Effective methylation triage of HPV positive women with abnormal cytology in a middle-income country

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Abstract
The S5-methylation test, an alternative to cytology and HPV16/18 genotyping to triage high-risk HPV-positive (hrHPV+) women, has not been widely validated in low-middle-income countries (LMICs). We compared S5 to HPV16/18 and cytology to detect cervical intraepithelial neoplasia Grade 2 or worse (CIN2+) and CIN3+ in hrHPV+ women selected from a randomized pragmatic trial of 2661 Colombian women with an earlier-borderline abnormal cytology. We included all hrHPV+ CIN2 and CIN3+ cases (n = 183) age matched to 183 <CIN2 hrHPV+. Baseline specimens were HPV-genotyped and tested by S5-methylation, blinded to cytology, histology and initial HPV results. We evaluated the test performance of predefined S5-classifier (cut-point 0.8) and a post hoc classifier at a different cut-point (3.1). S5 sensitivity for CIN2+ was 82% (95% confidence interval CI 76.4-87.5) and for CIN3+ 77.08% (95% CI 65.19-88.97). S5 sensitivity was higher than HPV16/18 sensitivity (48.1%, 95% CI 40.85-55.33) or cytology (31.21%, 95% CI 24.50-37.93) but with lower specificity (35%, 95% CI 28.1-42). At cut-point 3.1, S5 sensitivity for CIN2+ (55.2%, 95% CI 48-62.4) or CIN3+ (64.6%, 95% CI 51.0-78.1) was also superior to HPV16/18 (P < .05) or cytology (P < .0001). At this cut-point S5 specificity (76%, 95% CI 69.8-82.1) for <CIN2 was higher than HPV16/18 (67.21%, 95% CI 60.41-74.01, P = .0062) and similar to cytology (75.57%, 95% CI 69.34-81.79, P = 1). HPV16/18 plus cytology sensitivity was similar to S5 for CIN3+, however, false-positive rate was higher (50.27% vs. 24.04%). High sensitivity is crucial in LMICs, S5-methylation exceeded HPV16/18 or cytology sensitivity with comparable specificity for CIN2+ and CIN3+ in hrHPV-

Abbreviations: ADC, adenocarcinoma; ASC-US, atypical squamous cells of undetermined significance; CIN2+, cervical intraepithelial neoplasia Grade 2 or worse; CIN3+, cervical intraepithelial neoplasia Grade 3 or worse; hrHPV+, high-risk HPV-positive; LMICs, low-middle-income countries; LBC, liquid-based cytology.
positive Colombian women. Furthermore, S5 triage had comparable sensitivity and significantly fewer false positives than cytology and HPV16/18 combination.

KEYWORDS
biomarkers, cervical cancer, DNA methylation, HPV test, triage

1 INTRODUCTION

Cervical cancer is a leading cause of cancer death for women living in low and middle income countries (LMICs) where 95% of the approximately 570,000 new cases and 311,000 deaths (estimated by Globocan for 2018) occur. High-risk human papillomavirus (hrHPV), a common sexually transmitted infection worldwide is the cause of almost all cervical cancer. Only a small proportion of hrHPV infections persist and develop into cervical intraepithelial neoplasia (CIN) Grade 2 or 3 (CIN2 and CIN3) which may, if left untreated, progress to cancer.5

In 2018, the World Health Organization issued a call for action to eliminate cervical cancer by a comprehensive approach that includes increasing HPV vaccine coverage and screening of women aged more than 30 years with hrHPV testing followed by treatment of hrHPV-positive (hrHPV+) women that in the visual inspection are suspicious of cervical cancer precursor lesions.3 hrHPV DNA testing has greater sensitivity and negative predictive value for detecting cervical intraepithelial neoplasia Grade 2 or worse (CIN2+) and CIN3+ (more reproducible and definitive surrogate endpoint of cervical cancer risk) than cytology,4 which permits longer screening intervals and more cost-effective prevention programs.5 Because HPV-based screening provides 60% to 70% greater protection against invasive cervical carcinomas compared to cytology,6 it is expected that HPV-based screening would result in a lower incidence of and mortality from cervical cancer. hrHPV testing is the most effective approach for reducing cervical cancer mortality especially in LMICs, where improvement of quality of cytology remains a challenge and the number of life-time screening visits is low.5 However, hrHPV testing has low specificity, which can increase referrals to colposcopy, leading to more anxiety and overtreatment of women with nonprogressive disease.7

