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Performance of p16^{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

Tara J Wu,[1] Karen Smith-McCune,[2] Miriam Reuschenbach,[3] Magnus von Knebel Doeberitz,[3] May Maloba,[4] Megan J Huchko[2]

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For numbered affiliations see end of article.

Correspondence to
Tara J Wu;
Tara.Wu@ucsf.edu

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^{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^{INK4a} specimen collection and colposcopy. Lesions with unsatisfactory colposcopy or suspicious for cervical intraepithelial neoplasia 2+ (CIN2+; including CIN2/3 or invasive cervical cancer) 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^{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^{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^{INK4a} to detect biopsy-proven CIN2+. At a p16^{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^{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^{INK4a} prediction to detect or rule out CIN2+, and 729 (69.2%) cases of incorrect p16^{INK4a} prediction.

Conclusions: p16^{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.

Strengths and limitations of this study

- This study is the first to investigate p16^{INK4a} ELISA as a primary cervical cancer screening tool in an HIV-infected population.
- The very large sample size of 1054 HIV-infected women provides well-powered estimates of the sensitivity, specificity and predictive values of p16^{INK4a} ELISA.
- In order to evaluate p16^{INK4a} as a stand-alone biomarker for cervical cancer screening, our study uses methods that would be feasible and readily employable in low-resource settings, including performing non-directed p16^{INK4a} specimen collection instead of preferentially sampling acetowhite lesions via colposcopy. This non-directed sampling may have decreased the sensitivity and specificity of p16^{INK4a} for cervical intraepithelial neoplasia 2+ (CIN2+) detection, compared with prior studies.
- We ascertained disease using colposcopy with biopsy only of lesions that appeared suspicious for CIN2+, which may have led to underascertainment of true disease, and to a lower precision of our sensitivity and specificity estimates.

INTRODUCTION

HIV-related immunosuppression significantly increases the incidence and persistence of infection with oncogenic human papillomavirus (HPV),[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.[2–4] Moreover, both diseases disproportionately affect women in low-resource countries. In sub-Saharan Africa, there are 24.7 million HIV-infected people[5] and 70 000 new cases of invasive cervical cancer (ICC) each year.[6] Recent advances in HIV/AIDS care, especially improved access to highly active antiretroviral therapy (HAART), have decreased AIDS-related mortality.[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;[8 9] in other studies, it has been shown to increase or decrease risk of cervical disease, depending on HAART duration or adherence.[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.[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.[13–16] The WHO published updated guidelines for screen-and-treat strategies for cervical cancer control in low-resource countries in 2013.[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.[18 19] With rates of HPV positivity up to 75% in HIV-infected women,[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, $p16^{INK4a}$, is a cyclin-dependent kinase inhibitor overexpressed in HPV-transformed cells, but rarely in normal tissue.[21] As an adjunct to traditional stains, $p16^{INK4a}$ immunohistochemical (IHC) staining significantly improves interobserver agreement in histological assessment, and adjudication of CIN1 compared with CIN2+.[22] An ELISA has been developed using simple technology to measure $p16^{INK4a}$ levels from a swab of exfoliated cervical cells.[23] $p16^{INK4a}$ ELISA showed significantly improved specificity for CIN2+ lesions among a screening population compared with HPV testing.[23 24]

$p16^{INK4a}$ 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 $p16^{INK4a}$ 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.

MATERIALS AND METHODS

Study design

We conducted a cross-sectional study to define the sensitivity, specificity, positive and negative predictive values of $p16^{INK4a}$ 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.[25] Women receive HIV education talks at the time of enrolment with information about basic gynaecological care, cervical cancer and strategies for screening.[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.

Measurements and examination

After signing informed consent, participants underwent pelvic examination with $p16^{INK4a}$ 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.[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 $p16^{INK4a}$ 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 $p16^{INK4a}$ values at the two sites was 0.89, which confirmed that $p16^{INK4a}$ 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 $p16^{INK4a}$-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 $p16^{INK4a}$ protein. For quantification of solubilised $p16^{INK4a}$ values, calibration curves had been established according to the manufacturer's protocol. $p16^{INK4a}$ concentrations in individual samples were calculated as arbitrary units (U/mL) using the standardised curve. For specimens analysed in both laboratories, the $p16^{INK4a}$ values were averaged.

