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Distinct anal microbiome is correlated with anal cancer precursors in MSM with HIV

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Objectives: Anal cancer risk is elevated in MSM with HIV (MSMWH). Anal high-risk human papillomavirus (hr-HPV) infection is necessary but insufficient to develop high-grade squamous intraepithelial lesion (HSIL), the anal cancer precursor, suggesting additional factors. We sought to determine whether the microbiome of the anal canal is distinct by comparing it with the microbiome of stool. We also sought to determine whether changes in the anal microbiome are associated with HSIL among MSMWH.

Design: Cross-sectional comparison of the microbiome of the anal canal with the microbiome of stool in MSMWH and cross-sectional comparison of the anal microbiome of MSMWH with anal HSIL with the anal microbiome of MSMWH without anal HSIL.

Methods: Sterile swabs were used to sample the anus of MSMWH for microbiome and HPV testing, followed by high-resolution anoscopy. Stool samples were mailed from home. 16S sequencing was used for bacterial identification. Measures of alpha diversity, beta diversity, and differential abundance analysis were used to compare samples.

Results: One hundred sixty-six anal samples and 103 matching stool samples were sequenced. Beta diversity showed clustering of stool and anal samples. Of hr-HPV-positive MSMWH, 31 had HSIL and 13 had no SIL. Comparison of the microbiome between these revealed 28 different species. The highest-fold enrichment among MSMWH/hr-HPV/HSIL included pro-inflammatory and carcinogenic *Prevotella, Parasuterella, Hungatella, Sneathia,* and *Fusobacterium* species. The anti-inflammatory *Anaerostipes caccae* showed the greatest reduction among MSMWH/hr-HPV/HSIL.

Conclusion: The anal microbiome is distinct from stool. A pro-inflammatory and carcinogenic environment may be associated with anal HSIL.

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Keywords: anal cancer, high-grade squamous intraepithelial lesion, HIV, human papillomavirus, MSM, microbiome

Introduction

Infection with high-risk human papillomavirus (hr-HPV) is responsible for 90% of anal cancers [1]. Actively replicating HPV may lead to nonprecancerous anal low-grade squamous intraepithelial lesions (LSIL), while anal high-grade squamous intraepithelial lesions (HSIL) are the precursors to anal cancer. HPV 16 and 18 (HPV 16/18) are the hr-HPV types most strongly associated with anal HSIL and cancer [1].

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MSM with HIV (MSMWH) are at particularly high risk for anal cancer; the age-adjusted incidence of anal cancer in this group is estimated at 90 per 100 000 person-years, reflecting a 40-fold increase in risk compared with the general population [2]. Consistent with their increased risk of anal cancer, MSMWH are at an increased risk of both anal HPV infection and anal HSIL [3–5]; however, many individuals with anal hr-HPV infection do not have anal HSIL [6]. Thus, it is likely that additional factors are important in HSIL pathogenesis.

One potential contributor is the local microbiome. In cervical cancer, another HPV-associated malignancy, changes to the composition of the vaginal microbiome are associated with a higher risk of cervical HSIL [7–11]. In the distal gastrointestinal tract or anal canal, being MSM [12] and the presence of HIV [13] are both independently associated with expansion of Prevotella, a genus of pro-inflammatory bacteria [14,15]. Thus, it is possible that inflammatory bacteria contribute to the development of anal HSIL.

Our study aimed to characterize the anal microbiome of the patients at highest risk for anal cancer, MSMWH, via two main objectives: first, to determine whether the anal canal comprises a distinct microenvironment within the gastrointestinal tract by comparing anal swabs from MSMWH to stool samples, and second, to determine whether an association exists between the anal microbiome and anal HSIL. We specifically hypothesized that the microbiome of participants with anal HSIL would show enrichment with pro-inflammatory bacteria, in particular *Prevotella*, and depletion of anti-inflammatory bacteria.

Materials and methods

Study design

We conducted a cross-sectional study to compare the microbiome of the anal canal with the microbiome of stool in MSMWH. For our second objective, we compared the anal microbiome of MSMWH with anal HSIL to the anal microbiome of MSMWH without anal HSIL.

