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Posttreatment monitoring by *ASCL1/LHX8* methylation analysis in women with HIV treated for cervical intraepithelial neoplasia grade 2/3

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Objective: Women with HIV (WWH) have an increased risk to develop recurrent cervical intraepithelial neoplasia grade 2/3 (rCIN2/3) after treatment compared with HIV-negative women. Therefore, appropriate posttreatment monitoring of WWH is important. This study evaluates the performance of *ASCL1* and *LHX8* methylation analysis as posttreatment monitoring test in WWH treated for CIN2/3, as alternative to cytology or human papillomavirus (HPV) as follow-up test.

Design: Prospective observational cohort study.

Methods: WWH treated for CIN2/3 by large loop excision of the transformation zone (LLETZ) ($n = 61$) were invited for follow-up study visits at 1, 2.5 and 4 years after baseline. Baseline and follow-up cervical scrapes were tested for cytology, HPV and DNA methylation of *ASCL1* and *LHX8* genes. The performance of these strategies for the detection of rCIN2/3 was evaluated in the first follow-up cervical scrape.

Results: Thirteen (21.3%) rCIN2/3 lesions were detected within 4 years of follow-up. In women without rCIN2/3 in follow-up, methylation levels of *ASCL1* and *LHX8* decreased significantly after LLETZ treatment ($P = 0.02$ and 0.007, respectively). In women with rCIN2/3, methylation levels remained high after LLETZ treatment. The 4-year rCIN2/3 risk was 4.9% (95% CI: 0.6–16.5) for *ASCL1/LHX8-negative* women, 8.1% (95% CI: 1.7–21.9) for HPV-negative women and 7.7% (95% CI: 2.1–18.5) for cytology-negative women.

Conclusion: A negative *ASCL1/LHX8* methylation test in follow-up is associated with a low rCIN2/3 risk and could serve as an objective test of cure and well tolerated alternative for HPV and/or cytology screening in the posttreatment monitoring of WWH.

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Keywords: cytology, DNA methylation marker, HIV, human papilloma virus, recurrent cervical intraepithelial neoplasia

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Introduction

Women diagnosed with cervical intraepithelial neoplasia grade 2 or 3 (CIN2/3) are treated by ablative surgery (most commonly excision or cryotherapy) to prevent progression to cervical cancer [1,2]. Despite treatment, these women are at risk to develop recurrent disease [3]. Recurrent CIN2/3 (rCIN2/3) can be divided into residual lesions (i.e. incompletely excised) or lesions caused by a new contracted HPV infection (so-called incident or early-onset CIN2/3) [4–6]. Detection of residual CIN2/3 is of particular importance because of their potentially faster progression to cancer compared with incident CIN caused by newly contracted HPV infections [3,6]. Women with HIV (WWH) are known to have two-fold higher rCIN2/3 risk compared with HIVnegative women [7–9]. Therefore, appropriate posttreatment monitoring of WWH is especially important.

Cytology and HPV testing are most commonly used for posttreatment monitoring but the length and frequency of follow-up vary widely between countries [10]. In Europe, three subsequent negative cytology tests at 6, 12 and 24 months or two subsequent negative cytology and HPV tests at 6 and 24 months or combinations thereof are recommended. After that period, women are referred back to the screening program with intervals of3–5 years [4,11–17]. In sub-Saharan African WWH, HPV incidence rates are very high [18,19] and HPV testing will result in a low specificity for rCIN2/3 [20]. Co-testing with frequent follow-up visits may be a challenge in low-income and middle-income countries (LMIC), often short in supply of recourses and trained personnel. Moreover, frequent follow-up visits are by many women experienced as a burden, resulting in a high loss to follow-up.

Methylation of promotor regions of tumour suppressor genes is an epigenetic DNA alteration that leads to gene silencing and is a crucial event in the cervical carcinogenesis. Methylation levels increase with increasing CIN grade, are particularly high in advanced CIN lesions associated with a longstanding HPV infection and in cervical cancer [21–23]. Therefore, high methylation levels are assumed to be associated with CIN lesions with a high cancer progression risk [24]. Recent data provide further evidence for this hypothesis, showing that methylation-negative CIN2/3 more often regress compared with methylation-positive CIN2/3 (Kremer, Dick *et al.,* in preparation) and that a positive methylation test was able to predict progressive CIN2 [25]. Moreover, a negative methylation test was shown to provide reassurance against CIN3 and cervical cancer [26–28]. Methylation analysis of several host-cell genes, such as *CADM1, EPB41L3, FAM19A4, MAL* and *miR124-2* in cervical scrapes has been shown to provide a promising strategy for the detection of CIN3 and cervical cancer in WWH [29–32]. More recently, *ASCL1* and *LHX8* genes have been identified as promising triage markers in HPV-positive women in both cervical scrapes as self-collected specimens [33–35]. These markers have also been demonstrated to enable CIN3 and cervical cancer detection in WWH without prior HPV testing [36,37]. Furthermore, methylation analysis has potential as a followup test after treatment of CIN, as shown for *CADM1* and *MAL* genes [5].

Here, we present follow-up data of 61 WWH treated for high-grade CIN and evaluated *ASCL1* and *LHX8* methylation analysis as an alternative test of cure, aiming to simplify the follow-up scheme of posttreatment monitoring for WWH.

Methods

Study population

The initial study cohort consists of 355 WWH visiting the gynaecologic outpatient clinic for cervical screening, included between November 2014 and March 2015 at Tshwane District Hospital, Pretoria, South Africa. The study was approved by the Research Ethics Committee of the University of Pretoria, South Africa (protocol numbers 100/2012, 155/2014 and 422/2018). Detailed characteristics and inclusion criteria of the initial study have been described previously [30]. In short, all women underwent cytology screening, colposcopy and two mandatory cervical biopsies at baseline. Women with abnormal cytology (≥HSIL) or CIN2 or worse (CIN2+) on biopsy were treated by large loop excision of the transformation zone (LLETZ) or clinical cancer staging, according to local guidelines.

Figure 1 shows the study flowchart. For this study, we selected 92 women with a CIN2/3 detected at the baseline study visit. Women who did not receive LLETZ treatment or who were lost to follow-up after LLETZ treatment were excluded, resulting in a final study population of 61 women. All women in the study population were on antiretroviral treatment (ART) and their median $CD4^+$ cell count was 475 cells/μl (IQR: 276–744 cells/μl) at baseline.

