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Cervical Microbiome and Response to a Human Papillomavirus Therapeutic Vaccine for Treating High-Grade Cervical Squamous Intraepithelial Lesion

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Abstract

Human papillomavirus (HPV) infection is associated with the vast majority of cervical cancer cases as well as with other anogenital cancers. PepCan is an investigational HPV therapeutic vaccine for treating cervical high-grade squamous intraepithelial lesions. The present study was performed to test whether the cervical microbiome influences vaccine responses and to explore host factors as determinants of the cervical microbiome composition in women with biopsy-proven high-grade squamous intraepithelial lesions. In a recently completed Phase I clinical trial of PepCan, histological response rate of 45% (14 of 31 patients), a significant increase in circulating T-helper type 1 cells, and a significant decrease in HPV 16 viral load were reported. DNA, extracted from liquid cytology specimens collected before and after vaccinations, were amplified and then hybridized to a G4 PhyloChip assay to characterize the microbiome. We describe trends that certain bacterial taxa in the cervix may be enriched in non-responders in comparison to responders ($P_{adj} = .052$ for phylum *Caldithrix* and $P_{adj} = .059$ for phylum *Nitrospirae*). There was no difference in bacterial diversity between the 2 groups. A permutational analysis of variance performed for various demographic and immune parameters showed significant clustering with microbiome beta diversity for race, HPV 16 status, peripheral T-helper type 1 cells, and HLA-B40 ($P = .001$, .014, .037, and .024, respectively). Further analyses showed significant differences at the empirical Operational Taxonomic Unit level for race and HPV 16 status. As these results are from a small Phase I study, further studies are needed to examine the role of cervical microbiome in response to HPV therapeutic vaccines.

Keywords

cervical microbiome, therapeutic vaccine, cervical cancer, high-grade squamous intraepithelial lesions, human papillomavirus

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Introduction

High-risk human papillomaviruses (HPVs) are best known as the causative agent of cervical cancer, but they are also associated with other cancers of mucous membranes such as anal, oropharyngeal, penile, vaginal, and vulvar cancers.[1] Among the high-risk HPV types, HPV type 16 (HPV 16) is the most dominant in terms of the number of cancer cases.

An immune-based therapy for cervical precancers are needed, as the current standard of care surgical treatments are known to result in doubling of preterm deliveries in future pregnancies.[2] PepCan is an investigational HPV therapeutic vaccine, which consists of 4 current Good Manufacturing Practice synthetic peptides covering the HPV 16 E6 protein amino acid sequence and a *Candida* skin test reagent as a vaccine adjuvant to enhance immune response.[3,4] In this Phase 1 dose-escalation clinical trial, 34 women with biopsy-proven high-grade squamous intraepithelial lesions (HSILs) were given 4 intradermal injections of PepCan 3 weeks apart. Three months after the last

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injection, loop electrical excision procedure (LEEP) was performed as a standard of care treatment and to categorize recipients into responders (n = 14) and nonresponders (n = 17). Three subjects did not complete the study. We previously reported that the vaccine was safe,[3,4] can significantly decrease HPV 16 viral load,[3] and can significantly increase circulating T-helper type 1 (Th1) cells.[3,4]

Multiple studies have reported that antitumor response with checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) or PD-1 ligand is modulated by the composition of gut microbiota.[5-10] The use of broad-spectrum antibiotics resulting in disruption of healthy microbiota could be related to the failure of such immunotherapy.[7] Furthermore, different bacterial taxa that positively correlate with good clinical responses were described in studies examining differing patient populations.[5-10] For example, Gopalakrishnan et al[5] reported significantly higher alpha diversity and relative abundance of the *Ruminococcaceae* family in melanoma patients responding to the anti-PD-1 immunotherapy.[5] While the role of gut microbiome has been studied robustly, the microbiome of the disease site (eg, skin for melanoma patients) has not received as much attention and has not been investigated.[5] We intended to fill this gap by examining the cervical microbiome of women being treated for HSILs using our investigational therapeutic vaccine, PepCan.

HPV infection has been shown to influence the microbiome composition in the cervicovaginal milieu,[11-14] and differing abundance of *Lactobacillus* species has been reported based on race.[15,16] Therefore, we examined not only the role of cervical microbiome in relation to HPV therapeutic vaccine response in this study, but also explored whether various demographic/immune parameters and the HPV status may influence the cervical microbiome composition.

Methods

Patients

The patients whose cervical samples were studied were all participants of a Phase I clinical trial of an HPV therapeutic vaccine, PepCan, which has been reported previously.[3,4] Written informed consent was obtained from all participants. The vaccine contains 4 current Good Manufacturing Practice–grade synthetic peptides covering the HPV 16 E6 protein and Candin (Allermed, San Diego, CA), a colorless extract of *Candida albicans*, as a vaccine adjuvant.[17] This was a single-center, single-arm, dose-escalation, Phase I clinical trial (NCT00569231). Women with biopsy-proven HSILs were eligible for intradermal vaccination, which was administered in limbs (most commonly administered in forearms, and less commonly in the outer thigh) every 3 weeks for a total of 4 injections. The doses of the peptides tested were 50 μg/peptide, 100 μg/peptide, 250 μg/peptide, and 500 μg/peptide, and 6 patients were tested in each dose group. Each patient received the same peptide dose for all 4 injections. The amount of Candin, 0.3 mL, was held constant at the dose successfully used to treat common warts.[18] After completing the dose escalation phase, 10 additional patients were vaccinated at the 50 μg/peptide dose, which was shown to be optimal in terms of histological regression and HPV clearance.[3,4] Twelve weeks after the last vaccination, LEEP was performed. Although the goal of developing HPV therapeutic vaccines is to replace an excisional procedure such as LEEP, it was performed since the primary objective of the Phase I study was to assess safety. Furthermore, histological response to the vaccine could be assessed by examining the LEEP biopsies. Subjects with persistent HSIL were considered to be vaccine nonresponders while those without HSIL (complete) and those with HSIL measuring ≤ 0.2 mm^2 (partial) were considered to be responders. Forkhead box P3 (FOXP3) staining of LEEP sample was performed to quantify regulatory T-cell (T_{reg}) levels in the epithelium of cervical intraepithelial lesions and their corresponding stroma. Blood was drawn once before and twice after vaccination started (Figure 1), and was used to perform HLA typing (One Lambda, Canoga Park, CA), and immune profiling of circulating Th1 cells, T-helper type 2 (Th2) cells, and T_{regs}. A histological regression rate nearly double of a historical placebo group from another clinical trial of HPV therapeutic vaccine, and a significant decrease in HPV 16 viral load were observed.[3,4]

