Pilot Study of Markers for High-grade Anal Dysplasia in a Southern Cohort From the Women's Interagency Human Immunodeficiency Virus Study

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(See the Editorial Commentary by Susan Bennett on pages 1129–30.)

Background. Anal cancer rates have increased, particularly in human immunodeficiency virus (HIV)–infected (HIV⁺) women. We assessed factors associated with anal precancer in HIV⁺ and at-risk HIV-negative women from the Atlanta Women's Interagency HIV Study cohort.

Methods. All participants underwent high-resolution anoscopy and anal cytology and had anal and cervical samples collected. Specimens were tested for 37 human papillomavirus (HPV) types and for *FAM19A4* and *microRNA124-2* promoter methylation. Binary logistic regression and multivariate analysis were conducted with histologic anal high-grade squamous intraepithelial lesion (A-HSIL) as the dependent variable.

Results. Seventy-five women were enrolled: 52 (69%) were HIV⁺ with three-fourths having undetectable viral load; 64 (86%) were black; mean age was 49 ± 8 years. Forty-nine (65%) anal cytology samples were abnormal, and 38 (51%) of anal samples were positive for at least 1 of 13 high-risk HPV (hrHPV) types. Thirteen (18%) anal biopsies identified A-HSIL. Hypermethylation of *FAM19A4* and/or *microRNA124-2* was found in 69 (95%) anal samples and 19 (26%) cervical samples. In multivariate analyses, the odds of having A-HSIL were >6 times higher in women with anal hrHPV (adjusted odds ratio [aOR], 6.08 [95% confidence interval {CI}, 1.27–29.18], *P* = .02) and with positive cervical methylation (aOR, 6.49 [95% CI, 1.66–25.35], *P* = .007), but not significantly higher in women with positive anal methylation.

Conclusions. Anal hrHPV and promoter hypermethylation in the cervix show promise as biomarkers for anal cancer screening in HIV⁺ and at-risk HIV-negative women. Greater understanding of gene silencing by promoter hypermethylation in anal carcinogenesis is needed.

Keywords. anal cancer; high-grade squamous intraepithelial lesion (HSIL); HIV; women; methylation.

Anal cancer incidence has more than doubled during 1975–2015, from 0.8 to 1.9 cases per 100 000 population [1]. Human papillomavirus (HPV) has been detected in 80%–90% of anal cancers [2]. Oncogenic HPV types, particularly HPV-16, are associated with anal cancer and precancer (anal high-grade squamous intraepithelial lesion [A-HSIL]) [3–6]. Human immunodeficiency virus (HIV)–infected (HIV⁺) women have increased risk for persistent HPV infections [7–11] and an almost 8-fold increased risk of anal precancer compared with women in the general population

[12]. Mortality from anal cancer in the HIV⁺ population remains significant, with a 64% 5-year survival rate [13].

While no data currently show that identification and treatment of A-HSIL reduces incidence of anal cancer, the American Society for Colposcopy and Cervical Pathology recommends screening HIV⁺ women or those with lower genital tract neoplasia using anal cytology (AC) with triage to high-resolution anoscopy (HRA)–guided biopsy and A-HSIL treatment [14]. Feasibility of performing HRA on all women with abnormal AC is limited due to lack of providers trained in the procedure. Alternative noninvasive markers are needed to identify women at highest risk of A-HSIL.

A meta-analysis showed that HPV tests applied to anal samples have a pooled sensitivity and specificity of 91.3% (95% confidence interval [CI], 78.9%–96.7%) and 33.1% (95% CI, 22.2%–46.3%), respectively, for detecting A-HSIL or greater disease, although study populations were predominantly male [15]. DNA methylation, epigenetic modification resulting in tumor suppressor