Approaches to stratify (triage) hrHPV+ women include conventional (Pap smear), liquid-based cytology (LBC), without or with adjunctive p16/Ki67 double staining (herein p16/Ki67 cytology) and HPV16/18 genotyping. Reassurance provided by cytology against false negatives is low8 and a high proportion of women hrHPV+ and cytology negative at screening need further follow-up. p16/Ki67 has both higher sensitivity and specificity than cytology testing for triage of HPV-positive women and negative results have greater reassurance against CIN2+ than negative cytological results,9 but as with cytology, good results depend on expert visual interpretation of well-preserved morphological specimens from LBC, which is an expensive and subjective expertise not widely available in LMICs. PCR-based HPV16/18 genotyping is a robust, operator independent assay without extra requirements for sample preservation; however, it reaches a sensitivity of only 50% to 60%10 and misses all the CIN2+ in women positive for the other 11 hrHPV types.

Deregulated expression of hrHPV E6 and E7 genes can induce uncontrolled cell cycle progression and favors the development of persistent hrHPV infection and precancer. This process coincides with a decline in activity of the late capsid genes promoter (L1 and L2), which shows a higher methylation level in hrHPV+ women diagnosed with CIN2+.11 In addition, changes in the levels of methylation of CpG sites in promoters or introns of host-cell genes such as EPB41L3, JAM3, TERT, CADM1, MAL, mir124 and FAM19A4 are also associated with higher risk of cervical cancer and its precursor lesions.12 Methylation assays can be automated, have accurate quantitation, are robust to operator variations and can be performed in the same specimen as the screening hrHPV tests. These attributes offer opportunities to develop automated high throughput methylation tests with consequent simplification of currently available triage algorithms.

The S5 classifier is a test based on DNA methylation of the late regions L1 and L2 of HPV16, HPV18, HPV31 and HPV33 combined with the promoter region of human tumor suppressor gene EPB41L3 that identifies women with CIN2+.13 At sensitivities of 90% (95% confidence interval [CI] 87-92) or 74% (95% CI 59-85), S5 showed a specificity of 49% (95% CI 46-52) or 65% (95% CI 60-70) for the detection of CIN2+ in women who attended colposcopy13 or screening14 health services in London, United Kingdom, respectively. The S5 classifier showed similar performance in samples selected from women attending primary cervical screening in Canada.15
Although earlier studies in developed countries have shown that the performance of the S5 classifier to detect CIN2+ is superior to that of HPV16/18 genotyping and similar to complex triage strategies such as a combination of repeated LBC and HPV genotyping, there are few studies validating S5 in LMIC settings. Thus, we compared the performance of the S5 DNA methylation classifier with repeated conventional cytology at 6 and 12 months and baseline HPV16/18 genotyping for detection of 2-year endpoint CIN2+ and CIN3+ in hrHPV+ women selected from a pragmatic trial (atypical squamous cells of undetermined significance (ASC-US-COL) trial) that recruited women from routine opportunistic screening services of Colombia, a middle-income country.

2 | MATERIALS AND METHODS

2.1 | ASC-US-COL study design

ASC-US-COL is a three-arm, nonblinded, parallel group, pragmatic trial. Women aged 20 to 69 years (n = 2661) with a first time ASC-US cytology in the last 2 years were flagged in routine screening services and randomly allocated to receive immediate colposcopy (IC arm; n = 882), repeat cytology at 6 and 12 months (RC arm; n = 890) or an HPV test within 2 months of recruitment (HPV arm; n = 889). Colposcopy and biopsies according to clinician judgment were recommended for all women in the IC arm, for women with a repeat ASC-US or worse (ASC-US-positive) in the RC arm and for hrHPV+ women in the HPV arm. Hybrid Capture 2 HPV DNA test (HC2©, Qiagen, Germantown, MD) with a relative light unit/cutoff (RLU/Co) ratio of ≥1 for considering an HPV result positive, was conducted at the laboratory of Infection and Cancer, University of Antioquia, Colombia. All women received invitation for and 80% (n = 2132 women) attended the exit visit after 24 months of follow-up which included hrHPV and cytology tests. All women positive for either test were referred to a certified, well-trained colposcopist using a standardized and controlled protocol of biopsy sampling. At the end of the study, two blinded accredited experts confirmed histopathological diagnoses and baseline samples of women from IC and RC arms were tested for hrHPV by HC2© (Qiagen).