Collection of Cervical Cells and Processing

ThinPrep (Hologic, Marlborough, MA) was used to collect cervical cells at the screening visit (before vaccination) and at the exit visit (after vaccination). Three patients exited the study early for personal reasons, and therefore provided only prevaccination cervical samples. The central part of the broom-like device was inserted into the endocervix and rotated 360° to collect cells from the endocervix and the entire ectocervix. In addition, a cervical brush was used to collect more cells from the endocervix. Cells were equally distributed by vortexing and were stored at −80°C. DNA was isolated using the Qiagen QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer instructions. DNA was quantified using ND-1000 NanoDrop (NanoDrop Technologies, Wilmington, DE), and was used for HPV testing (Linear Array HPV Genotyping Test, Roche Molecular Diagnostics, Pleasanton, CA), HPV 16 viral load determination,[3] and microbiome analysis as described below.

Polymerase Chain Reaction Amplification and Hybridization

DNA was amplified by polymerase chain reaction (PCR; 30 cycles in duplicate) with a bacterial 16S rRNA gene degenerate forward primer (27F.1 5′-AGRGTTTGATCMT

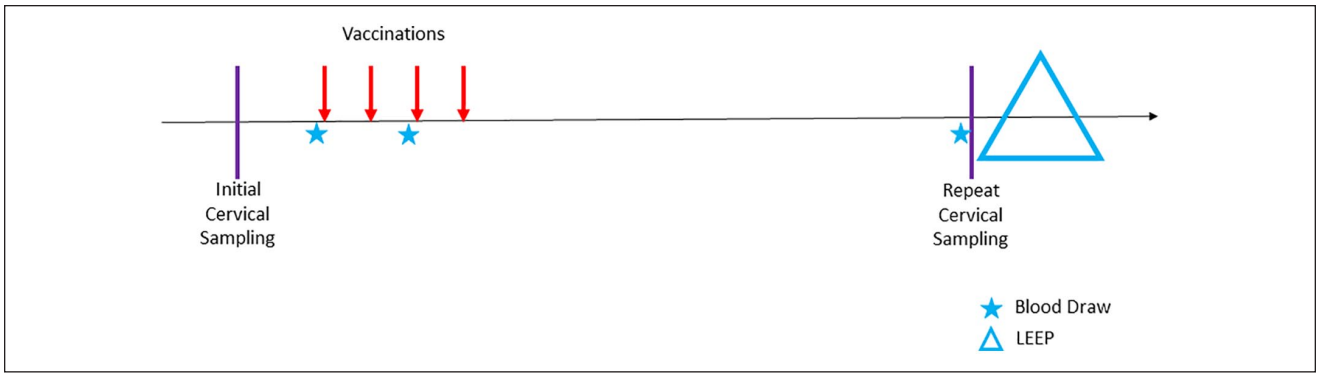

Figure 1. Study scheme showing that the initial cervical sample was taken prior to 4 vaccinations given 3 weeks apart in women with biopsy-proven high-grade squamous intraepithelial lesions (HSILs). Three months after the last vaccination, a repeat cervical sample was taken immediately prior to having loop electrical excision procedure (LEEP) performed based on which responders and nonresponders were described.

GGCTCAG-3′) and a nondegenerate reverse primer, 1492R.jgi (5′-GGTTACCTTGTTACGACTT-3′). Amplified products from each sample were concentrated with a solid-phase reversible immobilization method and quantified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA). PhyloChip Control Mix was added to 500 ng of PCR products from each sample. Assuming an average GC content of 54% (Greengenes database of 16S rRNA genes)[19] and an amplicon length of 1465 bp, 3.3×10^{11} molecules were assayed from each sample.[20] The amplicons were then fragmented, labeled with biotin, and hybridized to the PhyloChip Array (version G4).[21]

The arrays were washed, stained, and scanned using a GeneTitan scanner (ThermoFisher Scientific, Waltham, MA). Standard Affymetrix software (GeneChip Microarray Analysis Suite, ThermoFisher Scientific) was used to capture the scans. Only perfect-match probes with fluorescence intensity observed in at least 4 samples (n = 1111) were exported for rank-normalization in Sinfonietta software (Second Genome, Inc, South San Francisco, CA), and were used as input to empirical probe-set discovery. All probe sets contained 5 or more probes, and the empirical Operational Taxonomic Units (eOTU) tracked by a probe set were taxonomically annotated against the May 2013 release of Greengenes database.[22] Where standard taxonomic names have not been established, hierarchical taxon identifiers were used. Analyses were conducted on hybridization scores (HybScores), which are the mean normalized rank for all probes within an eOTU. The probes were ranked according to their scaled-background subtracted fluorescence intensities.

