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Validation of *ASCL1* and *LHX8* Methylation Analysis as Primary Cervical Cancer Screening Strategy in South African Women with Human Immunodeficiency Virus

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Background. Compared with women who are human immunodeficiency virus (HIV) negative, women with human immunodeficiency virus (WWH) have a higher human papillomavirus (HPV) prevalence and increased cervical cancer risk, emphasizing the need for effective cervical cancer screening in this population. The present study aimed to validate methylation markers *ASCL1* and *LHX8* for primary screening in a South African cohort of WWH.

Methods. In this post hoc analysis within the DIAgnosis in Vaccine And Cervical Cancer Screen (DiaVACCS) study, a South African observational multicenter cohort study, cervical scrape samples from 411 HIV-positive women were analyzed for hypermethylation of *ASCL1* and *LHX8* genes, HPV DNA, and cytology. Sensitivities, specificities, and positive and negative predictive values of primary methylation-based, HPV-based and cytology-based screening were calculated for the detection of cervical intraepithelial neoplasia of grade 3 or higher.

Results. Single markers *ASCL1* and *LHX8* resulted in a good performance for the detection of cervical intraepithelial neoplasia of grade 3 or higher, with sensitivities of 85.9% (95% confidence interval [CI], 78.2%–93.6%) and 89.7% (83.0%–96.5%), respectively, and specificities of 72.9% (67.3%–78.5%) and 75.0% (69.5%–80.5%). Combining markers *ASCL1* and *LHX8* resulted in a lower sensitivity compared with HPV testing (84.6% vs 93.6%, respectively; ratio, 0.90 [95% CI, .82–.99]) and a higher specificity (86.7% vs 78.3%; ratio 1.11 [1.02–1.20]) and reduced the referral rate from 46.8% to 33.4%. *ASCL1*/*LHX8* methylation had a significantly higher sensitivity than cytology (threshold, high-grade intraepithelial squamous lesion or worse), (84.6% vs 74.0%, respectively; ratio, 1.16 [95% CI, 1.01–1.32]) and similar specificity (86.7% vs 91.0%; ratio, 0.95 [.90–1.003]).

Conclusions. Our results validate the accuracy of *ASCL1*/*LHX8* methylation analysis for primary screening in WWH, which offers a full-molecular alternative to cytology- or HPV-based screening, without the need for additional triage testing.

Keywords. cervical cancer; cervical intraepithelial neoplasia; host cell DNA methylation analysis; cervical cancer screening; HIV.

Compared with women who are human immunodeficiency virus (HIV) negative, women with HIV (WWH) have a higher prevalence and incidence of cervical human papillomavirus (HPV) infection and cervical intraepithelial neoplasia (CIN) grade 1–3 and a higher incidence of cervical cancer [1–3]. Because most low- and middle income countries (LMICs) with a high prevalence of HIV have limited programs for cervical cancer prevention and control [4], cervical cancer is still the second most common cause of cancer deaths among women in LMICs. In fact, cervical cancer is the leading cause of cancer deaths among South African women [5]. These deaths are preventable when cervical cancer and its precursor lesions are detected and treated early. This underlines the need for cervical cancer prevention programs, especially among WWH. Although many international efforts have been taken to implement HPV vaccination, the percentage of vaccinated women in LMICs remains relatively low. Current cohorts in screening age were not offered an HPV vaccination at adolescent age, making

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cervical screening programs still necessary for the foreseeable future.

Currently, the World Health Organization recommends primary HPV testing as a strategy for cervical cancer screening in LMICs [6]. However, this strategy preferably requires additional triage owing to its suboptimal specificity. In a screen-and-treat strategy without triage testing, referral and treatment of all HPV-positive WWH would lead to overtreatment owing to the high HPV prevalence in WWH [7, 8]. There is thus a need for cervical prevention programs using a 1-step objective molecular test that can be applied on various specimen types, thereby promoting participation in the prevention program.

Hypermethylation analysis of promoter regions of specific host cell genes has been identified as a promising candidate cervical cancer screening test for identification of women with cervical cancer or CIN lesions with a high cancer progression risk (advanced CIN lesions) [9–15]. Methylation markers *ASCL1* and *LHX8*, originally identified in a genome-wide DNA methylation profiling study [16], have been shown to provide a promising primary screening strategy without prior HPV testing in WWH [17]. The current study aims to validate these methylation markers *ASCL1* and *LHX8* for primary screening in a South African cohort of WWH.