2.2 | Selection of methylation sub-study participants

Cases were women identified after the end of the ASC-US-COL trial as women who had hrHPV+ test results at baseline (recruitment visit) and with a colposcopy-directed biopsy diagnosis of CIN2, CIN3 or carcinoma in situ, adenocarcinoma (ADC) or squamous cell carcinoma (SCC) at any time during the 2-year follow-up. Controls were randomly selected regardless of arm allocation from women who were hrHPV+ at baseline, had a biopsy with a diagnosis of less than CIN2 (<CIN2) during the follow-up confirming that they were at low risk of cervical cancer and with enough remainder of archived baseline samples in specimen transport medium (STM; Qiagen) for further testing. Controls were individually matched to cases by age and time to diagnosis (±12 months). As shown in the flowchart (Figure 1), 185 cases (137 CIN2, 44 CIN3 and 4 SCC) and 185 matched controls (143 biopsies negative and 42 CIN1) were identified for the study. The CLART HPV4 (Clinical Array Technology, Genomica, Madrid, Spain) test was used for HPV genotyping at the Queen’s Medical Research Institute of The University of Edinburgh (Edinburgh, United Kingdom). Two CIN2 cases that were inadequate in the HPV genotyping test and their corresponding controls (biopsy negative) were excluded. There were no equivocal results in S5 methylation assays, therefore 183 pairs were evaluated by all tests.

2.3 | Methylation study specimen characteristics

Exfoliated cervical cells in the ASC-US-COL trial were collected in STM and immediately stored at −30°C. Samples were thawed and denatured at the time of HC2 testing. DNA was extracted from 200 μL of the residual content. Briefly, STM specimens were digested for 2 hours at 55°C in the presence of 200 μg/mL of proteinase K and 1% Laureth-12. Samples were centrifuged for 30 seconds at maximum speed in an Eppendorf microcentrifuge and then heated to 95°C for 10 minutes. After precipitation with 0.5 M ammonium-acetate and 70% ethanol, DNA was washed, dried and resuspended in 100 μL of TE buffer (10 mM TRIS/0.1 mM EDTA). DNA quality and quantity were assessed using Nanodrop and PCR amplification for the MID856 variant as internal control. Isolated DNA was stored at −30°C and shipped frozen on dry ice to Queen Mary University of London for S5 methylation testing.

2.4 | HPV genotyping

HPV genotyping was independently and blindly conducted on 5 μL of a 1:10 dilution of each specimen using the CLART HPV4 test (Genomica). This test uses biotinylated MY09/11 consensus primers and detects 13 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), previously misclassified low risk type HPV66 and 21 low-risk types (26, 53, 70, 73, 82, 85, 6, 11, 40, 42, 43, 44, 54, 61, 62, 71, 72, 81, 83, 84, 89). High proficiency of the detection of the PCR products by the low-density microarray platform CLART has been reported.

2.5 | S5 DNA methylation classifier testing

Methylation assays were based on end-point PCR and quantitative pyrosequencing of amplicons using primers for six target regions covering in total 22 CpG positions of human gene EPB41L3 and the late (L1 and L2) regions of HPV16, HPV18 and HPV31 and HPV33. As detailed previously. Briefly, 100 ng of DNA was used for bisulfite conversion where unmethylated cytosines were converted to uracil with the EZ-DNA methylation kit (Zymo Research, Irvine, CA).
Converted DNA (2 μL/sample) was added to PCR master mix and amplified by methylation independent PCR primers. Quality of the amplified DNA was confirmed on a QIAxel capillary electrophoresis instrument (QIAGEN). Twenty-five microliters of PCR product were used for pyrosequencing (PyromarkQ96 ID Platform, QIAGEN, Germany). The S5 score was defined as 30.9(EPB41L3) + 13.7(HPV16L1) + 4.3(HPV16L2) + 8.4(HPV18L1) + 22.4(HPV31L1) + 20.3(HPV33L2) using the percentage of individual CpG sites methylated as described previously.13

