

# *Prevotella* and *Gardnerella* Are Associated With Treatment Failure Following First-line Antibiotics for Bacterial Vaginosis

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**Background.** Bacterial vaginosis (BV) is a common vaginal dysbiosis that often recurs following first-line antibiotics. We investigated if vaginal microbiota composition was associated with BV recurrence.

**Methods.** We analyzed samples and data from 121 women who participated in 3 published trials evaluating novel interventions for improving BV cure, including concurrent antibiotic treatment of regular sexual partners (RSPs). Women diagnosed with BV received first-line antibiotics and self-collected vaginal swabs pretreatment and the day after finishing antibiotics (immediately posttreatment). 16S rRNA gene sequencing was performed on vaginal samples. Logistic regression explored associations between BV recurrence and features of the vaginal microbiota pre- and posttreatment.

**Results.** Sixteen women (13% [95% confidence interval {CI}, 8%–21%]) experienced BV recurrence within 1 month of treatment. Women with an untreated RSP were more likely to experience recurrence than women with no RSP ( $P = .008$ ) or an RSP who received treatment ( $P = .011$ ). A higher abundance of *Prevotella* pretreatment (adjusted odds ratio [AOR], 1.35 [95% CI, 1.05–1.91]) and *Gardnerella* immediately posttreatment (AOR, 1.23 [95% CI, 1.03–1.49]) were associated with increased odds of BV recurrence.

**Conclusions.** Having specific *Prevotella* spp prior to recommended treatment and persistence of *Gardnerella* immediately posttreatment may contribute to the high rates of BV recurrence. Interventions that target these taxa are likely required to achieve sustained BV cure.

**Keywords.** bacterial vaginosis; *Gardnerella*; *Prevotella*; regular sexual partner; vaginal microbiota.

Bacterial vaginosis (BV) is a common vaginal condition that affects approximately 30% of reproductive-aged women globally [1]. While the etiology of BV has not been established, the vaginal microbiota of women with BV is typically deficient in optimal *Lactobacillus* spp (eg, *Lactobacillus crispatus*), with high diversity and high abundance of anaerobes [2]. Recommended treatment for BV involves antibiotic therapy

with metronidazole or clindamycin, both of which provide broad coverage of anaerobes. One-month cure posttreatment is approximately 70%–85% [3]; however, up to 50% of women experience BV recurrence within 12 weeks following antibiotics [4]. BV recurrences are distressing for women [5] and result in repeat presentations to clinical services and repeated antibiotic use. Furthermore, the sequelae associated with BV carry significant implications for a woman's reproductive and sexual health and impose a large economic burden [1].

There has been little progress made toward improving long-term BV cure, partly because the mechanisms driving BV recurrence are multifactorial and incompletely understood [6]. Prior studies exploring the association between microbial signatures of the vaginal microbiota (ie, diversity metrics, community types, and individual taxa) and BV treatment outcomes [7–14] have produced mixed results regarding the contribution of bacterial diversity to treatment failure. Interestingly, a number of studies have reported a positive association between BV recurrence and high relative and absolute abundance of *Gardnerella* (or individual *Gardnerella* spp) pretreatment [10–12]. Furthermore, treatment success has been associated with significant reductions in *Gardnerella* spp

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posttreatment [7, 11]. Together these data highlight the importance of *Gardnerella* spp to BV pathogenesis [15]. However, prior studies have been limited by small numbers, and few have adjusted microbiota findings for confounding factors that may influence an individual's likelihood of recurrence [11, 13], such as posttreatment sexual practices [6].

To overcome limitations of past studies and to provide insight into the microbial drivers of BV recurrence, we aimed to determine if the vaginal microbiota composition immediately before or immediately after treatment was associated with BV recurrence within 1 month of recommended treatment, after adjustment for confounding factors.

## METHODS

### Study Populations and Study Procedures

We conducted a secondary analysis of vaginal microbiota data and accompanying clinical and behavioral data collected from women who participated in 3 clinical trials conducted at the Melbourne Sexual Health Centre (MSHC), Australia, that evaluated new interventions for improving BV cure: Strategies to prevent BV (SToPBV), StepUp-1, and StepUp-2. The parent trials have been described elsewhere [16–18]. In brief, SToPBV was an open-label randomized controlled trial of combined oral contraceptive pill use following first-line antibiotic treatment to prevent BV recurrence [16]. StepUp-1 and StepUp-2 were open-label single-arm pilot studies that assessed the acceptability and tolerability of concurrent male partner treatment for BV and its impact on the genital microbiota of couples [17, 18]. The [Supplementary Material](#) details the interventions assessed in the parent studies.

Across the 3 studies, women presenting to MSHC were eligible to participate if they were diagnosed with BV (defined as  $\geq 3$  Amsel criteria and Nugent score [NS] = 4–10) and received first-line treatment (oral metronidazole 400 mg twice daily for 7 days, or if contraindicated, clindamycin 2% vaginal cream applied intravaginally nocte for 7 nights or metronidazole 0.75% gel applied intravaginally nocte for 5 nights). Additional study-specific eligibility criteria were applied [16–18]. Of note, women were eligible for SToPBV if they were not currently using hormonal contraception and had no combined oral contraceptive pill-related contraindications. Women were eligible for the StepUp studies if they had a male regular sexual partner (RSP) of  $\geq 2$  months who was willing to receive treatment and had no additional sexual partners.

Women self-collected a high-vaginal swab for microbiota characterization and completed questionnaires concerning demographic, clinical, and behavioral information prior to commencing antibiotics at day 0 (termed pretreatment) and the day after finishing antibiotics at day 8 (termed immediately posttreatment). Posttreatment, women were followed longitudinally, with the follow-up duration differing between studies:

Women were followed for 6 months postenrollment in SToPBV, 1 month in StepUp-1, and 3 months in StepUp-2. However, the current study only used samples collected at day 0 and day 8 and metadata collected up to 1 month postenrollment ([Supplementary Figure 1](#)). At 1 month postenrollment, women completed a questionnaire and self-collected a vaginal smear at home that was assessed for BV using NS criteria. Women who had NS = 7–10 on a home smear or who experienced symptoms were encouraged to attend MSHC for BV assessment using combined Amsel and NS criteria.

