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

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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.
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.6 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.5-8 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 and to complement previously published 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
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.

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

| Characteristics | HPV(−)/Cyt(−) * | HPV(+) / Cyt(−) * | CIN3 * | Cervical cancer – FIGO a stage |
|-----------------|-----------------|------------------|--------|-----------------------------|
|                  | n = 110 (%)     | n = 110 (%)      | n = 204 (%) | 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.
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 unmethylated 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 HPV16L2 (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 weighted average defined in 2016 by Lorincz et al9:

\[
S5 = 30.9 \left( EPB41L3 \right) + 13.7 \left( HPV16L1 \right) + 4.3 \left( HPV16L2 \right) + 8.4 \left( HPV18L2 \right) + 22.4 \left( HPV31L1 \right) + 20.3 \left( HPV33L2 \right).
\]

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 (CSI), III (CIII) 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 predefined 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 α ≤ .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(−)/C0 (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 polimerase 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 60).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 weighted average defined in 2016 by Lorincz et al9:

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\]

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 (CSI), III (CIII) 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 predefined 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 α ≤ .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.
3.2 | 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 were 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 (CSIIR) 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 HPV(−)/Cyt(−) vs HPV(+)/Cyt(−) vs CIN3.
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](image-url)
# 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 |              |
| Adenocarcinoma         | 29/29 | 100.0      | 97.34–100.0 | 0.837 |
| 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 |              |
| Stage II               | 249/249 | 100.0      | 99.43–100.0 | 0.687 |
| 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 |              |
| HPV16                  | 379/379 | 100.0      | 99.36–100.0 | 0.465\(a\) |
| HPV18                  | 36/36 | 100.0      | 98.82–100.0 | 0.587\(b\) |
| 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 | 0.917 |
| FFPE tissue            | 160/160 | 100.0      | 98.76–100.0 |       |
| **Age (years)**        |      |            |         |              |
| <25                    | 7/7   | 100.0      | 64.87–100.0 |       |
| 25-29                  | 38/38 | 100.0      | 98.62–100.0 |       |
| 30-39                  | 133/133 | 100.0     | 99.32–100.0 | 0.989 |
| 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 |       |
| 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 | 0.892 |
| 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.

\(\text{\(P\) value between HPV-positive and HPV-negative subgroups.}\)

\(\text{\(P\) value among all subgroups in the HPV groups.}\)

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

Determined by performing Fisher’s exact test of independence.
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 $\chi^2 = 27.1, P < 0.0001$) 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, $P < 0.0001$). 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, $P < 0.0001$). 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, $P = 0.005$).

### 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 ($P < 0.0001$). 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, $P < 0.0001$). 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 ($P < 0.0001$), 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

| Histotype of cervical cancer                  | n/N   | Percentage | 95% CI       | P value |
|----------------------------------------------|-------|------------|--------------|---------|
| 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                                  | n/N   | Percentage | 95% CI       | P value |
|----------------------------------------------|-------|------------|--------------|---------|
| 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 statusc                                 | n/N   | Percentage | 95% CI       | P value |
|----------------------------------------------|-------|------------|--------------|---------|
| 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  | .465a   |
| HPV31                                        | 19/20  | 95.00      | 92.31-96.42  | .587b   |
| 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                                  | n/N   | Percentage | 95% CI       | P value |
|----------------------------------------------|-------|------------|--------------|---------|
| Cervical scrape                              | 371/384 | 96.61      | 91.70-98.62  | .917    |
| FFPE tissue                                  | 150/160 | 93.75      | 90.82-96.62  |         |

| Age (years)                                  | n/N   | Percentage | 95% CI       | P value |
|----------------------------------------------|-------|------------|--------------|---------|
| <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                            | n/N   | Percentage | 95% CI       | P value |
|----------------------------------------------|-------|------------|--------------|---------|
| 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.

<sup>a</sup>P value among all subgroups in the HPV-positive group.
<sup>b</sup>P value between HPV-positive and HPV-negative subgroups.
<sup>c</sup>hrHPV genotype grouping performed by hierarchical genotype attribution, as detailed in Section 2.
<sup>d</sup>Determined by performing Fisher’s exact test of independence.
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 = 92.39-98.30) at 3.70.

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, and the cancer CSI-IV groups in Figure 3. The Cuzick test for trend showed an increasing trend of EPB41L3 weight with disease severity, $z = 8.21$ ($P < .0001$). EPB41L3 weight plateaus at CSI II. Unconditional logistic regression models showed the strength of the association between EPB41L3 methylation, severity of lesion, and the relationship between EPB41L3 methylation was stronger for severity of lesion: $F = 367.50$, $P < .0001$ than age ($F = 81.00$, $P < .0001$). 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 CSI II+ 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. 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. 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, 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 and Canada 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). Additionally, the four-gene methylation marker panel comprising of the host genes JAM3, EPB41L3, TERT and C13ORF18 identified 94.20% (65/9) of cervical cancers. 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’. 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.

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. 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. 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. 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 ≤ .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%.

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 LMIC 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.

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