

VU Research Portal

Triage of HPV-positive women by methylation marker analysis

de Strooper, L.M.A.

2016

document version

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

citation for published version (APA)

de Strooper, L. M. A. (2016). *Triage of HPV-positive women by methylation marker analysis*. [, Vrije Universiteit Amsterdam].

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:

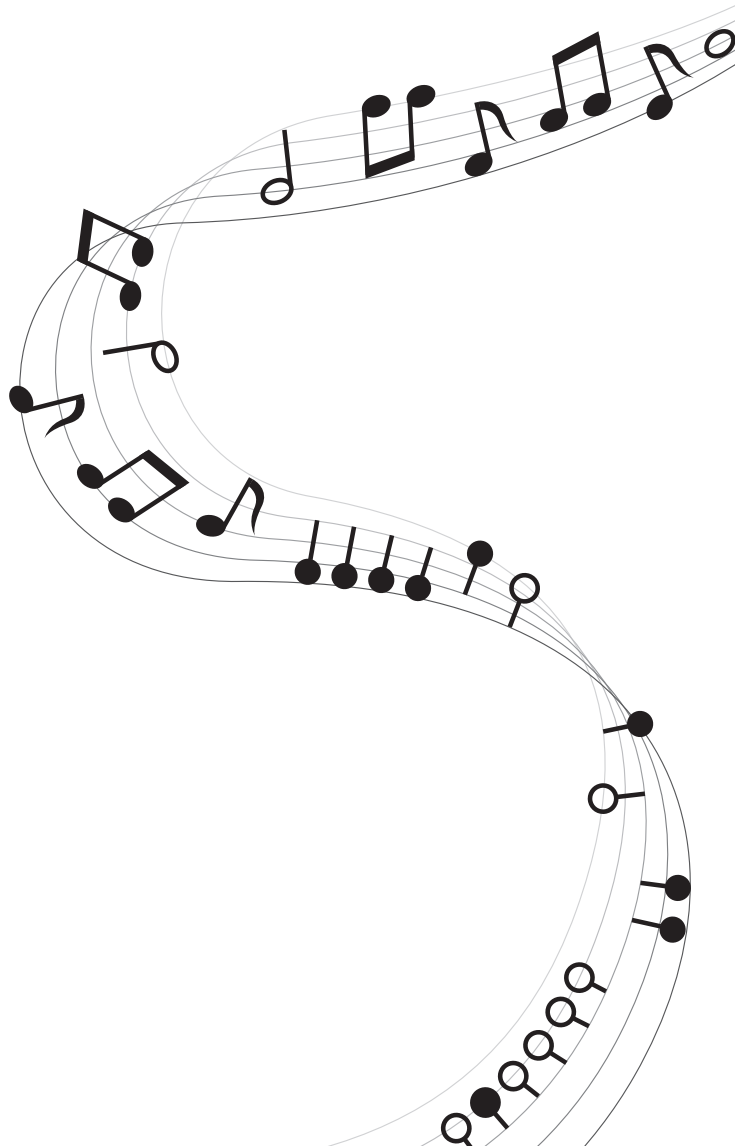
vuresearchportal.ub@vu.nl

Chapter 7

Validation of a DNA methylation test
for both lavage- and brush-based self-samples
to detect cervical (pre)cancer in HPV-positive women

Lise M.A. De Strooper
Viola M.J. Verhoef
Johannes Berkhof
Albertus T. Hesselink
Helena M.E. de Bruin
Folkert J. van Kemenade
Remko P. Bosgraaf
Ruud L.M. Bekkers
Leon F.A.G. Massuger
Willem J.G. Melchers
Renske D.M. Steenbergen
Peter J.F. Snijders
Chris J.L.M. Meijer
Daniëlle A.M. Heideman

Submitted for publication



ABSTRACT

Introduction:

DNA methylation analysis of cancer-related genes is an appropriate tool for HPV-positive women to identify those with cervical (pre)cancer (CIN3+) in need of treatment. However, clinical performance of methylation markers can be influenced by the sample type utilized. We describe a multiplex quantitative methylation-specific PCR that targets *FAM19A4* and *mir124-2* loci, to detect CIN3+ using both HPV-positive lavage- and brush self-samples.

Methods:

We determined methylation thresholds for clinical classification using HPV-positive training sets comprising lavage self-samples of 182 women (including 40 with CIN3+) and brush self-samples of 224 women (including 61 with CIN3+). Subsequently, independent HPV-positive validation sets of 389 lavage self-samples (including 78 with CIN3+), and 254 brush self-samples (including 72 with CIN3+) were tested using the preset thresholds. Furthermore, the clinical performance of combined methylation analysis and HPV16/18 genotyping was determined.

Results:

Training set analysis revealed similar *FAM19A4* and *mir124-2* thresholds for both self-sample types to yield highest CIN3+ sensitivity at 70% specificity. Validation set analysis resulted in a CIN3+ sensitivity of 70.5% (95% CI: 60.4-80.6) at a specificity of 67.8% (95% CI: 62.7-73.0) for lavage self-samples and a CIN3+ sensitivity of 69.4% (95% CI: 58.8-80.1) at a 76.4% (95% CI: 70.2-82.6) specificity for brush self-samples. In combination with HPV16/18 genotyping, CIN3+ sensitivity and specificity were 88.5% (95% CI: 81.4-95.6) and 46.0% (95% CI: 40.4-51.5) for lavage self-samples, and 84.7% (95% CI: 76.4-93.0) and 54.9% (95% CI: 47.7-62.2) for brush self-samples.

Conclusions:

FAM19A4/mir124-2 methylation analysis performs equally well in HPV-positive lavage- and brush self-samples to identify women with CIN3+. In combination with HPV16/18 genotyping, significantly higher CIN3+ sensitivities are obtained, at decreased specificity.

