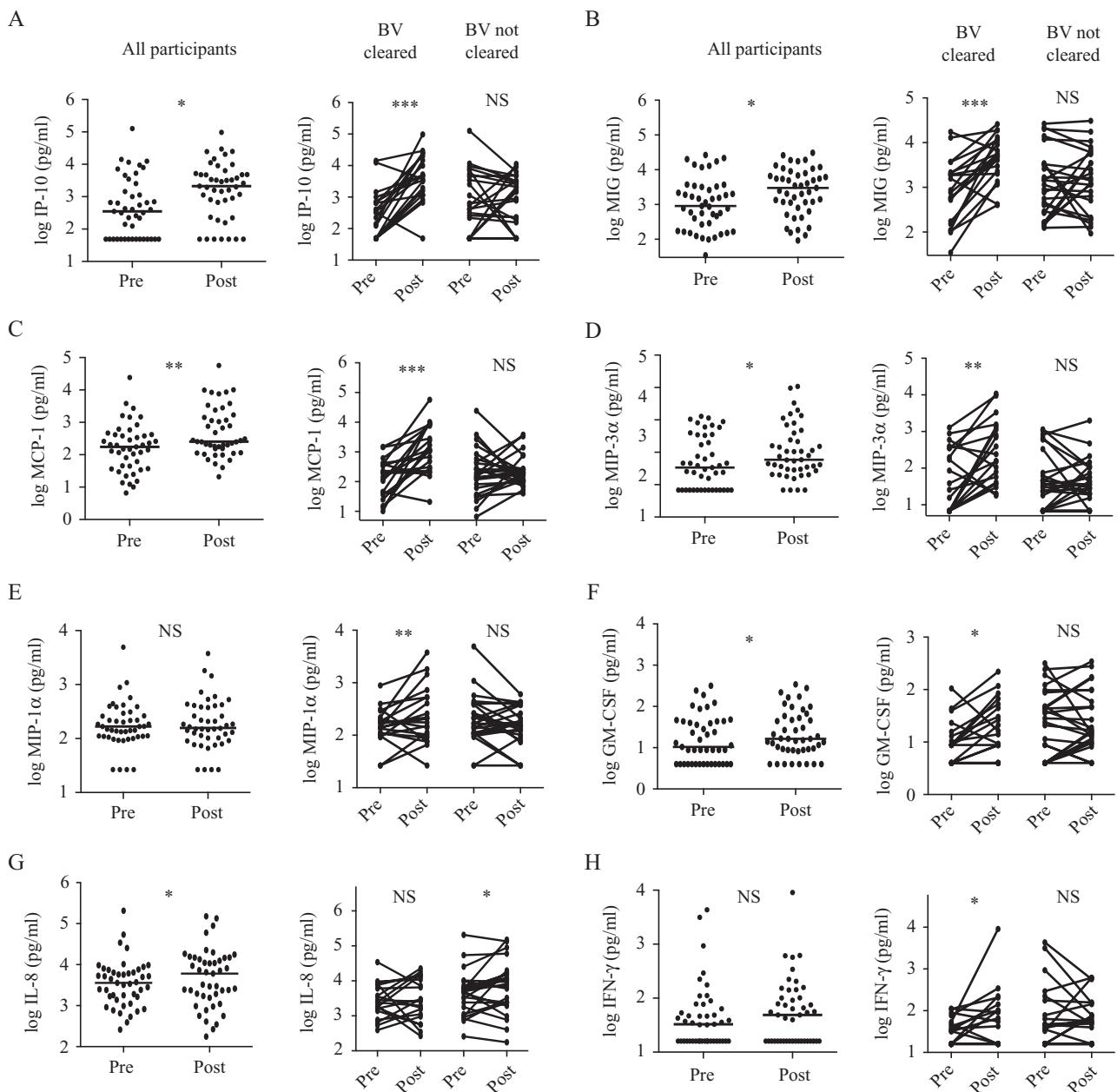


Figure 4. Marked increases in genital chemokines and cytokines after effective BV treatment. Graphs show \log_{10} -transformed cytokine and chemokine levels at baseline (Pre) and after treatment with metronidazole (Post) in women who screened positive for BV. n = 45 for analyses of all participants in panels on the left. In panels on the right, participants are stratified according to Nugent scores after BV treatment: n = 20 in BV cleared (Nugent score ≤ 3) and n = 25 in BV not cleared (Nugent score 4–10) groups. All analyses were performed using the Wilcoxon matched-pairs signed rank test. Abbreviations: BV, bacterial vaginosis; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; IP, interferon- γ inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; NS, not significant. * $P < .05$, ** $P < .01$, *** $P < .0001$.