METHODS

Study Population

The current study is a post hoc analysis of *ASCL1* and *LHX8* methylation in the HIV-positive women of the DIAgnosis in Vaccine And Cervical Cancer Screen (DiaVACCS) study, a South African observational multicenter cohort study designed to evaluate primary HPV testing and several triage algorithms for cervical cancer screening in South Africa [18]. Between December 2016 and March 2020, 1104 HIV-negative and HIV-positive women aged 25–65 years were recruited in gynecological outpatient or cervical cancer screening clinics at Tshwane District Hospital in Pretoria (site A), Kalafong Provincial Tertiary Hospital in Pretoria (site B), or Tygerberg Academic Hospital in Cape Town (site C), South Africa. Investigators invited women from the public, outpatient clinics, and antiretroviral therapy clinics to participate if they were eligible for screening according to national guidelines [19].

Inclusion criteria were healthcare-seeking behavior or request for a cervical cancer screening test and willingness and ability to receive test results by automated text messages or at clinic visits. Exclusion criteria were a current pregnancy, past hysterectomy, awareness of a cervical screening test in the preceding 5 years, current or previous treatment for gynecological cancer, or inability to undergo screening and treatment (if indicated). All study participants who were not aware of their HIV status underwent a rapid HIV test on study inclusion. The protocol was approved by the Faculty of Health Sciences research ethics committees of University of Pretoria (196/2014) and Stellenbosch University (reciprocal approval no. 2015), registered as a clinical trial (ClinicalTrials.gov NCT02956031), and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants. For this post hoc study, we included all HIV-positive women (n = 423) from the first 1000 study inclusions of the DiaVACCS study.

Sample Collection and Study Procedures

The DiaVACCS trial study procedures have been described in detail by Dreyer et al [18]. In short, all study participants underwent cytology and HPV DNA testing as screening tests. Cervical cells were collected for either conventional or liquid-based cytology (LBC) testing. A second cervical sample was collected using a Cervex Brush (Rovers Medical Devices) and stored in 20 mL of Thinprep PreservCyt solution (Hologic) for HPV DNA testing and molecular analysis. Subsequently, visual inspection of the cervix with acetic acid or Lugol's iodine was performed.

Women with any abnormality reported within 6 months of recruitment, based on visual inspection, HPV or cytology, were invited for colposcopy. A colposcopy-directed biopsy was performed in women with a visible lesion and 2 blind cervical biopsies at 6° and 12° were performed in those without a visible lesion. In addition, a subset of HPV-negative and cytology-negative women was selected for colposcopy and biopsy to comprise a negative control group. Colposcopy and biopsy were planned for a second visit if results of the visual inspection ere not positive at the initial study visit. Following poor attendance for this second visit, the research protocol was amended to include performance of a colposcopy and biopsy at the initial visit for all consenting participants. Large loop excision of the transformation zone (LLETZ) treatment was offered as part of the trial to all women who required this treatment based on national guidelines.

Cytology and Histology and End Points

Conventional cytology was initially performed at sites A and B, but all sites migrated to LBC at a later stage. Cytology results were classified according to the Bethesda 2001 classification [20]. All biopsy and LLETZ specimens were classified as no dysplasia, CIN grade 1, 2, or 3 (CIN1, CIN2, or CIN3), or invasive cervical cancer, according to international criteria [21]. Worst histology on either the cervical biopsy or LLETZ specimen was taken as study end point.

HPV Testing

The presence of HPV DNA in the LBC samples was determined with the Cobas 4800 HPV test (Roche Molecular Diagnostics), according to manufacturers' instructions.

DNA Isolation and Methylation Analysis

Vials containing 3 mL of cervical LBC material in Thinprep PreservCyt medium (original sample volume; 20 mL) were shipped to the Department of Pathology at the Amsterdam University Medical Centers, location Vrije Universiteit, Amsterdam, the Netherlands. DNA was isolated from the cervical LBC material using QIAamp DNA Blood Mini Kit (Qiagen). Isolated DNA was subjected to bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research). All procedures were performed according to manufacturer recommendations.

Quantitative methylation-specific polymerase chain reaction for *ASCL1* and *LHX8* genes has been described elsewhere [16, 17]. Double-stranded gBlocks Gene Fragments (Integrated DNA Technologies) containing the amplicon sequences of all targets and reference gene β-actin were used as technical quality control. Target DNA methylation values were normalized to β-actin and the gBlock using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta}\mathrm{Ct} \times 100$) to obtain ΔΔCt ratios [22]. Samples with a β-actin Ct value >30 were considered invalid.