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and DNA repair inactivation, plays an important role in cervical carcinogenesis [16]. Gene methylation has the potential to be applied as a risk biomarker in women with positive clinical HPV tests to identify those requiring colposcopy [17–21]. *FAM19A4* gene promoter methylation levels in cervical scrapes of women with HPV detected cervical intraepithelial neoplasia (CIN) 3 (ie, HSIL in current terminology) with comparable sensitivity and higher specificity than cytology [21], and a recent study showed an increasing level of *FAM19A4* methylation with increasing severity of cervical lesions [22]. Similarly, gene promoter methylation of *micro-RNA124-2* (*miR124-2*) strongly predicted CIN 3 [19]. Aberrant DNA methylation patterns have been identified in anal carcinomas compared to normal tissue [23, 24] and in high-risk (tumor >5 cm and/or nodal involvement) vs low-risk anal cancers [25]. However, methylation markers have not been explored in anal cancer screening strategies.

HPV exposure at 1 genital site increases risk for exposure at others, so women with cervical HPV may be more likely to have anal HPV, and HPV oncogenic changes may affect multiple genital sites (the “field effect”) [26]. In high-risk women, cervical findings may be sufficient to guide HRA referral. We assessed cytology, HPV genotyping, and DNA methylation of *FAM19A4* and *miR124-2* promoter regions, as predictors of A-HSIL/cancer in a population of HIV⁺ and at-risk HIV-negative (ARHIVN) women.

MATERIALS AND METHODS

Study Population

A cross-sectional pilot study was conducted within the Atlanta site of the Women’s Interagency HIV Study (WIHS), a cohort created in 2013 consisting of two-thirds HIV⁺ and one-third ARHIVN women. Women were classified as ARHIVN if they or their sexual partner reported ≥1 criteria within 5 years of enrollment: (1) intravenous or other illicit drug use; (2) sexually transmitted infection; (3) unprotected sex with ≥3 partners; (4) sex with ≥6 partners; (5) transactional sex; or (6) sex with known HIV⁺ partner. WIHS data collection and methods have been previously described [27]; in brief, participants complete questionnaires, blood draws, and physical/gynecological examinations during semiannual visits. All women within WIHS undergo colposcopy if they have (1) visible lesion(s) concerning for condyloma; (2) atypical squamous cells of undetermined significance (ASCUS) on cervical cytology without prior colposcopy; or (3) more significant cervical cytologic abnormalities, that is, low-grade squamous intraepithelial lesion (LSIL) or more severe, or atypical glandular cells. For this substudy, eligibility criteria included (1) no history of A-HSIL or anal cancer; (2) no active anal/rectal disease necessitating surgery; and (3) willing/able to undergo cervical/anal sampling and HRA. Participants were enrolled after informed consent obtained prior to the study visit. The Emory Institutional Review Board and the Grady Research Oversight Committee approved the study.

Data Collection

Data collected from core WIHS visits were abstracted and included sociodemographics, smoking status, alcohol consumption, sexual history, cervical cytology and histology, CD4 cell count, and HIV RNA load within 12 months of the study visit. Additional data collected at the study visit included cervical and anal HPV genotyping, *FAM19A4* and *miR124-2* gene promoter methylation, and anal cytology and histology.

Study Procedures and Specimen Collection

Investigators collected cervical and anal swabs prior to HRA (Figure 1). A cytobroom was rotated 3 times in the cervical os and agitated in a ThinPrep vial 3–5 times. Two saline-moistened Dacron swabs were sequentially inserted 3–5 cm into the anal canal and rotated for 30 seconds. The first anal swab was agitated for 15 seconds in SurePath liquid media for AC, and the second swab was placed in ThinPrep media for HPV genotyping and methylation analysis.

HRA was performed on all participants by a trained anoscopist. Abnormal anal areas were identified using 3% acetic acid and Lugol solution, biopsied, and placed in buffered formalin solution. AC and histology specimens were transported to the Grady Memorial Hospital Pathology Laboratory at room temperature within 24 hours of collection. Anal and cervical ThinPrep specimens were maintained at 4°C and transported to the Centers for Disease Control and Prevention within 72 hours of collection.

