MAJOR ARTICLE

Cancer Risk Stratification of Anal Intraepithelial Neoplasia in Human Immunodeficiency Virus–Positive Men by Validated Methylation Markers Associated With Progression to Cancer

Ramon P. van der Zee,[1,2,3] Olivier Richel,[4] Carel J. M. van Noesel,[5] Iuliana Ciocănea-Teodorescu,[6] Annina P. van Splunter,[1] Timo J. ter Braak,[1] Mayura Nathan,[7] Tamzin Cuming,[7] Michael Sheaff,[8] Alexander Kreuter,[9] Chris J. L. M. Meijer,[1] Wim G. V. Quint,[10] Henry J. C. de Vries,[3,11] Jan M. Prins,[2] and Renske D. M. Steenbergen[1]

[1]Department of Pathology, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam, Cancer Center Amsterdam, Amsterdam, The Netherlands, [2]Department of Internal Medicine, Division of Infectious Diseases, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam Institute for Infection and Immunity (AI&II), Amsterdam, The Netherlands, [3]Department of Dermatology, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, The Netherlands, [4]Department of Internal Medicine, Division of Infectious Diseases, Radboud University Medical Center, Nijmegen, The Netherlands, [5]Department of Pathology, Amsterdam University Medical Centers, University of Amsterdam, Cancer Center Amsterdam, Amsterdam, The Netherlands, [6]Department of Epidemiology and Biostatistics, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam, Amsterdam Public Health Research Institute, Amsterdam, The Netherlands, [7]Homerton Anal Neoplasia Service, Homerton University Hospital, London, United Kingdom, [8]Department of Cellular Pathology, Barts Health National Health Service (NHS) Trust, London, United Kingdom, [9]Department of Dermatology, Venerology, and Allergology, Helios St Elisabeth Hospital Oberhausen, University Witten/Herdecke, Oberhausen, Germany, [10]DDL Diagnostic Laboratory, Rijswijk, The Netherlands, and [11]Sexually Transmitted Infections Outpatient Clinic, Department of Infectious Diseases, Public Health Service of Amsterdam (GGD Amsterdam), Amsterdam, The Netherlands

(See the Editorial Commentary by Clifford and Alberts on pages 2164-6.)

Background. High-grade anal intraepithelial neoplasia (HGAIN; AIN2–3) is highly prevalent in HIV+ men, but only a minority of these lesions progress towards cancer. Currently, cancer progression risk cannot be established; therefore, no consensus exists on whether HGAIN should be treated. This study aimed to validate previously identified host cell DNA methylation markers for detection and cancer risk stratification of HGAIN.

Methods. A large independent cross-sectional series of 345 anal cancer, AIN3, AIN2, AIN1, and normal control biopsies of HIV+ men was tested for DNA methylation of 6 genes using quantitative methylation-specific PCR. We determined accuracy for detection of AIN3 and cancer (AIN3+) by univariable and multivariable logistic regression analysis, followed by leave-one-out cross-validation. Methylation levels were assessed in a series of 10 anal cancer cases with preceding HGAIN at similar anatomic locations, and compared with the cross-sectional series.

Results. Methylation levels of all genes increased with increasing severity of disease ($P < .05$). HGAIN revealed a heterogeneous methylation pattern, with a subset resembling cancer. *ZNF582* showed highest accuracy (AUC = 0.88) for AIN3+ detection, slightly improved by addition of *ASCL1* and *SST* (AUC = 0.89), forming a marker panel. In the longitudinal series, HGAIN preceding cancer displayed high methylation levels similar to cancers.

Conclusions. We validated the accuracy of 5 methylation markers for the detection of anal (pre-) cancer. High methylation levels in HGAIN were associated with progression to cancer. These markers provide a promising tool to identify HGAIN in need of treatment, preventing overtreatment of HGAIN with a low cancer progression risk.

Keywords. anal intraepithelial neoplasia; anal cancer; HIV; human papillomavirus; host cell DNA methylation markers.

Anal cancer is an increasing problem, especially in high-risk groups. Human immunodeficiency virus–positive (HIV+)

men who have sex with men (MSM) have the highest incidence rates and a 78-fold higher risk of developing anal cancer compared with the general population [1, 2]. Anal cancer mainly comprises squamous cell carcinoma (SCC) and is caused by a persistent human papillomavirus (HPV) infection [3]. Similar to cervical carcinogenesis, anal SCC is preceded by precursor lesions: anal intraepithelial neoplasia (AIN; graded 1–3), which can develop perianally and in the anal canal [4]. High-grade AIN (HGAIN; AIN2–3), also called anal high-grade squamous intraepithelial lesions, are highly prevalent (23–35%) in HIV+ MSM [1, 5], for which screening using high-resolution anoscopy (HRA)–guided biopsies is the gold standard [6, 7]. However, only a minority of HGAIN eventually progresses to cancer [1, 8]. Since we are unable to determine the risk of

progression, there are no consensus guidelines on screening or whether HGAIN should be treated. Current clinical practice in some countries of treating all HGAIN leads to considerable overtreatment of lesions that have a low risk of progression towards cancer. If we can better identify HGAIN with a high risk of progression towards cancer, we might be able to improve the effectiveness of anal cancer screening and treatment of HGAIN.

Host cell DNA methylation (ie, addition of methyl groups to cytosines in CpG-sites [hypermethylation]) is an epigenetic hallmark in HPV-induced carcinogenesis that can lead to inactivation of tumor suppressor genes [9]. Previously, we showed that host cell DNA methylation is associated with anal carcinogenesis and we identified several methylation markers for the detection of AIN3 and anal cancer [10]. These markers showed high methylation levels in anal SCC and a heterogeneous methylation pattern in HGAIN, displaying either high (cancer-like) or low methylation levels [10]. In analogy to our findings in the cervix, we hypothesized that precursor lesions with a cancer-like methylation pattern have a higher risk of progression towards cancer [11–13]. Providing further evidence of an association between high methylation levels and a high risk of progression would be an important step towards developing a risk-stratification tool.

