Evaluation of six methylation markers derived from genome-wide screens for detection of cervical precancer and cancer

Dick, Stèfanie; Verhoef, Lisanne; De Strooper, Lise Ma; Ciocănea-Teodorescu, Iuliana; Wisman, G Bea A; Meijer, Chris Jlm; Bleeker, Maaike Cg; Steenbergen, Renske Dm; Heideman, Daniëlle Am

Published in:
Epigenomics

DOI:
10.2217/epi-2019-0331

Publication date:
2020

Short Communication

For reprint orders, please contact: reprints@futuremedicine.com

Epigenomics

Evaluation of six methylation markers derived from genome-wide screens for detection of cervical precancer and cancer

Stèfanie Dick[‡,1], Lisanne Verhoef[‡,1], Lise MA De Strooper[1], Iuliana Ciocănea-Teodorescu[2], G Bea A Wisman[3], Chris JLM Meijer[1], Maaike CG Bleeker[1], Renske DM Steenbergen[‡,1] & Daniëlle AM Heideman*[,‡,1]

[1]Amsterdam UMC, Vrije Universiteit Amsterdam, Pathology, Cancer Center Amsterdam, De Boelelaan 1117, Amsterdam, The Netherlands

[2]Amsterdam UMC, Vrije Universiteit Amsterdam, Epidemiology & Biostatistics, De Boelelaan 1117, Amsterdam, The Netherlands

[3]University of Groningen, University Medical Centre Groningen, Gynaecologic Oncology, Cancer Research Center Groningen, Groningen, The Netherlands

*Author for correspondence: dam.heideman@amsterdamumc.nl

‡Authors contributed equally

Aim: To evaluate the triage performance of six host-cell DNA methylation markers derived from two genome-wide discovery screens for detection of cervical precancer (cervical intraepithelial neoplasia 3 [CIN]) and cancer. **Materials & methods:** Human papillomavirus-positive cervical scrapes of controls (≤CIN1; n = 352) and women diagnosed with CIN3 (n = 175) or cervical cancer (n = 50) were analyzed for methylation of *ASCL1*, *LHX8*, *ST6GALNAC5*, *GHSR*, *SST* and *ZIC1*. **Results:** Methylation levels increased significantly with disease severity (all markers $p < 0.001$). Three markers (*ASCL1*, *LHX8*, *ZIC1*) showed receiver operating characteristic curves with area under the curve >0.800 after leave-one-out cross-validation. Bi-marker panel *ASCL1*/*LHX8* had highest area under the curve (0.882), and detected 83.4% of CIN3 and all cervical cancers at specificity of 82.4%. **Conclusion:** All six methylation markers showed an equivalent, high performance for the triage of human papillomavirus-positive women using cervical scrapes with complementarity between markers.

First draft submitted: 5 November 2019; Accepted for publication: 9 June 2020; Published online: 17 September 2020

Keywords: *ASCL1* • cervical cancer • cervical intraepithelial neoplasia • cervical screening • DNA methylation markers • human genome methylation • human papillomavirus • *LHX8* • triage testing

Cervical cancer is caused by a persistent infection with human papillomavirus (HPV) [1]. Cervical cancer develops through precursor lesions, called cervical intraepithelial neoplasia (CIN), which are classified into CIN1, CIN2 and CIN3 depending on the severity of the dysplasia. The latter is considered as the most advanced precursor of cervical cancer. Cervical screening programs aim to detect and treat precancerous stages in order to prevent cervical cancer. HPV testing is more sensitive for the detection of CIN3 and cervical cancer compared with cytology and has a high negative predictive value and reproducibility [2–4]. Primary HPV testing has therefore been implemented or is scheduled for implementation in cervical screening programs in several countries. However, since most HPV infections are transient and do not cause clinically relevant disease [5], additional triage testing is required to identify HPV-positive women with progressive CIN lesions in need of treatment. Besides, triage testing is necessary to prevent overdiagnosis and overtreatment of women without clinically relevant CIN lesions. Several triage strategies have been evaluated [6–13], with reflex cytology, repeat cytology, HPV16/18-genotyping or a combination thereof being adopted in current HPV screening guidelines [14,15]. However, there is no consensus about the most appropriate triage strategy yet. An objective molecular test which could be automated and directly incorporated following a positive HPV result would be most beneficial.

Several studies have demonstrated the potential of DNA methylation analysis as an alternative triage method for the detection of CIN3 or worse (CIN3+) in HPV-positive women [16–23]. DNA methylation has been identified to play an important role in the development of cervical cancer [22–24]. A strong association was found between the

methylation levels of host-cell and/or viral genes and the severity of the lesion, reaching highest levels in cervical cancer [20,25–27]. Various DNA methylation marker panels have been proposed, including combinations of host-cell genes such as *ASTN1*, *CADM1*, *DLX1*, *EPB41L3*, *FAM19A4*, *ITGA4*, *MAL*, *miR124-2*, *PAX1*, *POU4F3*, *RXFP3*, *SOX1*, *SOX17*, *ZNF671* and/or CpG sites in the late regions of various HPV genomes [17,18,27–31]. In several studies these methylation markers have demonstrated to be a useful alternative to cytology or HPV16/18 genotyping for the detection of CIN3+ as a triage test [21]. A recent longitudinal evaluation in HPV-positive women from a screening cohort demonstrated a similarly low long-term CIN3+ risk upon a negative methylation test result as was found for a negative cytology result, underscoring the good diagnostic potential of methylation markers [32,33].

