Altered DNA methylation during HPV-induced cervical carcinogenesis

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CHAPTER 5

COMBINED CADM1 AND MAL PROMOTER METHYLATION ANALYSIS TO DETECT (PRE-)MALIGNANT CERVICAL LESIONS IN HIGH-RISK HPV-POSITIVE WOMEN

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ABSTRACT

Given the lower specificity for high-grade cervical lesions of high-risk HPV (hrHPV) testing compared to cytology, additional triage testing for hrHPV test-positive women is needed to detect high-grade cervical lesions. Here, we tested whether combined methylation analysis for CADM1 and MAL, both functionally involved in cervical carcinogenesis, could serve as such a triage marker. Four quantitative methylation-specific PCRs (qMSP), two for CADM1 (regions M12 and M18) and MAL (regions M1 and M2) each, were applied to 261 cervical tissue specimens ranging from no neoplasia to carcinoma. When qMSPs were combined and positivity for at least one of the qMSPs in the combination was taken into account, the highest positivity rates for CIN3 lesions (97%) and squamous cell- and adenocarcinomas (99%) were obtained by combining a single CADM1 marker with a single MAL marker.

Subsequent qMSP analysis of 70 GP5+/6+-PCR hrHPV-positive scrapings revealed that a two-marker panel consisting of CADM1-M18 and MAL-M1 was most discriminative, detecting 90% of women with CIN3 (n=30), whereas it showed a positive result in only 13.5% of women without cervical disease (n=40). Finally, we applied hrHPV GP5+/6+-PCR testing followed by CADM1-M18/MAL-M1 methylation analysis to a cohort of 79 women visiting the outpatient colposcopy clinic. hrHPV testing revealed a sensitivity of 97% and a specificity of 33% for CIN3+. Additional CADM1-M18/MAL-M1 methylation analysis on the hrHPV-positive women increased the specificity to 78% with a sensitivity of 70%. In conclusion, the methylation marker panel CADM1-M18 and MAL-M1 may serve as an alternative molecular triage tool for hrHPV-positive women.

INTRODUCTION

Testing for high-risk human papillomavirus (hrHPV) provides a superior protection against cervical (pre-)cancerous lesions compared to cytology, and is therefore an attractive primary cervical cancer screening tool [1-3]. However, hrHPV testing is accompanied with a lower specificity for high-grade cervical lesions, due to detection of transient, clinically irrelevant hrHPV infections. The consequence of the latter would be too many undesired adverse effects in the generally healthy population, such as anxiety and over-treatment, leading to pregnancy-associated morbidity. Furthermore, unnecessary high costs of the screening program would be the result. Therefore, triage testing is necessary to distinguish hrHPV-positive women with high-grade cervical intraepithelial neoplasia (CIN) lesions or cervical cancer in need of colposcopy from those without meaningful cervical disease. Currently, cytology is considered an appropriate triage tool when applied as reflex test to hrHPV-positive women [4]. However, cytology is a subjective method that can display highly variable outcomes. Particularly in case of pre-existing knowledge of hrHPV presence by the cytologist, minor cellular abnormalities might be overcalled, leading to a decline in specificity of cytology. Ideally, objective triage tools based on biomarker analysis should be available for this purpose.

Numerous studies have indicated the importance of promoter methylation of tumour suppressor genes in the development of cervical cancer, as was recently reviewed by Wentzensen *et al.* [5]. In addition, our group and others have provided proof of principle for a good performance of methylation analysis by methylation-specific PCR (MSP) on cervical scrapings [6-8] with methylation even detectable in archival smears taken up to seven years prior to cervical cancer diagnosis [9]. Collectively, this suggests that methylation markers have a high potential as molecular triage markers for hrHPV-positive women.

Yet, despite the fact that single methylation markers on cervical scrapings display high sensitivities of over 90% for cervical cancer, these are generally inferior to cytology when considering detection of high-grade CIN [6,8,10-14]. Combined methylation analysis of more genes, in which the sum of each assay is scored, may increase the sensitivity for high-grade CIN, as was demonstrated by Kahn *et al*. [8].

In separate studies, we have recently evaluated promoter methylation of two genes, i.e. Cell Adhesion Molecule 1 (CADM1; formerly referred to as TSLC1) and T-lymphocyte Maturation Associated Protein (MAL), during cervical carcinogenesis. These genes were frequently silenced by methylation in cervical cancer cell lines and appeared to possess tumour suppressor activity when their expression was reconstituted, indicating a functional contribution to cervical cancer development [6,9]. Moreover, methylation of CADM1 and MAL was also evident in CIN lesions and cervical cancers with increasing frequencies proportional to severity of cervical (pre-)malignant disease [6,9,15]. Interestingly, whereas in the HPV-mediated transformation process silencing of MAL by promoter methylation occurred rather early in HPV-immortalised, non-tumourigenic keratinocytes, methylation-mediated silencing of CADM1 was linked to later stages of transformation (i.e. cells with anchorage independence and tumourigenic phenotypes [6,9,15]. Since gene silencing by promoter methylation of each of these genes occurred at a distinct stage in the transformation process, we hypothesised that methylation analysis of these genes may at least be partly complementary in terms of CIN3+ detection. In view of the fact that, unlike hrHPV tests, methylation markers detect later events in the carcinogenic process, primary testing by methylation markers alone is unlikely to reach the excellent sensitivity and long term negative predictive value of hrHPV testing for high-grade cervical disease. Therefore, we focused in this study on its significance as a triage marker for hrHPV-positive women. Firstly, a comprehensive quantitative methylation-specific PCR (qMSP) analysis for both genes was performed on archival cervical tissue samples. Next, the potential

value of a combined CADM1 and MAL methylation analysis as triage tool for hrHPV positive women was evaluated, using a series of hrHPV-positive cervical scrapings of women with and without CIN3. Finally, a cross-sectional pilot study was conducted in a gynaecologic outpatient setting to examine the discriminative value of this marker panel for CIN3+.