Anal Cytology and Histology

All AC and histology specimens were interpreted by 2 pathologists with expertise in anal histopathology. AC was classified based on 2001 and 2014 Bethesda terminology for cervical cytology [28, 29]: NILM (negative for intraepithelial lesion and malignancy), ASCUS, LSIL, ASC-H (atypical squamous cells, cannot exclude HSIL), and HSIL. Anal histology results were reported using Lower Anogenital Squamous Terminology (LAST) classification [30]: benign/negative, LSIL (condyloma or anal intraepithelial neoplasia [AIN] grade 1), HSIL (AIN grade 2 or 3), or invasive squamous cell carcinoma. P16 staining was performed per LAST recommendations [30].

Extraction and Molecular Testing

Cells from a 12-mL aliquot of each sample were pelleted by centrifugation. Collection media was decanted and pellets were stored at –80°C until extraction using the Roche MagNA Pure LC instrument with the MagNA Pure LC DNA Isolation Kit III and external lysis step (Roche Applied Science, Mannheim, Germany). Each thawed pellet was suspended in 130 µL lysis buffer and 20 µL proteinase K from MagNA Pure LC reagents. Samples were incubated at 65°C for 1 hour and then added to the instrument, yielding 100 µL final extract volume. Extracts were maintained at 4°C for up to a week and stored at –80°C.

Figure 1. Study schema (N = 75 unless otherwise noted). Abbreviations: h/o, history of; HPV, human papillomavirus.

HPV detection and typing were performed with Research Use Only Linear Array HPV Genotyping Test (LA; Roche Molecular Diagnostics, Indianapolis, Indiana) as previously described [31]. The manufacturer's protocol was modified to use 5 μ L per 100 μ L reaction, and type-specific hybridization was automated. The LA test relies on L1 consensus polymerase chain reaction (PCR) and includes primers for β -globin to control for specimen adequacy. Type-specific hybridization allows qualitative detection of 36 types, HPV-52 with potential cross-reactivity, and positive control (β -globin). Ambiguous results for HPV-52 are resolved with a type-specific quantitative PCR [32]. Samples testing negative for HPV and β -globin were considered inadequate. Thirteen of the 14 HPV types included in clinical assays were considered high risk (hrHPV): 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, omitting HPV-66, which is not in International Agency for Research on Cancer HPV carcinogenicity group 1 or 2A [33, 34]. Additional HPV types detected by LA are 6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89, and IS39.

DNA (1.6–250 ng), quantified by Qubit Fluorometer (Life Technologies, Carlsbad, California), was treated with sodium bisulfite via the EZ DNA Methylation Kit (Zymo Research, Irvine, California) and collected in 12.5 μ L elution buffer. The PrecursorM+ kit from Self-Screen (www.self-screen.nl), a real-time multiplex methylation-specific PCR assay, was used for detection of *FAM19A4* and *miR124-2* promoter methylation level. PreCursor M+ uses primers and probes specific for *FAM19A4* and *miR124-2*, and for reference gene β -actin (*ACTB*) as sample quality control. Positive controls for all 3 genes serve as the calibrator for expressing differential methylation. PCR

reactions were performed on the Rotorgene-Q5-plex thermal cycler (Qiagen) following the Precursor M+ kit protocol using 2.5 μ L of bisulfite-treated DNA. Promoter methylation results were reported as the delta-delta ($\Delta\Delta$) cycle threshold (Ct) for each marker gene [35]. Methylation levels of target genes were normalized to *ACTB* and expressed relative to the calibrator. Clinical samples were considered methylation positive when the $\Delta\Delta$ Ct for *FAM19A4* was <10.36 and/or <6.50 for *miR124-2*, per the manufacturer's instructions. Repeat assays for 28 (19%) samples showed significant correlation of Ct using Pearson coefficient analyses: *FAM19A4* = 0.92 and *miR124-2* = 0.96.

