Performance of p16\textsuperscript{INK4a} ELISA as a primary cervical cancer screening test among a large cohort of HIV-infected women in western Kenya: a 2-year cross-sectional study

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ABSTRACT

Objective: A biomarker with increased specificity for cervical dysplasia compared with human papillomavirus (HPV) testing would be an attractive option for cervical cancer screening among HIV-infected women in resource-limited settings. p16\textsuperscript{INK4a} has been explored as a biomarker for screening in general populations.

Design: A 2-year cross-sectional study.

Setting: 2 large HIV primary care clinics in western Kenya.

Participants: 1054 HIV-infected women in western Kenya undergoing cervical cancer screening as part of routine HIV care from October 2010 to November 2012.

Interventions: Participants underwent p16\textsuperscript{INK4a} specimen collection and colposcopy. Lesions with unsatisfactory colposcopy or suspicious for cervical intraepithelial neoplasia 2+ (CIN2+) were biopsied. Following biopsy, disease status was determined by histopathological diagnosis.

Primary and secondary outcome measures: We measured the sensitivity, specificity and predictive values of p16\textsuperscript{INK4a} ELISA for CIN2+ detection among HIV-infected women and compared them to the test characteristics of current screening methods used in general as well as HIV-infected populations.

Results: Average p16\textsuperscript{INK4a} concentration in cervical samples was 37.4 U/mL. After colposcopically directed biopsy, 127 (12%) women were determined to have CIN2+. Receiver operating characteristic analysis showed an area under the curve of 0.664 for p16\textsuperscript{INK4a} to detect biopsy-proven CIN2+. At a p16\textsuperscript{INK4a} cut-off level of 9 U/mL, sensitivity, specificity, positive and negative predictive values were 89.0%, 22.9%, 13.6% and 93.8%, respectively. The overall p16\textsuperscript{INK4a} positivity at a cut-off level of 9 U/mL was 828 (78.6%) women. There were 325 (30.8%) cases of correct p16\textsuperscript{INK4a} prediction to detect or rule out CIN2+, and 729 (69.2%) cases of incorrect p16\textsuperscript{INK4a} prediction.

Conclusions: p16\textsuperscript{INK4a} ELISA did not perform well as a screening test for CIN2+ detection among HIV-infected women due to low specificity. Our study contributes to the ongoing search for a more specific alternative to HPV testing for CIN2+ detection.

INTRODUCTION

HIV-related immunosuppression significantly increases the incidence and persistence of infection with oncogenic human papillomavirus (HPV),\textsuperscript{1} leading to higher risk for development of cervical precancer and cancer. Compared with HIV-negative women, HIV-infected women have higher cervical cancer morbidity and mortality, including...
younger age of onset and more advanced malignancies at presentation, leading to lower survival rates.\textsuperscript{2–4} Moreover, both diseases disproportionately affect women in low-resource countries. In sub-Saharan Africa, there are 24.7 million HIV-infected people\textsuperscript{5} and 70,000 new cases of invasive cervical cancer (ICC) each year.\textsuperscript{5} Recent advances in HIV/AIDS care, especially improved access to highly active antiretroviral therapy (HAART), have decreased AIDS-related mortality.\textsuperscript{7} Studies on the impact of HAART on risk of cervical precancer or cancer have shown inconsistent results. In some studies, HAART has not been shown to reduce the risk of cervical precancer or cancer;\textsuperscript{8,9} in other studies, it has been shown to increase or decrease risk of cervical disease, depending on HAART duration or adherence.\textsuperscript{10–11} Therefore, it is possible that HIV-infected women in low-resource settings are now living longer, but remain at higher risk for cervical disease. Unfortunately, many of these settings offer limited or no access to cervical cancer screening programmes.\textsuperscript{12}

Cytology-based screening programmes, though successful in resource-replete settings, are not always the best option in low-resource settings due to costs, laboratory infrastructure and need for multiple visits.\textsuperscript{13–16} The WHO published updated guidelines for screen-and-treat strategies for cervical cancer control in low-resource countries in 2013.\textsuperscript{17} These guidelines preferentially recommend screening with HPV testing, followed by treatment for women who test positive. However, among HIV-infected women, high HIV-HPV co-infection rates lead to concerns about decreased specificity for cervical disease.\textsuperscript{18,19} With rates of HPV positivity up to 75\% in HIV-infected women,\textsuperscript{20} HPV testing could lead to an overburdening of the referral system or overtreatment in screen-and-treat settings. With limitations to current screening options, a resource-appropriate objective method for testing HIV-infected women is needed.

