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

Short title:
DNA methylation for triage HPV positive women

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Key words: HPV test; DNA methylation; cervical cancer; triage; biomarkers

List of 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

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Cancer Epidemiology

Novelty and Impact:

This is the first triage study among hrHPV+ women with head-to-head comparison of S5-methylation, HPV16/18, and conventional cytology from a LMIC country; we show clear clinical value for methylation triage. Advances in methylation technologies may allow implementation of these assays relatively soon in LMIC settings. This availability is likely to have an important impact on screening and triaging hrHPV+ women algorithms in regions where greater accuracy of triage and enhanced simplicity of follow-up are urgently needed.

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 pre-defined 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%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 ($p<0.05$) or cytology ($p<0.0001$). 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,$p=0.0062$) 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-positive Colombian women. Furthermore, S5 triage had comparable sensitivity and significantly fewer false positives than cytology and HPV16/18 combination.

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.[1] 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.[2]

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 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 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 60–70% greater protection against invasive cervical carcinomas compared with 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 non-progressive disease.[7]

Approaches to stratify (triage) hrHPV-positive 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 low[8] and a high proportion of women hrHPV-positive 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 50-60%[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 favours 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-positive 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 tumour suppressor

gene *EPB41L3* that identifies women with CIN2+.[13] At sensitivities of 90% (95% 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 colposcopy[13] or screening[14] health services in London, UK 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,[15] there are few studies validating S5 in LMIC settings[16]. 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-positive women selected from a pragmatic trial (ASC-US-COL trial) that recruited women from routine opportunistic screening services of Colombia, a middle-income country.

Material and Methods

ASC-US-COL study design.

ASC-US-COL is a three-arm, non-blinded, parallel group, pragmatic trial.[17] Women aged 20-69 years (n=2661) with a first time atypical squamous cells of undetermined significance (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 USA) with a relative light unit/cut-off (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).

Selection of methylation sub-study participants.

Cases were women identified after the end of the ASC-US-COL trial as women who had hrHPV-positive 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-positive 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, UK). 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.

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 200µL of the residual content. Briefly, STM specimens were digested for 2 h 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 min. After precipitation with 0.5M ammonium-acetate and 70% ethanol, DNA was washed, dried and resuspended in 100µL of TE buffer (10mM TRIS/0.1mM EDTA).[18] DNA quality and quantity were assessed using Nanodrop and PCR amplification for the MID856 variant as internal control.[19] Isolated DNA was stored at –30°C and shipped frozen on dry ice to Queen Mary University of London for S5 methylation testing.

HPV genotyping

HPV genotyping was independently and blindly conducted on 5µL of a 1:10 dilution of each specimen using the CLART® HPV4 test (Genomica®, Madrid, Spain). 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), one presumptive 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.[20]

S5 DNA methylation classifier testing.

Methylation assays were based on end-point PCR and quantitative pyrosequencing of amplicons using primers for 6 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.[14] Briefly, 100 ng of DNA was used for bisulphite 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 μL of PCR product was used for pyrosequencing (PyromarkQ96 ID Platform, Qiagen, Germany). The S5 score was defined as $30.9(\textit{EPB41L3}) + 13.7(\mathrm{HPV16_{L1}}) + 4.3(\mathrm{HPV16_{L2}}) + 8.4(\mathrm{HPV18_{L2}}) + 22.4(\mathrm{HPV31_{L1}}) + 20.3(\mathrm{HPV33_{L2}})$ using the percentage of individual CpG sites methylated as described previously.[13]

Statistical methods

The analysis was based on a pre-specified analytical plan. The primary hypothesis was that using the hrHPV-positive baseline samples, S5 at the standard pre-defined cut-point 0.8[13] 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 75% 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 <0.05 was considered significant.

Results

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-positive. 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 4 samples (2 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. 82 (44.8%) of CIN2+ were positive for HPV16, 10 (5.5%) for HPV18, 24 (13.1%) for HPV31 and 17 (9.3%) for HPV33.

***EPB4IL3*, HPV16L1, and S5 methylation patterns were related to the severity of CIN**

Median S5 methylation (Figure 2) increased proportionally (Cuzick trend test $\chi2=45.1$, $p<0.0001$) 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 $\chi2=23.47$, $p<0.001$) and HPV16L1 individually (Figure 3b, Cuzick trend test $\chi2=25.4$, $p<0.001$).

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

The ROC curve showed that S5 had an AUC of 0.70 (95% CI 0.64–0.75, $p<0.0001$) for CIN2+ and of 0.72 (95% CI 0.65–0.80, $p<0.0001$) 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 Table 2 and Table 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, $p<0.0001$) or cytology testing (75.6%, 95% CI 69.3–81.8, $p<0.0001$). At the cut-point of 3.1, (corresponds to setting the specificity of S5 for <CIN2 at 76% which is equal to cytology specficity) the S5 specifity was significantly higher than HPV16/18-genotyping ($p=0.0062$), while the sensitivity of S5 was 55.2% (95% CI 48.0–62.4), which remained significantly higher than sensitivity of HPV16/18 genotyping ($p=0.0164$) and of cytology ($p<0.0001$).

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, $p=0.0008$) or cytology (37.0%, 95% CI 23.3–50.6, $p=0.0003$). 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 ($p=0.0233$) and of cytology ($p=0.0088$). 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 (49.73% 95% CI 42.48–56.97) was lower than the corresponding specificity of S5 at the 3.1 cut-point ($p<0.0001$).