Study procedures

Baseline study visit

Cervical cells were collected using a Cervex Brush (Rovers Medical Devices B.V, Oss, the Netherlands), and after preparation of a conventional slide, stored in Thinprep PreservCyt solution (Hologic, Marlborough, Massachusetts, USA) for HPV and methylation analysis in the Netherlands. The conventional cytology slide was locally evaluated and used for patient management. Colposcopy was performed on all participants and two

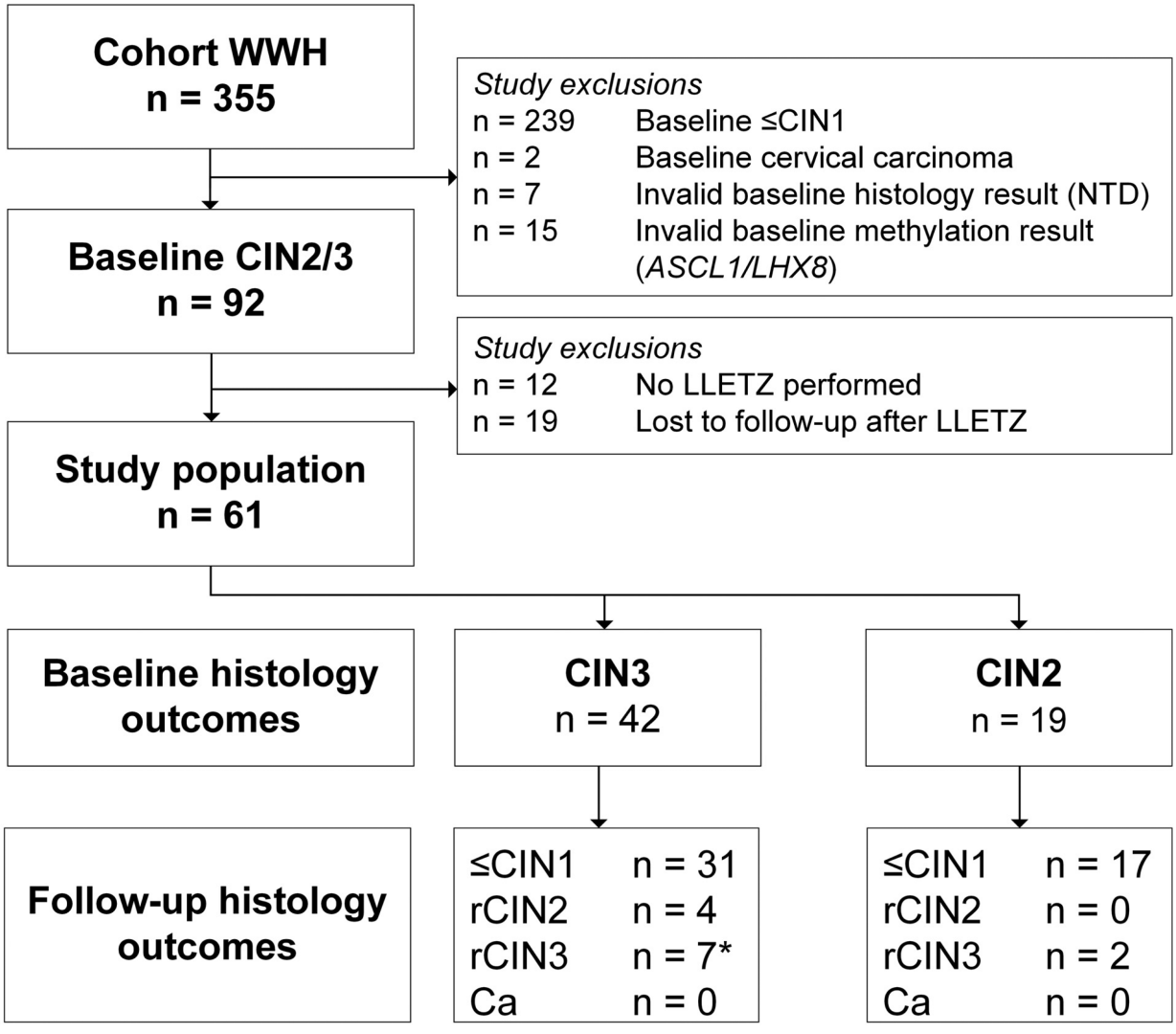

Fig. 1. Study flowchart. *One case of rCIN3 was excluded from analysis with HPV and methylation data as no follow-up cervical scrape was available. Ca, cervical carcinoma; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; NTD, not to determine; rCIN, recurrent CIN; WWH, women with HIV. Recurrent CIN was defined as a CIN2/3 diagnosed longitudinally after treatment of a baseline CIN2/3.

biopsies were taken from the most severe cervical lesion or, if no lesion present, two random biopsies (0600 and 1200 h) were collected. Histology of the biopsies was performed at the Department of Pathology, Amsterdam University Medical Centers, location VUmc.

Follow-up study visits

All study participants were invited for follow-up study visits at approximately 1, 2.5 and 4 years after baseline. During each follow-up visit, cervical cells were collected using a Cervex Brush and stored in Thinprep Preservcyt solution for local liquid-based cytology evaluation. All participants underwent colposcopy with biopsies in accordance with the baseline study visit. Women with abnormal cytology (≥HSIL) were treated by (re-) LLETZ, according to local guidelines.

High-risk human papilloma virus DNA and DNA methylation testing

High-risk HPV DNA testing and DNA methylation analysis of *ASCL1* and *LHX8* genes was performed on DNA isolated from cervical scrapes as described previously [30,36]. The presence of high-risk HPV DNA was determined using a clinically validated generic HPV test (GP5+/6+ PCR-EIA), that detects 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) [38,39]. Subsequent genotyping of EIA-positive samples was performed using a microsphere bead-based assay (Luminex) [40]. Samples testing EIA-negative were tested with β-globin PCR analysis to assure sample quality.

