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Combined *CADMI/MAL* methylation and cytology testing for colposcopy triage of high-risk HPV-positive women

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Running title

Colposcopy triage by cytology and *CADMI/MAL* methylation

Keywords

HPV testing, colposcopy triage, pap smear, DNA methylation, biomarkers

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ABSTRACT

Primary screening for high-risk human papillomavirus (hrHPV) requires a triage protocol. Repeat cytology testing at baseline and after 6-12 months has emerged as a reasonable triage approach, but carries the risk of loss to follow-up. Repeat cytology testing may be omitted if cytology is supplemented with another, complementary triage test at baseline. In this study, the performance of combined triage by cytology and DNA methylation analysis was assessed. In hrHPV-positive cervical scrapes (n=250), cytology (threshold: atypical squamous cells of undetermined significance (ASCUS)), bi-marker *CADMI/MAL* methylation testing (at different assay thresholds) and combinations of both, were evaluated for endpoints cervical intraepithelial neoplasia grade 2 or worse (CIN2+) and grade 3 or worse (CIN3+). At a predefined methylation threshold of 70% specificity for CIN3+, combined triage revealed a CIN3+ sensitivity of 86.8% (95% CI: 76.1-97.6) compared to 65.8% (95% CI: 50.7-80.9) for sole cytology triage testing. Corresponding CIN3+ specificity was 64.8% (95% CI: 58.1-71.5) for combined triage, and 78.6% (95% CI: 72.8-84.3) for sole cytology triage testing. For CIN2+, the sensitivity of combined triage testing was 84.5% (95% CI: 75.2-93.8) versus 65.5% (95% CI: 53.3-77.7) for sole cytology triage, with corresponding specificities of 69.9% (95% CI: 63.1-76.6) and 83.5% (95% CI: 78.0-89.0), respectively. In conclusion, combined triage reached substantially higher CIN2+/3+ sensitivities compared to sole cytology at a slight drop in specificity. Therefore, it is an attractive triage strategy for colposcopy of hrHPV-positive women with a high reassurance for cervical cancer and advanced CIN lesions.

INTRODUCTION

Due to its high sensitivity for high-grade cervical disease (i.e., cervical intraepithelial neoplasia grade 2/3, CIN2/3) and cervical cancer, testing for high-risk human papillomavirus (hrHPV) DNA is likely to become the primary method for cervical cancer screening in the near future (1–4). However, the main drawback of the hrHPV test is its lower specificity for CIN2/3 or worse (CIN2/3+) than cytology (5). To compensate for this limitation, different triage algorithms have been suggested aiming to reduce the number of hrHPV-positive women to be referred for colposcopy, thereby limiting over diagnosis and overtreatment. Cytology testing is nowadays considered a logical and feasible triage method. However, cytology testing at baseline alone has insufficient negative predictive value for CIN2/3+, and thus hrHPV-positive women with a negative baseline cytology test cannot be dismissed from further follow-up. Repeat cytology testing at baseline and after 6-12 months has emerged as an effective triage approach (6,7), but carries the risk of loss to follow-up. Repeat cytology testing may be omitted if cytology is supplemented with another, complementary triage test at baseline. One such test, i.e., HPV16/18 genotyping, seems to be useful in certain settings (6,7).

Measurement of DNA methylation of promoter regions of host cell tumor suppressor genes has shown promise as molecular triage test (8–10). In our previous study, combined analysis of *CADMI* (cell adhesion molecule 1) and *MAL* (T-lymphocyte maturation-associated protein) gene promoter methylation by quantitative methylation-specific PCR (qMSP) in hrHPV-positive cervical scrapes, revealed an equal sensitivity for CIN3+ as cytology at the same specificity (11). Levels of *CADMI* and *MAL* methylation in hrHPV-positive cervical scrapes increase proportionally to the degree and the duration of underlying high-grade cervical disease and are particularly high in scrapes of women with advanced CIN3 and cervical cancer (12). Previous data (11,13) suggest that DNA methylation analysis as triage test in hrHPV-positive women tends to be relatively more sensitive for CIN3 lesions and cervical cancer, whereas cytology is relatively more sensitive for CIN2 lesions. As such, DNA methylation analysis might be an interesting supplementary triage test to detect clinically relevant cervical lesions missed by cytology. In this study, we explored the performance of combined cytology and bi-marker *CADMI/MAL* methylation analysis at baseline compared to sole cytology testing at baseline, and assessed its potential as alternative triage strategy for hrHPV-positive women in cervical cancer screening.