A biomarker with increased specificity for detection of cervical intraepithelial neoplasia 2+ (CIN2+), including CIN2/3 and ICC, would decrease the referral burden and overtreatment inherent in an HPV-based screening programme. One such candidate, p16\textsuperscript{INK4a}, is a cyclin-dependent kinase inhibitor overexpressed in HPV-transformed cells, but rarely in normal tissue.\textsuperscript{21} As an adjunct to traditional stains, p16\textsuperscript{INK4a} immunohistochemical (IHC) staining significantly improves interobserver agreement in histological assessment, and adjudication of CIN1 compared with CIN2+.\textsuperscript{22} An ELISA has been developed using simple technology to measure levels from a swab of exfoliated cervical cells.\textsuperscript{23} p16\textsuperscript{INK4a} ELISA showed significantly improved specificity for CIN2+ lesions among a screening population compared with HPV testing.\textsuperscript{24–25}

p16\textsuperscript{INK4a} ELISA has not been widely studied in an HIV-infected population or in low-resource settings. We conducted a cross-sectional study in an HIV primary care setting in western Kenya to measure the sensitivity, specificity and predictive values of p16\textsuperscript{INK4a} ELISA for CIN2+. We sought to describe the test characteristics in a study that was adequately powered to examine the demographic, HIV-related and reproductive factors impacting test accuracy.

\textbf{MATERIALS AND METHODS}

\textbf{Study design}

We conducted a cross-sectional study to define the sensitivity, specificity, positive and negative predictive values of p16\textsuperscript{INK4a} ELISA among HIV-infected women undergoing cervical cancer screening as part of routine HIV care at the Family AIDS Care and Education Services (FACES) programme in Kisumu, Kenya.\textsuperscript{25} Women receive HIV education talks at the time of enrolment with information about basic gynaecological care, cervical cancer and strategies for screening.\textsuperscript{26} Women with serologically confirmed HIV infection, enrolled in HIV care and eligible for cervical cancer screening, were recruited for participation. Screening eligibility criteria included women who were 23 years or older, not pregnant, had no prior history of cervical cancer, had an intact uterus and cervix and had no lesion suspicious for cancer on examination.

\textbf{Measurements and examination}

After signing informed consent, participants underwent pelvic examination with p16\textsuperscript{INK4a} specimen collection using a collection brush swirled three times at the cervical os to capture exfoliated cells from the transformation zone. This was followed by visual inspection with acetic acid (VIA). After VIA, the initial clinician left the room and colposcopy was performed by a second trained clinician who was blinded to VIA results.

The study staff (one nurse and one clinical officer) were trained and certified to perform colposcopy independently and had each performed over 300 colposcopies before study initiation. Colposcopic assessment was carried out in four steps. Clinicians identified normal cervical anatomy before and after acetic acid, using a green filter and after application of Lugol’s iodine. Results were classified as normal, cervicitis, probable CIN1 or probable CIN2+. Women with a satisfactory colposcopy with no lesions were determined to have no disease.\textsuperscript{27} Women with unsatisfactory colposcopy, or visual impression of CIN2+ underwent cervical biopsy or endocervical curettage at the time of colposcopy. Disease status was determined by histopathological diagnosis in women who underwent biopsies.

Biopsy specimens were stored in 10\% buffered formalin at room temperature, and sent to the Department of Human Pathology Laboratory at the University of Nairobi for independent interpretation by two histopathologists. Specimens were read as normal, cervicitis, CIN1, CIN2/3 or invasive cancer. For women with two or more biopsies taken, the outcome was determined by the most severe diagnosis. For women with discrepant results from the same biopsy, final diagnosis was
determined by consultation and consensus between both histopathologists. Treatment decisions were based on histopathology results.

Demographic and clinical variables collected at the time of the visit included age, relationship status, number of partners, reproductive history, contraceptive use and current HAART regimen. Additional clinical variables were obtained from the paper file and electronic medical record. These included verification of HAART regimens, most recent CD4+ count, WHO stage, time since HIV diagnosis, duration of enrolment into HIV care and duration on HAART.

