Viewing Native American Cervical Cancer Disparities through the Lens of the Vaginal Microbiome: A Pilot Study

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ABSTRACT

Vaginal dysbiosis is implicated in persistent human papillomavirus (HPV) infection and cervical cancer. Yet, there is a paucity of data on the vaginal microbiome in Native American communities. Here, we aimed to elucidate the relationships between microbiome, HPV, sociodemographic, and behavioral risk factors to better understand an increased cervical cancer risk in Native American women. In this pilot study, we recruited 31 participants (16 Native American and 15 non-Native women) in Northern Arizona and examined vaginal microbiota composition, HPV status, and immune mediators. We also assessed individuals' sociodemographic information and physical, mental, sexual, and reproductive health. Overall, microbiota profiles were dominated by common *Lactobacillus* species (associated with vaginal health) or a mixture of bacterial vaginosis–associated bacteria. Only 44% of Native women exhibited *Lactobacillus* dominance, compared with 58% of non-Native women. Women with vaginal dysbiosis also had elevated vaginal pH and were more frequently infected with high-risk HPV. Furthermore, we observed associations of multiple people in a household, lower level of education, and high parity with vaginal dysbiosis and abundance of specific bacterial species. Finally, women with dysbiotic microbiota presented with elevated vaginal levels of proinflammatory cytokines. Altogether, these findings indicate an interplay between HPV, vaginal microbiota, and host defense, which may play a role in the cervical cancer disparity among Native American women. Future longitudinal studies are needed to determine the mechanistic role of vaginal microbiota in HPV persistence in the context of social determinants of health toward the long-term goal of reducing health disparities between non-Hispanic White and Native American populations.

Prevention Relevance: Cervical cancer disproportionally affects Native American women. Sociodemographic and behavioral factors might contribute to this disparity *via* alteration of vaginal microbiota. Here, we show the association between these factors and vaginal dysbiosis and immune activation, which can be implicated in high-risk HPV infection among Native American and other racial/ethnic populations.

Introduction

Human papillomavirus (HPV) infection and cervical cancer disproportionately impact Native American women relative to non-Hispanic White (NHW) women (1). According to Indian Health Service data from 1999 to 2009, Native American women had approximately a two-fold higher incidence and associated mortality rate than NHW women (2). In Arizona between 2016 and 2020, Hispanic and American Indian/Alaska Native (AI/AN) women had the highest age-adjusted rates of cervical cancer, with 7.8

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and 7.1 cases per 100,000 women, respectively, compared with 5.6 in NHW women (3). The associated mortality was also 1.5 times higher in Hispanic women compared with NHW, whereas data on AI/AN were suppressed because the number of cases was too small (3). This cervical cancer disparity is primarily attributed to a lack of screening, unequal access to healthcare, and quality of care. Yet, biologic factors within the local microenvironment, such as vaginal microbiome, might contribute to HPV infection, HPV persistence, and cervical carcinogenesis in Native American women.

Vaginal microbiome is a collection of microorganisms residing in the vagina (referred to as the vaginal microbiota) with "theater" of their activity (such as a spectrum of molecules produced by microorganisms and coexisting hosts structured by the surrounding environment; ref. 4). In most reproductive-age women, the lower reproductive tract (vagina and cervix) is colonized by one or few *Lactobacillus* species (including *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri/paragasseri*, and *Lactobacillus jensenii/mulieris*), which protect the host from invading pathogens, by lowering vaginal pH through lactic acid production and the secretion of antimicrobial compounds (5). However, when vaginal dysbiosis occurs, protective lactobacilli are depleted and replaced by a diverse consortium of anaerobic bacteria, such as *Gardnerella*, *Fannyhessea*, *Prevotella*, *Megasphaera*, and *Sneathia* (5). This dramatic shift in the microbiota composition may lead to bacterial vaginosis (BV) and manifest with symptoms, such as vaginal discharge, malodor, and elevated pH; yet, some women with *Lactobacillus*-depleted microbiota are asymptomatic (6).

Notably, vaginal dysbiosis has been implicated in an increased risk of acquisition of sexually transmitted infections (STI), including HPV (7). Growing evidence also suggests the causal link between dysbiosis and HPV persistence and development of cervical dysplasia (8–10). Intriguingly, women representing racial/ethnic minorities have higher rates of BV or vaginal dysbiosis (11), which consequently may increase their risk of cervical cancer. For example, in the United States, Black and Hispanic women have significantly higher rates of BV or vaginal dysbiosis (5, 11, 12). These differences in the microbiota composition among racial/ethnic groups are multifactorial and have been attributed to individuals' behavior, lifestyle, diet, socioeconomics, environment, genetic ancestry, and immune interactions (10).

However, our knowledge of the vaginal microbiota composition and HPV infection patterns in Native American communities is very limited (1). In fact, most prior microbiome studies do not include Native American women. In this pilot study, we elucidate the relationships between the vaginal microbiota, HPV, immune markers, and sociodemographic and lifestyle factors in a cohort of women from Northern Arizona to better understand an increased cervical cancer risk in Native American communities.

Materials and Methods

Ethics statement

This prospective observational study was approved by the Institutional Review Board at the University of Arizona (reference no. 1510171298). The study design was respectfully discussed with The Partnership for Native American Cancer Prevention (NACP) Outreach Core, the Community Advisory Board of the Native Americans for Community Action (NACA) Family Health Center, and the University of Arizona Tribal Consultation for guidance on cultural relevance and appropriateness. All patients provided written informed consent to participate, and the study was conducted in accordance with federal guidelines and regulations and the Declaration of Helsinki.

Study participants

Participants were recruited during their visit to the NACA Family Health Center (a non-Indian Health Service clinic) in Flagstaff, AZ, USA, between December 2020 and April 2022. The NACA clinic provides a variety of health and human services (including physicals, immunizations, disease screening, STI testing, women's health, and other medical services) to urban Native Americans, low-income and other underserved families, and individuals who experience health and socioeconomic disparities. This clinic provides services to Native and non-Native people in Northern Arizona regardless of their insurance and healthcare coverage. The NACA's mission is to provide preventive wellness strategies, empower, and advocate for Native people and others in need to create a healthy community based on Harmony, Respect, and Indigenous values. Recruitment strategies included flyers describing the study posted at the clinic, on the website as well as social media, postcards given to patients during their visits, and word of mouth from NACA staff to eligible participants. Thirty-one participants were enrolled and contributed to the study. We included individuals of any race or ethnicity aged 18 to 55 years who were premenopausal. Postmenopausal women were excluded because of the known impact of menopause on the vaginal microbiota composition (13). Data on tribal affiliation were not collected. Exclusion criteria included the following: being pregnant, currently menstruating, or postmenopausal; currently on antibiotics, antifungals, antivirals, or topical steroids; having a current vaginal infection (including BV), vulvar infection, urinary tract infection, or STI or within the previous 3 weeks; and having sexual intercourse within 48 hours. Demographic, socioeconomic, medical history, and sexual/reproductive health data were collected from culturally tailored, self-reported surveys (14). In addition, participants were administered the Perceived Stress Scale (PSS10) survey, developed by Cohen and colleagues (15), and a Patient Reported Outcome Measurement System survey, National Institutes of Health Toolbox Global Health v1.2, which includes measurements of physical health, mental health, general health and ability to carry out social activities.

T-scores were calculated to express final scores for the Global Health and PSS10 survey responses.