Recently, we used genome-wide approaches to identify novel host-cell DNA methylation markers associated with CIN3 and cervical cancer. Methylation markers *GHSR*, *SST* and *ZIC1* were identified using a methyl-binding domain-enriched DNA-based discovery screen on high-risk HPV-transformed cell lines and cervical tissue specimen [34] and methylation markers *ASCL1*, *LHX8* and *ST6GALNAC5* were discovered using an array-based methylation discovery screen on HPV-positive self-collected cervico-vaginal material [35]. These six methylation markers showed to be most promising for the triage of HPV-positive women in subsequent verification and validation series using a multiplex quantitative methylation-specific PCR (qMSP). DNA methylation markers *GHSR*, *SST* and *ZIC1* resulted in area under the curves (AUCs) of 0.87, 0.86 and 0.89, respectively, for CIN3+ detection in HPV-positive cervical scrapes [34]. DNA methylation markers *ASCL1*, *LHX8* and *ST6GALNAC5* resulted in a combined AUC of 0.88 and 0.90 for CIN3+ detection in HPV-positive lavage- and brush self-samples, respectively [35]. These markers were initially evaluated in different cervical sample series, comprising cervical scrapes and self-collected cervico-vaginal material. This study was designed as a next step to assess and compare the clinical performance of these methylation markers on the same cohort. The six recently identified host-cell DNA methylation markers were evaluated on HPV-positive cervical scrapes as a molecular triage method for the detection of CIN3 and cervical cancer for the application in HPV-based cervical screening.

Materials & methods

Clinical specimens

A series of HPV-positive cervical scrapes (n = 527) obtained from screening or gynecologic outpatient populations was used, comprising 175 scrapes from women who were histologically diagnosed with CIN3 (median age 36.5 years) and 352 scrapes from control women (median age 38.0 years). The controls consisted of women with no evidence of CIN2+ classified as CIN1 or less (≤ CIN1), including 225 women with no histology, 64 with normal histology (CIN0) and 63 with histologically proven CIN1. The histological diagnosis of CIN2 comprises a heterogeneous disease category and was therefore not evaluated in this study [36]. In addition, 50 HPV-positive cervical scrapes from women diagnosed with cervical cancer were used (median age 47.0 years; squamous cell carcinoma n = 40; adenocarcinoma n = 4; adenosquamous carcinoma n = 2; clear cell carcinoma n = 1; neuroendocrine carcinoma n = 2; gastric-type mucinous adenocarcinoma n = 1). HPV testing was performed using clinically validated high-risk HPV DNA assays [37]. This study followed the ethical guidelines of the Institutional Review Board of VU University Medical Centre and University Medical Cenre Groningen.

DNA isolation, modification & qMSP

DNA isolation, sodium bisulphite treatment and multiplex qMSPs were performed as described previously for markers *GHSR*, *SST* and *ZIC1* [34] and *ASCL1*, *LHX8* and *ST6GALNAC5* [35]. Multiplex qMSPs were designed as described by Snellenberg, *et al.* [38]. This assay type is able to detect small amounts of methylated DNA in a background of unmethylated DNA, targets simultaneously multiple genes and provides high sample throughput [38]. To verify DNA quality and successful bisulphite conversion, the housekeeping gene *β-Actin* (*ACTB*) was used as a reference gene in all qMSPs. Methylation levels were normalized to *ACTB* using the quantification cycle (Cq) values ($2^{-\Delta Cq} \times 100$) to obtain ΔCq ratios [39]. Part of the methylation data were derived from previous studies: data from 88 cervical scrapes, including 42 cancers and 46 CIN3, were reported before for methylation markers *GHSR*, *SST* and *ZIC1* [26,40].

Data & statistical analysis

Differences in DNA methylation levels between the disease categories were assessed using the Kruskal–Wallis omnibus test followed by *post hoc* pairwise Wilcoxon-Mann–Whitney U-tests with Bonferroni adjustment for multiple testing. Bivariate associations between markers were evaluated by Spearman correlation coefficients and presence of multicollinearity in the data was assessed using the variance inflation factor. To compare the individual

Table 1. Area under the curve received from logistic regression and leave-one-out cross-validation analysis for CIN3 detection of six DNA methylation markers (*ASCL1*, *LHX8*, *ST6GALNAC5*, *GHSR*, *SST*, *ZIC1*) and the bi-marker panel (*ASCL1*/*LHX8*).

Methylation marker	Non-CV AUC	Sensitivity (%)†	Specificity (%)†	LOOCV AUC	Sensitivity (%)†	Specificity (%)†
ASCL1	0.886	84.0	81.8	0.876	80.0	85.2
LHX8	0.852	72.0	84.4	0.845	73.1	82.7
ST6GALNAC5	0.788	67.4	81.2	0.784	66.3	82.1
GHSR	0.801	65.7	85.2	0.795	65.7	85.2
SST	0.774	65.7	80.1	0.768	65.7	79.8
ZIC1	0.843	70.9	85.2	0.836	70.3	85.5
Bi-marker panel (*ASCL1*/*LHX8*)	0.890	84.0	83.2	0.882	83.4	82.4

†Sensitivities and specificities are calculated for CIN3 detection and based on Youden's J-index.
AUC: Area under the curve; CIN: Cervical intraepithelial neoplasia; LOOCV: Leave-one-out cross-validation; Non-CV AUC: Non-cross-validated AUC.

discriminative performance of each marker in controls and CIN3, we performed simple (univariable) logistic regression for each marker separately. In order to evaluate the improvement in terms of prediction performance of a marker panel involving two or more markers, taking into account the multicollinearity in the data, we performed least absolute shrinkage and selection operator (LASSO) logistic regression. LASSO logistic regression has an inbuilt parameter (penalty parameter) that controls the number of variables eventually included in the model, balanced against model fit. The risk of CIN3 was calculated for each sample as a predicted probability (value ranging from 0 to 1). The predicted probabilities obtained were visualized using the receiver operating characteristics (ROC) curve. The model performance was evaluated by the AUC and corresponding sensitivity and specificity at the best threshold, which is the threshold that maximizes the sum of sensitivity and specificity (i.e., Youden J-index). Sensitivities at a threshold corresponding to a predefined specificity of 70 and 80% were calculated for all models. All models were trained to discriminate between controls and CIN3 and subsequently evaluated by leave-one-out cross-validation (LOOCV). Cancer samples were not included in the training, but once trained; the models were tested on these samples as well. All statistical analyses and visualizations were performed using square-root values of the ΔCq, and p-values were two-sided, with 0.05 as significance threshold. Statistical analyses were performed with R Statistical Software (version 3.5.1) in RStudio, using the following R-packages: pROC (v.1.13.0), gplots (v.3.0.1), ggplot2 (v.3.1.0) and glmnet (v2.0-16).

