Follow-up of high-risk HPV positive women by combined cytology and bi-marker CADM1/MAL methylation analysis on cervical scrapes


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ABSTRACT

Objectives
Triage of HPV screen-positive women is needed to identify those with underlying cervical intraepithelial neoplasia grade 2/3 or worse (CIN2/3+). Presently, cytology on a physician-taken cervical scrape at baseline is mostly accepted as triage test, but needs follow-up testing in order not to miss severe disease. Here, we evaluated the performance of combined cytology and bi-marker CADM1/MAL methylation analysis as triage test on physician-taken cervical scrapes of HPV positive women.

Methods
In this post-hoc analysis, we used 364 left-over HPV positive cytology triage samples of participants of a randomized controlled trial (PROHTECT-3: n=46,001, age 33-63 years) performed in the population-based cervical screening program. Study endpoints were CIN2+ and CIN3+ detection. Cytology testing with and without methylation marker analysis was evaluated with regard to sensitivity, specificity, positive and negative predictive value, and referral rate.

Results
Bi-marker CADM1/MAL methylation positivity increased proportionally with severity of the underlying lesion. Overall, cytology and bi-marker CADM1/MAL methylation analysis yielded similar performances with regard to CIN3+ detection, yet in combination a significantly higher sensitivity for CIN3+ (88.7%) was obtained at a specificity of 53.6% and a colposcopy referral rate of 53.6%. The combined strategy detected all six cervical cancers, whereas triage by cytology alone failed to detect two of them.

Conclusions
Cytology and bi-marker CADM1/MAL methylation analysis perform complementary for CIN2+/CIN3+ detection when used as triage tool on cervical scrapes of HPV positive women. This approach not only results in a higher CIN3+ sensitivity than cytology triage with an acceptable referral rate, but also seems to reduce the risk of missing cervical cancers and advanced high-grade lesions.
INTRODUCTION

HPV testing has a higher sensitivity, but lower specificity than cytology for detection of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) and grade 3 or worse (CIN3+). Therefore, it is essential to triage women with a positive HPV test to distinguish women with underlying CIN2+ who are in need of immediate referral for colposcopy from those without clinically relevant disease. The most widely studied triage test for HPV positive women is cytology. However, cytological assessment on cervical smears is subjective with a large intra- and inter-observer variability and a suboptimal sensitivity. Therefore, a proportion of cervical (pre)cancers will remain undetected because of false negative cytology results, which requires repeat testing or complementary methods allowing CIN2+ lesion detection in cervical smears of HPV positive women with normal cytology.

Data from recent studies suggest that HPV16 and HPV18 genotyping might be a valuable triage technique when added to cytology triage at baseline. However, HPV16/18 genotyping does not detect all cervical cancers since only approximately 70% of cancers is attributable to HPV16/18. Amongst novel, non-morphological candidate triage markers, DNA methylation analysis of tumor suppressor genes involved in cervical carcinogenesis has shown to be a promising alternative triage tool in HPV positive women. Methylation marker tests can easily be performed on the left-over DNA used for HPV testing. Previously, it has been shown that, depending on the assay threshold setting, bi-marker methylation panel CADM1/MAL could be as sensitive as cytology, or cytology combined with HPV16 and 18 genotyping, for the detection of CIN3+. Interestingly, methylation levels of these genes showed a steep increase associated with longer duration and increasing severity of CIN lesions and are extremely high in cervical scrapes of women with cervical cancer. This has led to the concept that methylation analysis would be more sensitive than cytology in detecting advanced high-grade CIN lesions and cervical cancers, with cytology being more sensitive in detecting earlier CIN2/3 lesions.

