



Clinical performance of methylation as a biomarker for cervical carcinoma in situ and cancer diagnosis: A worldwide study

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Funding information

Cancer Research UK, Funding Award/Number: C27045/A27046.

Abstract

The shift towards primary human papillomavirus (HPV)-based screening has necessitated the search for a secondary triage test that provides sufficient sensitivity to detect high-grade cervical intraepithelial neoplasia (CIN) and cancer, but also brings an improved specificity to avoid unnecessary clinical work and colposcopy referrals. We evaluated the performance of the previously described DNA-methylation test (S5) in detecting CIN3 and cancers from diverse geographic settings in high-, medium- and low-income countries, using the cut-off of 0.80 and exploratory cut-offs of 2.62 and 3.70. Assays were performed using exfoliated cervical specimens ($n = 808$) and formalin-fixed biopsies ($n = 166$) from women diagnosed with cytology-negative results ($n = 220$), CIN3 ($n = 204$) and cancer stages I ($n = 245$), II ($n = 249$), III ($n = 28$) and IV ($n = 22$). Methylation increased proportionally with disease severity (Cuzick test for trend, $P < .0001$). S5 accurately separated women with negative-histology from CIN3 or cancer ($P < .0001$). At the 0.80 cut-off, 543/544 cancers were correctly identified as S5 positive (99.81%). At cut-off 3.70, S5 showed a sensitivity of 95.77% with improved specificity. The S5 odds ratios of women negative for cervical disease vs CIN3+ were significantly higher than for HPV16/18 genotyping at all cut-offs (all $P < .0001$). At S5 cut-off 0.80, 96.15% of consistently high-risk human papillomavirus (hrHPV)-negative cancers (tested with multiple hrHPV-genotyping assay) were positive by S5. These cancers may have been missed in current primary hrHPV-screening programmes. The S5 test can accurately detect CIN3 and malignancy irrespective of geographic context and setting. The test can be used as a screening and triage tool. Adjustment of the S5 cut-off can be performed considering the relative importance given to sensitivity vs specificity.

Abbreviations: CI, confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; IQR, interquartile range; LMICs, low- and middle-income countries; OR, odds ratio; PCR, Polymerase Chain reaction; FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; LMICS, Low and Medium Income Countries.

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization. The authors gratefully recognise the patients who participated in this study. The study sponsors CRUK had no role in the study design, collection, analysis and interpretation of data, in the writing of the manuscript and in the decision to submit the manuscript for publication.

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KEYWORDS

cancer screening, cervical cancer, DNA methylation, high-risk human papillomavirus, HPV, molecular triage, triage

What's new?

In recent years, cervical cancer screening has shifted towards high-risk human papilloma virus (HPV) testing with triage of HPV-positive women through cytology. Here, the authors confirm that the S5 DNA-methylation test can accurately detect and predict high-grade cervical intra-epithelial neoplasia and cervical cancer, irrespective of the setting and geographic context. The S5 test cut-off can be adjusted to reflect the relative importance given to sensitivity versus specificity, and the S5 test-score is directly proportional to disease severity. An objective biomarker test like S5 coupled with minimally-invasive self-sampling strategies could potentially improve the utilisation of healthcare resources and save lives globally.

1 | INTRODUCTION

The implementation of cervical cancer prevention programmes by systematic cytology screening¹ has contributed to a reduction in cervical cancer-associated deaths in high-income countries.² Yet, cervical cancer is currently the fourth most common cancer in women and continues to increase worldwide, with 604 000 cases in 2020, accounting for 7.5% of all female cancer deaths.³ To allow a further reduction in the incidence of cervical cancer, screening has shifted towards high-risk human papillomavirus (hrHPV) testing with triage of HPV-positive women. Cervical cancer incidence, ranges from 5 to 50 per 100 000 women depending on setting and while hrHPV testing is highly sensitive for the detection of disease, specificity is less optimal given the benign trajectory of most infections. Triage generally relies on cytology as the preferred secondary test in HPV-positive women.⁴ However, being subjective, cytology has limitations and objective secondary triage tests are urgently needed to identify the minority of hrHPV-positive women with high-grade disease.⁴ Furthermore, triage tests that rely on molecular rather than morphological signatures (such as cytology) remove the requirement for specialised expertise.

Methylation biomarkers can offer an accurate alternative to detect clinically significant infection and associated disease and can identify women who have the highest risk of progressing into invasive cervical cancer.^{5,6} Aberrant DNA methylation has been reported to increase with cervical cancer disease progression,⁷ allowing this epigenetic event to be used as a temporal biomarker, with a potential to accurately predict whether hr-HPV infection will lead to cervical intra-epithelial neoplasia grade 2 or above (CIN2+) or disappear.^{5,8}

Several methylation biomarkers tests including our S5 DNA-methylation classifier, which tests for methylation on the host tumour suppressor gene *EPB41L3* and viral late genes (L1 and L2) of HPV16, HPV18, HPV31 and HPV33, can accurately separate women with CIN2/3 and cancer from those with CIN1 or normal cytology.⁹ The S5-classifier has demonstrated improved triage performance compared to hrHPV genotyping, cytology or the combination thereof and has been validated in a HPV-positive cohort of women as part of the Canadian FOCAL clinical trial, in the FRIDA screening trial in Mexico and the

Colombian ASC-US-COL trial.¹⁰⁻¹² Additionally, the S5-classifier demonstrated a potential prognostic utility, in its ability to identify women with progressive CIN2.⁸ Together, these data support the prospect of using the S5-classifier as a molecular tool to identify clinically significant cervical abnormalities and predicting their clinical course.

Validation of the S5-classifier in a large number of CIN3+ samples from both high-income and low- and middle-income countries (LMICs) is required to demonstrate that the methylation test can consistently detect cervical cancers worldwide. Extensive validation of the S5 classifier will support implementation of the test in global screening and disease-management systems, especially with the rise in acceptance of screening based on self-sampling.^{13,14} The main objective of the present study is to analyse the performance and consistency of S5 in detecting high-grade lesions and cervical cancers from diverse settings that reflect Asia, Europe, Africa and the Americas. The present study aims to complement previous works on the S5 DNA-methylation classifier.^{6,9,11,12,15}

2 | MATERIALS AND METHODS**2.1 | Study population**

Cervical swabs and biopsies were collected from a total of 973 patients aged 21 to 64 as described in the referenced papers.¹⁶⁻²² All samples included in the study were analysed by cytology or histology and had results of negative, CIN3 or invasive cervical cancer. We excluded CIN1 and CIN2 from our study because the CIN1 is a low-grade lesion and CIN2 is considered increasingly, a heterogenous lesion that does not serve as a robust histological indicator of high-grade disease. Our study focussed mainly on cervical cancer stages I and II in order to have a sharper view of the epigenetic contrast between CIN3 vs early cancer and to complement previously published data on S5 performance.

Details regarding patient characteristics are described in Table 1.