INTRODUCTION

A substantial subset of women does not participate in population-based cervical screening, which compromises the effectiveness of the screening programme¹. These non-responders are at increased risk of developing cervical cancer, indicating the need for recruitment of these women into screening^{1,2}. Offering devices for self-collection of (cervico-)vaginal specimens has prospects to increase screening compliance³⁻⁶. Self-collected (cervico-)vaginal specimens can be tested for the presence of DNA from high-risk types of human papillomavirus (HPV) in a laboratory (i.e., HPV self-sampling). When using validated PCR assays, HPV testing on self-samples can have similar accuracy for detecting cervical (pre) cancer (CIN3+) as HPV testing of physician-taken samples^{4,7-12}. Therefore, it is expected that HPV self-sampling with validated combinations of self-collection devices and HPV tests, will be increasingly adopted in future HPV-based screening programmes^{10,13,14}.

However, HPV testing cannot distinguish transient from persistent, clinically relevant high-risk HPV infections. Therefore, additional testing (i.e., triage) is essential to identify HPV-positive women with CIN lesions in need of treatment. This approach will reduce over-referral, unnecessary colposcopies, and overtreatment of women without clinically meaningful HPV infections. Currently, cytology, either or not combined with HPV16/18 genotyping analysis, is a widely accepted tool for colposcopy triage of HPV-positive women^{15,16}. However, cytology on self-collected (cervico-)vaginal specimens is unreliable¹⁷. Therefore, cytology triage would require an additional visit to a clinician for cervical scrape collection. The resulting prerequisite for cervical cytology for previous non-responder women, has been associated with loss to follow-up and has shown to extend the diagnostic track¹⁸. This process could be simplified substantially by triage testing directly on HPV-positive self-samples by non-morphological molecular methods.

Although HPV16/18 genotyping as molecular test is directly applicable to self-samples and detects women with increased cervical cancer risk, a non-negligible fraction of (pre)cancers associated with non-HPV16/18 high-risk HPV types will be missed when using genotyping alone¹⁹. As an alternative or additive tool, methylation markers that reflect molecular events in host cells contributing to cervical carcinogenesis are highly promising²⁰⁻²². Previous studies have revealed that promoter methylation of host cell genes such as *FAM19A4* and *mir124-2*, increases with cervical disease severity²³⁻²⁵. Methylation levels of *FAM19A4* and *mir124-2* are particularly high in women with cervical cancer and advanced high-grade lesions, the latter characterized by a longer duration (≥ 5 years) of a preceding high-risk HPV infection^{20,24,26,27}. Of interest, methylation analysis on HPV-positive self-collected lavage samples as direct molecular triage tool using *MAL* and *mir124-2* loci has reported to be clinically non-inferior to cytology triage on a subsequent physician-taken scrape in a recent randomized controlled trial¹⁸. In addition, methylation analysis on these samples could be combined with HPV16/18 genotyping to reach higher CIN3+ sensitivity¹⁹.

With an increasing interest in self-collection for HPV-based cervical screening programmes^{28,29}, it is of importance to gain more clinical performance data on methylation marker analysis of HPV-positive self-samples. Since performance of methylation markers can be influenced by the type of sample utilized, performance evaluation in different self-sample types is necessary to determine its broader utility. Unlike samples collected by lavage-based devices^{18,22}, evaluation of brush-collected self-samples by methylation analysis is restricted to feasibility studies so far²¹. Furthermore, no studies with large sample numbers have compared the performance of DNA methylation markers on HPV-positive lavage and brush self-samples.

Here, we conducted a post-hoc analysis on 1049 HPV-positive self-samples from previous PROTECT studies that had been collected by either lavage- and or brush-based self-collection devices^{5,18,30}. We evaluated a multiplex quantitative methylation-specific PCR (qMSP) that targets *FAM19A4* and *mir124-2* loci. The performance of the qMSP assay in terms of analytical sensitivity, analytical specificity, and daily-use reproducibility is described. In addition, the clinical performance of the assay, either or not combined with HPV16/18 genotyping, for detection of cervical (pre)cancer was determined on both self-sample types.

METHODS

Study populations

Lavage-collected self-samples

For the training set, 375 HPV-positive (GP5+/6+ PCR) cervico-vaginal lavage self-samples collected with the second generation Delphi-screener (Delphi-Bioscience, the Netherlands) from non-responder women participating in the PROTECT-3B trial were available. The flowchart of this training set is shown in Figure 1A (left panel). These samples are further referred to as lavage self-samples. The trial was registered in the trial register as NTR3350. Detailed characteristics, inclusion criteria and follow-up procedures of the PROTECT-3B trial have been described previously³⁰. Of the HPV-positive women, 15 women had no cytological or histological follow-up and were excluded from analysis. Of the remaining 360 women, 251 women reached a study-endpoint [i.e., either a histological outcome, classified as cervical cancer, cervical intraepithelial neoplasia (CIN) grade 3 (CIN3), CIN2, CIN1, or absence of CIN (CIN0)) or a combined normal cytology and HPV negative outcome]. Since CIN2 lesions often represent a misclassified CIN1 or CIN3, women with CIN2 lesions (n=36) were excluded from the training set. From the remaining 215 women, 182 had sufficient quantities of the self-sample with qualitatively adequate DNA left for qMSP analysis. Of them, 40 were histologically diagnosed with CIN3+ [i.e., 33 CIN3, 6 squamous cell carcinomas (SCCs), and 1 adenocarcinoma (AdCA)] with a median age of 38 years (range: 33-58). The remaining 142 women had no evidence of CIN2+ (also referred to as women with \leq CIN1). Of

these, 42 had histologically-confirmed absence of CIN, 36 had CIN1 and 64 women displayed HPV clearance combined with a normal cytology result during follow-up. The median age of women with \leq CIN1 was 38 years (range: 33-64).