Statistical Analysis

Log_{10}-transformed Ct ratios were visualized in box plots. The Kruskal-Wallis omnibus test was performed on each methylated gene to assess differences in methylation levels among disease categories. Following a significant result from the omnibus test, post hoc testing was performed using the Mann-Whitney *U* test. Bonferroni correction was used to correct *P* values for multiple testing, differences were considered significant at $P < .05$. The primary end point of the study was CIN of grade 3 or higher (CIN3+) at histology. Women without an histology end point who were screen negative (HPV negative and cytology negative) were considered controls.

The performance of the screening strategies was evaluated with respect to sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and referral rate (based on the percentage of test positivity). We considered 5 screening tests for primary screening: (1) *ASCL1* methylation, (2) *LHX8* methylation, (3) *ASCL*1 and *LHX8* methylation, (4) HPV DNA testing and (5) cytology (threshold, high-grade intraepithelial squamous lesion or worse [≥HSIL]). Previously defined thresholds at 80% specificity for CIN1 or lower grade were used for *ASCL1* and *LHX8* methylation positivity [17].

Results of screening tests 1 and 2 were labeled positive if methylation levels exceeded these thresholds for positivity. Screening test 3 was labeled positive if methylation levels of both *ASCL1* and *LHX8* genes exceeded the threshold for positivity. The result of screening test 4 was considered positive if the Cobas 4800 HPV test result was positive. The result of screening test 5 was considered positive when the cytology result was HSIL or worse (including atypical squamous cells, cannot exclude HSIL and atypical glandular cells of unknown significance). The number of women referred to detect 1 case of CIN3+ was calculated by dividing the number of screen positives by the number of true-positives. Relative sensitivities and specificities (ratios of the sensitivity or specificity of a test to the sensitivity or specificity of the reference test) were calculated with 95% confidence intervals (CIs) and visualized using forest plots, with HPV or cytology as the reference for all other strategies. A difference in sensitivity or specificity was considered significant if the 95% CI of the relative sensitivity or specificity was entirely >1 or <1.

RESULTS

Study Cohort and Baseline Findings

Of 423 HIV-positive women from the DiaVACCS study, 11 women were excluded owing to invalid methylation results and 1 was excluded because of an endometrial cancer diagnosis, resulting in a study population of 411 HIV-positive women (median age, 40 years; age range, 25–64 years). Baseline study population characteristics are shown in Table 1. Histology results were as follows: 79 women had no histology end point; 88 no CIN; 73, CIN1; 71, CIN2; 71, CIN3; and 7, squamous cell carcinoma. The overall HPV positivity was 48.4% (199 of 411) and increased from 36.4% in those with no CIN and 27.4% in CIN1 to 80.3% in CIN2, 93.0% in CIN3, and 100% in carcinoma. Twenty-two women tested positive at ≥1 screen test but were lost to follow-up. These women were excluded from further analysis, resulting in a final study population of 389 women.

Methylation Analysis

As shown in Figure 1, methylation levels of *ASCL1* and *LHX8* genes increased significantly from CIN1 to CIN2 and from CIN2 to CIN3. Supplementary Table 1 shows an overview of all histology, HPV, cytology, and methylation results. All 7 squamous cell carcinomas had positive HPV, cytology, *ASCL1,* and *LHX8* results. Figure 2 shows the proportions of *ASCL1* and *LHX8* genes testing positive in relation to the severity of underlying disease. The proportion of both markers testing positive increased from 9.6% in CIN1 to 45.1% in CIN2, 83.1% in CIN3, and 100% in carcinoma.

Performance of Screening Strategies

Next, we evaluated the performance of single *ASCL1*, single *LHX8,* and combined *ASCL1*/*LHX8* methylation analysis for the detection of CIN3+ and compared these with primary HPV screening and primary cytology (≥HSIL) screening.

Table 1. Baseline Study Population Characteristics

Characteristic	Study Participants, No. (%)[a] (n = 411)
Results of cytology	
NILM	243 (59.1)
ASC-US	13 (3.2)
LSIL	27 (6.6)
AGUS	1 (0.2)
ASC-H	17 (4.1)
HSIL	87 (21.2)
Carcinoma or suspicious/malignant cells	9 (2.2)
Inadequate	14 (3.4)
Results of histology	
Lost to follow-up	22 (5.4)
No histology, double screen negative	79 (19.2)
No CIN	88 (21.4)
CIN1	73 (17.8)
CIN2	71 (17.3)
CIN3	71 (17.3)
Squamous cell carcinoma	7 (1.7)
HPV results	
Positive	199 (48.4)
HPV-16/18	72 (17.5)
Non–HPV-16/18	127 (30.9)
Negative	212 (51.6)
Origin of sample[b]	
Tshwane	328 (79.8)
Tygerberg	31 (7.5)
Kalafong	52 (12.7)
Age, median (range), y	40 (25–64)
Age group, y	
25–34	89 (21.7)
35–44	197 (47.9)
45–54	97 (23.6)
55–64	28 (6.8)
ARV use and duration	
Yes	390 (94.9)
Duration of use <12 mo	53 (12.9)
Duration of use >12 mo	337 (82.0)
No	20 (4.9)
Unknown	1 (0.2)