abundance post-treatment) were also included (Figure 5 and Supplementary Table 2), given their prior association with HIV protection [8]. Baseline and metronidazole-induced changes in the relationships between microbial taxa and genital cytokines IL-1 α/β and the chemokines IP-10, MIG, MIP-3 α , MCP-1, and MIP-1 α were assessed. For participants with CST-III or -IV at baseline (Figure 5) or after treatment (Figure 5), the centroids of principal components (PCs) 1 and 2 were diametrically

opposed, indicating that these groups were distinct. Indeed, this distinction was driven by several key factors. Treatment-induced changes were seen in the levels of genital IL-1 α/β and the abundance of *Prevotella*, *Eggerthela*, and *Atopobium* clustered together, and these factors were each negatively correlated with PC1 ($r < -0.44$; $P < .001$ for all), while changes in the levels of genital IP-10, MIG, MCP-1, and MIP-3 α and the abundance of *L. iners* clustered together all correlated positively with PC1

Table 2. Stepwise Association Between the Degree of Bacterial Vaginosis (BV) Resolution and Changes in Genital Cytokines and Chemokines After Metronidazole Treatment

Cytokine Change	Efficacy of Metronidazole Treatment			Linear-by-Linear Association	P Value
	Nugent Score 0–3	Nugent Score 4–6	Nugent Score 7–10		
IP-10 increase	18 (90%)	5 (71.4%)	8 (44.4%)	13.0	<.0001
MCP-1 increase	17 (85%)	5 (71%)	10 (55%)	3.9	.048
MIP-3 α increase	16 (80%)	4 (57%)	8 (44.4%)	4.0	.046
MIG increase	18 (90%)	5 (71.4%)	5 (28%)	15.2	<.0001
IL-1 β decrease	16 (80%)	4 (57%)	8 (45%)	4.8	.025
# of participants	20	7	18		

Table shows the number (percentage) of participants in whom levels of genital cytokines/chemokines increased or decreased, as indicated, after metronidazole treatment. Data are stratified according to bacterial vaginosis treatment efficacy, indicated by Nugent score at follow-up: Nugent score 0–3 (n = 20), 4–6, (n = 7) and 7–10 (n = 18). Trends were assessed using a linear-by-linear association.

Abbreviations: BV, bacterial vaginosis; IL, interleukin; IP, interferon- γ inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein.

($r > 0.46$; $P < .001$ for all; **Figure 5**). These data demonstrate that baseline or treatment-induced changes in IL-1 α/β and gram-negative strict and facultative anaerobes were closely related, as were changes in genital chemokines and *L. iners* abundance.

Independent Confirmation of Bacterial Associations With Genital Cytokines and Chemokines

To validate these observations in an independent cohort and to better explore the chemokine/cytokine associations of a non-*iners* Lactobacillus spp.–dominated vaginal microbiota, we used data generated from an independent, cross-sectional cohort of HIV-uninfected, sexually transmitted infection–free ACB women in Toronto, Canada [20], where enrollment had not been restricted to women with BV. Among these participants, 22/51 carried an *L. iners*–dominated microbiota (CST-III; 42%), 20 carried a diversity-dominated microbiota (CST-IV; 39%), and the remainder (9/51, 19%) carried a microbiota dominated by another Lactobacillus spp.: *L. crispatus* in 7/9 (CST-I) and *L. gasseri* in 2/9 (CST-II). Consistent with findings from our BV trial, the relative abundances of BV-associated taxa (BVAB 1–3, Gardnerella, Sneathia, and Prevotella) were clustered with levels of genital IL-1 α and were positively correlated with PC1 ($r > 0.43$; $P < .0002$ for all); however, the abundance of *L. iners* was clustered with genital levels of IP-10, MIG, MIP-3 α , and MCP-1, and each of these factors negatively correlated with PC1 ($r < -0.48$; $P < .0003$ for all; **Figure 5**). The abundance of non-*iners* Lactobacillus spp. (largely *L. crispatus*) were not clustered with genital cytokines or chemokines and were negatively correlated with PC2 ($r = -0.56$; $P < .0001$), while PC2 was positively correlated with chemokines IP-10, MIG, MIP-3 α , and MCP-1 ($r > 0.28$; $P < .01$ for all) and cytokines IL-1 α/β ($r > 0.67$; $P < .0001$ for both).