2.6 | Statistical methods

The analysis was based on a prespecified analytical plan. The primary hypothesis was that using the hrHPV+ baseline samples, S5 at the standard predefined cut-point 0.813 can distinguish between <CIN2 and CIN2+ (includes CIN2, CIN3 and cancer) and also have a very high sensitivity for CIN3+. We also compared the sensitivity of S5 with the other tests at a specificity of 76% that corresponds to using a positivity threshold of 3.1. We used this threshold for S5 because it gave the same specificity as cytology, the currently recommended triage test for hrHPV+ women in the Colombian screening guidelines. The specificity of S5 for CIN3+ was calculated by omitting CIN2 cases because this is an intermediate category and it is not correct to combine it with the normal and CIN1 diagnoses, which have minimal risks for cervical cancer. The Cuzick test for trend was used to investigate continuous changes in methylation with increasing lesion severity. The sensitivity and specificity of cytology was determined using the highest-grade cytology from the 6 and 12-month samples after the initial entry ASC-US cytology, at an ASC-US-positive threshold. The performance of continuous risk scores was measured by area under the curve (AUC) with a Wilcoxon test and DeLong confidence intervals.21 Differences in sensitivities and specificities of S5 with the other tests were examined by McNemar’s test with continuity correction. Statistical analysis was conducted in R version 3.5.2.22 A P value of <.05 was considered significant.

3 | RESULTS

3.1 | Characteristics of study population

Figure 1 shows that 5 of the 2661 women included in the ASC-US-COL trial did not have an HC2 HPV DNA test result and that 364 (41%) women in the IC arm, 396 (44.5%) in the RC arm and 362 (40.7%) in the HPV arm were hrHPV+. Among the 1122 hrHPV+ women, 734 (65.4%) had an adequate histological diagnosis, 549 of which had a diagnosis of <CIN2 (404 negative and 145 CIN1) and 185 of CIN2+ (137 CIN2, 44 CIN3 and 4 cancers). After exclusion of four samples (two cases inadequate in the HPV genotyping test and corresponding controls, 34.2% (138/404), 31.0% (45/145), 98.5% (135/137) and 100% (48/48) of women with negative, CIN1, CIN2 or CIN3/SCC histopathological diagnosis were included in this
analysis. Table 1 describes the characteristics of the included 183 CIN2+ cases and 183 <CIN2 controls. Cases and controls had similar age, time to diagnosis (±12 months), age distribution at first sexual intercourse, number of sexual partners, distribution of cytological interpretations and arm allocation. HPV16 was confirmed in 131 (35.8%), HPV18 in 24 (6.6%), HPV31 in 47 (12.8%) and HPV33 in 23 (6.3%) of all samples. Eighty-two (44.8%) of CIN2+ were positive for HPV16, 10 (5.5%) for HPV18, 24 (13.1%) for HPV31 and 17 (9.3%) for HPV33.