Abbreviations: AGUS, atypical glandular cells of unknown significance; ARV, antiretroviral; ASC-H, atypical squamous cells, cannot exclude HSIL; ASC-US, atypical squamous cells of unknown significance; CIN, cervical intraepithelial neoplasia; CIN1, CIN2, and CIN3, CIN grade 1, 2, and 3; HPV, human papillomavirus; HSIL, high-grade intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

[a]Data represent no. (%) of study participants unless otherwise specified.

[b]Tshwane District Hospital (Pretoria, South Africa), Tygerberg Academic Hospital in (Cape Town, South Africa), and Kalafong Provincial Tertiary Hospital (Pretoria).

Table 2 shows sensitivities, specificities, PPVs, NPVs, referral rates, and the number of referrals needed to detect 1 CIN3+. Relative sensitivities and relative specificities with HPV testing (test 4) as the reference strategy are shown in Figure 3. The sensitivities and specificities of *ASCL1* as a single marker (test 1) and *LHX8* as a single marker (test 2) were similar to those of HPV. For *ASCL1* versus HPV, the sensitivity was 85.9% versus 93.6%, respectively (ratio, 0.92 [95% CI, .84–1.003]), and the specificity 72.9% versus 78.3% (ratio, 0.93 [.85–1.02]). For *LHX8* versus HPV, the sensitivity was 89.7% versus 93.6%, respectively (ratio, 0.96 [95% CI, .88–1.04]), and the specificity, 75.0% versus 78.3% (ratio, 0.96 [.87–1.05]). Combining *ASCL1* and *LHX8* (test 3; both markers positive) resulted in a lower sensitivity compared with HPV (84.6% versus 93.6%, respectively; ratio, 0.90 [95% CI .82–.99]) and a higher specificity (86.7% versus 78.3%; ratio, 1.11 [1.02–1.20]) without losing sensitivity for cervical cancer.

Figure 3*B* shows relative sensitivities and specificities, with cytology (≥HSIL) as a reference. *ASCL1* and *LHX8* (test 3) had significantly higher sensitivity than cytology (84.6% versus 74.0%, respectively; ratio, 1.16 [95% CI, 1.01–1.32]) and similar specificity (86.7% versus 91.0%; ratio, 0.95, [.90–1.003]). All other strategies had a significantly higher sensitivity than cytology, at a lower specificity. Supplementary Table 2 shows sensitivities, specificities, PPVs, NPVs, referral rates, and the number of referrals needed to detect 1 case of CIN3+ when *ASCL1* methylation, *LHX8* methylation, or cytology is used as a triage test for HPV-positive women. While the number of tests increases, the referral rates decrease for all triage strategies, at the cost of a small decrease in sensitivity.

DISCUSSION

In the current study, we evaluated primary methylation-based cervical cancer screening in WWH and demonstrated a good performance of methylation markers *ASCL1* and *LHX8* for the detection of CIN3+. Single-marker strategies with *ASCL1* and *LHX8* resulted in a similar performance for the detection of CIN3+ compared with HPV testing. Combining *ASCL1* and *LHX8* led to a lower sensitivity than HPV testing (84.6% vs 93.5%, respectively) but higher specificity (86.7% vs 78.3%) and reduced the referral rate from 46.8% to 33.4%. Compared with cytology (threshold, ≥HSIL), the combination of *ASCL1* and *LHX8* resulted in significantly higher sensitivity at a similar specificity.

Our findings are in line with those of Kremer et al, who found CIN3+ sensitivities of 67.2% for primary *ASCL1* methylation and 70.5% for primary *LHX8* methylation at a fixed specificity of 80%. Application of these thresholds to our independent cohort validated the accuracy of these markers for primary screening. It has been shown that methylation analysis is particularly sensitive for "advanced" cervical lesions, defined as CIN lesions associated with a persistent HPV infection (≥5 years) and also characterized by increased chromosomal aberrations [23–25]. Methylation analysis also has an extremely high sensitivity for cervical cancer (>98%) [26].