The current study aimed to (1) validate the accuracy of previously identified methylation markers in detecting anal cancer and HGAIN in a large, independent cross-sectional series of tissue samples of HIV+ men and (2) to determine their association with progression to cancer in an international, longitudinal series of anal cancer cases with documented preceding HGAIN. In addition to the 5 best-performing markers identified in our previous study (*ASCL1, SST, WDR17, ZIC1*, and *ZNF582*) [10], we also tested *LHX8* on anal samples, a recently discovered and well-performing marker in the cervix [14].

METHODS

Clinical Specimens and Ethics

This study involved a molecular analysis of a cross-sectional and a longitudinal series of formalin-fixed paraffin-embedded anal tissue samples.

The cross-sectional series, used for validation purposes, consisted of 345 samples, all from HIV+ men (Table 1). Anal SCC specimens (n = 30; including 2 verrucous carcinomas),

obtained between 1999 and 2018, were retrieved from the pathology archives of the Amsterdam University Medical Centers (Amsterdam UMC) and 5 other hospitals in the Netherlands (see Acknowledgments). A total of 209 AIN biopsies (AIN1, n = 37; AIN2, n = 98; AIN3, n = 74), obtained between 2008 and 2017 in 205 men, were retrieved from the Amsterdam UMC pathology archive. For 4 patients, 2 biopsies from different lesions and histological AIN grades were included. Normal control samples (n = 106) consisted of biopsies with normal or reactive anal epithelium. These biopsies were taken from nonsuspected anal epithelium in 106 HIV+ men who also had biopsies taken from suspected lesions during screening for anal (pre-) cancer at the Amsterdam UMC between 2016 and 2018 as described before [10].

The longitudinal series consisted of 40 biopsies of 10 patients (8 HIV+ men, 1 HIV-negative [HIV−] woman, 1 HIV− man) who developed (suspected) anal SCC over time. Each case comprised multiple consecutive biopsies taken from the same anatomic location, including 1 or more biopsies of histopathologically confirmed SCC or "highly suspicious for infiltrative growth" (endpoint) and all suitable and available biopsies preceding the endpoint diagnosis. To determine the corresponding anatomic location of the consecutive biopsies, medical records including HRA photo documentation and HPV genotyping results were reviewed. The biopsies, obtained between 2009 and 2019, were retrospectively identified and retrieved from the pathology archives of the Amsterdam UMC, and Onze Lieve Vrouwe Gasthuis, Amsterdam, the Netherlands; Homerton University Hospital, London, United Kingdom; and Helios St Elisabeth Hospital, Oberhausen, Germany.

This study followed the ethical guidelines of the Institutional Review Board of the Amsterdam UMC. Ethical approval was granted under reference number 05/031 (normal control samples) and 07/318 (AIN biopsies taken in the course of a triple-arm trial on AIN treatment) [15]. We adhered to the Code of Conduct for Responsible Use of Left-over Material of the Dutch Federation of Biomedical Scientific Societies and ethical approval was waived for use of archived biopsies (reference no. 17/151 [SCC], 18/341 [AIN biopsies], and 17/234 [longitudinal series]). For the longitudinal series, local ethical approval was granted by the NHS Health Research Authority, United Kingdom (IRAS ID 226196), and the Ethical Committee

Table 1.   Tissue Samples, All From Human Immunodeficiency Virus-positive Men, Used in the Cross-sectional Series, Including Anatomic Location

Histological Category	Total No. of Samples	Median Age, years	Anatomic Location, No. of Samples		
			Anal Canal	Perianal	Not Provided
Normal	106	53	105	1	0
AIN1	37	47	27	10	0
AIN2	98	48	91	7	0
AIN3	74	48	64	10	0
SCC	30	52	4	11	15
Total	345	50	291	39	15

Abbreviations: AIN1–AIN3, anal intraepithelial neoplasia (grades 1–3); Normal, normal control samples; SCC, anal squamous cell carcinoma.

of the University Witten/Herdecke, Germany (reference no. 166/2017).

Histopathological Review, Human Papillomavirus Genotyping, and DNA Methylation Analysis

Histopathological review, HPV genotyping, and DNA methylation analysis were performed as described before (see Supplementary Methods) [10]. In short, a board-certified pathologist (C. J. M. v. N.) confirmed histopathological classification of all samples and p16INK4A immunohistochemistry was used if indicated. Bisulfite-converted sample DNA was analyzed for 6 methylation markers (*ASCL1, LHX8, SST, WDR17, ZIC1*, and *ZNF582*) [10, 14] using 2 multiplex quantitative methylation-specific polymerase chain reaction (qMSP) assays, each targeting 3 genes and the reference gene, β-actin (*ACTB*) [16, 17].

Statistical Analysis

Differences in methylation levels across the different histological categories (normal, AIN1–3, SCC) were visualized using boxplots and tested for statistical significance using the Kruskal-Wallis test, followed by pairwise Mann-Whitney *U* tests, with Bonferroni correction. Differences in methylation levels for anal canal versus perianal biopsies within histological categories were assessed using the Mann-Whitney *U* test and differences in distributions of HPV16 versus non-HPV16 samples between positive and negative methylation results were assessed using the Fisher's exact test.

Evaluation of the diagnostic performance of the markers was performed as described before (see Supplementary Methods) [10]. In short, for the individual markers, we performed univariable (simple) logistic regression, while multivariable (multiple) logistic regression with forward and backward selection was used to obtain a marker panel. These analyses were performed on ≤AIN1 (normal control samples and AIN1) versus AIN3+ (AIN3 and anal SCC), which also allowed for comparison with our previous study. As additional confirmation, "normal" was compared with "anal SCC." Performance of the models was visualized using receiver operating characteristic (ROC) curves and assessed through the area under the curve (AUC), as well as through the sensitivity and specificity at the Youden's index (*J*) threshold (threshold that maximizes the sum of sensitivity and specificity). To evaluate the predictive performance of our models on samples outside the set, we performed leave-one-out cross-validation (LOOCV).