Results

Methylation levels & CIN3 detection by single methylation markers

Methylation levels of all six methylation markers increased significantly with increasing underlying disease severity, from controls to CIN3 and cervical cancer (Kruskal–Wallis test with *post hoc* pairwise Wilcoxon-Mann–Whitney U-tests, all p-values < 0.001; Figure 1). A significant positive association between all six markers was observed within the group of controls and CIN3, with Spearman coefficients between 0.64 and 0.83 (Supplementary Table 1) and variance inflation factors between 3.67 and 6.97.

The individual ability of the six markers to distinguish controls from CIN3 was evaluated by logistic regression and resulted in single marker models with AUCs between 0.774 and 0.886 (Table 1). Validation by LOOCV produced a similarly good performance of all markers with AUCs greater than 0.800 for *ASCL1*, *LHX8* and *ZIC1* (Figure 2 & Table 1). Corresponding sensitivities and specificities at the best threshold can be found in Table 1. Sensitivities at a threshold corresponding to a predefined specificity of 70 and 80% varied from 70.3 to 89.1% and 65.7 to 84.6%, respectively (Table 2). Adding age of the patient as a covariate to the models did not improve performance of the models (Supplementary Table 2).

Complementarity between methylation markers with a marker panel

LASSO logistic regression was used to evaluate whether a marker panel involving two or more markers could further improve CIN3 detection. This resulted in a bi-marker panel consisting of *ASCL1* and *LHX8* with an AUC of 0.890 (Table 1). LOOCV yielded a near identical AUC of 0.882, which corresponded to a CIN3 sensitivity of 83.4% and a specificity of 82.4% at the best threshold (Figure 3 & Table 1). At a predefined specificity of 70 and 80%, corresponding CIN3 sensitivities were 89.1 and 85.7%, respectively (Table 2).

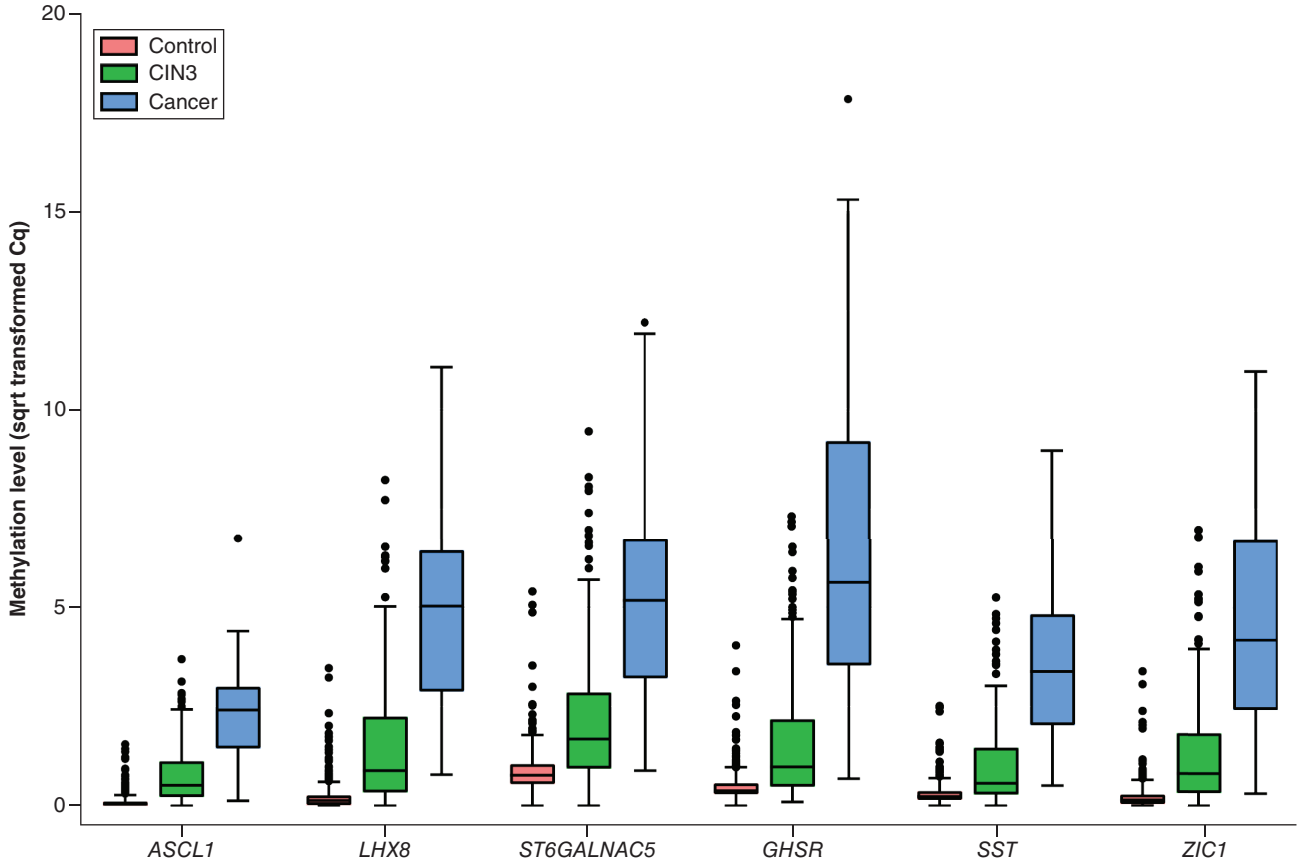

Figure 1. Methylation levels increase with severity of cervical disease. DNA methylation levels of six single methylation markers relative to that of the housekeeping gene *ACTB* (square-root transformed ΔCq; Y-axis) in HPV-positive cervical scrapes corresponding to different histological categories of underlying cervical disease are shown. Boxplots are based on a five-number summary (i.e., minimum, first quartile [Q1/25th percentile], median [Q2/50th percentile], third quartile [Q3/75th percentile] and maximum with outliers displayed as single points). Differences between histological categories were significant: Kruskal–Wallis test p-value < 0.001, pairwise Wilcoxon-Mann–Whitney U-test p-values < 0.001.
HPV: Human papillomavirus.