Quantitative methylation-specific PCR (qMSP) for *ASCL1* and *LHX8* genes was performed using

bisulphite-converted DNA as described previously [41]. Target DNA methylation values were normalized to reference gene β-actin and the calibrator using the comparative Ct method (2-ddct × 100) to obtain ddCt ratios [42]. Samples with a β-actin Ct value above 30 were considered invalid.

Study endpoints

Baseline conventional cytology slides were assessed using regular cervical screening procedures and classified according to the Bethesda 2001 classification [43]. All biopsies and LLETZ specimen were classified as no dysplasia, CIN1, CIN2, CIN3 or invasive cancer, according to international criteria [44]. Baseline histology diagnoses are based on the worst histology outcome on either the baseline cervical biopsy or the baseline LLETZ specimen. Longitudinal histology diagnoses are based on the worst histology outcome of either the follow-up cervical biopsy specimen or the follow-up LLETZ specimen. If no diagnosis could be made based on baseline biopsy or LLETZ specimen, a sample was excluded from analysis. A rCIN2/3 was defined as a CIN2/3 diagnosed longitudinally after treatment of a baseline CIN2/3. One case of rCIN3 was excluded from analysis with HPV and methylation data as no follow-up cervical scrape was available.

Statistical analysis

Square root-transformed Ct ratios from methylation testing were visualized in boxplots. The Wilcoxon matched-pairs signed-ranks test was conducted to compare baseline and follow-up methylation levels within women. The Mann–Whitney *U* test was conducted to assess differences in methylation levels among baseline and follow-up samples between women. Differences in lesion size were calculated using the Fisher's exact test. Previously defined thresholds were used for *ASCL1* and *LHX8* methylation positivity [36]. A sample was considered positive for the *ASCL1/LHX8* marker panel when it exceeds the thresholds for both *ASCL1* and *LHX8* genes. A sample was considered negative for the *ASCL1/LHX8* marker panel when it was negative for either one or both genes. The performance for rCIN2/3 detection was evaluated in terms of sensitivity, specificity, positive-predictive value (PPV), 1-negative-predictive value (1-NPV, absolute rCIN2/3 risk) and referral rate with exact 95% confidence intervals (Clopper-Pearson). Differences between sensitivities and specificities were calculated using the McNemar test. In case of 100% specificity of one of the tests, the exact McNemar test was used, using R package exact2x2. To compare PPVs and 1-NPVs, the method of Leisenring *et al.* [45] was used in R package DTComPair. A *p* value of 0.05 was considered statistically significant. All statistical analysis were performed with R (V4.0.3), SPSS Statistics (version 26; IBM Corp., Armonk, New York, USA), and Graphpad Prism (Version 8.2.1.; San Diego, California, USA).

Results

Study population

The study population consists of 61 HIV seropositive women with a baseline CIN2/3 lesion (19 CIN2, 42 CIN3, Fig. 1), who were all treated by LLETZ. These women were invited for follow-up visits at approximately 1, 2.5 and 4 years after baseline (T1, T2 and T3, respectively) and all women attended at least one followup visit. The median interval between LLETZ treatment and first follow-up visit after LLETZ was 1.1 years (IQR 0.7–1.8years). Median age was 38 years (IQR: 34.5–45 years).

Recurrent CIN2/3

In total, 13 rCIN2/3 lesions were detected within 4 years of follow-up, resulting in a rCIN2/3 percentage of 21.3% (13/61) up to 4 years of follow-up. No cervical cancer was detected during 4 years of follow-up. Seven rCIN3 and four rCIN2 were diagnosed after treatment for CIN3, whereas 2 rCIN3 were diagnosed after treatment for CIN2. Thirty-one women had CIN1 or less in follow-up after treatment for a CIN3 and 17 women had CIN1 or less in follow-up after treatment for CIN2. For one woman with rCIN3 after treatment for CIN3, no follow-up cervical scrape was available for HPV and methylation analysis; this sample was excluded from further analysis. The HPV genotyping results in women with rCIN2/3 are shown in Supplementary Table 1, http://links.lww.com/QAD/C457. Six out of 12 women with rCIN2/3 (50%) had a persistent HPV infection with the same, or in case of multiple infection at least one identical HPV type, three out of 12 women (25%) had a genotype switch (i.e. an incident HPV infection) and three out of 12 women (25%) tested high-risk HPV negative at follow-up, of whom one was HPV-positive (HPV51) at baseline. Four out of 12 women with rCIN2/3 (33%) tested HPV16-positive at baseline, of whom two had a persistent HPV16 infection.

Methylation levels in women with and without recurrent CIN2/3

Figure 2 shows *ASCL1* and *LHX8* methylation levels of the baseline sample and of the first available follow-up sample of women with CIN1 or less in follow-up and women with rCIN2/3 in follow-up. In women with CIN1 or less in follow-up, methylation levels of *ASCL1* and *LHX8* decreased significantly after LLETZ treatment ($P = 0.02$ and 0.007, respectively). In women with rCIN2/3 methylation levels remained high after LLETZ treatment. Women with rCIN2/3 had significantly higher *ASCL1* and *LHX8* methylation levels in their first follow-up scrape compared with women with CIN1 or less in follow-up ($P < 0.001$ and $P < 0.001$, respectively). Furthermore, baseline *ASCL1* and *LHX8* methylation levels of women with rCIN2/3 were higher compared with baseline methylation levels of women with CIN1 or less in follow-up ($P = 0.005$ and $P = 0.003$,