For the longitudinal series, methylation results were obtained by applying the multivariable logistic regression model fitted on the cross-sectional series and using the corresponding *J*-threshold.

In the AIN3+ analysis, the *J*-threshold is optimized for detecting SCC as well as all AIN3. For the purpose of trying to optimize identification of HGAINs that are likely to have a high risk of progression in the cross-sectional series, detection thresholds were adjusted to achieve maximum specificity. The adjustment was based on methylation levels of the (suspected) cancers and HGAIN in the longitudinal series, making sure none of these lesions were misclassified.

Statistical analyses were performed using R statistical software (version 3.5.1; R Foundation for Statistical Computing, Austria) with packages pROC and ggplot2 and IBM SPSS statistics software (version 24; IBM Corporation). Reported *P* values are 2-sided, with .05 as the significance threshold.

RESULTS

Human Papillomavirus Genotyping of Tissue Samples

All 209 AIN1–3 biopsies, 97% (29/30) of SCC, and 63% (67/106) of normal control samples were HPV positive (Supplementary Table 1). Of these HPV-positive biopsies, high-risk HPV (hrHPV) types [18, 19] were found in 30% of AIN1, 79% of AIN2, 89% of AIN3, and 90% of SCC. In HPV-positive normal control samples, 64% showed hrHPV positivity. Multiple HPV types were found in 37% of HPV-positive samples. HPV16 was the most predominant type, found in 39% of AIN2, 46% of AIN3, and 72% of HPV-positive anal SCC, and was the most frequent HPV type among the single infections.

Methylation Levels Across Histological Categories of Anal Disease

In the cross-sectional series, methylation levels of all markers differed significantly between histological categories ($P < 8 \times 10^{-19}$). For all consecutive AIN grades (AIN1–3) and SCC, methylation levels increased significantly with increasing severity of disease ($P < .05$) (Figure 1). Methylation levels did not significantly differ between anal canal and perianal biopsies in any histological category for any marker ($P > .07$) (Supplementary Table 2), although the proportion of perianal biopsies was generally low.

DNA methylation patterns of the 6 markers showed high methylation levels in all cancers and low methylation levels in the vast majority of normal control samples and AIN1 (Figure 2A). In AIN3, methylation levels varied greatly, exhibiting both low as well as high methylation levels similar to cancers. Whereas these biopsies were similar in histopathological grade, the methylation pattern proved to be heterogeneous. In AIN2, the same heterogeneous pattern was observed, although with a lower proportion of biopsies with cancer-like high methylation levels.

Diagnostic Performance of the Individual Methylation Markers

In the logistic regression analysis for AIN3+ detection (cases vs controls: AIN3+ [AIN3 and anal SCC; n = 104] vs ≤AIN1 [normal control samples and AIN1; n = 143]), all markers proved able to significantly distinguish cases from controls ($P < .001$). AUCs ranged from 0.84 to 0.89 (Table 2), with the highest AUC achieved by *ZNF582* (AUC = 0.89), followed by
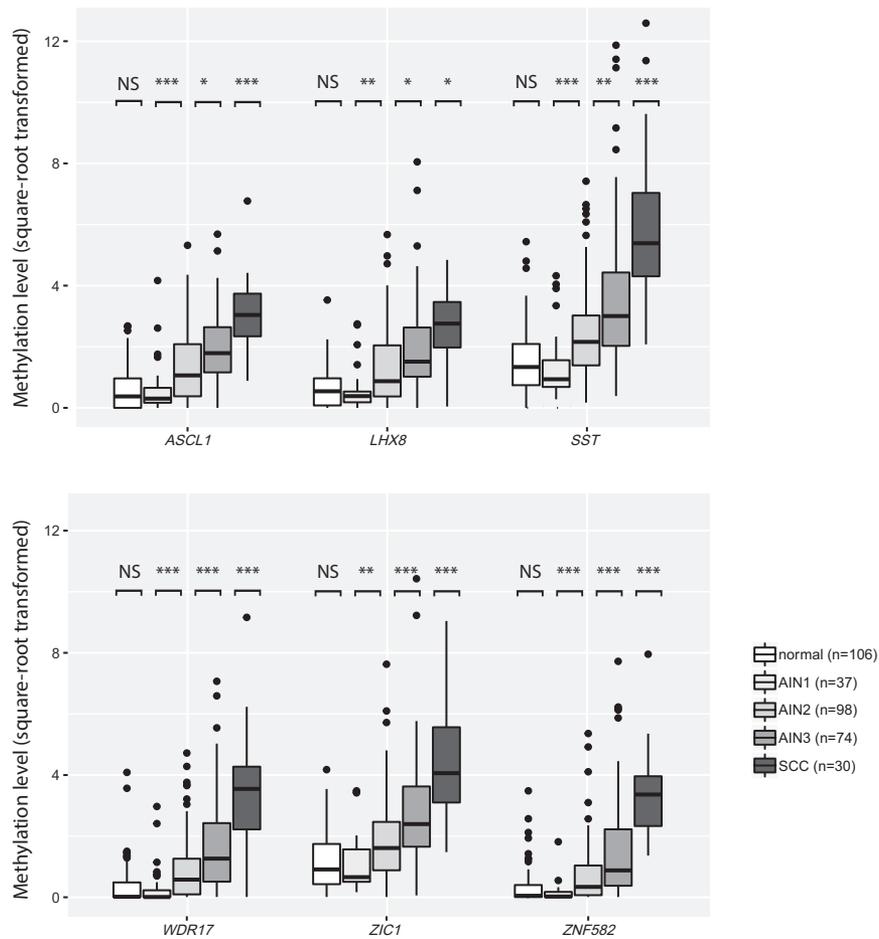
Figure 1. Methylation levels increased with severity of anal disease. DNA methylation levels relative to a reference gene β-actin (square-root transformed ΔΔCq ratios; *y* axis) in the different histological categories of anal tissue samples of HIV-positive men (*x* axis) for 6 markers: *ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582*. Differences between histological categories upon Kruskal-Wallis omnibus test, followed by post hoc testing using the Mann-Whitney *U* test and Bonferroni multiple testing correction: $*P < .05$, $**P < .01$, $***P < .001$. ● outlier sample. Abbreviations: AIN1–AIN3, anal intraepithelial neoplasia (grades 1–3); HIV, human immunodeficiency virus; normal, normal control samples; NS, not significant; SCC, squamous cell carcinoma.