Patient population

Participants were recruited at the Anal Neoplasia Clinic, Research and Education (ANCRE) Center at the University of California, San Francisco. Informed consent was obtained prior to enrollment. This study was approved by the Institutional Review Board of the University of California, San Francisco. MSMWH 18 years old and over were eligible. We restricted the study to individuals on antiretroviral therapy (ART) with undetectable HIV viral loads to minimize the effect of HIV on the composition of the anal microbiome. Additional exclusion criteria included conditions that could affect the composition of the gastrointestinal microbiome (obesity, a history of inflammatory bowel disease or colorectal cancer, antibiotic use within the last 3 months, and vegetarian or vegan diet [16,17]). Individuals on immunosuppressive drugs, with active evidence of proctitis, or with treatment of anal squamous intraepithelial lesions (SILs) within the last 6 months were also excluded.

Data and sample collection

Information on demographics and HIV, including $CD4^+$ T-cells and viral load, were obtained via electronic medical record review. To sample the anus, a sterile Dacron swab was inserted into the anal canal and material was dispersed in Thin-prep bottles for cytology and HPV testing. HPV testing was performed using HPV L1 MY09/MY11 DNA PCR as published previously [18]. Hr-HPV consisted of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 [19]. A second sterile Dacron swab was then inserted to sample the anal canal microbiome, placed in a tube containing guanidine thiocyanate in tris-acetate EDTA and zirconia beads, stirred for 1 min, and stored at -20°C. High-resolution anoscopy (HRA) with biopsies of visible lesions was then performed to determine the presence of anal HSIL.

For stool samples, participants were provided with a sampling kit designed for transport at ambient temperatures [20]. They were asked to use a sterile swab from the kit to sample used toilet paper at the time of their next bowel movement. This swab was placed in the same lysis and sterilization buffer used to store anal microbiome specimens and mailed to the ANCRE Center, where samples were stored at -20°C until processing.

DNA extraction and microbiome sequencing

DNA extraction and sequencing were performed in the uBiome laboratory in San Francisco, California, USA, as previously described [20]. Briefly, DNA was extracted using bead-beating in a class 1000 clean room by a guanidine thiocyanate silica column-based purification method using a liquid-handling robot [21,22]. The V4 region of the 16S gene was amplified using the following primer sequences: (515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT) [23]. Paired-end sequencing was performed on the Illumina NextSeq500 (Illumina, San Diego, California, USA).

Bioinformatic analysis and taxonomy annotation

De-multiplexing was performed using the Illumina BCL2FASTQ algorithm and reads were filtered using an average Q-score more than 30. Sequences were clustered using the Swarm algorithm [24], and the most abundant sequence per cluster was assigned the counts of all reads in the cluster. Chimera removal was performed using vsearch [25]. The SILVA database v 123 was used to

annotate taxonomy for reads that matched with at least 77% sequencing identity (at the phylum level). Ninety-five percent sequencing identity was used to annotate at the genus level. Species level identification was performed using 100% identity over 100% length against a hand-curated database of target 16S rRNA gene sequences and taxonomic annotations derived from the SILVA database [20].

Study endpoints

Our primary endpoints included differences in alpha diversity, beta diversity, and differential abundance analysis between [1] anal and stool samples and [2] between anal samples with HSIL and anal samples without SIL. For the comparison of anal samples in relation to HSIL, we restricted analysis to participants with hr-anal HPV infection to minimize the role of HPV as an effect modifier. We also excluded participants with LSIL but no HSIL to minimize the potential for misclassification of lesion severity. The presence of HSIL was based on HRA-guided biopsy. To be classified as having no SIL, anal cytology could not show SIL and there could be no lesions consistent with HSIL or LSIL on HRA.

Statistical analysis

To compare demographics and clinical characteristics, we used unpaired *t*-tests for normally distributed continuous variables and the Wilcoxon rank-sum test for non-normally distributed continuous variables. For categorical variables, we used chi-squared test, or Fisher's exact test when expected values were small.

For our first objective, we compared alpha diversity, beta diversity, and differential abundance between samples of the anal canal and stool in MSMWH. For our second objective, we compared alpha diversity, beta diversity, and differential abundance between hr-HPV positive MSMWH with HSIL and hr-HPV positive MSMWH with no SIL.

In addition to quantifying the richness of individual samples, to analyze alpha diversity, we used Pielou's index to capture community evenness and both Simpson and Shannon indices, which incorporate richness and evenness into one value. The Wilcoxon rank-sum test was used for comparison between groups.