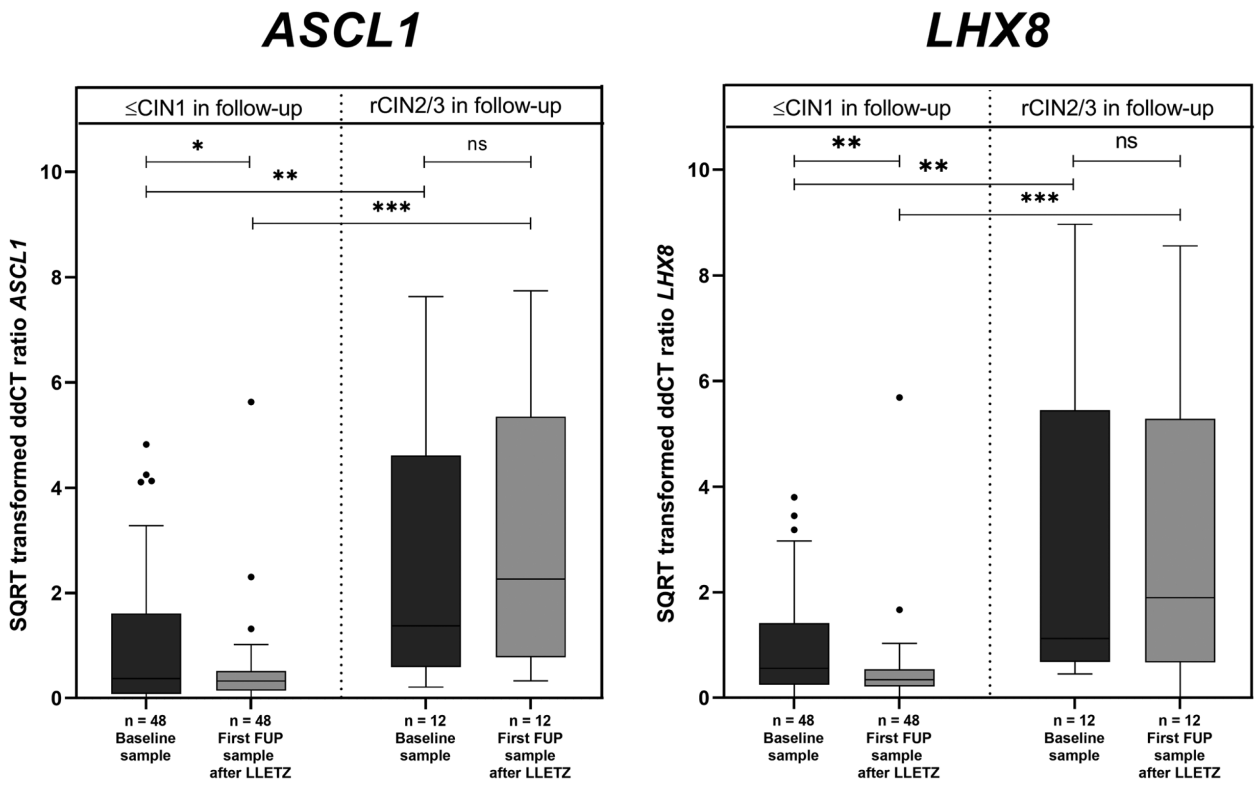

Fig. 2. Baseline and follow-up *ASCL1* and *LHX8* methylation levels of women with a baseline CIN2 or 3, treated by large loop excision of the transformation. CIN, cervical intraepithelial neoplasia; FUP, follow-up; LLETZ, large loop excision of the transformation zone; rCIN2/3, recurrent CIN2/3. **P* less than 0.05, ***P* less than 0.01, ****P* less than 0.001, ns, not significant

respectively). The lesion size during baseline colposcopy (reported as no lesion visible, <25% of transformation zone, 25–50% of transformation zone and 50–75% of transformation zone) was larger in women with rCIN2/3 compared with women without rCIN ($P = 0.019$), Supplementary Table 2, http://links.lww.com/QAD/C457. Supplementary Figure 1, http://links.lww.com/QAD/C457 shows the longitudinal change in individual *ASCL1* and *LHX8* methylation levels measured in baseline and all follow-up cervical scrapes, stratified for baseline and follow-up histology outcome. Individual cytology, HPV and methylation results on each timepoint are shown in Fig. 3.

Risk of recurrent CIN2/3

Table 1 shows the performance for the detection of rCIN2/3 of *ASCL1/LHX8* methylation analysis, HPV testing, cytology and combinations of these tests. For this analysis, we used the test result of the first available followup sample. The median interval between the first followup cervical scrape and the histological diagnosis of rCIN2/3 was 1.2 months (IQR 0–2.3 months). The absolute rCIN2/3 risk (1–NPV) was 4.9% (95% CI: 0.6–16.5) for ASCL1/LHX8-negative women, 8.1% (95% CI: 1.7–21.9) for HPV-negative women and 7.7% (95% CI: 2.1–18.5) for cytology-negative (cut-off ≥HSIL) women. Combining HPV and cytology as a follow-up test resulted in a rCIN2/3 risk of 2.9% (95% CI: 0.0–14.9) among test-negatives. Statistical comparison of methylation analysis to the other strategies revealed a significantly higher specificity and PPV for cytology, only when the cut-off was at least HSIL (Supplementary Table 3, http://links.lww.com/QAD/C457).

Discussion

In this study, we evaluated the performance of *ASCL1/LHX8* methylation analysis as a test of cure in WWH treated for CIN2/3. We showed that a negative *ASCL1/LHX8* methylation test after LLETZ treatment results in a low (4.9%) risk of developing rCIN2/3. Therefore, the *ASCL1/LHX8* methylation test could be used as a safe alternative to cytology to rule out recurrent disease.

Although host-cell DNA methylation analysis has been suggested as a biomarker for the detection of rCIN [46,47], very limited evaluations for posttreatment monitoring have been performed. Our findings are in line with evaluations of the SIMONATH-trial, in which Uijterwaal *et al.* [5] found a 2% rCIN3 risk in HIVnegative women with a *CADM1/MAL* methylationnegative follow-up test 6 or 12 months after