For the validation set, 515 HPV-positive (GP5+/6+ PCR) lavage self-samples collected with the first generation Delphi-screener (Delphi-Bioscience, the Netherlands), were available from non-responder women participating in the methylation triage arm of the PROHTECT-3A trial (NTR2606). The first and second generation Delphi-screener perform equal in DNA yield and HPV-detection³¹. The flowchart of the validation set is shown in Figure 1A (right panel). Detailed characteristics, inclusion criteria and follow-up procedures of the PROHTECT-3A trial have been described previously¹⁸. Of the 515 HPV-positive women, 408 women reached a study-endpoint (as detailed above) within 18 months of follow-up. Of the 408 women, 389 had sufficient quantities left of the self-sample with qualitatively adequate DNA for qMSP analysis. This series included 78 women who were histologically diagnosed with CIN3+ [i.e., 70 CIN3, 5 SCCs, 2 adenocarcinomas in situ (ACIS) and 1 AdCA] with a median age of 38 years (range: 33-58) and 41 women with CIN2 with a median age of 43 years (range: 33-58). The remaining 270 women had no evidence of CIN2+. Of these, 125 had histologically-confirmed absence of CIN and 76 had CIN1. The remaining 69 women displayed both HPV clearance and a normal cytology result at follow-up. The median age of this group was 43 years (range: 33-63).

Brush-collected self-samples

For the training set, 409 HPV-positive (GP5+/6+ PCR) vaginal brush self-samples collected with the Evalyn brush (Rovers, the Netherlands) from non-responder women participating in the PROHTECT-3B trial³⁰ were available. The flowchart of this training set is shown in Figure 1B (left panel). After self-sampling, the brushes were sent dry to the laboratory where they were placed into 1.5 ml PreservCyt medium (Hologic, USA) before further processing. Of the HPV-positive women, 18 women had no cytological or histological follow-up and were excluded from analysis. From the 391 HPV-positive women, those who reached a study-endpoint (as detailed above), except for those with CIN2 (n=25, as explained above), and of whom sufficient quantities were left of the self-sample with qualitatively adequate DNA for qMSP analysis, were included in the training set. The remaining series of 224 women included 61 women who were histologically diagnosed with a CIN3+ lesion (i.e., 53 CIN3, 5 SCC, and 3 AdCA) with a median age of 38 years (range: 33-59). The other 163 women had no evidence of CIN2+. Of these, 57 had histologically-confirmed absence of CIN, 34 had CIN1, and 72 women displayed HPV clearance combined with a normal cytology result at follow-up. The median age of women with \leq CIN1 was 38 years (range 33-63).

For the validation set, 541 HPV-positive (GP5+/6+ PCR) vaginal brush self-samples from non-responder women participating in the PROHTECT-2 trial (NTR1851), were available. These samples were collected with the VibaBrush (Rovers, the Netherlands). The flowchart

of the validation set is shown in Figure 1B (right panel). After self-sampling the brushes were placed in 1.5ml universal collection medium (Qiagen, USA) before sending to the laboratory. Detailed characteristics, inclusion criteria and follow-up procedures of the PROTECT-2 trial have been described previously⁷. From the 541 HPV-positive women, only those who reached a study-endpoint (as detailed above) within 36 months of follow-up, and of whom sufficient quantities were left of the self-sample with qualitatively adequate DNA for qMSP analysis, were included in the validation set (n=254). Of these, 72 women were histologically diagnosed with a CIN3+ lesion [i.e., 67 CIN3, 3 SCCs, 1 ACIS and 1 AdCA] and had a median age of 36 years (range: 31-61). 27 women were diagnosed with CIN2 and had a median age of 36 years (range: 31-56). The remaining 155 women had no evidence of CIN2+. Of these women, 24 had histologically-confirmed absence of CIN, 24 had CIN1, and 107 women displayed both HPV clearance and a normal cytology result at follow-up. The median age of this group was 36 years (range: 30-62). All PROTECT trials had ethical approval by the National Health Council and all participants gave informed consent.

DNA isolation and HPV genotyping

DNA from lavage and brush self-samples was isolated using the Nucleo-Mag 96 Tissue kit (Macherey-Nagel, Germany) and Microlab Star robotic system (Hamilton, Germany) according to manufacturers' protocol³². In the PROTECT-3 trials, all samples were tested for hrHPV DNA by the clinically validated hrHPV GP5+/6+ PCR (EIA HPV GP HR kit, Diassay, The Netherlands) according to the manufacturer's protocol³³. In PROTECT-3A, subsequent hrHPV genotyping was performed using Luminex suspension array technology³⁴. In the PROTECT-2 trial, samples were tested for hrHPV using the Hybrid Capture-2® (HC2, Qiagen, USA) according to manufacturer's protocol³⁵. All HC2 positive samples were subsequently tested by GP5+/6+ PCR and subjected to hrHPV genotyping by the reverse line blot assay³⁵. In the current study, only samples that were GP5+/6+ PCR-positive were used.

Bisulphite treatment and qMSP methylation analysis

Isolated DNA was subjected to bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research, USA) as described previously^{37,38}. Bisulphite-converted DNA was used as template for DNA methylation analysis. DNA methylation analysis was performed by a multiplex qMSP assay targeting *FAM19A4* and *mir124-2* loci, as well as *ACTB* as a sample quality control. Analyses were performed on an ABI 7500 real-time PCR-system (Applied Biosystems, USA). For each target, Quantification Cycle (Cq) values were measured at a fixed fluorescence threshold. The result of a sample is expressed in $\Delta\Delta Cq$ ratio being a measure for hypermethylation using the $2^{-\Delta\Delta Cq}$ method³⁹. A plasmid containing all amplicon sequences (i.e., *ACTB*, *FAM19A4*, and *mir124-2*) was used as amplification target to assess the analytical sensitivity. Serial 10-fold plasmid dilutions (range from 750,000 to 1 copy per reaction) were analysed in duplicate in two independent qMSP runs.