Laboratory methods

Specimens for p16\textsuperscript{INK4a} ELISA were collected on a cytobrush and transferred into a specimen collection vial with a prototypic sample lysis medium (MTM Laboratories, Heidelberg, Germany). Samples were heat stabilised at 100°C for 3 min within 30 min of collection and then stored in a −80°C freezer. Specimens were shipped on dry ice to the Department of Applied Tumor Biology at the German Cancer Research Center (DKFZ) in Heidelberg, Germany and/or the onsite FACES laboratory. A total of 460 (43.6%) women had samples sent to DKFZ, while 740 (70.2%) women had samples sent to the onsite FACES laboratory. Among the total cohort, 146 (13.9%) women had samples sent to both sites. The interclass correlation coefficient of p16\textsuperscript{INK4a} values at the two sites was 0.89, which confirmed that p16\textsuperscript{INK4a} ELISA would perform independently with valid and reproducible results in the FACES laboratory. In the laboratory, 100 μL aliquots of each processed sample were subjected in duplicate to a prototypic calorimetric sandwich-ELISA protocol (Cervatec, MTM Laboratories). Two p16\textsuperscript{INK4a}-specific monoclonal antibodies were used: a capture antibody coated to the solid phase of a microtitre plate and a tracer antibody conjugated to horseradish peroxidase for detection of captured p16\textsuperscript{INK4a} protein. For quantification of solubilised p16\textsuperscript{INK4a} values, calibration curves had been established according to the manufacturer’s protocol. p16\textsuperscript{INK4a} concentrations in individual samples were calculated as arbitrary units (U/mL) using the standardised curve. For specimens analysed in both laboratories, the p16\textsuperscript{INK4a} values were averaged.

Statistical methods

We based our sample size calculations on the CIN2+ prevalence of 7.2% previously seen in this clinic.\textsuperscript{26} We calculated that a sample size of 1100 women was needed to calculate as arbitrary units (U/mL) using the standardised curve. For specimens analysed in both laboratories, the p16\textsuperscript{INK4a} values were averaged.

We built receiver operating characteristic (ROC) curves to determine the p16\textsuperscript{INK4a} cut-off values with the highest combined sensitivity and specificity for CIN2+, as determined by the area under the curve (AUC). Using the value with the greatest AUC, sensitivity, specificity, positive and negative predictive values of p16\textsuperscript{INK4a} for detection of CIN2+ were calculated. Sensitivity analysis was performed for different outcomes, including CIN1+ (defined as CIN1, CIN2/3 and ICC) diagnosis by colposcopy or biopsy, CIN2+ diagnosis by only colposcopy or CIN1+ diagnosis by only colposcopy. Stratified analysis was performed by looking at test performance calculations after stratification by age (<35 or ≥35 years), HAART status, duration on HAART (<18 or >18 months) or most recent CD4+ count (<350 or >350 cells/dL).

In order to determine the performance of p16\textsuperscript{INK4a} at the determined cut-off threshold, we created a dichotomous variable named ‘p16\textsuperscript{INK4a} prediction’, with two possible outcomes: correct or incorrect. Correct was defined as accurately detected cases of CIN2+ (true positives) and non-CIN2+ (true negatives). Women were classified as correct if they had p16\textsuperscript{INK4a} greater than or equal to the determined threshold with biopsy-confirmed CIN2+ (positive gold standard result), or if they had p16\textsuperscript{INK4a} less than the threshold and no CIN2+ by colposcopy or biopsy (negative gold standard result). Women were classified as incorrect if the p16\textsuperscript{INK4a} value did not correctly predict the presence or absence of CIN2+. Women were classified as incorrect if they had p16\textsuperscript{INK4a} greater than or equal to the threshold and no CIN2+ by colposcopy or biopsy, or if they had p16\textsuperscript{INK4a} less than the threshold and biopsy-confirmed CIN2+. Student’s t-tests were performed for continuous variables, while χ\textsuperscript{2} tests were performed for categorical variables. Predictors found to be statistically significantly associated with correct p16\textsuperscript{INK4a} prediction on univariate analysis (p<0.05) were included in the multivariate logistic regression model. Fit of the regression model was tested using R\textsuperscript{2}. Statistical analysis was performed using Stata V.13 (StataCorp, College Station, Texas, USA).

RESULTS

Between 25 October 2010 and 30 November 2012, 1134 HIV-infected women undergoing cervical cancer screening at FACES were enrolled in the study and underwent p16\textsuperscript{INK4a} specimen collection and colposcopy with biopsy as indicated. Of these, 1054 (92.9%) had complete p16\textsuperscript{INK4a} values and colposcopy and biopsy results (figure 1). The average age of participants was 34.5 years (±SD 7.8), 824 (78.2%) were on HAART, average duration on HAART was 18.0 months and median most recent CD4+ count was 506 cells/dL (IQR 355–685; table 1).