Ethical approval was obtained from the Human Research and Ethics Committee of the Alfred Hospital, Melbourne, Australia [16–18]. All participants provided informed written consent.

### Study Outcome

The study outcome for the current study was BV recurrence within 1 month of enrollment (recurrence cases) or reaching 1 month without recurrence (cure cases). Recurrence was defined as  $\geq 3$  Amsel criteria and NS = 4–10. If a woman was unable to return to clinic, a NS = 7–10 on a home smear was accepted as a BV diagnosis. We investigated the association between BV recurrence and bacterial diversity and composition of the vaginal microbiota at 2 timepoints: (i) pretreatment at day 0 and (ii) immediately posttreatment at day 8. Women were eligible for inclusion if they provided a day 0 and/or day 8 microbiota sample and reached the study endpoint. If a woman provided only a day 0 or a day 8 sample they could still be included in the study, but they only contributed to the analysis at 1 timepoint. Of the 95 women recruited to SToPBV and 73 women recruited to the StepUp studies, 121 women (72 from SToPBV, 49 from StepUp) were eligible for inclusion in the current study.

### Microbiota Characterization

Sample storage, DNA extraction, amplification, and sequencing methodology are described in detail in the original publications [17–19]. Laboratory methods applied to SToPBV and StepUp-1 samples were identical, whereas aspects of the methodology for StepUp-2 (including DNA extraction and library preparation methods) differed. The vaginal microbiota was characterized by 16S ribosomal RNA (rRNA) gene sequencing of the V3–V4 regions using the Illumina MiSeq platform. DADA2 v1.20.0 was used for quality filtering, inferring amplicon sequence variants, chimera identification, merging of paired end reads and taxonomic assignment, as described previously [18]. Laboratory and bioinformatics methods are described in the [Supplementary Material](#).

### Statistical Analysis

We compared demographic, contraceptive, and behavioral characteristics between the 2 study populations (SToPBV and

StepUp), and between recurrence and cure cases.  $\chi^2$ , Fisher exact, and Wilcoxon rank-sum tests were used where appropriate.

Diversity and ordination analyses were performed using vegan software v2.6.2. Nonmetric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM), utilizing the Bray–Curtis metric, were used to visualize and test for differences in the pretreatment and immediate posttreatment composition of the vaginal microbiota between recurrence and cure cases.  $\alpha$ -diversity was calculated using the Shannon diversity index. Bray–Curtis distances calculated between paired pretreatment and posttreatment samples provided a measure of compositional change of the vaginal microbiota following treatment. We used logistic regression models to examine the association between BV recurrence and (i)  $\alpha$ -diversity of the pre- and posttreatment vaginal microbiota, as well as (ii) compositional change posttreatment. Models were adjusted for laboratory method to account for potential confounding due to differences in the methods used for SToPBV and StepUp-1 versus StepUp-2 (see [Supplementary Material](#)). Models were also adjusted for sexual partner type and their treatment status (no RSP vs treated RSP vs untreated RSP), as this characteristic was found to differ between recurrence and cure cases (see Results). We did not adjust models for study (SToPBV/StepUp-1/StepUp-2), as this variable was highly correlated with sexual partner type and treatment status (Cramer  $V = 0.7071$ ).

Analysis of compositions of microbiomes with bias correction (ANCOM-BC) version 1.6.2 [20] was used to identify differentially abundant taxa between recurrence and cure cases pre- and immediately posttreatment. ANCOM-BC analyses were adjusted for laboratory method and taxa that were present in  $\leq 10\%$  of samples were excluded. Bacterial taxa identified as differentially abundant by ANCOM-BC with a  $P$  value  $< .05$  were explored further using logistic regression. Logistic regression was used to examine the association between BV recurrence and the centered log-ratio (CLR)-transformed abundance of each taxon, adjusting for laboratory method and partner type/treatment status.

Finally, using an approach similar to Lee et al [9], we explored the relationship between BV recurrence and the ratio of nonoptimal to optimal bacteria present in the vagina pre- and posttreatment. The following ratios were examined: (i) total BV-associated bacteria to *Lactobacillus* spp (BVAB: *Lactobacillus*), (ii) *Gardnerella* to *Lactobacillus iners* (*Gardnerella:L iners*) and (iii) total pathobionts to *Lactobacillus* spp (pathobionts:*Lactobacillus*). Pathobionts are organisms that may become inflammatory and pathogenic under specific conditions, and high pretreatment pathobiont load has been associated with metronidazole treatment failure [12]. [Supplementary Table 1](#) lists the bacterial taxa included in each group. We calculated ratios using  $\log_{10}$ -transformed relative abundance data and evaluated the association between BV

recurrence and bacterial ratios using logistic regression models, adjusted as above.

RStudio version 2022.02.2 software running R version 4.2.0 was used for analyses.

## RESULTS

### Participant Characteristics and Factors Associated With BV Recurrence

[Table 1](#) summarizes baseline characteristics and sexual practices of the 121 women included in analyses. The pooled cohort of women had a median age of 28 years (interquartile range, 25–32 years). Approximately half of participants were born in Australia or New Zealand (56/121 [46%]), 24% were born in Europe (most commonly the United Kingdom and Ireland), and 22% were born in Asia (most commonly China, Hong Kong, Taiwan, and Southeast Asia). At baseline, 87 women (72%) reported having an RSP (most of whom were male [83/87]), 26 (21%) reported hormonal contraceptive use, and 13 (11%) had an intrauterine device in situ. Most women (89/121 [74%]) had a history of BV. Some characteristics differed between the StepUp and SToPBV cohorts (ie, having an RSP at baseline, baseline contraceptive practices, and posttreatment sexual practices; [Table 1](#)), which was primarily due to differences in the eligibility criteria applied in each study.