The cellular material for cervical liquid-based cytology samples was collected in PreservCyt medium (Hologic Corporation, Marlborough, MA) for storage until DNA extraction. A subset of

TABLE 1 Baseline characteristics of the 973 women from the study cohort

Characteristics	HPV(-)/Cyt(-) ^a n = 110 (%)	HPV(+)/Cyt(-) ^a n = 110 (%)	CIN3 ^a n = 204 (%)	Cervical cancer - FIGO ^a stage			
				Stage I ^a n = 245 (%)	Stage II ^a n = 249 (%)	Stage III ^a n = 28 (%)	Stage IV ^a n = 22 (%)
Histotype of cervical cancer							
Squamous cell carcinoma				230 (93.9)	236 (94.8)	26 (92.8)	19 (86.5)
Adenocarcinoma				14 (5.7)	10 (4.0)	2 (7.2)	2 (9.0)
Adenosquamous cell carcinoma				–	1 (0.4)	–	–
Neuroendocrine carcinoma				1 (0.4)	2 (0.8)	–	1 (4.5)
hr-HPV status ^a							
Consistently negative	110 (100)	–	10 (4.90)	14 (5.7)	10 (4.0)	2 (7.1)	–
HPV16+	–	35 (31.8)	120 (58.8)	160 (65.3)	179 (72.1)	20 (71.4)	19 (86.4)
HPV18+	–	14 (12.7)	7 (3.4)	19 (7.7)	15 (6.0)	1 (3.5)	–
HPV31+	–	6 (5.5)	30 (14.7)	8 (3.2)	9 (3.6)	2 (7.1)	1 (4.5)
HPV33+	–	6 (5.5)	7 (3.4)	9 (3.6)	8 (3.2)	–	–
Other hr-HPV+	–	49 (44.5)	30 (14.7)	35 (14.3)	28 (10.8)	3 (10.7)	2 (9.1)
Sample type							
Cervical scrape	110 (100)	110 (100)	204 (100)	142 (58.0)	192 (77.0)	28 (100)	22 (100)
FFPE tissue ^a	–	–	–	103 (42.0)	57 (22.9)	–	–
Age (years)							
<25	20 (18.18)	12 (10.9)	14 (6.9)	5 (2.0)	2 (0.8)	–	–
25-29	27 (24.5)	25 (22.7)	59 (28.9)	27 (11.0)	10 (4.0)	–	1 (4.5)
30-39	28 (25.4)	26 (23.6)	81 (39.7)	83 (33.8)	43 (17.3)	4 (14.2)	3 (13.6)
40-49	21 (19.0)	23 (20.9)	30 (14.7)	56 (22.8)	84 (33.8)	8 (28.5)	6 (27.4)
50-59	8 (7.2)	12 (10.9)	14 (6.9)	46 (18.7)	63 (25.4)	9 (32.1)	11 (50.0)
>60	6 (5.5)	12 (10.9)	6 (2.9)	28 (11.4)	47 (18.5)	7 (25.0)	1 (4.5)
Country of origin							
Bhutan	–	10 (9.0)	–	28 (11.4)	22 (8.8)	–	–
Colombia	16 (14.5)	4 (3.6)	50 (24.2)	21 (8.5)	25 (10.0)	–	–
Ethiopia	39 (35.5)	10 (9.0)	–	2 (0.8)	18 (7.2)	28 (100)	22 (100)
Georgia	–	–	–	40 (16.3)	2 (0.8)	–	–
India	–	10 (9.0)	–	12 (4.9)	38 (15.3)	–	–
Philippines	–	–	–	15 (6.1)	35 (14.1)	–	–
South Africa	–	–	–	2 (0.8)	47 (18.9)	–	–
Spain	16 (14.5)	4 (3.6)	50 (24.2)	28 (11.4)	22 (8.8)	–	–
United Kingdom	25 (22.7)	36 (32.7)	54 (27.1)	48 (19.5)	3 (1.2)	–	–
United States (New Mexico)	14 (12.7)	36 (32.7)	50 (24.2)	50 (20.4)	36 (14.5)	–	–

Note: All women were diagnosed as histology negative (healthy women), cervical intraepithelial neoplasia Grade 3 (CIN3) and cervical cancer stages I-IV. Abbreviations: FFPE, formalin-fixed paraffin-embedded; FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus.

specimens from Bhutan (n = 10), Ethiopia (n = 49), India (n = 10), Spain (n = 20), United Kingdom (n = 51), Colombia (n = 20) and United States (n = 50) were selected for negative cytology results. CIN3 samples originated from Spain (n = 50), United Kingdom (n = 54), Colombia (n = 50) and United States (n = 50). Cancer samples originated from Ethiopia (n = 70), South Africa (n = 49), Bhutan (n = 50), India (n = 50), Philippines (n = 50), Georgia (n = 42), Spain (n = 50), United Kingdom (n = 51), Colombia (n = 46) and

United States (n = 86). All CIN3 and cancer samples were collected from patients showing abnormal cytology and histology results through colposcopy referral and diagnosed according to specific country recommendations. Biopsy samples were collected and stored at –70°C (IARC and Scottish HPV Archive and Addis Ababa University); formalin-fixed paraffin-embedded (FFPE) samples were stored at room temperature (Bhutan-IARC and University of New Mexico) until DNA was extracted.

2.2 | S5-methylation assays

DNA was extracted using (a) QIASymphony DSP DNA Mini Kit (Qiagen, Hilden, Germany) for all samples in the IARC biobank (both PreservCyt and FFPE specimens); (b) Abbott M2000 system (Abbott Laboratories, Maidenhead, UK) for all samples in the Scottish HPV Archive biobank; (c) DNA from Ethiopian samples and FFPE tissue from University of New Mexico was manually extracted using DNeasy Blood & Tissue Kit (Qiagen). After extraction, DNA was quantified with the Qubit Flex Fluorometer (ThermoFisher Scientific, London, UK). One hundred nanograms of DNA was used in the bisulphite conversion reaction where un-methylated cytosines were converted to uracils with the EZ DNA methylation kit (Zymo Research, Irvine, CA). The converted DNA was amplified and pyrosequenced on a PyroMark Q96ID (Qiagen) for DNA methylation on CpG islands from *EPB41L3* and viral late genes (L1 and L2) of HPV16, HPV18, HPV31 and HPV33 as previously described.⁹ Percentage methylation was taken as the mean for CpG sites involved in each case.

2.3 | HPV genotyping

The clinical samples used in the study have been initially genotyped as part of previously approved research studies. The following technologies were used: GP5+/6+ PCR assay ($n = 641$),^{17,18,20,21} Abbott RealTime assay ($n = 126$), Roche LINEAR ARRAY HPV Genotyping assay ($n = 88$) and BD Onclarity assay ($n = 52$). Genotyping with Papilloplex High-Risk HPV test (GeneFirst, Oxford, UK) was additionally used to validate previous HPV genotyping in all samples investigated. This is a multiplexed polymerase chain reaction (PCR)-based system that simultaneously detects 13 hr-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and the low-risk type 66).²³ Genotyping with the Papilloplex High-Risk HPV test was run in house on the QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific) and targeted the L1 region of all the genotypes described. Papilloplex High-Risk HPV internal negative and positive controls were used as baselines and analysis was performed on the GeneFirst software.