In line with these findings, high CIN3+ sensitivities for methylation analysis are found and all 7 cervical carcinomas in this study are detected by both methylation markers. The

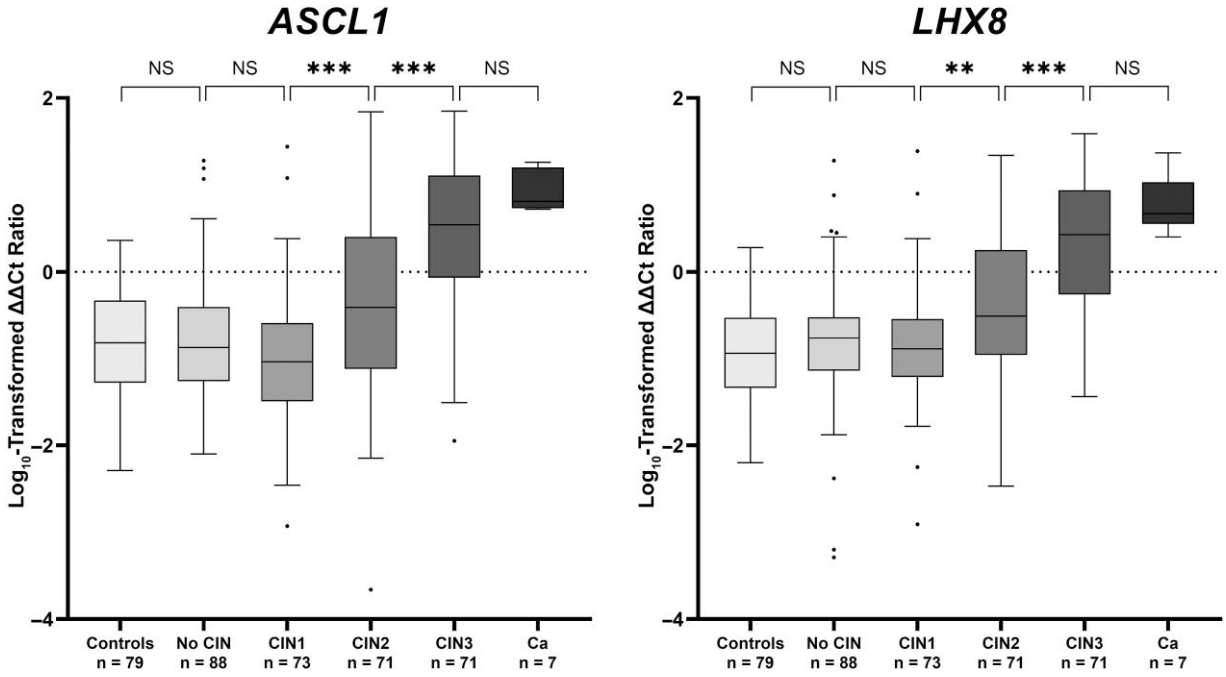

Figure 1. Methylation levels of *ASCL1* and *LHX8* genes increase with severity of disease. Methylation levels are represented by the $\log_{10}$-transformed ΔΔ cycle threshold (Ct) ratios (y-axis) in different histology groups. Abbreviations: Ca, cervical carcinoma; CIN, cervical intraepithelial neoplasia; CIN1, CIN2, and CIN3, CIN grade 1, 2, and 3; NS, not significant. $^{**}P < .01$; $^{***}P < .001$.