Statistical Analyses

Data were summarized using descriptive statistics with counts and percentages for categorical variables, and mean $\pm$ standard deviation or median with first and third quartiles for continuous variables. Statistical comparisons between HIV⁺ vs ARHIVN or between women with histologic A-HSIL vs other used χ^2 or Fisher exact tests for categorical variables and 2-sided 2-sample *t* tests or Wilcoxon rank-sum tests for continuous variables. Variables significant at $P < .2$ in bivariate analyses for histologic A-HSIL were included in multivariate analyses. Models using a penalized log-likelihood were created using backward selection ($P < .1$) of variables where the event of interest was histologic A-HSIL.

RESULTS

Demographic and Clinical Characteristics

One hundred ninety-seven (197) women within Atlanta WIHS were screened, 139 were enrolled, and 75 completed the study and were included in the analyses (Figure 1). Fifty-two (69%)

were HIV⁺ and 23 (31%) were ARHIVN. Mean age of women was 48.7 ± 8.5 years. Women were predominantly black (86.5%), had high school education or less (70.3%), and had annual incomes ≤\$12 000 (57.5%). Among HIV⁺ women, 50 (96%) had CD4 count >200 cells/μL and 40 (77%) had HIV RNA <20 copies/mL within the last 6 months. HIV⁺ women were more likely to have health insurance (including Ryan White funding) than ARHIVN (96.2% vs 59.1%, *P* = .0002) but were similar in other sociodemographic characteristics (Table 1).

Anal Cytology and Histology

Forty-nine women (65.3%) had abnormal anal cytology: 34 (45.3%) ASCUS, 11 (14.7%) LSIL, and 4 (5.3%) ASC-H, and this distribution was similar across HIV status. Thirty-seven (50.7%) women had normal HRA findings: 20 (27.4%) had a negative HRA exam without biopsies and 17 (23.3%) had normal biopsy results. Of the 36 (49.3%) women with abnormal anal histology, 23 (31.5%) had LSIL/condyloma and 13 (17.8%) had HSIL (Table 2). Anal histology findings did not differ between HIV⁺ and ARHIVN women.

Cervical Cytology and Histology From WIHS Core Visit

Most women had normal cervical cytology within 12 months of the study visit, and this was similar by HIV status: 80.8% vs 95.7% of HIV⁺ and ARHIVN women, respectively. Only 23 women (30.7%) underwent colposcopy within 12 months of the study visit: 13 (56.5%) had normal biopsy results, 9 (39.1%) had LSIL, and 1 (4.3%) had HSIL. HIV⁺ women were more likely

to undergo colposcopy and have abnormal biopsy results than ARHIVN women: 19 (82.6%) vs 4 (17.4%) and 10 of 10 (100%) vs 0, respectively (*P* = .05; Table 2).

Cervical and Anal HPV

Forty-nine (65.3%) women had detectable cervical HPV; 20 (26.7%) had hrHPV with 3 (4.0%) having HPV-16/18, and this was similar by HIV status. Overall, 57 (77.0%) of women had detectable anal HPV; 36 (48.7%) had hrHPV with 13 (17.6%) having HPV-16/18 detected. HIV⁺ women were more likely to have anal HPV-16/18 detected than ARHIVN women (23.5 vs 4.4%, *P* = .05; Table 2).

Cervical and Anal Methylation of *FAM19A4* and *miR124-2*

One hundred forty-eight of 150 samples (98.7%) had sufficient DNA and were evaluable for cervical and anal methylation. Of those, 98% (145/148) met quality criteria, defined as Ct value ≤30.0 for control gene *ACTB*, in concordance with published literature [20]. At least 1 gene promoter region (*FAM19A4* or *miR124-2*) was considered methylation positive in cervical samples from 19 (26.4%) women. This proportion did not differ by HIV status. In contrast, nearly all (69 [94.5%]) anal samples were methylation positive for at least 1 target (Table 2).