Statistical Analyses

Alpha diversity refers to the richness of individual cervical samples. It was calculated as a sum of unique eOTUs found in each sample, using the Phylogenetic Diversity.[23-25] In contrast, beta diversity was defined by intersample comparisons performed in a pairwise fashion. Beta diversity was expressed as a dissimilarity score and was stored in a distance dissimilarity matrix. Abundance-weighted sample pairwise differences were calculated using the Bray-Curtis dissimilarity.[26] The binary dissimilarity values were calculated with the Jaccard index.[27] Principal coordinate analysis was performed in which 2-dimensional ordinations were created to graphically summarize the inter-sample relationships. Permutational analysis of variance[28] was used to identify significant differences in the microbiome at the eOTU level in concordance with discrete categorical variable or in association with continuous variables. Fast Unifrac was applied to identify well-defined subcategories using the Biotype R package.[29,30] The difference between the 2 biotypes with regard to HPV 16 status or race was calculated using Fisher's exact test. The difference in representation of 20 families between biotypes 1 and 2 were calculated using the Wilcoxon rank sum test followed by a Benjamini-Hochberg correction. We limited the analyses of HLA types to those previously shown to be associated with HSIL compared with the general population in the United States, and those detected in at least 5 study participants.[3,4]

Differentially abundant phyla, families, genera, and eOTUs were identified using a Welch *t* test. Where samples could be paired across categories, a paired Welch *t* test was performed. *P* values were adjusted by Benjamini-Hochberg procedures to control for multiple testing[31] for eOTU abundance testing.

For covariates which had *P* values less than .05, the differences were further analyzed for 1111 eOTUs selected as described above. Quantile normalization was applied to raw hybridization intensity scores. Then, a generalized linear model was applied to each eOTU to assess the comparison of interest (eg, Caucasian vs non-Caucasian, HPV

Table 1. Baseline Characteristics of Patients[a].

Characteristics	Responders (N = 14)	Nonresponders (N = 17)	*P*
Age (years)			
Mean ± SD	31.3 ± 8.3	29.5 ± 4.6	.4632
Range	22-49	22-37	
Histology at entry, n (%)			
CIN2	6 (42.9%)	6 (35.3%)	.9524
CIN3	8 (57.1%)	11 (64.7%)	
Race, n (%)			
Caucasian	7 (50.0%)	5 (29.4%)	.4233
African American	6 (42.9%)	12 (70.6%)	
Other	1 (7.1%)	—	
HPV 16 status at entry, n (%)			
Positive	5 (35.7%)	7 (41.2%)	>.99

Abbreviations: HPV, human papillomavirus; SD, standard deviation.
[a]A 2-sample *t* test was used to assess age and Pearson's χ^2 tests were used to assess histology, race, and HPV status.

16-positive vs HPV 16-negative, percent peripheral Th1 cells, and HLA-B40-positive vs B40-negative), and a Benjamini-Hochberg correction was applied to adjust for multiple comparisons.

Results

Of 31 subjects who received vaccinations and completed the study (Figure 1), 14 (45%) were responders and 17 (55%) were nonresponders as determined by the histological findings in LEEP.[3,4] There were no significant differences between these 2 groups with regard to age, histological finding at entry, race, and HPV status at entry (Table 1). Alpha diversity eOTU richness ranged from 72 to 365 (mean = 232) eOTUs per sample. In all, 2 unique kingdoms, 46 phyla, 108 classes, 184 orders, 285 families, 414 genera, 586 species, and 1111 eOTUs were detected. The 10 most abundant phyla were *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, Planctomycetes, Cyanobacteria, Euryarchaeota, Chloroflexi*, and *Acidobacteria* (Figure 2A). The 10 most abundant families were *Prevotellaceae, Lactobacillaceae, Lachnospiraceae, Streptococcaceae, Mycoplasmataceae, Bacillaceae, Enterobacteriaceae, Spirochaetacea, Veillonellaceae*, and *Pasteurellaceae* (Figure 2B).

Among the 46 phyla examined, *Caldithrix* and *Nitrospirae* appeared to be enriched in nonresponders, approaching statistical significance after correction for multiple analyses (Figure 3; P_{adj} = .052 and .059, respectively). Three of the 1111 eOTUs observed in this study were enriched in the nonresponder group compared with the vaccine response group (at $P < .05$; Figure 4): *Prevotella* sp (*Prevotellaceae*), A17 sp (family *Pirellulaceae*), and an unclassified species (94otu38814) in the family *Sphingomonadaceae*. No significant differences were found between cervical microbiome diversity before and after vaccination with regard to alpha diversity, phyla (n = 46), families (n = 285), and eOTUs (n = 1111). There was no difference in alpha diversity between the 2 groups.

A permutational analysis of variance using distance matrices was performed for each variable of interest to determine if there was significant clustering with regard to the beta diversity of the samples (Table 2). *P* values of <.05 were found for race (Figure 5A), HPV 16 status (Figure 5B), peripheral Th1 cells, and HLA B40 or not. When these variables were further analyzed at the eOTU level, significant differences were found in 498 eOTUs with regard to race, and in 15 eOTUs with regard to HPV 16-positivity after adjusting for multiple comparisons (Supplementary Tables 1 and 2; available online).