Bray-Curtis distance was used to assess beta diversity and Kruskal's nonmetric multidimensional scaling was performed to visualize these results. Differences in mean and dispersion of beta diversity across groups was tested using permutational analysis of variance. For exploratory plotting, low-count taxa were amalgamated [26], and zero-replacement was performed using a Bayesian multiplicative method [27], and densities of log-normalized counts were plotted against variables of interest. Genera and species with less than 5 nonzero counts across the whole sample were excluded.

Differential abundance analysis identifies taxa-specific differences between samples. This analysis was carried out using the DESeq2 package [28], in conjunction with a robust normalization method to obtain base mean counts [29]. Fold-changes were calculated with no SIL as the reference category. All fold-changes were shrunk using normal priors [28]. and adjusted for age, race, tobacco use, and recent $CD4^+$ cell count. All *P* values were adjusted for multiple testing using the Benjamini-Hochberg method. Analysis was performed in the R programming environment [30].

Results

Baseline characteristics

Two hundred fifty-four participants were enrolled, 166 of whom had anal samples that were successfully sequenced. One hundred seventy-three of the 254 participants also provided stool samples, of which 170 were sequenced. Demographic and other characteristics are summarized in Table 1. Mean $CD4^+$ cell count was more than 500 cells/μl. The majority of participants were identified as white and a high proportion were active smokers. Sixty-eight of 166 participants with sequenced samples (41%) were hr-HPV positive. Of those with hr-HPV infection, 31 (46%) had HSIL, 23 (34%) had LSIL, and 13 (19%) had no SIL. SIL status could not be determined for one participant. Participants with hr-HPV infection with HSIL were more likely to be infected with HPV types 16/18 ($n = 20$, 67%) compared with participants with hr-HPV infection and no SIL ($n = 2$, 17%, $P < 0.01$).

We performed sensitivity analyses to identify differences in baseline characteristics between participants with and without sequenced anal and stool samples (Supplemental Table 1, http://links.lww.com/QAD/D206); we also compared baseline characteristics of participants by the presence of anal hr-HPV infection (Supplemental Table 2, http://links.lww.com/QAD/D207). Participants with sequenced samples were somewhat older than participants who did not have sequenced anal and stool samples (median age 60 years, IQR 52–65, versus median age 56, IQR 48–62, $P = 0.01$). Analysis of baseline characteristics by hr-HPV status showed a higher percentage of white or Caucasian participants (89 versus 73%) and a lower percentage of Asian participants (0 versus 9%) in the group without anal hr-HPV infection ($P = 0.01$).

Comparison of anal and stool samples

After excluding missing variables, 103 participants with both anal canal and stool samples were used to compare the anal and stool microbiomes. Paired analysis was not

Table 1. Baseline characteristics of participants according to analyzed microbiome samples.

	Anal versus stool	Hr-HPV positive		
		HSIL	No SIL	
	$N=103$	$N=30$	$N=12$	P
Age in years – median (IQR)	60 (52–65)	57 (45–62)	60 (51–66)	0.38
Race – no. (%)				
White or Caucasian	85 (82)	24 (80)	9 (75)	0.88
Black or African–American	4 (4)	1 (3)	0 (0)	
Asian	5 (5)	2 (7)	1 (8)	
Other	9 (9)	3 (10)	2 (17)	
Tobacco – no (%)				
Never	55 (53)	17 (57)	5 (42)	0.27
Active	12 (12)	8 (27)	2 (16)	
Past	36 (35)	5 (17)	5 (42)	
Recent $CD4^+$ cells per μl– median (IQR)	650 (460–820)	724 (619–853)	645 (498–853)	0.55
HPV 16/18 positive – no. (%)		20 (67)	2 (17)	<0.01

performed in order to maximize sample size. Days between anal canal and stool specimen collection were recorded in a subset of patients (median-2 days, IQR 1–4, $n=39$).

Anal samples showed significantly higher alpha diversity than stool samples for each metric at the species (Fig. 1a) and genus level (Fig. 1b). Bray-Curtis distance analysis showed significant differences in beta diversity between stool and anal microbiomes at both the genus (Fig. 2a) and species levels (Fig. 2b).