Biospecimen collection and processing

Participants provided two physician- or self-collected vaginal swabs based on participant preference. The first swab was collected using an ESwab collection system with liquid Amies medium (Cat. No. 480C, COPAN Diagnostics). The second swab was collected using FLOQSwabs nylon-flocked swab (Cat. No. 502CS01, COPAN Diagnostics) and used to measure vaginal pH by Hydrion pH test paper 4.5 to 7.0 (Cat. No. 334, Micro Essential Laboratory) followed by placing the swab in a tube containing 1 mL of sterile 0.9% saline solution (Cat. No. S5815, Teknova). Following the collection, vaginal specimens were immediately placed on ice and frozen at −20°C within 1 hour. For long-term storage, specimens were shipped on dry ice and transferred to −80°C. The first vaginal swabs were used for DNA extraction and subsequent HPV genotyping and 16S rRNA sequencing analyses. Swabs were thawed on ice, and total DNA was isolated using the DNeasy PowerSoil Pro Kit (Qiagen, RRID:SCR_008539) following the manufacturer's instructions. DNA samples were aliquoted and stored at −80°C for further analysis. The second vaginal swabs were used for the quantification of soluble proteins. Swabs were thawed on ice and vigorously shaken in saline solution. Samples were clarified by centrifugation (700× *g* for 10 minutes at 4°C) and aliquoted to avoid freeze/thaw cycles and stored at −80°C for further analysis.

HPV genotyping

Detection of 14 high-risk HPV (hrHPV) genotypes, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, HPV68, and one emerging possibly high-risk genotype, HPV53, was performed by isothermal amplification with real-time fluorescence detection on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, RRID:SCR_020239) using purified vaginal DNA samples and the AmpFire HPV High Risk Genotyping Assay (Cat. No. GHPVF100, Atila Biosystems) following the manufacturer's instructions.

Microbiome analysis

Microbiome analysis was performed using DNA extracted from vaginal swabs and 16S ribosomal RNA (rRNA) gene sequencing. The hypervariable region 4 (V4) of the 16S rRNA gene was amplified by PCR using 515F and 806R primers with Golay barcode tags on the forward primer (16). Amplicons were purified from agarose gel and quantified using the Quant-iT dsDNA High Sensitivity Assay (Cat. No. Q33120, Invitrogen, RRID:SCR_008452). Amplicons were pooled in equimolar concentrations, followed by quality verification with the Bioanalyzer DNA 1000 chip (Agilent Technologies, RRID:SCR_013575). The pool was combined with 1% PhiX control and sequenced on the MiSeq platform using the 600-cycle MiSeq Reagent Kit v3 (Illumina, RRID:SCR_016379). Bioinformatic analyses were performed using QIIME 2 (RRID:SCR_021258; ref. 17). Following demultiplexing, amplicon quality filtering, denoising and chimera removal, and amplicon sequence variant (ASV) definition were performed using DADA2 (q2-dada2 plugin) with no trimming or truncation of reads required because of high sequence quality (18). ASVs were taxonomically classified using q2-feature-classifier (19) using the 202 release of Genome Taxonomy Database (20) and the vaginal microenvironment-weighted classifier (21, 22) based on the STIRRUPS database (23), which increases the frequency at which species level identification can be obtained from 16S sequencing data (Supplementary Table S1). Any ASVs that were not classified as bacterial phylum or that were annotated as mitochondria were removed from the data as likely nonmicrobial features. Microbial differential abundance and microbiome composition analyses were performed using R (RRID:SCR_001905). For the microbial differential abundance analysis, microbiome tables from QIIME 2 were loaded with QIIME2R (https://github.com/jbisanz/qiime2R/blob/master/inst/CITATION). ASV counts were combined with taxonomy identification and filtered to species level for downstream analyses. The R package Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) package version 2.6.0 (24) was used to determine differentially abundant taxa between the groups based on age, body mass index (BMI), educational level, household size, marital status, self-reported race, vaginal pH, number of pregnancies, hrHPV status, perceived stress scores, general health scores, physical health *t*-scores, mental health *t*-scores, and ability to carry out social scores. The formula was based on the categorical or continuous groupings with structural zeroes included. *P* values were corrected for multiple comparisons using the FDR method (q value < 0.05 was considered significant). The output data was visualized with Prism 9.0 (GraphPad, RRID:SCR_002798). For the microbiome composition analysis, microbiome tables from QIIME 2 were converted with QIIME2R to relative abundances. Taxa were then filtered to remove those with less than 0.1% relative abundance within a sample and those with less than 5% prevalence across the samples. A hierarchical clustering of taxa at the species level was performed using ClustVis (RRID:SCR_017133; ref. 25) and based on Euclidean distance and Ward linkage. A heatmap was used to visualize the relative abundance data and patient-related annotations, including self-reported racial groups, household size, education level, number of pregnancies, HPV status, *Lactobacillus* dominance, bacterial species predominance, and vaginal pH. In addition, the correlation between vaginal microbiota species was calculated using Spearman's rank correlation analysis.

Quantification of soluble proteins

Levels of 42 proteins (EGF, eotaxin/CCL11, fibroblast growth factor 2 (FGF2), Flt3L, fractalkine/CX3CL1, G-CSF, granulocyte–macrophage colony-stimulating factor (GM-CSF), GROα/CXCL1, IFNα2, IFNγ, IL1α, IL1β, IL1Ra, IL6,

Table 1. Participant demographics and characteristics.

Characteristic	*N* = 31
Age [mean (SD)]	29.1 (6.6)
Range	18–46
Race[a] [*n* (%)]	
American Indian or Alaska Native	16 (51.6)
White/Caucasian	13 (41.9)
Asian	2 (6.5)
Mixed or multiracial	1 (3.2)
Refused to answer	1 (3.2)
Missing	1 (3.2)
Ethnicity [*n* (%)]	
Hispanic/Latina	5 (16.1)
Non-Hispanic	26 (83.9)
First language [*n* (%)]	
English	28 (90.3)
Non-English	3 (9.7)
Sexual orientation [*n* (%)]	
Heterosexual	25 (80.6)
Bisexual	5 (16.1)
Pansexual	1 (3.2)
Body mass index [mean (SD)]	28.0 (7.4)
Marital status [*n* (%)]	
Single	14 (45.2)
Living with partner	11 (35.5)
Married	3 (9.7)
Divorced	2 (6.5)
Missing	1 (3.2)
Completed education [*n* (%)]	
Less than high school	2 (6.5)
High school diploma, GED	4 (12.9)
Some college credit/no degree	10 (32.3)
Associate degree/technical school	3 (9.7)
Bachelor's degree	8 (25.8)
Master's or doctoral degree	4 (12.9)
Daily activity[a] [*n* (%)]	
Work full-time	22 (71.0)
Work part-time	4 (12.9)
Unemployed or laid-off	2 (6.5)
Keeping house or raising children full-time	5 (16.1)
Student	2 (6.5)
Disabled	1 (3.2)
Household income [*n* (%)]	
<$10,000	3 (9.7)
$10,000–$24,999	3 (9.7)
$25,000–$49,999	5 (16.1)
$50,000–$74,999	8 (25.8)
$75,000–$99,999	4 (12.9)
≥$100,000	3 (9.7)
Refuse to answer	5 (16.1)
Household size [including self; *n* (%)]	
1	2 (6.5)
2	9 (29.0)
3	10 (32.3)
4	5 (16.1)
5	3 (9.7)
6+	2 (6.5)
Antibiotics use [in the last 3 months; *n* (%)]	
Yes	6 (19.4)
No	25 (80.6)
History of smoking [at least 100 cigarettes in lifetime; *n* (%)]	
Yes	6 (19.4)
No	25 (80.6)

(Continued on the following page)