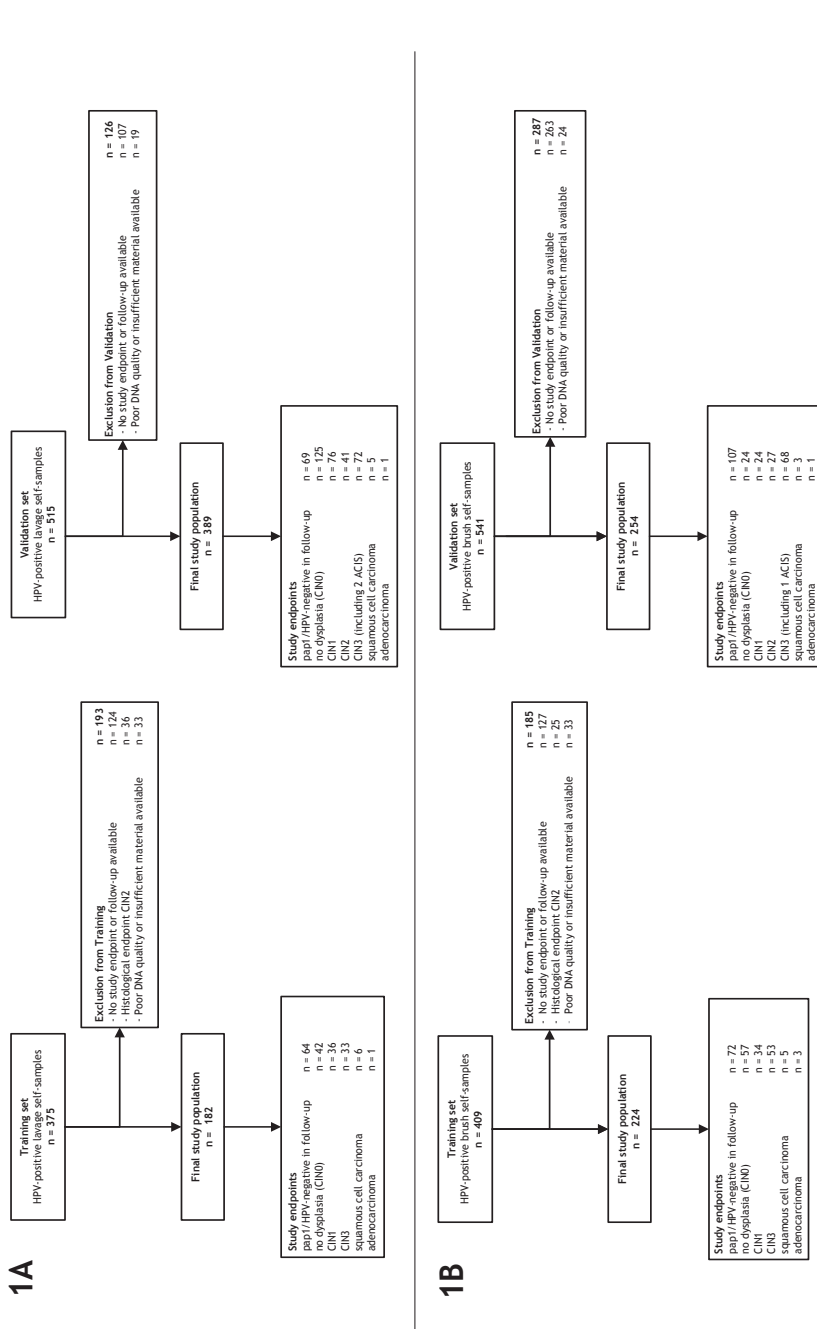


Figure 1. Overview of the study populations. The training and validation sets for lavage self-samples, (1A), and the training and validation sets for brush self-samples (1B) are presented. HPV= Human Papillomavirus, CIN= cervical intraepithelial neoplasia.

The analytical sensitivity was determined as the lowest dilution giving 4 out of 4 positive results ($Cq < 40$). To assess the analytical specificity, bisulphite-converted unmethylated DNA from primary keratinocytes and unmodified DNA (i.e., pool of non-bisulphite treated DNA from cervical samples) were analysed in quadruplicate. The reproducibility of the assay based on $\Delta\Delta Cq$ values was evaluated by duplicate measurement in independent qMSP runs of bisulphite-converted DNA of 30 HPV-positive cervical samples.

Data and statistical analysis

First, both training sets (lavage self-samples and brush self-samples, separately) were used to define clinical decision points, i.e., thresholds for positivity of the triage assay. At a predefined specificity value of 70% for CIN3+, an optimization procedure was performed to calculate the maximum corresponding CIN3+ sensitivity over all possible threshold values for the combined marker panel. The defined methylation thresholds converted the test result into a categorical variable leading to an optimal CIN3+ sensitivity at 70% CIN3+ specificity level. The thresholds were subsequently evaluated in the two independent validation sets (lavage and brush, respectively). The primary outcome was CIN3+ detection and the secondary outcome was CIN2+ detection. In the validation sets, all samples were additionally evaluated for the clinical performance of *FAM19A4/mir124-2* methylation analysis combined with HPV16/18 genotyping. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined for outcome CIN3+ and CIN2+ with 95% Wald confidence intervals. The colposcopy referral rate was calculated as the proportion of hrHPV positive women who had a positive triage test result. Analyses were performed in the R package (version 2.15). Additional calculations were performed in IBM SPSS Statistics 20 and Excel.

RESULTS

Analytical performance

For assessing DNA methylation of *FAM19A4* and *mir124-2*, a multiplex qMSP assay was used. The analytical sensitivity of the assay was 2.5 copies of methylated target per PCR reaction for both *FAM19A4* and *mir124-2*, and the reference gene *ACTB*. The assay revealed no positive signals with bisulphite-converted unmethylated DNA nor non-bisulphite treated DNA of cervical samples, showing a high analytical specificity. The assay demonstrated a high reproducibility on HPV-positive cervical samples with Pearson correlation coefficients between the $\Delta\Delta Cq$ values of 0.988 for *FAM19A4* (Figure 2A) and 0.970 for *mir124-2* (Figure 2B).