When Fast Unifrac was applied to identify subcategories analogous to community state types, 2 distinct biotypes were elucidated (Figure 6A). The biotype 1 and biotype 2 differed significantly based on HPV 16 status ($P = .0088$) and race (Caucasian vs non-Caucasian, $P = .0021$). The families significantly over-represented in biotype 1 were *Enterococcaceae, Mycoplasmataceae, Bacillaceae, Pasteurellaceae, Clostridiaceae, Phycisphaeraceae, Staphylococcaceae, Corynebacteriaceae, Lactobacillaceae, Pirellulaceae, Streptococcaceae*, and *Enterobactericeae*, and those underrepresented were *Prevotellaceae, Lachnospiraceae, Micrococcaceae, Porphyromonadaceae*, and *Veillonellaceae* (Figure 6B).

Discussion

To our knowledge, this study of women with HSILs was the first to examine the effects of the cervical microbiome in response to therapeutic HPV vaccination. This is of clinical interest since others have shown the impact of the gut microbiome on patient's responses to checkpoint inhibitors,[5-10] but not necessarily that of a local microbiome, for example, that of the skin in melanoma patients. We studied the cervical microbiome in patients with biopsy-proven HSILs prior to receiving an investigational HPV therapeutic vaccine, and have described trends of phyla *Caldithrix* and *Nitrospirae* being enriched in the nonresponder group (Figure 3; P_{adj} = .052 and .059, respectively). Furthermore, 3 eOTUs were enriched in the vaccine nonresponder group, including *Prevotella* sp (*Prevotellaceae*), A17 sp (family *Pirellulaceae*), and an unclassified species (94otu38814) in the family *Sphingomonadaceae* (Figure 4). Therefore, a local microbiome could potentially influence the mounting of immune response to consequences of local disease (ie, HSIL).

Huang et al[32] conducted a study comparing the cervicovaginal community profiles among the cervix, posterior fornix, and vaginal canal of nonpregnant women, women in

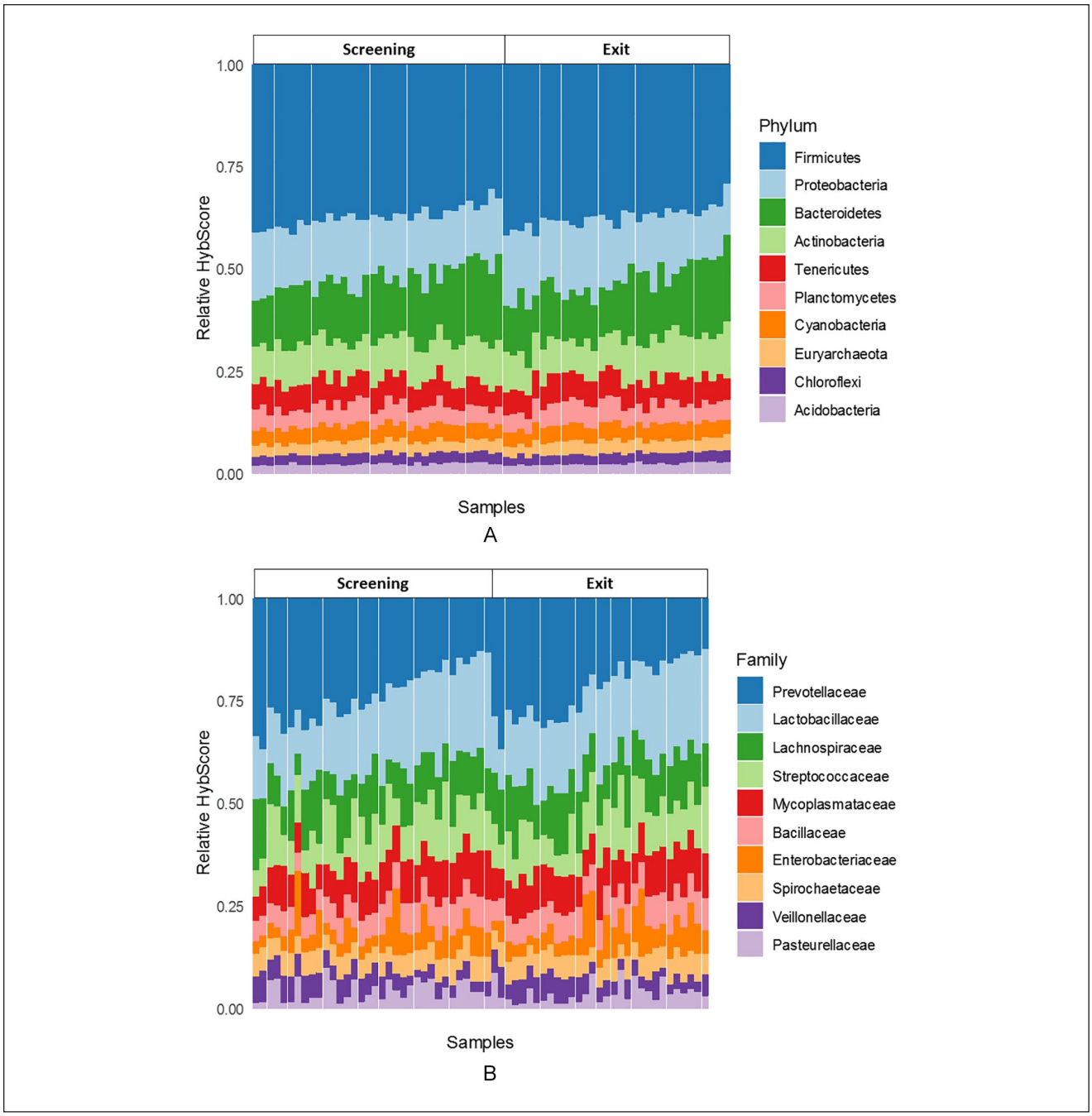

Figure 2. Stacked bar plot of phylogenetic composition of the 10 most common bacterial taxa at (A) the phylum and (B) family levels based on relative HybScores in cervical ThinPrep samples collected at the screening visits (prevaccination) and the exit visits (after 4 vaccinations).