We investigated the following HPV categories: (a) HPV16 positive, (b) HPV18 positive, (c) HPV31 positive, (d) HPV33 positive and (e) other hr-HPV positive (non-HPV16/18/31/33). We followed a hierarchical attribution of HPV genotypes, namely in cases with multiple hrHPV-infections the more prevalent genotype in cancer was attributed dominant status (eg, a sample positive for both HPV16 and HPV18 was placed in category 1).

2.4 | Statistical analysis

We validated the performance sensitivity of the S5 classifier on CIN3 and cervical cancer samples from a global population of samples. We used the mean of methylation scores for selected CpG sites: three for *EPB41L3* (438, 427, 425), two for HPV16L1 (6367, 6389), six for HPV18L2 (4256, 4261, 4265, 4269, 4275, 4282), two for HPV31L1

(6352, 6367) and three for HPV33L2 (5557, 5560, 5566) as previously described.^{9,15,24-26} Proportion of methylation on HPV16L2 was calculated using the following three CpG sites: 4275, 4259 and 4238 as they were the most reproducible sites in this region. The S5-methylation score was calculated by using the following weighed average defined in 2016 by Lorincz et al⁹:

$$S5 = 30.9 (EPB41L3) + 13.7 (HPV16L1) + 4.3 (HPV16L2) + 8.4 (HPV18L2) + 22.4 (HPV31L1) + 20.3 (HPV33L2).$$

The main groups being compared were HPV negative cytology negative: HPV(-)/Cyt(-), HPV-positive cytology negative: HPV(+)/Cyt(-), CIN3 and cervical cancer including stages I (CSI), II (CSII), III (CSIII) and IV (CSIV) as per Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage classification. We hypothesised that uniformity in proportions and/or levels of methylation status among the different lesion categories may imply prognostic value as a clinical marker.

We compared differences in methylation levels between groups using Kruskal-Wallis and Dunn's multiple comparison tests and the Cuzick test for trend to assess any methylation trend with disease progression. We evaluated the S5 diagnostic potential using the pre-defined cut-off of 0.80. In addition, we explored cut-off points more suited for LMIC, using a previously defined alternative cut-off and Youden-J index.¹² McNemar's test with continuity correction was used for differences in sensitivity and specificity.

We also used unconditional logistic regression to study the relationship between methylation in the invasive cervical cancer group and the covariates – stage of cancer, type, age, demographics and HPV status. Additionally, we calculated the odds ratios (ORs) for the associations between HPV16/18 positivity, S5 classifier positivity at different cut-offs and CIN3 or cervical cancer (CSI-IV, FIGO stage unknown included) diagnosis. All *P* values were two-sided with $\alpha \leq .05$ considered significant. Analysis was performed with GraphPad Prism v8.0 as well as R v 3.4.1 for Cuzick tests, ORs and for unconditional logistic regression analyses.

3 | RESULTS

3.1 | Characteristics and selection criteria

We present a cross-sectional retrospective study including 973 women from 10 countries to evaluate the S5 methylation classifier performance to detect CIN3 and cervical cancer. The present study aims to complement previous work on the S5 DNA-methylation classifier.^{6,9,11,12,15} We selected 220 women cytology negative or HPV(-/+)/Cyt(-), 204 women diagnosed with CIN3 and 544 with invasive cervical cancer as described in Figure 1. Baseline characteristics of the women are presented in Table 1. The invasive cervical cancer group is further divided into squamous cell carcinomas ($n = 510$), adenocarcinomas ($n = 29$), adenosquamous cell carcinoma ($n = 1$) and neuroendocrine small cell carcinoma ($n = 4$). The median age for women with negative cytology was 38 years [interquartile range (IQR), 30-47], for CIN3 was 31 years (IQR, 28-39) and for women with invasive cervical cancer was 45 years (IQR, 38-55).