Selected results of the differential abundance analyses for species in the stool and anal samples are summarized in Table 2. After adjusting for age, race, tobacco use, and recent $CD4^+$ cell count, 31 species were associated with significant fold-changes in abundance between stool and anal sites. Compared with stool samples, anal samples

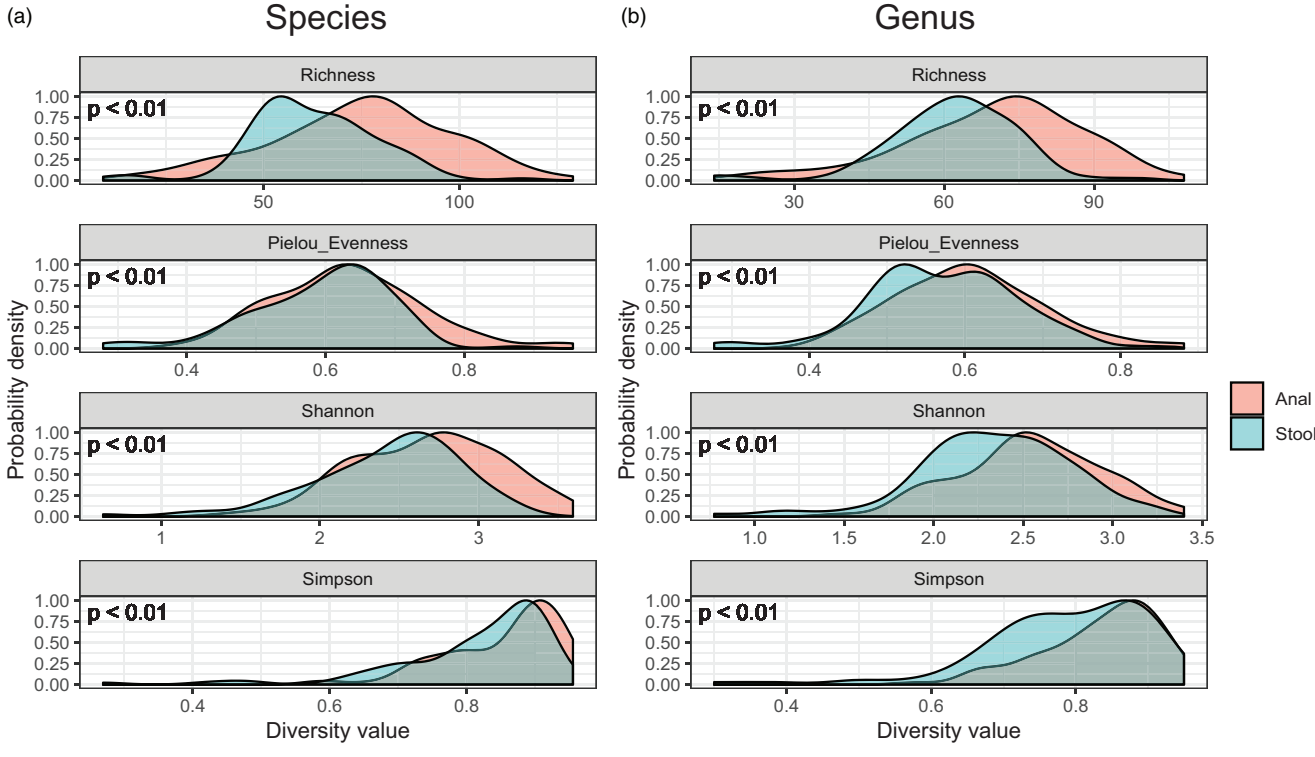

Fig. 1. Comparison of alpha diversity between anal (red) and stool (green) samples using different indices. The x-axis for richness represents the total number of taxa. Pielou's index ranges from 0 to 1 with 0 signifying no evenness and 1 complete evenness. Increasing Shannon and Simpson indices reflect increasing diversity.

