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


ABSTRACT

**Background** Cytology is a widely used method of triaging women who test positive for human papillomavirus (HPV). However, self-sampled specimens, which can substantially increase participation in screening programmes, are not suitable for accurate cyto logical assessment. We investigated whether direct DNA methylation-based molecular triage on self-sampled cervicovaginal specimens was non-inferior to cytology triage on additional physician-collected cervical samples in the detection of cervical intraepithelial neoplasia grade 2 (CIN2) or worse in women who did not attend cervical screening programmes.

**Methods** In this randomised controlled non-inferiority trial, we invited women (aged 33–63 years) registered as non-attendees of cervical screening in the Netherlands in 2007 to submit a self-collected cervicovaginal sample for HPV testing. Using a computer-generated sequence, we randomly allocated women who tested positive for high-risk hrHPV on a self-sample to either triage by cytology on an additional physician-taken smear or direct triage on the self-sample by methylation analysis of MAL and miR-124-2 genes (1:1; stratified by age and region, with block sizes by age group). Triage-positive women in either group were referred for colposcopy. The primary endpoint was detection of CIN2 or worse, analysed by intention to treat. The non-inferiority margin was 0.80. This study is registered in the Primary Trial Register of the Netherlands, number NTR6026.

**Findings** We invited 46,001 women to participate, 12,819 of whom returned self-sampled material; 1,038 samples tested positive for high-risk HPV. Between Nov 1, 2010, and Dec 31, 2011, after exclusion of women who were ineligible, we enrolled and randomly allocated 515 women to methylation triage and 509 to cytology triage. The detection of CIN2 or worse with methylation triage was non-inferior to that with cytology triage (90 [17%] of 515 women vs 75 [15%] of 509 women; relative risk 1.19, 95% CI 0.90–1.57). Referral for colposcopy was more common in the molecular group (284 [55%] women) than in the cytology group (149 [29%] women; p<0.0001). Mean time to CIN2 or worse diagnosis was shorter in the molecular triage group (96 days, range 44–101) than in the cytology triage group (158 days, 71–222; p=0.00084).

**Interpretation** DNA methylation analysis of MAL and miR-124-2 genes on HPV-test-positive self-samples is non-inferior to cytology triage in the detection of CIN2 or worse, opening the way to full molecular screening.
INTRODUCTION

In high-income countries, population-wide, cytology-based cervical cancer screening has decreased cervical cancer incidence and mortality.\(^1\) Almost half of the cases of invasive cervical cancer in high-income countries are in the 30% of women who do not attend cervical screening (i.e., non-attendees).\(^1,\text{3}^\) Offering self-collection of cervico-vaginal material for high-risk HPV (hrHPV) testing in a laboratory (hereafter referred to as ‘HPV self-sampling’) has been shown to be effective in recruiting non-attendees into screening programmes,\(^4,\text{9}^\) with response rates varying from 8.7% up to 39.0%.\(^10\) As such, HPV self-sampling can substantially increase compliance to screening programmes.

Women testing positive for hrHPV on self-sampled specimens require additional triage testing because specificity of hrHPV testing is too low to justify direct referral for colposcopy for all screen-positive women.\(^11,\text{12}^\) Cytology is an accepted standard method of examination in triage for hrHPV-positive women.\(^13,\text{14}^\) However, because cytology is not reliable on self-sampled material, triage by cytology requires an extra visit to a physician for a cervical smear.\(^15,\text{16}^\) This visit is often unwelcome to the women, delays the diagnostic work-up, and leads to loss to follow-up.\(^4,\text{5}^\) These drawbacks could be circumvented by direct molecular triage on HPV-positive cervicovaginal self-samples, provided that molecular testing is at least as sensitive as cytology for the detection of cervical intraepithelial neoplasia grade 2 (CIN2) or worse.

DNA methylation analysis of the promoter region of tumour-suppressor genes involved in cervical carcinogenesis can provide effective molecular triage of hrHPV positive women.\(^17,\text{20}^\) Methylation marker analysis is accurate on self-sampled specimens.\(^21,\text{22}^\) Combined methylation marker analysis of two genes — MAL and miR-124-2 — on HPV-positive self-collected cervicovaginal lavage material could distinguish CIN2 or worse and CIN3 or worse with minimum sensitivities of 71.3% (for CIN2 or worse) and 77.0% (for CIN3 or worse), at specificity of 50%, thus exceeding the sensitivity of combined HPV16 and HPV18 genotyping.\(^22\) Application of this panel would therefore allow direct triage for colposcopy of women with an HPV-positive self-sample.