Predictors of Histologic Anal HSIL

Compared to women without histologic A-HSIL (*n* = 60), those with A-HSIL (*n* = 13) were more likely to drink >7

Table 1. Demographic and Clinical Characteristics

Characteristic	Overall (N = 75) ^a	HIV-infected (n = 52)	HIV-negative (n = 23)	<i>P</i> Value ^b
Age, y, mean ± SD	48.7 ± 8.5	49.1 ± 8.1	47.7 ± 9.6	.06
Race/ethnicity (n = 74)				.47
Black	64 (86.5)	46 (88.5)	18 (81.8)	
White	4 (5.4)	3 (5.8)	1 (4.6)	
Hispanic	3 (4.0)	2 (3.9)	1 (4.6)	
Other	3 (4.0)	1 (1.9)	2 (9.1)	
High school education or less (n = 74)	52 (70.3)	37 (71.2)	15 (68.2)	.79
Income ≤\$12 000/y (n = 73)	42 (57.5)	31 (60.8)	11 (50.0)	.39
Unemployed (n = 74)	54 (73.0)	41 (78.9)	13 (59.1)	.08
Has health insurance (n = 74)	63 (85.1)	50 (96.2)	13 (59.1)	.0002
Alcohol consumption (n = 74)				.58
Abstainer	30 (40.5)	22 (42.3)	8 (36.4)	
0–7 drinks/week	35 (47.3)	25 (48.1)	10 (45.5)	
>7 drinks/week	9 (12.2)	5 (9.6)	4 (18.2)	
Current smoker (n = 74)	43 (58.1)	30 (57.7)	13 (59.1)	.91
History of anal sex (ever)	16 (21.3)	11 (21.2)	5 (21.7)	1.00
Male partners in last 6 mo, median, IQR	1 (0–1)	1 (1–2)	1 (0–1)	.10
CD4 count >200 cells/μL	NA	50 (96.2)	NA	NA
HIV RNA <20 copies/mL	NA	40 (76.9)	NA	NA

Data are presented as No. (%) unless otherwise indicated. Values in bold indicate statistical significance at *P* < .05.

Abbreviations: HIV, human immunodeficiency virus; IQR, interquartile range; NA, not applicable; SD, standard deviation.

^aData available for 75 women unless otherwise noted.

^bStatistical comparison conducted using χ^2 or Fisher exact tests for categorical variables and 2-sided 2-sample *t* tests or Wilcoxon rank-sum tests for continuous variables.

Table 2. Anal and Cervical Characteristics by Human Immunodeficiency Virus Status^a

Characteristic	Overall (N = 75 ^a)	HIV-infected (n = 52)	HIV-negative (n = 23)	PValue ^b
Anal cytology				.61
NILM	26 (34.7)	19 (36.5)	7 (30.4)	
ASCUS	34 (45.3)	21 (40.4)	13 (56.5)	
LSIL	11 (14.7)	9 (17.3)	2 (8.7)	
ASC-H	4 (5.3)	3 (5.8)	1 (4.4)	
Anal histology (n = 73)				.85
No biopsies	20 (27.4)	15 (30.0)	5 (21.7)	
Benign	17 (23.3)	11 (22.0)	6 (26.1)	
LSIL/condyloma	23 (31.5)	16 (32.0)	7 (30.4)	
HSIL	13 (17.8)	8 (16.0)	5 (21.7)	
Anal HPV ^c (n = 74)				
Any	57 (77.0)	42 (82.4)	15 (65.2)	.10
hrHPV	36 (48.7)	28 (54.9)	8 (34.8)	.11
HPV-16/18	13 (17.6)	12 (23.5)	1 (4.4)	.05
Anal methylation positive (n = 73)				
Any (<i>FAM19A4</i> or <i>miR124-2</i>)	69 (94.5)	49 (98.0)	20 (87.0)	.09
<i>FAM19A4</i> only	69 (94.5)	36 (72.0)	16 (69.6)	.09
<i>miR124-2</i> only	52 (71.2)	49 (98.0)	20 (87.0)	1.00
Cervical cytology				.47
NILM	64 (85.3)	42 (80.8)	22 (95.7)	
ASCUS	7 (9.3)	6 (11.5)	1 (4.4)	
LSIL	2 (2.7)	2 (3.9)	0 (0)	
ASC-H	2 (2.7)	2 (3.9)	0 (0)	
HSIL	0 (0)	0 (0)	0 (0)	
Cervical histology				.05
No colposcopy performed	49 (65.3)	31 (59.6)	18 (78.3)	
Normal	13 (17.3)	9 (17.3)	4 (17.4)	
LSIL	9 (12.5)	9 (17.3)	0	
HSIL	1 (1.4)	1 (2.0)	0	
Missed ^d	3 (4.0)	2 (3.9)	1 (4.4)	
Cervical HPV ^c				
Any	49 (65.3)	36 (69.2)	13 (56.5)	.30
hrHPV	20 (26.7)	16 (30.8)	4 (17.4)	.23
HPV-16/18	3 (4.0)	3 (5.8)	0 (0)	.55
Cervical methylation positive (n = 72)				
Any (<i>FAM19A4</i> or <i>miR124-2</i>)	19 (26.4)	13 (25.5)	6 (28.6)	.78
<i>FAM19A4</i> only	19 (26.4)	13 (25.5)	6 (28.6)	.78
<i>miR124-2</i> only	5 (6.9)	4 (7.8)	1 (4.8)	1.00

