Bacterial Communities Associated With Abnormal Nugent Score in Postmenopausal Versus Premenopausal Women

Caroline M. Mitchell,^{1,a} Sujatha Srinivasan,^{2,a} Nanxun Ma,³ Susan D. Reed,⁴ Michael C. Wu,³ Noah G. Hoffman,⁵ Daniel J. Valint,² Sean Prohl,² Tina L. Fiedler,² Kathy J. Agnew,⁴ Katherine A. Guthrie,³ and David N. Fredricks²

¹Vincent Center for Reproductive Biology, Massachusetts General Hospital, Boston, Massachusetts, USA, ²Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, ³Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, ⁴Department of Obstetrics and Gynecology, University of Washington, Seattle, Washington, USA, and ⁵Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA

The Nugent score is the reference standard for bacterial vaginosis (BV) diagnosis but has not been validated in postmenopausal women. We compared relative abundances from 16S ribosomal RNA gene sequencing of vaginal microbiota with Nugent score in cohorts of premenopausal (n = 220) and postmenopausal (n = 144) women. In premenopausal women, 33 taxa were significantly correlated with Nugent score, including the classic BV-associated taxa *Gardnerella*, *Atopobium*, *Sneathia*, *Megasphaera*, and *Prevotella*. In postmenopausal women, 11 taxa were significantly associated with Nugent score, including *Prevotella* but no other BV-associated genera. High Nugent scores should not be used to infer BV in postmenopausal women.

Keywords. bacterial vaginosis; vaginal microbiota; menopause; vaginitis.

Bacterial vaginosis (BV) is a syndrome characterized by lower abundance of vaginal lactobacilli and higher diversity and quantity of non-*Lactobacillus* species. The clinical diagnostic criteria for BV (Amsel criteria) developed in 1983 used data from a cohort of 397 college-age women in Seattle, Washington [1]. The reference standard for BV diagnosis in research settings (Nugent and Hillier) is Gram stain scoring of bacterial morphotypes from vaginal smear samples, developed using data from 200 pregnant women from 5 centers around the United States [2]. These 2 diagnostic modalities do not always

agree; Amsel criteria have a lower sensitivity for BV compared with Nugent score [3].

Molecular characterization of vaginal microbiota using 16S ribosomal RNA (rRNA) gene sequencing provides an additional lens through which to view the vaginal bacterial community, with more detailed resolution of the species present. Amsel criteria, Gram stain of vaginal smear samples, and taxonomic profiles by 16S rRNA gene sequencing measure different features and are not always concordant when identifying BV (Amsel) or a BV-like microbiota (Gram stain; 16S rRNA gene sequencing). Some argue for a category of “molecular BV,” characterized by high relative abundance of *Gardnerella*, low abundance of *Lactobacillus*, and increased community diversity [4].

Lactobacillus dominance of the vaginal microbiota is less common in postmenopausal than in premenopausal women, likely owing to lower systemic estrogen leading to less vaginal epithelial glycogen to provide nutrients for lactobacilli [5]. It is unclear how non-*Lactobacillus*-dominant microbiota in postmenopausal women relates to non-*Lactobacillus*-dominant microbiota observed in premenopausal women with BV, and the bacterial morphotypes used for Gram stain scoring. Clue cells on wet mount, one clinical criterion for BV diagnosis, are also relatively uncommon in postmenopausal women [6]. We compared 16S rRNA gene sequencing results and Nugent scoring of Gram-stained vaginal fluid smear samples to assess whether an abnormal Nugent score in postmenopausal women resembles “molecular BV” and can be used to infer a diagnosis of BV.

METHODS

We used data from 2 studies: the MsFLASH (Menopause Strategies: Finding Lasting Answers for Symptoms and Health) Vaginal Health Trial (ClinicalTrials.gov NCT02516202) (postmenopausal women) and a cohort of women presenting to a sexual health clinic in Seattle, Washington (premenopausal women). Both studies had human subjects approval, and participants signed informed consent [7, 8]. We included the second cohort to demonstrate that Nugent score and molecular microbiologic findings are concordant within a premenopausal group of women, as expected.

The MsFLASH Vaginal Health Trial was a randomized trial of treatments for postmenopausal vaginal discomfort between June 2016 and April 2017 [7]. Eligible women reported moderate to severe vulvovaginal itching, burning, irritation, or pain with vaginal penetration, and were negative for BV (using modified Amsel criteria not including pH), vulvovaginal candidiasis and trichomoniasis by wet mount. Recent use of hormone therapy was an exclusion. In a subset

Received 10 September 2020; editorial decision 15 October 2020; accepted 22 October 2020; published online October 27, 2020.

^aC. M. M. and S. S. contributed equally to this work.

Presented in part: North American Menopause Society Annual Meeting, Chicago, Illinois, 25–28 September 2019.