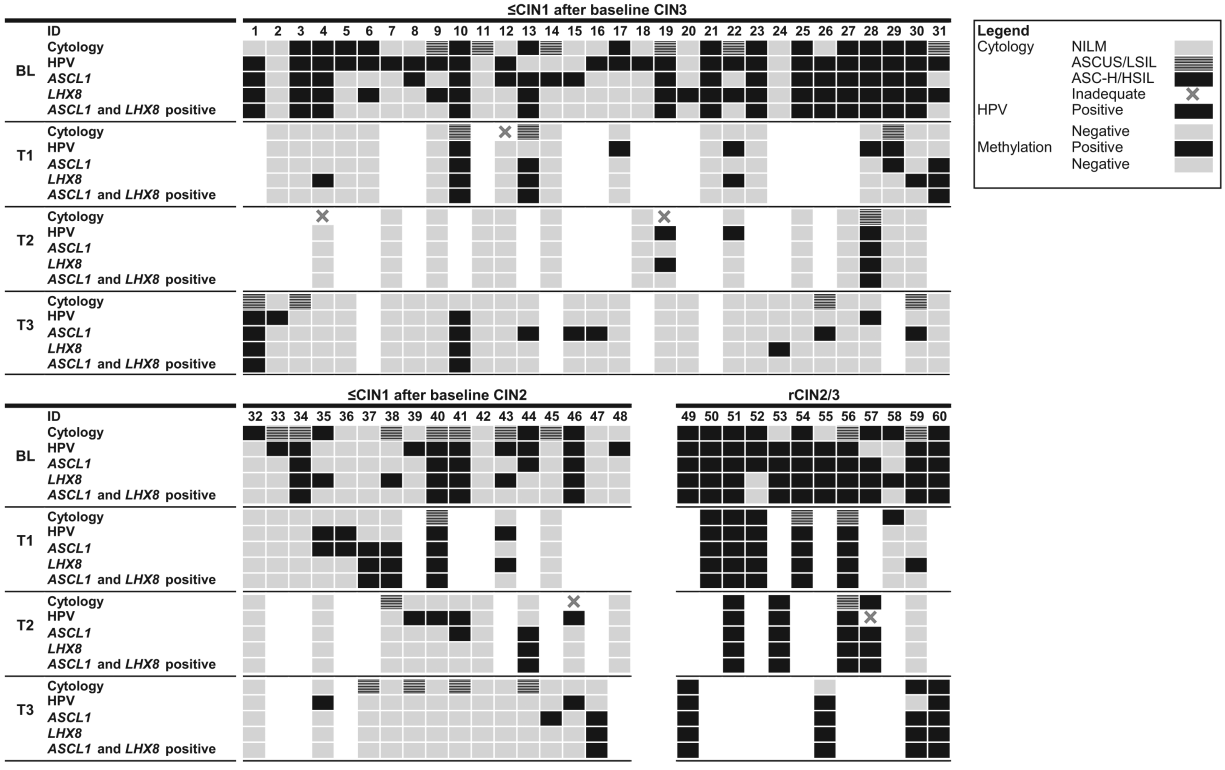

Fig. 3. Overview of individual cytology, methylation and human papilloma virus test results per study visit. BL, baseline; T1, follow-up study visit 1 (1 year); T2, follow-up study visit 2 (2.5year); T3, follow-up study visit 3 (4 years).

treatment. We showed that methylation levels significantly decreased in women with CIN1 or less after treatment for CIN2/3, whereas methylation levels remain increased in women with rCIN2/3. In addition, we found that women with rCIN2/3 had significantly higher methylation levels at the baseline study visit compared with women without rCIN2/3, which may be explained by larger baseline CIN2/3 lesions in women who developed rCIN2/3. Larger CIN lesions are at risk of incomplete excision [48] and the risk of rCIN is significantly greater with involved margins on excisional treatment [49]. Moreover, it has been shown that lesion size is associated with disease severity [50,51] and that invasive squamous carcinoma more often arises in large CIN3 lesions [52].

Cytology is currently used as follow-up test after treatment in South Africa. Women with at least HSIL cytology are treated with re-LLETZ and women with ASCUS/LSIL cytology are advised to be rescreened until a normal cytology result is reached before they return to regular screening intervals [53]. However, in clinical practice these women are often lost to follow-up as an active recall system is lacking. In the present

Table 1. Performance of follow-up strategies for the detection of rCIN2/3.

	Sensitivity			Specificity			PPV		1-NPV		Referral rate
Follow-up strategy	*n*1/N1	%	95% CI	*n2/N2*	%	95% CI	%	95% CI	%	95% CI	%
ASCL1/LHX8	10/12	83.3%	(51.6–97.9)	39/48	81.3%	(67.4–91.1)	52.6%	(28.9–75.6)	4.9%	(0.6–16.5)	31.7%
HPV	9/12	75.0%	(42.8–94.5)	34/48	70.8%	(55.9–83.1)	39.1%	(19.7–61.5)	8.1%	(1.7–21.9)	38.3%
Cytology (≥HSIL)	8/12	66.7%	(34.9–90.1)	48/48	100.0%	(92.6–100)	100.0%	(63.1–100)	7.7%	(2.1–18.5)	13.3%
Cytology (≥ASCUS)	10/12	83.3%	(51.6–97.9)	41/48	85.4%	(72.2–93.9)	58.8%	(32.9–81.6)	4.7%	(0.6–15.8)	28.3%
HPV and/or cytology (≥HSIL)	11/12	91.7%	(61.5–99.8)	34/48	70.8%	(55.9–83.1)	44.0%	(24.4–65.1)	2.9%	(0.0–14.9)	41.7%
HPV and/or cytology (≥ASCUS)	11/12	91.7%	(61.5–99.8)	31/48	64.6%	(49.5–77.8)	39.3%	(21.5–59.4)	3.1%	(0.0–16.2)	46.7%

The first available follow-up sample was used. Cytology with threshold high-grade squamous intraepithelial lesion or worse (≥HSIL, includes atypical squamous cells-cannot exclude HSIL); cytology with threshold atypical squamous cells of unknown significance (≥ASCUS). 95% CI, 95% confidence interval; *n*1, number of screen-positive disease cases; *N*1, total number of disease cases; *n*2, number of screen-negative nondisease cases; *N***2,** total number of nondisease cases; NPV, negative-predictive value; PPV, positive-predictive value.

study, *ASCL1/LHX8* methylation analysis had a similar performance compared with the other screening strategies but showed a lower specificity and PPV compared with cytology, only when the cut-off was ≥HSIL. However, these women were treated based on an abnormal cytology result, which may have caused a preferential effect in favour of cytology. A negative cytology test (cut-off <HSIL) resulted in a rCIN2/3 risk of 7.7% not only with a low referral rate (13.3%) but also with moderate sensitivity (66.7%). Cytology with cut-off ASCUS demonstrated a similar performance as *ASCL1/LHX8* methylation analysis. Yet, a methylation-based follow-up strategy may have several advantages over cytology: it is objective, can be directly applied to both cervical scrapes and self-collected samples and could be adapted to intermediate and low-resource settings, without the need of experienced cyto-pathologists. Our results show that the *ASCL1/LHX8* methylation test provided a low rCIN2/3 risk (4.9%) among testnegatives, in combination with a high rCIN2/3 detection rate (83.8%) and a referral rate of 31.7%. *ASCL1/LHX8* methylation analysis could thus be a safe alternative to cytology for posttreatment monitoring in which methylation-negative women could be directly referred back to screening at 3-yearly intervals advised as for WWH in South Africa, thereby simplifying the follow-up scheme [53]. The choice of follow-up strategy will depend on local resources available and availability of well trained cytotechnicians. Automated workflows for methylation analysis are currently being developed, which combined with robust and user friendly assays can generate results within a day, thereby also impacting costs [54,55].