Table 1. Participant demographics and characteristics. (Cont'd)

Characteristic	*N* = 31
Number of sexual partners in lifetime [*n* (%)]	
0	1 (3.2)
1–2	11 (35.5)
3–5	4 (12.9)
6–7	1 (3.2)
8+	12 (38.7)
Missing	2 (6.5)
Number of sexual partners in last year [*n* (%)]	
0	3 (9.7)
1–2	23 (74.2)
3–5	1 (3.2)
Missing	4 (12.9)
Contraception use[a] [*n* (%)]	
Condoms	10 (32.3)
Birth control pills	6 (19.4)
Intrauterine device	4 (12.9)
Contraception injection	3 (9.7)
Contraception implant	1 (3.2)
None	11 (35.5)
Number of pregnancies [*n* (%)]	
0	20 (64.5)
1	6 (19.4)
2	1 (3.2)
3	1 (3.2)
5+	3 (9.7)
Vaccinated against HPV[b] [*n* (%)]	
Yes	16 (51.6)
No	10 (32.3)
Do not know	5 (16.1)
Most recent Pap smear [*n* (%)]	
Within the last year	13 (41.9)
More than 1 year ago, but within the last 3 years	7 (22.6%)
More than 3 years ago, but within the last 5 years	8 (25.8)
Never had a Pap smear	2 (6.5)
Missing	1 (3.2)

Abbreviations: GED, general education development; SD, standard deviation.
[a]Not mutually exclusive.
[b]Received at least one dose.

IL8/CXCL8, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL15, IL17A, IL17E/IL25, IL18, IL22, IL27, IP10/CXCL10, M-CSF, MCP1/CCL2, MCP3/CCL7, MDC/CCL22, MIG/CXCL9, MIP1α/CCL3, MIP1β/CCL4, platelet-derived growth factor (PDGF)–AA, PDGF-AB/BB, RANTES/CCL5, sCD40L, TGF-α, TNFα, TNFβ, and VEGF-A) were measured in vaginal swab samples using the Milliplex MAP Human Cytokine Chemokine Panel A Magnetic Bead Immunoassay (Cat. No. HCYTA60K, Millipore, RRID:SCR_008983) in accordance with the manufacturer's protocols. Data were collected with a Bio-Plex 200 instrument (Bio-Rad, RRID:SCR_018026) and analyzed using Bio-Plex Manager 6.0 software (Bio-Rad, RRID:SCR_014330). A five-parameter logistic regression curve fit was used to determine the concentration. All samples were assayed in duplicate. The concentration values below the detection limit were substituted with 0.5 of the minimum detectable concentration provided in the manufacturer's instructions. Logarithmic transformation was applied to normalize the data.

Statistical analyses

Statistical differences between continuous variables were determined using a two-sample independent *t* test, whereas differences between categorical variables were determined using Fisher's exact test or χ^2 test. *P* values less than 0.05 were considered significant. Statistical analyses were performed using Prism 10 software (GraphPad, RRID: SCR_002798).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The 16S rRNA gene sequences were not deposited in the NCBI database due to limited consent, the presence of possible human reads within the dataset, and Indigenous data sovereignty. Instead, we included a table with ASV counts and corresponding taxonomic annotations in the supplementary materials (Supplementary Table S1).

Results

Study population

In this pilot, cross-sectional study, a total of 31 participants were recruited and enrolled at the Native Americans for Community Action (NACA) clinic in Flagstaff, AZ. **Table 1** shows patients' demographics, socioeconomics, and other characteristics. The mean age of participants was 29.1 years old (ranging from 18 to 46). About race or ethnicity, participants predominantly identified themselves as Native American (or AI/AN; 51.6%), White (41.9%), or Hispanic/Latina (16.1%). The majority of participants shared that they spoke English as a first language (90.3%) and were heterosexual (80.6%). About socioeconomics, many of the participants identified that they were single (45.2%) or living with a partner (35.5%), had some college education (32.3%) or had a bachelor's degree (25.8%), were working full-time (71.0%), part-time (12.9%), or keeping house/raising children full-time (16.1%), living with at least two other people in a household (64.5%), and earning less than $75,000 per household (61.3%). Most participants identified that they did not use antibiotics within the last 3 months (80.6%) and did not smoke (80.6%). The data showed that the majority of women were sexually active in the last year (77.4%). Many participants did not use any contraception (35.5%). Participants who used contraceptives most frequently reported the use of condoms (32.3%) or birth control pills (19.4%). About parity, the majority of participants were nulliparous (64.5%) followed by individuals who were primiparous (19.4%). About prophylactic vaccination and screening, most women received at least one dose of the HPV vaccine (51.6%) and had a Pap smear within the last 3 years (64.5%).

HPV status

First, we determined the distribution of high-risk HPV genotypes in our cohort. High-risk HPV genotypes were detected in 22.6% of participants, including five Native American women and two NHW women. Four participants (12.9%) were infected with a single high-risk genotype and three participants (9.7%) were infected with two different high-risk genotypes. Detected HPV genotypes included HPV39, HPV45, HPV52, HPV53, HPV58, and HPV59. Notably, the current nonavalent HPV vaccine does not target half of the genotypes detected in this cohort (such as HPV39, HPV53, and HPV59; **Table 1**).

Vaginal microbiome composition

To examine the vaginal microbiota communities, we utilized the 16S rRNA gene sequencing technique. We were able to amplify DNA of sufficient quantity and quality (at least 4,000 reads) for microbiome analysis from 28 out of 31 vaginal swab samples. We calculated the relative abundance of bacterial taxa on the genus (**Fig. 1A**) and species (**Fig. 1B**; Supplementary Fig. S1) levels and compared taxonomic compositions between Native American women and women of other races (referred to as non-Native American). Composition bar plots were used to visualize the vaginal microbial profiles of each participant. Overall, vaginal microbiota profiles were similar between Native American and non-Native American women. In some women, vaginal microbiota was dominated by health-associated *Lactobacillus* species (mainly *Lactobacillus crispatus* or *Lactobacillus iners*), whereas in other women vaginal microbiota were depleted of health-associated *Lactobacillus* species and consisted of a mixture of common BV-associated bacteria (*Gardnerella*, *Fannyhessea*, *Prevotella*, *Sneathia*, and *Megasphaera*). When analyzed across all participants, BV-associated species positively correlated with each other, whereas *L. crispatus* but not *L. iners* negatively correlated with BV-associated bacteria (Supplementary Fig. S2). In addition, we also observed that pathobionts and opportunistic pathogens correlated with each other; however, these bacterial species were not highly abundant in our cohort.

Next, we compared the relative abundance of health-associated *Lactobacillus* species between Native American and non-Native American women, and we did not observe any significant differences ($P = 0.9363$) between the groups (Supplementary Fig. S3). We also analyzed *Lactobacillus* abundance in the context of vaginal pH and high-risk HPV status. Women with normal vaginal pH (<4.5) had significantly ($P = 0.0094$) higher levels of *Lactobacillus* spp. when compared with women with abnormal vaginal pH (≥4.5; Supplementary Fig. S3). Similarly, HPV-negative women also tended ($P = 0.0682$) to have higher levels of *Lactobacillus* spp. compared with HPV-positive women (Supplementary Fig. S3). Once we categorized microbial profiles based on the *Lactobacillus* dominance (defined as ≥80% relative abundance of *Lactobacillus*; ref. 26), we also found no significant differences between the racial groups (**Fig. 2A**). Only 44% of Native American women exhibited *Lactobacillus* dominance compared with 58% of non-Native women. Vaginal pH was significantly ($P = 0.0017$) associated with *Lactobacillus* dominance with 91% of women with normal pH having *Lactobacillus*-dominant microbiota (**Fig. 2B**). In contrast, high-risk HPV infection was associated with *Lactobacillus*-depleted microbiota, although this association did not reach significance ($P = 0.1031$). Women infected with high-risk HPV also tended to have microbiome depleted of *Lactobacillus* (**Fig. 2C**). Seventy-one percent of HPV-positive women exhibit non-*Lactobacillus*-dominant microbiota, whereas only 33% of HPV-negative women had vaginal dysbiosis. Overall, these data show a strong association of vaginal dysbiosis with abnormal vaginal pH regardless of race/ethnicity, which might contribute to HPV infection.