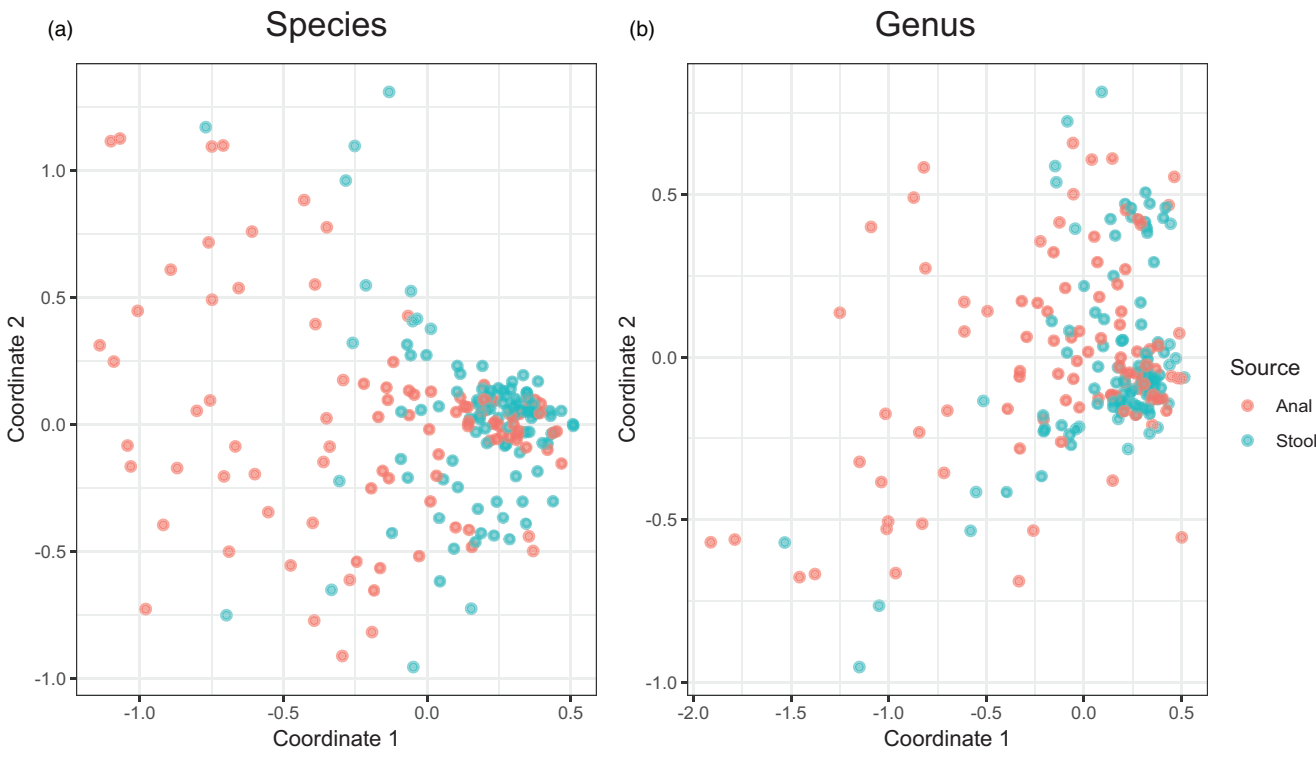

Fig. 2. Comparison of beta diversity between anal (red) and stool (green) samples. Multidimensional scaling plots reflect distinct clustering of anal and stool samples at both the species (a) and genus (b) levels.

were enriched with various anaerobic gram-negative *Prevotella* species, as well as multiple anaerobic gram-positive cocci from the Clostridiales order, including *Peptoniphilus* species, *Anaerostipes hadrus, Casaltella massiliensis*, *Peptostreptococcus anaerobius,* and *Finegoldia magna*.

Comparison of anal samples from hr-human papilloma virus-positive participants with high-grade squamous intraepithelial lesion and no squamous intraepithelial lesion

After excluding samples with missing variables, the anal microbiome was compared between 30 participants with HSIL and 12 with no SIL. A statistically significant increase in richness was noted in participants with HSIL compared with no SIL; however, other measures of alpha diversity were not significantly different (Supplemental Figure 1, http://links.lww.com/QAD/D204). Multidimensional scaling and the Bray-Curtis dissimilarity did not identify distinct clustering between participants with HSIL and no SIL (Supplemental Figure 2, http://links.lww.com/QAD/D205); however, differential abundance analysis identified statistically significant differences in the composition of 28 species between the two groups (Table 3). The largest increase in abundance within the HSIL group was noted for *Prevotella disiens.* Other bacteria with notable fold-increases included *Parasutterella excrementihominis, Streptococcus agalactiae,* and *Hungtella hathewayi*. Notable decreases in differential abundance in participants with HSIL included *Anaerostipes caccae* and two *Prevotella* species, *Prevotella ihumii* and *Prevotella bivia*.

