Characterisation of anal intraepithelial neoplasia and anal cancer in HIV-positive men by immunohistochemical markers p16, Ki-67, HPV-E4, and DNA methylation markers

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Supplementary materials and methods

Longitudinal sample series

Samples of the longitudinal series, obtained between 2009-2019, were retrospectively identified and retrieved from the pathology archives of the Amsterdam UMC, and OLVG, Amsterdam, the Netherlands; Homerton University Hospital, London, United Kingdom; and Helios St Elisabeth Hospital, Oberhausen, Germany, as described previously in more detail.[1]

Ethical considerations

We adhered to the Declaration of Helsinki and Code of Conduct for Responsible Use of Left-over Material of the Dutch Federation of Biomedical Scientific Societies. Ethical approval was granted under reference numbers 07/318 (AIN biopsies) and 05/031 (normal control samples) and waived under reference number 17/151 (SCC) and 17/234 (longitudinal series) by the Institutional Review Board of the Amsterdam UMC.[1, 2] For the longitudinal series, additional local ethical approval was granted by the NHS Health Research Authority, United Kingdom (IRAS ID 226196), and the Ethical Committee of the University Witten/Herdecke, Germany (reference no. 166/2017).[1]

HPV testing

HPV testing on these series was performed and reported previously.[1, 2] In short, HPV detection and genotyping were performed using SPF_{10} DNA enzyme immunoassay (DEIA) (Labo Biomedical Products B.V., Rijswijk, The Netherlands), followed by reverse hybridisation Line Probe $Assay_{25}$ ($LiPA_{25}$; version 1, Biomedical Products B.V., Rijswijk, The Netherlands). DEIA-positive samples that were negative for 25 HPV types on the $LiPA_{25}$ strip are referred to as undetermined.

Methylation analysis

DNA methylation analysis on these series was performed using quantitative methylation-specific PCR (qMSP) on bisulphite-converted sample DNA and reported previously.[1, 2] In the cross-sectional and longitudinal series methylation markers involved in HPV-induced carcinogenesis were evaluated using multiplex qMSP assays, each targeting multiple host cell genes and the reference gene, β-actin. Methylation values of the targets were normalised using the comparative Cq method ($2^{-(\Delta)\Delta Cq}$ x 100), resulting in (Δ)ΔCq ratios.[3] Using multivariable logistic regression analysis optimal methylation marker panels (combining *ASCL1, ST6GALNAC3, WDR17, ZIC1, ZNF582* for cross-sectional and *ASCL1*, *SST*, *ZNF582* for longitudinal series) were identified for the detection of [AIN3+] (AIN3 and anal SCC). The methylation result (i.e. the outcome of the multivariable logistic regression model for the panels) is expressed as predicted probabilities. The predicted probability values range from 0 to 1 and represent the risk for [AIN3+]; 0 indicates no risk and 1 indicates high risk. For samples with predicted probabilities above the Youden's Index (*J*)-threshold (threshold that maximises the sum of sensitivity and specificity), the methylation result was considered methylation 'positive'. Although the methylation marker panels and *J*-threshold slightly differed between the series (≥0.38 for cross-sectional and ≥0.43 for longitudinal), the cross-validated diagnostic performance for [AIN3+] detection of the panels was similar.[1, 2]

Supplementary tables

Supplementary table 1. Scoring of p16 and Ki-67 stratified by study diagnosis and E4 status.

Study diagnosis	Total	p16 score										Ki-67 score							
		No staining (score 0)		Patchy (score 1)		≤lower 1/3 (score 2)		≤lower 2/3 (score 3)		>lower 2/3 (score 4)		Normal basal (score 0)		≤lower 1/3 (score 1)		≤lower 2/3 (score 2)		>lower 2/3 (score 3)	
Normal	8																		
E4 negative	8	4	50.0%	4	50.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	8	100.0%	0	0.0%	0	0.0%
E4 positive	0	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
AIN1	26																		
E4 negative	7	1	14.3%	4	57.1%	2	28.6%	0	0.0%	0	0.0%	0	0.0%	3	42.9%	4	57.1%	0	0.0%
E4 positive	19	3	15.8%	15	78.9%	1	5.3%	0	0.0%	0	0.0%	2	10.5%	8	42.1%	7	36.8%	2	10.5%
AIN2	45																		
E4 negative	26	0	0.0%	0	0.0%	6	23.1%	11	42.3%	9	34.6%	1	3.8%	4	15.4%	14	53.8%	7	26.9%
E4 positive	19	0	0.0%	0	0.0%	3	15.8%	10	52.6%	6	31.6%	0	0.0%	0	0.0%	9	47.4%	10	52.6%
AIN3	15																		
E4 negative	14	0	0.0%	0	0.0%	0	0.0%	1	7.1%	13	92.9%	0	0.0%	0	0.0%	3	21.4%	11	78.6%
E4 positive	1	0	0.0%	0	0.0%	0	0.0%	0	0.0%	1	100.0%	0	0.0%	0	0.0%	0	0.0%	1	100.0%