Table 2. Corresponding sensitivity at a predefined specificity of 70 and 80% of six DNA methylation markers (*ASCL1*, *LHX8*, *ST6GALNAC5*, *GHSR*, *SST*, *ZIC1*) and the bi-marker panel (*ASCL1*/*LHX8*).

Methylation marker	Predefined specificity 70%		Predefined specificity 80%	
	Sensitivity (%)†	Cancer detection rate	Sensitivity (%)†	Cancer detection rate
ASCL1	89.1	50/50	84.6	49/50
LHX8	82.9	50/50	74.9	50/50
ST6GALNAC5	73.1	49/50	67.4	49/50
GHSR	73.1	50/50	68.6	50/50
SST	70.3	50/50	65.7	50/50
ZIC1	81.1	50/50	73.7	49/50
Bimarker panel (*ASCL1*/*LHX8*)	89.1	50/50	85.7	50/50

†Sensitivities and specificities are calculated for CIN3 detection.
CIN: Cervical intraepithelial neoplasia.

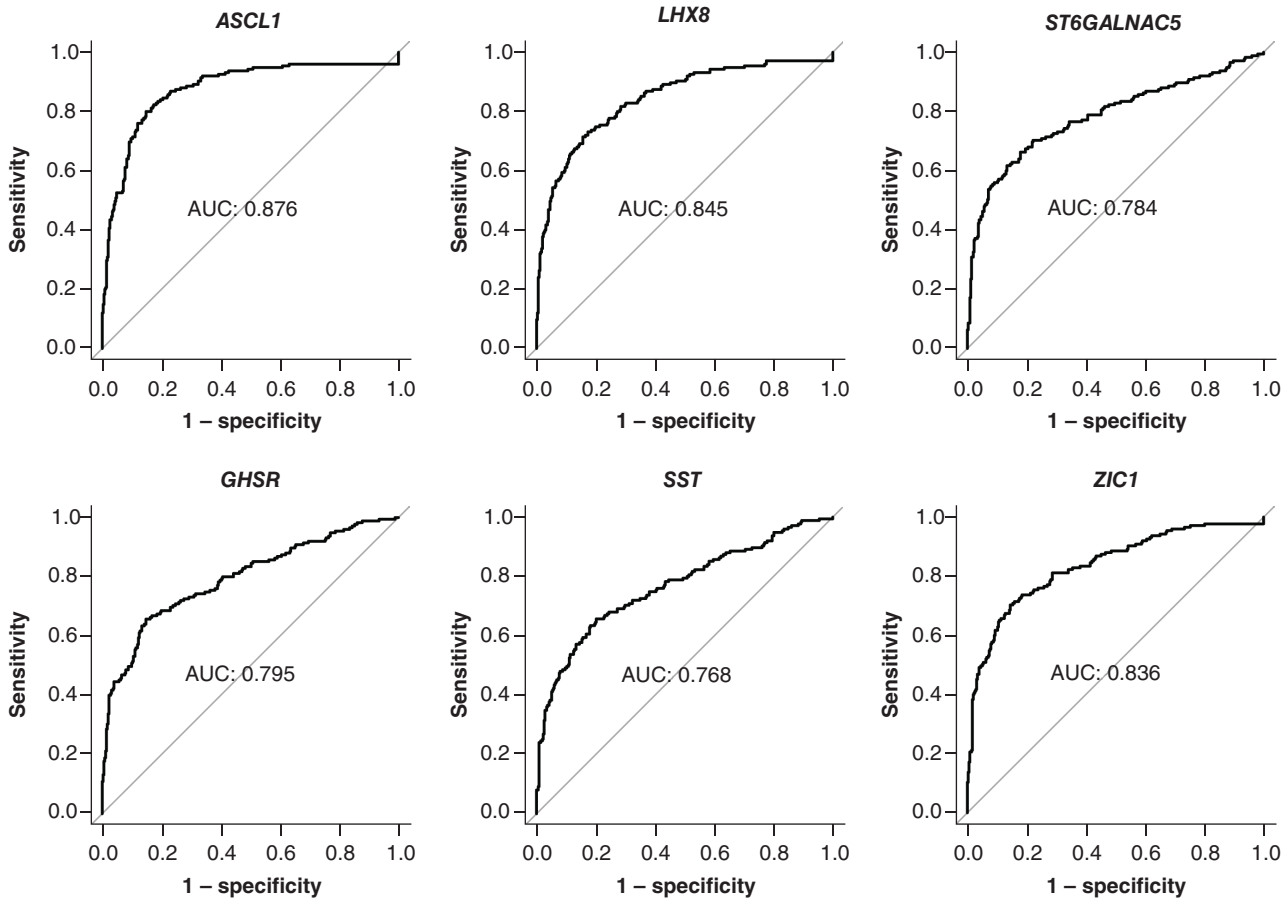

Figure 2. Clinical performance of six single DNA methylation markers (*ASCL1*, *LHX8*, *ST6GALNAC5*, *GHSR*, *SST*, *ZIC1*) for CIN3 detection in HPV-positive women. Cross-validated receiver operating characteristic curves and corresponding AUCs.
AUC: Area under the curve; CIN: Cervical intraepithelial neoplasia.

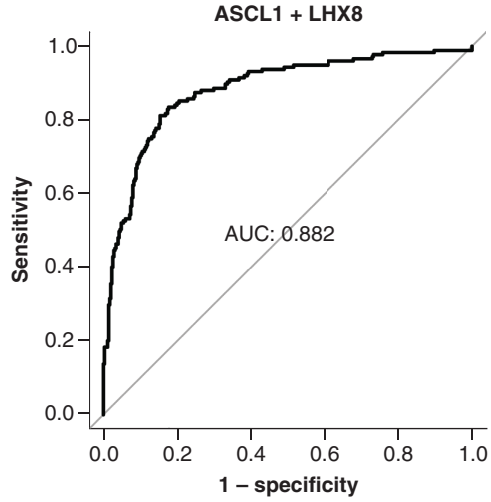

Figure 3. Clinical performance of the bi-marker panel *ASCL1*/*LHX8* for CIN3 detection in human papillomavirus-positive women. Cross-validated receiver operating characteristic curve and corresponding AUC.
AUC: Area under the curve; CIN: Cervical intraepithelial neoplasia.