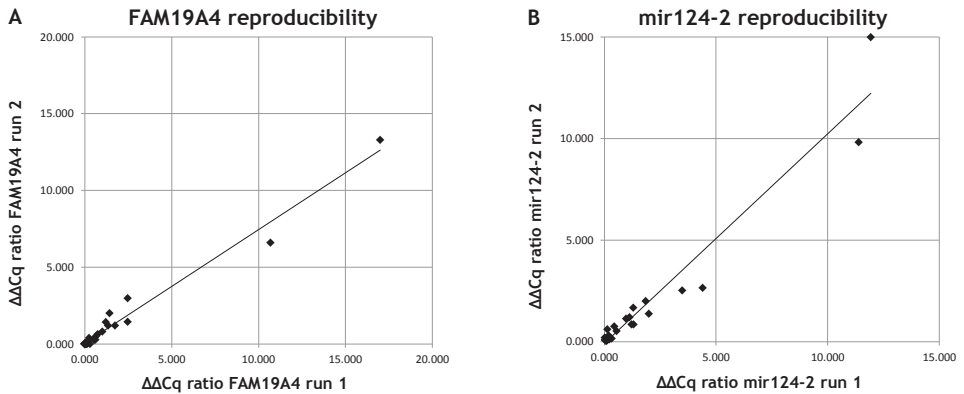


Figure 2. Reproducibility of the $\Delta\Delta Cq$ ratios of *FAM19A4* (A) and *mir124-2* (B) between two independent qMSP runs.

Clinical performance

Using the training sets of HPV-positive lavage and brush self-samples separately, methylation thresholds for *FAM19A4* and *mir124-2* were determined that resulted in a maximum CIN3+ sensitivity at a preset specificity of 70%. Of interest, these thresholds did not differ between the different sample types (i.e., HPV-positive lavage compared to brush self-samples). According to the thresholds, samples with $\Delta\Delta Cq$ ratios above the respective threshold for at least one of the targets (*FAM19A4* and/or *mir124-2*) were rated as test-positive, while samples with $\Delta\Delta Cq$ ratios of both targets below their threshold were rated as test-negative. The use of these thresholds resulted in CIN3+ sensitivities of 75.0% (95% CI: 61.6-88.4) and 72.1% (95% CI: 60.9-83.4) in the training sets of HPV-positive lavage self-samples and brush self-samples, respectively.

Next, the clinical performance of the multiplex qMSP assay was validated on independent sets of HPV-positive lavage and brush self-samples. Sensitivities, specificities, PPV, NPV and referral rate for colposcopy for endpoints CIN3+ and CIN2+ are presented in Table 1. At the predefined threshold of 70%, a CIN3+ sensitivity of 70.5% (95% CI: 60.4-80.6) at a specificity of 67.8% (95% CI: 62.7-73.0) was obtained for HPV-positive lavage self-samples. For CIN2+, a sensitivity of 63.9% (95% CI: 55.2-72.5) at 70.7% (95% CI: 65.3-76.2) specificity was obtained. In the validation set of HPV-positive brush self-samples, a CIN3+ sensitivity of 69.4% (95% CI: 58.8-80.1) at a specificity of 76.4% (95% CI: 70.2-82.5), and a CIN2+ sensitivity of 59.6% (95% CI: 49.9-69.3) at 78.1 (95%CI: 71.5-84.5) specificity was obtained. Of note, all women with cervical carcinomas (lavage, n=13; brush, n=12) were scored methylation positive on their self-sample by the *FAM19A4/mir124-2* methylation marker assay at the predefined 70% specificity threshold.

| Endpoint | Self-sample | Triage marker | n1 / N1 | Sensitivity (%) | (95%CI) | n2 / N2 | Specificity (%) | (95%CI) | PPV (%) | (95%CI) | NPV (%) | (95%CI) | Referral rate (%) |
|----------|-------------|---|----------|-----------------|---------------|-----------|-----------------|---------------|---------|---------------|---------|---------------|-------------------|
| CIN3+ | lavage | FAM19A4/mir124-2 methylation | 55 / 78 | 70.5 | (60.4 - 80.6) | 211 / 311 | 67.8 | (62.7 - 73.0) | 35.5 | (28.0 - 43.0) | 90.2 | (86.4 - 94.0) | 39.8 |
| | brush | FAM19A4/mir124-2 methylation | 50 / 72 | 69.4 | (58.8 - 80.1) | 139 / 182 | 76.4 | (70.2 - 82.6) | 53.8 | (43.6 - 63.9) | 86.3 | (81.0 - 91.6) | 36.6 |
| | lavage | HPV16/18 genotyping | 51 / 78 | 65.4 | (54.8 - 75.9) | 202 / 311 | 65.0 | (59.7 - 70.3) | 31.9 | (24.7 - 39.1) | 88.2 | (84.0 - 92.4) | 41.1 |
| | brush | HPV16/18 genotyping | 50 / 72 | 69.4 | (58.8 - 80.1) | 129 / 182 | 70.9 | (64.3 - 77.5) | 48.5 | (38.9 - 58.2) | 85.4 | (79.8 - 91.1) | 40.6 |
| CIN2+ | lavage | FAM19A4/mir124-2 methylation and/or HPV16/18 genotyping | 69 / 78 | 88.5 | (81.4 - 95.6) | 168 / 311 | 46.0 | (40.4 - 51.5) | 29.1 | (23.3 - 34.9) | 94.1 | (90.3 - 97.8) | 60.9 |
| | brush | FAM19A4/mir124-2 methylation and/or HPV16/18 genotyping | 61 / 72 | 84.7 | (76.4 - 93.0) | 100 / 182 | 54.9 | (47.7 - 62.2) | 42.7 | (34.6 - 50.8) | 90.1 | (84.5 - 95.7) | 56.3 |
| CIN2+ | lavage | FAM19A4/mir124-2 methylation | 76 / 119 | 63.9 | (55.2 - 72.5) | 191 / 270 | 70.7 | (65.3 - 76.2) | 49.0 | (41.2 - 56.9) | 81.6 | (76.7 - 86.6) | 39.8 |
| | brush | FAM19A4/mir124-2 methylation | 59 / 99 | 59.6 | (49.9 - 69.2) | 121 / 155 | 78.1 | (71.6 - 84.6) | 63.4 | (53.6 - 73.2) | 75.2 | (68.5 - 81.8) | 36.6 |
| | lavage | HPV16/18 genotyping | 67 / 119 | 56.3 | (47.4 - 65.2) | 177 / 270 | 65.6 | (59.9 - 71.2) | 41.9 | (34.2 - 49.5) | 77.3 | (71.9 - 82.7) | 41.1 |
| | brush | HPV16/18 genotyping | 64 / 99 | 64.6 | (55.2 - 74.1) | 116 / 155 | 74.8 | (68.0 - 81.7) | 62.1 | (52.8 - 71.5) | 76.8 | (70.1 - 83.6) | 40.6 |
| CIN2+ | lavage | FAM19A4/mir124-2 methylation and/or HPV16/18 genotyping | 94 / 119 | 79.0 | (71.7 - 86.3) | 127 / 270 | 47.0 | (41.1 - 53.0) | 39.7 | (33.4 - 45.9) | 83.6 | (77.7 - 89.4) | 60.9 |
| | brush | FAM19A4/mir124-2 methylation and/or HPV16/18 genotyping | 78 / 99 | 78.8 | (70.7 - 86.8) | 90 / 155 | 58.1 | (50.3 - 65.8) | 54.5 | (46.4 - 62.7) | 81.1 | (73.8 - 88.4) | 56.3 |