Here, we assessed the performance of the bi-marker CADM1/MAL methylation assay with cytology, and the potential complementarity using a set of cervical scrapes collected in liquid-based cytology medium of HPV positive women participating in the cytology group of the PROHTECT-3 study.
MATERIAL AND METHODS

Study population
The study population consisted of HPV positive women who participated in the PRotection by Offering HPV Testing on self-sampled Cervico-vaginal specimens Trial-3 (PROHTECT-3) and were assigned to the cytology triage group. The PROHTECT-3 study was a randomised controlled trial (Trial register: NTR6026) that was approved by the national review board (Ministry of Public Health No 2010/04WBO) and informed consent was obtained from all participants. A detailed description of this trial has been published previously. Briefly, 46,001 registered non-attendees of regular cervical screening program from the year 2007, who lived in the regions Noord-Holland, Flevoland, Utrecht and Gelderland, were offered self-sampling lavage devices. Trial inclusion was between November 2010 and December 2011. Exclusion criteria were previous hysterectomy and a history of CIN2+ or abnormal cytology in the preceding two years. Women of the cytology triage group who tested HPV positive (n= 509) were referred to the physician for a cervical scrape. Left-over material of this liquid based physician-taken triage smears was used for our current post-hoc analysis. In total, HPV positive liquid based cytology samples of 364 women could be used for bi-marker CADM1/MAL methylation analysis.

Cervical scrapes were taken by a Cervex brush (Rovers Medical devices B.V., Oss, The Netherlands) and collected in a vial with PreservCyt medium. This vial was sent to the department of Pathology of the VU University Medical Center for hrHPV testing and liquid based cytology preparations. HRHPV testing was performed with the hrHPV GP5+/6+ PCR using Diassay EIA HPV GP HR kit according to instructions of the manufacturer (Diassay, Voorburg, The Netherlands). Cytology was read and classified according to the CISOE-A classification, the standard classification system for cytology in the Netherlands, which can be translated into the Bethesda classification. Cytological results were grouped as normal or abnormal; results classified as atypical squamous cells of undetermined significance (ASC-US) or worse were considered abnormal and indicative for colposcopy referral. If no abnormalities were seen at colposcopy, we advised the gynecologist to take two random biopsies according to the study protocol. Women with normal cytology were offered an exit test after 6 months, which consisted of cytology and hrHPV co-testing. At that occasion, women were referred for colposcopy if any of both tests was abnormal (i.e., abnormal cytology and/or hrHPV positive). Women with double negative results (i.e., normal cytology and hrHPV-negative) were sent back to regular screening. All women with a double negative exit test or a histological outcome before the end of the study (i.e. January 2013) reached the study endpoint. Women without a study-endpoint at January 2013, which is at least 12 months after entering the study, were labeled ‘no CIN2+ detected’. Histology was read according to current guidelines in different pathology laboratories and results were registered in the nationwide histopathology and cytopathology registry in the Netherlands (PALGA, Houten, The Netherlands). Follow-up data of hrHPV positive women were retrieved from the PALGA database, physicians and gynaecologists. All cytological and histological findings recorded before January 2013 were included in our analysis to assure a minimal follow-up of one year for each woman.
Methylation marker analysis on cervical scrapes

For this study, left-over material of the HPV positive physician-collected liquid based cytology samples was used to isolate DNA as described before. Extracted DNA was subjected to sodium bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Orange, US) as described previously. Subsequently, bisulfite treated DNA was used for real-time, quantitative methylation-specific PCR (qMSP) for the CADM1 gene and the MAL gene on an ABI 7500 real-time PCR system (Applied Biosystems) with the bisulfite converted housekeeping gene beta-actin as internal reference. Primers and probes for qMSP have been described earlier. Cycle threshold (Ct) values were fixed at a given fluorescence threshold (i.e., 0.01). Ct ratios between Ct values of the target gene and housekeeping gene were calculated as described earlier. Samples that tested negative for both beta-actin and the target genes were scored as invalid.