SST (AUC = 0.88) and *WDR17* (AUC = 0.87). *ZNF582* was the only marker that classified all (30/30) cancers as methylation positive at the *J*-threshold (≥0.35), corresponding to an AIN3+ sensitivity and specificity of 76% and 87%, respectively. Using this threshold, *ZNF582* classified 66% (49/74) of AIN3, 36% (35/98) of AIN2, 3% (1/37) of AIN1, and 16% (17/106) of normal control samples as methylation positive (Figure 2A: [Detection by *ZNF582*], black indicators). Upon leave-one-out cross-validation, we obtained AUC values from 0.82 to 0.88, with *ZNF582* again achieving the highest AUC (AUC = 0.88) (Figure 3; Table 2) for AIN3+ detection. *ZNF582* remained the only marker classifying all cancers as methylation positive at the *J*-threshold (≥0.32), corresponding to a sensitivity and specificity of 77% and 86%, respectively.

In the analysis for anal SCC detection (cases vs controls: anal SCC [n = 30] vs normal control samples [n = 106]), similar results were obtained (Figure 4; Table 3). *ZNF582* attained the highest AUC (AUC = 0.99), with AUCs for the other markers ranging from 0.91

to 0.98. At the *J*-threshold (≥0.17) this resulted in a sensitivity of 100% and specificity of 95%. At this threshold, *ZNF582* was the only marker able to classify all cancers as methylation positive, along with 36% (27/74) of AIN3, 19% (19/98) of AIN2, 3% (1/37) of AIN1, and 5% (5/106) of normal control samples. After cross-validation, AUCs ranged from 0.90 to 0.99 with *ZNF582* performing best, providing a sensitivity of 100% and specificity of 95% at the *J*-threshold (≥0.16).

Identification and Diagnostic Performance of a Marker Panel

In the multivariable logistic regression, both forward and backward selection for AIN3+ detection yielded the same model consisting of *ASCL1, SST*, and *ZNF582* as the optimal marker panel, with an AUC of 0.90 (Figure 3; Table 4). Using the *J*-threshold (≥0.43), this panel provided a sensitivity of 78% and specificity of 90%. At this threshold, all cancers (30/30) were classified as methylation positive, 69% (51/74) of AIN3, 42% (41/98) of AIN2, 8% (3/37) of AIN1, and 10% (11/106) of normal control samples (Figure 2B: [Detection by optimal
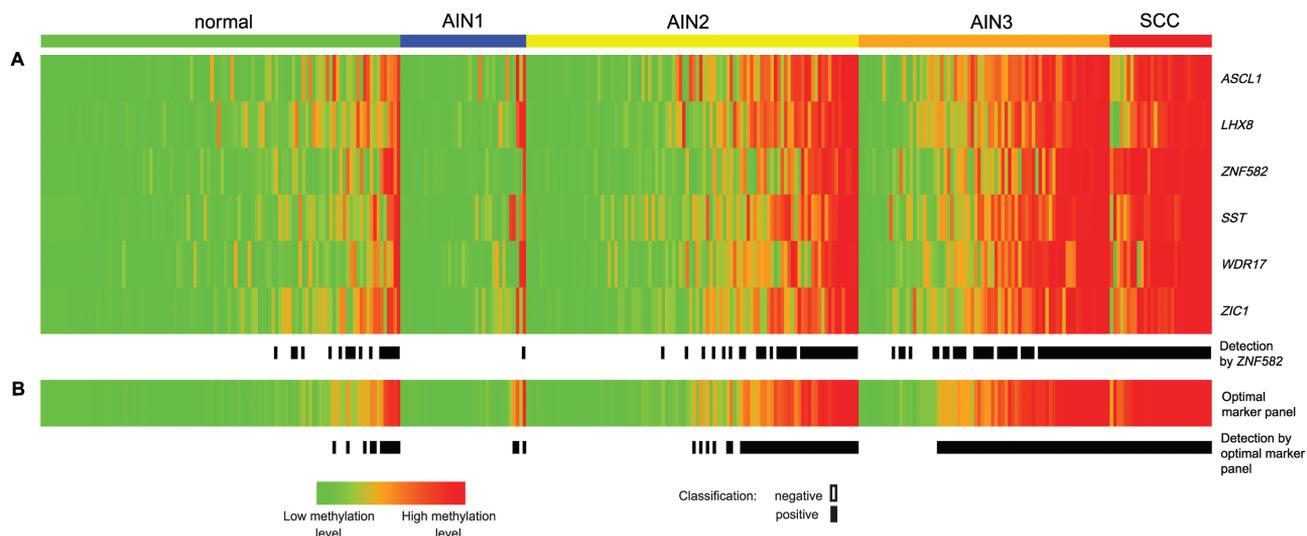
Figure 2. *A*, DNA methylation pattern of the 6 individual methylation markers (rows: *ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582*; based on LOOCV univariable regression for AIN3+ detection), per histological category in cross-sectional series. Methylation result per sample (column) in the different histological subgroups is displayed in color according to the PP from green (low methylation levels; PP of 0) to red (high methylation levels; PP of 1). In each group, samples are ordered based on their average predicted probability. Black boxes [detection by *ZNF582*] represent samples classified as methylation positive using *ZNF582* alone (at the non-CV Youden index threshold ≥0.35). *B*, DNA methylation pattern of the optimal marker panel (*ASCL1, SST, ZNF582*; based on LOOCV multivariable regression for AIN3+ detection) and methylation positivity using the panel. Black boxes [detection by optimal marker panel] represent samples classified as methylation positive using the optimal marker panel (at the non-CV Youden index threshold ≥0.43). Endpoint: AIN3+ (AIN3 and anal SCC). Abbreviations: AIN1–AIN3, anal intraepithelial neoplasia (grades 1–3); LOOCV, leave-one-out cross-validated; non-CV, non–cross-validated; normal, normal control samples; PP, predicted probability; SCC, squamous cell carcinoma.