Discussion

This study shows a distinct anal canal microbiome compared with stool among a large cohort of MSMWH. This finding is consistent with the anal canal being anatomically and functionally separate from the colorectal tract. The presence of a distinct anal canal microbiome corroborates two previous small studies. The first demonstrated distinct clustering of bacteria from anal swabs and stool samples of 23 MSMWH [31], while the second demonstrated distinct clustering of bacteria from anal and rectal swabs (the latter collected via rigid sigmoidoscopy) in 54 men without HIV [32]. Our study leverages in-depth taxonomic annotations to identify novel species and genus-level associations in a sample of over 100 MSMWH. The results show an increase of anaerobic bacteria, particularly of *Prevotella* and anaerobic Gram-positive cocci, in the anal canal compared with stool. The presence of a discrete anal canal microbiome highlights the need to sample this area specifically when considering the role of the microbiome in anal HPV pathogenesis.

The study also identified several important characteristics of the anal canal microbiome in relation to anal HSIL. As

Table 2. Differential abundance of microbiota in stool versus anal canal, by species.

Species	Base mean count (Stool)	Fold-change in anal sample	*P* *
		> 50X increase	
Finegoldia magna	1230.84	366.48	<0.01
Streptococcus pneumoniae	520.45	253.41	<0.01
Prevotella bivia	631.80	192.45	<0.01
Blautia obeum	156.60	140.34	<0.01
Peptoniphilus urinimassiliensis	66.13	112.75	<0.01
Escherichia coli	494.11	91.05	<0.01
Peptostreptococcus anaerobius	294.65	86.47	<0.01
Anaerococcus vaginalis	118.09	84.81	<0.01
Faecalibacterium prausnitzii	2848.39	82.32	<0.01
Holdemanella biformis	171.65	77.59	<0.01
Blautia schinkii	187.95	68.91	<0.01
Prevotella disiens	405.05	59.92	<0.01
		10–50X increase	
Slackia isoflavoniconvertens	44.27	41.63	<0.01
Blautia massiliensis	287.75	37.87	<0.01
Senegalimassilia anaerobia	78.65	28.02	<0.01
Prevotella corporis	222.54	27.27	<0.01
Lachnospira pectinoschiza	121.00	16.72	<0.01
Prevotella buccalis	107.80	11.13	<0.01
		0–10X increase	
Prevotella copri	7212.08	6.69	<0.01
		Decrease	
Negativibacillus massiliensi	31.47	0.88	<0.01
Dialister propionicifaciens	121.09	0.71	<0.01
Streptococcus anginosus	121.43	0.70	<0.01
Corynebacterium singulare	107.32	0.61	<0.01
Staphylococcus aureus	67.68	0.60	0.01
Collinsella aerofaciens	872.02	0.58	0.01
Roseburia faecis	821.44	0.53	0.02
Fusobacterium equinum	756.91	0.52	0.02
Peptoniphilus lacrimalis	48.50	0.52	0.03
Anaerostipes hadrus	220.98	0.49	0.04
Casaltella massiliensis	42.00	0.45	0.04
Prevotella timonensis	109.3	0.43	0.05

*Adjusted for multiple comparisons.

with cervical HSIL [7–11], an association was detected between anal HSIL and bacterial richness. Although differences in other measures of alpha diversity were not statistically significant, greater richness may reflect the expansion of pro-inflammatory bacteria. A role for *Prevotella* species in anal HPV pathogenesis is of special interest. In the cervix, depletion of *Lactobacilli* and enrichment with microaerophilic and anaerobic bacteria, including *Prevotella,* is associated with hr-HPV infection, HSIL, and cervical cancer [9–11]. HIV and being MSM are both risk factors for anal cancer [4] and are associated with enrichment of Prevotella of the distal gastrointestinal tract [12,13]; therefore, enrichment with *Prevotella* species MSMWH may contribute to anal HSIL. Our study specifically noted a three-fold increase in *Prevotella disiens* among participants with anal HSIL as well as a concomitant increase in *Prevotella bergensis.* However, moderate decreases in *Prevotella ihumii* and *bivia* were seen. A better understanding of the interplay between specific bacterial species is needed, as different bacteria within the same genus may possess distinct properties.