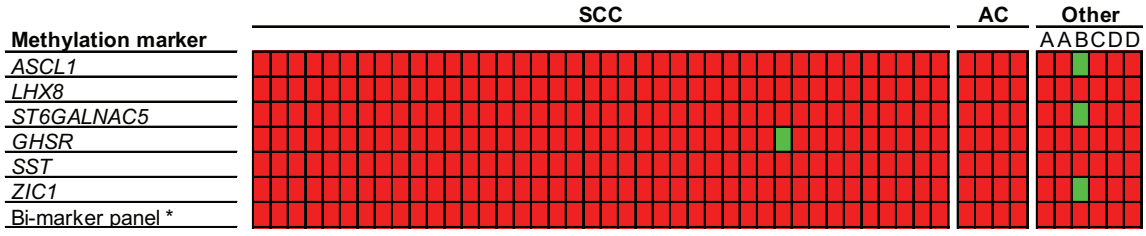

Figure 4. DNA methylation results in human papillomavirus-positive cervical scrapes of women diagnosed with cervical cancer. Positive and negative samples are shown in red and green, respectively. Other histotypes: **(A)** adenosquamous carcinoma; **(B)** clear cell carcinoma; **(C)** mucinous adenocarcinoma, gastric type; **(D)** small-cell neuroendocrine carcinoma.
*Bi-marker panel: *ASCL1*/*LHX8*.
AC: Adenocarcinoma; SCC: Squamous cell carcinoma.

Cancer detection

In Figure 4 the performance of the single markers and the bi-marker panel, at the best threshold, for the detection of cervical cancer stratified by histotype is shown. The bi-marker panel detected all 40 squamous cell carcinomas and ten other cancer histotypes. Four single markers misclassified one of the cervical cancers, involving two different cancer cases, one squamous cell carcinoma and one clear cell carcinoma. Cancer detection rate at the threshold corresponding to a predefined specificity of 70 and 80% is reported in Table 2. All cancers were detected by the bi-marker panel at both 70 and 80% specificity.

Discussion

DNA methylation of host-cell genes is a common event in cervical carcinogenesis [22,23]. In recent years, multiple promising host-cell DNA methylation markers for the detection of CIN3+ in HPV-positive women have been identified by targeted and genome-wide approaches [18,19,31,34,35,41,42]. However, it is difficult to mutually compare the diagnostic performance of markers due to different techniques and the use of different study material. For the application in HPV-based cervical screening, this study evaluated six recently identified methylation markers for CIN3 detection by a direct head-to-head comparison on HPV-positive cervical scrapes. We demonstrated that all single markers, derived from two different genome-wide discovery screens, had a similarly good performance for the detection of CIN3 (AUC: 0.774–0.886). Upon validation by LOOCV, nearly identical AUCs were obtained, indicating the stability of the prediction models. Three single markers (*ASCL1*, *LHX8*, *ZIC1*) showed particularly good performance as quantified by cross-validated AUCs greater than 0.800 for CIN3 detection. LASSO logistic regression showed a bi-marker panel consisting of *ASCL1* and *LHX8* to be most discriminative for CIN3 detection with a cross-validated AUC of 0.882, resulting in a sensitivity of 83.4% and specificity of 82.4%. Of note, *ASCL1* and *LHX8* had a complementary performance resulting in the detection of all cervical cancers, including not only squamous cell carcinomas, but also adenocarcinomas and rare cancers. This further demonstrates the potential of methylation analysis, since non-squamous cell cancer histotypes are often missed by cytology.

The concept of increased DNA hypermethylation during cervical carcinogenesis is reflected by the gradual increase of the methylation levels of all six markers with disease severity, reaching highest levels in cervical cancer. Data on a well-studied methylation marker panel with host-cell genes *FAM19A4* (currently known as *TAFA4*) and *miR124-2* recently showed that nearly all cervical cancers, including early-stage cancers, from a large worldwide series are detected by methylation analysis [43]. Our findings support the high methylation positivity rate in cervical cancer. Though FIGO stage was only available for a subset of samples in our study, all cervical scrapes of women with known as Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage I cancer (n = 26) scored methylation-positive for the bi-marker panel. These findings underscore the potential of the methylation test for use in population-based screening where most cervical cancers are early-stage. The six methylation markers are potentially not (fully) cervix-specific, as they have been described in other cancers as well [44,45]. Analysis of cervical scrapes may thereby have added value for the detection of other, non-cervix gynecologic cancers. This concept has been corroborated for other methylation markers by recent studies [46–48].

Methylation analysis has shown before to particularly detect high-grade CIN lesions associated with a persistent HPV infection of at least 5 years, as a surrogate for a more advanced lesion [27,49]. In a recent methylation study on HPV-positive cervical scrapes, including host-cell genes *GHSR*, *SST* and *ZIC1*, it was found that 72% of CIN3 showed a methylation profile similar to cervical cancer [26]. These methylation-positive CIN3 lesions are therefore considered to have a substantial higher short-term risk of progression to cancer. The sensitivity of 80% for CIN3 as found in our study corresponds to these previous findings, and suggests that a methylation-negative result may be indicative of lesions with a low chance of progression to cervical cancer. This hypothesis is further supported by a recent study evaluating a methylation panel of host-cell and viral genes that showed the ability to differentiate between regressive and progressive CIN2 in young women [50]. Hence, methylation analysis may stratify between immediate treatment and close surveillance, preventing overtreatment and the associated cervical morbidity, which is especially relevant for women of childbearing age [51]. This association between methylation status and regression probability is subject of further studies [52].