marker panel], black indicators). In the methylation-positive AIN3, significantly more cases were HPV16 positive than non–HPV16 positive as compared with methylation-negative AIN3 (*P* = .0001). Upon cross-validation at the *J*-threshold (≥0.44), we found an AUC of 0.89 with a sensitivity of 76% and specificity of 90% (Table 4), again with all cancers being classified as methylation positive. For anal SCC detection, multivariable logistic regression was not possible due to the relatively small number of SCC cases.

Association of Methylation Positivity With Progression to Cancer
In the longitudinal series of 10 cases with (suspected) anal cancer and preceding HGAIN, all HPV types detected in (suspected) cancers were also found in the corresponding preceding

HGAIN biopsies (Supplementary Table 3). In all cases, all HGAIN biopsies preceding the biopsies with endpoint diagnosis (suspected) SCC consistently showed high methylation levels, comparable to the highest levels in AIN3 and cancers in the cross-sectional series, even when taken several years before endpoint diagnosis (Figure 5). In addition, all of these biopsies were scored as methylation positive using the aforementioned 3-gene marker panel (*ASCL1, SST*, and *ZNF582*) at the *J*-threshold (Supplementary Table 3), as well as using *ZNF582* alone (data not shown).

Defining Detection Threshold Boundaries for Clinical Application
For the purpose of optimizing identification of HGAINs that are likely to have a high risk of progression to cancer, the detection threshold of

Table 2. Logistic Regression Analysis on Diagnostic Performance for AIN3+ Detection: Univariable Regression of the 6 Individual Markers (*ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582*)

Methylation Marker	*ASCL1*	*LHX8*	*SST*	*WDR17*	*ZIC1*	*ZNF582*
AUC (non-CV) (95% CI)	.87 (.82–.91)	.84 (.78–.89)	.88 (.83–.92)	.87 (.82–.91)	.87 (.82–.91)	.89 (.85–.93)
Sensitivity, %	81	80	78	71	76	76
Specificity, %	85	80	87	90	84	87
Missed SCC	2	3	1	2	1	0
AUC (LOOCV)	.86	.82	.87	.84	.86	.88
Sensitivity, %	81	80	77	71	76	77
Specificity, %	84	80	87	89	84	86
Missed SCC	2	3	1	2	1	0

Non–CV, including 95% CI and LOOCV AUCs, are reported. Sensitivity and specificity are for the Youden index threshold. Endpoint: AIN3+ (AIN3 and anal SCC) in anal tissue samples of HIV-positive men.