Statistical analysis

Cytology was dichotomised into normal or abnormal (≥ASC-US). Samples were scored positive for methylation when either one or both markers had an outcome above predefined qMSP thresholds. These thresholds were set as Ct ratios that gave rise to CIN3+ specificity value of 70% of the bi-marker panel in an earlier study. Triage strategies were evaluated based on cytology, methylation marker analysis, and the combination of the two triage tests. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), together with 95% Wald confidence intervals (95% CIs), were calculated with regard to CIN2+ and CIN3+. Sensitivity and specificity of the triage strategies were compared with the McNemar test. Moreover, referral rates were measured for each triage strategy. The primary outcome measure of this study was histologically confirmed CIN2+ and CIN3+ measured at a minimum of 12 to a maximum of 24 months after intake. SPSS 20 software and STATA version 11 were used for statistical analysis.

RESULTS

For this study, 364 physician-taken cervical smears were used of women from the cytology triage group of the PROHTECT-3 study who tested HPV positive on the cervical smear and had both a valid cytology and methylation marker test result. The median age of these women was 42 years (interquartile range (IQR) 38-48 years). In total, six women were diagnosed with cervical cancer, 56 with CIN3, 28 with CIN2 and 274 had no evidence of CIN2+ within at least twelve months after inclusion. The latter group consisted of 64 women with CIN1, 77 without CIN in the biopsy, 40 women with a double negative exit-test (i.e., HPV negative and normal cytology), and 93 who did not reach a formal study endpoint (i.e., no histology result or no double negative exit-test). For the three different triage strategies, i.e. cytology testing, bi-marker CADM1/MAL methylation analysis, and combined cytology and bi-marker CADM1/MAL methylation analysis, the number of positive triage tests in HPV positive women and per study-endpoint are given in Table 1.
Table 1. Positivity of triage strategies per histological outcome in 364 women tested hrHPV positive on their cervical scrape

<table>
<thead>
<tr>
<th></th>
<th>no CIN2+ detected</th>
<th>CIN2</th>
<th>CIN3</th>
<th>Cancer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n= 274</td>
<td>n= 28</td>
<td>n= 56</td>
<td>n= 6</td>
<td>n= 364</td>
</tr>
<tr>
<td>Cytology</td>
<td>59</td>
<td>20</td>
<td>44</td>
<td>4</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>21.5%</td>
<td>71.4%</td>
<td>78.6%</td>
<td>66.7%</td>
<td>34.9%</td>
</tr>
<tr>
<td>CADM1/MAL methylation</td>
<td>76</td>
<td>11</td>
<td>37</td>
<td>6</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>27.7%</td>
<td>39.3%</td>
<td>66.1%</td>
<td>100.0%</td>
<td>35.7%</td>
</tr>
<tr>
<td>Cytology and/or CADM1/MAL methylation</td>
<td>118</td>
<td>22</td>
<td>49</td>
<td>6</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>43.1%</td>
<td>78.6%</td>
<td>87.5%</td>
<td>100.0%</td>
<td>53.6%</td>
</tr>
</tbody>
</table>

CIN = cervical intraepithelial neoplasia (grade 2 or grade 3 or higher)  
CI = confidence interval  

With cytology, 34.9% of all HPV positive women had an abnormal test result, whereas 35.7% tested positive for the bi-marker CADM1/MAL marker panel. As depicted in Figure 1, methylation positivity showed a steep increase with increasing severity of underlying disease from 39.3% in CIN2 to 66.1% in CIN3 and 100% in carcinomas. Conversely, cytology positivity in the various disease categories revealed a more flat curve with percentages of 71.4% in CIN2, 78.6% in CIN3, and 66.7% in cervical carcinomas.