Of the 121 women, 16 (13% [95% confidence interval {CI}, 8%–21%]) experienced BV recurrence. Only 1 characteristic differed significantly between recurrence and cure cases ([Table 2](#)). Women who reported sex with an untreated RSP during follow-up were significantly more likely to experience BV recurrence compared to women who had no RSP (9/28 [32%] vs 3/44 [7%],  $P = .008$ ) and women who had an RSP treated through StepUp-1/2 (9/28 [32%] vs 4/49 [8%],  $P = .011$ ). Interestingly, there was no difference in the proportion of women who experienced recurrence between those with a treated RSP and those with no RSP (4/49 vs 3/44,  $P = 1.000$ ). No other characteristics, including treatment prescribed, treatment adherence, or history of BV, differed significantly between recurrence and cure cases.

### Overall Composition and Diversity of the Vaginal Microbiota of Women Who Experienced BV Recurrence and Women Who Did Not

Two hundred thirty specimens were included in analyses ([Supplementary Figure 2](#)); 109 women provided both day 0 and day 8 samples, 5 provided a day 0 sample only, and 7 provided a day 8 sample only. As expected, pretreatment specimens were predominantly high in diversity with high relative abundance of BV-associated bacteria including *Gardnerella* spp, *Prevotella* spp, *Fannyhessea vaginae* (previously *Atopobium vaginae*), and *Megasphaera* spp, with low relative abundance of lactobacilli. Immediately posttreatment, most specimens were low in diversity and dominated by *L iners*.

**Table 1. Demographic, Contraceptive, and Behavioral Characteristics of the Study Population (N = 121)**

| Characteristic  | Total (N = 121) | StepUp <sup>a</sup> (n = 49) | SToPBV (n = 72) | P Value <sup>b</sup> |
|---|-----------------|------------------------------|-----------------|----------------------|
| <b>Characteristics reported at baseline</b>             |                 |                              |                 |                      |
| Age, y, median (IQR)                                    | 28 (25–32)      | 29 (26–33)                   | 28 (24–30)      | .064                 |
| <b>Region of birth</b>                                  |                 |                              |                 |                      |
| Australia/New Zealand                                   | 56 (46)         | 25 (51)                      | 31 (43)         | .960                 |
| Asia  | 27 (22)         | 11 (22)                      | 16 (22)         |                      |
| Europe  | 29 (24)         | 10 (20)                      | 19 (26)         |                      |
| Middle East or Africa                                   | 3 (2)           | 1 (2)                        | 2 (3)           |                      |
| North America   | 3 (2)           | 1 (2)                        | 2 (3)           |                      |
| South America   | 3 (2)           | 1 (2)                        | 2 (3)           |                      |
| <b>Past history of BV</b>                               |                 |                              |                 |                      |
| No  | 32 (26)         | 9 (18)                       | 23 (32)         | .096                 |
| Yes   | 89 (74)         | 40 (82)                      | 49 (68)         |                      |
| <b>Any hormonal contraceptive use</b>                   |                 |                              |                 |                      |
| No  | 95 (79)         | 23 (47)                      | 72 (100)        | < .001               |
| Yes   | 26 (21)         | 26 (53) <sup>c</sup>         | 0 (0)           |                      |
| <b>Any IUD use</b>                                      |                 |                              |                 |                      |
| No  | 108 (89)        | 36 (73)                      | 72 (100)        | < .001               |
| Yes   | 13 (11)         | 13 (27) <sup>d</sup>         | 0 (0)           |                      |
| <b>Any smoking</b>                                      |                 |                              |                 |                      |
| No  | 75 (63)         | 38 (79)                      | 37 (51)         | .002                 |
| Yes   | 45 (38)         | 10 (21)                      | 35 (49)         |                      |
| Lifetime No. of male partners, median (IQR)             | 15 (7–25)       | 15 (7–30)                    | 15 (7–23)       | .547                 |
| Lifetime No. of female partners, median (IQR)           | 0 (0–1)         | 0 (0–0)                      | 0 (0–2)         | .328                 |
| <b>Current RSP</b>                                      |                 |                              |                 |                      |
| No  | 34 (28)         | 0 (0)                        | 34 (47)         | < .001               |
| Yes <sup>e</sup>  | 87 (72)         | 49 (100)                     | 38 (53)         |                      |
| <b>Characteristics between day 0 and study endpoint</b> |                 |                              |                 |                      |
| <b>Any hormonal contraceptive use</b>                   |                 |                              |                 |                      |
| No  | 64 (53)         | 23 (47)                      | 41 (57)         | .279                 |
| Yes   | 57 (47)         | 26 (53)                      | 31 (43)         |                      |
| <b>Any smoking</b>                                      |                 |                              |                 |                      |
| No  | 76 (63)         | 38 (79)                      | 38 (53)         | .003                 |
| Yes   | 44 (37)         | 10 (21)                      | 34 (47)         |                      |
| <b>Any penile–vaginal sex</b>                           |                 |                              |                 |                      |
| No  | 29 (24)         | 2 (4)                        | 27 (37.5)       | < .001               |
| Yes   | 92 (76)         | 47 (96)                      | 45 (62.5)       |                      |
| <b>Condom use for penile–vaginal sex</b>                |                 |                              |                 |                      |
| Always/no penile–vaginal sex                            | 44 (36)         | 2 (4)                        | 42 (58)         | < .001               |
| Not always  | 77 (64)         | 47 (96)                      | 30 (42)         |                      |
| <b>Any receptive oral sex</b>                           |                 |                              |                 |                      |
| No  | 47 (39)         | 11 (22)                      | 36 (50)         | .002                 |
| Yes   | 74 (61)         | 38 (78)                      | 36 (50)         |                      |
| <b>Any anal sex</b>                                     |                 |                              |                 |                      |
| No  | 105 (87)        | 41 (84)                      | 64 (89)         | .406                 |
| Yes   | 16 (13)         | 8 (16)                       | 8 (11)          |                      |
| <b>Any sex with RSP</b>                                 |                 |                              |                 |                      |
| No  | 47 (39)         | 3 (6)                        | 44 (61)         | < .001               |
| Yes   | 74 (61)         | 46 (94)                      | 28 (39)         |                      |
| <b>Any new sexual partner</b>                           |                 |                              |                 |                      |
| No  | 98 (81)         | 47 (96)                      | 51 (71)         | < .001               |
| Yes   | 23 (19)         | 2 (4)                        | 21 (29)         |                      |

**Table 1. Continued**

| Characteristic                                      | Total (N = 121) | StepUp <sup>a</sup> (n = 49) | SToPBV (n = 72) | P Value <sup>b</sup> |
|---|-----------------|------------------------------|-----------------|----------------------|
| <b>Gender of sexual partner(s) during follow-up</b> |                 |                              |                 |                      |
| Male  | 91 (91)         | 48 (98)                      | 43 (84)         | <b>.051</b>          |
| Female  | 4 (4)           | 0                            | 4 (8)           |                      |
| Male and female                                     | 1 (2)           | 1 (2)                        | 4 (8)           |                      |

Data are presented as No. (%) unless otherwise indicated. Missing data from up to 9 women for some variables. P values < .05 are bolded to indicate statistically significant associations.