Abbreviations: AIN3, anal intraepithelial neoplasia (grade 3); AUC, area under the receiver operating characteristic curve; CI, confidence interval; HIV, human immunodeficiency virus; LOOCV, leave-one-out cross-validated; nonCV, non–cross-validated; SCC, anal squamous cell carcinoma.
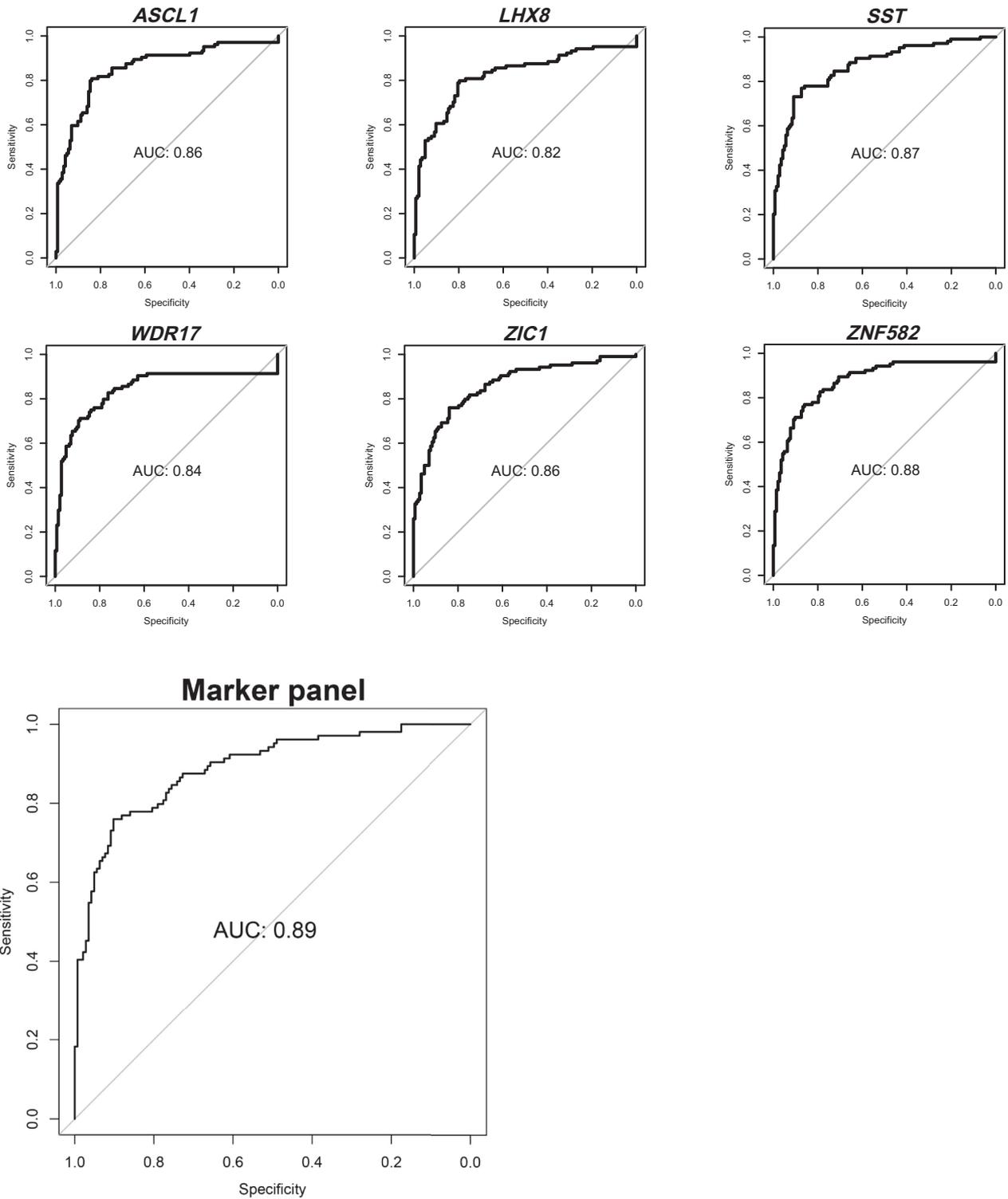
Figure 3. Diagnostic performance visualized with ROC curves of the logistic regression analysis for AIN3+ detection: univariable regression (LOOCV) for the 6 individual methylation markers (*ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582*) and multivariable regression (LOOCV) for the optimal marker panel (*ASCL1, SST, ZNF582*). Endpoint: AIN3+ (AIN3 and anal SCC). Abbreviations: AIN2–3, anal intraepithelial neoplasia (grade 2–3); AUC, area under the ROC curve; LOOCV, leave-one-out cross-validated; ROC, receiver operating characteristic.

the 3-gene marker panel could be adjusted to a maximum of ≥0.52, at which point all cancers and preceding HGAINs in the longitudinal series were scored as methylation positive, without missing any cancers in the cross-sectional series. At this threshold, 61% (45/74) of AIN3, 37% (36/98) of AIN2, 8% (3/37) of AIN1, and 8% (9/106) of normal control samples scored methylation positive.
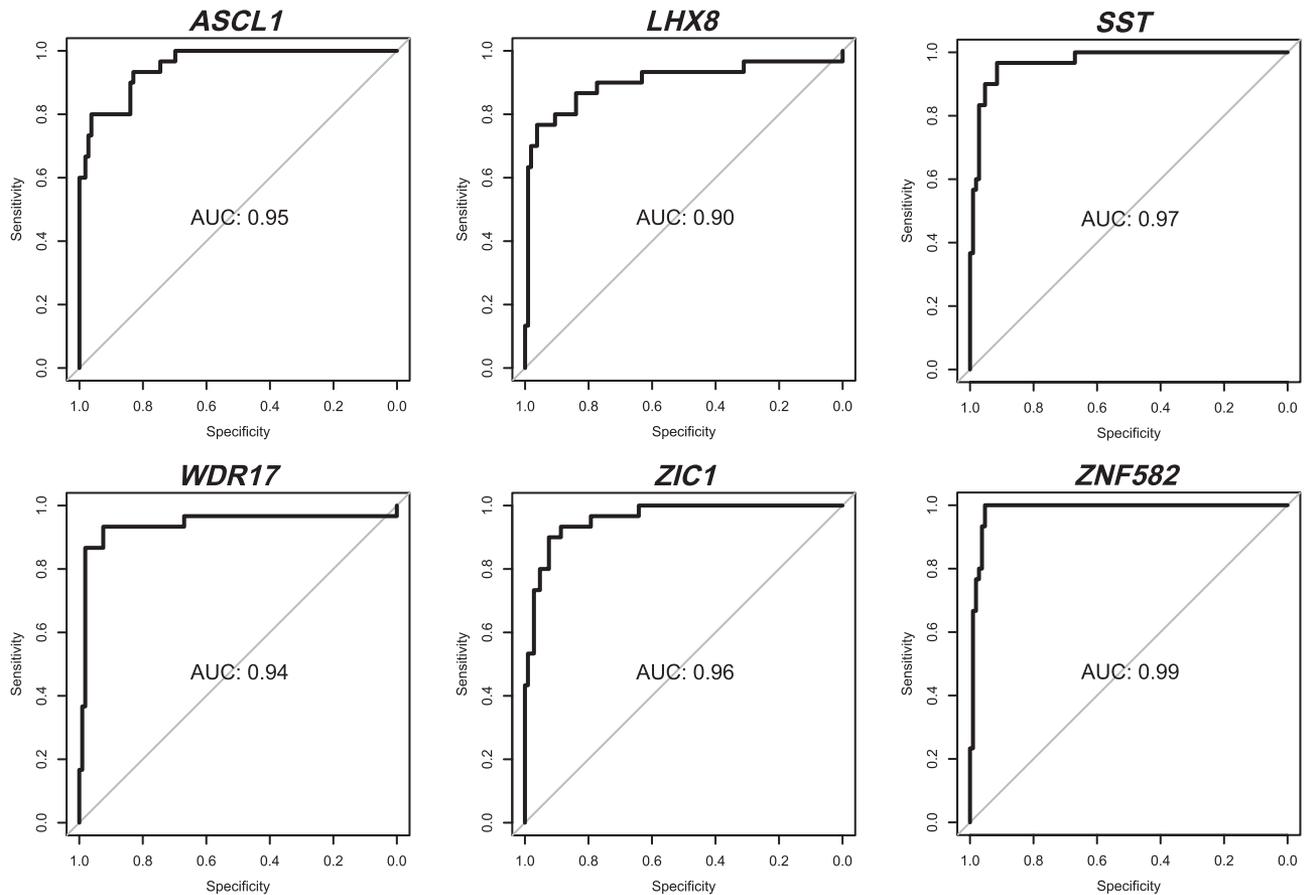
Figure 4. Diagnostic performance visualized with ROC curves of the univariable regression analysis (LOOCV) for anal SCC detection (normal vs SCC) for the 6 individual methylation markers (*ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582*). Endpoint: anal SCC. Abbreviations: AUC, area under the ROC curve; LOOCV, leave-one-out cross-validated; ROC, receiver operating characteristic; SCC, squamous cell carcinoma.