At colposcopy, 203 (19.3%) women were biopsied on suspicion of CIN2+; of these, 126 (62.1%) had CIN2/3 confirmed on biopsy and 1 (0.5%) had stage IA1 ICC
1134 women underwent p16INK4a collection and colposcopically-directed biopsy

80 excluded:
Missing p16INK4a values: 20
Missing colposcopy results: 15
Missing biopsy results: 24
No demographic information available: 2
Missing p16INK4a ID linked to CCID: 10
Duplicate p16INK4a values: 5
Duplicate p16INK4a IDs for same CCID: 4

1054 used for final analysis

No CIN2+ by colposcopy: 851 (80.7%)
Normal: 720 (84.6%)
Cervicitis: 5 (0.6%)
CIN1+: 126 (14.8%)

No CIN2+ by biopsy: 76 (33.4%)
Normal: 27 (13.3%)
Cervicitis: 15 (7.4%)
CIN1+: 34 (16.7%)

CIN2+ by colposcopy: 203 (15.3%)

CIN2+ by biopsy: 127 (62.6%)
CIN2/3: 126 (62.1%)
ICCs: 1 (0.5%)

DISCUSSION

p16INK4a ELISA showed potential to be an effective cervical cancer screening test in low-resource settings based on its molecular function and performance on IHC and in prior studies in general populations. However, in our cohort of 1054 HIV-infected women, the assay did not

Figure 1 Flow sheet of study enrolment, eligibility and outcomes. CCID, Cross-Sectional Study ID; CIN2+, cervical intraepithelial neoplasia 2+.
perform well as a screening test. Our study provides well-powered estimates of the sensitivity and specificity of p16\(^{INK4a}\) ELISA among HIV-infected women. We observed a similar sensitivity but much lower specificity compared with current screening methods used in the general population (high-risk HPV (HR-HPV) testing, Pap smear and VIA). Specificities are \(\sim 73.9\%\) for HR-HPV testing, 94.6% for Pap smear and 80.0% for

| Table 1 Baseline characteristics of women screened with p16\(^{INK4a}\) ELISA (n=1054) |
|---------------------------------|------------------|------------------|-----------------|
| Characteristic                  | n*               | Mean/median or n | SD (IQR) or per cent† |
| Age (mean, years)               | 1054             | 34.5             | 7.8             |
| Relationship status             | 1043             |                  |                 |
| Single                          | 76               |                  | 7.3%            |
| Married                         | 565              |                  | 54.1%           |
| Separated                       | 105              |                  | 10.1%           |
| Widowed                         | 297              |                  | 28.5%           |
| Number of current partners      | 1053             | 269              | 25.6%           |
| 0                               |                  | 771              | 73.2%           |
| >1                              |                 | 13               | 1.2%            |
| Number of previous partners     | 1047             | 3                | (2–4)           |
| Number of lifetime partners     | 1053             | 4                | (3–5)           |
| Age of first intercourse (mean, years) | 1048 | 17.3             | 2.7             |
| Reproductive history            |                  |                  |                 |
| Gravidity (median)              | 1054             | 3                | (2–4)           |
| Parity (median)                 | 1053             | 3                | (1–4)           |
| Currently experiencing vaginal symptoms‡ | 1050 | 397              | 37.8%           |
| Amenorrhoea                     | 1050             | 165              | 15.7%           |
| Postmenopausal§                 | 1050             | 98               | 9.3%            |
| Had vaginal delivery            | 1042             | 953              | 91.5%           |
| Had caesarean section           | 1052             | 129              | 12.3%           |
| History of STI                  | 1046             | 116              | 11.1%           |
| Current contraceptive use       |                  |                  |                 |
| Any contraception               | 1054             | 402              | 38.1%           |
| Hormonal contraception          | 1054             | 314              | 29.8%           |
| Duration of contraceptive use   | 380              | 33.1             | 38.0            |
| Contraception by type           | 402              |                  |                 |
| Oral contraceptives             | 31               |                  | 7.7%            |
| Injectable (Depo Provera)       | 200              |                  | 49.8%           |
| Implant (Jadelle or Norplant)   | 83               |                  | 20.6%           |
| Intrauterine device in situ (copper) | 10   |                 | 2.5%            |
| Female sterilisation            | 63               |                  | 15.7%           |
| Condom only                     | 15               |                  | 3.7%            |
| HIV-related characteristics     |                  |                  |                 |
| Time since first HIV diagnosis  | 1054             | 45.1             | 29.7            |
| WHO stage                       | 1053             |                  |                 |
| 1                               | 292              |                  | 27.7%           |
| 2                               | 310              |                  | 29.5%           |
| 3                               | 377              |                  | 35.8%           |
| 4                               | 74               |                  | 7.0%            |
| Most recent CD4+ count (median, cells/dL) | 1054 | 506              | (355–685)       |
| <200                            | 91               |                  | 8.6%            |
| 200–349                         | 165              |                  | 15.7%           |
| 350–499                         | 255              |                  | 24.2%           |
| ≥500                            | 543              |                  | 51.5%           |
| On HAART                        | 1054             | 824              | 78.2%           |
| Time from HIV diagnosis to HAART initiation (mean, months) | 805 | 26.0             | 26.3            |
| Duration on current HAART (mean, months) | 1054 | 18.0             | 21.0            |

