Methylation markers for anal cancer screening: a repeated cross-sectional analysis of HIV-positive individuals, 2015-2016

Anjali Vasavada

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Rachel L. Winer

Stephen E. Hawes

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Department of Epidemiology

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Anjali Vasavada

University of Washington

Abstract

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Anjali Vasavada

Chair of the Supervisory Committee:

Rachel L. Winer

Department of Epidemiology

Background: HIV-positive individuals remain at risk for anal cancer and will likely benefit from research that prioritizes early detection of anal cancer and optimized screening practices. We assessed host deoxyribonucleic acid (DNA) methylation markers for detecting high-grade anal intraepithelial neoplasia (AIN) versus low-grade AIN or normal samples in HIV-positive individuals in Seattle, Washington.

Methods: HIV-positive individuals ≥18 years of age and with assigned male sex at birth were recruited at anal cancer screening, diagnosis, or treatment visits at the Harborview Medical Center Madison Clinic in Seattle, Washington between 2015 and 2016. Anal brush samples were collected by an anoscopist for HPV genotyping and pyrosequencing methylation analysis. Demographic and clinical data, as well as anal cancer screening, diagnosis, and treatment results, were collected through chart abstraction and review of electronic medical record (EMR) data. The exposures were the mean percent of DNA methylation for genes ASCL1, PAX1, FMN2 and ATP10A. The primary outcome was AIN grade, dichotomized as normal/low grade-AIN and high-grade AIN, determined by clinically available histology and cytology results from

the same visit. Generalized estimating equation (GEE) logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CI) to assess the associations between methylation and high-grade disease, adjusted for age, HIV viral load and CD4 count. The performance of a marker panel was assessed by measuring sensitivity and specificity of a model at the Youden's index threshold using genes that were individually associated with high-grade AIN detection. A sensitivity analysis measuring high-grade disease extent was conducted using ordinal GEE logistic regression.

Results: 125 samples were analyzed from 85 participants. The mean age of participants was 50.3 (SD 10.2) years, their mean CD4 count was 568 (SD 348) cells/µl and 94.2% had an undetectable HIV viral load. Methylation of ASCL1 (aOR per 1 unit increase in mean percent methylation 1.07, 95% CI 1.01, 1.13) and FMN2 (aOR per 1 unit increase in mean percent methylation 1.12, 95% CI 1.06, 1.19) were significantly associated with high-grade AIN versus low-grade AIN/normal. Mean percent methylation of PAX1 and ATP10A did not show significant associations with high-grade disease. A marker panel combining ASCL1 and FMN2 had a sensitivity of 90.4% for high-grade AIN and a specificity of 45.6%. Methylation of ASCL1 (OR 1.06, 95% CI 1.01, 1.11) and FMN2 (OR 1.07, 95% CI 1.02, 1.10) were each positively associated with increasing extent of high-grade disease, indicating a dose-response effect.

Discussion: Increasing levels of DNA methylation of ASCL1 and FMN2 were positively associated with high-grade AIN detection. With further validation studies, self-sampling host gene methylation testing has promise to be an effective screening and triage tool for anal cancer.

INTRODUCTION

Although anal cancer is rare in the United States (9,449 new cases; 0.5% of all new cancer cases in 2022[1]), incidence has been steadily rising over the past two decades. Incidence rates in high-risk populations, such as people living with HIV, men who have sex with men (MSM), and individuals diagnosed with HPV-related gynecological precancerous lesions, are four to fifty times higher than in the general population[1], with anal squamous cell carcinoma (ASCC) being one of the most common cancers in people living with HIV[2].

Anal intraepithelial neoplasia (AIN) is a premalignant lesion for ASCC, with roughly 10% of high-grade AIN (HGAIN) progressing to anal cancer. AIN is more commonly found in high-risk patients (e.g. HIV positive individuals, men who have sex with men), and development is driven by HPV infection, primarily HPV 16[3]. High-grade AIN lesions are highly prevalent (30-35%) in HIV infected MSM[4]. HPV vaccination is expected to be the long-term solution to ASCC, but the effects and impact of mass HPV vaccination will likely not be evident for decades[5]. In the interim, HIV-positive individuals, both vaccinated and unvaccinated, remain at risk for anal cancer and will likely benefit from research that prioritizes early detection of anal cancer and optimized screening practices.

However, research remains sparse on anal cancer screening and diagnosis and treatment of pre-cancerous lesions to inform clinical practice guidelines. Current screening strategies, including anal cytology and HPV testing, have suboptimal performance characteristics[7,8] High-resolution anoscopy-guided biopsies are burdensome, expensive, and complicated, and a scarcity of trained providers presents a barrier to diagnosis[9]. Until recently, there was uncertainty within the scientific and clinical community that screening to detect and treat AIN is effective for preventing anal cancer[10]. Preliminary results from the Anal Cancer/HSIL Outcomes Research (ANCHOR) study, released in 2022, indicate that the treatment of high-grade anal dysplasia (HSIL) significantly reduces the incidence of ASCC compared to close monitoring alone[11]. Screening could be optimized through the use of additional biomarkers that could be incorporated into risk stratification tools for clinicians to identify individuals who may or may not benefit from treatment, increased surveillance or diagnostic follow up. For example, methylation has potential as a biomarker to distinguish between lesion grades or identify high-grade lesions that should be treated.