Self-sampling is increasingly offered in cervical cancer screening programs as an alternative or additive to physician-taken cervical scrapes to reduce screening barriers and increase screening participation [14,53,54]. The candidate host-cell methylation markers *ASCL1* and *LHX8* were originally identified on self-collected cervicovaginal samples and validated in independent series of HPV-positive self-samples with AUCs between 0.88 and 0.90 for CIN3 detection [35]. Previous research demonstrated that the performance of methylation markers on cervical scrapes and self-collected samples is not necessarily similar [55], as these two sample types can differ in cellular composition. Of interest, this study validated *ASCL1* and *LHX8* on HPV-positive cervical scrapes with an equally well performance compared with the previous results from HPV-positive self-samples. This highlights compatibility of this marker panel with both self-collected and physician-collected cervical samples, which is highly beneficial for a triage test. In addition, *ASCL1* and *LHX8* have been evaluated in a South African, HIV-positive study cohort as a primary screening tool for the detection of CIN3+ [56]. Both markers showed a good performance with an AUC of 0.79 for *ASCL1* and 0.81 for *LHX8*. These data support the diagnostic potential of *ASCL1* and *LHX8*, although direct extrapolation is not feasible due to the HIV status and the absence of a well-organized screening program in South Africa.

Strengths of our study are the large sample size and the evaluation of all six methylation markers on the same sample series allowing direct comparison between markers. Another strength is the evaluation and validation of all models for CIN3 detection, consistent with the purpose of cervical screening. A limitation of our study is the absence of histological confirmation in part of the controls. In a restricted analysis including only controls with histologically proven outcome (n = 127), no difference in prediction performance was obtained (data not shown). Another limitation is that LOOCV was used for validation of the models. Further validation in independent cohorts including CIN2 lesions is warranted.

Conclusion

All six single markers showed an equivalent, high diagnostic performance for CIN3 detection. *ASCL1* and *LHX8* showed complementarity in the detection of cervical cancer. These markers were originally identified on self-collected samples and have now been demonstrated to have a similar good performance on cervical scrapes.

Future perspective

Cervical screening programs, currently based on cytology or primary HPV testing plus cytology and/or HPV genotyping, could be improved by the implementation of methylation-based triage strategies. Methylation analysis of host-cell genes using qMSP provides a promising objective triage strategy for HPV-positive women. This study confirms the triage potential of six methylation markers, supporting full molecular cervical screening compatible with both physician- and self-collected samples. Triage of HPV-positive women by methylation markers enables the detection of clinically relevant CIN lesions, and importantly reduces overreferral and overtreatment of women with a low cervical cancer risk. Host-cell methylation-based triage strategies, such as *ASCL1*/*LHX8* methylation testing, detect all cervical cancers independent of HPV genotype, histotype or sample type.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2019-0331

Financial & competing interest disclosure

This project was supported by grants of the Dutch Cancer Society (KWF VU 2014-7238; KWF 11337). DAM Heideman, RDM Steenbergen and CJLM Meijer are minority shareholders of self-screen B.V., a spin-off company of VUmc. Self-screen B.V. holds patents related to the work (i.e., high-risk HPV test and methylation markers for cervical screening) and has developed and manufactured HPV and methylation assays, which are licensed to QIAGEN (QIAscreen® HPV PCR Test and QIAsure® Methylation Test). DAM Heideman has been on the speakers bureau of QIAGEN and serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb. CJLM Meijer has received speakers fee from GSK, QIAGEN and SPMSD/Merck and served occasionally on the scientific advisory board (expert meeting) of GSK, QIAGEN and SPMSD/Merck. CJLM Meijer has a very small number of shares of QIAGEN and holds minority stock in Self-Screen B.V. Until April 2016 he was minority shareholder of Diassay B.V. CJLM Meijer is part-time director of Self-screen B.V. since September 2017. Self-screen B.V. was supported by the Valid-screen project, funded by the SME Instrument in the Horizon 2020 Work Program of the European Commission (Valid-screen 666800). LMA Strooper is currently an employee of Bayer on work that is not related to the manuscript. GBA Wisman is inventor on several patents related to the work, is scientific advisor for CC Diagnostics and has received a grant from CC Diagnostics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials & methods discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Summary points

- *ASCL1*, *LHX8*, *ST6GALNAC5*, *GHSR*, *SST* and *ZIC1* have been discovered by genome-wide approaches as novel DNA methylation markers for cervical intraepithelial neoplasia 3 (CIN) and cervical cancer detection.
- This study was designed to assess and compare the triage performance of these methylation markers for the application in human papillomavirus (HPV)-based cervical screening.
- A series of HPV-positive cervical scrapes was tested for methylation of *ASCL1*, *LHX8*, *ST6GALNAC5*, *ZIC1*, *GHSR*, *SST* and *ZIC1* and marker performance was compared.
- Methylation levels of all six markers in cervical scrapes increased significantly with underlying disease severity.
- The performance for the detection of CIN3 was comparable between markers with cross-validated AUCs >0.800 for *ASCL1*, *LHX8* and *ZIC1*.
- The bi-marker panel consisting of *ASCL1* and *LHX8* resulted in the highest cross-validated AUC of 0.882 for the detection of CIN3.
- The bi-marker panel *ASCL1*/*LHX8* detected all cervical cancers independent of HPV genotype or histotype.
- Performance of *ASCL1* and *LHX8* on cervical scrapes in this study was comparable with earlier results on HPV-positive self-collected samples.
- This study confirms the diagnostic potential of six recently identified methylation markers with an equivalent and complementary high diagnostic performance for CIN3+ on HPV-positive cervical scrapes.
- The *ASCL1*/*LHX8* marker panel holds promise for full molecular cervical screening compatible with both physician- and self-collected samples.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Walboomers JM, Jacobs MV, Manos MM *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 189(1), 12–19 (1999).
2. Ronco G, Dillner J, Elfstrom KM *et al.* Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet* 383(9916), 524–532 (2014).
3. Arbyn M, Ronco G, Anttila A *et al.* Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. *Vaccine* 30(Suppl. 5), F88–F99 (2012).
4. Koliopoulos G, Nyaga VN, Santesso N *et al.* Cytology versus HPV testing for cervical cancer screening in the general population. *Cochrane Database Syst. Rev.* 8, CD008587 (2017).