Impact of sociodemographic and lifestyle factors on vaginal microbiome

Because the vaginal microbiota composition can be impacted by multiple factors, we investigated the relationship between *Lactobacillus* dominance and other self-reported sociodemographic and behavioral data. There was no

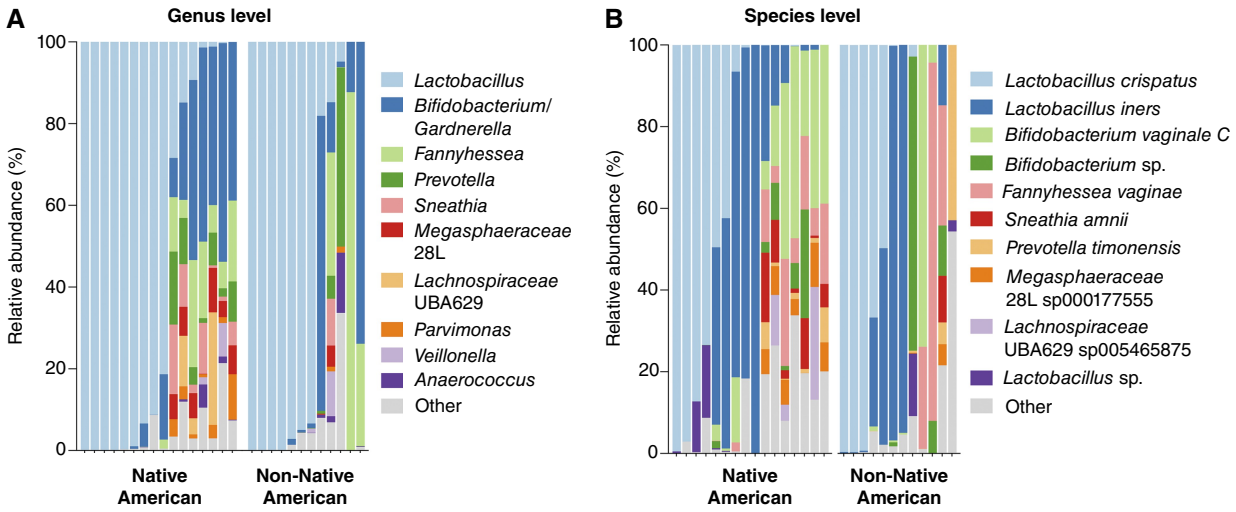

Figure 1.

Vaginal microbiota composition of Native American women in our study. Bar plots show the relative abundance of taxa assigned to the **A,** genus or **B,** species level grouped based on race. Vaginal microbiota profiles were similar between Native American and non-Native American women from Northern Arizona. The most prevalent *Lactobacillus* species included *Lactobacillus crispatus* and *Lactobacillus iners*. Dysbiotic *Lactobacillus*-depleted profiles consisted of typical communities of anaerobic bacteria associated with bacterial vaginosis (BV), including *Gardnerella vaginalis* [assigned as *Bifidobacterium vaginale* in the Genome Taxonomy Database (GTDB)], *Fannyhessea vaginae* (formerly known as *Atopobium vaginae*), *Sneathia vaginalis* (formerly known as *Sneathia amnii*), *Prevotella timonensis*, *Megasphaera lornae* (assigned as 28L sp00017755 in GTDB), and *Clostridiales* genomosp. BVAB1 (assigned as UBA629 sp005465875 in GTDB).

significant association between participants' age and *Lactobacillus* dominance ($P = 0.7036$; **Fig. 2D**). However, participants with BMI equal to or greater than 25 tended to have more frequently *Lactobacillus*-depleted microbiota compared with participants with BMI less than 25 ($P = 0.0992$; **Fig. 2E**). In addition, we observed significant associations between the vaginal microbiota composition and participants' household size (**Fig. 2F**), an education level (**Fig. 2G**) and the number of pregnancies (**Fig. 2H**). Women living in smaller households (three or fewer people including study participant; $P = 0.0441$), women with a higher level of education (with college or associate/technical degree; $P = 0.0093$), as well as nulliparous women ($P = 0.0497$) exhibited more often *Lactobacillus* dominance. Individuals who were married or living with a partner also tended ($P = 0.0542$) to have more frequently *Lactobacillus*-dominant microbiota compared with women who were single, divorced/separated, or widowed (**Fig. 2I**). We did not observe any significant associations of *Lactobacillus* dominance with employment status, household income, antibiotic use, history of smoking, number of sexual partners, contraception use, or HPV vaccination status (Supplementary Table S2). In addition, we examined associations of the vaginal microbiota composition with self-reported measurements of physical health, mental health, general health, carrying out social activities, and perceived stress. There were no significant associations of *Lactobacillus* dominance with any of these health measurements (Supplementary Table S3). Furthermore, neither HPV status (Supplementary Table S4) nor race (Supplementary Table S5) related to higher health T-scores, except for Native American women reporting better general health compared with non-Native Americans ($P = 0.0013$).

Differentially abundant vaginal taxa

To identify microbial features that were differential between sociodemographic groups and to understand the role of the vaginal microbiome in risk factors associated with HPV infection, we applied ANCOM-BC (24). Participants who identified as Native American had profiles with significantly enriched in *Gemella* sp002871655 ($q < 0.0001$) and *Megasphaeraceae* 28L sp000177555 ($q < 0.0001$) as well as *Aerococcus christensenii* ($q < 0.0001$), *Prevotella amnii* ($q < 0.0001$), and most enriched in *Sneathia amnii* ($q < 0.0001$; **Fig. 3A**). These signatures may be related to health disparities observed in HPV infection and cervical cancer. Investigation of vaginal pH, in which elevated pH is associated with vaginal dysbiosis, revealed that eight vaginal species were statistically significantly depleted in abnormal vaginal pH (>4.5), and 24 species were enriched (**Fig. 3B**). Participants with abnormal pH had significantly enriched [log fold change (LFC) of 1] levels of *Prevotella timonensis* ($q < 0.00001$), *Fannyhessea vaginae* ($q < 0.00001$), *Megasphaereaceae* 28L sp000177555 ($q < 0.00001$), *Sneathia amnii* ($q < 0.00001$), *Parvimonas* sp001552895 ($q < 0.00001$), *Dialister* sp001553355 ($q < 0.00001$), *Prevotella* sp000479005 ($q < 0.00001$), unclassified *Bifidobacterium* species ($q < 0.00001$), *Peptoniphilus* A sp900538655 ($q < 0.00001$), and *Anaerococcus marasmi* ($q < 0.00001$) compared with those with normal vaginal pH (≤ 4.5; **Fig. 3B**). However, profiles with an abnormal pH had significant depletion (LFC of 1) of *Limosilactobacillus reuteri* ($q < 0.0001$), *Limosilactobacillus*