CIN= cervical intraepithelial neoplasia; CI= confidence interval; PPV= positive predictive value; NPV= negative predictive value; n1= number of test-positive disease cases; N1= total number of disease cases; n2= number of test-negative non-disease cases; N2= total number of non-disease cases

Table 1. Clinical performance of FAM19A4/mir124-2 methylation marker analysis, HPV16/18 genotyping and the combination of both triage tests in the validation sets stratified by self-sampling type.

After combining *FAM19A4/mir124-2* methylation analysis with HPV16/18 genotyping, CIN3+ sensitivities increased and specificities decreased for both self-sample types. In lavage self-samples, a CIN3+ sensitivity of 88.5% (95% CI: 81.4-95.6) at a specificity of 46.0% (95% CI: 40.4-51.5) was observed. In brush self-samples, a CIN3+ sensitivity of 84.7% (95% CI: 76.4-93.0) at a specificity of 54.9% (95% CI: 47.7-62.2) was observed. For CIN2+, a similar tendency is seen (Table 1).

DISCUSSION

In this study, we evaluated *FAM19A4/mir124-2* methylation analysis for lavage- and brush-based self-samples to detect cervical (pre)cancer in HPV-positive women. In comparison to the *MAL/mir124-2* triage test that was prospectively evaluated in HPV-positive lavage self-samples in a screening setting¹⁸, the current assay shows a similar CIN3+ sensitivity at a higher specificity. Since the *FAM19A4/mir124-2* assay features likewise clinical performance in HPV-positive brush-collected self-samples, it can be considered as a universal triage test for HPV-positive (cervico-)vaginal self-samples collected by different self-collection devices. The combination of HPV-testing and methylation marker analysis has thereby opened the way to full molecular screening. In combination with HPV16/18 genotyping, significantly higher sensitivities were obtained, yet at the cost of decreased specificities.

This study is the first to evaluate in large sample series whether DNA methylation analysis is equally applicable to both HPV-positive lavage- and brush-collected self-samples for CIN2/3+ detection. Differences in clinical performance of methylation markers between self-sample types have been reported before⁴⁰. These differences are most likely related to intrinsic underlying variability in cellular composition and proportion of hypermethylation-positive cervical cells indicative for CIN lesions between various types of self-samples. Previously, only lavage self-samples have been extensively investigated by methylation marker analysis, showing feasibility²² and good clinical performance^{18,40}. For brush samples, a feasibility study has been published²¹, but no clinical performance data in large series have been reported. The equal clinical performance of *FAM19A4/mir124-2* methylation analysis in HPV-positive lavage and brush self-samples as assessed on large sample series herein, makes this assay a promising direct molecular triage tool for self-samples, independent of the collection device used.

The qMSP assay used in this study allows fast and reliable read-out of multiple methylation markers and a reference gene in one assay. As such, the methylation test saves clinical material, time and costs and improves quality control. Using a dichotomized outcome, the assay provides a clinical decision point to refer an HPV-positive woman for colposcopy or not. The advantages of our study are the evaluation of large, independent series of different self-sample types, and the use of a standardized assay.

To the best of our knowledge, no other methodology is currently available that can be reliably applied directly to self-collected (cervico-)vaginal specimens, and has similar clinical sensitivity and clinical specificity figures for the triage of HPV-positive women following self-sampling as the assay described herein. Cytology is a widely accepted triage test for HPV-positive women, but cytology triage following HPV self-sampling requires an additional cervical scrape taken by a physician^{17,18}. Furthermore, it has been proposed that methylation analysis has a high detection sensitivity for cancer and advanced lesions with a high short-term progression risk for cancer²⁴. In opposition, cytology detects with a moderate sensitivity all morphological cellular abnormalities associated with most, but not all, CIN2/3 and cancer²⁰. Indeed, methylation analysis has shown to detect all cervical carcinomas^{24,27}, as confirmed herein for limited numbers (n=13 and n=12 for lavage and brush self-samples respectively).

Nonetheless, part of CIN2 and few of CIN3 lesions is likely to remain undetected when a methylation marker-based triage strategy is used. Based on our previous work, these lesions are likely early-onset or incident lesions with a low progression-risk to invasive cancer²⁴. As clinicians prefer to also detect these early-onset CIN2/3 lesions, combined molecular triage by methylation marker analysis and HPV16/18 genotyping may be considered for triage of women with an HPV-positive self-sample¹⁹. Indeed, CIN3+ and CIN2+ sensitivities increased when adding HPV16/18 genotyping to methylation analysis. These findings are in line with Verhoef et al.¹⁹ showing that combined molecular triage by *MAL/mir124-2* and HPV16/18 genotyping on HPV-positive lavage self-samples leads to significantly higher sensitivities for CIN3+, yet at the cost of a lower specificity.