Abbreviations: BV, bacterial vaginosis; IQR, interquartile range; IUD, intrauterine device; RSP, regular sexual partner; SToPBV, Strategies to prevent BV.

<sup>a</sup>Women from StepUp-1 and StepUp-2 were combined as the eligibility criteria applied in these studies were identical and participant characteristics did not differ between the 2 studies.

<sup>b</sup>The  $\chi^2$  or Fisher exact test was used for categorical data, and Wilcoxon signed-rank test was used for continuous variables to examine differences between StepUp and SToPBV participants.

<sup>c</sup>At baseline, 13 women reported current oral contraceptive use, 6 reported using a levonorgestrel IUD, 6 reported using a contraceptive implant, and 1 reported using a hormonal injection (Depo-Provera).

<sup>d</sup>At baseline, 6 women reported using a levonorgestrel IUD and 7 women reported using a copper IUD.

<sup>e</sup>Four women in SToPBV reported a female RSP at baseline; all other women reported a male RSP.

NMDS and ANOSIM showed no difference in the overall composition of the vaginal microbiota between recurrence and cure cases either pretreatment at day 0 (ANOSIM:  $R = 0.070$ ,  $P = .166$ ; Figure 1A) or immediately posttreatment at day 8 (ANOSIM:  $R = 0.037$ ,  $P = .318$ ; Figure 1B). Similarly, the  $\alpha$ -diversity of the vaginal microbiota did not differ pretreatment (adjusted odds ratio [AOR], 1.99 [95% CI, .77–6.71],  $P = .203$ ) or posttreatment (AOR, 1.37 [95% CI, .60–2.97],  $P = .435$ ; Table 3, Figure 1C) between recurrence and cure cases. Furthermore, there was no difference in the degree of compositional change of the vaginal microbiota following treatment between recurrence and cure cases (AOR, 0.21 [95% CI, .02–3.01],  $P = .235$ ; Table 3, Figure 1D).

#### Individual Bacterial Taxa Associated With BV Recurrence

ANCOM-BC was used to identify bacterial taxa that were differentially abundant pre- and posttreatment between recurrence and cure cases. Pretreatment, the mean abundance of *Prevotella* spp (representing *Prevotella* amplicon sequence variants that were not able to be classified at the species level) was significantly higher in women who experienced recurrence compared to those who were cured at 1 month posttreatment (log fold change [LFC] = 1.44, standard error [SE] = 0.56,  $P = .011$ ).

Immediately posttreatment, the mean abundance of *Gardnerella* was increased in recurrence versus cure cases (LFC = 1.90, SE = 0.86,  $P = .028$ ). Conversely, the mean abundance of both *L. crispatus* and *Lactobacillus fornicialis* was decreased in women who experienced recurrence compared to those who did not (*L. crispatus* LFC = -1.55, SE = 0.40,  $P = .0001$ ; *L. fornicialis* LFC = -1.19, SE = 0.34,

**Table 2. Baseline and Longitudinal Characteristics by Bacterial Vaginosis Recurrence Status (n = 121)**

| Characteristic  | Cure (n = 105) | Recurrence (n = 16) | P Value <sup>a</sup> |
|---|----------------|---------------------|----------------------|
| <b>Study</b>  |                |                     |                      |
| StepUp studies  | 45 (43)        | 4 (25)              | .274                 |
| SToPBV  | 60 (57)        | 12 (75)             |                      |
| <b>Past history of BV</b>                               |                |                     |                      |
| No  | 30 (29)        | 2 (12)              | .232                 |
| Yes   | 75 (71)        | 14 (88)             |                      |
| <b>Antibiotic prescribed</b>                            |                |                     |                      |
| Oral metronidazole                                      | 92 (88)        | 14 (87.5)           | 1.000                |
| Intravaginal clindamycin                                | 12 (11)        | 2 (12.5)            |                      |
| Intravaginal metronidazole                              | 1 (1)          | 0 (0)               |                      |
| <b>100% treatment adherence<sup>b</sup></b>             |                |                     |                      |
| No  | 10 (10)        | 2 (12)              | .662                 |
| Yes   | 94 (90)        | 14 (88)             |                      |
| <b>Characteristics between day 0 and study endpoint</b> |                |                     |                      |
| <b>Any hormonal contraceptive use<sup>c</sup></b>       |                |                     |                      |
| No  | 55 (52)        | 9 (56)              | .796                 |
| Yes   | 50 (48)        | 7 (44)              |                      |
| <b>Any IUD use<sup>d</sup></b>                          |                |                     |                      |
| No  | 94 (90)        | 13 (81)             | .396                 |
| Yes   | 11 (10)        | 3 (19)              |                      |
| <b>Any douching</b>                                     |                |                     |                      |
| No  | 94 (90)        | 15 (94)             | 1.000                |
| Yes   | 11 (10)        | 1 (6)               |                      |
| <b>Any smoking</b>                                      |                |                     |                      |
| No  | 68 (65)        | 8 (50)              | .271                 |
| Yes   | 36 (35)        | 8 (50)              |                      |
| <b>Any penile–vaginal sex</b>                           |                |                     |                      |
| No  | 28 (27)        | 1 (6)               | .114                 |
| Yes   | 77 (73)        | 15 (94)             |                      |
| <b>Condom use for penile–vaginal sex</b>                |                |                     |                      |
| Always/no penile–vaginal sex                            | 38 (36)        | 6 (38)              | 1.000                |
| Not always  | 67 (64)        | 10 (62)             |                      |
| <b>Any receptive oral sex</b>                           |                |                     |                      |
| No  | 42 (40)        | 5 (31)              | .590                 |
| Yes   | 63 (60)        | 11 (69)             |                      |
| <b>Any anal sex</b>                                     |                |                     |                      |
| No  | 93 (89)        | 12 (75)             | .225                 |
| Yes   | 12 (11)        | 4 (25)              |                      |
| <b>Any sex with an RSP</b>                              |                |                     |                      |
| No  | 44 (42)        | 3 (19)              | .100                 |
| Yes   | 61 (58)        | 13 (81)             |                      |
| <b>RSP treatment details</b>                            |                |                     |                      |
| No RSP  | 41 (39)        | 3 (19)              | <b>.007</b>          |
| Treated RSP   | 45 (43)        | 4 (25)              |                      |
| Untreated RSP   | 19 (18)        | 9 (56)              |                      |
| <b>Any new sexual partner</b>                           |                |                     |                      |
| No  | 85 (81)        | 13 (81)             | 1.000                |
| Yes   | 20 (19)        | 3 (19)              |                      |