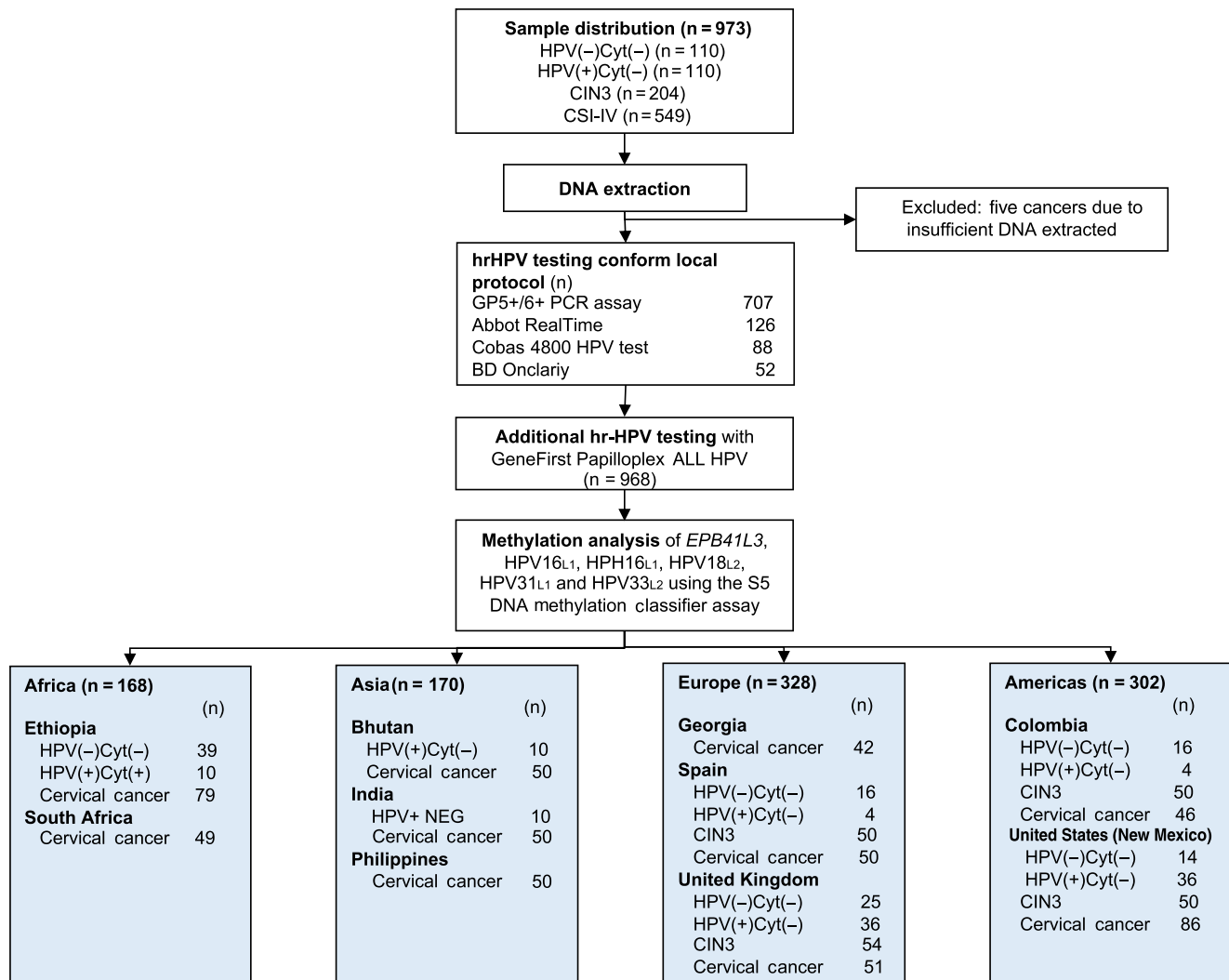


FIGURE 1 Study design flow chart

3.2 | hrHPV detection

The Papilloplex High-Risk HPV genotyping data were in 98.23% [95% confidence interval (CI) = 96.38%-99.99%] agreement with previous genotyping methodologies used (Figure 1). The grouped prevalence of 13 types of hrHPV plus HPV66 (now regarded as a low-risk HPV) was 95.09% (95% CI = 92.13%-97.89%) in the CIN3 group and 95.21% (95% CI = 92.45%-98.12%) in the cancer group. Each type of HPV in women infected by multiple HPV types was counted individually as described in Table 1 and Figure S1. In the cancer group, 374 women were consistently positive for HPV16 (68.91%), 37 women for HPV18 (6.91%), 21 women for HPV31 (3.90%), 16 women for HPV33 (3.01%) and 68 women for other hr-HPV types (12.41%). A total of 25 (4.78%) cervical cancers tested negative for any hr-HPV type covered by the assays. HPV genotyping was in agreement with methylation data on HPV16/18/31/33 infection in 95.38% (95% CI = 91.38%-98.33%) of cases.

3.3 | Increasing trend in the S5 methylation scores with disease severity

The S5 methylation scores were clustered according to severity of cervical abnormality. An outline of the methylation scores per country is provided in Figure S2. Median methylation score was 0.66 (95% CI = 0.60-0.78) in HPV negative and cytology negative women [HPV(-)/Cyt(-)], 0.91 (95% CI = 0.86-0.94) in HPV positive and cytology negative women [HPV(+)/Cyt(-)], 5.64 (95% CI = 3.99-6.88) in CIN3, 17.58 (95% CI = 15.99-20.33) in cervical cancer stage I (CSI), 23.71 (95% CI = 21.67-25.24) in cervical cancer stage II (CSII), 24.44 (95% CI = 22.10-32.14) in cervical cancer stage III (CSIII) and 24.95 (95% CI = 22.34-32.85) in cervical cancer stage IV (CSIV). The distribution of the S5 scores based on the disease diagnosis and hr-HPV status is shown in Figure 2A. Kruskal-Wallis and Dunn's multiple comparisons tests show significant separation in the following paired comparisons: HPV(-)/Cyt(-) vs CIN3 ($P < .0001$), HPV(-)/Cyt(-) vs CSI-IV (all $P < .0001$), HPV(+)/Cyt(-) vs CIN3 ($P < .0001$), HPV(+)/Cyt(-) vs

CSI-IV (all $P < .0001$), CIN3 vs CSI-IV (all $P < .0001$). No other significant differences were identified. The S5-classifier scores increased significantly with the severity of lesions (Cuzick test for trend: $z = 9.23$, $P < .0001$).

3.4 | S5-classifier sensitivity in cervical cancers at the 0.80 predefined cut-off

The S5-classifier methylation score was successfully measured in all 544 women diagnosed with cervical cancer which were included in the study. A total of 543 out of 544 cancer patients tested positive for S5 at 0.80, yielding a sensitivity of 99.81% (95% CI = 98.34-99.96). Table 2 shows the S5 sensitivity stratified per histology, FIGO stage, hrHPV status, hrHPV type, sample type, age and country of origin. At the 0.80 cut-off, cervical cancers which were consistently hrHPV negative when tested with multiple hrHPV genotyping assays were 96.15% (95% CI = 94.38-98.25) identified by the S5 classifier. The performance of the S5 classifier

was uniform among all groups considered. There were no significant differences in S5 sensitivity among histology, FIGO stage, hrHPV type, sample type, age and country of origin (Fishers' test, all $P > .05$).

3.5 | S5 classifier cut-off adjusted per country to optimise triage capacity

Cervical cancer incidence is directly linked to the availability of screening in a particular country. Hence the importance to introduce different modalities for the implementation of a molecular triage reflecting the country clinical setting. We investigated the false positive rates in women with HPV(-)/Cyt(-), HPV(+)/Cyt(-), CIN3 and CSI-IV at the UK-predefined cut-off of 0.80, the Youden-J index cut-off based on the S5 methylation scores of cervical cancers: 2.62 and the previously explored LMIC cut-off of 3.70.¹² For all groups analysed, the false positive rate decreased with the increase in cut-off