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, $p=0.0001$) and CIN3+ (Table S2, 0.75, 95%CI 0.67–0.79, $p=0.0033$) 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=0.0014 for CIN2+ and 0.53, 95% CI 0.46–0.49, p<0.0001 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[23] recommends visual assessment for treatment (VAT) 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 gynaecological 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-positive. Re-calling 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-positive women, but HPV self-sampling,[24] 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. HPV18 has been included with HPV16 as a combination triage because it is a highly prevalent virus in squamous cancers and adenocarcinomas, although

not in CIN3. However, HPV16/18 reaches a sensitivity of only 50-60% 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 L1 gene in all HPV genotypes for prediction of CIN2+ or CIN3+ range from 0.65 to 0.95[25] and that markers of host DNA methylation, with specificity set at 70%, have a sensitivity for CIN2+ and CIN3+ of 68.6% (95% CI 62.9–73.8) and 71.1% (95% CI 65.7–76.0), and positive predictive values of 53.4% (95% CI 44.4–62.1) and 35.0% (95% CI 28.9–41.6) respectively[26]. Methylation also exhibited higher specificity than cytology (at an ASC-US threshold) and higher sensitivity than HPV16/18 to detect CIN2+ and CIN3+ among hrHPV-positive. Because of the heterogeneity of assays and targets to estimate the level of methylation, and because some studies did not have histopathology verification, there is need of further confirmation of these conclusions. In contrast to cell-based methods, DNA methylation can be reflexed using the same cervical exfoliates used for HPV testing and the molecular methods offer the possibility for self-sampling. These characteristics make a methylation-based test a very good candidate for immediate triage and treatment where necessary when self-sampling is used as the primary test. However there are very few studies validating methylation markers in women from LMICs[25, 26], which are the countries with the highest cervical cancer incidence and mortality rates.

Here we have evaluated the S5 classifier, a multigene methylation test, which has very well validated procedures and parameters for estimating the methylation levels and that has shown good performance in UK[14], Canada[15] and Mexico.[16] Our study included 366 hrHPV+ baseline samples from women who had two years of active follow-up culminating in colposcopically-

directed biopsy diagnoses if positive for either hrHPV or cytology tests. These women had most of the high-grade cervical precancerous endpoints identified from among the 2661 participants of ASC-US-COL study.[17] To address potential bias in methylation levels by age at diagnosis or time of follow-up, our controls were age- and time to diagnosis matched and randomly chosen among all baseline hrHPV+ women. The genotyping and methylation assays, as well as the verification of the histological diagnoses, were conducted independently and blindly after the end of the study. To date there are very few reports comparing the performance of cytology and HPV16/18 genotyping with DNA methylation assays to detect CIN2+ and CIN3+ in hrHPV+ women.[15, 27-30] The study allowed us to objectively compare the performance of S5 for CIN2+ and CIN3+ detection versus twice repeated cytology testing and HPV16/18 in hrHPV+ women in an LMIC setting.

The AUCs of the continuous S5 classifier for CIN2+ and CIN3+ were not different (0.70 vs. 0.72; p=0.6218, DeLong's test) and were similar to those obtained in a population-based HPV cervical screening clinical trial in Canada,[15] but lower than the AUC (0.82) seen in women attending colposcopy in UK.[13] At a specificity set at 76.0% that corresponds to using S5 at a cut-point of 3.1, the sensitivity of S5 for CIN2+ and for CIN3+ were 55.2% and 64.6% respectively, which were significantly higher than the sensitivities of HPV16/18 (p=0.0164 and p=0.0233, respectively) or cytology (p<0.0001 and p=0.0088, respectively). Performance of S5 was similar to the comparator triage tests and their combination regarding the specificity. Considering that we are comparing different triage tests 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 centres, 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 pre-specified 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 cut-offs 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.[14-16] Currently S5 DNA methylation test is labour intensive and costly. The recent developments of affordable and scalable next generation sequencing assays[35] 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.

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, GeneFirst, SelfScreen, Qiagen, Hiantis and Hologic. No conflicts of interest were disclosed by the other authors.

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).

Contributors

ATL and GIS conceived the study which was designed with assistance from ATR, BN, MCA and JC. ATR and KC did the methylation and the HPV genotyping laboratory tests. AB designed the statistical methods and AB and GIS conducted the statistical analysis. MAC, KMC and ATR, reported the data of clinical management, hrHPV testing and methylation analysis. All authors contributed to the writing of the manuscript and read and approved the final version.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* 2018;**68**: 394-424.

2. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. *Lancet* 2007;**370**: 890-907.

3. Ghebreyesus TA. Global call for action towards the elimination of cervical cancer., vol. 2019: World Health Organization, 2018.

4. Koliopoulos G, Nyaga VN, Santesso N, Bryant A, Martin-Hirsch PP, Mustafa RA, Schünemann H, Paraskevaidis E, Arbyn M. Cytology versus HPV testing for cervical cancer screening in the general population. *Cochrane Database Syst Rev* 2017;**8**: CD008587.

5. Mezei AK, Armstrong HL, Pedersen HN, Campos NG, Mitchell SM, Sekikubo M, Byamugisha JK, Kim JJ, Bryan S, Ogilvie GS. Cost-effectiveness of cervical cancer screening methods in low- and middle-income countries: A systematic review. *Int J Cancer* 2017;**141**: 437-46.

6. Ronco G, Dillner J, Elfström KM, Tunesi S, Snijders PJ, Arbyn M, Kitchener H, Segnan N, Gilham C, Giorgi-Rossi P, Berkhof J, Peto J, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet* 2014;**383**: 524-32.

7. Rijkaart DC, Berkhof J, van Kemenade FJ, Coupe VM, Hesselink AT, Rozendaal L, Heideman DA, Verheijen RH, Bulk S, Verweij WM, Snijders PJ, Meijer CJ. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *Int J Cancer* 2012;**130**: 602-10.

8. Demarco M, Lorey TS, Fetterman B, Cheung LC, Guido RS, Wentzensen N, Kinney WK, Poitras NE, Befano B, Castle PE, Schiffman M. Risks of CIN 2+, CIN 3+, and Cancer by Cytology and Human Papillomavirus Status: The Foundation of Risk-Based Cervical Screening Guidelines. *J Low Genit Tract Dis* 2017;**21**: 261-7.