different stages of pregnancy, and postpartum women. Based on their results, it has been proposed that the cervicovaginal microbial communities were homogenous across these 3 anatomic sites although there was a substantial person-to-person variation.[32]

Our data presented above showed overabundance of *Caldithrix, Nirtospirae, Prevotella* sp A17 sp (family *Pirellulaceae*), and an unclassified species (94otu38814) in vaccine nonresponders, suggesting that certain bacterial taxa may influence immunological responses to vaccination. *Prevotella* has been best characterized as having a role in gynecological and general health.[33,34] *Prevotella* has been shown to be the key vaginal microbial species influencing host menopause, bacterial vaginosis, cervicitis, HPV status,

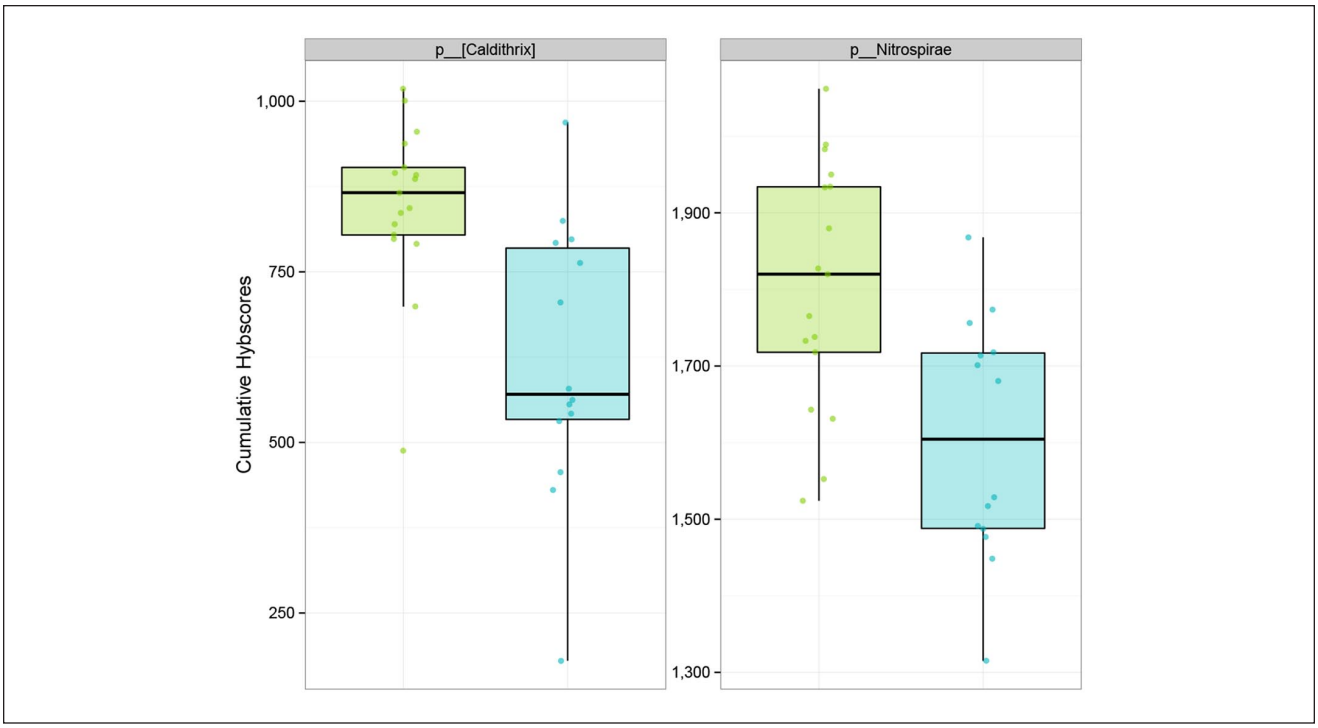

Figure 3. Phyla (*Caldithrix* and *Nitrospirae*) differing between vaccine nonresponders (green) and responders (blue) approaching statistical significance after adjustment for multiple comparisons (P_{adj} = .052 and .059, respectively). HybScores were added for all eOTUs within each phylum.

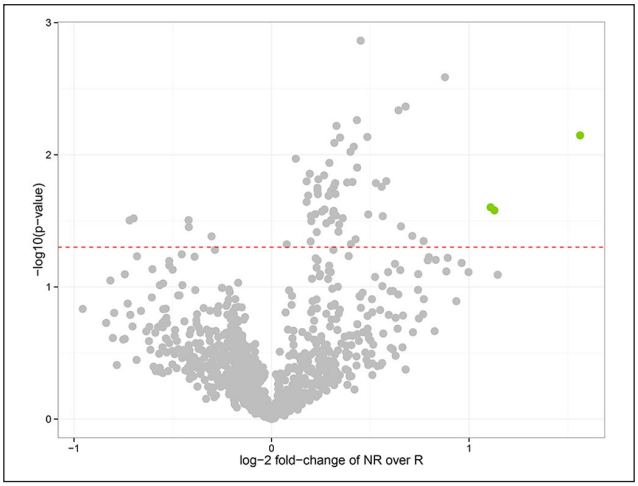

Figure 4. Three eOTUs with $P < .05$ were enriched in the vaccine non-responder group including *Prevotella* sp (*Prevotellaceae*), A17 sp (family *Pirellulaceae*), and an unclassified species (94otu38814) in the family *Sphingomonadaceae*.

body mass index, and hormone therapy.[34,35] Therefore, it is possible that the abundance of *Prevotella* may be creating an environment conducive for HPV infection to take hold, thereby dampening the effect of therapeutic vaccination. The potential mechanisms for *Caldithrix* and *Nitrospirae* are difficult to speculate, as *Caldithrix* has been reported to be a nitrate-reducing, thermophilic, anaerobic bacterium[36] and *Nitrospirae* has been shown to alleviate nitrite toxicity by converting nitrite into nitrate in aquatic organisms.[37] As classification of bacterial taxa is a dynamic process, it is possible that organisms identified in this study are closely related, but are of distinct taxa.