Statistical methods

We based our sample size calculations on the CIN2+ prevalence of 7.2% previously seen in this clinic.[26] We calculated that a sample size of 1100 women was needed to achieve a $p16^{INK4a}$ ELISA anticipated sensitivity between 70% and 80% with 95% CI 5% to −5%, comparable to that of cytology screening and VIA. To attain an anticipated specificity between 80% and 90% with 95% CI 5% to −5%, 500 women needed to be examined. Power analysis was performed using Power Analysis and Sample Size Software (PASS): V.2008 (National Council for Social Studies, Kaysville, Utah).

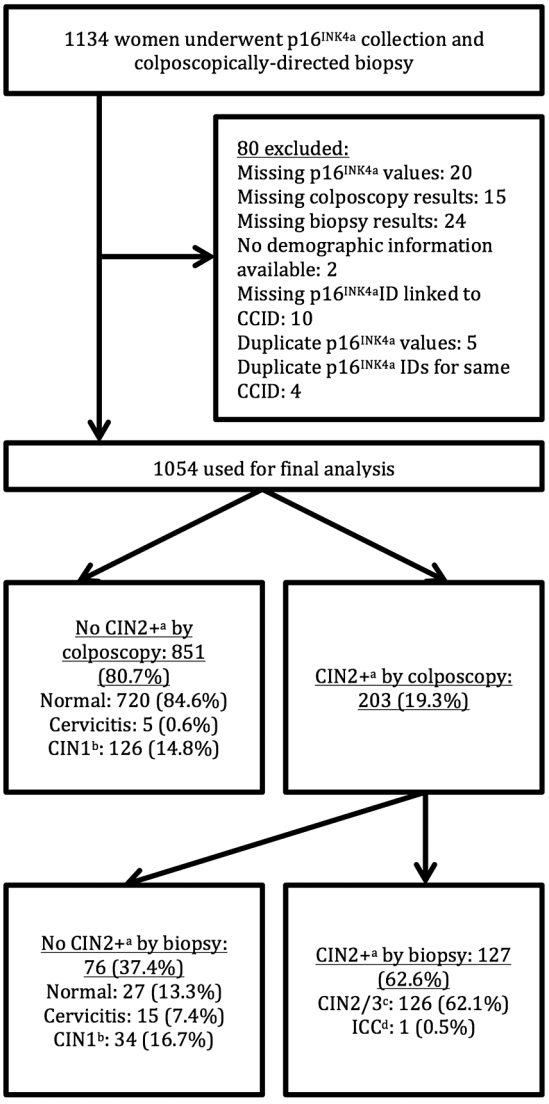
We built receiver operating characteristic (ROC) curves to determine the $p16^{INK4a}$ cut-off values with the highest combined sensitivity and specificity for CIN2+, as determined by the area under the curve (AUC). Using the value with the greatest AUC, sensitivity, specificity, positive and negative predictive values of $p16^{INK4a}$ 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 $p16^{INK4a}$ at the determined cut-off threshold, we created a dichotomous variable named '$p16^{INK4a}$ 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 $p16^{INK4a}$ greater than or equal to the determined threshold with biopsy-confirmed CIN2+ (positive gold standard result), or if they had $p16^{INK4a}$ less than the threshold and no CIN2+ by colposcopy or biopsy (negative gold standard result). Women were classified as incorrect if the $p16^{INK4a}$ value did not correctly predict the presence or absence of CIN2+. Women were classified as incorrect if they had $p16^{INK4a}$ greater than or equal to the threshold and no CIN2+ by colposcopy or biopsy, or if they had $p16^{INK4a}$ less than the threshold and biopsy-confirmed CIN2+. Student's t-tests were performed for continuous variables, while χ^2 tests were performed for categorical variables. Predictors found to be statistically significantly associated with correct $p16^{INK4a}$ prediction on univariate analysis ($p<0.05$) were included in the multivariate logistic regression model. Fit of the regression model was tested using R^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 $p16^{INK4a}$ specimen collection and colposcopy with biopsy as indicated. Of these, 1054 (92.9%) had complete $p16^{INK4a}$ 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



Footnotes

[a]Cervical intraepithelial neoplasia 2+ (CIN2+) [includes CIN2/3 and invasive cervical cancer]
[b]Cervical intraepithelial neoplasia 1 (CIN1)
[c]Cervical intraepithelial neoplasia 2/3 (CIN2/3)
[d]Invasive cervical cancer (ICC)

Figure 1 Flow sheet of study enrolment, eligibility and outcomes. CCID, Cross-Sectional Study ID; CIN2+, cervical intraepithelial neoplasia 2+.