MATERIALS AND METHODS

Cervical scrapes of hrHPV-positive women

Cytology and methylation data for *CADMI* and *MAL* genes of a consecutive series of 250 hrHPV GP5+/6+-PCR-positive cervical scrapes were used (11). The scrapes were from women who participated in population-based screening using the same screening and referral algorithm as in the intervention arm of the POBASCAM trial (14). In short, co-testing for hrHPV and cytology on the cervical scrapes at baseline was performed. Cytology was assessed according to the CISOE-A classification that can be easily converted into either the British or the 2001 Bethesda system (15). Borderline or mild dyskaryosis (BMD) corresponds to atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells that cannot exclude high-grade squamous intraepithelial lesions (ASC-H), or low-grade squamous intraepithelial lesions (LSIL). Moderate or worse dyskaryosis (>BMD) corresponds to high-grade squamous intraepithelial lesions (HSIL). All women with >BMD cytology were directly referred for colposcopy; hrHPV-positive women with BMD cytology were advised to repeat cytology and hrHPV testing at 6 and 18 months. These women were referred for colposcopy at 6 months, if they had >BMD cytology, or BMD cytology in

combination with a positive hrHPV test result. The women were referred at 18 months if they had >BMD cytology and/or an hrHPV-positive test result. Women with a positive hrHPV test result and normal cytology at baseline were advised to repeat cytology and hrHPV testing at 6 and 18 months. They were referred at 6 months if they had >BMD cytology, and were referred at 18 months if they had >BMD cytology and/or a positive hrHPV test result.

Methylation data for *CADMI* and *MAL* genes were generated by qMSP analysis as described before (11). In short, left-over DNA that was used for HPV testing was treated first with sodium bisulphite, and resulting converted DNA was subjected to qMSP for *CADMI/MAL* and *ACTB* as reference gene. Ct ratios were calculated (using the formula $2^{[Ct(ACTB) - Ct(target)]} \times 100$) to quantify the methylation level of the target genes. Samples with a Ct >40 were considered negative for methylation of the respective target gene, and samples with a Ct value of the *ACTB* >32 were considered invalid and excluded from analysis because of an indication of poor DNA quality or recovery after bisulphite treatment.

A total of 234 hrHPV-positive scrapes had both valid cytology and qMSP results of the bi-marker panel *CADMI/MAL*. These included 38 scrapes of women with a CIN3+ lesion (i.e., 34 CIN3, 1 adenocarcinoma in situ (ACIS), and 3 squamous cell carcinomas (SCCs)) with a median age of 34.5 years (range 25-61), and 20 women with a CIN2 lesion (median age of 33.5 years; range 24-49). Histology was assessed on colposcopy-guided biopsies that were taken according to standard procedures in the Netherlands (www.oncoline.nl) within 36 months of follow-up. The remaining 176 women had no evidence of CIN2+, further referred to as ≤CIN1, within the same follow-up time (including 15 CIN1 and 10 histologically-confirmed absence of CIN). The median age of this group was 40 years (range 19-62).

Data and statistical analysis

Specimens were recorded as positive for methylation when either or both markers had a qMSP outcome above a predefined threshold. Thresholds have previously been set as Ct ratios giving rise to CIN3+ specificity values of ≥20%, ≥30%, ≥40%, ≥50%, ≥60%, ≥70%, and ≥80% (11). Receiver operating characteristic (ROC) curves (for endpoints CIN2+ and CIN3+) were constructed for the methylation marker panel combined with cytology (i.e., recording positive if either methylation or cytology testing or both were above their threshold). The threshold used for cytology positivity was ASCUS (i.e., BMD) (15). The ROC curve was compared with the CIN2+ and CIN3+ sensitivities and specificities of cytology. The positive predictive values (PPVs), negative predictive values (NPVs) and their 95% Wald confidence intervals (95% CIs) were calculated for endpoints CIN2+ and CIN3+, and referral rates for colposcopy (with 95% CIs) were determined by dividing the number of women with a positive triage test result by the number of hrHPV-positive women. For the latter analyses, the threshold that was used to score the bi-marker *CADMI/MAL* methylation assay positive comprised the validated Ct ratios that corresponded to a CIN3+ specificity of ≥70% (11). All calculations were performed in Microsoft Excel (2010) and SPSS (version 20).