• **Shows that human papillomavirus (HPV) testing gives a greater protection against cervical cancer compared with cytology testing.**

5. Doorbar J, Quint W, Banks L *et al.* The biology and life-cycle of human papillomaviruses. *Vaccine* 30(Suppl. 5), F55–F70 (2012).

6. Rijkaart DC, Berkhof J, Van Kemenade FJ *et al.* Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *Int. J. Can.* 130(3), 602–610 (2012).
7. Naucler P, Ryd W, Tornberg S *et al.* Efficacy of HPV DNA testing with cytology triage and/or repeat HPV DNA testing in primary cervical cancer screening. *J. Natl Cancer Inst.* 101(2), 88–99 (2009).
8. Luttmer R, De Strooper LM, Berkhof J *et al.* Comparing the performance of FAM19A4 methylation analysis, cytology and HPV16/18 genotyping for the detection of cervical (pre)cancer in high-risk HPV-positive women of a gynecologic outpatient population (COMETH study). *Int. J. Can.* 138(4), 992–1002 (2016).
9. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. *J. Med. Virol.* 76(Suppl. 1), S49–S55 (2016).
10. Babion I, Snoek BC, Novianti PW *et al.* Triage of high-risk HPV-positive women in population-based screening by miRNA expression analysis in cervical scrapes; a feasibility study. *Clin. Epigenetics* 10, 76 (2018).
11. Clarke MA, Cheung LC, Castle PE *et al.* Five-year risk of cervical precancer following p16/Ki-67 dual-stain triage of HPV-positive women. *JAMA Oncol.* 5(2), 181–186 (2019).
12. Stanczuk GA, Baxter GJ, Currie H *et al.* Defining optimal triage strategies for hrHPV screen-positive women – an evaluation of HPV 16/18 genotyping, cytology, and p16/Ki-67 cytoimmunochemistry. *Canc. Epidemiol. Biomarkers Prev.* 26(11), 1629–1635 (2017).
13. Luttmer R, De Strooper LM, Steenbergen RD *et al.* Management of high-risk HPV-positive women for detection of cervical (pre)cancer. *Expert Rev. Mol. Diagn.* 16(9), 961–974 (2016).
14. Polman NJ, Ebisch RMF, Heideman DA *et al.* Performance of human papillomavirus testing on self-collected versus clinician-collected samples for the detection of cervical intraepithelial neoplasia of grade 2 or worse: a randomised, paired screen-positive, non-inferiority trial. *Lancet Oncol.* 20(2), 229–238 (2019).

- **First randomized controlled clinical trial which demonstrates that HPV testing on self-collected samples is non-inferior to HPV testing on cervical scrapes.**

15. Massad LS, Einstein MH, Huh WK *et al.* 2012 updated consensus guidelines for the management of abnormal cervical cancer screening tests and cancer precursors. *Obstet. Gynecol.* 121(4), 829–846 (2013).
16. Lorincz AT. Virtues and weaknesses of DNA methylation as a test for cervical cancer prevention. *Acta Cytol.* 60(6), 501–512 (2016).
17. Schmitz M, Wunsch K, Hoyer H *et al.* Performance of a methylation specific real-time PCR assay as a triage test for HPV-positive women. *Clin. Epigenetics* 9, 118 (2017).
18. Lorincz AT, Brentnall AR, Scibior-Bentkowska D *et al.* Validation of a DNA methylation HPV triage classifier in a screening sample. *Int. J. Can.* 138(11), 2745–2751 (2016).
19. Boers A, Wang R, Van Leeuwen RW *et al.* Discovery of new methylation markers to improve screening for cervical intraepithelial neoplasia grade 2/3. *Clin. Epigenetics* 8, 29 (2016).
20. Cook DA, Krajden M, Brentnall AR *et al.* Evaluation of a validated methylation triage signature for human papillomavirus positive women in the HPV FOCAL cervical cancer screening trial. *Int. J. Can.* 144(10), 2587–2595 (2019).
21. Kelly H, Benavente Y, Pavon MA, De Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (CIN2+): a systematic review and meta-analysis. *Br. J. Canc.* 121(11), 954–965 (2019).

- **Provides a detailed overview of the current literature regarding methylation analysis and its clinical implications.**

22. Wentzensen N, Sherman ME, Schiffman M, Wang SS. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol. Oncol.* 112(2), 293–299 (2009).

- **Provides a detailed overview of the current literature regarding methylation analysis and its clinical implications.**

23. Steenbergen RD, Snijders PJ, Heideman DA, Meijer CJ. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat. Rev. Canc.* 14(6), 395–405 (2014).

- **Provides a detailed overview of the current literature regarding methylation analysis and its clinical implications.**

24. Szalmas A, Konya J. Epigenetic alterations in cervical carcinogenesis. *Semin. Canc. Biol.* 19(3), 144–152 (2009).
25. Bierkens M, Wilting SM, Van Wieringen WN *et al.* Chromosomal profiles of high-grade cervical intraepithelial neoplasia relate to duration of preceding high-risk human papillomavirus infection. *Int. J. Can.* 131(4), E579–E585 (2012).
26. Verlaat W, Van Leeuwen RW, Novianti PW *et al.* Host-cell DNA methylation patterns during high-risk HPV-induced carcinogenesis reveal a heterogeneous nature of cervical pre-cancer. *Epigenetics* 13(7), 769–778 (2018).
27. De Strooper LM, Meijer CJ, Berkhof J *et al.* Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. *Canc. Prev. Res.* 7(12), 1251–1257 (2014).
28. Eijsink JJ, Lendvai A, Deregowski V *et al.* A four-gene methylation marker panel as triage test in high-risk human papillomavirus positive patients. *Int. J. Can.* 130(8), 1861–1869 (2012).
29. Hesselink AT, Heideman DA, Steenbergen RD *et al.* Combined promoter methylation analysis of CADM1 and MAL: an objective triage tool for high-risk human papillomavirus DNA-positive women. *Clin. Canc. Res.* 17(8), 2459–2465 (2011).
30. Clarke MA, Wentzensen N, Mirabello L *et al.* Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. *Canc. Epidemiol. Biomarkers Prev.* 21(12), 2125–2137 (2012).