Enrichment with *Fusobacterium* and *Sneathia* species was also noted in participants with anal HSIL. Expansion of *Fusobacterium* species is noted in colorectal cancer [33]. In the cervix, risk for HSIL and invasive squamous cell carcinoma has been specifically linked to both these groups [7,9]. *Sneathia* have also been previously linked to anal hr-HPV infection in MSM [34].

Several other bacteria may also play a role in the pathogenesis of anal HSIL. *Streptococcus agalactiae,* a bacterium that can cause both local inflammation and invasive systemic disease [35], and *Parasutterella excrementihominis* and *Hungtella hathewayi*, two species linked to worsening inflammation in Crohn's disease [36,37], were detected at significantly increased levels in participants with anal HSIL. This was accompanied by a decrease of *Anaerostipes caccae,* a butyrate-producing clostridial species with anti-inflammatory properties [38].

These bacteria may contribute to anal HSIL via several different mechanisms. Overall, our results suggest a model wherein, as in the cervix and among those with hr-HPV infection, a diverse mixture of pro-inflammatory and/or carcinogenic bacteria is associated with anal HSIL. These shifts in composition may be associated with metabolic pathways that impact HPV pathogenesis. Recently, an increase in cobalamin and succinyl-CoA, which are associated with solid organ tumors, has been reported in

Table 3. Differential abundance of species in anal samples of participants with Hr-HPV infection, by HSIL status.

	Base mean count	Fold-change in HSIL compared with no SIL	*P* *
		Increase	
Prevotella disiens	1095.90	2.93	<0.01
Parasutterella excrementihominis	28.29	1.56	<0.01
Streptococcus agalactiae	987.09	1.45	<0.01
Hungatella hathewayi	26.83	1.45	<0.01
Megasphaera massiliensis	145.39	1.42	<0.01
Granulicatella elegans	354.32	1.42	<0.01
Sneathia amnii	131.21	1.40	<0.01
Bacteroides fragilis	947.22	1.37	<0.01
Acidaminococcus timonensis	85.92	1.31	<0.01
Sutterella wadsworthensis	275.99	1.29	<0.01
Butyricimonas virosa	48.94	1.23	<0.01
Parvimonas micra	125.73	1.23	<0.01
Prevotella bergensis	4.64	1.23	<0.01
Anaerococcus vaginalis	213.9	1.19	<0.01
Dialister invisus	21.20	1.17	<0.01
Fusobacterium equinum	1068.09	1.16	<0.01
Bacteroides acidifaciens	4.17	1.12	<0.01
Desulfovibrio piger	97.81	1.03	<0.01
Atopobium vaginae	40.28	1.02	<0.01
Tyzzerella nexilis	151.31	1.01	<0.01
		Decrease	
Acidaminococcus intestini	15.43	0.97	0.01
Dialister pneumosintes	29.21	0.84	0.01
Brevibacterium paucivorans	12.53	0.80	0.01
Prevotella ihumii	166.78	0.80	0.01
Coprobacillus cateniformis	46.84	0.80	0.01
Prevotella bivia	369.23	0.77	0.02
Eubacterium coprostanoligenes	11.53	0.67	0.03
Mycoplasma hominis	40.10	0.62	0.03
Anaerostipes caccae	2.88	0.56	0.04

*Adjusted for multiple comparisons.

anal samples from MSMWH with anal HSIL [39]. However, the role of an altered microbiome in increasing levels of these compounds remains to be elucidated, as does the role of these compounds in potentiating development of HSIL.

Increased abundance of *Prevotella* at mucosal sites is linked to local and systemic inflammatory diseases including periodontitis and rheumatoid arthritis via the activation of pro-inflammatory T-helper 17 cells [14]. *Hungatella* is similarly associated with induction of T-helper 17 cells [37]. Depletion of *Anaerostipes caccae* may further contribute to a pro-inflammatory milieu via decreases in butyrate, a short-chain fatty acid that stimulates production of regulatory T-cells and anti-inflammatory cytokines [38]. Chronic inflammation can disrupt epithelial tight-junctions, which may facilitate HPV entry into the basal cell of the epithelium, and lead to tissue damage and further release of growth-stimulating cytokines and chemokines [8]. Bacteria with invasive properties including *Streptococcus agalactiae* may similarly facilitate epithelial disruption [35]. Lastly, Fusobacteria, including *Sneathia,* can activate the WNT signaling pathway, a key survival and proliferation pathway that is dysregulated in colorectal carcinogenesis and in cervical cancer [8].