Data are presented as No. (%) unless otherwise indicated. P values <.05 are bolded to indicate statistically significant associations.

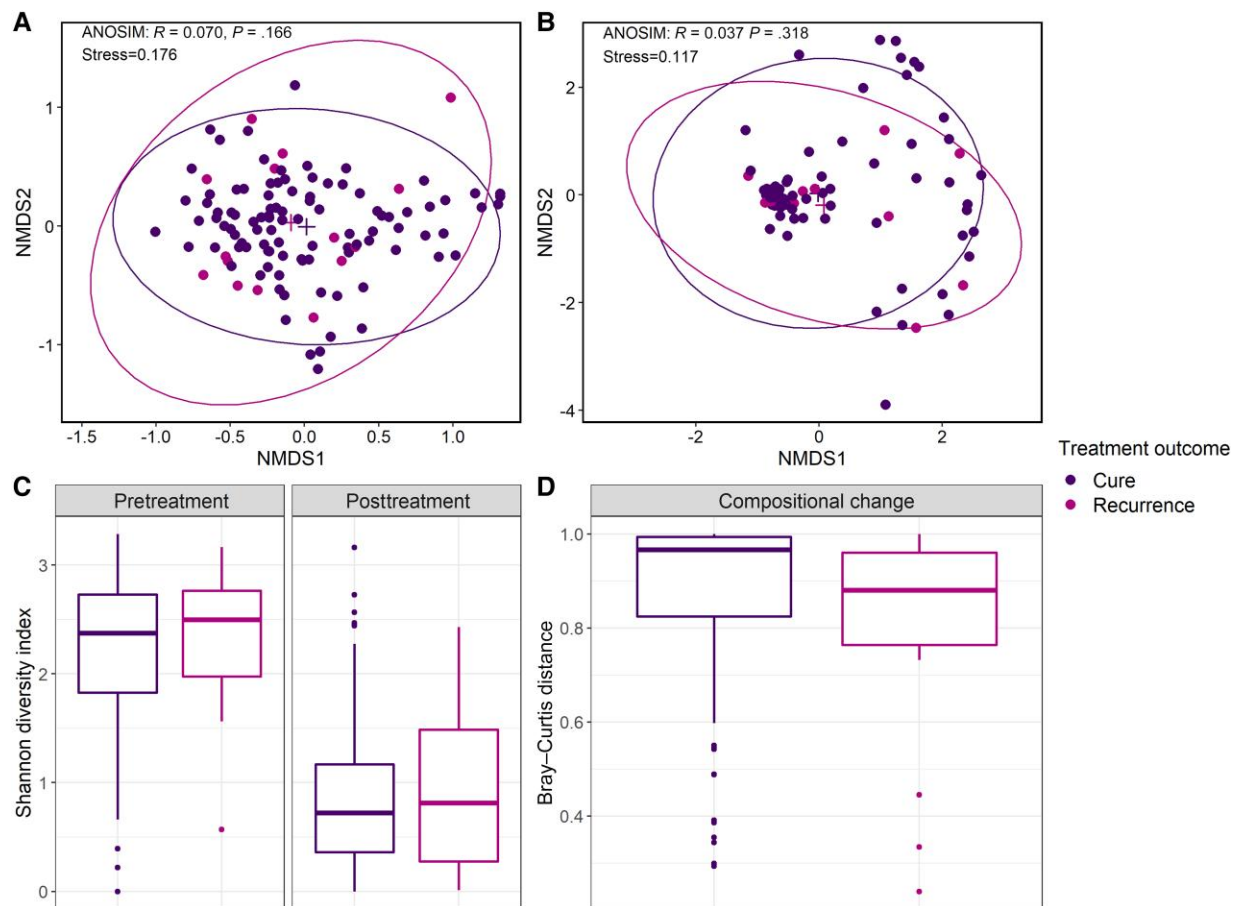
Abbreviations: BV, bacterial vaginosis; IUD, intrauterine device; RSP, regular sexual partner; SToPBV, Strategies to prevent BV.

<sup>a</sup>The  $\chi^2$  or Fisher exact test was used for categorical data and Wilcoxon signed-rank test was used for continuous variables.

<sup>b</sup>Antibiotics prescribed included oral metronidazole (n = 106), intravaginal clindamycin (n = 14), and intravaginal metronidazole (n = 1). One woman did not report adherence data.

<sup>c</sup>Between day 0 and month 1, 44 women reported current oral contraceptive use, 6 reported using a levonorgestrel IUD, 6 reported using a contraceptive implant, and 1 reported using a hormonal injection (Depo-Provera).

<sup>d</sup>Between day 0 and month 1, 6 women reported using a levonorgestrel IUD and 8 women reported using a copper IUD. Of the 3 women with an IUD in situ who experienced recurrence, 2 reported using a copper IUD and 1 reported using a levonorgestrel IUD.