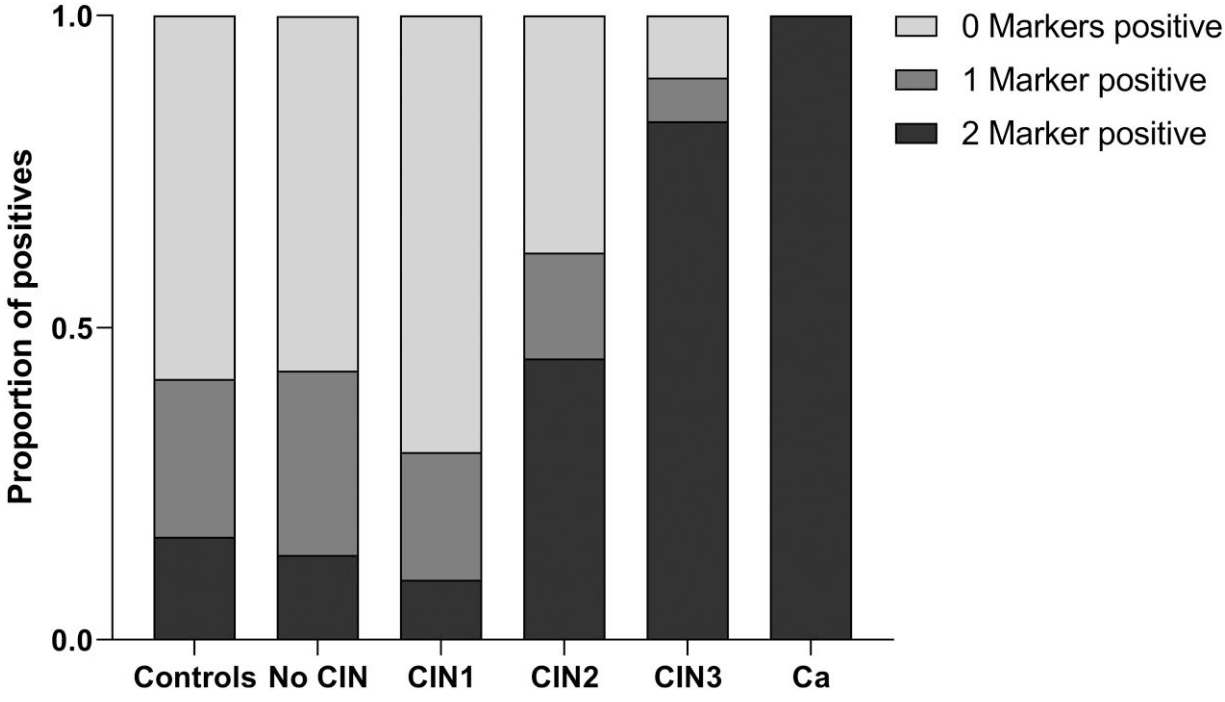

Figure 2. Proportion of hypermethylated *ASCL1* and *LHX8* genes testing positive in relation to severity of underlying cervical disease. The proportions of samples testing positive for 0, 1, or 2 markers within the different histology subgroups (x-axis) are represented on the y-axis. Abbreviations: Ca, cervical carcinoma; No CIN, CIN1, CIN2, and CIN3, cervical intraepithelial neoplasia, grade 0, 1, 2, and 3.

Table 2. Performance of Primary Screening Tests for the Detection of Cervical Intraepithelial Neoplasia of Grade 3 or Higher in Women With Human Immunodeficiency Virus

Test	Positivity, No. Positive/Total No. (%)			Sensitivity for CIN3+ (95% CI), %	Specificity for ≤CIN1 (95% CI), %	PPV for CIN3+, %	NPV for CIN3+, %	Referral Rate, %	Referrals Needed to Detect 1 CIN3+ Case
	Carcinoma	CIN3	CIN2						
1. *ASCL1*	7/7 (100.0)	60/71 (84.5)	38/71 (53.5)	85.9 (78.2–93.6)	72.9 (67.3–78.5)	39.4 (21.3–46.8)	95.0 (92.1–97.9)	43.7	2.5
2. *LHX8*	7/7 (100.0)	63/71 (88.7)	38/71 (53.5)	89.7 (83.0–96.5)	75.0 (69.5–80.5)	41.7 (34.2–49.1)	96.4 (93.9–98.8)	43.2	2.4
3. *ASCL1* and *LHX8*	7/7 (100.0)	59/71 (83.1)	32/71 (45.1)	84.6 (76.6–92.6)	86.7 (82.4–91.0)	50.8 (42.2–59.4)	95.4 (92.8–97.9)	33.4	2.0
4. HPV	7/7 (100.0)	66/71 (93.0)	57/71 (80.3)	93.6 (88.2–99.0)	78.3 (73.1–83.5)	40.1 (33.0–47.2)	97.6 (95.5–99.7)	46.8	2.5
5. Cytology[a] (≥HSIL)	7/7 (100.0)	50/70 (71.4)	28/69 (40.6)	74.0 (64.2–83.8)	91.0 (87.3—94.7)	53.8 (44.3–63.3)	92.7 (89.6–95.8)	28.0	1.9

Abbreviations: CI, confidence interval; CIN≤1, CIN2, CIN3, and CIN3+, cervical intraepithelial neoplasia, grade 1 or below, grade 2, grade 3, and grade 3 or higher; HPV, human papillomavirus; ≥HSIL, high-grade intraepithelial lesion or worse; NPV, negative predictive value; PPV, positive predictive value.