9. Wentzensen N, Clarke MA, Bremer R, Poitras N, Tokugawa D, Goldhoff PE, Castle PE, Schiffman M, Kingery JD, Grewal KK, Locke A, Kinney W, et al. Clinical Evaluation of Human Papillomavirus Screening With p16/Ki-67 Dual Stain Triage in a Large Organized Cervical Cancer Screening Program. *JAMA Intern Med* 2019;**179**: 881-8.

10. Stanczuk GA, Baxter GJ, Currie H, Forson W, Lawrence JR, Cuschieri K, Wilson A, Patterson L, Govan L, Black J, Palmer T, Arbyn M. Defining Optimal Triage Strategies for hrHPV Screen-Positive Women-An Evaluation of HPV 16/18 Genotyping, Cytology, and p16/Ki-67 Cytoimmunochemistry. *Cancer Epidemiol Biomarkers Prev* 2017;**26**: 1629-35.

11. Clarke MA, Wentzensen N, Mirabello L, Ghosh A, Wacholder S, Harari A, Lorincz A, Schiffman M, Burk RD. Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. *Cancer Epidemiol Biomarkers Prev* 2012;**21**: 2125-37.

12. Vasiljević N, Scibior-Bentkowska D, Brentnall AR, Cuzick J, Lorincz AT. Credentialing of DNA methylation assays for human genes as diagnostic biomarkers of cervical intraepithelial neoplasia in high-risk HPV positive women. *Gynecol Oncol* 2014;**132**: 709-14.

13. Brentnall AR, Vasiljevic N, Scibior-Bentkowska D, Cadman L, Austin J, Cuzick J, Lorincz AT. HPV33 DNA methylation measurement improves cervical pre-cancer risk estimation of an HPV16, HPV18, HPV31 and EPB41L3 methylation classifier. *Cancer Biomark* 2015;**15**: 669-75.

14. Lorincz AT, Brentnall AR, Scibior-Bentkowska D, Reuter C, Banwait R, Cadman L, Austin J, Cuzick J, Vasiljević N. Validation of a DNA methylation HPV triage classifier in a screening sample. *Int J Cancer* 2016;**138**: 2745-51.

15. Cook DA, Krajden M, Brentnall AR, Gondara L, Chan T, Law JH, Smith LW, van Niekerk DJ, Ogilvie GS, Coldman AJ, Warman R, Reuter C, et al. Evaluation of a validated methylation triage signature for human papillomavirus positive women in the HPV FOCAL cervical cancer screening trial. *Int J Cancer* 2019;**144**: 2587-95.

16. Hernández-López R, Lorincz AT, Torres-Ibarra L, Reuter C, Scibior-Bentkowska D, Warman R, Nedjai B, Mendiola-Pastrana I, León-Maldonado L, Rivera-Paredez B, Ramírez-Palacios P, Lazcano-Ponce E, et al. Methylation estimates the risk of precancer in HPV-infected women with discrepant results between cytology and HPV16/18 genotyping. *Clin Epigenetics* 2019;**11**: 140.

17. Baena A, Agudelo M, Lopez C, Ramirez A, Castañeda K, Bedoya A, Riveros M, Posada G, Borrero M, Buitrago C, Suescun D, Gomez L, et al. Comparison of immediate colposcopy, repeat conventional cytology and hrHPV testing for the clinical management of ASC-

US cytology in routine health services of Medellin, Colombia: The ASCUS-COL Trial. *International Journal of Cancer* 2020.

18. Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlée F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000;**38**: 357-61.

19. Santos NP, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos AK, Pereira R, Gusmao L, Amorim A, Guerreiro JF, Zago MA, Matte C, Hutz MH, Santos SE. Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. *Hum Mutat* 2010;**31**: 184-90.

20. Bonde J, Ejegod DM, Cuschieri K, Dillner J, Heideman DAM, Quint W, Pavon Ribas MA, Padalko E, Christiansen IK, Xu L, Arbyn M. The Valgent4 protocol: Robust analytical and clinical validation of 11 HPV assays with genotyping on cervical samples collected in SurePath medium. *J Clin Virol* 2018;**108**: 64-71.

21. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988;**44**: 837-45.

22. R-Core-Team. R: A Language and Environment for Statistical Computing Vienna, Austria: R Foundation for Statistical Computing, 2016.

23. WHO guidelines for the use of thermal ablation for cervical pre-cancer lesionsed., 2019.

24. Lazcano-Ponce E, Lorincz AT, Cruz-Valdez A, Salmerón J, Uribe P, Velasco-Mondragón E, Nevarez PH, Acosta RD, Hernández-Avila M. Self-collection of vaginal specimens for human papillomavirus testing in cervical cancer prevention (MARCH): a community-based randomised controlled trial. *Lancet* 2011;**378**: 1868-73.

25. Bowden SJ, Kalliala I, Veroniki AA, Arbyn M, Mitra A, Lathouras K, Mirabello L, Chadeau-Hyam M, Paraskevaidis E, Flanagan JM, Kyrgiou M. The use of human papillomavirus DNA methylation in cervical intraepithelial neoplasia: A systematic review and meta-analysis. *EBioMedicine* 2019;**50**: 246-59.

26. Kelly H, Benavente Y, Pavon MA, De Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (CIN2+): a systematic review and meta-analysis. *Br J Cancer* 2019;**121**: 954-65.

27. Leeman A, Del Pino M, Marimon L, Torné A, Ordi J, Ter Harmsel B, Meijer CJLM, Jenkins D, Van Kemenade FJ, Quint WGV. Reliable identification of women with CIN3+ using hrHPV genotyping and methylation markers in a cytology-screened referral population. *Int J Cancer* 2019;**144**: 160-8.

28. van Leeuwen RW, Oštrbenk A, Poljak M, van der Zee AGJ, Schuuring E, Wisman GBA. DNA methylation markers as a triage test for identification of cervical lesions in a high risk human papillomavirus positive screening cohort. *Int J Cancer* 2019;**144**: 746-54.