A persistent HPV infection was found in 50% of women with rCIN2/3, suggesting an incompletely excised lesion. Twenty-five percent had an incident HPV infection characterized by a HPV genotype switch and 25% tested HPV negative. The use of HPV as follow-up test resulted in the highest rCIN2/3 risk after a negative test of 8.1%, the lowest specificity and the highest referral rate. This low specificity because of the high HPV prevalence in subSaharan Africa makes this strategy less practical [19]. Combining HPV with cytology (cut-off ≥HSIL) increases the safety of the strategy with an increase in sensitivity and a low rCIN2/3 risk of 2.9%; however, at the cost of a decrease in specificity and a higher referral rate.

The strengths of this study are the collection of histology specimens during each follow-up visit, the high attendance rates at follow-up and the long follow-up period of 4 years. Baseline cervical scrapes were taken before biopsy collection, enabling a good comparison between methylation levels before and after treatment. A limitation may be seen in that women were treated based only on an abnormal cytology or biopsy result but not based on a positive high-risk HPV test or methylation result. This may have influenced the results in favour of cytology. Numbers of rCIN2/3 in this study are low because of the size of the initial study population, and therefore, a larger implementation study of methylation analysis in posttreatment surveillance is warranted. However, inclusion of large numbers while minimizing loss to follow-up remains challenging in LMIC.

In conclusion, this study shows that the *ASCL1/LHX8* methylation test is associated with a low risk of rCIN2/3 after LLETZ treatment among test-negatives, while maintaining a high detection rate of rCIN2/3. Therefore, methylation analysis is potentially a safe, objective and reproducible alternative for HPV screening and/or cytology screening in posttreatment monitoring in WWH.

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Author's contributions: Principal investigators: C.J.L.M. M., G.D. Study design: M.Z., W.W.K., C.J.L.M.M., G.D. Data collection and processing: F.J.V., M.Z., W.W.K., D. A.M.H., M.C.G.B., E.B., C.V., A.L. Data management: W.W.K., F.J.V. Laboratory experiments: W.W.K., F.J.V. Statistical analysis: F.J.V., B.I.L.W. Data interpretation: F.J. V., R.D.M.S., C.J.L.M.M., G.D. Writing first draft of manuscript: F.J.V., R.D.M.S., C.J.L.M.M. All authors were involved in writing the article and had final approval of the submitted and published version.

Conflicts of interest

C.J.L.M.M., R.D.M.S. and D.A.M.H. are minority shareholders of Self-screen B.V., a spin-off company of VUmc. Self-screen B.V. develops, manufactures and licences high-risk HPV and methylation marker assays for cervical cancer screening and holds patents on these tests. C.J.L.M.M. is part-time director of Self-screen BV. He has been on the speakers bureau and served occasionally on the scientific advisory board (expert meeting) of GSK, Qiagen, SPMSD/Merck. He has been co-investigator on a SPMSD sponsored trial, of which his institute received research funding; has a very small number of shares of MDxHealth and previously of Qiagen. D.A.M.H. has been on the speaker's bureau of Qiagen and serves occasionally on the scientific advisory boards of Pfizer and

Bristol-Myers Squibb. All other authors have no competing interests.