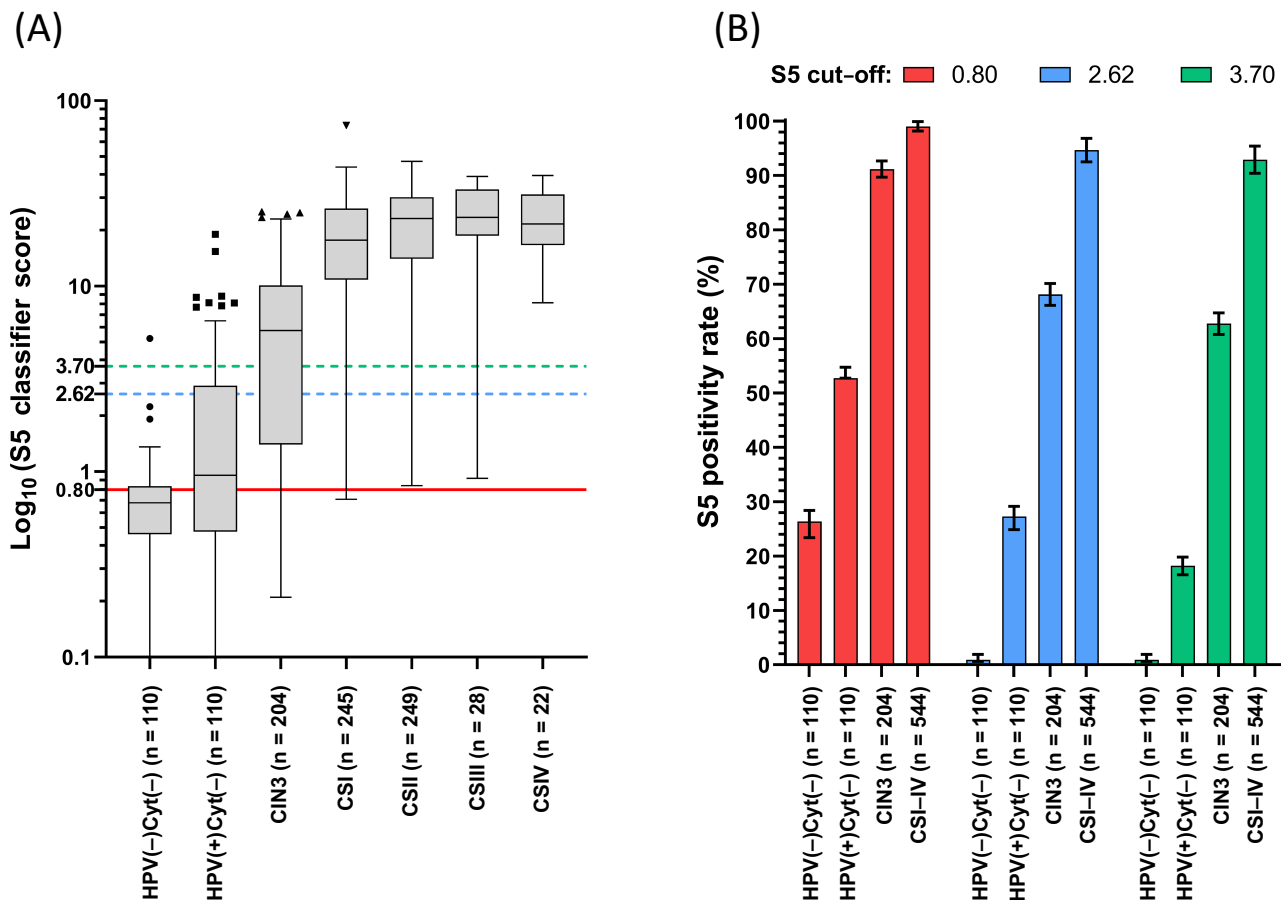


FIGURE 2 S5 performance in the study group: (A) Distribution of the S5 scores based on the histopathological diagnostic of the patient. Data were plotted as log 10 of the S5 score according to lesion grade: HPV(-)/Cyt(-), HPV(+)/Cyt(-), CIN3 and all stages of cervical cancer (CSI-IV). The S5 classifier was significantly different between the following group comparisons: HPV(-)/Cyt(-) vs CIN3 ($P < .0001$), HPV(-)/Cyt(-) vs CSI-IV (all, $P < .0001$), HPV(+)/Cyt(-) vs CIN3 ($P < .0001$), HPV(+)/Cyt(-) vs CSI-IV (all, $P < .0001$) and CIN3 vs CSI-IV (all, $P < .0001$). Other comparisons were not significant [HPV(-)/Cyt(-) vs HPV(+)/Cyt(-), among CSI-IV]. The proposed cut-offs for analysis are: 0.80 (red), 2.62 (blue) and 3.70 (green). The top of box represents the upper quartile (p75), bottom the lower quartile (p25) and the line the median (p50). The upper whisker extends to the largest point of the inter-quartile range from the upper quartile. The lower whisker extends to the smallest point of the inter-quartile range from the lower quartile. The Cuzick test for trend was highly significant ($P < .0001$). (B) Cumulative S5 positivity per lesion grade at the three cut-offs: 0.80 (red), 2.62 (blue) and 3.70 (green). Median values are shown by each bar and error bars show 95% CI of the median. CI, confidence interval; CIN, cervical intraepithelial neoplasia (of Grade 3); CSI-IV, cervical cancer stages I-IV; HPV, human papillomavirus; HPV(-)/Cyt(-), HPV negative and cytology negative; HPV(+)/Cyt(-), HPV positive and cytology negative

TABLE 2 S5 classifier sensitivity at the 0.80 cut-off in a cervical cancer referral group, stratified per histotype of cervical carcinoma, FIGO stage, hrHPV status, sample type, age or country of origin

	S5 sensitivity at cut-off 0.80			
	n/N	Percentage	95% CI	P value ^c
Histotype of cervical carcinoma				
Squamous cell carcinoma	509/510	99.80	99.10-99.95	.837
Adenocarcinoma	29/29	100.0	97.34-100.0	
Adenosquamous cell carcinoma	1/1	100.0	12.8-100.0	
Neuroendocrine carcinoma	4/4	100.0	26.86-100.0	
FIGO stage				
Stage I	244/245	99.60	99.25-99.83	.687
Stage II	249/249	100.0	99.43-100.0	
Stage III	28/28	100.0	97.34-100.0	
Stage IV	22/22	100.0	96.45-100.0	
HPV status^d				
HPV-positive	518/518	100.0	99.46-100.0	.465 ^a .587 ^b
HPV16	379/379	100.0	99.36-100.0	
HPV18	36/36	100.0	98.82-100.0	
HPV31	20/20	100.0	96.45-100.0	
HPV33	17/17	100.0	93.12-100.0	
Other hr-HPV	66/66	100.0	98.93-100.0	
HPV-negative	25/26	96.15	94.38-98.25	
Sample type				
Cervical scrape	383/384	99.73	98.34-99.96	.917
FFPE tissue	160/160	100.0	98.76-100.0	
Age (years)				
<25	7/7	100.0	64.87-100.0	.989
25-29	38/38	100.0	98.62-100.0	
30-39	133/133	100.0	99.32-100.0	
40-49	153/154	99.39	98.74-99.86	
50-59	129/129	100.0	99.22-100.0	
>60	83/83	100.0	99.02-100.0	
Country of origin				
Bhutan	50/50	100.0	98.89-100.0	.892
Colombia	46/46	100.0	98.87-100.0	
Ethiopia	70/70	100.0	98.98-100.0	
Georgia	42/42	100.0	98.84-100.0	
India	50/50	100.0	98.89-100.0	
Philippines	50/50	100.0	98.89-100.0	
South Africa	49/49	100.0	98.88-100.0	
Spain	50/50	100.0	98.89-100.0	
United Kingdom	50/51	98.03	95.99-99.05	
United States (New Mexico)	86/86	100.0	99.12-100.0	
Total	543/544	99.81	98.34-99.96	

Note: FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; n = number of positive samples in a specified group; N = group total.

Abbreviations: CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus.

^aP value between HPV-positive and HPV-negative subgroups.

^bP value among all subgroups in the HPV groups.

^chrHPV genotype grouping performed by hierarchical genotype attribution, as detailed in materials and methods.

^dDetermined by performing Fisher's exact test of independence.

	Variables	OR	95% CI	z value	P value ^a
CIN3	HPV16/18	2.86	1.77-4.62	4.30	Reference
	S5 0.80	4.50	2.71-7.46	5.83	<.0001
	S5 2.62	5.63	3.26-9.73	6.19	<.0001
	S5 3.70	6.42	3.67-11.24	6.52	<.0001
	HPV16/18 and S5 0.80 ^b	3.26	2.01-5.30	4.79	<.0001
	HPV16/18 and S5 2.62 ^b	3.56	2.12-5.98	4.80	<.0001
	HPV16/18 and S5 3.70 ^b	5.01	2.82-8.90	5.49	<.0001
Cervical cancer	HPV16/18	4.80	3.13-7.36	7.19	Reference
	S5 0.80	20.94	7.89-51.71	7.22	<.0001
	S5 2.62	36.21	20.9-62.73	12.80	<.0001
	S5 3.70	45.55	24.67-73.38	13.49	<.0001
	HPV16/18 and S5 0.80 ^b	6.32	4.08-9.80	8.25	<.0001
	HPV16/18 and S5 2.62 ^b	9.87	6.11-15.96	9.35	<.0001
	HPV16/18 and S5 3.70 ^b	14.90	8.69-25.56	9.81	<.0001

TABLE 3 Univariate and bivariate odds ratios (ORs) with 95% confidence intervals (CIs) for the associations between the different clinical outcomes and HPV16/18 and/or S5 cut-offs in a HPV(+)/Cyt(-) baseline

Abbreviations: CIN, cervical intraepithelial neoplasia (of Grade 3); HPV, human papillomavirus.