DNA methylation is a type of epigenetic change characterized by the addition of a methyl group to a cytosine located 5' of a guanine in gene promoters. When promoter regions of tumor suppressor genes are hypermethylated, this generally leads to their inactivation[12]. Progression of HPV-induced precancerous lesions such as high-grade AIN to invasive carcinoma is driven by an accumulation of genetic and environmental aberrations, resulting in the activation of oncogenes[13]. Host cell DNA methylation is a well-studied, important epigenetic hallmark in HPV-induced cervical carcinogenesis[14]. Though there is limited research on anal carcinogenesis, DNA methylation has potential as a useful biomarker for anal cancer screening[15]. Recent research has identified that DNA hypermethylation of several host cell genes, including ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582, CADM1 and MAL, is associated with anal carcinogenesis in HIV-positive MSM, and that high methylation levels are associated with progression toward cancer[16-18].

Prior research involving genome-wide host methylation profiling of anal and cervical carcinoma identified 17 common overlapping hypermethylated genes that are associated with

anal cancer progression, including ASCL1, ATP10A, PAX1, and FMN2[19]. This study aimed to explore the differences in host gene methylation (ASCL1, PAX1, FMN2 and ATP10A) between high-grade AIN versus low-grade AIN or normal samples from HIV-positive individuals in Seattle, Washington.

RESEARCH DESIGN AND METHODS

Study Design, Setting & Population

The study included HIV-positive individuals undergoing anal cancer screening, diagnosis, or treatment at the Harborview Medical Center Madison Clinic in Seattle, Washington between 2015 and 2016. Patients seeking care with or without anal symptoms, who were assigned male sex at birth, ≥18 years of age and living with HIV were eligible. Participants were recruited during clinical visits, and all study data were collected during clinical visits. Enrolled participants who were scheduled for additional clinical visits during the study period (for screening, diagnosis, or treatment) could contribute multiple observations for the study (up to four).

Recruitment Strategy & Enrollment

Research coordinators reviewed daily Harborview Medical Center Madison Clinic schedules to identify eligible patients scheduled for anal cancer screening, diagnosis, or treatment. At the clinic visit, potentially eligible patients received an explanation of the study from the research coordinator and completed screening questions to confirm eligibility. If a patient agreed to participate, the research coordinator administered written informed consent and assigned the patient a study ID number to which their samples were linked. Enrolled participants were given $10 per study visit.

Study Procedures

Data Collection

Anal samples were collected from study participants with an Anex® Brush (Rovers Medical Devices) into ThinPrep® vials by an anoscopist. Samples were used for HPV genotyping and methylation analysis. Additional data were collected through chart abstraction and review of electronic medical records (EMRs), including age, race, ethnicity, HIV viral load, CD4 count, previous anal disease, previous non-study screening, follow-up, and treatment. Race and ethnicity data were collected for required National Institutes of Health (NIH) reporting and to describe patient population characteristics. Outcome data were collected through EMR review of histology and cytology results collected as part of clinical care, when available. At the end of study, an anoscopist performed medical record quality control review of disease extent and impression for all high resolution anoscopy (HRA) and infra-red coagulation (IRC) visits up to and including last study visit date.

Processing of Specimens

Anal samples were processed at the HPV Research Group laboratory located at the Harborview Research and Training Building in Seattle, Washington. DNA was isolated from anal brush samples in ThinPrep® vials using QIAmp Blood DNA mini kit. An aliquot of extracted DNA was HPV-genotyped by PCR using a liquid bead microarray assay described previously[22]. For methylation analyses, residual DNA samples were quantified by Applied Biosystems 7900HT Fast Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA), and 50 µL was aliquoted into microcentrifuge tubes and shipped on dry ice to EpigenDx for methylation

analysis. DNA methylation analysis was done by pyrosequencing per EpigenDx protocols. For DNA methylation analysis of genes ASCL1 (2 different regions, 7 and 9 CpGs), PAX1 (8 CpGs), FMN2 (6 CpGs) and ATP10A (12 CpGs), 200-500ng of extracted genomic DNA was bisulfate treated using an EZ DNA Methylation Kit. PCR tests were performed using 1-4 µL of bisulfite treated DNA. PCR product was bound to Streptavidin Sepharose HP, after which the PCR products were purified and washed, and re-washed with the Pyrosequencing Vacuum Prep Tool. Primer was annealed to the purified PCR products, and then sequenced by Pyrosequencing on the PSQ96 HS System. The methylation status of each CpG site was calculated as the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites within the targeted region of each gene.