### Table 1 Description of study population

| Characteristic                                | Control (n = 183) | Case (n = 183) | P valuea |
|-----------------------------------------------|-------------------|---------------|----------|
| Number                                        | n (%)             | n (%)         |          |
| Age (years)                                   |                   |               |          |
| 20-29                                         | 91 (49.73)        | 80 (43.72)    | .2164    |
| 30-39                                         | 50 (27.32)        | 66 (36.07)    |          |
| 40-49                                         | 34 (18.58)        | 26 (14.21)    |          |
| ≥50                                           | 8 (4.37)          | 11 (6.01)     |          |
| Time to histological diagnosis (months)       |                   |               | .0604    |
| 1-12                                          | 109 (59.56)       | 98 (53.55)    |          |
| 13 to >18                                     | 74 (40.43)        | 85 (46.44)    |          |
| Age of first sexual intercourse (years)       |                   |               | .0595    |
| ≤15                                           | 42 (22.95)        | 58 (31.69)    |          |
| 16-19                                         | 102 (55.74)       | 100 (54.64)   |          |
| ≥20                                           | 39 (21.31)        | 25 (13.66)    |          |
| Number of sexual partners                     |                   |               | .659     |
| 1-3                                           | 94 (51.37)        | 87 (47.54)    |          |
| 4-5                                           | 40 (21.86)        | 47 (25.68)    |          |
| ≥6                                            | 49 (26.78)        | 49 (26.78)    |          |
| hrHPV types frequency                         |                   |               |          |
| HPV 16                                        | 49 (26.78)        | 82 (44.81)    | .0005    |
| HPV 18                                        | 14 (7.65)         | 10 (5.46)     | .5264    |
| HPV 31                                        | 23 (12.57)        | 24 (13.11)    | 1        |
| HPV 33                                        | 6 (3.28)          | 17 (9.29)     | .0312    |
| Other hrHPV typesb                            | 142 (77.6)        | 124 (67.76)   | .0461    |
| Cytology                                      |                   |               | .1678    |
| NEG                                           | 133 (72.68)       | 119 (65.03)   |          |
| LSIL                                          | 23 (12.57)        | 28 (15.3)     |          |
| HSIL                                          | 0 (0)             | 3 (1.64)      |          |
| ASC-US                                        | 20 (10.93)        | 20 (10.93)    |          |
| ASC-H-AGC/ASC-H                              | 0 (0)             | 3 (1.64)      |          |
| Missing                                       | 7 (3.83)          | 10 (5.46)     |          |
| Arm                                           |                   |               | .6674    |
| RC                                            | 58 (31.69)        | 65 (35.71)    |          |
| IC                                            | 64 (34.97)        | 57 (31.32)    |          |
| HPV                                           | 61 (33.33)        | 60 (32.97)    |          |

Abbreviations: ASC-H, atypical squamous cells, cannot exclude HSIL; ASC-H-AGC, atypical squamous cells, cannot exclude HSIL or atypical glandular cells; ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus test; HSIL, high grade squamous intraepithelial lesion; IC, immediate colposcopy; LSIL, low grade squamous intraepithelial lesion; NEG, negative; RC, repeat cytology.

3.2 | **EPB4IL3, HPV16**_L1_ and S5 methylation patterns were related to the severity of CIN

Median S5 methylation (Figure 2) increased proportionally (Cuzick trend test $\chi^2 = 45.1, P < .0001$) with histopathological diagnosis: 1.0 in histology Negative (n = 138), 1.4 in CIN1 (n = 45), 3.4 in CIN2 (n = 135), 7.1 in CIN3 (n = 44) and 10.8 in cancer (n = 4). Figure 3 shows similar patterns of methylation levels increasing by lesion severity for **EPB4IL3** (Figure 3A, Cuzick trend test $\chi^2 = 23.47,$
$P < .001$) and HPV16L1 individually (Figure 3B, Cuzick trend test $\chi^2 = 25.4, P < .001$).

### 3.3 Performance of the S5 classifier to detect CIN2+ or CIN3+

The receiver operating characteristic curve showed that S5 had an AUC of 0.70 (95% CI 0.64-0.75, $P < .0001$) for CIN2+ and of 0.72 (95% CI 0.65-0.80, $P < .0001$) for CIN3+ (Figure 4). Cross tabulation of the number of cases or controls with negative or positive test results and the comparison of sensitivities and specificities of the tests to detect CIN2+ or CIN3+ are shown in Tables 2 and 3, respectively. For CIN2+, the sensitivity of HPV16/18 was 48.1% (95% CI 40.8-55.3) and of cytology was 31.2% (95% CI 24.5-37.9). S5 at the predefined cut-point of 0.8 had a higher sensitivity (82.0%, 95% CI 76.4-87.5) but significantly lower specificity (35.0%, 95% CI 28.1-41.9) than HPV16/18 (67.2%, 95% CI 60.4-74.0, $P < .0001$) or cytology testing (75.6%, 95% CI 69.3-81.8, $P < .0001$). At the cut-point of 3.1 (corresponds to setting the specificity of S5 for <CIN2 at 76% which is equal to cytology specificity), the S5 specificity was significantly higher than HPV16/18 genotyping ($P = .0062$), while the sensitivity of S5 was 55.2% (95% CI 48.0-62.4), which remained significantly higher than sensitivity of HPV16/18 genotyping ($P = .0164$) and of cytology ($P < .0001$).