^aP value indicating an increased ORs compared to the reference HPV16/18. P value determined using Fisher's test of independence.

^bBivariate OR for the associations between HPV16/18 and S5 cut-offs (0.80, 2.62 and 3.70) and CIN3 and cervical cancer.

(Figure 2B). The most important decrease was observed in the HPV(-)/Cyt(-) and HPV(+)/Cyt(-) groups. At a cut-off of 0.80, the false positive rate in HPV(-)/Cyt(-) women was 26.32% (95% CI = 23.90-29.94). This may be acceptable for a country desiring very high sensitivity, where a strong healthcare system could accommodate the rather common false positives. The false positive rate decreased to 0.92% (95% CI = 0.36-1.82) at both 2.62 and 3.70 cut-offs (McNemar test $\chi^2 = 27.1$, $P < .0001$) which would better suit countries with lower screening capacity. However, the false positive rate of S5 in the HPV(+)/Cyt(-) was 52.74% (95% CI = 49.71-55.63) at 0.80 and showed a significant decrease trend to 27.22% (95% CI = 24.94-29.53) at cut-off 2.62 and 18.26% (95% CI = 16.62-20.24) at cut-off 3.70 (Cuzick test for trend, $P < .0001$). These possible adjustments can allow a customization of the S5 cut-off according to the country of clinical implementation.

Although we observed a significant decrease in false positive rate with the increase of the cut-off, a similar but less pronounced decrease trend was observed in S5 positivity for CIN3 and cancer detection. CIN3 sensitivity decreased from 87.26% (95% CI = 84.42-89.93) at 0.80 to 62.74% (95% CI = 60.13-65.25) at 3.70 (Cuzick test for trend, $P < .0001$). Further, the S5 sensitivity for cancer decreased from 99.81% (95% CI = 98.34-99.96) at 0.80 to 95.77% (95% CI = 92.39-97.40) at 3.70 (Cuzick test for trend, $P = .005$).

3.6 | Diagnostic potential of S5 classifier compared to HPV16/18 testing

Table 3 presents the associations between HPV16/18 and S5 classifier positivity at different cut-offs for the identification of CIN3+, compared to the HPV(+)/Cyt(-). HPV16/18 positivity was strongly

associated with CIN3+ development. The univariate OR of HPV16/18 positivity for CIN3 was 2.86 (95% CI = 1.77-4.62), while for cancer the OR was approximately two times higher: 4.80 (95% CI = 3.13-7.96). The univariate ORs for all S5 cut-offs were higher than the univariate ORs for HPV16/18 ($P < .0001$). Increased ORs were observed for the bi-variable associations of HPV16/18 and the S5 test regardless of the cut-off or the geographic location (all, $P < .0001$). This indicates stronger associations between the combination of HPV16/18 positivity and S5 positivity and CIN3+ development. Although the ORs for the bi-variable analysis of HPV16/18 positivity and S5 0.80 cut-off were significantly higher than the univariate HPV16/18 ORs ($P < .0001$), the highest associations for CIN3 + development were observed for S5 positivity at the 3.70 cut-off (OR = 5.63, 95% CI = 3.26-9.73 for CIN3; and OR = 45.55, 95% CI = 24.67-73.38 for cervical cancer).

3.7 | S5-classifier performance in detecting cervical cancers at the 3.70 cut-off

When the cut-off was increased to 3.70, the false positive rate in HPV(+)/Cyt(-) was 9.54% (95% CI = 8.49-10.76), which was approximately 4-fold lower than at the 0.80 cut-off (39.54%, 95% CI = 37.20-41.86). The decrease in the false positive rate correlates to an increase in specificity of the S5 classifier. Estimates of specificity are presented in Table S3 for cervical cancer compared to HPV(-)/Cyt(-) women or HPV(+)/Cyt(-) women (approximating a currently relevant triage population). For both cases, increasing the cut-off from 0.80 to 3.70 markedly improved specificity at a cost of sensitivity (Table S3). In an HPV(-)/Cyt(-) population, specificity rose from 65.12% (95% CI = 54.59-74.35) at 0.80 to 100% (95.19-100.0) at 3.70.

TABLE 4 S5 classifier sensitivity at the 3.70 cut-off in a cervical cancer referral group, stratified per histotype of cervical cancer, FIGO stage, hrHPV status, sample type, age or country of origin

	S5 sensitivity at cut-off 3.70			
	n/N	Percentage	95% CI	P value ^c
Histotype of cervical cancer				
Squamous cell carcinoma	491/510	96.22	91.32-98.35	
Adenocarcinoma	28/29	96.55	91.71-99.23	
Adenosquamous cell carcinoma	1/1	100.0	91.81-100.0	.837
Neuroendocrine carcinoma	4/4	100.0	94.24-100.0	
FIGO stage				
Stage I	230/245	93.87	89.42-96.91	
Stage II	242/249	97.18	93.62-99.00	
Stage III	27/28	96.42	90.32-99.42	.687
Stage IV	22/22	100.0	97.76-100.0	
HPV status^d				
HPV-positive	510/518	98.45	92.72-99.46	
HPV16	372/379	98.15	92.52-99.32	
HPV18	34/36	94.44	91.60-98.72	.465 ^a
HPV31	19/20	95.00	92.31-96.42	.587 ^b
HPV33	16/17	94.11	90.22-95.41	
Other hr-HPV	61/66	92.42	81.35-94.15	
HPV-negative	19/26	73.07	56.85-86.82	
Sample type				
Cervical scrape	371/384	96.61	91.70-98.62	
FFPE tissue	150/160	93.75	90.82-96.62	.917
Age (years)				
<25	6/7	85.71	65.55-90.22	
25-29	36/38	94.73	90.45-98.65	
30-39	128/133	96.24	91.24-98.75	
40-49	148/154	96.10	92.54-98.12	.989
50-59	122/129	94.57	89.79-96.12	
>60	81/83	97.59	94.05-98.92	
Country of origin				
Bhutan	47/50	94.00	92.32-96.82	
Colombia	40/46	86.95	78.35-92.92	
Ethiopia	68/70	97.14	94.92-98.96	
Georgia	40/42	95.23	92.98-97.35	
India	48/50	96.00	94.12-97.59	.892
Philippines	48/50	96.00	94.12-97.59	
South Africa	49/49	100.0	98.88-100.0	
Spain	48/50	96.00	94.12-97.59	
United Kingdom	48/51	94.11	93.72-96.59	
United States (New Mexico)	84/86	97.67	94.72-98.68	
Total	521/544	95.77	92.39-97.40	

Note: FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; n = number of positive samples in a specified group; N = group total.