Measurement of Exposures and Outcomes

The exposures were defined as the mean percent of DNA methylation for genes ASCL1, PAX1, FMN2 and ATP10A. The primary outcome of interest was AIN grade, dichotomized as normal/low grade-AIN and high-grade AIN, determined by available histology and cytology results from clinical samples collected at the same visit. Histology data was categorized as high-grade AIN when results were HSIL or AIN2-3, and normal/low grade otherwise. Cytology data was categorized as high-grade AIN when results were HSIL, and normal/low grade otherwise. If histology results were not available in the EMR, then cytology results were used to determine outcome status, and vice versa. If both cytology and histology results were available, the result that was more severe was used to define the outcome. If neither cytology nor histology was performed, or if all available cytology and histology results were unsatisfactory, the study visit was excluded from analysis.

The secondary outcome of interest for a sensitivity analysis was disease extent in high-grade samples using HRA impression data abstracted by an anoscopist. The percent of HSIL disease was calculated by using the maximum of the intra-anal and perianal lesions, with four outcome groups categorized as cytology/histology <high-grade (e.g., low-grade/normal), <25% high-grade, 25%-75% high-grade, and >75%-100% high-grade.

Statistical Analysis

Multiple study visits per subject, as available in the data, were used to assess differences in methylation between high-grade AIN versus low-grade AIN or normal samples. First, to visualize methylation levels for genes ASCL1, PAX1, FMN2 and ATP10A, boxplots were generated of mean percent methylation to assess comparisons between patients with high-grade AIN and low-grade AIN/normal samples. Then, mean percent methylation of ASCL1, PAX1, FMN2 and ATP10A were each evaluated as continuous variables in separate models, using logistic GEE regression with an exchangeable correlation structure to account for clustering within study participants due to repeated study visits. Confounders considered a priori included CD4 count (continuous; most recently available result), HIV viral load (detectable, undetectable, unknown: most recently available result), and age (continuous; measured at each visit).

Crude and adjusted estimates and standard errors were exponentiated to obtain odds ratios and 95% confidence intervals. Multivariable GEE logistic regression was used to explore a composite gene panel of the candidate genes studied to measure if methylation of a combination of genes was associated with high-grade AIN. The composite gene panel included only combinations of genes that were individually associated with the outcome after adjustment

for covariates, and panel models were constructed in a stepwise manner with the marker most significantly associated with the outcome first. Sensitivity and specificity of the marker panel was assessed at the Youden's index threshold (threshold that maximizes the sum of sensitivity and specificity)[20]. To further evaluate the predictive performance of the marker panel, leave one out cross validation (LOOCV) was performed[21]. Performance of each model was visualized using receiver operation characteristic (ROC) curves, and the area under the curve was assessed to evaluate the sensitivity and specificity of the marker(s).

A sensitivity analysis was performed to explore a dose-response effect with increasing high-grade disease extent. To assess the relationship between methylation of each marker and high-grade disease extent, an ordinal GEE regression was conducted with the outcome variable categories ordered as <HSIL, <25% disease, 25-75% disease, and >75%-100% disease.

Statistical analyses were performed on R Statistical Software version 4.0.2 with packages geepack, multgee and pROC. Reported p-values are 2-sided, with 0.05 as the significance threshold.

RESULTS

Study Sample and Demographic Characteristics

85 participants were included in the sample, contributing a total of 133 study visits. More than half of the participants (59.5%) contributed only one study visit. Most participants (83.5%) were non-Hispanic; 1.2% were American Indian/Alaska Native, 4.7% Asian, 11.8% Black, 70.6% White and 11.8% Unknown (Table 1). At first visit, the mean age of the participants was 50.3 years (standard deviation (SD) 10.2 years), the mean CD4 count was 568 (SD 348) cells/µl and 94.2% of participants had an undetectable HIV viral load at baseline. Sixty-five participants (76.5%) had high-risk HPV infection (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, or 68) at baseline. Approximately one-third of participants (34.1%) had an HPV-16 infection, and 11.8% of participants had an HPV-18 infection. Fifty-seven participants (67.1%) had non-HPV16/18 high-risk HPV detected.

Human Papillomavirus Genotyping of Cytology and Histology Samples

Of the total 133 study samples collected, 8 were not included in the analysis because they did not have a corresponding satisfactory sample collected for cytology or histology or were unsatisfactory for methylation evaluation. Of the 125 included in the analysis, 49 were from individuals with concurrent high-grade AIN and 76 from individuals with low-grade AIN/normal (Table 2). Of those with high-grade AIN, 30 were <25%, 11 were 25-75% and 4 were >75%-100% high-grade disease. Most (94%; 46/49) high-grade AIN and 75% (57/76) of low-grade AIN/normal samples were positive for any high-risk HPV (HR-HPV) (Table 3). HR-HPV types 16 and 18 were found in 76% (35/49) of samples categorized as high-grade AIN, and 33% (25/76) of samples categorized as low-grade AIN/normal. Non-16 or 18 HR-HPV was found in 84% (41/49) of high-grade AIN samples, and 68% (52/76) of low-grade AIN/normal samples.