Correspondence: Caroline M. Mitchell, Vincent Center for Reproductive Biology, Massachusetts General Hospital, 55 Fruit St, Boston, MA 02114 (Caroline.mitchell@mgh.harvard.edu).

The Journal of Infectious Diseases® 2021;223:2048–52

© The Author(s) 2020. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com. DOI: 10.1093/infdis/jiaa675

of 144 women, DNA extraction and polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene were performed as described elsewhere [9]. This analysis included women with both Gram stain and PCR data at enrollment and 12-week visits.

Data for premenopausal women were drawn from enrollment visits in a previously described cohort of 220 women attending the Public Health Seattle and King County Sexually Transmitted Diseases Clinic between September 2006 and June 2010. Participants were evaluated for BV using Amsel criteria, confirmed by Nugent scoring. Sequencing was performed using 454 pyrosequencing of the V3–V4 region of the 16S rRNA gene, and taxonomic assignment was performed using a custom, hand-curated sequence database in addition to RDP (Ribosomal Database Project) [8].

Gram-positive rods (*Lactobacillus* morphotypes), gram-negative or gram-variable rods (*Gardnerella/Bacteroides* morphotypes), and curved gram-negative rods (*Mobiluncus* morphotypes) per high-powered microscope field ($\times 1000$) of a vaginal fluid Gram stain were enumerated using the Nugent and Hillier methods [2]. Slides for both studies were scored by the same person (K. J. A.).

For statistical analysis, taxa present in <5% of samples were removed, bayesian multiplicative replacement was used to desparsify the taxon counts, and data were center log-ratio transformed. To estimate associations between bacterial taxa and overall Nugent score or individual component scores, we used linear models and linear mixed models with a random intercept, for data from premenopausal and postmenopausal women, respectively. We controlled for multiple testing using the Benjamini-Hochberg method and set significance at false discovery rate (q value) <0.1. Bacterial taxa were analyzed at the genus level, with the exception of *Lactobacillus iners*. *L. iners* is often noted in women with BV [8] and shown to be associated with the *Gardnerella/Bacteroides* morphotype [10]; hence we analyzed *L. iners* separately from other *Lactobacillus* species.

RESULTS

Of 144 postmenopausal women included in the microbiota substudy, 131 (91%) were white and 6 (4%) were black, with a mean age of 61 years (Supplementary Table 1). One woman had BV diagnosed at week 12 based on modified Amsel criteria. Among 220 premenopausal women, 97 (44%) were white and 75 (34%) black, with mean age of 29 years. In the premenopausal cohort, 98 (43%) had BV by Amsel criteria; 47% had an abnormal discharge, 36% had >20% clue cells, 66% had an elevated vaginal pH, and 40% had a positive whiff test result. A small proportion had yeast (8%) or trichomoniasis (7%) diagnosed.

At enrollment, the Nugent score distribution differed between premenopausal and postmenopausal women; the score was normal (0–3) in 41% versus 20%, respectively, intermediate (4–6) in 6% versus 64%, and abnormal (indicating BV) (7–10) in 53% versus 16%, recognizing that the population

of premenopausal women was enriched for BV as part of the parent study design. No curved gram-negative rods, otherwise known as *Mobiluncus* morphotypes (but which often include the uncultivated bacterium, BV-associated bacterium 1 [10]), were seen in postmenopausal women. Hence none had Nugent scores of 9–10. *Mobiluncus curtisii* sequences were present by PCR in postmenopausal samples; however these were not sufficiently abundant to be visualized on a Gram-stained slide. Among postmenopausal women with a Nugent score of 0–3, *Lactobacillus*-dominant communities were less frequent than in premenopausal women by 16S rRNA gene sequencing (78.8% vs 97.8%, respectively; $P < .001$) (Figure 1).

To assess how well Nugent scores represented the abundance of BV-associated taxa in the 2 cohorts, we compared the relative abundance of individual taxa across the range of Nugent scores within each cohort. We found multiple taxa to be significantly associated with Nugent scores, but these differed between premenopausal and postmenopausal women (Figure 2 and Supplementary Tables 2A and 3A). Not surprisingly, *Lactobacillus* was negatively associated with Nugent score in both groups. The group “*Lactobacillus*” included all species except *L. iners* and was present in 47% of postmenopausal and 66% of premenopausal women. Considered separately, *L. iners* was not associated with Nugent score in postmenopausal women but was negatively associated in premenopausal women (Supplementary Table 3A). *L. iners* was more prevalent in premenopausal (90%) than in postmenopausal (36%) women. None of the classic BV-associated taxa associated with Nugent score in premenopausal women (eg, *Gardnerella*, *Atopobium*, *Sneathia*, and *Megasphaera*) were significantly associated with Nugent score in postmenopausal women. *Prevotella* and *Dialister* were associated with Nugent scores in both postmenopausal and premenopausal women. The total number of taxa significantly associated with Nugent score ($P < .05$; false discovery rate, <0.1) was lower in postmenopausal ($n = 11$) than in premenopausal ($n = 33$) women.