* N was different due to missing demographic information.
†Mean with SD was used to describe normally distributed variables. Median with IQR was used to describe non-normally distributed variables.
‡Vaginal symptoms included abnormal discharge, itching or pain with intercourse.
§Postmenopausal was not a subset of amenorrhoea.

HAART, highly active antiretroviral therapy; STI, sexually transmitted infection.
VIA among the general population. Compared with sensitivity and specificity values from studies of HR-HPV testing in HIV-infected women, p16 INK4a ELISA performed with similar sensitivity but lower specificity.\(^29\)\(^{30}\) Specificities from these studies were 55.7% and 77.4%, respectively.

Differences between our study and other p16\(^{INK4a}\) ELISA studies can be attributed to characteristics of the study setting and population. Our setting was an HIV care clinic in East Africa, compared with a general clinic population in developed countries. In order to evaluate the performance of p16\(^{INK4a}\) as a stand-alone biomarker for cervical cancer screening, we collected the specimen prior to VIA or colposcopy. Prior studies have preferentially sampled acetowhite lesions, which may have increased the accuracy of p16\(^{INK4a}\). In addition, HIV-infected women may have intrinsic differences in p16\(^{INK4a}\) expression, reflected in the higher mean p16\(^{INK4a}\) concentration seen among our population. Overall, our mean p16\(^{INK4a}\) concentration was 37.4 U/mL, compared with 33 U/mL in a previous study among the general population. Specifically, we had much higher p16\(^{INK4a}\) concentrations within the subset of women with non-CIN2+. Our mean p16\(^{INK4a}\) concentration among women with normal cervical examinations (32.6 U/mL) was four times higher than that among women with normal cervical examinations in the other study (8.7 U/mL).\(^23\) Our higher p16\(^{INK4a}\) levels among HIV-positive women may reflect higher rates of infection with HR-HPV subtypes in HIV-infected women compared with HIV-negative women. There is higher p16\(^{INK4a}\) in infections by HR-HPV subtypes, compared with low-risk subtypes.\(^31\)\(^32\) It is also possible that higher p16\(^{INK4a}\) levels reflect higher HPV viral loads among HIV-positive women, even when comparing HIV-positive and HIV-negative women infected by the same HPV subtype. A recent study showed that among HIV-positive and HIV-negative women with normal Pap tests but who tested positive for a HR-HPV subtype (HPV 16), HIV-positive women were still found to have a 1.4–2.2 times higher risk for developing CIN2+ in 5 years;\(^33\) this may be attributed to HIV-positive women having higher

Figure 2  ROC curves (sensitivity vs 1—specificity) in primary and sensitivity analyses (n=1054). (A) Base case: true positives limited to biopsy-proven CIN2+. CIN2+ includes CIN2/3 and ICC. All other diagnoses were considered negative. AUC=0.6639. (B) Sensitivity analysis (1): true positives included any biopsy-proven or visual impression of CIN1+. CIN1+ includes CIN1, CIN2/3 and ICC. AUC=0.6078. (C) Sensitivity analysis (2): true positives included any visual impression of CIN2+ by colposcopy. AUC=0.6445. (D) Sensitivity analysis (3): true positives included any visual impression of CIN1+ by colposcopy. AUC=0.6133. AUC, area under the curve; CIN2+, cervical intraepithelial neoplasia 2+; ICC, invasive cervical cancer; ROC, receiver operating characteristic.
Table 2  Test characteristics of p16\textsuperscript{INK4a} ELISA for biopsy-proven CIN2+\textsuperscript{*} in primary and stratified analyses (n=1054)

| Primary analyses: sensitivity and specificity of p16\textsuperscript{INK4a} ELISA at varying cut-off levels |
|--------------------------------------------------|
| p16\textsuperscript{INK4a} ELISA cut-off levels (U/mL) | Sensitivity (%) | Specificity (%) |
| 7 | 90.6 | 18.2 |
| 8 | 89.8 | 20.6 |
| 9 | 89.0 | 22.9 |
| 10 | 85.8 | 26.9 |