Likewise to the observations with CIN2+, the sensitivity for CIN3+ of S5 at the cut-point 0.8 (77.1%, 95% CI 65.2-89.0) was higher than the sensitivity of HPV16/18 genotyping (50%, 95% CI 35.9-64.1, $P = .0008$) or cytology (37.0%, 95% CI 23.3-50.6, $P = .0003$). At the cut-point of 3.1, the S5 sensitivity for CIN3+ was 64.6% (95% CI 51.0-78.1) which remained significantly higher than the sensitivity of HPV16/18 ($P = .0233$) and of cytology ($P = .0088$). The combination of cytology with HPV16/18 increased sensitivity to 63.4% (95% CI 56.4-70.4) for CIN2+ and to 64.6% (95% CI 51.0-78.1) for CIN3+ and exceeded the sensitivity of S5 for CIN2+ but not for CIN3+. However, the specificity of the combination of these tests for <CIN2 (49.7%, 95% CI 42.4-56.9) was lower than the corresponding specificity of S5 at the 3.1 cut-point ($P < .0001$).

Combining sensitivity and specificity, the accuracy of S5 at a cut-point of 3.1 for detection of both CIN2+ (Table S1, 0.66, 95% CI 0.60-0.70, $P = .0001$) and CIN3+ (Table S2, 0.75, 95% CI 0.67-0.79, $P = .0033$) was significantly higher than the accuracy of the HPV16/18 test (0.58, 95% CI 0.52-0.63 for CIN2+; 0.63, 95% CI 0.57-0.70 for CIN3+) or when this test was combined with cytology (0.57, 95% CI 0.51-0.62, $P = .0014$ for CIN2+ and 0.53, 95% CI 0.46-0.49, $P < .0001$ for CIN3+).
DISCUSSION

Because HPV testing has higher sensitivity and negative predictive value than cytology for detection of cervical high-grade lesions, it has the potential to increase the detection of disease and lengthen the screening intervals. The World Health Organization recommends visual assessment for treatment after positive HPV DNA testing for populations living in remote areas where there are few opportunities to screen women at proper intervals and for follow-up after screening. Most HPV infections are transient so immediate ablative treatments can lead to unnecessary gynecological harms for women with low risk of disease. For this reason, primary screening with hrHPV testing requires other triage tests to identify women at higher-risk of high-grade disease among those who are hrHPV+. Recalling women for a second test after screening is challenging or impossible in LMICs; therefore, risk stratification should ideally occur at the screening visit in these settings. Hence, these settings have unique needs for triage strategies. Currently, LBC or conventional cytology, p16/Ki67 cytology and HPV16/18 are recommended tests for triage hrHPV+ women, but HPV self-sampling, a strategy that increases screening coverage of women living in rural and dispersed environments, precludes the use of cell-based tests. HPV16, the genotype associated with the greatest elevated risk of cervical high-grade lesions, offers an alternative for immediate identification of women who should be referred for diagnosis and treatment. HPV16/18 reaches a sensitivity of only 50% to 60% and women positive for the other 11 hrHPV types require further management with cytology.