**Figure 1.** Diversity metrics of the vaginal microbiota of women who experienced bacterial vaginosis recurrence and those that remained cured at 1 month postenrollment. Nonmetric multidimensional scaling plots of the overall composition of the vaginal microbiota pretreatment (A) and immediately posttreatment (B). Box plots showing the bacterial diversity (measured using the Shannon diversity index) of the vaginal microbiota of cure and recurrence cases pretreatment and immediately posttreatment (C). Box plots displaying differences in the compositional change of the vaginal microbiota following treatment (measured using Bray–Curtis distances calculated between paired pretreatment and immediate posttreatment samples) between cure and recurrence cases (D). Abbreviations: ANOSIM, analysis of similarity; NMDS, nonmetric multidimensional scaling.

$P = .0006$ ). There was no significant difference in the abundance of *L iners* between recurrence and cure cases at day 0 (LFC = 0.08, SE = 0.71,  $P > .05$ ) or day 8 (LFC =  $-1.60$ , SE = 1.02,  $P > .05$ ). ANCOM-BC findings were mostly consistent with findings from a second differential abundance analysis method (Supplementary Material). Supplementary Table 2 presents the prevalence and relative abundance of taxa identified as differentially abundant by ANCOM-BC. Of note, *L crispatus* was present in low relative abundance both pretreatment and immediately posttreatment in most participants, except for 8 cure cases who had a *L crispatus* relative abundance  $>50\%$  at day 8.

In logistic regression analyses adjusted for partner type/treatment status and laboratory method, a high CLR-transformed abundance of *Prevotella* spp pretreatment (AOR, 1.35 [95% CI, 1.05–1.91],  $P = .041$ ; Table 3) and a high CLR-transformed abundance of *Gardnerella* immediately

posttreatment (AOR, 1.23 [95% CI, 1.03–1.49],  $P = .026$ ; Table 3) were both associated with increased odds of BV recurrence. No other taxa were associated with BV recurrence.

#### Bacterial Ratios Associated With BV Recurrence

Finally, we explored the relationship between BV recurrence and the ratio of nonoptimal to optimal bacteria present in the vaginal microbiota pretreatment and immediately posttreatment. Pretreatment, the ratios of BVAB:*Lactobacillus* and *Gardnerella*:*L iners* were not associated with BV recurrence (Table 4). Immediately posttreatment however, higher levels of total BVAB relative to *Lactobacillus* spp and higher levels of *Gardnerella* relative to *L iners* were both associated with increased odds of BV recurrence (AOR, 1.38 [95% CI, 1.06–1.81],  $P = .017$  and AOR, 1.44 [95% CI, 1.09–1.95],  $P = .012$ ; Table 4). The ratio of pathobionts:*Lactobacillus* was not associated with recurrence at either timepoint.

**Table 3. Associations Between Bacterial Vaginosis Recurrence and Characteristics of the Vaginal Microbiota Pretreatment and Immediately Posttreatment**

| Characteristic                                  | Cure, Median (IQR) <sup>a</sup> | Recurrence, Median (IQR) <sup>a</sup> | AOR (95% CI) <sup>b</sup> | P Value     |
|---|---------------------------------|---------------------------------------|---------------------------|-------------|
| Compositional change (Bray–Curtis) <sup>c</sup> | 0.97 (0.82–0.99)                | 0.88 (0.76–0.96)                      | 0.22 (.02–2.96)           | .357        |
| Pretreatment (day 0)                            |                                 |                                       |                           |             |
| α-diversity (Shannon diversity)                 | 2.37 (1.83–2.73)                | 2.49 (1.98–2.76)                      | 1.99 (.77–6.71)           | .203        |
| <i>Gardnerella</i> spp                          | 7.07 (6.44–7.66)                | 6.69 (6.44–7.09)                      | 1.15 (.80–1.86)           | .514        |
| <i>Prevotella</i> spp <sup>d</sup>              | 4.99 (2.44–6.03)                | 5.92 (5.52–6.54)                      | 1.35 (1.05–1.91)          | <b>.041</b> |
| <i>Lactobacillus crispatus</i>                  | −0.86 (−1.22 to −0.51)          | −0.88 (−1.13 to −0.44)                | 0.89 (.52–1.25)           | .576        |
| <i>Lactobacillus fornicalis</i>                 | −0.87 (−1.23 to −0.56)          | −0.88 (−1.13 to −0.44)                | 0.78 (.33–1.25)           | .418        |
| Immediately posttreatment (day 8)               |                                 |                                       |                           |             |
| α-diversity (Shannon diversity)                 | 0.72 (0.36–1.17)                | 0.81 (0.28–1.49)                      | 1.37 (.60–2.97)           | .435        |
| <i>Gardnerella</i> spp                          | 2.33 (−0.17 to 5.18)            | 5.41 (1.18–7.83)                      | 1.23 (1.03–1.49)          | <b>.026</b> |
| <i>Prevotella</i> spp <sup>d</sup>              | −0.22 (−0.36 to −0.11)          | −0.20 (−0.38 to −0.13)                | 1.00 (.72–1.31)           | .983        |
| <i>Lactobacillus crispatus</i>                  | −0.22 (−0.48 to −0.11)          | −0.30 (−0.52 to −0.16)                | 0.71 (.29–1.03)           | .228        |
| <i>Lactobacillus fornicalis</i>                 | −0.18 (−0.39 to −0.08)          | −0.30 (−0.52 to −0.16)                | 0.67 (.30–1.03)           | .168        |

P values <.05 are bolded to indicate statistically significant associations.

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; IQR, interquartile range.

<sup>a</sup>Values for individual bacterial taxa represent the centered log-ratio transformed abundance.

<sup>b</sup>Regression models were adjusted for partner type (treated regular sexual partner [RSP], untreated RSP, no RSP) and laboratory method.

<sup>c</sup>Compositional change was measured using Bray–Curtis distances calculated between paired pretreatment and immediate posttreatment samples, where a value of 0 indicates no compositional change following treatment and a value closer to 1 indicates substantial change following treatment. Only women who provided both day 0 and day 8 samples (n = 109) were included in this analysis.