CONCLUSION

In conclusion, *FAM19A4/mir124-2* methylation analysis is a universal triage assay for HPV-positive women that can be used in both HPV-positive lavage and brush (cervico-)vaginal self-samples for the identification of women with (pre)cancer in need of treatment. In combination with HPV16/18 genotyping, significantly higher CIN3+ sensitivities are obtained, at a decrease in specificity. Direct molecular triage testing on HPV-positive self-samples, independent of the collection device used, can optimize the screening programme by obviating the need for a cytology triage visit to a physician.

ACKNOWLEDGEMENTS

This project was supported by grants from the Dutch Cancer Society (KWF VU 2009-4522 and KWF VU 2014-7238), the European community Program Health-FP7 Program (CoheaHr-Health-F3-2013-603019) and the European Research Council (ERC advanced 2012-AdG, proposal 322986 Mass-care). The PROHTECT-2 trial was sponsored by the Screening Organization Noord-Holland and Flevoland, and IKA (Comprehensive Cancer Center Amsterdam). The PROHTECT-3 and PROHTECT-3B trials were sponsored by Screening Organizations Midden-West and Oost, and Stichting Achmea Gezondheidszorg. We are grateful to all PROHTECT participants who contributed to this study. We would like to acknowledge all PROHTECT study team members, general practitioners, gynaecologists, pathologists, technicians, secretarial services and the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA) for their contribution. We also would like to thank A. van Splunter for excellent technical assistance.

REFERENCES

1. Bos AB, Rebolj M, Habbema JDF, van Ballegooijen M. Nonattendance is still the main limitation for the effectiveness of screening for cervical cancer in the Netherlands. *Int. J. Cancer* 2006; 119(10):2372-2375.
2. Peto J, Gilham C, Fletcher O, Matthews FE. The cervical cancer epidemic that screening has prevented in the UK. *Lancet* 2004; 364(9430):249-256.
3. Bais AG, van Kemenade FJ, Berkhof J, Verheijen RHM, Snijders PJF, Voorhorst F, et al. Human papillomavirus testing on self-sampled cervicovaginal brushes: an effective alternative to protect nonresponders in cervical screening programs. *Int. J. Cancer* 2007; 120(7):1505-1510.
4. Gök M, Heideman DAM, van Kemenade FJ, Berkhof J, Rozendaal L, Spruyt JWM, et al. HPV testing on self collected cervicovaginal lavage specimens as screening method for women who do not attend cervical screening: cohort study. *BMJ* 2010; 340:c1040.
5. Gök M, Heideman DAM, van Kemenade FJ, de Vries ALM, Berkhof J, Rozendaal L, et al. Offering self-sampling for human papillomavirus testing to non-attendees of the cervical screening programme: Characteristics of the responders. *Eur. J. Cancer* 2012; 48(12):1799-1808.
6. Racey CS, Withrow DR, Gesink D. Self-collected HPV Testing Improves Participation in Cervical Cancer Screening: A Systematic Review and Meta-analysis. *can j public Heal.* 2013; 104(2):e159-e166.
7. Gök M, van Kemenade FJ, Heideman DAM, Berkhof J, Rozendaal L, Spruyt JWM, et al. Experience with high-risk human papillomavirus testing on vaginal brush-based self-samples of non-attendees of the cervical screening program. *Int. J. Cancer* 2012; 130(5):1128-1135.
8. Giorgi Rossi P, Marsili LM, Camilloni L, Iossa A, Lattanzi A, Sani C, et al. The effect of self-sampled HPV testing on participation to cervical cancer screening in Italy: a randomised controlled trial (ISRCTN96071600). *Br. J. Cancer* 2011; 104(2):248-254.
9. Lazcano-Ponce E, Lorincz AT, Cruz-Valdez A, Salmerón J, Uribe P, Velasco-Mondragón E, et al. Self-collection of vaginal specimens for human papillomavirus testing in cervical cancer prevention (MARCH): a community-based randomised controlled trial. *Lancet* 2011; 378(9806):1868-1873.
10. Snijders PJF, Verhoef VMJ, Arbyn M, Ogilvie G, Minozzi S, Banzi R, et al. High-risk HPV testing on self-sampled versus clinician-collected specimens: a review on the clinical accuracy and impact on population attendance in cervical cancer screening. *Int. J. Cancer* 2013; 132(10):2223-2236.
11. Gyllensten U, Sanner K, Gustavsson I, Lindell M, Wikström I, Wilander E. Short-time repeat high-risk HPV testing by self-sampling for screening of cervical cancer. *Br. J. Cancer* 2011; 105(5):694-697.
12. van Baars R, Bosgraaf RP, ter Harmsel BWA, Melchers WJG, Quint WGV, Bekkers RLM. Dry storage and transport of a cervicovaginal self-sample by use of the Evalyn Brush, providing reliable human papillomavirus detection combined with comfort for women. *J. Clin. Microbiol.* 2012; 50(12):3937-3943.