Collectively, the data from these 2 distinct cohorts demonstrate relationships between IL-1 α/β and BV-associated gram-negative strict and facultative anaerobes, and between *L. iners* (but not other vaginal Lactobacillus spp.) and genital chemokines.

DISCUSSION

Multiple epidemiological studies have associated BV with an increased risk of HIV acquisition [1], and more recent in-depth microbiota studies based on 16S rRNA gene amplicon confirmed that HIV susceptibility was enhanced by a vaginal microbiota that lacks *Lactobacillus crispatus* [8], likely due to the induction of inflammatory cytokines and the subsequent mucosal recruitment of HIV-susceptible CD4+ T cells [8, 19, 20, 23]. Therefore, it seems intuitive that BV treatment and/or prevention would reduce HIV acquisition risk, but substantial barriers exist to this clinical strategy. First, BV generally recurs quickly after standard antibiotic treatment [24], and the intermittent provision of metronidazole did not reduce the incidence of HIV in a large, community-based trial [25]. Furthermore, the short-term ability of BV therapy to ameliorate the mucosal immune changes that enhance HIV susceptibility has not been assessed. Therefore, we studied the impact of standard metronidazole therapy on the genital immune milieu, cellular HIV susceptibility, and the microbiota. BV treatment moderately reduced endocervical CD4+ T cells' susceptibility to HIV. While effective therapy reduced the pro-inflammatory cytokine IL-1, it also dramatically increased genital chemokines, including IP-10, MIG, MIP-3 α , MCP-1, and MIP-1 α , and the abundance of vaginal *L. iners*; several of these factors have been directly linked to HIV/simian immunodeficiency virus (SIV) acquisition [16, 18, 26, 27]. Therefore, further studies are indicated before metronidazole treatment can be recommended as an HIV prevention tool.

Current BV treatment regimens are something of a blunt instrument, causing a rapid and profound reduction in multiple strict and facultative vaginal bacteria, with a more gradual reduction in *G. vaginalis* and increases in Lactobacillus species [28]. These pleomorphic effects mean that it is not possible to link the mucosal immune effects of BV treatment to its impact on a single bacterial species or taxa, but factor analysis from our 2 datasets demonstrate very interesting relationships. The baseline abundance and/or change in abundance of various