Performance of Individual Methylation Markers

Methylation analysis could not be run for all four genes for some samples, likely attributed to cellular DNA fragmentation in the processing stage. The total number of observations per gene was 112 for ATP10A, 122 for ASCL1, 109 for PAX1, and 120 for FMN2.

In visualizing the distribution of mean methylation in boxplots and conducting univariate unadjusted analyses, mean methylation levels were greater in high-grade versus low grade/normal groups for genes PAX1, ASCL1 and FMN2. For ATP10A, the methylation level was similar between high grade and low grade/normal samples (Figure 1, Table 4). In the multivariate analysis, adjusted for age, CD4 count and HIV viral load, increasing methylation of ASCL1 (OR per 1 unit increase in mean percent methylation 1.07, 95% CI: 1.01, 1.13) and FMN2 (OR per 1 unit increase in mean percent methylation 1.12, 95% CI: 1.06, 1.19) were both associated with the detection of a high-grade cytology/histology versus low-grade/normal result (Table 4). Neither ATP10A nor PAX1 was significantly associated with high-grade disease. Areas under the curve (AUCs) for adjusted models ranged from 0.583 to 0.731, with the highest AUC achieved by FMN2 (AUC = 0.731) followed by ASCL1 (AUC = 0.664) (Figure 2).

Identification and Performance of a Marker Panel

A marker panel consisting of FMN2 and ASCL1 was created, adjusting for age, CD4 count and HIV viral load. The marker panel consisted of 119 samples with complete data for methylation of ASCL1 and FMN2. The model was constructed in a stepwise manner, with FMN2 included first due to greater significance, and ASCL1 included second. The marker panel model had an AUC of 0.742 (95% CI: 0.639, 0.819), indicating better performance for prediction of high-grade disease than FMN2 or ASCL1 alone, which had an AUC of 0.731 and 0.664, respectively. Using a Youden index threshold (≥0.48), this panel provided a sensitivity of 90.4% and specificity of 45.6%.

Performance of Individual Markers with High Grade Disease Extent

Using a GEE ordinal model to investigate the association between methylation of ATP10A, ASCL1, PAX1 and FMN2 and high-grade disease extent, ATP10A and PAX1 did not show an association between methylation level and disease extent categories (Table 6). Methylation of FMN2 (OR for increase in extent category per 1 unit increase in mean percent methylation 1.07, 95% CI: 1.02, 1.10) and ASCL1 (OR for increase in extent category per 1 unit increase in mean percent methylation 1.06, 95% CI 1.01, 1.11) were each significantly associated with high-grade disease extent, after adjusting for CD4 count, age and HIV viral load. For every one unit increase in mean methylation of FMN2 and ASCL1, the odds of having an increased high-grade disease extent category increased by 7% and 6%, respectively.

DISCUSSION

In this study, we presented findings to enhance the current landscape of research surrounding methylation and anal cancer. Using anal swab samples from HIV-positive individuals, we provided data that showed positive associations between increasing mean methylation of ASCL1 and FMN2 and high-grade AIN detection. Further, the results of our marker panel showed that the combined use of these markers can detect samples with high-grade disease with a sensitivity of 90.4% and specificity of 45.6%. The secondary analysis of a dose-response effect with increasing high-grade disease extent showed an association between the methylation of ASCL1 and FMN2 with increasing high-grade disease extent, affirming results from the primary analysis.

Cervical and anal cancer are both caused by oncogenic human papillomaviruses[23]. Cervical cytology-based screening has led to dramatic decreases of cervical cancer incidence and mortality over the past decades and remains the widely used screening approach for cervical

cancer[23]. The success of cervical cancer screening has led to its use as a template for anal cancer screening in high-risk groups – current screening algorithms are based on anal cytology assessment, followed by high-resolution anoscopy (HRA) biopsy and histology as a diagnostic tool[24]. However, performance of anal cytology as a screening test for anal cancer prevention has been demonstrated to vary based on the population[25]. A meta-analysis evaluating the performance of anal cytology identified a sensitivity and specificity of detection of high-grade AIN at 77.3% and 55.5%, respectively[26]. Similarly, HR-HPV DNA detection has been widely adopted for cervical cancer screening[24,26]. However, the application of HR-HPV DNA detection as a primary screening test for anal cancer in high-risk populations may be limited, due to the high prevalence of anal HR-HPV, which we also observed within our own study (Table 1). Thus, using HR-HPV DNA testing for anal cancer screening would result in high sensitivity but poor specificity[26,27]. Another potential screening tool is dual immunostaining for p16 and Ki-67, which are widely studied biomarkers[28] that have received FDA-approval as a cervical cancer screening triage test[27]. In a meta-analysis, the pooled sensitivity of p16/Ki-67 for detection of anal precancer and cancer was 56.6% with a pooled specificity of 62.3%[26]. As such, there are no optimal screening modalities for anal cancer that have both high sensitivity and specificity, and no universally accepted guidelines for anal cancer screening.