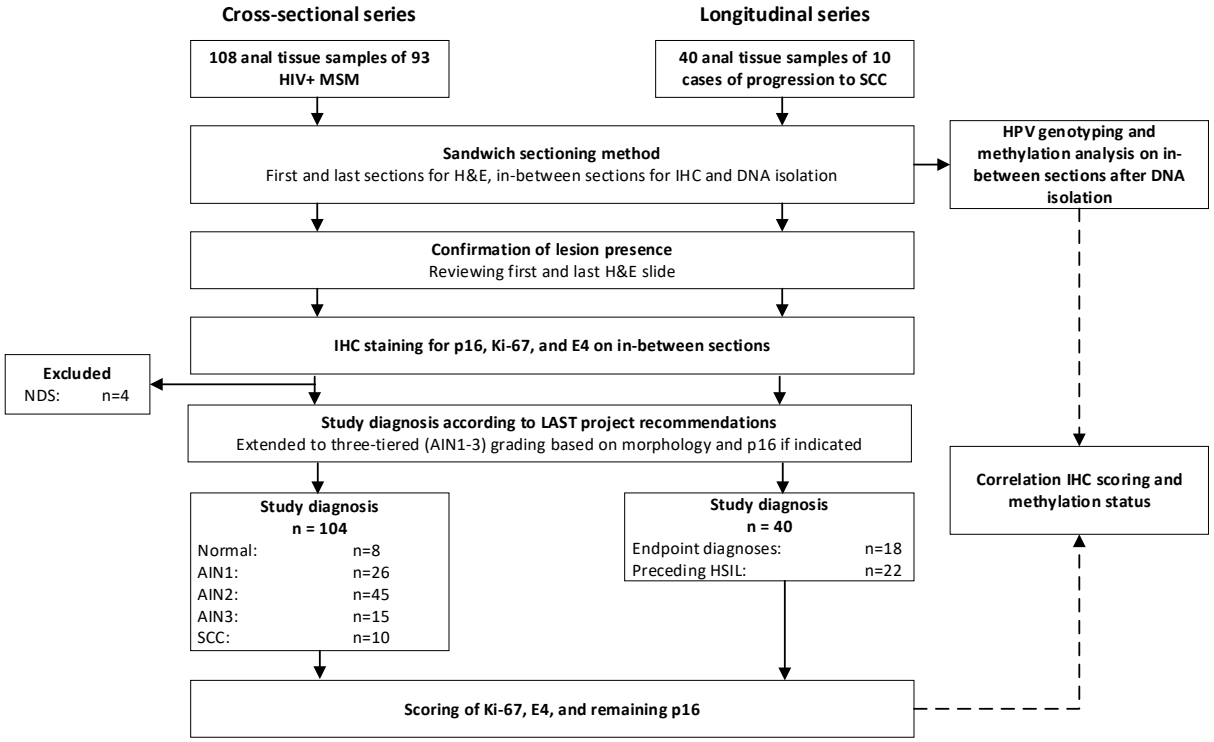
Data are numbers, %. Abbreviations: AIN: anal intraepithelial neoplasia (grades 1-3); Normal: normal control samples.

Supplementary table 2. Scoring of p16 and Ki-67 stratified by study diagnosis and methylation status.