We did the PRotection by Offering HPV Testing on self-sampled Cervico-vaginal specimens Trial-3, (PROHTECT-3) to assess whether direct molecular triage with the bi-methylation marker panel MAL and miR-124-2 is non-inferior to indirect cytology triage on physician-collected cervical samples in the detection of CIN2 or worse in women with HPV-positive self-collected lavage specimens.
METHODS

Study design and participants
In the Dutch cervical screening programme, women aged 30-60 are invited every 5 years by one of five regional screening organisations for a cervical smear at the general practitioner’s office. Non-compliant invitees are registered as non-attendees in the databases of the screening organisations. For this randomised controlled trial, we invited 46,001 women (age 33-63 years) living in Noord-Holland, Flevoland, Utrecht and Gelderland, who were registered as non-attendees in 2007 to participate. We enrolled women between Nov 1, 2010, and Dec 31, 2011. Exclusion criteria were previous hysterectomy and a history of CIN2 or worse, or abnormal cytology in the preceding 2 years.

We sent non-attendees a pre-invitation letter allowing opt-out of this trial, and those who did not opt out within 3 weeks subsequently received a self-sampling lavage device (Delphi screener; Delphi Bioscience, Scherpenzeel, Netherlands), an explanation letter, an informed consent form, a pictorial and written instruction form, collection tube, seal bag, and free return envelope as described elsewhere. Women were asked to return the collection tube, containing their self-sampled material, together with a signed informed consent form to the laboratory for hrHPV testing. During the study period, a website, an email address, and telephone number were available for trial participants for additional information and questions about the study.

We did the trial at the VU University Medical Center (VUmc), Radboud University Nijmegen Medical Centre (RUNMC), and the Screening Organisations Mid-West (Midden-West) and East (Oost). We report our study results according to the 2010 CONSORT guidelines. This study was approved by a national review board (Ministry of Public Health No 2010/04WBO).

Randomisation and masking
LR randomly assigned women who tested hrHPV-positive on self-sampling to one of two triage groups in a 1:1 ratio using a computer-generated sequence: the molecular triage group, in which women received methylation-based triage testing directly on the self-sampled material, and the cytology triage group, in which women were advised to visit their physician for a cervical scrape. We stratified randomisation for age (seven categories: 30–34 years, 35–39 years, 40–44 years, 45–49 years, 50–54 years, 55–59 years, and 60–64 years) and region (two categories: Midden-West and Oost). Cytotechnicians were not informed about the HPV status of the women, whereas physicians and gynaecologists were informed about the HPV and triage test results.

Procedures
Participating women sent their self-collected cervicovaginal lavage material in their supplied 25 mL vial to the laboratory. We did hrHPV GP5+/6+-PCR testing using the Diassay EIA HPV GP HR kit (Diassay, Voorburg, Netherlands) as described previously. To find out whether sufficient material had been sampled we first did a visual inspection of the sample vial after centrifugation to check for the presence of a cell pellet. If no cell pellet was visible we regarded a sample as having possible poor material yield and subsequently did a β-globin PCR for assessing DNA presence.
Of the remaining samples, every ninth sample was selected for β-globin PCR. In case of a negative β-globin PCR, samples were considered invalid and a new self-sampling kit was sent. If women did not resubmit another sample, the overall result was classified as not determined. All women who submitted self-sampled material and their physicians (if known and consented by the women) received a letter with the laboratory test results and follow-up advice. We asked women who tested hrHPV-negative to participate in the next round of regular screening.

Women with a positive triage test (i.e., positive methylation test or abnormal cytology) were referred for a colposcopy-directed biopsy by the investigators of the VUmc, whereas women with a negative triage test (i.e., negative methylation test or normal cytology) were re-invited after 6 months for an exit test. This exit test consisted of cytology and hrHPV co-testing, and if at least one of these tests was abnormal (i.e., abnormal cytology or hrHPV-positive), women were referred for colposcopy-directed biopsy. In case of a double-negative exit test (i.e., normal cytology and hrHPV negative), women were referred back to regular screening. To motivate women to comply with the study, we sent reminders 16 weeks after HPV self-sampling and contacted their physicians.