References

1. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. **Human papillomavirus and cervical cancer.** *Lancet* 2007; **370**:890–907.
2. Castle PE, Murokora D, Perez C, Alvarez M, Quek SC, Campbell C. **Treatment of cervical intraepithelial lesions.** *Int J Gynaecol Obstet* 2017; **138 Suppl 1**:20–25.
3. Soutter WP, Sasieni P, Panoskaltsis T. **Long-term risk of invasive cervical cancer after treatment of squamous cervical intraepithelial neoplasia.** *Int J Cancer* 2006; **118**:2048–2055.
4. Bleeker MC, Meijer CJ, Berkhof J. **Follow-up after treatment for cervical intraepithelial neoplasia.** *BMJ* 2012; **345**:e7186.
5. Uijterwaal MH, van Zummeren M, Kocken M, Luttmer R, Berkhof J, Witte BI, *et al*. **Performance of CADM1/MAL-methylation analysis for monitoring of women treated for high-grade CIN.** *Gynecol Oncol* 2016; **143**:135–142.
6. Strander B, Andersson-Ellstrom A, Milsom I, Sparen P. **Long term risk of invasive cancer after treatment for cervical intraepithelial neoplasia grade 3: population based cohort study.** *BMJ* 2007; **335**:1077.
7. Looker KJ, Ronn MM, Brock PM, Brisson M, Drolet M, Mayaud P, Boily MC. **Evidence of synergistic relationships between HIV and human papillomavirus (HPV): systematic reviews and meta-analyses of longitudinal studies of HPV acquisition and clearance by HIV status, and of HIV acquisition by HPV status.** *J Int AIDS Soc* 2018; **21**:e25110.
8. Denslow SA, Rositch AF, Firnhaber C, Ting J, Smith JS. **Incidence and progression of cervical lesions in women with HIV: a systematic global review.** *Int J STD AIDS* 2014; **25**:163–177.
9. Debeaudrap P, Sobngwi J, Tebeu PM, Clifford GM. **Residual or recurrent precancerous lesions after treatment of cervical lesions in human immunodeficiency virus-infected women: a systematic review and meta-analysis of treatment failure.** *Clin Infect Dis* 2019; **69**:1555–1565.
10. Cuschieri K, Bhatia R, Cruickshank M, Hillemanns P, Arbyn M. **HPV testing in the context of posttreatment follow up (test of cure).** *J Clin Virol* 2016; **76 Suppl 1**:S56–S61.
11. Kocken M, Helmerhorst TJ, Berkhof J, Louwers JA, Nobbenhuis MA, Bais AG, *et al*. **Risk of recurrent high-grade cervical intraepithelial neoplasia after successful treatment: a longterm multicohort study.** *Lancet Oncol* 2011; **12**:441–450.
12. NHS cervical screening: programme and colposcopy management, Public Health England 2021 [cited 26 October 2021; Available at: https://www.gov.uk/government/publications/cervical-screening-programme-and-colposcopy-management/3-colposcopic-diagnosis-treatment-and-follow-up [Accessed 26 October 2021].
13. Hillemanns P, Friese K, Dannecker C, Klug S, Seifert U, Iftner T, *et al*. **Prevention of cervical cancer: guideline of the DGGG and the DKG (S3 Level, AWMF Register Number 015/027OL, December 2017)-part 2 on triage, treatment and followup.** *Geburtshilfe Frauenheilkd* 2019; **79**:160–176.
14. Cervix cancer prevention, Nationellt vårdprogram, Regionala Cancer Centrum, Sweden, Kapitel 17 Uppföljning efter dysplasibehandling. 26 October 2021. Available at: https://kunskapsbanken.cancercentrum.se/globalassets/vara-uppdrag/prevention-tidig-upptackt/gynekologisk-cellprovskontroll/vardprogram/nationellt-vardprogram-cervixcancerprevention.pdf [Accessed 26 October 2021].
15. Veileder i gynekologisk onkologi (2021), Nasjonalt Handlingsprogram for Gynekologisk Kreft Norway, 7. Premaligne lidelser i cervix uteri, 7.4 Oppfølging og kontroller. October 26, 2021. Available at: https://www.legeforeningen.no/contentassets/710975c4941b4cbfb89ed2f93259a420/kap-7-premaligne-lidelser-i-cervix-uteri.pdf [Accessed 26 October 2021].
16. Bjornerem MS, Sorbye SW, Skjeldestad FE. **Recurrent disease after treatment for cervical intraepithelial neoplasia-the importance of a flawless definition of residual disease and length of follow-up.** *Eur J Obstet Gynecol Reprod Biol* 2020; **248**:44–49.
17. Kennisinstituut van de Federatie van Medisch Specialisten, The Netherlands, Richtlijn CIN, AIS en VAIN, Chapter 9.3 Na behandelde CIN2-3. 26 October 2021. Available at: https://richtlijnendatabase.nl/richtlijn/cin_ais_en_vain/follow_up/na_-behandelde_cin2_3.html.
18. De Vuyst H, Lillo F, Broutet N, Smith JS. **HIV, human papillomavirus, and cervical neoplasia and cancer in the era of highly active antiretroviral therapy.** *Eur J Cancer Prev* 2008; **17**:545–554.
19. De Vuyst H, Alemany L, Lacey C, Chibwesha CJ, Sahasrabuddhe V, Banura C, *et al*. **The burden of human papillomavirus infections and related diseases in sub-Saharan Africa.** *Vaccine* 2013; **31 Suppl 5**:F32–F46.
20. De Vuyst H, Mugo NR, Franceschi S, McKenzie K, Tenet V, Njoroge J, *et al*. **Residual disease and HPV persistence after cryotherapy for cervical intraepithelial neoplasia grade 2/3 in HIV-positive women in Kenya.** *PLoS One* 2014; **9**:e111037.
21. De Strooper LM, Meijer CJ, Berkhof J, Hesselink AT, Snijders PJ, Steenbergen RD, Heideman DA. **Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions.** *Cancer Prev Res (Phila)* 2014; **7**:1251–1257.
22. Bierkens M, Hesselink AT, Meijer CJ, Heideman DA, Wisman GB, van der Zee AG, *et al*. **CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease.** *Int J Cancer* 2013; **133**:1293–1299.
23. Verlaat W, Van Leeuwen RW, Novianti PW, Schuuring E, Meijer C, Van Der Zee AGJ, *et al*. **Host-cell DNA methylation patterns during high-risk HPV-induced carcinogenesis reveal a heterogeneous nature of cervical precancer.** *Epigenetics* 2018; **13**:769–778.
24. Steenbergen RD, Snijders PJ, Heideman DA, Meijer CJ. **Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions.** *Nat Rev Cancer* 2014; **14**:395–405.
25. Louvanto K, Aro K, Nedjai B, Butzow R, Jakobsson M, Kalliala I, *et al*. **Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia.** *Clin Infect Dis* 2020; **70**:2582–2590.
26. De Strooper LMA, Berkhof J, Steenbergen RDM, Lissenberg-Witte BI, Snijders PJF, Meijer C, *et al*. **Cervical cancer risk in HPV-positive women after a negative FAM19A4/mir124-2 methylation test: a post hoc analysis in the POBASCAM trial with 14year follow-up.** *Int J Cancer* 2018; **143**:1541–1548.
27. Dick S, Kremer WW, De Strooper LMA, Lissenberg-Witte BI, Steenbergen RDM, Meijer C, *et al*. **Long-term CIN3 + risk of HPV positive women after triage with FAM19A4/miR124-2 methylation analysis.** *Gynecol Oncol* 2019; **154**:368–373.
28. Vink FJ, Lissenberg-Witte BI, Meijer C, Berkhof J, van Kemenade FJ, Siebers AG, *et al*. **FAM19A4/miR124-2 methylation analysis as a triage test for HPV-positive women: cross-sectional and longitudinal data from a Dutch screening cohort.** *Clin Microbiol Infect* 2021; **27**:125.e1–125.e6.
29. Kremer WW, van Zummeren M, Breytenbach E, Richter KL, Steenbergen RDM, Meijer C, Dreyer G. **The use of molecular markers for cervical screening of women living with HIV in South Africa.** *AIDS* 2019; **33**:2035–2042.
30. Van Zummeren M, Kremer WW, Van Aardt MC, Breytenbach E, Richter KL, Rozendaal L, *et al*. **Selection of women at risk for cervical cancer in an HIV-infected South African population.** *AIDS* 2017; **31**:1945–1953.
31. Kelly HA, Chikandiwa A, Warman R, Segondy M, Sawadogo B, Vasiljevic N, *et al*. **Associations of human gene EPB41L3 DNA methylation and cervical intraepithelial neoplasia in women living with HIV-1 in Africa.** *AIDS* 2018; **32**:2227–2236.
32. De Vuyst H, Franceschi S, Plummer M, Mugo NR, Sakr SR, Meijer CJ, *et al*. **Methylation levels of CADM1, MAL, and MIR124-2 in cervical scrapes for triage of HIV-infected, high-risk HPV-positive women in Kenya.** *J Acquir Immune Defic Syndr* 2015; **70**:311–318.
33. Verlaat W, Snoek BC, Heideman DAM, Wilting SM, Snijders PJF, Novianti PW, *et al*. **Identification and validation of a 3-gene methylation classifier for HPV-based cervical screening on self-samples.** *Clin Cancer Res* 2018; **24**:3456–3464.
34. Dick S, Verhoef L, De Strooper LM, Ciocanea-Teodorescu I, Wisman GBA, Meijer CJ, *et al*. **Evaluation of six methylation markers derived from genome-wide screens for detection of cervical precancer and cancer.** *Epigenomics* 2020; **12**:1569–1578.