<sup>d</sup>Represents *Prevotella* amplicon sequence variants that were not able to be classified at the species level.

## DISCUSSION

In this study, we identified specific vaginal taxa that were associated with BV recurrence within 1 month of treatment. Specifically, we found that a higher abundance of *Prevotella* spp immediately prior to treatment and of *Gardnerella* spp immediately following treatment were both associated with increased odds of BV recurrence. Additionally, women who experienced recurrence had increased levels of BV-associated bacteria (primarily representing *Gardnerella*) relative to *Lactobacillus* spp (primarily representing *L. iners*) present in their vaginal microbiota immediately posttreatment compared to women who did not experience recurrence. Neither bacterial diversity nor the degree of compositional change following treatment were associated with BV recurrence. These data provide insight into key taxa that may play a role in treatment failure following recommended BV therapy.

*Prevotella* and *Gardnerella* are commonly recovered from the vaginal microbiome of women with BV, and species from both genera possess characteristics that are important for BV initiation and persistence. In vitro, *Prevotella bivia* has been shown to produce ammonia and amino acids which enhance the growth of other BV-associated bacteria including *Gardnerella* spp and *Peptostreptococcus* spp [21, 22]. *Gardnerella* in turn produces amino acids, which stimulates growth of *P. bivia* [21]. In addition, both *Gardnerella* spp and *Prevotella* spp produce sialidases [23, 24], and there is evidence that *Prevotella* may be a dominant source of vaginal sialidase.

One early study found that sialidase activity in women with BV was attributable to *Prevotella* spp in a majority of cases [25], and another reported that among women with molecular BV, *Prevotella* was a key taxon that was enriched in the presence of cervicovaginal sialidase activity [26]. Sialidases degrade the cervicovaginal mucus [27], which assists bacterial adherence to vaginal epithelium and formation of biofilms [28]. Dense polymicrobial biofilms are a characteristic feature of BV [29] and likely contribute to treatment failure and BV recurrence [30]; not only do they act as a barrier to reduce antibiotic penetration, but quorum sensing between organisms present in a biofilm can result in activation of resistance mechanisms including expression of efflux pumps [31]. Indeed, BV biofilms have been shown to persist following metronidazole treatment [32]. *Gardnerella* spp typically dominate BV biofilms [29, 33]. However, other organisms including *F. vaginae* are known to co-occur [29, 33], and both *F. vaginae* and *P. bivia* can integrate into established *Gardnerella* biofilms in vitro and may contribute to ongoing maintenance of the biofilm [34, 35]. Therefore, it is possible that our observed association between BV recurrence and high pretreatment abundance of *Prevotella* spp reflects the presence of a highly developed multispecies biofilm that is refractory to treatment.

The observed association between recurrence and high abundance of *Gardnerella* spp and high BVAB:*Lactobacillus* ratio immediately posttreatment may also suggest treatment failure due to the presence of a dense biofilm, or alternatively

**Table 4. Associations Between Bacterial Vaginosis Recurrence and the Ratio of Nonoptimal to Optimal Bacteria Present in the Vaginal Microbiota Pretreatment and Immediately Posttreatment**

| Ratio   | Cure, Median (IQR) <sup>a</sup> | Recurrence, Median (IQR) <sup>a</sup> | AOR (95% CI) <sup>b</sup> | P Value     |
|---|---------------------------------|---------------------------------------|---------------------------|-------------|
| Pretreatment (day 0)                            |                                 |                                       |                           |             |
| BVAB <sup>c</sup> : <i>Lactobacillus</i>        | 1.40 (0.61–2.25)                | 1.53 (0.95–2.08)                      | 1.17 (.78–1.81)           | .445        |
| <i>Gardnerella:L. iners</i>                     | 0.97 (0.16–1.97)                | 0.95 (0.46–1.43)                      | 1.15 (.77–1.74)           | .511        |
| Pathobionts <sup>d</sup> : <i>Lactobacillus</i> | –1.35 (–2.22 to –0.23)          | –1.55 (–1.80 to –0.83)                | 0.99 (.61–1.56)           | .976        |
| Immediately posttreatment (day 8)               |                                 |                                       |                           |             |
| BVAB <sup>c</sup> : <i>Lactobacillus</i>        | –2.15 (–2.76 to –0.38)          | –0.28 (–1.63 to 0.31)                 | 1.38 (1.06–1.81)          | <b>.017</b> |
| <i>Gardnerella:L. iners</i>                     | –2.01 (–3.71 to –0.42)          | –0.43 (–1.90 to 0.22)                 | 1.44 (1.09–1.95)          | <b>.012</b> |
| Pathobionts <sup>d</sup> : <i>Lactobacillus</i> | –2.63 (–3.02 to –1.43)          | –2.28 (–2.54 to –0.99)                | 1.14 (.87–1.45)           | .314        |

P values <.05 are bolded to indicate statistically significant associations.

Abbreviations: AOR, adjusted odds ratio; BVAB, bacterial vaginosis–associated bacteria; CI, confidence interval; IQR, interquartile range; *L. iners*, *Lactobacillus iners*.

<sup>a</sup>Ratios were calculated using log<sub>10</sub>-transformed relative abundance data. A positive ratio reflects a higher abundance of nonoptimal bacteria (BVAB or pathobionts) relative to optimal bacteria (*Lactobacillus* spp or *L. iners*). A negative ratio reflects a lower abundance of nonoptimal bacteria relative to optimal bacteria.

<sup>b</sup>Logistic regression models were adjusted for partner type (treated regular sexual partner [RSP], untreated RSP, no RSP) and laboratory method.

<sup>c</sup>BVAB: *Gardnerella*, *Dialister*, *Prevotella*, *Finegoldia*, *Atopobium*, *Fannyhessea*, *Peptoniphilus*, *Aerococcus*, *Sneathia*, *Megasphaera*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Mobiluncus*, *Peptococcus*, *Fusobacterium*, *Saccharibacteria* (BVAB TM7), BVAB1-3.