Assessments

We did liquid-based cytology on cervical scrapes taken with a Cervex Brush (Rovers Medical Devices BV, Oss, Netherlands). The brush was collected in a vial with PreservCyt medium, which was sent to the Department of Pathology of the VUmc for making thin layer preparations. Cytology preparations were read according to the CISOE-A classification, which can be translated into the Bethesda system. We grouped cytological results as normal, borderline or mild dyskaryosis, or moderate dyskaryosis or worse, as described elsewhere. We did DNA methylation marker analysis for MAL and miR-124-2 genes. The clinical performance of this MAL/miR-124-2 panel has been described previously in a training set of 355 hrHPV-positive self-sampled cervicovaginal specimens. The investigators in that study noted that between three different methylation marker panels, the MAL/miR-124-2 panel was most robust in detecting CIN3 or worse. Here, we did the methylation assay with a preplanned protocol as a prospective component of the study, as previously described. Briefly, we treated extracted DNA from the self-sample with sodium bisulphite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). Bisulphite-treated DNA was subsequently used for real-time, quantitative methylation-specific PCR for the MAL gene, using the MAL-M1 marker, and the miR-124-2 gene, using the miR-124-2 marker, on an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Primers and probes for quantitative methylation-specific PCR are described elsewhere. As a reference, we did PCR for the bisulphite-converted housekeeping gene β-actin. Cycle threshold values were measured at a fixed fluorescence threshold (i.e., 0.01). Cycle threshold ratios between cycle threshold values of the housekeeping gene and the target gene were calculated as previously described. When β-actin-negative samples were also negative for their target genes, we scored them as invalid. The thresholds used for scoring samples as methylation positive—the threshold for colposcopy referral—were a cycle threshold ratio 0.33 for MAL-M1 marker and 0.064 for miR-124-2. Women were referred when either of these markers had a ratio value above the threshold. Findings from a previous study using these thresholds showed the highest sensitivity for CIN3 or worse at a predefined specificity of 50%.
Outcomes

Women reached the study endpoint if they had a double negative exit test or a histological outcome. Histology was assessed in different pathology laboratories in the Netherlands according to recommended guidelines and recorded in the nationwide network and registry of histopathology and cytopathology in the Netherlands (PALGA, Houten, The Netherlands). The primary outcome measure was detection of CIN2 or worse. Other predefined outcome measures were detection of CIN3 or worse, colposcopy referral, and compliance to follow-up. We also did a post-hoc analysis of time to histological diagnosis of CIN2 or worse. We counted all women who submitted self-sampled specimens between Nov 1, 2010, and Dec 31, 2011, as participants in this study. We retrieved follow-up outcomes for all participants from the PALGA database and, if necessary, from their physicians and gynaecologists. All cytological and histological findings were included in our analysis when recorded before January, 2013, to ensure a follow-up of at least 12 months for each woman.

Statistical analysis

Analyses were done by intention to treat, unless otherwise specified. We calculated risk ratios for the detection of CIN2 or worse and CIN3 or worse in the molecular and cytology triage group with 95% Cornfield confidence intervals and sensitivity and positive predictive value (PPV) of both triage strategies with 95% Wald CIs, reporting both unadjusted and adjusted sensitivities. We corrected the adjusted sensitivities for the number of women who did not comply with the triage test or the exit test using formulae by Begg and Greenes. We analysed differences in time to diagnosis and in mean age with the unpaired t-test. We did a logistic regression analysis to estimate the effects of age on hrHPV positivity. We tested the effect of age cohort on detection of CIN2 or worse and CIN3 or worse with a Breslow-Day homogeneity test. We assessed the sample size using calculations for non-inferiority trials. More precisely, a study of 45 000 women has 80% power to show non-inferiority for detection of CIN2 or worse at a non-inferiority threshold of 80% if the participation is set at 30%, the hrHPV positivity is set at 10%, and the significance level is set at 5%. We used SPSS (version 20) and STATA (version 11) for statistical analyses. This study is registered in the Primary Trial Register of the Netherlands, number NTR6026.

Role of the funding source

The sponsors had no role in data collection, data analysis, data interpretation, study design, or writing the paper. CJLMM and VMJV had full access to all data. CJLMM had the final responsibility for the decision to submit for publication.
RESULTS

We invited 46,001 women to participate, 12,819 of whom returned their self-sampled material and a signed informed consent form. Self-samples from 1,038 women tested hrHPV-positive—after exclusion of women who were ineligible for inclusion, we randomly allocated 515 women to methylation triage and 509 to cytology triage (Figure, Table 1). Women who opted out were, on average, older than those who did not (mean age 48.7 years vs 44.9 years; p<0.0001). Of those who did not opt out, women who responded were, on average, younger than those who did not (mean age 44.7 years vs 45.0 years; p=0.00043). The proportion of hrHPV-positive women decreased with age at a 5-year odds ratio of 0.87 (95% CI 0.83–0.90; p<0.0001).