[a]Ten women had an inadequate cytological result and were excluded from the analysis concerning cytology.

good performance of *ASCL1*/*LHX8* methylation (strategy 3), with an increased specificity compared with HPV-based screening, indicates that *ASCL1*/*LHX8* methylation is an interesting objective primary screening tool that could limit referral rates while detecting the majority of CIN3 cases, and a negative test outcome shows a high reassurance against cervical

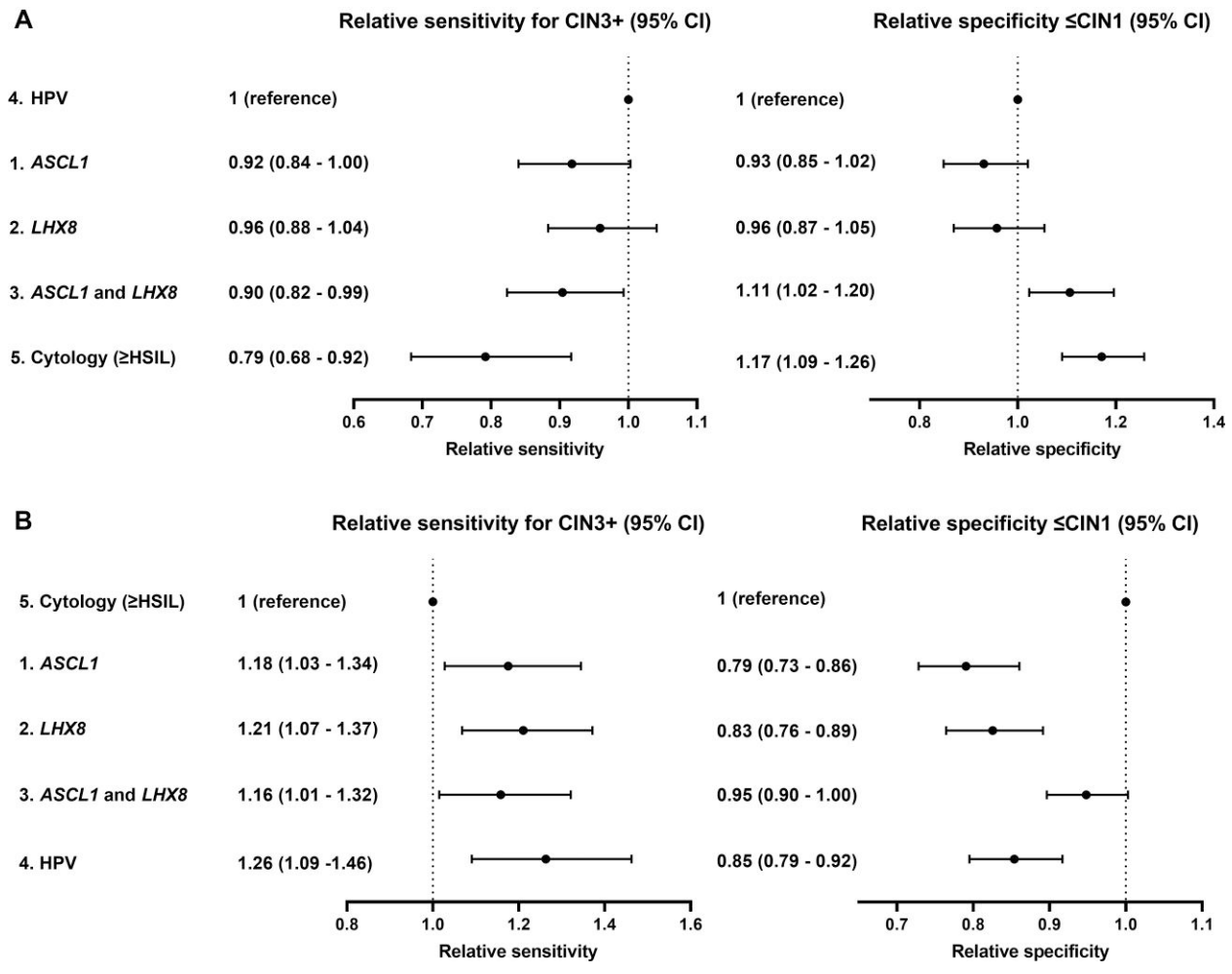

Figure 3. Forest plots showing the relative sensitivity and specificity for the detection of cervical intraepithelial neoplasia (CIN) of grade 3 or higher (CIN3+) of different primary screening tests compared with human papillomavirus (HPV) (*A*) and cytology (threshold, high-grade squamous intraepithelial lesion or worse [≥HSIL]) (*B*). Abbreviations: CI, confidence interval; ≤CIN1, CIN grade 1 or below.

cancer. In addition, methylation analysis has been shown to be compatible with self-collected cervicovaginal material and is therefore an interesting strategy that could improve adherence to screening [16, 27–29]. The presently investigated markers *ASCL1* and *LHX8* were originally identified and validated on self-collected vaginal samples [16].