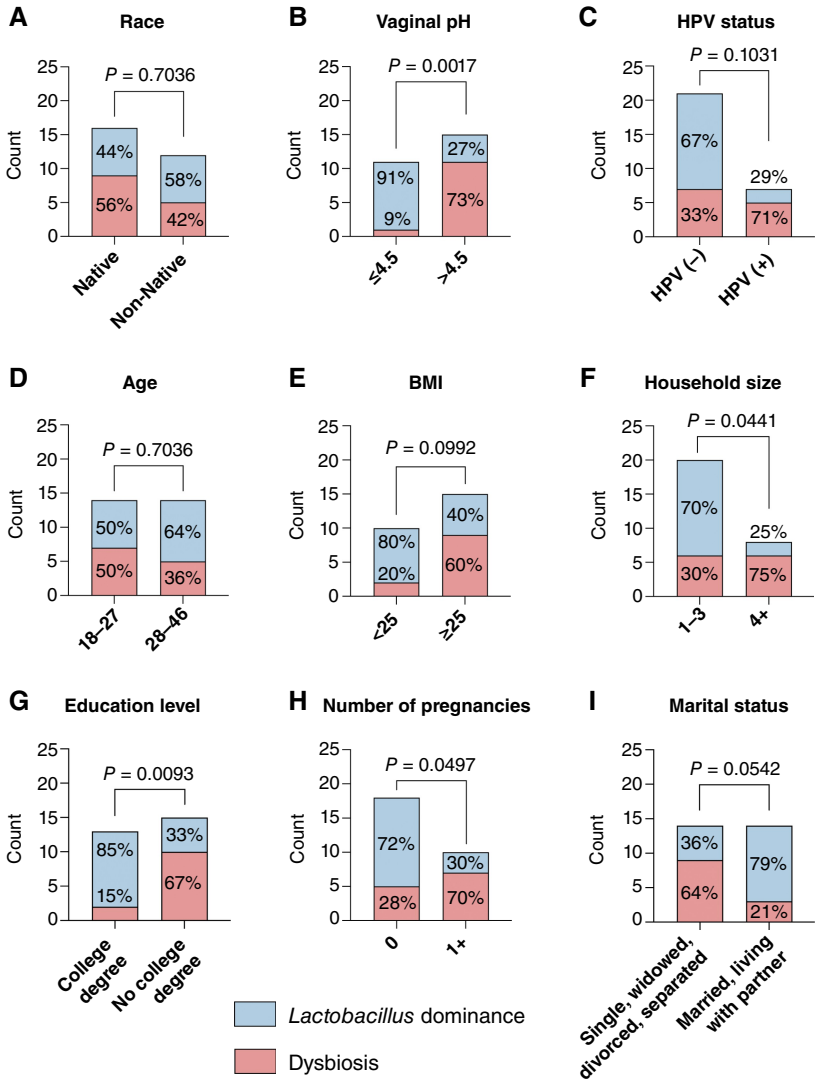

Figure 2.

Associations of *Lactobacillus* dominance with race, vaginal pH, HPV status, and socioeconomic and lifestyle factors. Stacked bar plots show the number of participants with *Lactobacillus*-dominant microbiota or dysbiotic *Lactobacillus*-depleted microbiota among the groups based on **A,** race, **B,** vaginal pH, **C,** HPV status, **D,** age, **E,** BMI, **F,** size of household, **G,** education level, **H,** number of pregnancies, and **I,** marital status. *P* values were calculated using Fisher's exact test. Vaginal microbiota profiles were dominated by *Lactobacillus* species in 44% of Native American women, which was similar to levels observed in non-Native American participants (58%). *Lactobacillus* dominance was highly associated with low vaginal pH (≤4.5). HPV-positive women also tended to have lower *Lactobacillus* abundance compared with HPV-negative women. Vaginal microbiota profiles were also more frequently dominated by *Lactobacillus* species in participants with BMI < 25, living in smaller households (1–3 people), having a college (bachelor's, master's, and doctorate) or associate/technical degree, who were nulliparous, married or living with a partner. No difference was observed between the younger and older participants (dichotomized based on the median age).

coleohominis ($q < 0.0001$), and *Lactobacillus jensenii* ($q < 0.0001$; **Fig. 3B**). Although a small sample size in this pilot cohort, one taxon was significantly enriched in participants who were hrHPV-positive, *Bifidobacterium vaginale C* ($q < 0.0001$; **Fig. 3C**), also known as *Gardnerella vaginalis*. Participants with a high BMI of 35+ had enrichment in *Prevotella timonensis* ($q < 0.0001$) and an unclassified *Lactobacillus* species ($q < 0.0001$) compared with profiles of participants with a BMI of <25. Vaginal profiles of 35+ were also depleted in *Streptococcus oralis* E ($q < 0.0001$), an unclassified *Bifidobacterium* species ($q < 0.0001$), *Staphylococcus aureus* ($q < 0.0001$) and *Lactobacillus jensenii* ($q < 0.0001$; **Fig. 3D**). Interestingly, we observed that the vaginal microbiota of participants with a college degree were highly enriched in *Lactobacillus cripatus* ($q < 0.0001$) and depleted in *Bifidobacterium vaginale C* ($q < 0.0001$) and *Fannyhessea vaginae* ($q < 0.0001$; **Fig. 3E**). Other dysbiotic bacteria were also enriched in vaginal profiles from participants who reported households with four or more individuals, including *Gemella* sp002871655 ($q < 0.0001$) and *Megasphaeraceae* 28L sp000177555 ($q < 0.0001$; **Fig. 3F**). No significant taxa were observed for categorical groupings of marital status and the number of pregnancies. Additional analyses on continuous variables of age, ability to carry out social scores, general health scores, mental health *t*-scores, physical health *t*-scores, and perceived stress *t*-scores also observed no significant differences.

Vaginal immune mediator profiles

To characterize vaginal immune mediator profiles, we quantified concentrations of 42 cytokines, chemokines, and growth factors in vaginal swab samples. Unsupervised hierarchical clustering analysis was utilized to visualize relative levels of immune markers and compare the protein profiles among participants (**Fig. 4A**). The generated heatmap and dendrogram revealed two distinct clusters:

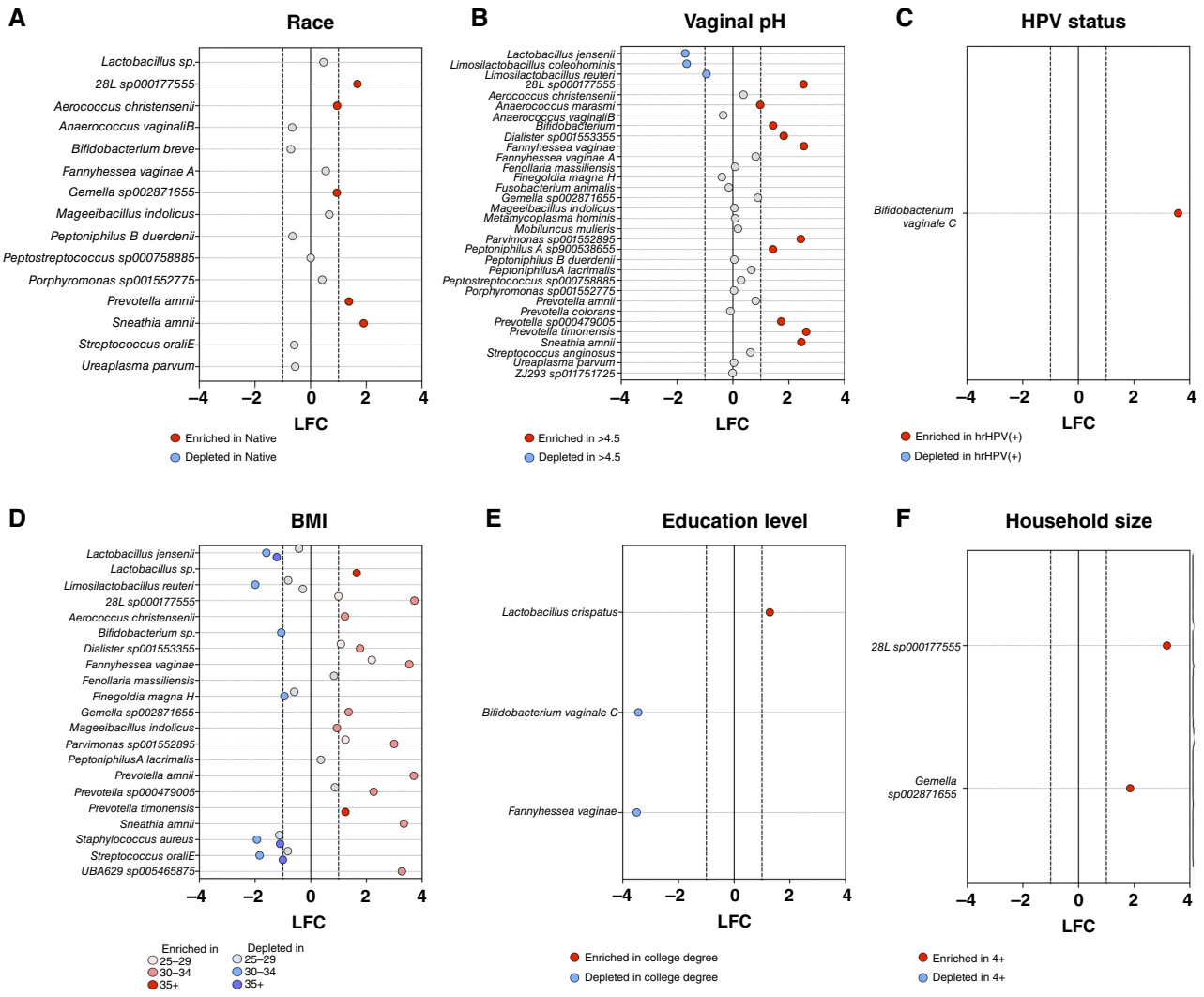

Figure 3.

Microbial features associated with sociodemographic factors, vaginal pH, and HPV status. Differentially abundant taxa between the groups based on **A,** race, **B,** vaginal pH, **C,** HPV status, **D,** BMI, **E,** education level, and **F,** household size were identified at the species level using ANCOM-BC. *P* values were corrected for multiple comparisons using the FDR method; taxa with $q < 0.05$ are depicted. Red and blue dots indicate taxa with LFC > 1 and LFC < −1, respectively. Numerous dysbiotic vaginal species are enriched in participants with abnormal vaginal pH, lower education level, who live in larger households, identify themselves as Native American, have higher BMI, and are hrHPV-positive, whereas *Lactobacillus* and *Limosilactobacillus* species were enriched in women with normal pH, higher education level and lower BMI.

cluster 1 with relatively low levels of immune mediators and cluster 2 with elevated levels of immune mediators. To further characterize these clusters, patient-related demographic metadata (race, ethnicity, and BMI), as well as vaginal pH, vaginal microbiota composition, and high-risk HPV status data, were also plotted on the heatmap. The distribution of demographic metadata did not significantly ($P > 0.05$) differ between the clusters. Yet, cluster 2 had a significantly ($P = 0.0216$) higher rate of patients with abnormal vaginal pH (85%; **Fig. 4B**) as well tended ($P = 0.1248$) to have more patients with dysbiotic *Lactobacillus*-depleted microbiota (65%; **Fig. 4C**) in contrast to cluster 1 with only 28% and 27% of participants, respectively, exhibiting these negative cervicovaginal microenvironment features.

Subsequently, we compared individual concentrations of proteins between women with *Lactobacillus*-dominant microbiota and women with dysbiotic *Lactobacillus*-depleted microbiota. Four proinflammatory cytokines, IL1β, IL12 (p70), IL15, and TNFα, and one regulatory cytokine, IL12 (p40), were significantly ($P < 0.05$) increased in women with vaginal dysbiosis compared with women with *Lactobacillus* dominance (**Fig. 5A**). No chemokines were significantly altered between the groups (**Fig. 5B**). One growth factor, FGF2, was also increased in women with dysbiosis (**Fig. 5C**). Furthermore,

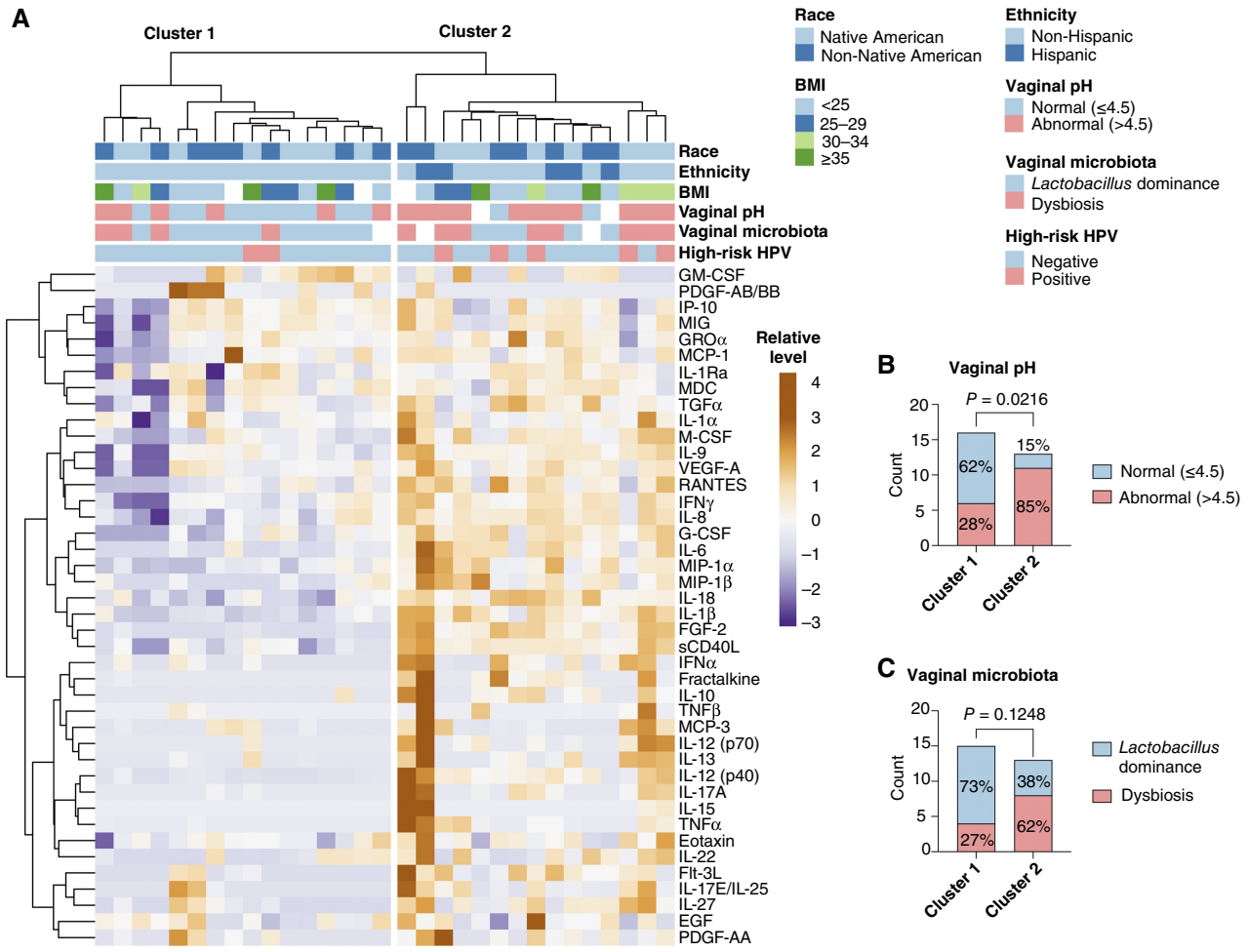

Figure 4.