(figure 1). Colposcopy and biopsy results were combined to determine the final number of women with normal results, cervicitis or CIN1. For example, women were considered to have CIN1 if they had CIN1 on colposcopy alone (no biopsy), if they had unsatisfactory colposcopy and CIN1 confirmed on biopsy or if they had the impression of CIN2+ on colposcopy and CIN1 confirmed on biopsy. Among the 1054 participants, a total of 747 (70.9%) women had normal results, 160 (15.2%) women had CIN1, 126 (11.9%) women had CIN2/3, 20 (1.9%) women had cervicitis and 1 (0.1%) woman had stage IA1 ICC.

The mean concentration of $p16^{INK4a}$ protein in cervical samples was 37.4 U/mL (±SD 42.0). The mean $p16^{INK4a}$ in the 747 women with normal results on colposcopic impression or biopsy was 32.6 U/mL (±SD 36.2). Mean $p16^{INK4a}$ in the 160 women with CIN1 on colposcopic impression or biopsy was 38.7 U/mL (±SD 41.5). Mean $p16^{INK4a}$ in the 126 women with biopsy-confirmed that CIN2/3 was 63.4 U/mL (±SD 59.7). The $p16^{INK4a}$ value in the woman with stage IA1 ICC was 210.0 U/mL. In these stratified results, women with cervicitis (mean $p16^{INK4a}$=34.4 U/mL±SD 42.5) were excluded in the calculation of mean $p16^{INK4a}$ values, as they did not constitute normal results nor any form of premalignant disease.

ROC analysis showed an AUC of 0.6639 for $p16^{INK4a}$ to detect biopsy-proven CIN2+ (figure 2A). The sensitivity analyses changing the outcome to any biopsy-proven or visual impression of CIN1+ (figure 2B) decreased the AUC to 0.6078. The AUC was 0.6445 for colposcopic impression only (without biopsy confirmation) of CIN2+ (figure 2C) and 0.6133 for colposcopic impression only of CIN1+ (figure 2D).

The $p16^{INK4a}$ cut-off value with the highest combined sensitivity (89.0%) and specificity (22.9%) for biopsy-proven CIN2+ was 9 U/mL (table 2). The positive predictive value was 13.6% and negative predictive value was 93.8%. Overall, the $p16^{INK4a}$ positivity with the selected 9 U/mL cut-off level was 828 (78.6%) women; in comparison, biopsy-proven CIN2+ was found in only 127 (12%) women. When stratified by age (<35 or ≥35 years), HAART status, average duration on HAART (≤18 or >18 months) or most recent CD4+ count (≤350 or >350 cells/dL), no significant differences were found in regard to sensitivity, specificity or predictive values (table 2).

Using a threshold $p16^{INK4a}$ level of 9 U/mL to predict CIN2+ diagnosis, there were 325 (30.8%) cases of correct $p16^{INK4a}$ prediction and 729 (69.2%) cases of incorrect $p16^{INK4a}$ prediction. On univariate analysis, age, relationship status, number of current partners, prior pregnancies, deliveries and delivery type, whether women were experiencing vaginal symptoms, amenorrhoea, menopause and time since HIV diagnosis were associated with correct $p16^{INK4a}$ prediction ($p<0.05$; table 3), and therefore were included in the final logistic regression model. An R^2 value (range from 0 to 1) to measure the goodness-of-fit of our model to the data was calculated to be 0.0553 (p=0.001), indicating that our model gave little predictive information about the outcome of correct versus incorrect $p16^{INK4a}$ prediction.