Changes in the levels of methylation of HPV DNA and CpG sites in promoters or introns of host-cell genes are associated with higher risk of cervical cancer and precursor lesions. Recent meta-analysis and systematic reviews have shown that AUC values for methylation of

**FIGURE 4** Receiver operating characteristic (ROC) plot of S5 classifier for (A) CIN2+ and (B) CIN3+. As comparison sensitivity and specificity cut-points of S5 classifier at cutoffs of ≥0.8, or ≥3.1. Cytology (positive = worst cytology repeated at 6 or 12 months after first ASC-US cytology, at a threshold, ASC-US or above), HPV16/18 and cytology plus HPV16/18 are depicted. The CIN2 cases were excluded in the analysis for the CIN3 endpoint, as CIN2 would be treated as false positive when included with <CIN2 [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 2** Number and percentages of <CIN2 controls and CIN2+ and CIN3+ cases with negative or positive tests results

|                  | <CIN2 (n = 183) | CIN2+ (n = 183) | CIN3+ (n = 48) |
|------------------|----------------|----------------|---------------|
|                  | TN n (%)       | FP n (%)       | TP n (%)      | FN n (%)      | TP n (%)   | FN n (%)   |
| S5 different cutoff                        |               |                |               |
| S5 ≥0.8a       | 64 (34.97)    | 119 (65.03)    | 150 (81.97)   | 33 (18.03)    | 37 (77.08) | 11 (22.92) |
| S5 ≥3.1b       | 139 (75.96)   | 44 (24.04)     | 101 (55.19)   | 82 (44.81)    | 31 (64.58) | 17 (35.42) |
| HPV16/18       | 123 (67.21)   | 60 (32.79)     | 88 (48.09)    | 95 (51.91)    | 24 (50)    | 24 (50)    |
| Cytologyc      | 133 (75.57)   | 43 (24.43)     | 54 (31.21)    | 119 (68.79)   | 17 (36.96) | 29 (63.04) |
| Cytology plus HPV16/18d | 91 (49.73)   | 92 (50.27)     | 116 (63.39)   | 67 (36.61)    | 31 (64.58) | 17 (35.42) |

Abbreviations: FN, false negative; FP, false positive; TN, true negative; TP, true positive.

Notes: Positive tests cutoffs: aS5 score ≥0.8 or bS5 score ≥3.1, cfirst ≥ASC-US result of cytology repeated at 6 or 12 months after first time ASC-US cytology. Two CIN3+ and 8 CIN2 cases and 7 <CIN2 controls have missed results for cytology. dPositive for any of the two tests.
| Test                        | CIN2 (n = 183) | CIN2+ (n = 183) | CIN3+ (n = 48) |
|-----------------------------|---------------|----------------|---------------|
| Specificity, % (95% CI)     | P value* S5 ≥ 0.8 vs other tests | P value* S5 ≥ 3.1 vs other tests | P value* S5 ≥ 0.8 vs other tests | P value* S5 ≥ 3.1 vs other tests |
| S5 ≥ 0.8<sup>a</sup>        | 34.97 (28.06-41.88) | NA            | 81.97 (76.40-87.54) | NA |
| S5 ≥ 3.1<sup>b</sup>        | 75.96 (69.76-82.15) | <.0001        | 55.19 (47.99-62.40) | <.0001 |
| HPV16/18                    | 67.21 (60.41-74.01) | <.0001        | 48.09 (40.85-55.33) | <.0001 |
| Cytology                    | 75.57 (69.34-81.79) | <.0001        | 31.21 (24.50-37.93) | <.0001 |
| Cytology plus HPV16/18<sup>d</sup> | 49.73 (42.48-56.97) | <.0001 | 63.39 (56.41-70.37) | <.0001 |

Abbreviation: NA, not applicable; same test comparison or compared in previous row.

Notes: Positive tests cutoffs: <sup>a</sup>S5 score ≥ 0.8 or <sup>b</sup>S5 score ≥ 3.1, <sup>c</sup>first ASC-US result of cytology repeated at 6 or 12 months after first time ASC-US cytology, 2 CIN3+ and 8 CIN2 cases and 7 < CIN2 controls have missed results for cytology, <sup>d</sup>Positive for any of the two tests. Only paired results were included in the McNemar test.

*P value of McNemar test for comparison of S5 at cutoff 0.8 or 3.1 with each other tests.