35. Verhoef L, Bleeker MCG, Polman N, Steenbergen RDM, Meijer C, Melchers WJG, *et al*. **Performance of DNA methylation analysis of ASCL1, LHX8, ST6GALNAC5, GHSR, ZIC1 and SST for the triage of HPV-positive women: results from a Dutch primary HPV-based screening cohort.** *Int J Cancer* 2021; **150**:440–449.
36. Kremer WW, Van Zummeren M, Novianti PW, Richter KL, Verlaat W, Snijders PJ, *et al*. **Detection of hypermethylated genes as markers for cervical screening in women living with HIV.** *J Int AIDS Soc* 2018; **21**:e25165.
37. Kremer WW, van Zummeren M, Heideman DAM, Lissenberg-Witte BI, Snijders PJF, Steenbergen RDM, *et al*. **HPV16-related cervical cancers and precancers have increased levels of host cell DNA methylation in women living with HIV.** *Int J Mol Sci* 2018; **19**:3297.
38. van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. **GP5R/6R PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes.** *J Clin Microbiol* 2002; **40**:779–787.
39. Snijders PJ, van den Brule AJ, Jacobs MV, Pol RP, Meijer CJ. **HPV DNA detection and typing in cervical scrapes.** *Methods Mol Med* 2005; **119**:101–114.
40. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T. **Bead-based multiplex genotyping of human papillomaviruses.** *J Clin Microbiol* 2006; **44**:504–512.
41. Floore A, Hesselink A, Ostrbenk A, Alcaniz E, Rothe B, Pedersen H, *et al*. **Intra-and inter-laboratory agreement of the FAM19A4/mir124-2 methylation test: results from an international study.** *J Clin Lab Anal* 2019; **33**:e22854.
42. Livak KJ, Schmittgen TD. **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method.** *Methods* 2001; **25**:402–408.
43. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, *et al*. **Forum Group Members, Bethesda 2001 Workshop. The 2001 Bethesda System: terminology for reporting results of cervical cytology.** *JAMA* 2002; **287**:2114–2119.
44. Wright TC, Ronett BM, Kurman RJ. **Precancerous lesions of the cervix.** In: Kurman R, Hedrick Ellenson L, Ronnet B,editors. *Blaustein's pathology of the female genital tract Cham* Springer; 2019. pp. 239-313.
45. Leisenring W, Alonzo T, Pepe MS. **Comparisons of predictive values of binary medical diagnostic tests for paired designs.** *Biometrics* 2000; **56**:345–351.
46. Terra AP, Murta EF, Maluf PJ, Caballero OL, Brait M, Adad SJ. **Aberrant promoter methylation can be useful as a marker of recurrent disease in patients with cervical intraepithelial neoplasia grade III.** *Tumori* 2007; **93**:572–579.
47. Teixeira da Costa Lodi C, Michelin MA, Miranda Lima MI, Murta EFC, Braga LDC, Montes L, *et al*. **Predicting cervical intraepithelial neoplasia recurrence in HIV-infected and-noninfected women by detecting aberrant promoter methylation in the CDH1, TIMP3, and MGMT genes.** *Arch Gynecol Obstet* 2018; **298**:971–979.
48. Arbyn M, Simoens C, Goffin F, Noehr B, Bruinsma F. **Treatment of cervical cancer precursors: influence of age, completeness of excision and cone depth on therapeutic failure, and on adverse obstetric outcomes.** *BJOG* 2011; **118**:1274–1275.
49. Arbyn M, Redman CWE, Verdoodt F, Kyrgiou M, Tzafetas M, Ghaem-Maghami S, *et al*. **Incomplete excision of cervical precancer as a predictor of treatment failure: a systematic review and meta-analysis.** *Lancet Oncol* 2017; **18**:1665–1679.
50. Kierkegaard O, Byrjalsen C, Frandsen KH, Hansen KC. **Frydenberg M. Diagnostic accuracy of cytology and colposcopy in cervical squamous intraepithelial lesions.** *Acta Obstet Gynecol Scand* 1994; **73**:648–651.
51. Jarmulowicz MR, Jenkins D, Barton SE, Goodall AL. **Hollingworth A, Singer A. Cytological status and lesion size: a further dimension in cervical intraepithelial neoplasia.** *Br J Obstet Gynaecol* 1989; **96**:1061–1066.
52. Tidbury P, Singer A, Jenkins D. **CIN 3: the role of lesion size in invasion.** *Br J Obstet Gynaecol* 1992; **99**:583–586.
53. Botha MH, Dreyer G. **Guidelines for cervical cancer screening in South Africa.** *South Afr J Gynaecol Oncol* 2017; **9**:8–12.
54. Rausch S, Hasinger O, Konig T, Schlegel A, Weiss G. **An automated high throughput solution for DNA extraction and bisulfite-conversion from high volume liquid biopsy specimens: sample preparation for epigenetic analysis.** *BMC Res Notes* 2019; **12**:551.
55. Verhoef L, Floore A, Doorn S, Cuschieri K, Bhatia R, Hesselink B, *et al*. **Direct bisulphite conversion of cervical samples for DNA methylation analysis.** *Epigenetics* 2021:1–7[Epub ahead of print].