DISCUSSION

$p16^{INK4a}$ 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

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		
0		269	25.6%
1		771	73.2%
>1		13	1.2%
Number of previous partners (median)	1047	3	(2–4)
Number of lifetime partners (median)	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 (mean, moths)	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 (mean, months)	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.

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).[28] Specificities are ~73.9% for HR-HPV testing, 94.6% for Pap smear and 80.0% for

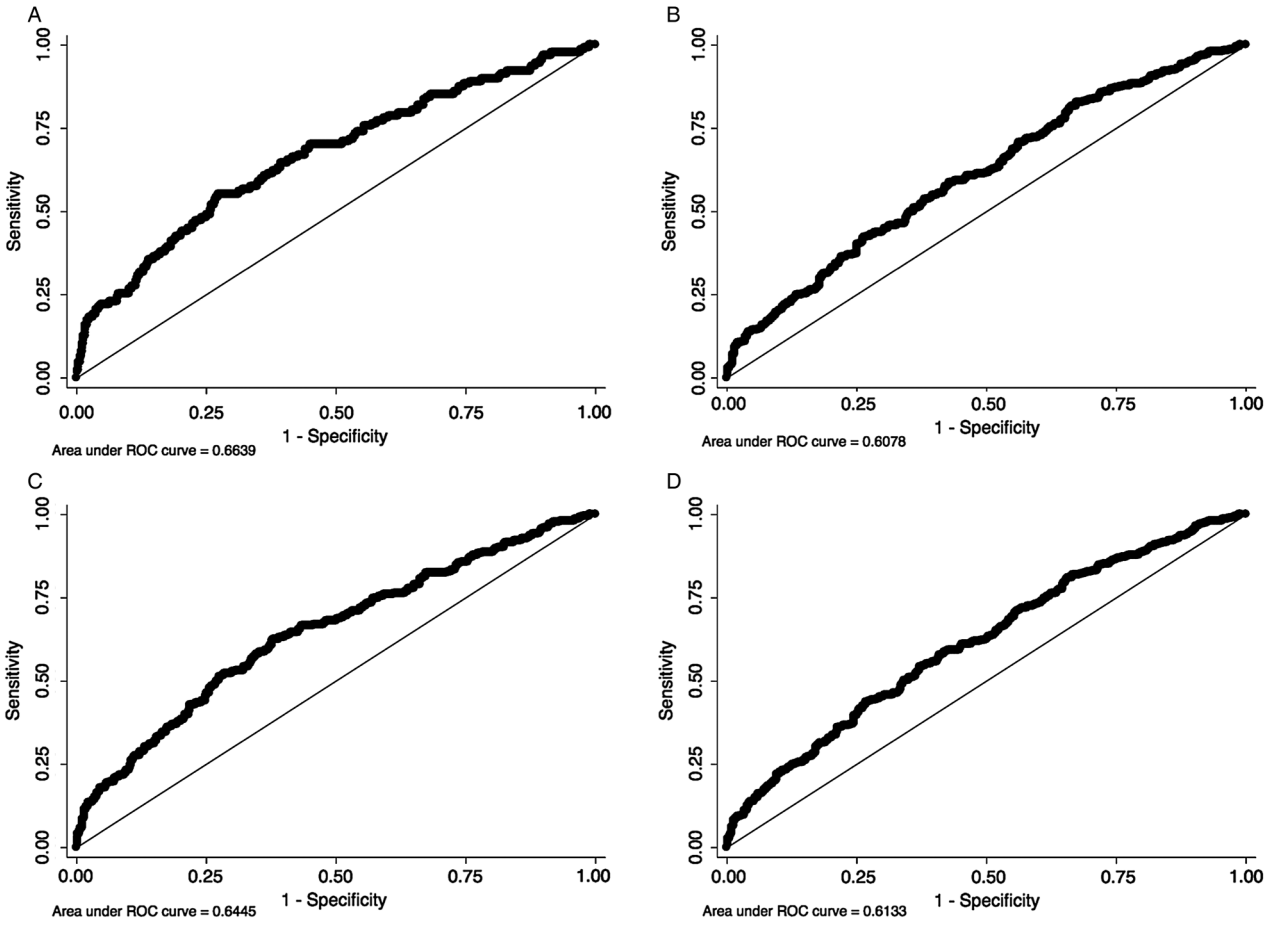


Figure 2 ROC curves (sensitivity vs 1—specificity) in primary and sensitivity analyses (n=1054). (A) Base case: true positives limited to biopsy-proven CIN2+. CIN2+ includes CIN2/3 and ICC. All other diagnoses were considered negative. AUC=0.6639. (B) Sensitivity analysis (1): true positives included 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.