Here, we have evaluated the S5 classifier, a multigene methylation test, which has validated procedures and parameters for estimating the methylation levels and that has shown good performance in the United Kingdom, Canada, and Mexico. Our study included 356 hrHPV baseline samples from women who had 2 years of active follow-up culminating in colposcopically directed biopsy and randomization chosen among all baseline hrHPV women. The women had most of the high-grade cervical precancerous endpoints identified from among the 2641 participants of ASC-US-COL study. To date, there are very few reports comparing the performance of S5 for CIN2+ and CIN3+ detection vs twice repeated cytology testing and HPV16/18 genotyping with hrHPV+, using the same cervical exfoliates used for HPV testing and the molecular methods used for HPV testing, and the molecular methods used for HPV testing, and the molecular methods used for HPV testing.
among women with above the average risk for CIN2+ (previous ASC-US cytology and hrHPV+), S5 offers a good triage alternative since this test decreased the false positive rate by near 9% (Table 3) and exhibited higher sensitivity than HPV16/18, cytology or combination of these two tests for both CIN2+ and CIN3+ endpoints. This characteristic of S5 is especially valuable for remote areas of LMICs, where higher sensitivity is crucial to identify at-risk women in fewer screening visits and decreasing the use of resources to follow-up women with low risk of disease.

In our study, cytology had a specificity of 75%, but was the test with by far the highest false negative rate. We recognize that the sensitivity of our cytology was much lower than the performance of this test seen in specialist centers, but several studies have demonstrated that the sensitivity in LMICs including some Latin American countries may be as low as 30%.31-34 It is worth noting that despite many efforts to improve cytology quality and sensitivity, difficulties in quality control and delays in diagnosis still prevail.5

It is recognized that borderline and mild cytology have low reproducibility. Furthermore, since we matched controls to cases by age and hrHPV status, the distribution of cytology grades of controls may be biased. Thus, our results must be interpreted with the knowledge that ASC-US diagnoses and cytology grades distribution may not be comparable to other clinical settings. The appropriate sensitivity/specificity combination (and the corresponding decision threshold) of the methylation tests for the application of triaging hrHPV+ women has not been defined. In one meta-analysis the pooled sensitivity and specificity for CIN2+ was estimated irrespective of threshold used to define methylation positivity.25 In the other, the threshold corresponded to a specificity of 70%.26 We used the prespecified 0.8 cut-point that corresponded to a sensitivity of 90% (95% CI 87-92) and a specificity of 49% (95% CI 46-52) in a previous study with S5,13 and conducted a post hoc analysis setting the specificity at 76% that corresponded at 3.1 cut-point. Future work is planned to assess the performance of S5 as a triage for HPV-positive tests to determine appropriate cutoffs within a screening population. Also, further work is needed to determine the performance of the S5 methylation assay in vaccinated populations.

Despite the differences in study designs and the proportion of CIN2+ and CIN3+ cases included, the consistency of our results are remarkably similar to what has been observed previously.13 The S5 methylation test accurately identified women with higher risk of cervical high-grade disease and cancer among those who were hrHPV+. Even further, our study demonstrated that S5 outperforms both cytology and HPV16/18 CIN2+ and CIN3+ detection in hrHPV+ women.15-16 Currently S5 DNA methylation test is labor intensive and costly. The recent developments of affordable and scalable next-generation sequencing assays35 strengthens our proposal that the S5 DNA methylation classifier test may be an acceptable strategy for the triage of hrHPV+ in LMICs. Strategies combining screening and immediate clinical management are urgently needed for low-resource settings where infrastructure for follow-up after screening is limited. Our results warrant further clinical validation of S5 in large prospective population-based screening trials.

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CONFLICT OF INTERESTS
Dr. Cuschieri’s institution has received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: Cepheid, Genomica, LifeRiver, Euroimmun,
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**DISCLOSURE**
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.

**DATA AVAILABILITY STATEMENT**
The dataset excluding personal identifiers will be available to proper academic parties on request from the corresponding author in accordance with the data sharing policies of Queen Mary University of London (UK).

**ETHICS STATEMENT**
All women from ASC-US-COL trial signed informed consent for use of samples and data for future studies. The ethics committees of Sede de Investigación Universitaria (SIU) and School of Medicine from the Universidad de Antioquia, Colombia, approved the study (Resolution 08-036-171).

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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