Cytology screening is still the norm in South Africa. A cutoff of ≥HSIL is used for immediate treatment by LLETZ and repeated cytology is advised in women with atypical squamous cells of unknown significance or a low-grade squamous intraepithelial lesion [30]. However, women with such findings are often lost to follow-up owing to the absence of an active recall system. In our study, cytology (threshold, ≥HSIL) led to the lowest referral rate of 28.0%. However, *ASCL1*/*LHX8* methylation (strategy 3) increased the referral rate only to 33.4% with a specificity similar to that of cytology (threshold, ≥HSIL) but at a significantly 10% higher sensitivity (84.6% vs 74.0%). In addition, cytology remains a subjective test and requires involvement of limited trained cytotechnicians [31].

Although the utility of HPV testing as a primary screening tool is recognized, HPV-based cervical screening has not been widely introduced in South Africa. The burden of HPV infection in women in South Africa is high, especially in WWH, and will lead to healthcare challenges in the triage of screen-positive women in an HPV-based screening program [8, 31, 32]. The World Health Organization screening guidelines recommend triage testing in a primary HPV screening program whenever possible and allow for "test-and-treat" if triage is not feasible [6]. However, a test-and-treat strategy in WWH will be associated with considerable overreferral and overtreatment, requiring more trained personnel. The present study found a referral rate of 46.8% for HPV-positive women, indicating the need for a more specific screening strategy.

Evaluations of methylation markers for cervical cancer screening in WWH have shown promising results [14, 33–36]. To the best of our knowledge, ours is the first study to validate methylation analysis as a primary screening strategy in a large population-based multicenter cohort study of WWH. A limitation is that a minority of women had no histological end point. Although their screen results were double negative (both HPV and cytology negative), underlying cervical disease cannot completely be ruled out. Another limitation is the potential preferential effect in favor of cytology and HPV, owing to management based on positive cytological and/or HPV results and not based on a positive methylation result. The methylation analyses were performed in an expert laboratory in the Netherlands. In future, the aim is to evaluate test performance locally in an implementation study to truly represent field conditions. Robust, automated, and user-friendly workflows for methylation assays that can generate results within a day at limited costs are needed before implementation in cervical screening in LMICs can be realized. Recent developments have shown that combined DNA extraction and bisulfite conversion is feasible, can be automated, and yields reliable methylation tests results [37]. These technical improvements will shorten test procedures and facilitate methylation testing, making primary methylation testing affordable also for LMICs.

To conclude, the current study validated the accuracy of *ASCL1* and *LHX8* methylation analysis for primary screening in WWH. Combined analysis of *ASCL1* and *LHX8* methylation analysis is a primary, objective full-molecular screening strategy that is applicable to cervical scrape samples and cervicovaginal self-collected samples and is an useful alternative to primary cytology or primary HPV screening, without the need for an additional visit to the physician for triage testing.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Principal investigator: G. D. Study design: C. J. L. M. M., K. L. R., R. D. M. S., and G. D. Data collection and processing: C. V., L. C. S., F. H. v. d. M., M. H. B., and G. D. Data management: F. J. V. and C. V. Laboratory experiments: F. J. V. and S. D. Statistical analysis: F. J. V. and B. I. L. W. Data interpretation: F. J. V., C. J. L. M. M., R. D. M. S., and G. D. First draft of manuscript: F. J. V., C. J. L. M. M., and R. D. M. S. All authors were involved in writing the manuscript and had final approval of the submitted and published versions.

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Potential conflicts of interest. C. J. L. M. M., and R. D. M. S. are minority shareholders of Self-screen, a spin-off company of Amsterdam UMC, location Vrije Universiteit; Self-screen develops, manufactures and licenses high-risk HPV and methylation marker assays for cervical cancer screening and holds patents on these tests. A research use only version of the methylation assay has been made available by Self-screen (www.self-screen.nl). C. J. L. M. M. is the part-time chief executive officer of Self-screen and served occasionally on scientific advisory boards and/or speakers bureaus for GlaxoSmithKline (GSK), Qiagen, Sanofi Pasteur MSD/Merck, and Asieris Pharma/Ismar Healthcare. He also reports consulting fees from Qiagen, GSK, Sanofi Pasteur MSD/Merck, Asieris Pharma/Ismar Healthcare, paid to the author, and support for attending meetings and/or travel, paid to the author, from Qiagen, GSK, Sanofi Pasteur MSD/Merck, and Self-screen. He formerly had a very small number of shares of MDXHealth and Qiagen. R. D. M. S. is an inventor on patents on methylation markers for cervical cancer (patents are licensed to Self-screen). All other authors report no potential conflicts. All authors have submitted the

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