Research exploring host gene methylation in cervical cancer has indicated that methylation markers have high sensitivity to detect high-grade cervical intraepithelial neoplasia (CIN) in patients with HPV infection[29-31]. Methylation markers in these studies, including host genes FAM19A4, CADM1 and MAL, have since been clinically validated to explore the prognostic value of using methylation markers to detect high-grade CIN in individuals with HR-HPV-positive cervical scrapes and self-collected cervico-vaginal specimens[29,32,33]. Given the promising prognostic value of methylation markers in cervical cancer screening, much of the research surrounding anal methylation has been extrapolated from cervical research.

The existing literature on methylation in anal cancer has focused mainly on viral DNA methylation, establishing that heterogenous patterns of the HPV16 upstream regulatory region has a possible role for detection of high-grade disease, but data remain inconclusive to support that viral methylation is significantly associated with high-grade detection[34,35]. Recent cross-sectional studies on host-cell methylation and anal carcinoma in HIV-positive individuals have identified genes, including ASCL1 and FMN2, that are significantly associated with detection of high-grade disease. Results from these studies indicate that methylation markers could be used to identify high risk pre-cancerous lesions in biopsy tissue and swab samples, with analyses showing high sensitivity and specificity for prediction of high-grade disease[6,19]. The results of our study support prior findings that increased methylation of both FMN2 and ASCL1 is cross-sectionally associated with high-grade disease, with sensitivity values that are in line with similar studies. Other studies have identified various other potential biomarkers for detecting high-grade anal lesions in HIV-positive patients, including, but not limited to, CADM1, IGSF4, and DAPK1[18,36,37].

The use of biomarkers such as methylation could help to streamline patients that need additional follow-up after screening. HRA is widely regarded as the best method for anal pre-cancer diagnosis due to its high positive predictive value in detecting high-grade AIN[38]. Though HRA-guided biopsies with histopathological evaluation is the current gold standard for identifying AIN, physicians require a significant amount of time to achieve competency in utilizing HRA[39], and physicians need to complete comprehensive training and certification in

HRA in order to be covered by major U.S. insurance companies[40]. Research has indicated that it can take up to two years for anoscopists to be able to accurately detect high grade anal intraepithelial lesions[41]. Given these barriers, there are a limited number of providers that are well trained in anoscopy and can accurately detect high-grade lesions. Therefore, it would be valuable to triage patients with abnormal cytology via methylation assays or other biomarkers, or a combination of markers, to identify which patients would need and benefit from HRA.

Further, there is potential for methylation assays to be used in a cost-effective manner to prioritize patients for screening. HRA can cost up to $193[42], and many insurers in the United States only cover HRA if an abnormal lesion is detected on cytology in a patient who is part of an at-risk population; referrals to HRA for anal symptoms suspicious of dysplastic progression are not covered by insurance[40]. Testing anal samples for methylation markers could potentially serve as a risk stratification tool to determine the need for additional follow up, or could be an alternative approach to cytology testing, which can cost up to $95[42]. Given the high cost of HRA, it may be valuable to run biomarker tests on self-collected anal swabs to determine if a patient requires further testing or HRA to diagnose pre-cancerous lesions. Research supports that anal self-sampling is acceptable and feasible with patient populations.[43,44]Studies have shown comparable sensitivity between self-collected anal cytology samples and clinician-collected cytology samples for detecting high-grade AIN,[45] and moderate agreement between self-collected and clinician-collected anal samples for detecting HR-HPV[46]. Boers et al. have detailed the success of conducting methylation analyses on self-collected cervico-vaginal samples[47], indicating the potential of self-collected samples for methylation analyses to be a patient-centered option, or an avenue to reduce burden on clinic staff during screening.

To the best of our knowledge, this is the first study to measure the association between methylation and extent of high-grade disease. Adding the disease extent impression from an HRA visit to high-grade cytology/histology results provided valuable information to better understand if there is a trend in percent methylation in increasing disease extent groups; the dose-response analysis affirms the results the primary analysis of an association between methylation and high-grade versus low-grade/normal samples. Our study showed associations with methylation data obtained from anal brush samples which could indicate potential utility in screening, contrasting with several studies that use methylation data from histopathological samples[16,17,19]. Our study is one of two[18] that evaluated anal brushes/swabs for methylation data; obtaining data with high sensitivity from an anal brush sample is encouraging, because if methylation markers like FMN2 and ASCL1 are to be used as a screening tool, they should be sensitive enough to detect high grade disease without collection of tissue samples, which require more specialized equipment and clinical staff.