29. Clarke MA, Gradissimo A, Schiffman M, Lam J, Sollecito CC, Fetterman B, Lorey T, Poitras N, Raine-Bennett TR, Castle PE, Wentzensen N, Burk RD. Human Papillomavirus DNA Methylation as a Biomarker for Cervical Precancer: Consistency across 12 Genotypes and Potential Impact on Management of HPV-Positive Women. *Clin Cancer Res* 2018;**24**: 2194-202.

30. Luttmer R, De Strooper LM, Berkhof J, Snijders PJ, Dijkstra MG, Uijterwaal MH, Steenbergen RD, van Kemenade FJ, Rozendaal L, Helmerhorst TJ, Verheijen RH, Ter Harmsel WA, et al. Comparing the performance of FAM19A4 methylation analysis, cytology and HPV16/18 genotyping for the detection of cervical (pre)cancer in high-risk HPV-positive women of a gynecologic outpatient population (COMETH study). *Int J Cancer* 2016;**138**: 992-1002.

31. Lazcano-Ponce EC, Alonso de Ruiz P, Lopez-Carrillo L, Najera-Aguilar P, Avila-Ceniceros R, Escandon-Romero C, Cisneros MT, Hernandez-Avila M. Validity and

reproducibility of cytologic diagnosis in a sample of cervical cancer screening centers in Mexico. *Acta Cytol* 1997;**41**: 277-84.

32. Nanda K, McCrory DC, Myers ER, Bastian LA, Hasselblad V, Hickey JD, Matchar DB. Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: a systematic review. *Ann Intern Med* 2000;**132**: 810-9.

33. Ferreccio C, Bratti MC, Sherman ME, Herrero R, Wacholder S, Hildesheim A, Burk RD, Hutchinson M, Alfaro M, Greenberg MD, Morales J, Rodriguez AC, et al. A comparison of single and combined visual, cytologic, and virologic tests as screening strategies in a region at high risk of cervical cancer. *Cancer Epidemiol Biomarkers Prev* 2003;**12**: 815-23.

34. Murillo R, Wiesner C, Cendales R, Piñeros M, Tovar S. Comprehensive evaluation of cervical cancer screening programs: the case of Colombia. *Salud Publica Mex* 2011;**53**: 469-77.

35. Wagner S, Roberson D, Boland J, Kreimer AR, Yeager M, Cullen M, Mirabello L, Dunn ST, Walker J, Zuna R, Porras C, Cortes B, et al. Evaluation of TypeSeq, a Novel High-Throughput, Low-Cost, Next-Generation Sequencing-Based Assay for Detection of 51 Human Papillomavirus Genotypes. *J Infect Dis* 2019;**220**: 1609-19.

Figure legends

Figure 1. Flowchart

Figure 2. Boxplot distribution of S5 risk score according to the lesion group. The median and interquartile range are depicted by boxes. Cuzick trend test $\chi 2 = 45.1$ ($p<0.001$).

Figure 3. Boxplot distribution of (a) EPB41L3 methylation (Cuzick trend test $\chi 2 = 23.47$, $p<0.001$) and (b) HPV16-L1 methylation (Cuzick trend test $\chi 2 = 25.40$, $p<0.001$) by histology.

Figure 4. ROC plot of S5 classifier for (a) CIN2+ and (b) CIN3+. As comparison sensitivity and specificity cutpoints of S5 classifier at cut-offs of ≥0.8, ≥1.4 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

Table 1. Description of study population

Characteristic	Control (n=183)	Case (n=183)	p value*
Number	n (%)	n (%)	-
Age (years)			0.2164
20–29	91 (49.73)	80 (43.72)	
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)			0.0604
1–12	109 (59.56)	98 (53.55)	
13–>18	74 (40.43)	85 (46.44)	
Age of first sexual intercourse (years)			0.0595
≤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			0.659
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)	0.0005
HPV 18	14 (7.65)	10 (5.46)	0.5264
HPV 31	23 (12.57)	24 (13.11)	1
HPV 33	6 (3.28)	17 (9.29)	0.0312
Other hrHPV types¥	142 (77.6)	124 (67.76)	0.0461
Cytologyʢ			0.1678
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			0.6674
RC	58 (31.69)	65 (35.71)	
IC	64 (34.97)	57 (31.32)	
HPV	61 (33.33)	60 (32.97)	

*Person's chi-squared test. The percentage is shown in column. NEG= negative, LSIL= Low Grade Squamous Intraepithelial Lesion, HSIL= High Grade squamous intraepithelial lesion, ASC-US= Atypical Squamous Cells of Undetermined Significance, ASC-H-AGC= Atypical squamous cells, cannot exclude HSIL or Atypical Glandular Cells, ASC-H = Atypical squamous cells, cannot exclude HSIL. RC= Repeat cytology, IC= Immediate colposcopy, HPV = Human Papillomavirus test. ¥Other hrHPV types = HPV 35, 39, 45, 51, 52, 56, 58, 59. ʢWorst cytology result after ASC-US index cytology.