Data are presented as No. (%) unless otherwise indicated. Values in bold indicate statistical significance at $P < .05$.

Abbreviations: ASC-H, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of undetermined significance; HIV, human immunodeficiency virus; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

^aData available for 75 women unless otherwise noted.

^bStatistical comparison conducted using χ^2 or Fisher exact test.

^cColumn does not equal 100% due to hrHPV and HPV-16/18 being subcategories of any HPV.

^dColposcopy indicated due to abnormal cervical cytology but missed appointment.

alcoholic beverages per week (30.8% vs 8.5%, $P = .01$) and to have detectable anal hrHPV (84.6% vs 42.4%, $P = .01$), in particular HPV-16/18 (38.5% vs 13.6%, $P = .05$), [Table 3](#). Twelve women with A-HSIL (92.3%) had ≥ 1 anal HPV type detected, and 11 (84.6%) had ≥ 1 anal hrHPV type. Four women with A-HSIL (30.8%) had HPV-16, 8 (61.5%) had HPV types 45, 51, 52, or 68, and 2 (15.4%) had HPV-18. Eleven women (84.6%) with A-HSIL had cervical HPV detected, and 6 (46.2%) had ≥ 1 hrHPV type. Six women with A-HSIL (46.2%) had ≥ 1

concordant HPV type in the anus and cervix, and 3 (50%) had concordant hrHPV types. Eleven women (84.6%) with A-HSIL had ≥ 1 type included in the 9-valent HPV vaccine (6, 11, 16, 18, 31, 33, 45, 52, 58).

Women with A-HSIL were more likely to have abnormal cervical cytology (38.5% vs 10.0%, $P = .007$), abnormal cervical histology (30.8% vs 10.0%, $P = .05$), and positive cervical methylation (61.5% vs 19.3%, $P = .004$) ([Table 3](#)). In contrast, positive anal methylation for *FAM19A4* or *miR124-2* did not

Table 3. Univariable Results: Histologic Anal High-grade Squamous Intraepithelial Lesion Versus Other^a