We found no difference in alpha diversity of cervical microbiome between responders and nonresponders prior to vaccination. This is in contrast to other studies of gut microbiome, which showed that higher alpha diversity is associated with better response to anti-PD-1 immunotherapy.[5,8-10] Use of antibiotics has also been shown to be associated with reduced response to anti-PD-1 immunotherapy,[7] suggesting a positive role of a healthy, diverse gut microbiome. Such a difference between the state of cervical and gut microbiomes in association with immune-based therapy may be a reflection of the difference in the baseline healthy states of these microbiota. More diverse gut microbiota are known to be associated with better response to immune checkpoint inhibitor therapy,[5,8-10] while a less diverse bacterial make-up comprised of a small number of species from the *Lactobacillus* genus is considered to be a "healthy" state.[38] Therefore, there may be no advantages immunologically of having a diverse array of bacteria in the cervicovaginal milieu.[39]

Table 2. Clustering Between Subject Characteristics and Variations in Cervical Bacterial Composition.

Variables	*P*	Classes or Range	Sample Count
Age	.147	22.3 to 49.5 years old	34
Race	**.001**	African American, Caucasian, and Hispanic	21, 12, 1
Histological response	.34	Responder and nonresponder	14, 17
HPV clearance	.554	Cleared at least one HPV type, no	19, 12
Peripheral Treg	.228	0.1% to 2.4% CD4 cells	29
Peripheral Th1	**.037**	2.6% to 40.2% CD4 cells	29
Peripheral Th2	.846	15.5% to 94.6% CD4 cells	28
HLA B15	.191	B15 positive, B15 negative	7, 27
HLA B40	**.024**	B40 positive, B40 negative	5, 29
HLA C3	.488	C3 positive, C3 negative	9, 25
HLA DQ3	.371	DQ3 positive, DQ3 negative	19, 15
Cervical Treg in epithelium[1]	.996	3 to 800 cells/mm^2	30
Cervical Treg in stroma[1]	.385	13 to 2240 cells/mm^2	30
Dose[1]	.633	50 µg, 100 µg, 250 µg, and 500 µg	14, 6, 6, 5
HPV 16 or not[2]	**.014**	HPV 16 positive, HPV 16 negative	22, 43
Vaccination[2]	.375	Pre-vaccination, post-vaccination	34, 31

Abbreviations: HPV, human papillomavirus; Th, T-helper type; HLA, human leukocyte antigen.
A permutational analysis of variance using distance matrices was performed for each variable of interest to determine if it significantly contributed to the beta diversity of the samples.
Baseline characteristics and cervical samples were examined for all variables with these exceptions: [1]Samples from the exit visits were examined for "cervical Treg in epithelium," "cervical Treg in stroma," and "dose" and [2]both samples were included for "HPV 16 or not" and "vaccination."
Bold values indicate $P < .05$.

In this study, cervical microbiome was characterized using the PhyloChip, a microarray-based platform that analyzes all 9 variable regions (V1-V9) of the 16S rRNA gene. This capability allows for detection of 16S rRNA sequences that have limited variation in one of their variable regions, whereas high-throughput, next-generation sequencing studies typically only amplify 1 or 2 variable regions (eg, V4, V3-V4). This technology has been previously used to identify differentially abundant microbes in other microbiome studies and results were concordant with V4 sequencing-based analyses.[40] Each method has its own limitations as to how its results could accurately represent bacterial composition of the samples being studied. Particular PCR primers used would naturally have stronger binding to certain taxa over others, possibly resulting in overrepresentation or underrepresentation of certain taxa. The use of hybridization signal as outputs used by PhyloChip could possibly contribute to over- and underrepresentations similarly due to the properties of particular probes used. As it would be difficult to characterize the effects due to use of different methodologies, it would be important to use the same methods within and across studies that should be compared.

It was expected that the cervical microbiome would be a low diversity community. For example, a total of 49 operational taxonomic units (by sequence) were identified in vaginal swab samples from 169 women.[41] In this study, a total of 72 to 365 eOTUs were observed per sample, with 1111 eOTUs observed across all samples. Additionally, 27 of these eOTUs were annotated at the strain-level, although they were not statistically significantly different between the nonresponder and responder groups.