Referral to colposcopy was 1.9 times higher in the methylation triage group than in the cytology triage group (p<0.0001; Figure). 465 (91%) of the 509 women in the cytology triage group attended triage testing within 16 weeks of self-sampling and 503 (99%) attended by the end of the study. The study endpoint (i.e., a double-negative exit test or a histological outcome) was reached in 408 women (79%) in the methylation group and 368 (72%) women in the cytology group (p=0.0097). For those who had a positive screening result, 253 (89%) of 284 women in the molecular triage group, and 137 (92%) of 149 women in the cytology triage group attended the follow-up appointment.

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics</th>
<th>Cytology triage (n=509)</th>
<th>Methylation triage (n=515)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>42.4 (33-63)</td>
<td>42.6 (33-63)</td>
</tr>
<tr>
<td>30-34 years</td>
<td>128 (25%)</td>
<td>126 (24%)</td>
</tr>
<tr>
<td>35-39 years</td>
<td>128 (25%)</td>
<td>129 (25%)</td>
</tr>
<tr>
<td>40-44 years</td>
<td>94 (18%)</td>
<td>94 (18%)</td>
</tr>
<tr>
<td>45-49 years</td>
<td>63 (12%)</td>
<td>63 (12%)</td>
</tr>
<tr>
<td>50-54 years</td>
<td>51 (10%)</td>
<td>50 (10%)</td>
</tr>
<tr>
<td>55-59 years</td>
<td>27 (5%)</td>
<td>37 (7%)</td>
</tr>
<tr>
<td>60-64 years</td>
<td>18 (4%)</td>
<td>16 (3%)</td>
</tr>
<tr>
<td>Region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid-west</td>
<td>411 (81%)</td>
<td>414 (80%)</td>
</tr>
<tr>
<td>East</td>
<td>98 (19%)</td>
<td>101 (20%)</td>
</tr>
<tr>
<td>Mean time to previous cytology smear, years (IQR)</td>
<td>6.1 (2.9-8.6)</td>
<td>6.0 (2.8-8.4)</td>
</tr>
</tbody>
</table>

Data are n (%) unless otherwise specified.
Figure 1. Trial profile
Of 284 (55%) women who tested positive in the methylation triage group (Table 2), five (2%) were diagnosed with squamous cell carcinoma, one (0.4%) with adenocarcinoma, 49 (17%) with CIN3, and 35 (12%) with CIN2. 50 (18%) women had CIN1 lesions and 96 (34%) had no CIN—48 (17%) women did not comply with follow-up advice, of whom 17 had both an hrHPV-negative test and normal smear in follow-up and 31 were lost to follow-up (i.e., had no study endpoint). Of 149 (29%) women in the cytology group with abnormal cytology (Table 2), five (3%) were diagnosed with squamous cell carcinoma, 48 (32%) with CIN3 lesions, and 22 (15%) with CIN2. Moreover, 27 (18%) women had a CIN1 lesion and 32 (21%) were diagnosed with no CIN. 15 (10%) cytology-positive women did not comply with colposcopy advice; three had both normal cytology and a negative hrHPV test in follow-up, and 12 were lost to follow-up. CIN2 or worse detection with methylation triage was non-inferior to cytology triage: the relative risk (RR) of detection of CIN2 or worse with methylation triage versus cytology triage was 1.19 (95% CI 0.90–1.57), with the lower bound of the CI above the hypothesised non-inferiority margin of 0.80. CIN3 or worse detection was similar between groups (RR 1.03; 0.72–1.47), although the CIs were wider than with CIN2 or worse. We detected no significant effect of age cohort on the detection of CIN2 or worse (p=0.13), or CIN3 or worse (p=0.54) lesions.

The mean time from arrival of self-sampled specimen in the laboratory to CIN2 or worse diagnosis was longer in women with an abnormal cytology test (158 days, range 71–222) than in women with a positive methylation test (96 days, 44–101; p=0.00084).