Characteristic	Anal HSIL (n = 13)	Other (n = 60)	PValue ^b
HIV infected	8 (61.5)	42 (70.0)	.53
Age, y, mean ± SD	48.5 ± 9.2	48.6 ± 8.4	1.0
Race/ethnicity ^c			.41
Black	11 (84.6)	52 (88.1)	
White	1 (7.7)	3 (5.1)	
Hispanic	0 (0)	3 (5.1)	
Other	1 (7.7)	1 (1.7)	
Alcohol consumption ^d			.01
Abstainer	1 (7.7)	28 (47.5)	
0–7 drinks/week	8 (61.5)	26 (44.1)	
>7 drinks/week	4 (30.8)	5 (8.5)	
Current smoker ^c	10 (76.9)	31 (52.4)	.13
History of anal sex (ever)	3 (23.1)	12 (20.0)	.72
Male partners in last 6 mo, median, IQR	1 (1–2)	1 (0–1)	.10
Anal cytology			.10
Normal	2 (15.4)	23 (38.3)	
ASCUS/LSIL	9 (69.2)	35 (58.3)	
ASC-H	2 (15.4)	2 (3.3)	
Anal HPV ^c			
Any HPV	12 (92.3)	44 (74.6)	.27
hrHPV	11 (84.6)	25 (42.4)	.01
HPV-16/18	5 (38.5)	8 (13.6)	.05
Anal methylation positive ^c (<i>FAM19A4</i> or <i>miR124-2</i>)	11 (84.6)	57 (96.6)	.15
Cervical cytology			.007
Normal	8 (61.5)	54 (90.0)	
ASCUS	3 (23.1)	4 (6.7)	
LSIL	2 (15.4)	0 (0)	
ASC-H/HSIL	0 (0)	2 (3.3)	
Cervical colposcopy			.05
No colposcopy	5 (38.5)	43 (71.7)	
Normal	4 (30.8)	8 (13.3)	
LSIL/condyloma	4 (30.8)	5 (8.3)	
HSIL	0 (0)	1 (1.7)	
Missed ^d	0 (0)	3 (5.0)	
Cervical HPV			
Any HPV	11 (84.6)	36 (60.0)	.12
hrHPV	6 (46.2)	14 (23.3)	.17
HPV-16/18	2 (15.4)	1 (1.7)	.08
Cervical methylation positive ^e (<i>FAM19A4</i> or <i>miR124-2</i>)	8 (61.5)	11 (19.3)	.004

Data are presented as No. (%) unless otherwise indicated. Values in bold indicate statistical significance at $P < .05$.

Abbreviations: ASC-H, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of undetermined significance; HIV, human immunodeficiency virus; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; IQR, interquartile range; LSIL, low-grade squamous intraepithelial lesion; SD, standard deviation.

^a“Other” indicates anoscopy result other than HSIL, including no biopsies, benign, condyloma, or LSIL.

^bStatistical comparison conducted using χ^2 or Fisher exact tests for categorical variables and 2-sided 2-sample t tests or Wilcoxon rank-sum tests for continuous variables.

^cData available for 59 women in “Other” group.

^dColposcopy indicated due to abnormal cervical cytology but missed appointment.

^eData available for 57 women in “Other” group.

differ significantly between women with and without A-HSIL: 84.6% vs 96.6%, respectively. In multivariate analyses, the odds of having A-HSIL were >6 times higher in women with anal hrHPV (adjusted odds ratio [aOR], 6.08 [95% CI, 1.27–29.18], $P = .02$) and with positive cervical methylation (aOR, 6.49 [95% CI, 1.66–25.35], $P = .007$) (Table 4).

DISCUSSION

This pilot study evaluated predictive markers for anal precancer (A-HSIL) in a southern US cohort of HIV⁺ and ARHIVN women. To our knowledge, this is the first study to assess association of both cervical and anal DNA methylation of *FAM19A4* and *miR124-2* gene promoters with A-HSIL.

Table 4. Logistic Regression Models for Anal High-grade Squamous Intraepithelial Lesion Histology

Variable	Estimate	SE	PValue	OR (95% CI)
Anal hrHPV (ref: No)	1.80	0.80	.024	6.08 (1.27–29.18)
Cervical methylation positive (<i>FAM19A4</i> or <i>miR124-2</i>) (ref: No)	1.87	0.69	.007	6.49 (1.66–25.35)

Abbreviations: CI, confidence interval; hrHPV, high-risk human papillomavirus; OR, odds ratio; ref, reference; SE, standard error.

^aModel of best fit created using a penalized log-likelihood with backward selection ($P < .1$) of variables where the event of interest was histologic anal high-grade squamous intraepithelial lesion.