This study, and study conclusions are limited by the small sample size. Further, because study samples were collected at clinically-indicated screening, diagnosis or treatment visits, we do not have multiple study visit data from all participants in the study, which necessitated a repeated cross-sectional design, rather than a longitudinal design. Future research should prioritize longitudinal data to explore methylation patterns across the longitudinal course of anal carcinogenesis. We do not have confirmatory histology on all participants as we relied on clinically-indicated histology or cytology results, so some participants had to be excluded from analysis. Another limitation is that the number of candidate genes we analyzed was limited by available resources and funding, precluding analysis of other genes that may be worth exploring. An additional limitation is that we lacked samples from individuals with cancer, so we

cannot directly conclude that increased methylation of FMN2 or ASCL1 is associated with progression to cancer. Lastly, we acknowledge that that by focusing on HIV-positive individuals with male sex assigned at birth, we cannot directly extrapolate the data to the general population or to other risk groups, such as HIV-negative individuals, HIV-positive individuals who identify as female, or individuals with a history of HPV-related cancer.

Though the research surrounding methylation in relation to anal cancer detection is increasing, this remains a sparse field of research, and this study provides valuable data to inform the clinical potential of using methylation to optimize anal cancer screening. Further validation studies can evaluate additional genes not explored in this analysis to identify optimal marker panels. Larger, clinic-based randomized control trials can also be conducted to determine the utility of methylation assays as part of anal cancer screening processes, if feasible and ethical to conduct in patient populations. With recent data from the ANCHOR study indicating that the treatment of HSIL in people living with HIV reduces cancer risk[11], it is especially important now to validate the scientific and clinical utility of methylation assays to detect high-grade lesions and identify which high-grade lesions need to be escalated to treatment. The use of methylation analyses has promise to be a cost effective, non-invasive, patient-friendly screening tool to enhance the landscape of anal cancer screening.

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Table 1. Baseline Characteristics of HIV-positive individuals Seeking Anal Cancer Screening, Diagnosis or Treatment in Seattle, Washington, 2015-2016, N=85

Characteristic	N (%)
Mean Age, years (SD)	50.3 (10.2)
Ethnicity	
Hispanic	11 (12.9)
Non-Hispanic	71 (83.5)
Unknown	3 (3.5)
Race	
American Indian/Alaska Native	1 (1.2)
Asian/Pacific Islander	4 (4.7)
Black	10 (11.8)
White	60 (70.6)
Unknown	10 (11.8)
Number of Visits	
1	51 (59.5)
2	21 (25.0)
3	12 (14.3)
4	1 (1.2)
Mean CD4 Count (cells/µl) (SD)	568 (348)
HIV Viral Load[1]	
Undetectable	80 (94.2)
Detectable	3 (3.5)
Unknown	2 (2.3)
HPV Type[2]	
Any high-risk (HR)-HPV[3]	65 (76.5)
Any HR-HPV (excluding HPV16 or HPV18)	57 (67.1)
HPV 16	29 (34.1)
HPV 18	10 (11.8)

[1]Viral load is categorized as undetectable for individuals with <20 copies/mL and detectable otherwise per clinical guidelines[48].

[2]The number of observations do not add up to 85 for this variable because individuals can test positive for multiple HPV types and therefore be categorized into more than one HPV type or type-group.

[3]14 high-risk HPV types were included: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

Abbreviations: High grade squamous intraepithelial lesion (HSIL); Low grade squamous intraepithelial lesion (LSIL); Anal intraepithelial neoplasia, grades 1-3 (AIN1-3); High risk HPV (HRHPV)

Table 2. Distribution of Lesion Grade and High-Grade Disease Extent in clinical samples (n=133)

Characteristic	**N (%)**
Lesion Grade (Combined Cytology/Histology Result)	
Normal	14 (11%)
LSIL/AIN1	62 (46%)
HSIL/AIN2-3	49 (37%)
No Specimen Collected or Unsatisfactory for Evaluation	8 (6%)
High-Grade Disease Extent[1]	
<HSIL	76 (57%)
<25%	30 (23%)
25%-75%	11 (8%)
<75%-100%	4 (3%)
No Specimen Collected or Unsatisfactory for Evaluation	12 (9%)

Abbreviations: High grade squamous intraepithelial lesion (HSIL); Low grade squamous intraepithelial lesion (LSIL); Anal intraepithelial neoplasia, grades 1-3 (AIN1-3)

[1]High-grade disease extent was collected from HRA impression notes; the percent disease extent is the maximum percent HSIL from both the intra-anal and perianal lesions.