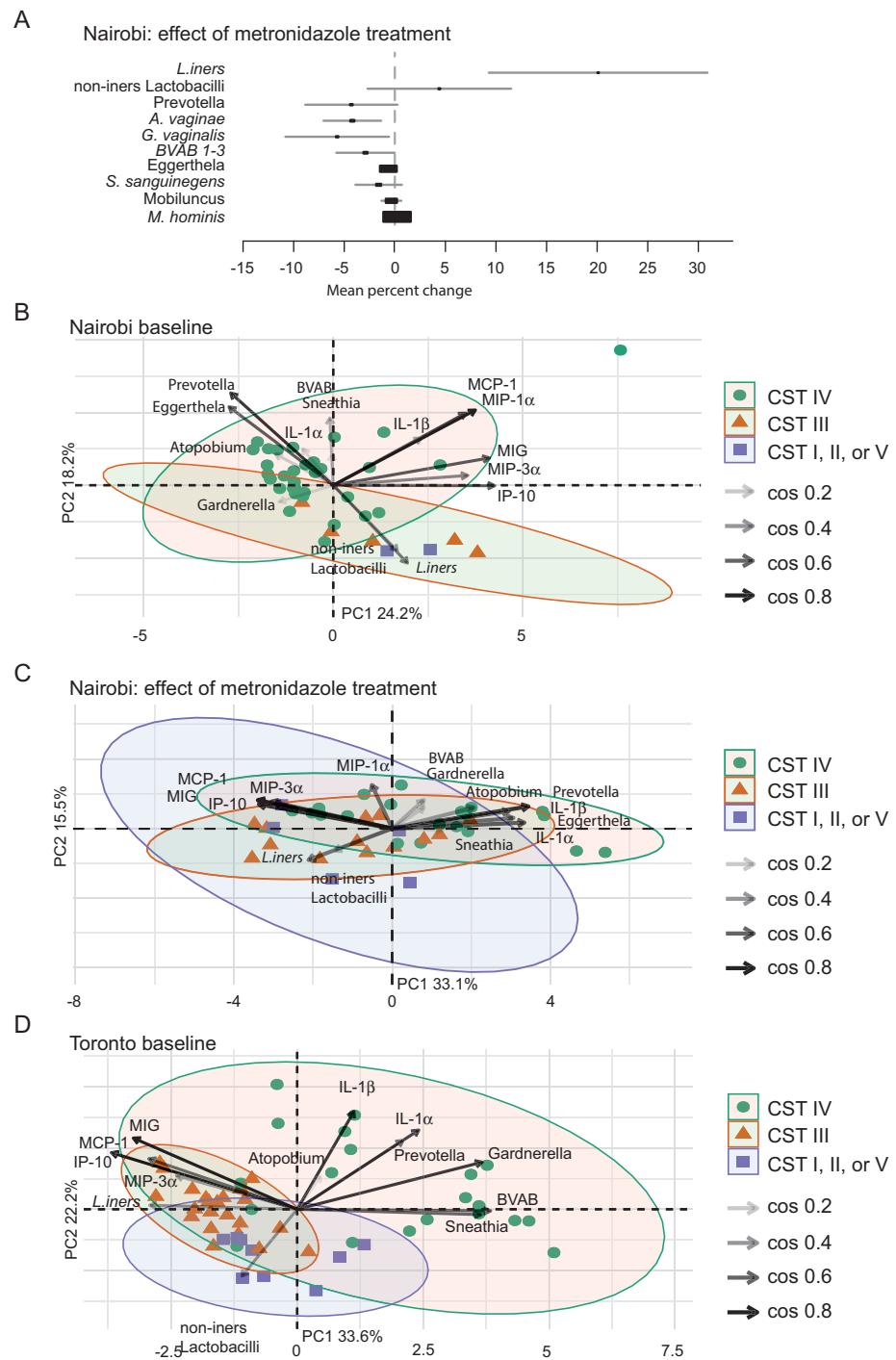


Figure 5. Association between various bacterial taxa and genital cytokines/chemokines. (A) The forest plot represents metronidazole-induced changes in the relative abundance of key microbial taxa linked to HIV acquisition risk in our Nairobi-based clinical trial. PC analysis biplots represent the association between the relative abundance of various bacterial taxa and \log_{10} -transformed levels in genital cytokine/chemokines (B) at baseline and (C) due to metronidazole-induced changes in Kenyan women. (D) Relationships between baseline microbial and immune parameters are explored in an independent cross-sectional cohort of ACB women from Toronto, Canada. Each data point (B, C, and D) represents the projection of each participant on the first 2 PCs. Arrows correspond to eigenvectors; arrow length indicates the variance across the dataset for the variable; and the angle between the arrows describes the correlation between the variables. The cos2 value of a variable indicates its contribution in driving the input data into principal components. Variables with large cos2 values contribute more to the distance separating the data points in a principal component analysis. The colored shapes represent individual study participants and their CST assignment (see legend), both (B and D) at baseline and (C) after BV treatment. Ellipses represent the 95% confidence interval of the centroid along the 2 PCs for participants within each CST group. (A–C) n=41; (D) n = 52. (B) An ellipse could not be generated for the CST-I, -II, or -V groups, due to low frequencies of participants (n = 2). (A) Non-iners Lactobacillus spp. include all other Lactobacillus spp. identified by sequencing (*crispatus*, *gasseri*, *jensenii*, *vaginalis*, *coleohominis*, *salivarius*, *mucosae*, *fermentum*, and *casei*), while the group Prevotella includes *P. genogroup 1–7*, *P. bivia*, *P. disiens*, and *P. melaninogenica*. Mobiluncus genera include *M. mulieris* and *M. curtisi*. Abbreviations: ACB, African, Caribbean and other Black; BV, bacterial vaginosis; BVAB, BV associated bacterium; CST, community state type; HIV, human immunodeficiency virus; IL, interleukin; IP, interferon- γ inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; PC, principal components.