![Figure 1: Bi-marker CADM1/MAL methylation analysis compared to cytology testing in cervical scrapes in relation to underlying disease](image-url)
Table 2. Performance of triage tests performed on the physician-taken scrape of 364 HPV positive women

<table>
<thead>
<tr>
<th></th>
<th>CIN3+</th>
<th></th>
<th></th>
<th></th>
<th>CIN2+</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>PPV</td>
<td>NPV</td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>PPV</td>
<td>NPV</td>
</tr>
<tr>
<td></td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>Cytology</td>
<td>77.4</td>
<td>73.8</td>
<td>37.8</td>
<td>94.1</td>
<td>75.6</td>
<td>78.5</td>
<td>53.5</td>
<td>90.7</td>
</tr>
<tr>
<td></td>
<td>(67.0-87.8)</td>
<td>(68.9-78.8)</td>
<td>(29.4-46.2)</td>
<td>(91.1-97.1)</td>
<td>(66.7-84.4)</td>
<td>(73.6-83.3)</td>
<td>(44.9-62.2)</td>
<td>(87.0-94.4)</td>
</tr>
<tr>
<td>CADM1/MAL methylation</td>
<td>69.4</td>
<td>71.2</td>
<td>33.1</td>
<td>91.9</td>
<td>60.0</td>
<td>72.3</td>
<td>41.5</td>
<td>84.6</td>
</tr>
<tr>
<td></td>
<td>(57.9-80.8)</td>
<td>(66.1-76.3)</td>
<td>(25.0-41.2)</td>
<td>(88.4-95.4)</td>
<td>(49.9-70.1)</td>
<td>(67.0-77.6)</td>
<td>(33.1-50.0)</td>
<td>(80.0-89.2)</td>
</tr>
<tr>
<td>Cytology and/or CADM1/MAL methylation</td>
<td>88.7</td>
<td>53.6</td>
<td>28.2</td>
<td>95.9</td>
<td>85.6</td>
<td>56.9</td>
<td>39.5</td>
<td>92.3</td>
</tr>
<tr>
<td></td>
<td>(80.8-96.6)</td>
<td>(48.0-59.3)</td>
<td>(21.9-34.5)</td>
<td>(92.9-98.9)</td>
<td>(78.3-92.8)</td>
<td>(51.1-62.8)</td>
<td>(32.6-46.3)</td>
<td>(88.3-96.3)</td>
</tr>
</tbody>
</table>

CIN = cervical intraepithelial neoplasia (grade 2 or grade 3 or higher)
CI = confidence interval
Relative to cytology, methylation marker testing resulted in the detection of 9 extra CIN2+ lesions, including 2 CIN2, 5 CIN3 and 2 carcinomas. Moreover, 17 women without a CIN2+ detected tested methylation marker positive but negative for cytology. With the combined strategy, 53.6% of women tested triage positive.

Performances of the triage strategies with regard to sensitivity, specificity, PPV and NPV for CIN3+ and CIN2+ are shown in Table 2. CIN3+ sensitivities of methylation marker testing (69.4%) and cytology (77.4%) were not significantly different (p= 0.36). CIN3+ specificity was also similar in both strategies (71.2% vs 73.8%, respectively) (p= 0.51). With combined cytology and methylation marker testing, a significantly higher sensitivity for CIN3+ was observed compared with cytology alone (i.e., 88.7% vs 77.4%, p= 0.016). The specificity for CIN3+ of this combined strategy was significantly lower than that of sole cytology (53.6% vs 73.8%, p< 0.001). Combined cytology with methylation marker testing revealed a PPV of 28.2% and NPV of 95.6% for CIN3+, with a colposcopy referral rate of 53.6%. Table 2 shows the performance of the various triage strategies with regard to CIN2+ detection. Also with regard to CIN2+, combined cytology and methylation testing showed a higher sensitivity (85.6% vs 75.6%, p= 0.004) with a lower specificity (56.9 vs 78.5%, p< 0.001) than cytology only.