A typical healthy cervicovaginal bacterial community is reported to be composed of a small number of species within genus *Lactobacillus*.[38] This is believed to be due to production of lactic acid by *Lactobacillus* species leading to protection against genital infections.[15,42-44] As would be expected, such *Lactobacillus* dominance was not observed in our study (Figure 2), as all but one of the patients were HPV-positive and all had biopsy-proven HSILs.[3,4] Such observation is in line with the findings by others who described that prevalent HPV infection can decrease the abundance of *Lactobacillus* species with a concomitant increase in anaerobes.[11] Others have also described that HPV-positive women have different microbial profiles compared with HPV-negative women.[12-14] The *Lactobacillus* dominance has also been shown to decrease with increased severity of cervical disease.[45] Another possible explanation for the differential abundance of *Lactobacillus* species in our study is the fact that *Lactobacillus* dominance has been reported in women of resource-rich settings. In one study, 90% of Caucasian women had *Lactobacillus*-dominant cervicovaginal microbiota. On the other hand, only 80% of Asian and 60% of Hispanic and African American women did.[15] In another study of young, healthy South African women, only 37% had *Lactobacillus*-dominant cervicovaginal community.[16]

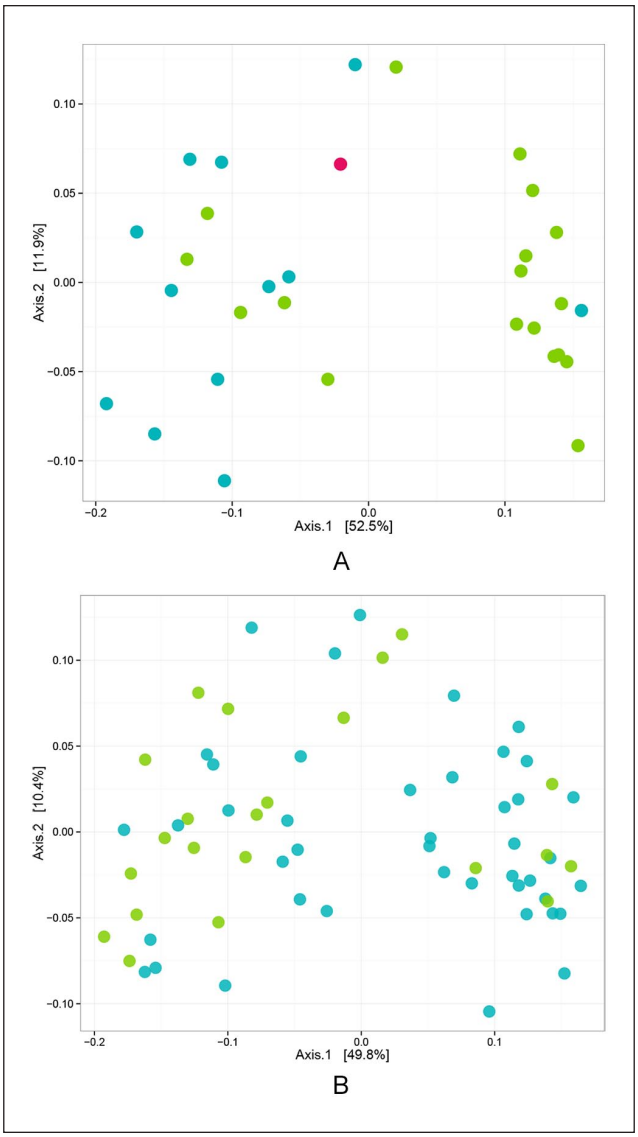

Figure 5. Significant associations with microbiome beta diversity were shown for race ($P = .001$) and human papillomavirus 16 (HPV 16) positivity ($P = .014$). Dimensional reductions of the Bray-Curtis dissimilarity between microbiome samples, using the principal coordinate analysis (PCoA) ordination method are shown for (A) race (Caucasian [blue], African American [green], and Hispanic [magenta]) prior to vaccination, and for (B) HPV 16 positivity (HPV 16-positive [green] and HPV 16-negative [blue]) for all samples.

The potential role of race on microbial composition was not only suggested by the differential abundance of *Lactobacillus* species in this study (as discussed above), but also by significant difference in beta diversity based on race (Table 2). Indeed, significant differences in relative abundance of 498 eOTUs were found based on race (Supplementary Table 1). However, there was no difference in racial distribution between the responders and nonresponders (Table 1), suggesting that, while race does influence the composition of cervical microbiome, it does not have effect on vaccine response.

Significant differences in beta diversity were also observed between HPV 16-positive and HPV 16-negative samples with 15 eOTUs being significantly different in abundance (Supplementary Table 2). Varied abundance in bacterial composition in women infected with HPV 16, 52, or 58 has been reported previously,[46] suggesting the possibility that we may be seeing effects between viruses and bacteria. It could be that viral infections have strain-specific effects on bacterial communities. Alternatively, bacterial communities may possibly shape the risk of acquiring or have influence on durations of strain-specific viral infections. As the 2 biotypes revealed using Fast Unifrac significantly separate on the basis of HPV 16 status and race, the influences of these factors on cervical microbiome warrant further investigations (Figure 6A).

Conclusions

It may be possible for some bacterial taxa such as *Calditrix, Nitrospirae*, and *Prevotella* to influence the response to HPV therapeutic vaccine. However, similar analyses would need to be repeated with a larger sample size to obtain additional data. The ongoing Phase II clinical trial (NCT02481414) would provide such opportunity. It is possible that the observed cervical microbiome composition associated with nonresponsiveness may be a marker for some underlying immune phenotypes. In other words, the observed possible association could reflect a shared cause rather than a direct causal relationship. In this study, we did not observe an effect of vaccination on the composition of the cervical microbiome. Furthermore, race and infection with HPV 16 appear to influence the beta diversity of cervical microbiome. The major limitation of this study was the small sample size, which is not unexpected given that the clinical trial was in Phase I. Recently, Ilhan and colleagues[47] reported a study in which they examined the interplay among microbiota, HPV, and cervical disease status using cervicovaginal metabolic profiling. Non–*Lactobacillus*-dominant communities, particularly in patients with HSILs, showed altered amino acid and nucleotide metabolisms. Future studies should not only be performed with a larger sample size, but should also probe the mechanisms underlying the association of cervical bacterial communities with vaccine nonresponsiveness using such metabolic profiling. If the role of certain bacterial taxa and their associated metabolites