Sensitivity for the detection of CIN2 or worse and CIN3 or worse, adjusted for loss to follow-up, was similar in both groups (Table 3). PPV was higher in the methylation group than in the cytology group for both CIN2 or worse (p<0.0001) and CIN3 or worse (p=0.00021; Table 3). More women in the methylation group were diagnosed with CIN1 than in the cytology group (50 with methylation vs 27 with cytology; RR 1.83, 95% CI 1.16–2.88).

In the methylation triage group, 230 (45%) women with a negative triage test were advised to visit their physician for an exit test. At this exit assessment, 24 (10%) had CIN3, ten (4%) had CIN2, 29 (13%) had CIN1, and 37 (16%) had no CIN; 55 (24%) women had both normal cytology and an hrHPV-negative result and 75 (33%) women did not reach a study endpoint. In the cytology triage group, 351 (69%) women were advised to undergo an exit test after a negative triage test. At this exit assessment, two (1%) had squamous cell carcinoma, 13 (4%) had CIN3, 11 (3%) had CIN2, 47 (13%) had CIN1, and 67 (19%) had no CIN; 91 (26%) women had both normal cytology and an hrHPV-negative result and 120 women did not reach a study endpoint.

The cumulative RR for lesion detection in women in the methylation versus the cytology group was 1.21 (95% CI 0.96–1.53) for CIN2 or worse and 1.15 (0.85–1.55) for CIN3 or worse. The cumulative RR for CIN1 detection was much the same in both groups (79 with methylation vs 74 with cytology; RR 1.06, 0.79–1.41).
<table>
<thead>
<tr>
<th>Strategy</th>
<th>Relative detection</th>
<th>CIN2 or worse</th>
<th>CIN3 or worse</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology triage group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=509)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>-</td>
<td>32 (6%)</td>
<td>27 (5%)</td>
<td>75 (15%)</td>
</tr>
<tr>
<td>- Cytology + hrHPV</td>
<td></td>
<td>67 (13%)</td>
<td>47 (9%)</td>
<td>26 (5%)</td>
</tr>
<tr>
<td>- Cytology + hrHPV</td>
<td></td>
<td>99 (19%)</td>
<td>74 (15%)</td>
<td>101 (20%)</td>
</tr>
<tr>
<td>Methylation triage group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=515)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylation</td>
<td>-</td>
<td>96 (19%)</td>
<td>50 (10%)</td>
<td>90 (17%)</td>
</tr>
<tr>
<td>- Cytology + hrHPV</td>
<td></td>
<td>37 (7%)</td>
<td>29 (6%)</td>
<td>34 (7%)</td>
</tr>
<tr>
<td>- Methylation + hrHPV</td>
<td></td>
<td>133 (26%)</td>
<td>79 (15%)</td>
<td>124 (24%)</td>
</tr>
</tbody>
</table>

Data are n or n(%)
CIN = cervical intraepithelial neoplasia (numbers indicate grade)
hrHPV = high-risk human papillomavirus

| Table 3. Sensitivity and positive predictive value of cytology triage and methylation triage |
|-----------------------------------------------|---------------------------------|-------------------------|-------------------------|-------------|
|                                              | Sensitivity                     | Positive predictive value |                         |
|                                              | Unadjusted*                    | Adjusted*               |                         |
|                                              | n/N % (95% CI)                 | n/N % (95% CI)          |                         |
| CIN2 or worse                                |                                 |                         |                         |
| Cytology triage                              | 75/101 74.3% (65.7-82.8)       | 75/149 50.3% (42.3-58.4) |                         |
| Methylation triage                           | 90/124 72.6% (64.7-80.4)       | 90/284 31.7% (26.3-37.1) |                         |
| CIN3 or worse                                |                                 |                         |                         |
| Cytology triage                              | 53/68 77.9% (68.1-87.8)        | 53/149 35.6% (27.9-43.3) |                         |
| Methylation triage                           | 55/79 69.6% (59.5-79.8)        | 55/284 19.4% (14.8-24.0) |                         |

CIN = cervical intraepithelial neoplasia (numbers indicate grade)
*Adjusted for the number of women who did not comply with the triage or exit test
DISCUSSION