DISCUSSION

This study showed that the bi-marker CADM1/MAL methylation test is complementary to cytology testing in detecting CIN2+/CIN3+ as triage tool on cervical scrapes of HPV positive women. This complementary effect could be attributed to the fact that cytology detects CIN2, CIN3, and cancer with similar sensitivity, while the frequency of methylation positive samples increases proportionally to severity of underlying lesions. Therefore, the combined strategy could provide a better re-assurance for absence of cervical cancer and its advanced precursors. As shown in Figure 1, our findings are in line with the recently suggested hypothesis that both tests do not detect exactly the same lesions; methylation marker testing has the preference for detecting the more advanced CIN3+ lesions, whereas cytology tends to also detect early CIN2/CIN3 lesions.\textsuperscript{19,24} The combination of cytology testing with methylation marker analysis resulted in a significantly higher sensitivity, at the cost of a lower, but acceptable specificity (i.e., 53.6%) than triage with cytology only.

Recently, de Strooper et al. showed that the bi-marker CADM1/MAL methylation panel tested positive in cervical smears of all 79 carcinomas investigated, indicating that bi-marker CADM1/MAL methylation analysis can be a safe triage test for excluding cancer in test negative women.\textsuperscript{25} Indeed, in the current study all carcinomas were detected at baseline by CADM1/MAL methylation testing, whereas two of six cervical cancers had a normal cytology result at baseline. In the PROHTECT-3 study these cancers were ultimately detected due to an abnormal smear at the exit-test. The seven extra CIN2/3 lesions detected by methylation analysis likely represent advanced CIN2+ lesions with a high short term risk for cervical cancer.\textsuperscript{19} Thus, by combining the bi-marker CADM1/MAL methylation panel with cytology a very sensitive baseline triage strategy for HPV
positive women is obtained, which might obviate the need for an extra follow-up visit at 6-12 months for women who are triage test negative at baseline. This approach prevents the risk of loss to follow-up, which is substantial (approximately 20%-40%) in case of repeat testing, as was shown in earlier studies.126-28

Of course, to further support the hypothesis that high-grade lesions testing negative for both cytology and bi-marker CADM1/MAL methylation analysis are early CIN2/CIN3 lesions with a relative low short-term progression risk to cervical cancer,19 additional proof is needed, for example by demonstrating no or limited chromosomal aberrations in these lesions.29,30

It should be noticed that our results were obtained in the Netherlands with a high quality standard of cytology. In countries without a well-organized cytology infrastructure, addition of molecular triage testing with methylation markers to cytology would probably lead to an even higher additive value in terms of re-assurance of not missing advanced lesions and cancers. Although we restricted the analysis of this study to cervical scrapings of women from the cytology triage group of the PROHTECT-3 study, HPV positive women were initially identified by a positive self-collected sample. At the end only eighty percent of all analyzed HPV self-sampling positive women tested also HPV positive on their cervical smear. Such a difference in HPV positivity between self-samples and physician-collected cervical scrapes was also found in earlier studies. This difference may, at least in part, reflect the presence of vaginal HPV infections in self-sampled material and the fact that HPV infections could have been cleared between the moment of self-sampling and subsequent physician-taken sampling.27,27

Strengths of our study are the large size, the design and the performance within a representative non-responder population in the setting of the regular cervical screening program. A limitation of this study is the relatively short duration of follow-up varying from 12 months to a maximum of 24 months per woman, while women in the current regular cervical program have an invitation interval of three to five years. However, the evaluated triage tests could still be used as baseline triage in women who tested HPV positive on their cervical smear, since we used a rather comprehensive exit test (HPV testing plus cytology testing) in our study, resulting in a low chance of missing CIN2+ lesions.

In conclusion, cytology and methylation marker testing had a complementary performance as triage test in HPV positive women with regard to CIN2+ and CIN3+ detection. With this combined strategy, higher sensitivities were reached at the cost of a lower specificity compared to cytology only as triage test on cervical smears of HPV positive women. This resulted in a feasible and safe baseline triage strategy with an acceptable referral rate in this group of women with a relatively high risk for CIN2+. 
REFERENCES


Cytology and methylation markers on cervical scrapes


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