Among postmenopausal women, 8 taxa were significantly associated with the *Gardnerella/Bacteroides* morphotype score (Supplementary Table 2B), including *Prevotella* but not *Gardnerella*. In premenopausal women, 28 taxa were associated with the *Gardnerella/Bacteroides* morphotype score, including *Gardnerella* and *Prevotella* (Supplementary Table 3B). As expected, the top taxon associated with the *Lactobacillus* morphotypes were *Lactobacillus* sequences in postmenopausal and premenopausal women. *L. iners* was also significantly associated with the *Lactobacillus* morphotype (Supplementary Tables 1C and 2C).

DISCUSSION

Our data demonstrate that among postmenopausal women with vaginal symptoms, classic BV-associated genera, such as *Gardnerella*, *Megasphaera*, *Atopobium*, and *Sneathia*, are not

Figure 1. Relative abundance of bacterial taxa identified in postmenopausal women by Illumina MiSeq sequencing and premenopausal women by 454 pyrosequencing of the V3–V4 region of the 16S ribosomal RNA gene. Results are grouped by menopausal status and Nugent score category. The top 25 bacterial taxa across both data sets and any genus with >20% relative abundance in any single sample are shown. Taxa indicated with triangles were noted only in postmenopausal women, and bacterial vaginosis–associated bacterium (BVAB) 1 was noted only in premenopausal women.

associated with an abnormal Nugent score (7–10). When we performed the same comparison in premenopausal women presenting with vaginal symptoms, these genera are strongly associated with elevated Nugent score, as expected. In both cohorts, *Lactobacillus* species are negatively associated with Nugent score, also as expected. These findings suggest that although symptomatic postmenopausal women with an abnormal Gram stain have a diverse, non-*Lactobacillus*–dominant vaginal bacterial community, that community is different from that in premenopausal women with BV, and a high Nugent score should not be used to infer BV status in this population.

The high prevalence of intermediate microbiota in our postmenopausal cohort is consistent with findings of other investigations. Gram-stained smear samples from 921 postmenopausal Italian women (36% using hormone therapy) identified a high rate of intermediate Nugent scores (47.5%), primarily characterized by the absence of *Lactobacillus*, *Gardnerella*/*Bacteroides*, and *Mobiluncus* morphotypes [6]. Only 8.5% had both an abnormal Nugent score (≥ 4) and clue cells, which would be more indicative of BV. A US community-based study of women aged 57–85 years examined 1899 swab samples and identified BV in 23%–38% with the Nugent method. Similar to our results, few women had scores in the 9–10 range [11].

Figure 2. Association between Nugent score (as a continuous variable) and relative abundance of individual bacterial taxa identified using regression with linear mixed models for postmenopausal (A) and premenopausal (B) women. The top 10 most significantly associated taxa are shown. For the full list of taxa associated in both cohorts, see [Supplementary Tables 2A and 3A](#).

Among 20 Canadian postmenopausal women, 70% had intermediate or BV scores, but only 52% of those with Nugent BV had *Gardnerella* detected with species-specific PCR [12].

BV is a syndrome characterized by diverse, heterogeneous vaginal communities of anaerobic and facultative bacteria, with a lower abundance of lactobacilli. No individual pathogens fulfill Koch's postulates as the causal agent, but several genera and species are consistently associated with the diagnosis. Commercial molecular diagnostic tests for BV commonly target *Gardnerella*, *Atopobium*, *Megasphaera*, BV-associated bacterium 2, and *Lactobacillus*. All of these taxa were associated with Nugent score in our premenopausal cohort, but only *Lactobacillus* was associated in our postmenopausal group.

The one common BV-associated genus correlated with Nugent score in both groups was the gram-negative genus *Prevotella*. While *Prevotella* is often found in women with BV, when evaluated as a diagnostic marker for BV it had low sensitivity (50%) [3]. In cohorts of African women, communities with vaginal microbiota notable for *Prevotella* clustered separately from women with BV and more classic high abundance of *Gardnerella* [13]. The Nugent scoring system includes a category for *Gardnerella* or *Bacteroides* morphotypes. In 1990, the genus *Prevotella* was created from species previously considered *Bacteroides*—at approximately the same time as the Nugent score was established [14]. We have demonstrated that in premenopausal women, *Porphyromonas* and *Prevotella* are associated with the *Bacteroides* morphotype [10], and we hypothesize that in postmenopausal women many of the morphotypes counted toward the *Gardnerella/Bacteroides* score may be *Prevotella*, though this bears formal testing.