<sup>d</sup>Pathobionts: *Campylobacter*, *Enterobacter*, Enterobacteriaceae, *Enterococcus*, *Escherichia/Shigella*, *Haemophilus*, *Klebsiella*, *Morganella*, *Neisseria*, *Proteus*, *Staphylococcus*, *Streptococcus*.

resistance to first-line therapy, which has been documented for metronidazole [36–39]. Our finding that high *Gardnerella* abundance posttreatment may contribute to recurrence is supported by a previous 16S rRNA gene sequencing study of 41 women that found that sustained cure was associated with lower relative abundance of *Gardnerella* posttreatment [10]. A quantitative polymerase chain reaction (qPCR)–based analysis of the same samples showed that persistently high levels of *Gardnerella* genomospecies GS07 pre- and posttreatment predicted treatment failure [11]. Furthermore, an analysis of 33 women found that sustained cure at 1 month posttreatment was associated with a marked decrease in *Gardnerella vaginalis* absolute abundance (quantified using qPCR) following intravaginal metronidazole [7]. Collectively, these data suggest that posttreatment persistence of *Gardnerella* spp contributes to treatment failure. Metronidazole has limited ability to disrupt established *Gardnerella* biofilms [40], and in vitro studies have found that the minimum inhibitory concentration for both metronidazole and clindamycin is higher for biofilm-forming *G. vaginalis* isolates compared to planktonic isolates [41, 42]. Furthermore, 2 recent studies both reported that while metronidazole and clindamycin had some in vitro activity against single-species *Gardnerella* biofilms, neither antibiotic significantly altered the composition of multispecies biofilms [42, 43]. Together these data suggest that antibiofilm agents may be needed to effectively treat BV [30], particularly in the setting of persistent infections. It is also possible that the high abundance of *Gardnerella* immediately posttreatment is a marker of other factor(s) (eg, presence of an intrauterine device, smoking, or douching) or perhaps other organisms that are driving disease recurrence.

*Prevotella* spp are also commonly detected in the cutaneous penile and male urethral microbiota [44–46], and prior studies

conducted by our group and others have reported the relative abundance of specific *Prevotella* spp to be moderately to strongly correlated between the vaginal and male genital microbiota of couples [17, 18, 47]. Therefore, an alternative hypothesis for our findings is that the high abundance of *Prevotella* spp pretreatment may also reflect women who are at increased risk of recurrence due to ongoing exposure to an untreated RSP. There is now substantial microbiological and epidemiological evidence that supports a role for sexual transmission in both the acquisition and recurrence of BV [6]. Notably, a recent study demonstrated that detection of several BV-associated bacteria in the penile urethra (including *Prevotella amnii* and *G. vaginalis*) was contingent upon prior penile–vaginal sex, and that these organisms could persist in the urethra >60 days after exposure [46]. In our study, women who reported sex with an untreated RSP during follow-up experienced BV recurrence more commonly compared to women with no RSP or women who had an RSP who received partner treatment. Of note, the proportion of women with a treated RSP who experienced recurrence (8%) was comparable to women with no RSP (7%). These findings suggest that combined oral and topical male partner treatment reduces BV recurrence at 1 month post-enrollment to the level of a woman without an RSP. A multisite randomized controlled trial evaluating this intervention is ongoing (ACTRN12619000196145) and will provide the necessary data on its efficacy for preventing BV recurrence over a 3-month follow-up period.

This study analyzed pooled samples and data from 3 well-characterized cohorts of women undergoing BV treatment. Study strengths included the availability of detailed metadata including sexual practices, contraceptive practices, and treatment adherence, allowing us to investigate the relationship between these factors and treatment outcome, and adjust analyses



for potential confounders. Furthermore, we investigated several features of the vaginal microbiota, providing a comprehensive analysis. Additionally, the sample size of our study was larger than prior studies that have investigated the role of the vaginal microbiota in BV treatment outcome [7–14, 48].

There are limitations to this study. The relatively small number of women who experienced BV recurrence may have influenced our ability to identify associations between diversity metrics and BV recurrence. Sequencing short fragments of the 16S rRNA gene provides limited resolution beyond the genus level, and we were unable to identify the specific species (and strains) of *Gardnerella* and *Prevotella* that may contribute to treatment failure. Furthermore, 16S rRNA gene sequencing generates relative abundance data, which may not accurately reflect the absolute abundance of individual bacteria [49, 50]. A quantitative method would provide additional insight into the contribution of key taxa to posttreatment recurrence. Finally, the laboratory methods were not identical between the studies; however, we adjusted for this in regression analyses.

Our findings suggest that key taxa may contribute to the high rates of treatment failure following recommended BV therapy. Future studies should prioritize understanding the role of specific *Prevotella* and *Gardnerella* strains in BV recurrence, as well as their concordance between sexual partners. Additionally, in vivo studies investigating the role of *Prevotella* spp in biofilm formation and persistence are needed, as much of the work in this space is in vitro. Importantly, our results suggest there are 2 dominant factors driving posttreatment recurrence: persistence of disease due to presence of a multispecies biofilm and/or antibiotic resistance, and reinfection from an untreated sexual partner. Ultimately, interventions that target these factors are needed to achieve sustained BV cure.

#### Supplementary Data

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

#### Notes

**Author contributions.** E. L. P., L. A. V., and C. S. B. conceived and designed this secondary analysis. E. L. P. analyzed the sequencing data. A. M. S., with statistical support from E. L. P. and L. A. V., performed statistical analyses. J. A. D. and G. L. M. performed and supervised the laboratory work. L. A. V. and C. S. B. conceived of the original trials from which the specimens were derived. L. A. V., C. S. B., E. L. P., C. K. F., J. A. D., G. L. M., and J. S. H. contributed to study design and/or implementation of 1 or more of the original trials. L. A. V., C. K. F., S. M. G., and C. S. B. acquired funding

for the microbiota analyses. G. F. performed Nugent scoring in the parent trials. E. L. P., A. M. S., L. A. V., and C. S. B. wrote the original draft. All authors critically reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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**Data availability.** The raw sequencing data are publicly available in the National Center for Biotechnology Information Sequence Read Archive (accession numbers PRJNA592384, PRJNA398590, and PRJNA735440).

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