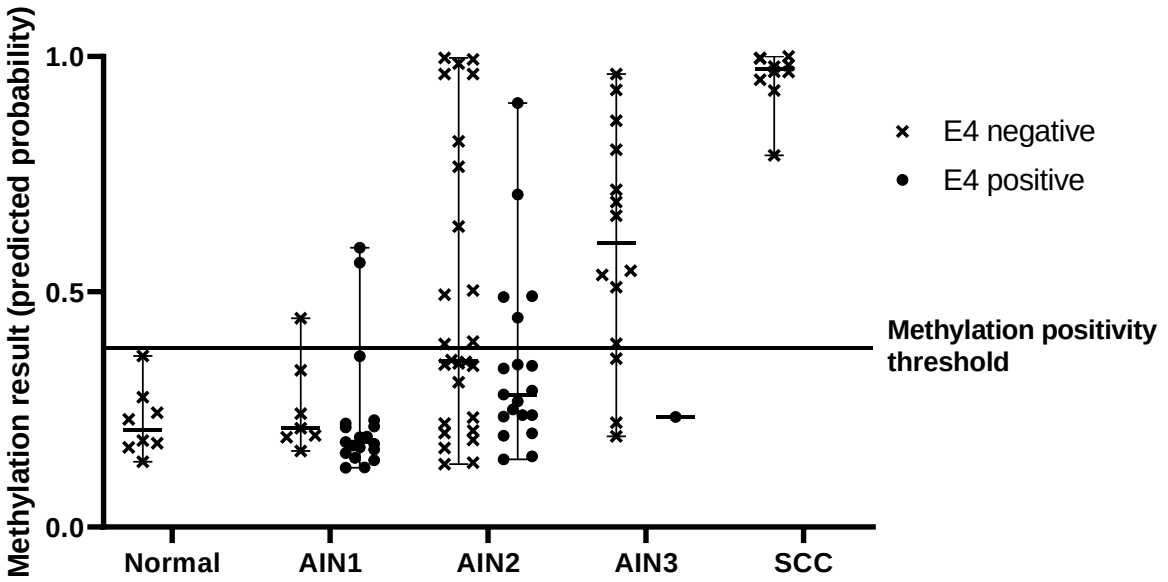
Study diagnosis	Total	p16 score										Ki-67 score							
		No staining (score 0)		Patchy (score 1)		≤lower 1/3 (score 2)		≤lower 2/3 (score 3)		>lower 2/3 (score 4)		Normal basal (score 0)		≤lower 1/3 (score 1)		≤lower 2/3 (score 2)		>lower 2/3 (score 3)	
Normal	8																		
Methylation negative	8	4	50.0%	4	50.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	8	100.0%	0	0.0%	0	0.0%
Methylation positive	0	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
AIN1	26																		
Methylation negative	23	4	17.4%	16	69.6%	3	13.0%	0	0.0%	0	0.0%	2	8.7%	9	39.1%	10	43.5%	2	8.7%
Methylation positive	3	0	0.0%	3	100.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	2	66.7%	1	33.3%	0	0.0%
AIN2	45																		
Methylation negative	28	0	0.0%	0	0.0%	2	7.1%	14	50.0%	12	42.9%	0	0.0%	4	14.3%	11	39.3%	13	46.4%
Methylation positive	17	0	0.0%	0	0.0%	7	41.2%	7	41.2%	3	17.6%	1	5.9%	0	0.0%	12	70.6%	4	23.5%
AIN3	15																		
Methylation negative	4	0	0.0%	0	0.0%	0	0.0%	0	0.0%	4	100.0%	0	0.0%	0	0.0%	2	50.0%	2	50.0%
Methylation positive	11	0	0.0%	0	0.0%	0	0.0%	1	9.1%	10	90.9%	0	0.0%	0	0.0%	1	9.1%	10	90.9%

Data are numbers, %. Abbreviations: AIN: anal intraepithelial neoplasia (grades 1-3); Normal: normal control samples.

Supplementary figures



Supplementary figure 1. Schematic overview of study procedures. Abbreviations: AIN: anal intraepithelial neoplasia (grades 1-3); HSIL: high-grade squamous intraepithelial lesion; H&E: Haematoxylin & Eosin; IHC: immunohistochemistry; LAST project: Lower Anogenital Squamous Terminology Standardisation Project; NDS: non-diagnostic sample (IHC staining uninterpretable due to technical staining issue or no more lesion being present in the slide); Normal: normal control samples; SCC: anal squamous cell carcinoma.



Supplementary figure 2. Methylation result per sample stratified by E4 status and study diagnosis.

Methylation result, i.e. predicted probability (PP; values ranging from low [PP=0] to high [PP=1], representing the risk for AIN3+), methylation positivity threshold based on Youden's Index (*J*)-threshold. Samples above the threshold (PP≥0.38) are considered methylation positive. E4 status ('negative': no staining [0] and focal [1]; or 'positive': extensive [2]). Abbreviations: AIN: anal intraepithelial neoplasia (grades 1-3); SCC: anal squamous cell carcinoma.

Supplementary references

1. van der Zee RP, Richel O, van Noesel CJM, Ciocanea-Teodorescu I, van Splunter AP, Ter Braak TJ, Nathan M, Cuming T, Sheaff M, Kreuter A, Meijer C, Quint WGV, et al. Cancer Risk Stratification of Anal Intraepithelial Neoplasia in Human Immunodeficiency Virus-Positive Men by Validated Methylation Markers Associated With Progression to Cancer. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2021;**72**: 2154-63.

2. van der Zee RP, Richel O, van Noesel CJM, Novianti PW, Ciocanea-Teodorescu I, van Splunter AP, Duin S, van den Berk GEL, Meijer C, Quint WGV, de Vries HJC, Prins JM, et al. Host Cell Deoxyribonucleic Acid Methylation Markers for the Detection of High-grade Anal Intraepithelial Neoplasia and Anal Cancer. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2019;**68**: 1110-7.

3. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008;**3**: 1101-8.