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

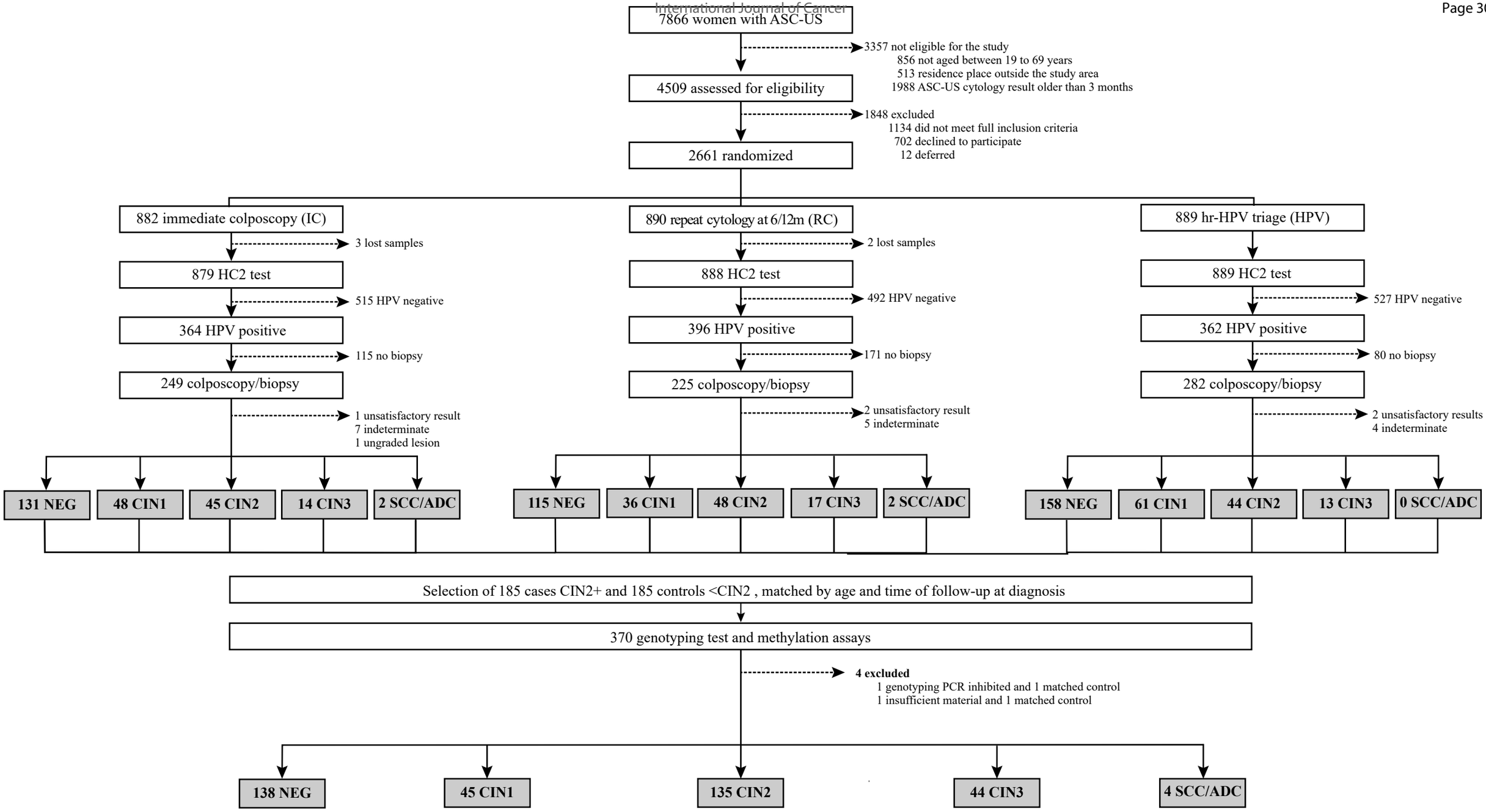
	<CIN2 (n=183)		CIN2+ (n=183)		CIN3+ (n=48)	
	TN n (%)	FP n (%)	TP n (%)	FN n (%)	TP n (%)	FN n (%)
S5 different cut-off						
S5 ≥0.8*	64 (34.97)	119 (65.03)	150 (81.97)	33 (18.03)	37 (77.08)	11 (22.92)
S5 ≥3.1¥	139 (75.96)	44 (24.04)	101 (55.19)	82 (44.81)	31 (64.58)	17 (35.42)
HPV16/18	123 (67.21)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

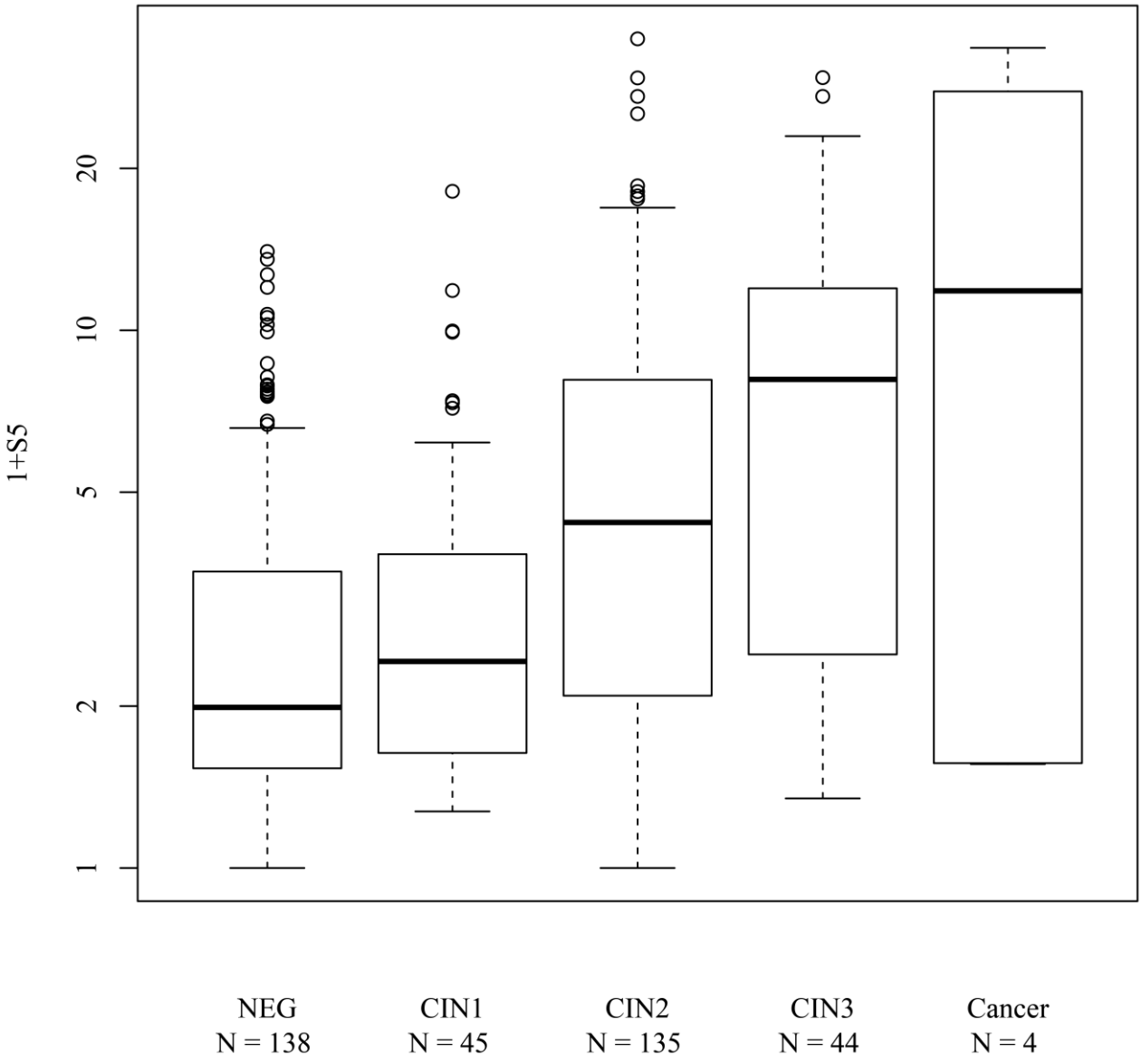
Positive tests cut-offs: *S5 score ≥0.8 or ¥S5 score ≥3.1, γFirst ≥ASC-US result of cytology repeated at 6 or 12 months after first time ASC-US cytology. 2 CIN3+ and 8 CIN2 cases and 7 <CIN2 controls have missed results for cytology. £Positive for any of the two tests. TP=true positive. FN=false negative. TN=true negative. FP=false positive.