Abbreviations: CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus.

^aP value among all subgroups in the HPV-positive group.

^bP value between HPV-positive and HPV-negative subgroups.

^chrHPV genotype grouping performed by hierarchical genotype attribution, as detailed in Section 2.

^dDetermined by performing Fisher's exact test of independence.

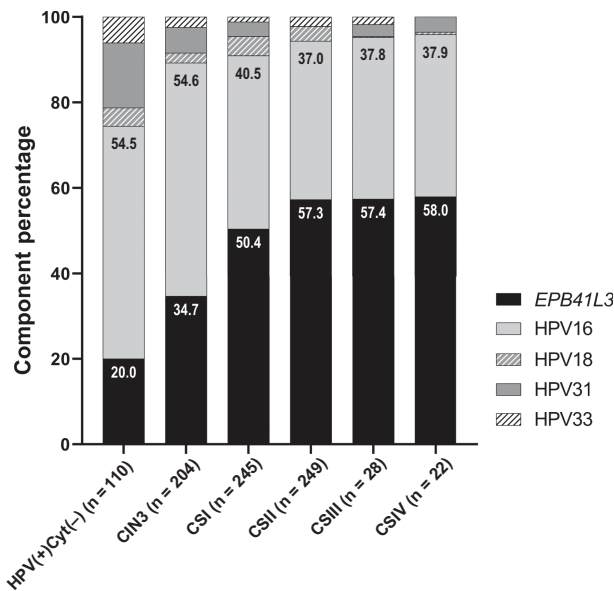


FIGURE 3 S5 component breakdown in HPV+ NEG, CIN3 and CSI-IV samples. The weight of the methylation on the S5 components: EPB41L3, HPV16, HPV18, HPV31 and HPV33 has been calculated for each group. Percentages of EPB41L3 and HPV16 weights in the classifier are noted at the top of the corresponding bars. HPV methylation becomes less important vs EPB41L3 as the lesions become more advanced; however, EPB41L3 weight in the classifier does not change after Stage II cancer. CIN, cervical intraepithelial neoplasia (of Grade 3); CSI-IV, cervical cancer stages I-IV; HPV, human papillomavirus

The same trend was observed in an approximated triage population. Here, specificity increased from 50.60% (95% CI = 43.11-58.06) at 0.80 to 83.33% (95% CI = 76.97-88.21) at 3.70.

A total of 521 out of 544 women with any cancer type tested positive for S5 at the 3.70 cut-off, yielding a sensitivity of 95.77% (95% CI = 92.39-97.40). A lower proportion of the hrHPV-negative cancer group tested S5 positive at a cut-off 3.70 compared to the hrHPV-positive cancer group: 73.07% (95% CI = 56.85-86.82) vs 98.45% (95% CI = 92.72-99.46) (Fishers' test, $P < .0001$) as described in Table 4. There were no other significant differences in S5 sensitivity among histology, FIGO stage, hrHPV type, sample type, age and country of origin. These results indicate a highly consistent performance of the S5 classifier in patients from different continents with respect to detection of cancer.

3.8 | S5 classifier components proportion vary with disease severity

On a component basis, individual EPB41L3 methylation was observed to increase in a sigmoidal curve with disease progression. The Cuzick test for trend was significant for HPV(-)/Cyt(-), CIN3, CSI and CSII ($P < .0001$). The second most important component of the S5 classifier, HPV16 methylation, showed an increasing trend with disease progression (Cuzick test for trend, both, $P < .001$) as well as a steep

increase from CIN3 to CSI-IV. Additionally, both HPV18 and HPV33 methylation showed a linear increase with disease progression (Cuzick test for trend, both, $P < .01$) as described in Figure S3.

The weight of each component of the S5-classifier was plotted for HPV(+)/Cyt(-), CIN3 and the cancer CSI-IV groups in Figure 3. The Cuzick test for trend showed an increasing trend of EPB41L3 weight with disease lesion, $z = 8.21$ ($P < .0001$). EPB41L3 weight plateaus at CSII. Unconditional logistic regression models showed the strength of the association between EPB41L3 methylation, severity of lesion and age. The relationship between EPB41L3 methylation was stronger for severity of lesion: $F = 367.50$, $P < .0001$ than age ($F = 81.0$, $P < .0001$). This indicates that host EPB41L3 methylation might have a good potential to predict disease progression, independent of increasing natural epigenetic methylation levels occurring with age.

Interestingly, the relative proportion of the HPV components of the S5-classifier decreased slightly with severity of lesion (Cuzick test for trend, $z = -6.52$, $P < .0001$). HPV16 had the highest weight out of all viral components; however, this was 1.8 times lower than the weight of EPB41L3 in CSII+ specimens.

4 | DISCUSSION

To our knowledge, our study represents one of the most comprehensive assessments of viral and host cell DNA methylation data in invasive cervical cancer to date, particularly given its multi-site dimension(s) and number of cases of high grade and invasive disease.²⁷ We show that there is a strong increasing trend of S5 DNA methylation score with cervical disease severity in our global collection of samples. Our results also show a very high S5 sensitivity for CIN3+ at the predefined cut-off of 0.80, while there was high to moderate sensitivity at the exploratory cut-offs of 2.62 and 3.70, respectively.

We found an S5 test sensitivity of 91.18% (186/204) for CIN3 and 99.81% (543/544) for cervical cancer detection at the predefined cut-off of 0.80.⁹ At this cut-off, S5 sensitivity for cervical cancer was higher than the sensitivity of HPV DNA testing: 95.21% (518/544) (McNemar test $\chi^2 = 5.08$, $P = .032$). Additionally, at the S5 cut-off of 3.70, previously demonstrated useful in LMIC settings,¹² we found a sensitivity of 62.74% (128/204) for CIN3 and 95.77% (521/544) for cervical cancer.

This study complements previous results in Colombia,¹⁰ Canada¹¹ and Mexico,¹² by including data on cancers from additional countries and thus describing a larger study for cervical cancer identification. A recent Dutch study of 519 cervical cancer samples, FAM19A4/miR124-2 methylation analysis yielded a sensitivity of 98.30% (510/519).²⁷ Additionally, the four-gene methylation marker panel comprising of the host genes JAM3, EPB41L3, TERT and C13ORF18 identified 94.20% (65/69) of cervical cancers.²⁸ S5 demonstrated a slightly higher sensitivity of 99.81% (543/544) compared to the above-mentioned tests ($P = .047$ and $P = .029$, respectively). Most importantly, S5 detected 25 out of 26 hrHPV-negative cancers, which were not explored in other studies.