Primary analyses: sensitivity, specificity, positive and negative predictive values of p16\textsuperscript{INK4a} ELISA at the optimal cut-off level

| p16\textsuperscript{INK4a} ELISA test performance (%) | p16\textsuperscript{INK4a} cut-off level=9 U/mL (%) |
|--------------------------------------------------|
| Sensitivity | 89.0 |
| Specificity | 22.9 |
| Positive predictive value | 13.6 |
| Negative predictive value | 93.8 |
| Overall p16\textsuperscript{INK4a} positivity | 78.6 |
| Prevalence of CIN2+ | 12.0 |

Stratified analyses: test characteristics of p16\textsuperscript{INK4a} ELISA stratified by age-related and HIV-related characteristics

| p16\textsuperscript{INK4a} ELISA test performance (%) | Age (years) (%) | HAART status (%) | Average duration on HAART (months) (%) | Most recent CD4+ count (cells/dL) (%) |
|--------------------------------------------------|-----------------|------------------|---------------------------------------|-------------------------------------|
| Sensitivity | >35 | <35 | Yes | No | >18 | ≤18 | >350 | ≤350 |
| Specificity | 86.1 | 90.5 | 90.6 | 83.9 | 86.11 | 90.11 | 87.5 | 91.5 |
| Positive predictive value | 20.5 | 24.9 | 22.0 | 26.1 | 22.37 | 23.74 | 23.8 | 19.6 |
| Negative predictive value | 9.9 | 16.7 | 13.3 | 15.0 | 9.72 | 16.1 | 11.3 | 20.4 |
| Sensitivity | ≥35 | <35 | Yes | No | >18 | ≤18 | >350 | ≤350 |
| Specificity | 93.5 | 94.0 | 94.7 | 91.2 | 94.3 | 93.5 | 94.5 | 91.1 |

\textsuperscript{*}CIN2+ includes CIN2/3 and ICC. CIN2+, cervical intraepithelial neoplasia 2+; ICC, invasive cervical cancer.
Table 3  Univariate analyses of factors associated with correct p16\(^{\text{INK4a}}\) prediction of CIN2+