DISCUSSION

The most important outcomes of this study were the validation of the accuracy of 5 host cell DNA methylation markers from our previous study [10] for the detection and cancer risk stratification of HGAIN in HIV+ men, and the finding that high methylation levels in precursor lesions are indeed associated with progression to cancer.

Using qMSP, we found that the diagnostic performance for AIN3+ detection was good and robust for all markers, as it not only held up after cross-validation (AUC = 0.82–0.88) but duplicated the performance of the same markers in our previous smaller study (AUC = 0.82–0.89), in which we analyzed 148 tissue samples [10]. Furthermore, we confirmed *ZNF582* to be the most potent marker. The addition of genes *ASCL1* and *SST* to *ZNF582*, forming a marker panel, slightly improved the performance for AIN3+ detection (AUC = 0.89) over *ZNF582* alone. Importantly, both the marker panel as well as *ZNF582* alone did not miss any cancers. *LHX8*, the newly tested marker, showed a similarly good performance as the markers in the previous study but did not have additive value. We acknowledge that omitting AIN2 in our analyses implicates an overestimation of specificity.

Consistent with our previous study, we established that histopathologically similar HGAIN samples display a heterogeneous methylation pattern, with both high (cancer-like) and low methylation levels. We hypothesized that the precursor lesions displaying high methylation levels were those having a higher risk of progression towards cancer. In this study, using a longitudinal series of biopsies from patients who developed (suspected) anal cancer over time, we were able to demonstrate that HGAIN preceding cancer consistently displayed methylation levels similar to cancers, even when biopsies were taken up to several years before cancer diagnosis. This supports our assumption that high methylation levels in HGAIN are associated with progression to cancer.

Hypermethylation of tumor suppressor genes appears to be an early event in HPV-induced carcinogenesis, with hypermethylation occurring up to at least 3.5 years before cancer diagnosis in one of our cases (Figure 5; Supplementary Table 3), making for an ideal screening tool [9, 20]. In comparable HPV-induced cervical (pre-) cancers, similarly high cancer-like methylation levels were found in a subset of precursor lesions of known high short-term cancer risk, comprising cervical intraepithelial neoplasia grade 2 or 3 with

Table 3. Univariable Logistic Regression Analysis on Diagnostic Performance for Anal Squamous Cell Carcinoma (SCC) Detection (Normal vs SCC) of the 6 Individual Markers (*ASCL1, LHX8, SST, WDR17, ZIC1, ZNF582*)

Methylation Marker	*ASCL1*	*LHX8*	*SST*	*WDR17*	*ZIC1*	*ZNF582*
AUC (non-CV) (95% CI)	.96 (.93–.99)	.91 (.85–.98)	.98 (.95–1.00)	.95 (.90–1.00)	.96 (.94–.99)	.99 (.97–1.00)
Sensitivity, %	93	77	97	93	93	100
Specificity, %	83	96	92	93	90	95
Missed SCC	2	7	1	2	2	0
AUC (LOOCV)	.95	.90	.97	.94	.96	.99
Sensitivity, %	93	77	97	93	90	100
Specificity, %	83	96	92	92	92	95
Missed SCC	2	7	1	2	3	0

Non–CV, including 95% CI and LOOCV AUCs, are reported. Sensitivity and specificity are for the Youden index threshold. Endpoint: anal SCC in anal tissue samples of HIV-positive men.
Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; HIV, human immunodeficiency virus; LOOCV, leave-one-out cross-validated; non-CV, non–cross-validated.

Table 4. Logistic Regression Analysis on Diagnostic Performance for AIN3+ Detection: Multivariable Regression for Optimal Marker Panel (*ZNF582, ASCL1, SST*)

Marker Panel	Non-CV	LOOCV
AUC (95% CI)	.90 (.86–.94)	.89
Sensitivity, %	78	76
Specificity, %	90	90
Missed SCC	0	0

Non–CV, including 95% CI and LOOCV AUCs, are reported. Sensitivity and specificity are for the Youden index threshold. Endpoint: AIN3+ (AIN3 and anal SCC) in anal tissue samples of HIV-positive men.
Abbreviations: AIN3, anal intraepithelial neoplasia (grade 3); AUC, area under the receiver operating characteristic curve; CI, confidence interval; HIV, human immunodeficiency virus; LOOCV, leave-one-out cross-validated; non-CV, non–cross-validated; SCC, anal squamous cell carcinoma.

a persistent hrHPV infection (≥5 years) and/or a chromosome 3q gain [9, 11, 20–22].