Almost two-thirds of participants had abnormal anal cytology and 18% had histologic A-HSIL, which did not differ by HIV status. Prior published data of WIHS participants from cities outside the southern United States (Chicago, San Francisco, New York) showed lower rates, with only 31% of HIV⁺ and 8% of ARHIVN women having abnormal anal cytology and histology [5]. While our HIV⁺ participants had similar high rates of detectable anal and cervical hrHPV (>80% and >50%, respectively) as previously published WIHS data, we saw much higher anal and cervical hrHPV rates in our ARHIVN population: 34.8% vs 17% and 17.4% vs 8%, respectively [5]. Reasons for discrepant findings between studies may include different cohort characteristics (older age and lower socioeconomic status in our cohort) as well as the fact that the southern United States is disproportionately affected with the highest rates of HIV-associated morbidity and mortality in the nation [36].

Factors associated with A-HSIL in bivariate analyses included >7 alcoholic beverages weekly, abnormal cervical cytology and histology within 12 months, detectable anal hrHPV (particularly HPV-16/18), and positive cervical DNA methylation of either *FAM19A4* or *miR124-2* gene promoter regions. Both anal hrHPV and positive cervical methylation markers remained statistically significant predictors in multivariable logistic regression analyses with 6-fold increased odds of A-HSIL.

Frequent alcohol consumption has been associated with persistent cervical hrHPV in Korean women [37] and increased prevalence of genital hrHPV in men, likely due to impaired host defenses [38]. Previous studies show that women with history of high-grade cervical neoplasia and cervical cancer [39] have higher anal cancer risk. The association between positive cervical methylation markers for *FAM19A4* or *miR124-2* and A-HSIL is new and likely reflects concurrent cervical disease and/or the field effect of HPV infection and oncogenesis. Only 1 (1.4%) woman had cervical HSIL, whereas 1 or more cervical methylation markers were positive in 19 (26.4%) of participants. Several factors may contribute to this discrepancy. Colposcopy findings from the prior 12 months may not accurately reflect current disease. In addition, the positive threshold established by the manufacturer may need recalibration in this older US population.

Consistent with prior data, detectable anal hrHPV was significantly associated with A-HSIL [5]. While the utility of using anal hrHPV detection as an anal cancer screening strategy in men is unclear given high anal hrHPV prevalence rates (particularly in

men who have sex with men), anal hrHPV testing may be a viable option for screening high-risk women. The fact that >80% of women with A-HSIL had ≥ 1 vaccine-preventable HPV type in the anus reaffirms the need for primary prevention, including increasing HPV vaccine availability to vulnerable populations. Most women in our study were older than the routine age established for catch-up vaccination, but vaccination may be worth considering to reduce risk in this population.

The high prevalence of positive methylation of *FAM19A4* and/or *miR124-2* in anal DNA resulted in a lack of correlation with A-HSIL. Anal samples may include more keratinized cells than cervical samples, and cellular differentiation is known to impact methylation. Methylation of other host genes may be more reflective of anal oncogenesis.

Our study had several limitations. First, our sample size was small and included relatively few cases of A-HSIL, limiting our ability to conduct extensive multivariable analyses. In addition, the cross-sectional design prevented us from following anal disease over time, as almost one-fourth of A-HSIL may regress spontaneously in men [40]. Data from this pilot study will support a larger longitudinal study to identify women at highest risk for developing clinically significant A-HSIL. Furthermore, to minimize participant risk, cervical cytology and histology data were retrospectively collected from WIHS core visits, which may not accurately reflect current cervical pathology. Nonetheless, by limiting analysis of cervical cytology and histology data to within 12 months of the study visit and assessing cervical DNA methylation markers, these data represent a robust assessment of cervical disease.

In conclusion, detection of anal hrHPV and/or positive promoter methylation of *FAM19A4*/*miR124-2* genes in cervical samples appear to be promising noninvasive screening strategies for A-HSIL in both HIV⁺ and ARHIVN women. Greater understanding of gene silencing by promoter methylation in anal carcinogenesis is needed.

Notes

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