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

Table 2 Test characteristics of p16^{INK4a} ELISA for biopsy-proven CIN2+* in primary and stratified analyses (n=1054)

Primary analyses: sensitivity and specificity of p16^{INK4a} ELISA at varying cut-off levels		
p16^{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^{INK4a} ELISA at the optimal cut-off level	
p16^{INK4a} ELISA test performance (%)	**p16^{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^{INK4a} positivity	78.6
Prevalence of CIN2+	12.0

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

*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^{INK4a} prediction of CIN2+

Variable	Correct p16^{INK4a} prediction*		Incorrect p16^{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%	
Amenorrhoea					
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%	226	31%	
Duration of contraception use (months)	29.05	6.65	34.64	4.66	0.20
Contraception by type					
Oral contraceptives	9	8%	22	8%	0.32
Injectable (Depo Provera)	60	51%	140	49%	
Implant (Jadelle or Norplant)	19	16%	64	23%	
Intrauterine device in situ (copper)	3	3%	7	2%	
Female sterilisation	18	15%	45	16%	
Condom only	8	7%	7	2%	
HIV-related characteristics					
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

Table 3 Continued

Variable	Correct $p16^{INK4a}$ prediction* Mean or n	SD or per cent	Incorrect $p16^{INK4a}$ prediction† 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%	
Time from HIV diagnosis to HAART initiation (months)	23.86	3.22	26.96	2.2	0.12
Duration on current HAART (months)	16.48	2.17	18.66	1.56	0.12

*Correct $p16^{INK4a}$ prediction included women whose $p16^{INK4a}$ value accurately predicted the presence or absence of CIN2+, which included CIN2/3 and ICC. Women were classified as correct if they had $p16^{INK4a} \geq 9$ U/mL and CIN2+, or if they had $p16^{INK4a} < 9$ U/mL and non-CIN2+.
†Incorrect $p16^{INK4a}$ prediction included women whose $p16^{INK4a}$ value did not accurately predict the presence or absence of CIN2+. Women were classified as incorrect if they had $p16^{INK4a} \geq 9$ U/mL and non-CIN2+, or if they had $p16^{INK4a} < 9$ U/m and CIN2+.
CIN2+, cervical intraepithelial neoplasia 2+; HAART, highly active antiretroviral therapy; ICC, invasive cervical cancer; STI, sexually transmitted infection.

HPV viral loads. The higher overall $p16^{INK4a}$ concentration in HIV-positive women indicates that it is critical to set an optimal $p16^{INK4a}$ 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ 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 is the common validation method in similar studies,[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 $p16^{INK4a}$ ELISA. While $p16^{INK4a}$ IHC staining as an adjunct to H&E staining significantly improves interobserver agreement and adjudication of CIN1 vs CIN2+,[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 $p16^{INK4a}$ as a biomarker or other novel methods are warranted to improve first-line screening options for the vulnerable population of HIV-infected women. $p16^{INK4a}$ cytology methods such as CINtec Plus, an immunocytochemistry assay detecting $p16^{INK4a}$ 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),[34] and also recently showed promising results among a resource-limited screening population in Kenya.[35]

On the basis of our findings, $p16^{INK4a}$ 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 $p16^{INK4a}$ 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+.

Author affiliations
[1]School of Medicine, University of California San Francisco, San Francisco, California, USA
[2]Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, San Francisco, California, USA
[3]Department of Applied Tumor Biology, Clinical Cooperation Unit, Institute of Pathology, University of Heidelberg, German Cancer Research Cancer (DKFZ), Heidelberg, Germany
[4]Family AIDS Care and Education Services, Kenya Medical Research Institute, Kisumu, Kenya

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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: MvKD 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 $p16^{INK4a}$ 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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