Vaginal profiles of cytokines, chemokines, and growth factors. **A,** heatmap reflects relative levels of proteins in vaginal swab samples. Unsupervised hierarchical clustering was used to assess the similarity between protein profiles. Two main clusters based on Euclidean distance and Ward linkage were observed. **B,** Stacked bar plots show the distribution of patients with normal and abnormal pH or **C,** *Lactobacillus*-dominant and dysbiotic *Lactobacillus*-depleted microbiota between the clusters. *P* values were calculated using the Fisher exact test. Unsupervised hierarchical clustering analysis indicates that immune marker levels associate with vaginal pH and *Lactobacillus* dominance.

women with *Lactobacillus* dominance had increased levels of two immune markers, GM-CSF and PDGF-AB/BB, when compared with women with dysbiosis (**Fig. 5A** and **C**).

Discussion

Emerging evidence strongly suggests that dysbiosis or polymorphic microbiomes are implicated in the development of cancer (10, 27). In the lower female reproductive tract, dysbiosis occurs when protective lactobacilli are replaced by a polymicrobial consortium of anaerobic bacteria (6). In cervical cancer, these dysbiotic bacterial communities have been linked to HPV acquisition and persistence, as well as the development and progression of cervical neoplasia (8, 9). Indeed, numerous cross-sectional studies have shown that women infected with HPV or with cervical neoplasia more frequently harbor *Lactobacillus*-depleted microbiota (26, 28–32). In addition, only a few longitudinal studies have demonstrated that women with vaginal dysbiosis have higher odds of HPV persistence and disease progression compared with women with *Lactobacillus* dominance (33–36).

Because racial/ethnic minorities have higher rates of dysbiosis (likely because of socioeconomic, behavioral/lifestyle factors at individual, relational, community, and societal levels; ref. 37), the vaginal microbiota might be an important driver of adverse health outcomes and contribute to cervical cancer health disparities in these populations (10). In our previous study, we demonstrated that Hispanic/Latina women from the Phoenix (AZ) metropolitan area had frequently *Lactobacillus*-depleted microbiota, which was strongly

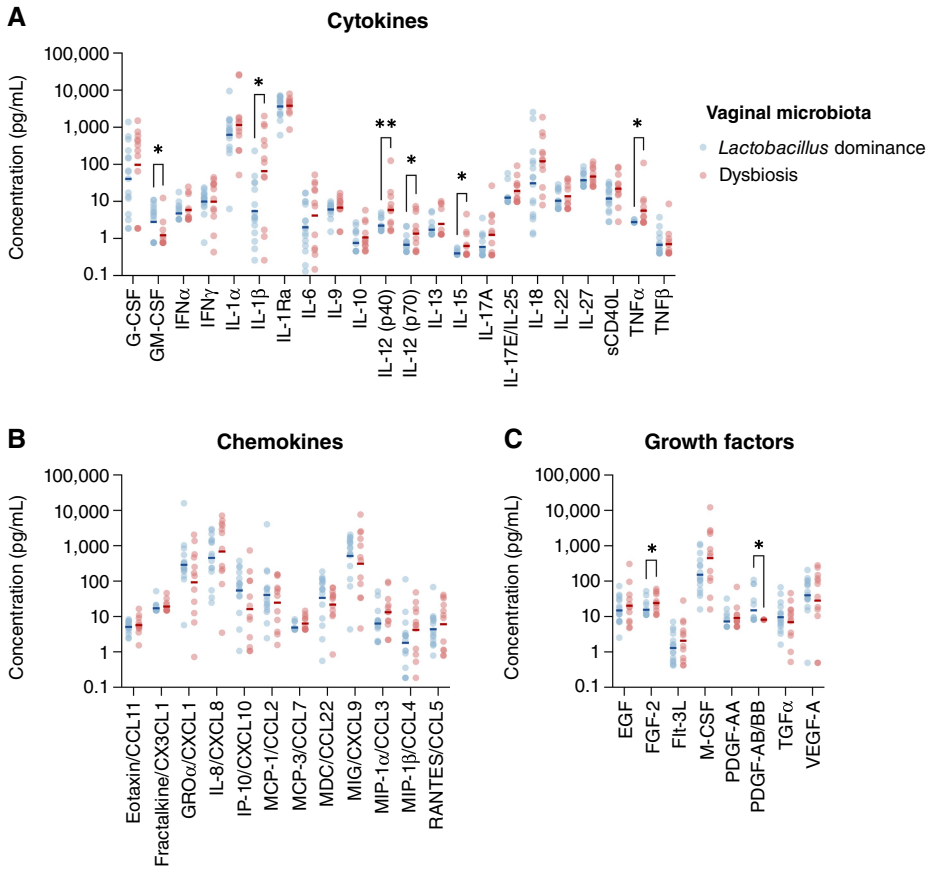

Figure 5.

Vaginal levels of immune markers in the context of *Lactobacillus* dominance. Scatter plots show the concentration of **A,** cytokines, **B,** chemokines, and **C,** growth factors in vaginal swab samples in women with *Lactobacillus*-dominant or dysbiotic *Lactobacillus*-depleted microbiota. *P* values were calculated using two sample independent *t* tests (*, $P < 0.05$; **, $P < 0.01$). Women with dysbiotic *Lactobacillus*-depleted microbiota exhibited increased vaginal levels of IL1β, IL12 (p40), IL12 (p70), IL15, TNFα, and FGF2, when compared with women with *Lactobacillus*-dominant microbiota. In addition, GM-CSF and PDGF-AB/BB were significantly decreased in women with dysbiosis.

associated with HPV status, severity of cervical disease, and elevated cervicovaginal levels of proinflammatory cytokines (26). Yet, the relationship between HPV and vaginal microbiota in Native American women is still unrecognized (1).

In this pilot study, we recruited Native and non-Native women of reproductive age. The microbiome analysis revealed that the majority of participants in our cohort (Native and non-Native) exhibited higher rates of vaginal dysbiosis (66% and 42%, respectively) compared with previously reported NHW cohorts (up to 90%; refs. 5, 12). This is in accordance with a small study of 70 Native American women from the Northwestern Plains (38), which reported vaginal dysbiosis in 60% of their participants. About predominant *Lactobacillus* species, women in our cohort were frequently colonized by *L. crispatus* or *L. iners*, which are the most common species of vaginal lactobacilli and also found in the Northwestern Plains cohort (38). Intriguingly, these species might not equally benefit the host: *L. crispatus* has been associated with optimal health outcomes, whereas the role of *L. iners* in health and disease remains controversial (5). For example, a recent meta-analysis revealed that *L. crispatus* correlated with decreased detection of hrHPV and cervical dysplasia, whereas *L. iners* did not (9).