Table 3. Diagnostic performance of three triage tests and selected combinations for detection of CIN2+ and CIN3+

	<CIN2 (n=183)			CIN2+ (n=183)			CIN3+ (n=48)		
	Specificity, % (95%CI)	p value[α] S5 ≥0.8 vs. other tests	p value[α] S5 ≥3.1 vs. other tests	Sensitivity, % (95%CI)	p value[α] S5 ≥0.8 vs. other tests	p value[α] S5 ≥3.1 vs. other tests	Sensitivity, % (95%CI)	p value[α] S5 ≥0.8 vs. other tests	p value[α] S5 ≥3.1 vs. other tests
S5 different cut-off									
S5 ≥0.8[*]	34.97 (28.06–41.88)	NA	NA	81.97 (76.40–87.54)	NA	NA	77.08 (65.19–88.97)	NA	NA
S5 ≥3.1[¥]	75.96 (69.76–82.15)	<0.0001	NA	55.19 (47.99–62.40)	<0.0001	NA	64.58 (51.05–78.11)	0.0412	NA
HPV16/18	67.21 (60.41–74.01)	<0.0001	0.0062	48.09 (40.85–55.33)	<0.0001	0.0164	50 (35.86–64.14)	0.0008	0.0233
Cytology[γ, e]	75.57 (69.34–81.79)	<0.0001	1	31.21 (24.50–37.93)	<0.0001	<0.0001	36.96 (23.30–50.61)	0.0003	0.0088
Cytology plus HPV16/18[£]	49.73 (42.48–56.97)	0.0015	<0.0001	63.39 (56.41–70.37)	<0.0001	0.0213	64.58 (51.05–78.11)	0.1489	1

Positive tests cut-offs: *S5 score ≥0.8 or [¥]S5 score ≥3.1, [γ]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. Only paired results were included in the McNemar test. [£]Positive for any of the two tests. [α]p value of McNemar test for comparison of S5 at cut-off 0.8 or 3.1 with each other tests. NA: Not Applicable: same test comparison or compared in previous row.


7866 women with ASC-US
3357 not eligible for the study
856 not aged between 19 to 69 years
513 residence place outside the study area
1988 ASC-US cytology result older than 3 months
4509 assessed for eligibility
1848 excluded
1134 did not meet full inclusion criteria
702 declined to participate
12 deferred
2661 randomized
882 immediate colposcopy (IC)
3 lost samples
879 HC2 test
515 HPV negative
364 HPV positive
115 no biopsy
249 colposcopy/biopsy
1 unsatisfactory result
7 indeterminate
1 ungraded lesion
131 NEG
48 CIN1
45 CIN2
14 CIN3
2 SCC/ADC
890 repeat cytology at 6/12m (RC)
2 lost samples
888 HC2 test
492 HPV negative
396 HPV positive
171 no biopsy
225 colposcopy/biopsy
2 unsatisfactory result
5 indeterminate
115 NEG
36 CIN1
48 CIN2
17 CIN3
2 SCC/ADC
889 hr-HPV triage (HPV)
889 HC2 test
527 HPV negative
362 HPV positive
80 no biopsy
282 colposcopy/biopsy
2 unsatisfactory results
4 indeterminate
158 NEG
61 CIN1
44 CIN2
13 CIN3
0 SCC/ADC
Selection of 185 cases CIN2+ and 185 controls <CIN2 , matched by age and time of follow-up at diagnosis
370 genotyping test and methylation assays
4 excluded
1 genotyping PCR inhibited and 1 matched control
1 insufficient material and 1 matched control
138 NEG
45 CIN1
135 CIN2
44 CIN3
4 SCC/ADC