Table 3. HPV Results by Highest Grade from Histology/Cytology Results (n=125)

	Any HR-HPV	**HR-HPV (non 16/18)**	**HPV16**	**HPV18**
LSIL/AIN1 & Normal (n=76)	57 (75%)	52 (68%)	17 (22%)	8 (11%)
HSIL/AIN2-3 (n=49)	46 (94%)	41 (84%)	27 (55%)	8 (16%)

Abbreviations: High grade squamous intraepithelial lesion (HSIL); Low grade squamous intraepithelial lesion (LSIL); Anal intraepithelial neoplasia, grades 1-3 (AIN1-3); High risk HPV (HRHPV)

Figure 1. Boxplots Comparing Mean Percent Methylation between High-Grade versus Low-Grade/Normal Anal Cytology/Histology Results Across Candidate Biomarkers ~~N=125~~

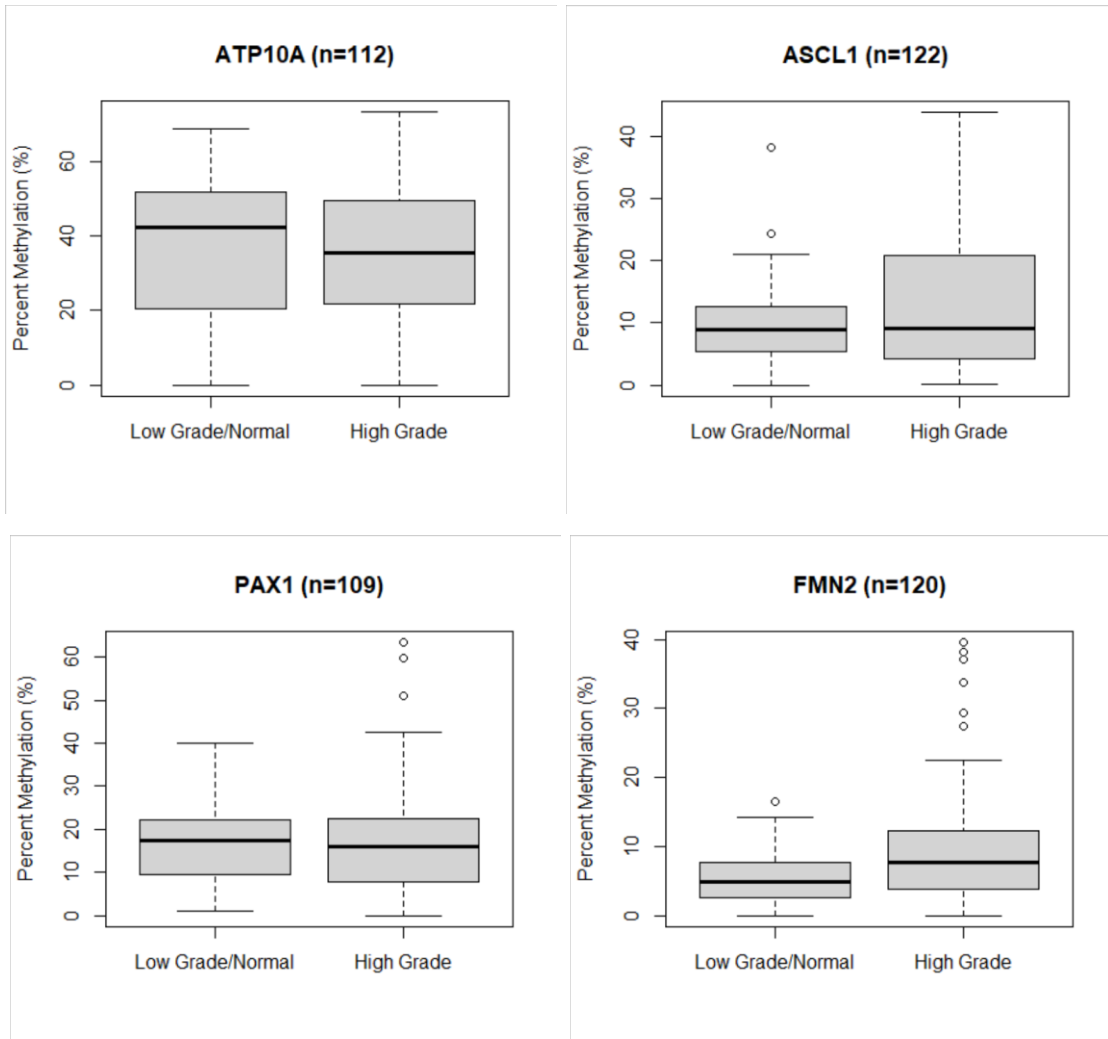


Table 4. Odds Ratios for associations between methylation levels[1] in candidate genes and detection of high-grade versus low-grade/normal anal cytology/histology results in HIV-positive individuals