13. Ogilvie GS, Patrick DM, Schulzer M, Sellors JW, Petric M, Chambers K, et al. Diagnostic accuracy of self collected vaginal specimens for human papillomavirus compared to clinician collected human papillomavirus specimens: a meta-analysis. *Sex. Transm. Infect.* 2005; 81(3):207-212.
14. Arbyn M, Verdoodt F, Snijders PJF, Verhoef VMJ, Suonio E, Dillner L, et al. Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis. *Lancet. Oncol.* 2014; 15(2):172-183.
15. Dijkstra MG, van Niekerk D, Rijkaart DC, van Kemenade FJ, Heideman DAM, Snijders PJF, et al. Primary hrHPV DNA testing in cervical cancer screening: how to manage screen-positive women? A POBASCAM trial substudy. *Cancer Epidemiol. Biomarkers Prev.* 2014; 23(1):55-63.
16. Rijkaart DC, Berkhof J, van Kemenade FJ, Coupé VMH, Hesselink AT, Rozendaal L, et al. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *Int. J. Cancer* 2012; 130(3):602-610.
17. Garcia F, Barker B, Santos C, Brown EM, Nuño T, Giuliano A, et al. Cross-sectional study of patient- and physician-collected cervical cytology and human papillomavirus. *Obstet. Gynecol.* 2003; 102(2):266-272.
18. Verhoef VMJ, Bosgraaf RP, van Kemenade FJ, Rozendaal L, Heideman DAM, Hesselink AT, et al. Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): a randomised controlled non-inferiority trial. *Lancet Oncol.* 2014; 15(3):315-322.
19. Verhoef VMJ, Heideman DAM, van Kemenade FJ, Rozendaal L, Bosgraaf RP, Hesselink AT, et al. Methylation marker analysis and HPV16/18 genotyping in high-risk HPV positive self-sampled specimens to identify women with high grade CIN or cervical cancer. *Gynecol. Oncol.* 2014; 135(1):58-63.
20. Steenbergen RDM, Snijders PJF, Heideman DAM, Meijer CJLM. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat. Rev. Cancer* 2014; 14(6):395-405.
21. Boers A, Bosgraaf RP, van Leeuwen RW, Schuurung E, Heideman DAM, Massuger LFAG, et al. DNA methylation analysis in self-sampled brush material as a triage test in hrHPV-positive women. *Br. J. Cancer* 2014; 111(6):1095-1101.
22. Eijsink JJH, Yang N, Lendvai A, Klip HG, Volders HH, Buikema HJ, et al. Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study. *Gynecol. Oncol.* 2011; 120(2):280-283.
23. Steenbergen RDM, Ongenaert M, Snellenberg S, Trooskens G, van der Meide WF, Pandey D, et al. Methylation-Specific Digital Karyotyping of HPV16E6E7 expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. *J. Pathol.* 2013; 231(1):53-62.
24. De Strooper LMA, Meijer CJLM, Berkhof J, Hesselink AT, Snijders PJF, Steenbergen RDM, et al. Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. *Cancer Prev. Res.* 2014; 7(12):1251-1257.
25. Wilting SM, van Boerdonk RAA, Henken FE, Meijer CJLM, Diosdado B, Meijer GA, et al. Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol. Cancer* 2010; 9:167.

26. Bierkens M, Hesselink AT, Meijer CJLM, Heideman DAM, Wisman GBA, van der Zee AGJ, 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(6):1293-1299.
27. De Strooper LMA, van Zummeren M, Steenbergen RDM, Bleeker MCG, Hesselink AT, Wisman GBA, et al. CADM1, MAL and miR124-2 methylation analysis in cervical scrapes to detect cervical and endometrial cancer. *J. Clin. Pathol.* 2014; 67(12):1067-1071.
28. van der Veen N, Carpay M, van Delden J, Brouwer E, Grievink L, Hoebee B, et al. Rijkstinstituut Voor Volksgezondheid En Milieu. Uitvoeringstoets Wijziging Bevolkingsonderzoek Baarmoederhalskanker. *RIVM Rapport 225121002/2013*.
29. Medical service advisory committee. National Cervical Screening Program Renewal: Executive summary. *Report November 2013*. MSAC application no. 1276.
30. Bosgraaf RP, Verhoef VMJ, Massuger LFAG, Siebers AG, Bulten J, de Kuyper-de Ridder GM, et al. Comparative performance of novel self-sampling methods in detecting high-risk human papillomavirus in 30,130 women not attending cervical screening. *Int. J. Cancer* 2015; 136(3):646-655.
31. Verhoef VMJ, Dijkstra MG, Bosgraaf RP, Hesselink AT, Melchers WJG, Bekkers RLM, et al. A second generation cervico-vaginal lavage device shows similar performance as its preceding version with respect to DNA yield and HPV DNA results. *BMC Womens. Health* 2013; 13:21.
32. Hesselink AT, Heideman DAM, Steenbergen RDM, Coupé VMH, Overmeer RM, Rijkaart D, et al. Combined promoter methylation analysis of CADM1 and MAL: an objective triage tool for high-risk human papillomavirus DNA-positive women. *Clin. cancer Res.* 2011; 17(8):2459-2465.
33. Jacobs M V, Snijders PJ, van den Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J. Clin. Microbiol.* 1997; 35(3):791-795.
34. Schmitt M, Bravo IG, Snijders PJF, Pawlita M, Waterboer T, Gissmann L. Bead-Based Multiplex Genotyping of Human Papillomaviruses. *J. Clin. Microbiol.* 2006; 44(2):504-512.
35. Terry G, Ho L, Londesborough P, Cuzick J, Mielzynska-Lohnas I, Lorincz A. Detection of high-risk HPV types by the hybrid capture 2 test. *J. Med. Virol.* 2001; 65(1):155-162.
36. Gravitt PE, Peyton C, Apple R, Wheeler CM. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. - PubMed - NCBI. *J. Clin. Microbiol.* 1998; 10:3020-7.
37. Overmeer RM, Henken FE, Snijders PJF, Claassen-Kramer D, Berkhof J, Helmerhorst TJM, et al. Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J. Pathol.* 2008; 1:388-397.
38. Overmeer RM, Henken FE, Bierkens M, Wilting SM, Timmerman I, Meijer CJLM, et al. Repression of MAL tumour suppressor activity by promoter methylation during cervical carcinogenesis. *J. Pathol.* 2009; 219(3):327-336.
39. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 2008; 3(6):1101-1108.

40. Hesselink AT, Heideman DAM, Steenbergen RDM, Gök M, van Kemenade FJ, Wilting SM, et al. Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. *Int. J. Cancer* 2014; 135(4):880-886.