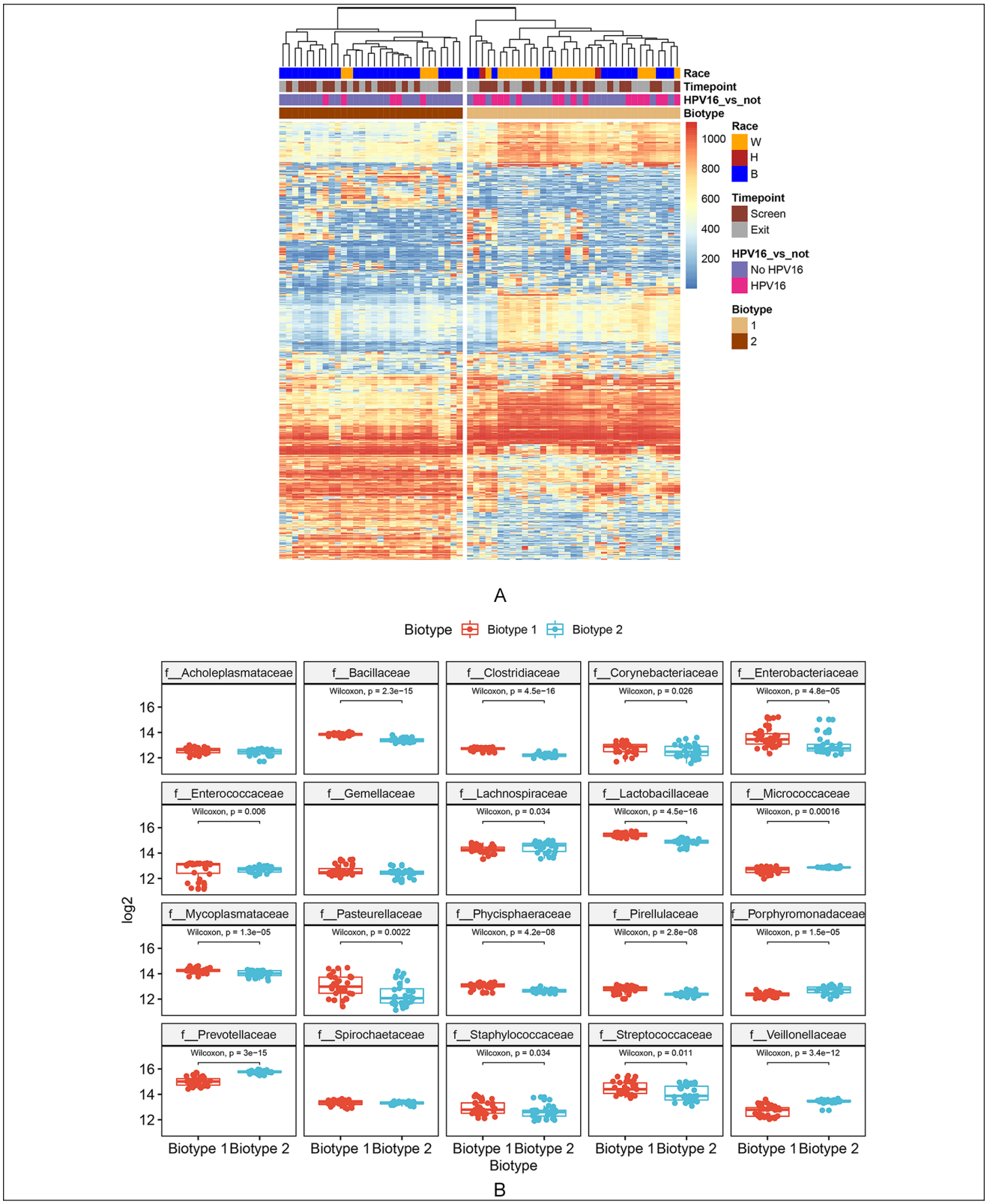

Figure 6. Fast UniFrac analysis revealing biotype 1 and biotype 2, which differ significantly based on human papillomavirus 16 (HPV 16) status ($P = .0088$) and race ($P = .0021$). (A) A heat map showing individual samples with regard to race, time point, and HPV 16 status. Each line represents eOTU and it abundance based on HybScore. (B) Box plots showing representation of 20 families between biotypes 1 and 2.

with vaccine nonresponsiveness were to be confirmed, it may be worthwhile to consider modulating the cervical microbiome to enhance vaccine efficacy in the future.

Authors' Note

This work was partly presented at the 2018 American Society for Clinical Oncologists Annual Meeting held in Chicago, IL, in June 2018.

The data in the form of (1) raw CEL files, (2) a csv file with the rank-normalized HybScore and taxonomic classification per eOTU, and (3) a short readme file are publicly available under the study name "UAMS_MNakagawa_2019_IntgCancTher.tgz" within the Phylochip datasets available at https://greengenes.secondgenome.com/.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Mayumi Nakagawa is one of the inventors named in a patent titled "Human Papilloma Virus Therapeutic Vaccine." Cheryl-Emiliane Chow and Kathryn Iverson are employed by Second Genome, Inc, and hold stock options in the company. Luisa Chan is an employee of Thermo Fisher Scientific, and is a former employee of Second Genome, Inc. She also holds stock options in the latter company.

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Supplemental Material

Supplemental material for this article is available online.

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