Strengths of this study include performance of HRA on all participants to accurately detect anal HSIL, a carefully selected patient population to minimize confounders, comparison of HSIL to no SIL with exclusion of LSIL from the analysis to minimize disease classification error, and restriction of our HSIL/no SIL analysis to participants with hr-anal HPV given the key role of hr-HPV as an effect modifier in determining the presence of HSIL.

This study also had several limitations. Although participants were instructed to submit stool samples immediately after their study visit, samples were not excluded when submission was delayed. It is thus possible for differences between the anal and stool microbiomes to result from sampling at different time-points. However, among participants for whom the date of stool collection is known, the majority submitted stool samples within 4 days of their study visit, suggesting a lesser role for sampling at different time-points.

This was a cross-sectional study, and associations between specific bacteria and HSIL do not necessarily reflect a cause-and-effect relationship. Microbiota were analyzed only once for each participant, and the degree of individual variability is not known. Due to sample size limitations, the study may have been underpowered to

detect differences in diversity and differential abundance; furthermore, there may exist limitations in the accuracy of species-level detection with 16S rRNA sequencing [40] Although our HSIL/no SIL microbiome analysis was restricted to participants with hr-anal HPV, sample size limitations precluded restriction of this analysis to those with HPV 16/18 specifically. Thus, we cannot entirely exclude a role for differential distribution of HPV 16/18 as a contributor to alterations of the microbial composition. However, at least one study of the cervix noted that HSIL persistence was associated with microbiome alterations even when HPV 16/18 distribution was similar between comparison groups [11], thus suggesting that the local microbial composition remains relevant in HSIL pathogenesis. Lastly, it is unclear how generalizable these studies are to broader patient populations, including MSMWH not on ART, given the careful selection of participants to minimize confounding.

In summary, this study shows a discrete anal microbiome in MSMWH and highlights the need to sample this area specifically when considering a role of the microbiome in HPV pathogenesis. Although our study did not detect a clear difference in beta diversity between participants with HSIL and no SIL, differential abundance analysis identified differences in the composition of bacterial species that may contribute to a pro-inflammatory and carcinogenic milieu. Additional larger and longitudinal studies are necessary to further understand the interplay between the microbiome and anal HSIL over time.

Acknowledgements

Dr Cristina Brickman oversaw all aspects of the study and was responsible for study design, data analysis, writing and submission of the manuscript.

Dr Melissa Agnello coordinated the efforts between UCSF and the uBiome Outcomes Research and Epidemiology team. She participated in next-generation sequencing and microbiome analyses.

Nabeel Imam, Rodolfo Pino, and Dr Pamela Camejo contributed to bioinformatic analysis of the sequencing libraries and performed the statistical analyses used to determine alpha diversity, beta diversity, and differential abundance of microorganisms.

Lauren Carroll oversaw the work of the uBiome Outcomes Research and Epidemiology team. She participated in study design and data analysis.

Dr Aung Chein contributed to the identification of and consenting potential participants, and to the overall conduct of the study at UCSF.

Dr Joel Palefsky oversaw all aspects of the study and of Dr Brickman's work, including study design, data analysis, and writing of the manuscript.

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The NIH had no role in writing of the manuscript. The uBiome Outcomes Research and Epidemiology team assisted with data analysis, including sequencing of samples and measurements of alpha diversity, beta diversity, and differential abundance. Drs Brickman and Palefsky had access to all data from uBiome and held full responsibility over manuscript writing and the decision to submit for publication. uBiome did not provide them with financial remuneration for this collaboration.

Conflicts of interest

Drs. Brickman, Chein, and Palefsky have no conflicts relevant to the study. Dr Melissa Agnello, Nabeel Imam, Dr Pamela Camejo, Rodolfo Pino, and Lauren N. Carroll were employees at uBiome, Inc, at the time of this work and received compensation.

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