BV-associated strict or facultative gram-negative anaerobes was most tightly linked to levels of the pro-inflammatory cytokines IL-1 α and IL-1 β in both the prospective Kenyan trial of BV treatment and the cross-sectional study of ACB women in Canada. Since pro-inflammatory cytokines can reduce epithelial barrier integrity and potentially recruit CD4+ T cells and other HIV targets [11, 12, 19], this is clearly a beneficial treatment outcome. However, the resolution of BV was strongly associated with elevated genital chemokines that may also increase susceptibility: IP-10 and MIP-1 α have been directly linked to HIV acquisition in women [16], both MIG and IL-8 have been linked to the recruitment of CD4+ targets and HIV acquisition in men [18], MIP-3 α recruits activated CD4+ T cells to the cervical mucosa in primate models [27], and chemokine receptor signaling was recently implicated in the dissemination of an HIV infection to the draining lymph nodes after establishment of a vaginal infection [29].

In our Nairobi-based BV-treatment trial, the changes that we observed in vaginal IP-10, MIG, MIP-3 α , and MCP-1 levels were not linked to alterations in strict and facultative anaerobes, but were, in fact, most closely linked to the genital abundance of *L. iners*. Indeed, given that *L. iners* is the predominant Lactobacillus spp. in the vaginal microbiota of ACB women, this may explain why ACB women who have BV (i.e., who lack lactobacilli) consistently demonstrate reduced genital IP-10 levels [20, 23]. These results imply that BV therapy may have different immune effects, depending on the dominant Lactobacillus species that is restored; restoration of an *L. iners*-dominated microbiota may not have the same beneficial effects on HIV susceptibility as the restoration of a vaginal microbiota dominated by *L. crispatus* or other non-*iners* Lactobacillus spp. The impact of these chemokine alterations on the epithelial barrier function and the mucosal recruitment of HIV target cells after treatment in the context of different vaginal microbiota will be important areas for future study.

Despite the very clear effects of BV therapy on genital immunology, our study does have several limitations. We assessed the impact of BV treatment on HIV entry and some T-cell parameters, but could not simultaneously characterize other important immune cell subsets, including Th17 cells, $\gamma\delta$ 1 T cells, dendritic cells, or macrophages. Th17 cells are important, early, genital HIV targets [30–32]; $\gamma\delta$ 1 T cells are significant producers of IL-17 [33, 34], are abundant in the endocervix, and their numbers decline substantially in women with BV [35, 36]. Moreover, the treatment-induced reduction in cellular HIV susceptibility was not mediated through any discernable changes in the microbiota, and a direct impact of metronidazole on T cells after 1 month may also be unlikely due to its short serum half-life (8 h) [37]. Nevertheless, future studies may formally test this possibility and also perform a more comprehensive microbial/immune assessment, including the time course of cytokines/chemokines and their relationship with HIV target

cells, and the composition and function of the vaginal microbiota. Approximately a quarter of our study participants with BV at screening were BV-free at enrollment or carried a lactobacillus-dominated microbiota prior to metronidazole treatment. Indeed, treatment-independent rapid fluctuations in the vaginal microbiota have been observed, especially between CST-III and CST-IV microbiota [21, 38], which were carried by 95% of our participants at enrollment. Even in the minority (20%) of participants carrying a lactobacillus-dominated microbiota at enrollment, in all but 2 cases, this was *L. iners*-dominated, which has been associated with increased bacterial diversity and a moderately-increased risk of HIV acquisition [8]. Therefore, we included all study participants in our analysis of microbial/immune associations. Participant compliance with metronidazole treatment could not be confirmed, but while low uptake could be 1 reason for low BV clearance rates after 1 month, our results are in keeping with prior clinical trials showing suboptimal efficacy and rapid recurrence of BV after treatment [24, 39]. Our sample size was also limited, to test whether co-factors such as HSV-2 infection, depot medroxyprogesterone acetate (DMPA) usage, or stage of the menstrual cycle affected HIV entry; however, our prospective study design enabled us to control for such inter-individual differences and to isolate any intra-individual effect directly to the metronidazole treatment itself. Our input cell number in pseudovirus assays was not kept constant; however, we have previously shown that, within the range of cytobrush-derived CD4+ T cells assessed in this study, input cell number has no impact on HIV entry [40]. Rather, the frequency of highly-susceptible cells is the critical determinant of viral entry [41].

Overall, the divergent effects of current BV therapy on genital immune parameters of HIV susceptibility suggest that further work is needed before the screening and treatment of asymptomatic BV can be considered for HIV prevention, and that novel approaches to treating BV will be needed, including probiotic approaches to restore an *L. crispatus*-predominant microbiota. This may be particularly true in populations where *L. iners* is the predominant vaginal Lactobacillus species, such as sub-Saharan Africa and ACB communities in North America.

Supplementary Data

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

Notes

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