Our study also revealed that the interplay between HPV, vaginal microbiota composition, and immune activation may play a role in Native American women, similar to our findings in Hispanic women (26). In this cohort of women, individuals infected with hrHPV were more likely to harbor *Lactobacillus*-depleted microbiota. Women with dysbiosis had elevated vaginal pH and increased cervicovaginal levels of key cytokines (e.g., IL1β and TNFα), which have been previously reported to be elevated in women with BV or vaginal dysbiosis (6). These findings further suggest that the host-microbe interactions in the cervicovaginal microenvironment might result in chronic inflammation, which, in consequence, can promote cervical carcinogenesis. The exact mechanisms by which chronic inflammation contributes to HPV-mediated carcinogenesis are still unclear; thus further investigations are required to identify specific microbial signatures or/and communities contributing to an inflammatory state in the cervicovaginal microenvironment.

Because the vaginal microbiome is a dynamic ecosystem that can be disturbed by multiple factors, we investigated associations of the vaginal microbiota composition with collected patient-related metadata within the entire cohort. Noteworthily, we found that regardless of race/ethnicity, women with higher education levels were more likely colonized by *Lactobacillus* species (particularly with *L. crispatus*). This aligns with other studies investigating the vaginal microbiota in pregnant African American women (39) and

nonpregnant premenopausal Brazilian (40) or Finnish women (41). The Brazilian study also identified other factors associated with *Lactobacillus*-depleted microbiota: smoking and a higher number of sexual partners (40). We did not observe these associations in our cohort; however, we might not have adequate sample size to detect these effects with only a few participants who were smokers or had more than two sexual partners within the last year. In addition, we found that women who were married or cohabiting with a partner had higher odds of *Lactobacillus* dominance in accordance with a previous study of women undergoing cervical cancer screening in Helsinki, Finland (41). On the other hand, living in larger households and history of pregnancy were associated with higher rates of vaginal dysbiosis. It is well established that the vaginal microbiota is frequently disturbed postpartum (42, 43) and multiple reports showed the influence of parity on the vaginal microbiome (44–46). For example, three independent studies of predominantly African American pregnant women (44), pregnant Finnish women (45), and nonpregnant Belgian women (46) showed significant associations of high parity with vaginal dysbiosis. This phenomenon could be explained by a cumulative effect of multiple pregnancies and increased microbiota diversity after live birth but warrants further investigation (44). The same reports also found the effect of obesity on the vaginal microbiota (44, 46). We observed a similar trend with BMI in our cohort. Other studies in nonpregnant populations also consistently show an association between obesity with a lower likelihood of *Lactobacillus* dominance and an increased risk of BV (47). It is postulated that hormone and immune system alterations in obese individuals may create an environment favoring dysbiotic bacteria, yet the mechanistic link between obesity and vaginal dysbiosis is still incompletely understood (47). Intriguingly, high BMI has also been linked to decreased detection of precancerous cervical lesions, and, consequently, increased risk of cervical cancer development (48). Finally, we did not detect any meaningful impact of self-reported measurements of general, physical, or mental health (including perceived stress) on the vaginal microbiota composition. This conflicts with a previous report on Northwestern Plains Native American women showing an association of psychosocial stress tied to historic loss-associated trauma with increased odds of vaginal dysbiosis (38). However, the use of different survey instruments to measure stress/trauma might explain these conflicting results. Because there is growing evidence of the role of psychosocial stress in modulating immune function and microbiota composition, additional larger translational studies with comprehensive evaluation of various stress/trauma measurements are required to further validate these associations in Native American women.

Herein, we also identified several microbial features associated with sociodemographic factors. Intriguingly, the vaginal microbiota of Native American women were highly enriched in *Sneathia vaginalis* (formerly known as *S. amnii*). In our previous study of Hispanic and non-Hispanic women, we found that this bacterium was associated with all stages of cervical carcinogenesis (HPV infection, dysplasia, and invasive carcinoma; ref. 26), as well as Hispanic ethnicity and abnormal vaginal pH (26). In addition, our systematic review of microbiome studies in Hispanic/Latina populations in North and South America revealed *Sneathia* to be consistently increased in women across cervical carcinogenesis (49). Though we did not observe a significant increase in *Sneathia* in HPV-positive individuals in this pilot study (likely because of the small sample size), *Sneathia* was more abundant in women who identified as Native American and had abnormal vaginal pH and higher BMI. Overall, *Sneathia* might be a microbial marker associated with HPV-related carcinogenesis across populations disproportionally affected by cervical cancer and requires further *in vitro* studies using human organotypic cell culture models (e.g., three-dimensional cervical epithelial cell model based on a rotating wall vessel bioreactor technology; ref. 50) to evaluate mechanisms by which this bacterium impacts HPV infection and the progression of disease.

Although this pilot study begins to advance our knowledge of HPV and the vaginal microbiome in Native American women, it is important to acknowledge its limitations, including a relatively small sample size (because of the COVID19 pandemic) and the cross-sectional design, thus we may not have had the power to detect effects of some factors (e.g., smoking or sexual activity) on vaginal dysbiosis in our cohort and we cannot generalize our findings. It is also important to note that patients recruited in our study were recruited in urban settings in Northern Arizona. More studies are needed across urban and rural populations, as well as geographic regions, to account for differences in experiencing adverse social determinants of health, gaps in the access and quality of healthcare, and other socioeconomic and/or cultural differences between these racial/ethnic groups and populations (1). Despite these limitations, we were able to identify associations of the vaginal microbiota with HPV and immune markers, as well as sociodemographic and lifestyle/behavioral factors, which provides a more comprehensive understanding of individual risk factors related to increased cervical cancer risk. In the future, longitudinal studies—which include Native American women—are required to move from association to causation. Investigation of social determinants of health contributing to geographic and racial/ethnic differences in the cervical cancer burden is needed to adequately address this health disparity. In addition, features of structural racism (a system of policies that support an unfair advantage for some people and unfair treatment of others based on their race/ethnicity) should be investigated in the context of cervical cancer. Finally, an improved understanding of the mechanistic role of the vaginal microbiota in HPV-related carcinogenesis is still needed to identify potential intervention strategies to increase health equity in Native American populations.

Authors' Disclosures

P. Łaniewski reports grants from the National Institutes of Health (NIH), National Cancer Institute (NCI) during the conduct of the study. D.J. Roe reports grants from the NIH NCI during the conduct of the study. M.M. Herbst-Kralovetz reports grants from the NIH NCI during the conduct of the study, as well as personal fees from Freya Biosciences outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

P. Łaniewski: Data curation, formal analysis, supervision, validation, investigation, visualization, methodology, writing–original draft. **T.R. Joe:** Data curation, formal analysis, investigation, visualization, methodology, writing–review and editing. **N.R. Jimenez:** Data curation, formal analysis, investigation, visualization, methodology, writing–review and editing. **T.L. Eddie:** Resources, writing–review and editing. **S.J. Bordeaux:** Resources, writing–review and editing. **V. Quiroz:** Resources, writing–review and editing. **D.J. Peace:** Resources, writing–review and editing. **H. Cui:** Formal analysis, methodology, writing–review and editing. **D.J. Roe:** Resources, formal analysis, methodology, writing–review and editing. **J.G. Caporaso:** Resources, formal analysis, supervision, funding acquisition, validation, investigation, writing–review and editing. **N.R. Lee:** Resources, formal analysis, supervision, funding acquisition, validation, investigation, writing–review and editing. **M.M. Herbst-Kralovetz:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, project administration, writing–review and editing.

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Note

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