For clinical application and resolving current overtreatment, the methylation test should ideally identify all HGAIN lesions with a high risk of progression towards cancer, while ignoring HGAIN lesions with a low risk of progression and not missing any cancers. Given the quantitative nature of the methylation test, thresholds can be adjusted to the clinical need. We provided a threshold for the methylation marker panel with maximum specificity, by which the panel identified all cancers in both the cross-sectional and in the longitudinal series, as well as all HGAIN lesions preceding cancer in the longitudinal series. With this threshold, 61% of AIN3 and 37% of AIN2 are identified as methylation positive. Currently, in some countries, treatment of all AIN2–3 lesions is recommended. Using this marker panel as a treatment decision-making tool and treating only the methylation-positive AIN2–3, treatment could be withheld in up to 39% of AIN3 and 63% of AIN2 cases. For future studies, determining the optimal detection threshold for clinical decision making with regard to treatment of HGAIN lesions is vital, providing long-term assurance that HGAIN lesions with negative methylation results will not progress to cancer and thus treatment for these lesions can be safely withheld.

We recognize that retrospective selection of HGAIN preceding anal cancer for the longitudinal series might render this series prone to error. Although anatomic locations of HGAIN preceding cancer were thoroughly reviewed and HPV genotypes matched, we cannot rule out that some of these lesions might not have been the exact same lesion, particularly in cases with long intervals between biopsies, such as in case 9 (Figure 5; Supplementary Table 3). Moreover, in cases with short intervals between HGAIN and endpoint diagnosis we cannot rule out that the preceding biopsies might already have been cancers that were misdiagnosed as HGAIN, rather than reflecting actual progression towards cancer. However, this mimics real clinical practice with regard to the difficulty in diagnosing anal cancer. In any case, our findings reinforce the idea that hypermethylation is an early event in anal carcinogenesis and emphasizes the need for clinical decision making based on more objective and reproducible biomarkers as compared with histopathology alone.

While several studies on DNA methylation in anal carcinogenesis have been published [23–29], we are the first to validate methylation markers in a large, independent cross-sectional series, as well as the first to provide evidence that high methylation levels are associated with progression to cancer in a longitudinal series.

In conclusion, we validated the accuracy of 5 host cell DNA methylation markers for the detection of anal (pre-) cancer in HIV+ men and established an optimal marker panel consisting of the genes *ASCL1*, *SST*, and *ZNF582*. High methylation levels in HGAIN were associated with progression to cancer. Therefore, these markers may provide a promising tool to identify which HGAIN lesions are in need of treatment, preventing overtreatment of HGAIN that has a low cancer progression risk. Prospective clinical studies are now warranted to assess the safety and efficacy of treatment decision making for HGAIN based on methylation analysis.

Figure 5. DNA methylation pattern of longitudinal series per case (row).

Case	Sex	HIV status	Age at endpoint diagnosis (year)		Preceding HGAIN						Endpoint diagnosis		
1	M	pos	62	Methylation result:					1	2	3		
				Diagnosis:					AIN3	AIN3	≈ SCC		
				t=					-12M	-7M	0		
2	M	pos	59	Methylation result:					1	2	3a	3b	
				Diagnosis:					AIN3	AIN2	≈ SCC	AIN2	
				t=					-5.5M	-2M	0	0	
3	F	neg	49	Methylation result:						1	2		
				Diagnosis:						AIN3	SCC		
				t=						-5M	0		
4	M	neg	60	Methylation result:					1	2	3		
				Diagnosis:					AIN3	≈ SCC	SCC		
				t=					-5M	-2.5M	0		
5	M	pos	51	Methylation result:						1	2		
				Diagnosis:						AIN3	≈ SCC		
				t=						-3M	0		
6	M	pos	47	Methylation result:						1	2		
				Diagnosis:						AIN2	SCC		
				t=						-5M	0		
7	M	pos	51	Methylation result:	1	2	3	4	5	6	7a	7b	7c
				Diagnosis:	AIN3	AIN2	AIN3	AIN2	AIN2	AIN3	≈ SCC	SCC	SCC
				t=	-28M	-20M	-18M	-16.5	-9.5M	-6M	0	0	0
8	M	pos	58	Methylation result:			1a	1b	2	3	4a	4b	4c
				Diagnosis:			AIN3	AIN2	AIN3	AIN2	≈ SCC	≈ SCC	≈ SCC
				t=			-9M	-9M	-2.5M	-2M	0	0	0
9	M	pos	62	Methylation result:				1	2	3	4		
				Diagnosis:				AIN2	AIN2	≈ SCC	SCC		
				t=				-42M	-30M	-0.5M	0		
10	M	pos	62	Methylation result:				1	2	3	4		
				Diagnosis:				AIN2	AIN2	AIN2	≈ SCC		
				t=				-24M	-13M	-3M	0		

Figure 5. DNA methylation pattern of longitudinal series per case (row). Numbers represent biopsy number corresponding to Supplementary Table 3 divided into Endpoint diagnosis and Preceding HGAIN. Endpoint diagnosis is SCC or suspected SCC: lesion with high suspicion for infiltrative growth (≈ SCC). In some cases, multiple biopsies were taken at the same time point, indicated with a letter (eg, 1a, 1b, etc). Methylation result per sample is displayed in color (similarly to the cross-sectional series; Figure 2) according to their PP from green (low methylation levels; PP of 0 [not applicable in longitudinal series]) to red (high methylation levels; PP of 1) at the non-CV marker panel Youden index threshold (≥0.43) for AIN3+ detection. All endpoint diagnosis samples and preceding HGAIN were classified as methylation positive. AIN1–AIN3, anal intraepithelial neoplasia (grades 1–3); F, female; HGAIN, high-grade anal intraepithelial neoplasia; HIV, human immunodeficiency virus; M, male; neg, negative; non-CV, non–cross-validated; pos, positive; PP, predicted probability; SCC, squamous cell carcinoma; *t*, time in months (M) before endpoint diagnosis.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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