1+S5
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2
5
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20
NEG
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CIN1
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CIN2
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CIN3
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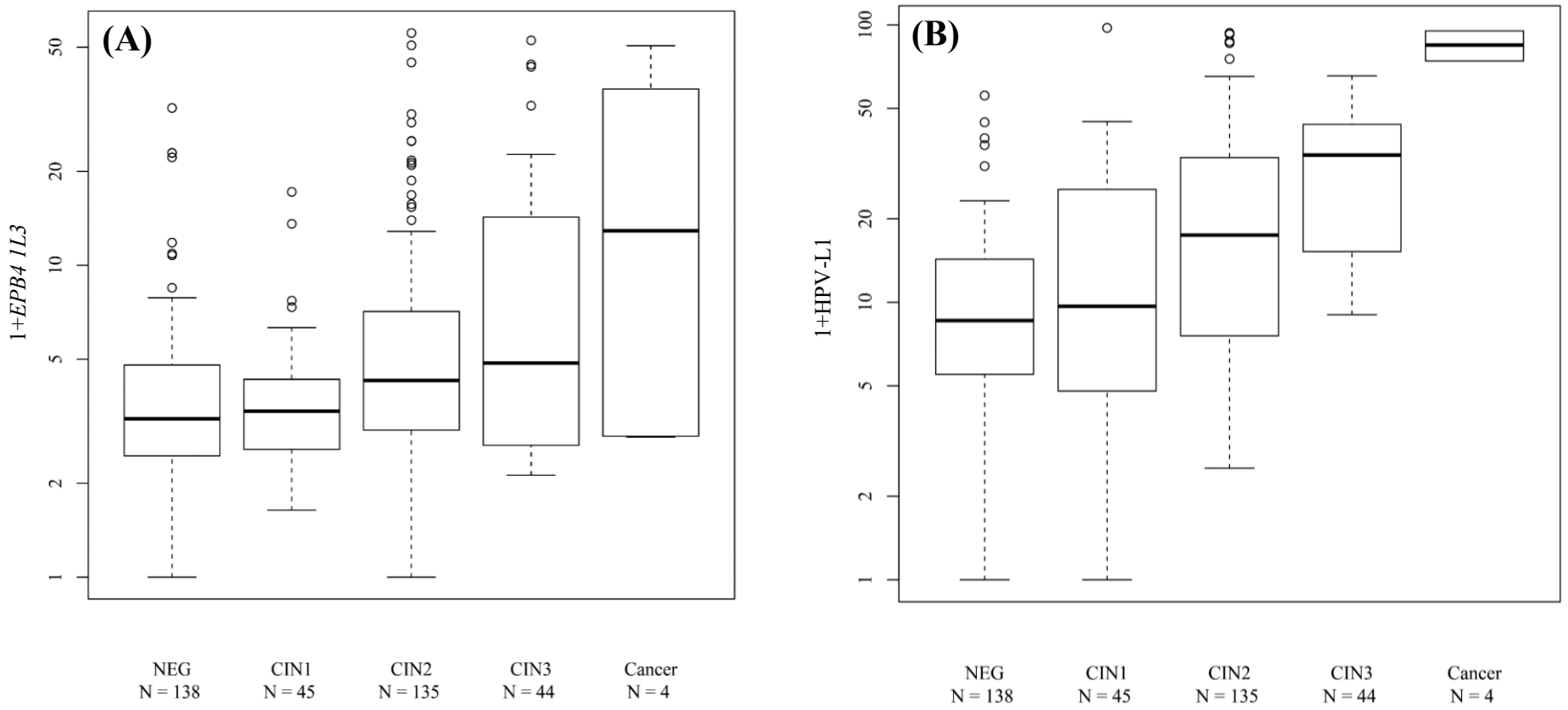
Cancer
N = 4