RESULTS

We determined the performance of combined triage by the bi-marker *CADMI/MAL* methylation assay (using validated thresholds (11)) and cytology (threshold of ASCUS (i.e., BMD)) in hrHPV-positive cervical scrapes for detection of CIN2+ and CIN3+, respectively. ROC curve analysis revealed that, relative to sole cytology and sole methylation testing, combined testing for both parameters yielded higher sensitivities for CIN2+ at specificities that were similar to those of sole methylation testing (Figure 1A). Similar findings were evident for CIN3+, although difference of ROC curves between the combined triage and

methylation analysis alone was less pronounced (Figure 1B). At the threshold for the bi-marker *CADMI/MAL* methylation assay that corresponded with $\geq 70\%$ CIN3+ specificity in our previous study (11), combined cytology and methylation analysis revealed a CIN3+ sensitivity of 86.8% (95% CI: 76.1-97.6) compared to 65.8% (95% CI: 50.7-80.9) for sole cytology triage testing. Corresponding CIN3+ specificity was 64.8% (95% CI: 58.1-71.5) for combined triage, and 78.6% (95% CI: 72.8-84.3) for sole cytology triage test (Table 1). For CIN2+, the sensitivity of combined cytology and methylation marker triage testing was 84.5% (95% CI: 75.2-93.8) vs. 65.5% (95% CI: 53.3-77.7) for sole cytology triage, with corresponding specificities of 69.9% (95% CI: 63.1-76.6) and 83.5% (95% CI: 78.0-89.0), respectively. The PPV for CIN2+ was 48.0% (95% CI: 38.3-57.7) for combined triage and 56.7% (95% CI: 44.9-68.6) for sole cytology. For CIN3+ outcome, these figures were 32.4% (95% CI: 23.3-41.4) and 37.3% (95% CI: 25.7-48.9; Table 1), respectively. The NPV for CIN2+ was 93.2% (95% CI: 88.9-97.5) for combined triage and 88.0% (95% CI: 83.1-92.9) for sole cytology. For CIN3+ outcome, these figures were 96.2% (95% CI: 93.0-99.5) and 92.2% (95% CI: 88.2-96.3; Table 1), respectively. The referral rate for CIN2+ in case of combined triage was 43.6% (95% CI: 37.4-50.0), compared to 28.6% (95% CI: 23.3-34.7) for sole cytology testing (Table 1).

DISCUSSION

We explored the value of triage testing of hrHPV-positive women by combined cytology and bi-marker *CADMI/MAL* methylation analysis in a population-based cervical screening population, and demonstrated a complementary effect of these triage tools. Combined triage reached substantially higher CIN2+/3+ sensitivities compared to sole cytology at a slight drop in specificity. Therefore, it is an attractive candidate triage tool for hrHPV-positive women.

Since increased methylation of *CADMI* and *MAL* was found to parallel increasing severity and duration of cervical disease (12), it is likely that additive methylation analysis particularly provides an extra safety net in terms of not missing women with advanced CIN disease or cervical cancer (10). Studies indeed have shown that methylation assays applied to cervical scrapes or self-collected specimens detected all cervical carcinomas (11,16,17). In addition, a higher detection rate of CIN3 among women of older age, which may reflect a higher duration of lesion existence, has been reported for methylation analysis (16,17). The preference of methylation analysis for more advanced lesions is further supported by the fact that, relative to sole cytology, the gain in sensitivity of the combined analysis is higher for CIN3+ than for CIN2+ (Table 1). Conversely, relative to methylation testing solely, combined triage testing tends to a higher increase in sensitivity for CIN2+ and less for CIN3+ at a similar specificity (Figures 1A and 1B). This indicates that, in addition to overlap, both assays in part detect different lesions, with cytology having a better sensitivity for CIN2 lesions and methylation analysis for CIN3+ lesions. As such, combined cytology and methylation marker testing by the *CADMI/MAL* panel is an attractive triage strategy for colposcopy of hrHPV-positive women, with a combined negative test providing a high reassurance for absence of cervical cancer and advanced CIN lesions. On the other hand, still a small number of CIN2+/3+ lesions is not detected by combined cytology and methylation triage of hrHPV-positive women. Possible reasons for non-detection of these cases by both cytology and methylation analysis could be incorrect sampling of the cervical scrapes, or the presence of early onset CIN2/3 lesions with a low cancer progression risk that might have limited numbers of abnormal cells and/or low methylation levels in their corresponding scrape. The addition of other potential molecular markers, or identification of even better differentially methylated genes, to be identified by genome-wide methods, might improve the diagnostic accuracy of molecular triage testing in the future. Furthermore, it should be noted

that the complementary effect of methylation marker analysis to cytology was observed in a setting where quality of cytology is high (18). Accordingly, molecular triage by methylation markers might even be more advantageous in settings with less adequate cytology infrastructure, or may even be a promising alternative to cytology (10). Methylation analysis can be performed on the same DNA isolate as used for HPV DNA testing, and has an objective read-out. Multiplex PCR technologies furthermore allow analyzing multiple methylation targets and an internal control in a multiplex reaction using a single aliquot of DNA, thereby saving material, time and costs, and improving throughput and quality control (19). A prototype version of a commercial, standardized multiplex qMSP test including *CADMI* and *MAL* targets, has recently been developed (PreCursor-M assay, Self-screen B.V, Amsterdam, The Netherlands).