In both cohorts we observed significant associations with higher Nugent scores for taxa that, like *Prevotella*, are associated with but not diagnostic for BV, including *Dialister*, *Anaerococcus*, and *Peptoniphilus*. These are genera previously identified at higher abundances in women with BV [15]. Detection of *Peptoniphilus lacrimalis* was associated with treatment failure in premenopausal women with BV [16]. Of note, *Streptococcus* was significantly positively associated with higher Nugent scores and was highly prevalent (71% of samples) in our postmenopausal cohort, but it was negatively associated with Nugent scores in premenopausal women and far less prevalent (17% of samples) in this cohort (Supplementary Tables 2 and 3). The results suggest that while the vaginal microbiota in postmenopausal women with an abnormal Nugent score is not “optimal,” it is not the same as that in premenopausal women with BV.

The current study is one of a few to compare differences in taxa within Nugent score groups between premenopausal and postmenopausal women with vaginal symptoms. Postmenopausal women enrolled in the parent trial had to be negative for BV by Amsel criteria, but few women were

excluded based on this criterion, and only 1 woman had BV according to Amsel criteria during the trial. All Nugent scores were assigned by a single reader, which is a significant strength. Because different sequencing technologies were used in the 2 studies, which leads to variation in sequencing depth and detection of minority taxa, we elected not to perform a direct comparison of bacterial relative abundance between cohorts. The cohorts may not be representative of the general US population, but they are likely representative of both premenopausal and postmenopausal women with vaginal symptoms.

The absence of lactobacilli, a high pH, and symptoms of vaginal discomfort after menopause often do not reflect a similar microbial community, as is found in premenopausal women with BV. A high Nugent score should not be used to infer BV status in postmenopausal women reporting moderate to severe vaginal symptoms.

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 copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. This work was solely conducted by the authors; the funding agency had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Financial support. This work was funded by the National Institute on Aging (grant 5R01AG048209) and the National Institute of Allergy and Infectious Diseases (grant R01 AI061628), National Institutes of Health.

Conflict of interest statement. C. M. M. reports receiving grant funding from Merck and has served as a consultant to Scynexis. S. S. has received speaking honoraria from Lupin. S. D. R. receives grant funding from Bayer. T. L. F. and D. N. F. have developed intellectual property related to the use of polymerase chain reaction for diagnosing bacterial vaginosis and receive royalties from BD. All other authors report no potential conflicts.

References

1. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. *Am J Med* **1983**; 74:14–22.
2. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J Clin Microbiol* **1991**; 29:297–301.

3. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol* **2007**; 45:3270–6.
4. McKinnon LR, Achilles SL, Bradshaw CS, et al. The evolving facets of bacterial vaginosis: implications for HIV transmission. *AIDS Res Hum Retroviruses* **2019**; 35:219–28.
5. Brotman RM, Shardell MD, Gajer P, et al. Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause* **2014**; 21:450–8.
6. Cauci S, Driussi S, De Santo D, et al. Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. *J Clin Microbiol* **2002**; 40:2147–52.
7. Mitchell CM, Reed SD, Diem S, et al. Efficacy of vaginal estradiol or vaginal moisturizer vs placebo for treating postmenopausal vulvovaginal symptoms: a randomized clinical trial. *JAMA Intern Med* **2018**; 178:681–90.
8. Srinivasan S, Hoffman NG, Morgan MT, et al. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* **2012**; 7:e37818.
9. Mitchell CW, Watson L, Mitchell AJ, et al. Vaginal microbiota and mucosal immune markers in women with vulvovaginal discomfort. *Sexually Transm Dis* **2020**; 47:269–74.
10. Srinivasan S, Morgan MT, Liu C, et al. More than meets the eye: associations of vaginal bacteria with Gram stain morphotypes using molecular phylogenetic analysis. *PLoS One* **2013**; 8:e78633.
11. Hoffmann JN, You HM, Hedberg EC, Jordan JA, McClintock MK. Prevalence of bacterial vaginosis and *Candida* among postmenopausal women in the United States. *J Gerontol B Psychol Sci Soc Sci* **2014**; 69(suppl 2):S205–14.
12. Burton JP, Reid G. Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. *J Infect Dis* **2002**; 186:1770–80.
13. Anahtar MN, Byrne EH, Doherty KE, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* **2015**; 42:965–76.
14. Shah HN, Collins DM. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* **1990**; 40:205–8.
15. Srinivasan S, Munch MM, Sizova MV, et al. More easily cultivated than identified: classical isolation with molecular identification of vaginal bacteria. *J Infect Dis* **2016**; 214(suppl 1):S21–8.
16. Marrazzo JM, Thomas KK, Fiedler TL, Ringwood K, Fredricks DN. Relationship of specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. *Ann Intern Med* **2008**; 149:20–8.