| Variable                                      | Correct \(\text{p16}^{\text{INK4a}}\) prediction* | Incorrect \(\text{p16}^{\text{INK4a}}\) prediction† | p Value  |
|-----------------------------------------------|--------------------------------------------------|--------------------------------------------------|----------|
|                                               | Mean or n  SD or per cent                         | Mean or n  SD or per cent                         |          |
| Age                                           | 33.04  0.76                                       | 35.1  0.58                                       | 0.0001   |
| Relationship status                           |                                                  |                                                  |          |
| Single                                        | 36  11%                                          | 40  6%                                           | 0.01     |
| Married                                       | 176  54%                                         | 389  54%                                         |          |
| Separated                                     | 30  9%                                           | 75  10%                                          |          |
| Widowed                                       | 81  25%                                          | 216  30%                                         |          |
| Number of current partners                    | 0.81  0.04                                        | 0.73  0.03                                        | 0.01     |
| Number of previous partners                   | 3.7  0.26                                         | 3.99  0.39                                        | 0.36     |
| Number of lifetime partners                   | 4.62  0.3                                        | 4.74  0.4                                         | 0.73     |
| Age at first intercourse                      | 17.41  0.31                                      | 17.21  0.18                                       | 0.26     |
| Reproductive history                          |                                                  |                                                  |          |
| Gravidity                                     | 2.87  0.22                                        | 3.65  0.16                                        | 0.0001   |
| Parity                                        | 2.38  0.2                                         | 3.15  0.15                                        | 0.0001   |
| Currently experiencing vaginal symptoms       |                                                  |                                                  |          |
| No                                            | 178  55%                                         | 475  65%                                         | 0.00     |
| Yes                                           | 145  45%                                         | 252  35%                                         |          |
| Amenorrhoe                                     |                                                  |                                                  |          |
| No                                            | 284  88%                                         | 601  83%                                         | 0.03     |
| Yes                                           | 39  12%                                          | 126  17%                                         |          |
| Menopause                                     |                                                  |                                                  |          |
| No                                            | 305  94%                                         | 647  89%                                         | 0.01     |
| Yes                                           | 18  6%                                           | 80  11%                                         |          |
| Number of vaginal deliveries                  | 2.52  0.21                                        | 3.4  0.15                                        | 0.0001   |
| Number of caesarean sections                  | 0.3  0.07                                         | 0.14  0.03                                        | 0.0001   |
| History of STI                                |                                                  |                                                  |          |
| No                                            | 282  88%                                         | 648  90%                                         | 0.36     |
| Yes                                           | 40  12%                                          | 76  10%                                         |          |
| Current contraceptive use                     |                                                  |                                                  |          |
| Any contraception                             |                                                  |                                                  |          |
| No                                            | 208  64%                                         | 444  61%                                         | 0.34     |
| Yes                                           | 117  36%                                         | 285  39%                                         |          |
| Hormonal contraception                        |                                                  |                                                  |          |
| No                                            | 237  73%                                         | 503  69%                                         | 0.20     |
| Yes                                           | 88  27%                                          | 228  31%                                         |          |
| Duration of contraception use (months)        | 29.05  6.65                                       | 34.64  4.66                                       | 0.20     |
| Contraception by type                         |                                                  |                                                  |          |
| Oral contraceptives                           |                                                  |                                                  |          |
| No                                            | 9  8%                                            | 22  8%                                          | 0.32     |
| Yes                                           | 60  51%                                          | 140  49%                                         |          |
| Injectable (Depo Provera)                     | 19  16%                                          | 64  23%                                         |          |
| Implant (Jadelle or Norplant)                 | 3  3%                                            | 7  2%                                           |          |
| Intrauterine device in situ (copper)           | 18  15%                                          | 45  16%                                         |          |
| Female sterilisation                          | 8  7%                                            | 7  2%                                           |          |
| Condom only                                   |                                                  |                                                  |          |
| Time since first HIV diagnosis (months)       | 42.21  3.15                                       | 46.36  2.18                                       | 0.04     |
| Most advanced WHO stage                       |                                                  |                                                  |          |
| 1                                             | 100  31%                                         | 192  26%                                         | 0.51     |
| 2                                             | 91  28%                                          | 219  30%                                         |          |
| 3                                             | 111  34%                                         | 266  37%                                         |          |
| 4                                             | 22  7%                                           | 52  7%                                          |          |
| Most recent CD4+ count (cells/dL)             | 518  28                                           | 543  20                                           | 0.16     |
| Most recent CD4+ count by category (cells/dL) |                                                  |                                                  |          |
| <200                                          | 33  10%                                         | 58  8%                                           | 0.61     |
| 201–350                                       | 51  16%                                         | 114  16%                                         |          |
| 351–500                                       | 73  22%                                         | 182  25%                                         |          |
| >500                                          | 168  52%                                         | 375  51%                                         |          |

Continued
HPV viral loads. The higher overall p16\textsuperscript{INK4a} concentration in HIV-positive women indicates that it is critical to set an optimal p16\textsuperscript{INK4a} threshold.

Differentiation between CIN2+ and non-CIN2+ had the greatest AUC compared with differentiation between CIN1+ and non-CIN1+ (figure 2A, B). We did not differentiate between different types of high-grade CIN (CIN2 vs CIN3) in data collection, because management more significantly differs between women with CIN1 and CIN2+, treatment guidelines in a screen-and-treat strategy are similar for women diagnosed with CIN2 and CIN3. In our study, there is a dose-dependent increase in mean p16\textsuperscript{INK4a} values from HIV-positive women with normal results (32.6 U/mL) to those with progressive levels of cervical dysplasia and cancer (38.7 U/mL for CIN1 to 63.4 U/mL for CIN2/3 to 210 U/mL for ICC). This suggests that p16\textsuperscript{INK4a} is a promising biomarker in measuring progression of cervical disease. However, it may be difficult to choose a numerical threshold to distinguish CIN1 from CIN2+ in a population of women with high rates of HPV positivity and low-grade dysplasia.