31. Hansel A, Steinbach D, Greinke C *et al.* A promising DNA methylation signature for the triage of high-risk human papillomavirus DNA-positive women. *PLoS ONE* 9(3), e91905 (2014).
32. De Strooper LMA, Berkhof J, Steenbergen RDM *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 14 year follow-up. *Int. J. Can.* 143(6), 1541–1548 (2018).
33. Dick S, Kremer WW, De Strooper LMA *et al.* Long-term CIN3+ risk of HPV positive women after triage with FAM19A4/miR124-2 methylation analysis. *Gynecol. Oncol.* 154(2), 368–373 (2019).
34. Verlaat W, Snijders PJF, Novianti PW *et al.* Genome-wide DNA methylation profiling reveals methylation markers associated with 3q gain for detection of cervical precancer and cancer. *Clin. Canc. Res.* 23(14), 3813–3822 (2017).

•• **Describes the discovery of methylation markers *GHSR*, *SST* and *ZIC1*.**

35. Verlaat W, Snoek BC, Heideman DA *et al.* Identification and validation of a 3-gene methylation classifier for HPV-based cervical screening on self-samples. *Clin. Canc. Res.* 24(14), 3456–3464 (2018).

•• **Describes the discovery of methylation markers *ASCL1*, *LHX8* and *ST6GALANC5*.**

36. Carreon JD, Sherman ME, Guillen D *et al.* CIN2 is a much less reproducible and less valid diagnosis than CIN3: results from a histological review of population-based cervical samples. *Int. J. Gynecol. Pathol.* 26(4), 441–446 (2007).
37. Arbyn M, Snijders PJ, Meijer CJ *et al.* Which high-risk HPV assays fulfil criteria for use in primary cervical cancer screening? *Clin. Microbiol. Infect.* 21(9), 817–826 (2015).
38. Snellenberg S, De Strooper LM, Hesselink AT *et al.* Development of a multiplex methylation-specific PCR as candidate triage test for women with an HPV-positive cervical scrape. *BMC Canc.* 12, 551 (2012).
39. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3(6), 1101–1108 (2008).
40. Snoek BC, Splunter APV, Bleeker MCG *et al.* Cervical cancer detection by DNA methylation analysis in urine. *Sci. Rep.* 9(1), 3088 (2019).
41. Clarke MA, Luhn P, Gage JC *et al.* Discovery and validation of candidate host DNA methylation markers for detection of cervical precancer and cancer. *Int. J. Can.* 141(4), 701–710 (2017).
42. Brebi P, Maldonado L, Noordhuis MG *et al.* Genome-wide methylation profiling reveals Zinc finger protein 516 (ZNF516) and FK-506-binding protein 6 (FKBP6) promoters frequently methylated in cervical neoplasia, associated with HPV status and ethnicity in a Chilean population. *Epigenetics* 9(2), 308–317 (2014).
43. Vink FJ, Meijer C, Clifford GM *et al.* FAM19A4/miR124-2 methylation in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Int. J. Can.* doi:10.1002/ijc.32614 (2019) (Epub aheah of print).
44. Tommasi S, Karm DL, Wu X, Yen Y, Pfeifer GP. Methylation of homeobox genes is a frequent and early epigenetic event in breast cancer. *Breast Canc. Res.* 11(1), R14 (2009).
45. Li YF, Hsiao YH, Lai YH *et al.* DNA methylation profiles and biomarkers of oral squamous cell carcinoma. *Epigenetics* 10(3), 229–236 (2015).
46. De Strooper LM, Van Zummeren M, Steenbergen RD *et al.* CADM1, MAL and miR124-2 methylation analysis in cervical scrapes to detect cervical and endometrial cancer. *J. Clin. Pathol.* 67(12), 1067–1071 (2014).
47. Wu TI, Huang RL, Su PH, Mao SP, Wu CH, Lai HC. Ovarian cancer detection by DNA methylation in cervical scrapings. *Clin. Epigenetics* 11(1), 166 (2019).
48. Chang CC, Wang HC, Liao YP *et al.* The feasibility of detecting endometrial and ovarian cancer using DNA methylation biomarkers in cervical scrapings. *J. Gynecol. Oncol.* 29(1), e17 (2018).
49. Bierkens M, Hesselink AT, Meijer CJ *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. Can.* 133(6), 1293–1299 (2013).
50. Louvanto K, Aro K, Nedjai B *et al.* Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia. *Clin. Infect. Dis.* doi:10.1093/cid/ciz677 (2019) (Epub ahead of print).
51. Kyrgiou M, Athanasiou A, Kalliala IEJ *et al.* Obstetric outcomes after conservative treatment for cervical intraepithelial lesions and early invasive disease. *Cochrane Database Syst. Rev.* 11, CD012847 (2017).
52. Kremer WW, Berkhof J, Bleeker MC *et al.* Role of FAM19A4/miR124-2 methylation analysis in predicting regression or non-regression of CIN2/3 lesions: a protocol of an observational longitudinal cohort study. *BMJ Open* 9(7), e029017 (2019).
53. Gok M, Heideman DA, Van Kemenade FJ *et al.* Offering self-sampling for human papillomavirus testing to non-attendees of the cervical screening programme: characteristics of the responders. *Eur. J. Canc.* 48(12), 1799–1808 (2012).
54. Arbyn M, Smith SB, Temin S, Sultana F, Castle P. Detecting cervical precancer and reaching underscreened women by using HPV testing on self samples: updated meta-analyses. *BMJ* 363, k4823 (2018).
55. Hesselink AT, Heideman DA, Steenbergen RD *et al.* Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. *Int. J. Can.* 135(4), 880–886 (2014).
56. Kremer WW, Van Zummeren M, Novianti PW *et al.* Detection of hypermethylated genes as markers for cervical screening in women living with HIV. *J. Int. AIDS Soc.* 21(8), e25165 (2018).