Our findings show that direct triage by combined promoter methylation analysis of MAL and miR-124-2 genes on hrHPV-positive self-samples of non-attendees of organised cervical screening is feasible. Direct molecular triage on self-samples has similar sensitivity for CIN2 or worse as indirect cytology triage on a physician-taken smear. Direct molecular triage detects CIN2 or worse lesions earlier than cytology triage, is easier for patients because they do not have to visit a physician, and is logistically simpler because no reminder letters have to be sent. 509 visits to the physician were avoided by methylation marker testing at the cost of 135 extra colposcopy referrals. Of the extra referrals, 15 led to a diagnosis of CIN2 or worse, meaning that 120 of the 135 extra colposcopy referrals could be considered as unnecessary. Thus, a drawback of the methylation marker test in this trial is its lower PPV compared with cytology triage. Nonetheless, the PPV of methylation triage (19.4% for CIN3 or worse) is still higher than the risk threshold for CIN3 or worse of 10% as has been previously proposed to identify women in need of direct colposcopic assessment. A related issue is whether the capacity of gynaecology departments is sufficient to meet the increase in colposcopy referrals in the Netherlands. Our study population of non-attendees comprises about a fifth of the total Dutch population of non-responders. In view of the fact that the number of active gynaecologists in the country is about 1,000, this direct triage strategy would result in no more than one extra woman for colposcopy on average per gynaecologist per year.

We did our study in the Netherlands, where the standard of cytology screening is high. Because substantially more women were referred to colposcopy with molecular triage compared with cytology triage, while detection of CIN2 or worse and CIN3 or worse were much the same with both methods, cytology triage is still an attractive triage test in countries with a good quality of cytology screening. In countries without a quality-assured cytology infrastructure, methylation marker testing might be an alternative triage method for women testing hrHPV-positive on self-samples. Moreover, large variability in cytology results has been seen in some high-income and medium-income countries, and an objective methylation marker analysis could lead to more homogeneous results.

Although findings from our previous study showed that the marker panel in our study performed well as a triage test for hrHPV-positive women, we realise that it has not been assessed by other groups. Additionally, methylation markers discovered by other groups, which include both DNA methylation of host-cell genes and HPV DNA methylation, have not been tested on self-sampled specimens in a screening setting. Nevertheless, with the aid of bisulphite treatment and the sample control (i.e., β-actin housekeeping gene) to establish proper quantity, the methylation markers tested in this study have previously shown good reproducibility on clinical samples.

In our study, most hrHPV-positive women complied with the advice to visit a gynaecologist after an abnormal triage test. Also, in the cytology triage group, 99% of the hrHPV-positive women had a cytological triage test taken, indicating that a positive hrHPV test encourages former non-attendees to visit a physician. A plausible explanation for this finding is that an hrHPV-positive test
changes the perception of the risk of cervical disease and motivates women to visit the physician. In the Netherlands, the number of general-practice physicians is high—in countries with fewer general-practice physicians, direct triage with methylation testing seems the most obvious approach to ensure good compliance.

Moreover, the 99% compliance to cytology triage is very high for former non-attendees. In our study, the compliance increased from 91% to 99% by sending a reminder letter to women and their physicians. However, in a routine setting, this compliance could be somewhat lower as already seen in two earlier studies in non-responders to cervical screening in the Netherlands.4,5

Strengths of our study are its large size, the randomised design, the setting within a representative non-responder population in a country with organised screening, and the availability of a nationwide registry for cytology and histological diagnoses. Local histological diagnoses were done in line with clinical practice in the Netherlands, but some variation of histological classification in different participating laboratories cannot be ruled out. However, concordance of original CIN3 or worse diagnoses in an earlier trial has been shown to be 97%,35 suggesting low inter-laboratory variation.

A limitation of our study is that we did not further characterise the detected CINs. Although a similar number of CIN2 or worse was detected with both triage tests, further research is needed to assess whether the CIN2 or worse lesions in the methylation group are similar to those in the cytology group. In an earlier study,36 we showed that promoter methylation levels proportionally increase with the duration and degree of CIN lesions, and are very high in samples of women with cervical cancer. Blinded methylation testing of self-samples of the two women whose carcinomas were missed by cytology at baseline showed a positive test result (data not shown). Moreover, no carcinomas were missed by methylation marker analysis in the molecular triage group. If substantiated in other studies, this finding would greatly underscore the value of molecular triage over cytology triage. Furthermore, methylation marker analysis can also be used on cervical smears and therefore as a triage test for women with an HPV-positive cervical smear.17

Our findings suggest that methylation marker analysis is an objective method for direct molecular triage of HPV-positive self-collected cervicovaginal specimens. This molecular approach obviates the need for a visit to a physician and reduces time to CIN2 or worse diagnosis - but at the cost of more colposcopy referrals. HPV self-sampling combined with molecular triage opens the possibility of full molecular cervical cancer screening minimising the need for visiting a physician.
REFERENCES