We explored the performance of the S5 classifier at three cut-offs: 0.80, 2.62 and 3.70. At cut-off 0.80, 26.32% of HPV(-)Cyt(-)

women tested positive for S5, which indicates either a potential specificity issue of the tool in our selected group of HPV(–)Cyt(–) women or a higher than expected prevalence of occult CIN in these women. The lowest false positive rate was observed at the 3.70 cut-off (0.92%), a cut-off at which 95.77% (521/544) of cancer cases were still identified. A similar trend was observed with CIN3 cases (Table S2). Due to the referral nature of the samples included, exact specificity values could not be calculated; however, estimated values are provided in Table S3.

Our data on S5 sensitivity combined with our earlier results from studies in the United Kingdom, Canada, Mexico and Colombia suggest that the prevalence of HPV infection as well as the difference in screening capacity and performance of populations can affect disease prevalence, thereby arguably the optimal cut-off of S5 could be made ‘setting specific’.^{9–12} An increased cut-off with a lower number of false positives rate would maximise the detection of cancer, which is favourable in countries with minimal screening resources such as in LMICs. Indeed, in settings with no or patchy screening programmes, an increased cut-off might be justified.^{11,12,29}

The major strengths of our study are its size, the incorporation of sample set that reflects diverse settings from 10 different countries spanning five continents. Our study highlights the general trend of increasing DNA methylation with disease progression.

Although HPV infection is an important co-factor in cervical cancer development, a small proportion of cervical cancer samples in the study tested hr-HPV negative as confirmed by HPV testing with multiple assays. Though rare, these cases represent a challenge for detection in the current primary hr-HPV screening programme. Our data show that nearly all of these cancers (25/26) were identified by the S5 classifier at a cut-off of 0.80. Regardless of the cut-off examined, performance of the S5 classifier was uniform among the stratified groups: histology, FIGO stage, hrHPV status, hrHPV type, sample type, age and country of origin.

A limitation to our study is that all CIN3 and cervical cancer cases come from referral populations and do not accurately represent those that may be apparent in the screening population or those that do not present to clinics. The proportion of rare histological subtypes in our study was also small, so this element would benefit from further investigation. Moreover, much more emphasis was placed on cancers FIGO stage I and II as previously published data indicate that aberrant methylation is an early event in cervical carcinogenesis.^{14,29} An intentional limitation of our study is that we excluded CIN1 and CIN2 which would be present in a real-world setting. Addition of these samples to our study in realistic proportions would likely lower the sensitivity and specificity of the S5 test. There is a further limitation in our selection of the cytology negative women who were presumed to have no disease on the basis of cytological testing. Although we divided these women into HPV+ and HPV– groups, these women may not be representative of the routine screening populations in many geographic locations including in Europe and the United States. Therefore, the aim of our present work was to assess a larger panel of CIN3+ samples to confirm the sensitivity and robustness of the assay for the detection of significant disease.

The present findings highlight the major contribution of host *EPB41L3* methylation in the S5 score. We showed that the relationship between *EPB41L3* methylation was approximately 4.5 times stronger for severity of lesion than age ($P < .0001$). This indicates that host *EPB41L3* methylation might have a strong potential to predict disease progression, independent of increasing natural epigenetic methylation levels occurring with age. This is in line with previously published data on the S5 where it better identified women with CIN2 that were more likely to progress to higher stages of the disease.⁸ Additionally, the weight of *EPB41L3* methylation shows an increasing trend ($P < .0001$, Cuzick test for trend), up to cervical cancer FIGO stage II, where it plateaus. However, the strength of this observation is limited by the decreased number of cervical cancer samples of FIGO stage III and IV, included in the study.

The COVID-19 pandemic points towards a shift to self-sampling for hrHPV primary screening to reduce the burdens on the healthcare professionals and access women who do not respond to screening invitations. Having the possibility to triage hrHPV-positive women from the same self-collected specimen would bring many advantages including a reduction in logistical issues associated to systematic screening as well as reducing the subjectivity of cytology.^{30–34} A pilot study tested the accuracy of S5 classifier in cervical self-samples. S5 showed a statistically significant separation between <CIN2 and CIN2 + samples for both urine and cervical self-samples ($P \leq .0001$). At the pre-defined cut-off of 0.80, the sensitivity for cervical self-samples was 71% and specificity 68% and for urine samples was 66% and specificity 72%.³³

In conclusion, our study shows that the S5 classifier at a cut-off of 0.80 identifies more than 90% CIN3 cases and almost 100% of cervical cancers, independent of histology, FIGO stage hrHPV status, hrHPV genotype, sample type and geographical origin. Adjustment of the cut-off leads to an increase in specificity with only a small decrease in sensitivity. The 3.70 cut-off could allow for a better triage modality for LIMC where screening is not performed as systematically as in higher income countries. Additionally, high methylation levels on the host gene component of the S5 classifier, *EPB41L3* is associated with higher severity of the disease, indicating prognostic potential. Thus, considering the growing acceptability of self-sampling, our results support the utility of the S5 classifier as a credible tool for enhanced risk stratification of women in cancer screening programmes.

ACKNOWLEDGEMENTS

We acknowledge Dr Daniel Seifu from the Department of Biochemistry, Global Health Equity, Kigali, Rwanda, Dr Yirgu Gebrehiwot from the Department of Obstetrics and Gynecology, Addis Ababa University, Addis Abbaba, Ethiopia and Dr Vanessa Tenet from the International Agency for Research on Cancer (IARC), World Health Organization; Lyon France for their contribution in collecting the samples included in the study. We also acknowledge Cancer Research UK who funded part of this study (Cancer Prevention programme Grant, C27045/A27046/CRUK_/CancerResearchUK/UnitedKingdom).

CONFLICT OF INTEREST

Kate Cuschieri reports her employer having received research funding or gratis consumables to support research from the following in the last 3 years: Cepheid, Furoimmun, Genefirst, Selfscreen, Hiantis, Seegene, Roche, Abbott, Hologic. Cosette M. Wheeler reports receiving the following outside the submitted work: cooperative agreements and grants from the US National Institutes of Health for research on cancer prevention and sexually transmitted infections, reagents and equipment from Roche Molecular Systems, Roche/Ventana Medical Systems, Hologic and Genera Biosystems, research funding from Hologic and Becton Dickinson (BD) and personal fees from BD. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The samples used for this study have received ethical and research approvals from: (a) The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) for all samples from Bhutan, Colombia, Georgia, India and Philippines, South Africa and Spain; (b) The Scottish HPV Archive, MRC Centre for Reproductive Health, The University of Edinburgh for all samples from the United Kingdom; (c) Institutional Review Board of College of Health, Addis Ababa University for all samples from Ethiopia; (d) University of New Mexico Human Research Review Committee for all samples from United States – New Mexico. The study protocol has been approved by Queen Mary University of London's Ethical Committee (MTA-2018-ICE-0426, MTA-2018-ICE-1353, 11/06/2018).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Banila C, Lorincz AT, Scbior-Bentkowska D, et al. Clinical performance of methylation as a biomarker for cervical carcinoma in situ and cancer diagnosis: A worldwide study. *Int. J. Cancer.* 2021;1-13. doi: 10.1002/ijc.33815