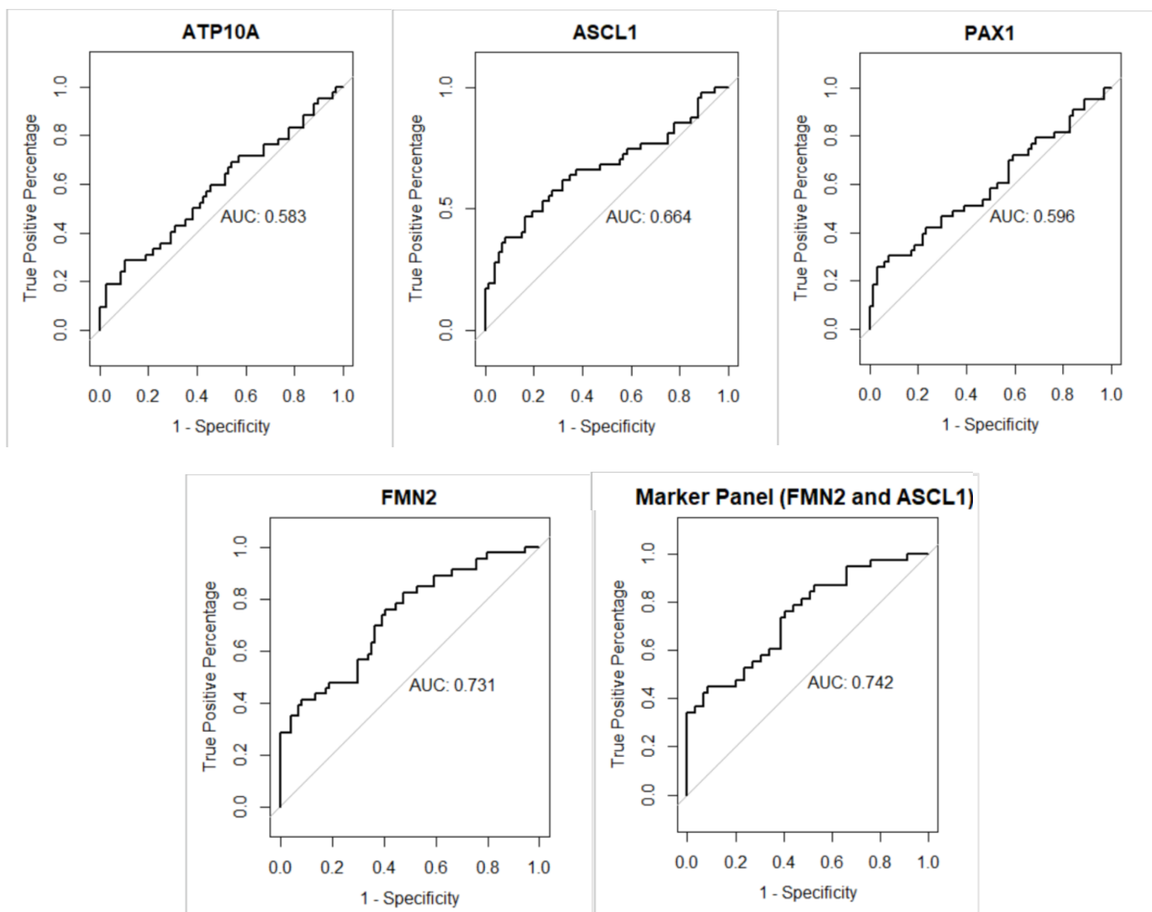
Biomarker	Unadjusted OR per 1-unit increase in mean percent methylation	95% CI	Adjusted OR[2] per 1-unit increase in mean percent methylation	95% CI
ATP10A (n=112)	0.99	(0.97, 1.01)	1.00	(0.98, 1.03)
ASCL1 (n=122)	1.04	(0.99, 1.09)	1.07	(1.01, 1.13)*
PAX1 (n=109)	1.00	(0.97, 1.04)	1.01	(0.98, 1.05)
FMN2 (n=120)	1.11	(1.05, 1.19)**	1.12	(1.06, 1.19)***

**P* < .05, ***P* < .01, ****P* < .001

[1]The methylation level at each CpG is the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites within the targeted region of each gene.

[2]Model adjusted for CD4 count, age, HIV viral load

Figure 2. Receiver Operating Characteristic (ROC) curve for models assessing association between percentage methylation of candidate genes and detection of high-grade versus low-grade/normal anal cytology/histology results in HIV-positive individuals



Abbreviations: Area under the receiver operating curve (AUC)

Table 5. Logistic Regression Analysis on Diagnostic Performance for HSIL/AIN2-3 Detection: Multivariable Regression for Optimal Marker Panel (ASCL1, FMN2) (n=119)

Marker Panel (ASCL1, FMN2)[1]	Non-CV
AUC (95% CI)	0.742 (0.639, 0.825)
Sensitivity, %	90.4%
Specificity, %	45.6%

[51]Model adjusted for CD4 count, age, HIV viral load

Abbreviations: Area under the receiver operating curve (AUC); Non-cross validated (Non-CV)

Table 6. Ordinal Logistic Regression Analysis for associations between methylation levels and high-grade disease extent (<HSIL, <25% HSIL, 25-75% HSIL, >75% HSIL)

Biomarker	Adjusted OR[2] per increase in extent category per 1 unit increase in mean percent methylation	95% CI
ATP10A (n=108)	0.98	(0.95, 1.01)
ASCL1 (n=118)	1.06	(1.01, 1.11)*
PAX1 (n=106)	1.01	(0.96, 1.05)
FMN2 (n=116)	1.07	(1.02, 1.10)**

*$P < .05$, **$P < .01$, ***$P < .001$
[2]Model adjusted for CD4 count, age, HIV viral load