(A)
1+EPB4 IL3
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Cancer
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(B)
1+HPV-L1
1
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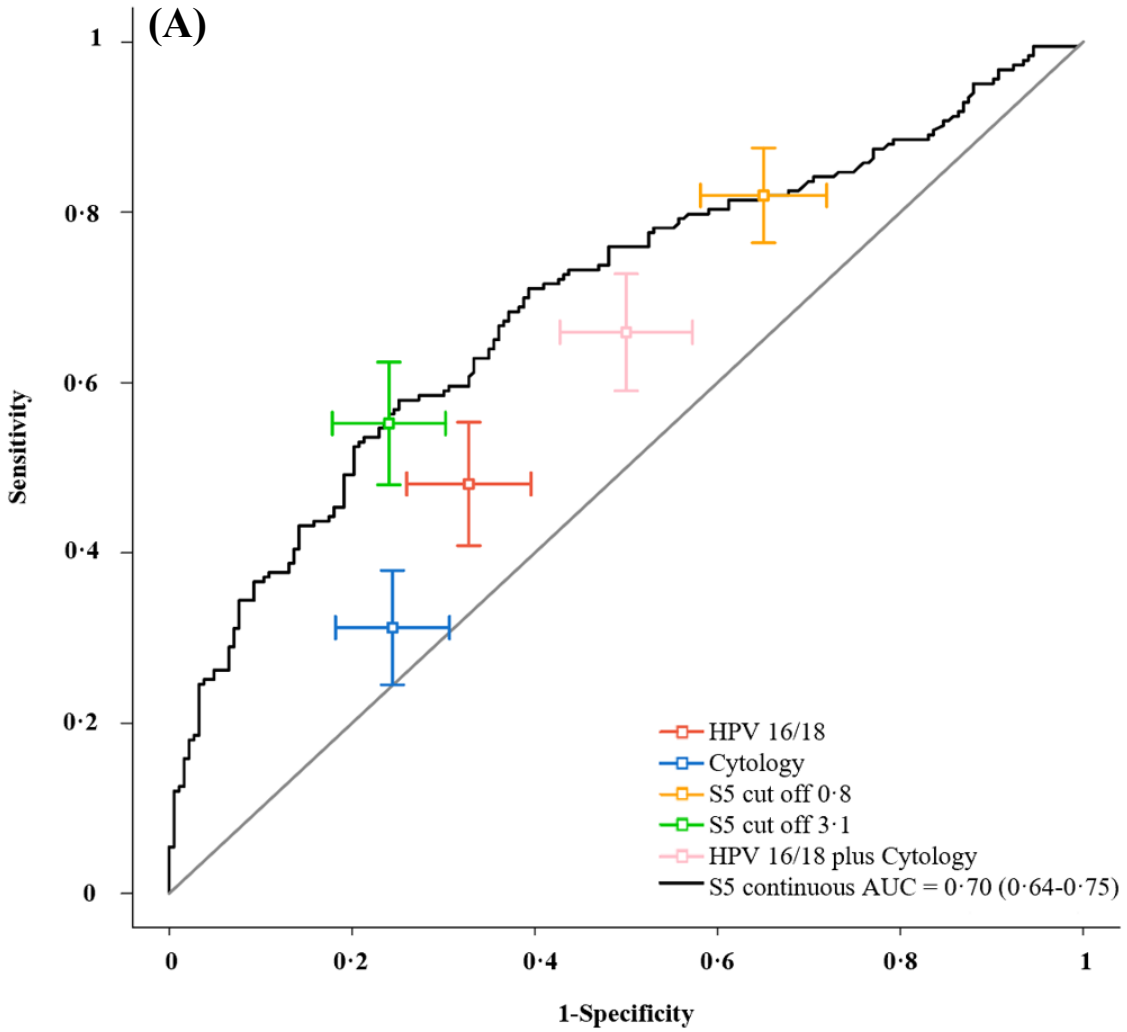
(A)
Sensitivity
1-Specificity
0
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0·4
0·6
0·8
1
HPV 16/18
Cytology
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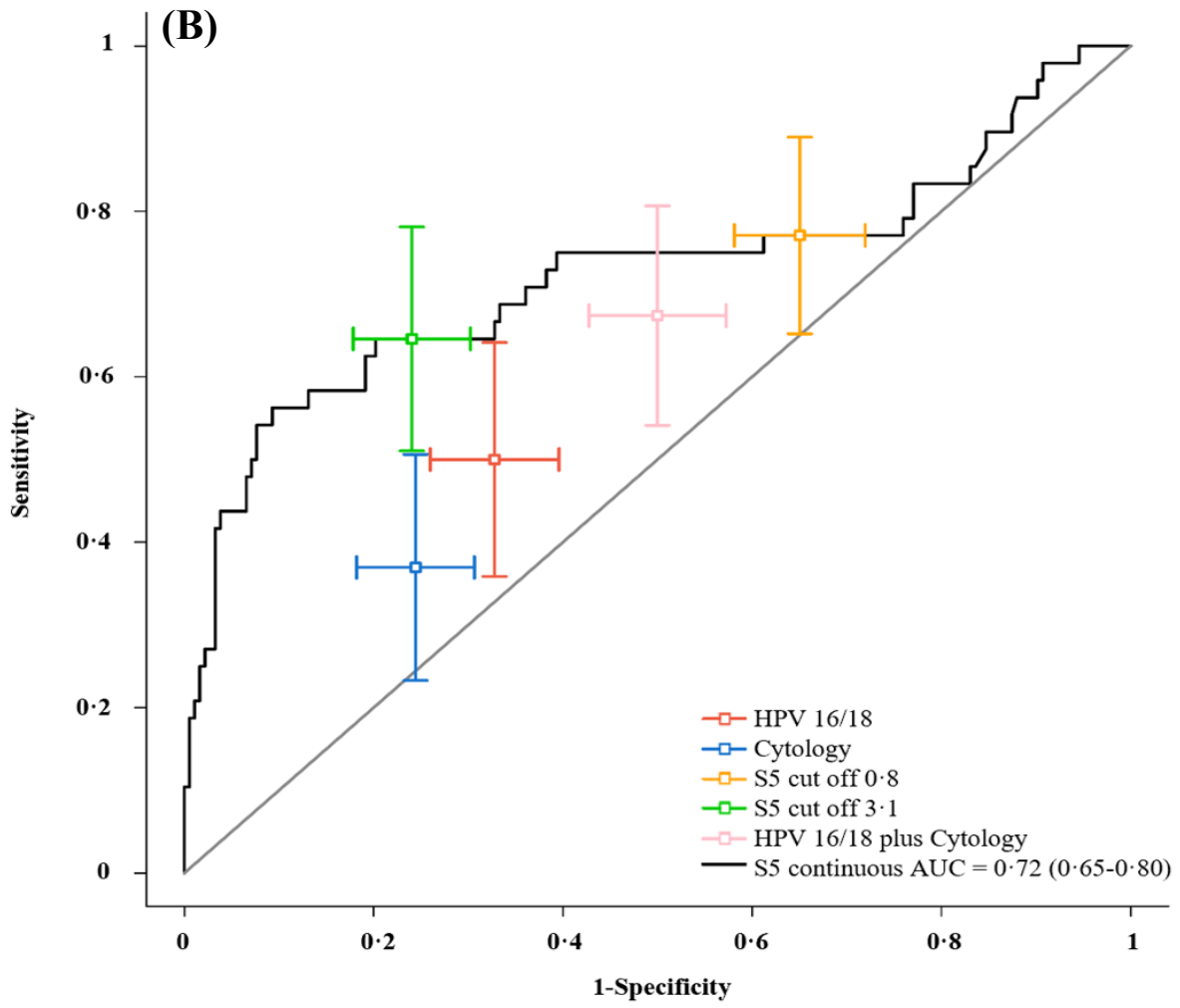
S5 cut off 3·1
HPV 16/18 plus Cytology
S5 continuous AUC = 0·70 (0·64-0·75)


(B)
Sensitivity
1-Specificity
0
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0·4
0·6
0·8
1
HPV 16/18
Cytology
S5 cut off 0·8
S5 cut off 3·1
HPV 16/18 plus Cytology
S5 continuous AUC = 0·72 (0·65-0·80)