In conclusion, combined cytology and methylation marker testing by the *CADMI/MAL* panel, is an attractive triage strategy for colposcopy of hrHPV-positive women with a high reassurance for cervical cancer and advanced CIN lesions.

Conflict of interest

C.J.L.M. Meijer, P.J.F. Snijders, R.D.M. Steenbergen and D.A.M. Heideman are shareholders of Self-Screen BV, a spin-off company of VU University Medical Center. All other authors declare that they have no conflicts of interest.

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TABLE

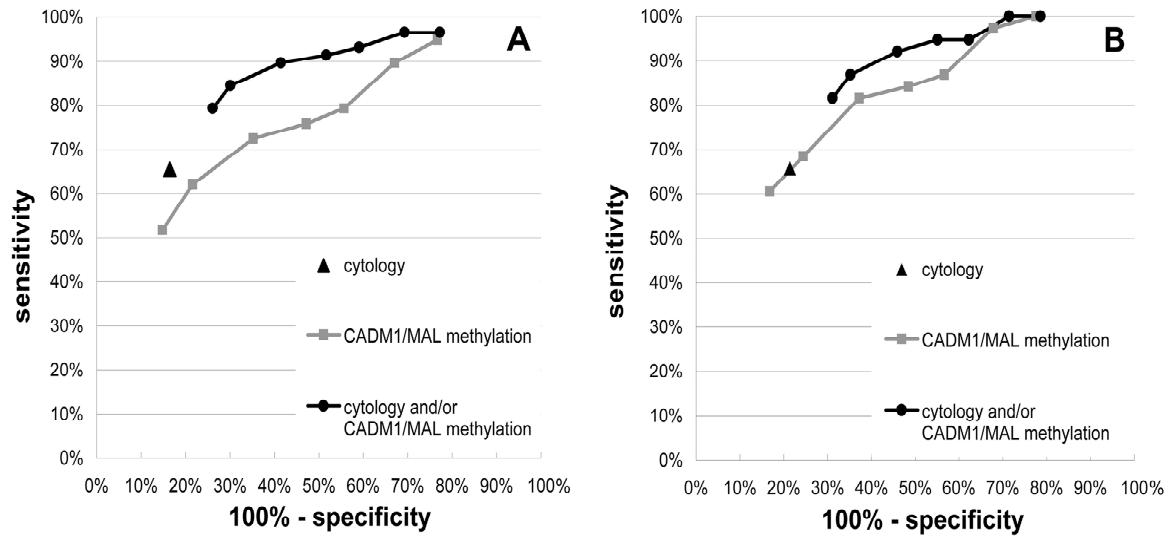
Table 1. Sensitivity, specificity, PPV and NPV for endpoints CIN2+ and CIN3+, and referral rates for colposcopy for different triage strategies

Triage marker	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	Referral Rate (%) (95% CI)
CIN2+ (A)					
cytology	65.5 (53.3-77.7)	83.5 (78.0-89.0)	56.7 (44.9-68.6)	88.0 (83.1-92.9)	28.6 (23.3-34.7)
<i>CADMI/MAL</i> (spec \geq 70%)	62.1 (49.6-74.6)	78.4 (72.3-84.5)	48.6 (37.3-60.0)	86.3 (80.9-91.6)	31.6 (26.0-37.8)
cytology and/or <i>CADMI/MAL</i> (spec \geq 70%)	84.5 (75.2-93.8)	69.9 (63.1-76.6)	48.0 (38.3-57.7)	93.2 (88.9-97.5)	43.6 (37.4-50.0)
CIN3+ (B)					
cytology	65.8 (50.7-80.9)	78.6 (72.8-84.3)	37.3 (25.7-48.9)	92.2 (88.2-96.3)	28.6 (23.3-34.7)
<i>CADMI/MAL</i> (spec \geq 70%)	68.4 (53.6-83.2)	75.5 (69.5-81.5)	35.1 (24.3-46.0)	92.5 (88.4-96.6)	31.6 (26.0-37.8)
cytology and/or <i>CADMI/MAL</i> (spec \geq 70%)	86.8 (76.1-97.6)	64.8 (58.1-71.5)	32.4 (23.3-41.4)	96.2 (93.0-99.5)	43.6 (37.4-50.0)

Abbreviations: PPV= positive predictive value, NPV= negative predictive value, CI= confidence interval

FIGURE

Figure 1. Colposcopy triage by combined cytology and bi-marker *CADM1/MAL* methylation analysis in cervical scrapes of 234 hrHPV-positive women.



Shown are the ROC curves for CIN2+ (A) and CIN3+ (B) of combined cytology and bi-marker *CADM1/MAL* methylation analysis (black line, dots) and sole bi-marker *CADM1/MAL* methylation analysis (grey line, squares). In addition, the point estimate of cytology is projected (triangle).