Limitations include intrinsic study characteristics such as the global p16\textsuperscript{INK4a} specimen collection, rather than directed collection from acetowhite areas, which could have improved our test performance. The collection protocol was adopted to simulate performance by providers without training in VIA, as would be the case in low-resource settings. Although we performed biopsy only for positive colposcopic findings as the common validation method in similar studies,\textsuperscript{29, 30} this may have led to underascertainment of true CIN2+ cases, which meant that our test characteristics may have underestimated true sensitivity and overestimated true specificity of the p16\textsuperscript{INK4a} ELISA. While p16\textsuperscript{INK4a} IHC staining as an adjunct to H&E staining significantly improves interobserver agreement and adjudication of CIN1 vs CIN2+,\textsuperscript{22} we limited our cohort assessment to H&E staining to maintain a dichotomous result for calculations. Although these additional procedures would have increased the accuracy of our test estimates, we decided not to perform them due to the unacceptability of added invasive procedures and widespread use of H&E alone for disease ascertainment and management.

Given these limitations, additional studies investigating the utility of p16\textsuperscript{INK4a} as a biomarker or other novel methods are warranted to improve first-line screening options for the vulnerable population of HIV-infected women. p16\textsuperscript{INK4a} cytology methods such as CINtec Plus, an immunocytochemistry assay detecting p16\textsuperscript{INK4a} and Ki-67 proteins, may be appropriate. This dual-stained cytology method showed higher specificity and a positive predictive value for CIN2+ than HPV testing among a general population in European countries with abnormal cytology results (including atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesions),\textsuperscript{34} and also recently showed promising results among a resource-limited screening population in Kenya.\textsuperscript{35}

On the basis of our findings, p16\textsuperscript{INK4a} ELISA measured in exfoliated cervical cells did not perform well as a cervical cancer screening test among HIV-infected women. We found a low specificity and positive predictive value for CIN2+. The low specificity of p16\textsuperscript{INK4a} ELISA could result in an overburdening of the referral system and overtreatment in screen-and-treat settings. With the additional considerations for cervical cancer screening among HIV-infected women in low-resource settings, our study contributes to the ongoing search for a feasible alternative with high sensitivity and specificity for CIN2+.

### Table 3 Continued

| Variable                              | Correct p16\textsuperscript{INK4a} prediction\(^*\) | Incorrect p16\textsuperscript{INK4a} prediction\(^†\) |
|---------------------------------------|------------------------------------------------------|-----------------------------------------------------|
|                                       | Mean or n | SD or per cent | Mean or n | SD or per cent | p Value |
| HAART status                          |           |               |           |               |         |
| Not on HAART                          | 78        | 24%           | 152       | 21%           | 0.25    |
| On HAART                              | 247       | 76%           | 577       | 79%           | 0.12    |
| Time from HIV diagnosis to HAART init (months) | 23.86   | 3.22          | 26.96     | 2.2           |         |
| Duration on current HAART (months)    | 16.48     | 2.17          | 18.66     | 1.56          | 0.12    |

\(^*\)Correct p16\textsuperscript{INK4a} prediction included women whose p16\textsuperscript{INK4a} value accurately predicted the presence or absence of CIN2+, which included CIN2/3 and ICC. Women were classified as correct if they had p16\textsuperscript{INK4a} ≥ 9 U/mL and CIN2+, or if they had p16\textsuperscript{INK4a} < 9 U/mL and non-CIN2+.

\(^†\)Incorrect p16\textsuperscript{INK4a} prediction included women whose p16\textsuperscript{INK4a} value did not accurately predict the presence or absence of CIN2+. Women were classified as incorrect if they had p16\textsuperscript{INK4a} ≥ 9 U/mL and non-CIN2+, or if they had p16\textsuperscript{INK4a} < 9 U/mL and CIN2+.

CIN2+, cervical intraepithelial neoplasia 2+; HAART, highly active antiretroviral therapy; ICC, invasive cervical cancer; STI, sexually transmitted infection.

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Contributors TJW carried out the data collection and organised the data, performed data analyses and interpretation, and drafted the manuscript. KS-M designed the study and performed data interpretation. MR and MvKD performed data interpretation. MM carried out the data collection. MJH designed the study, carried out the data collection, performed data interpretation and supervised the study. All authors provided critical revision of the manuscript on various drafts and also read and approved the final manuscript.

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Competing interests All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf and declare: MKD was shareholder and member of the board of MTM Laboratories (Heidelberg, Germany) at the time the study was initiated. MTM is a company, acquired by Roche in 2011, that makes p16\textsuperscript{INK4a} ELISA kits, which were donated for this study and evaluated in this research.

Ethics approval Ethical review boards at the University of California, San Francisco and Kenya Medical Research Institute (KEMRI) approved the study protocol in accordance with the US Department of Health and Human Services.

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