MATERIALS AND METHODS

Archival tissue specimens

We used 261 formalin-fixed, paraffin-embedded cervical specimens including CIN1 (n=64), CIN3 (n=59), cervical squamous cell carcinomas (SCC; n=94) and cervical adenocarcinomas (AdCA; n=25). In addition, 19 normal cervical specimens were obtained from women treated for a non-malignant disorder, without a history of abnormal cytology or any form of cancer. All biopsies were obtained during the course of routine clinical practice and stored at the Department of Pathology (VU University Medical Center, Amsterdam, the Netherlands). Per histological subgroup, the women had the following median ages: 46 years (range 34-70) in the normal group; 33 (range 22-60) years in the CIN1 group; 36 years (range 24-74) in the CIN3 group; 55 years (range 25-79) in the SCC group; 39 years (range 28-79) in the AdCA group.

Cervical scrapings were obtained from the population-based cervical screening trial POBASCAM, registered as an International Standard Randomised Controlled Trial under number ISRCTN20781131 [16]. From that study, we randomly selected 40 cervical scrapings of GP5+/6+-PCR hrHPV-positive women with normal cytology without evidence of CIN disease up to the next screening round (after 5 years) and 30 scrapings classified as moderate dyskaryosis or worse of hrHPV-positive women with a CIN3 diagnosis within 18 months of follow-up. The median age of hrHPV-positive women with normal

cytology was 33.5 (range 21-52) years and 31.5 (range 25-55) years of hrHPV-positive women with abnormal cytology.

Cross-sectional pilot analysis

Between June 2, 2008 and April 6, 2009, all women visiting the colposcopy outpatient clinic of the VU University Medical Center, Amsterdam, the Netherlands, who met the inclusion criteria listed below, were asked to participate in this pilot study. Women, aged 18 to 70, with abnormal cervical cytology within 120 days prior to colposcopy and an intact cervix (no history of treatment involving damage to the transformation zone of the cervix) were eligible for the pilot study. Furthermore, sufficient knowledge of the Dutch language and being able to understand the content of the study was compulsory. Exclusion criteria were cervical cytology suspicious for carcinoma, current pregnancy or pregnancy within the last three months before enrolment and any event in which colposcopy could not be performed.

Preceding any study-related procedures, a signed informed consent was obtained from every patient. Initially a total of 103 women were recruited of which 79 women remained eligible after excluding 24 women based on the exclusion criteria. The mean age of these women was 34 years (ranging from 18 to 60 years).

Prior to colposcopy, a cervical scraping was taken and collected in 5 mL Universal Collection Medium (UCM; kindly provided by Digene Corp., Gaithersburg, MD, USA). Upon arrival in the laboratory, samples were concentrated to 1 mL UCM and firstly used for DNA extraction for hrHPV GP5+/6+-PCR testing followed by CADM1-M18 and MAL-M1 promoter methylation analysis in case of a positive hrHPV test. In addition, in case of hrHPV positivity liquid-based cytology (LBC) was performed on 1/10 of the cervical scraping when sufficient material was available. A previous study has shown that LBC on samples collected in UCM is equally accurate as the conventional Pap scraping in terms of detecting CIN lesions and cervical

cancer [17]. For the latter 100 µL of UCM sample was carefully diluted in 20 mL PreservCyt (Cytyc Corporation, Marlborough, Mass, USA) and processed using a ThinPrep processor, followed by ThinPrep Papanicolaou staining. All slides were blindly scored by an experienced cytopathologist (FJvK). The results were reported according to the CISOE-A classification, the standard classification system for cytology in the Netherlands, which can be translated into the Bethesda classification [18].

Colposcopies were performed by experienced gynaecologists, according to the Dutch colposcopy guidelines [19]. Biopsies were sampled from the most suspicious cervical locations. In case no lesion could be visualised, a biopsy from the transformation zone at the 12 o'clock position was taken to minimise the chance of missing lesions and to reduce ascertainment bias. An endocervical curettage was performed when the transformation zone was not entirely visible. In case of a histological diagnosis of CIN2 or worse, women were treated by Large Electrosurgical Excision Procedure (LEEP), according to the regular management protocol used at the Department of Obstetrics and Gynaecology of the VU University medical center.

A total of 40 women were treated by LEEP and from 39 women only biopsies (average 1.6 per women: range 1-3) were taken.

All sections were seen by three pathologists and histological outcomes were based on cervical biopsies or LEEP samples, depending on the sample that showed the most severe histological abnormality. In case of discordance, a consensus diagnosis was made. The main outcome measure was the number of histologically confirmed cases of high-grade cervical disease (CIN3+), detected by hrHPV and methylation markers. Ninety-five % confidence intervals (95%CI) were calculated using the SPSS software package (version 15.0, Chicago, IL, USA).

The study was approved by the Medical Ethics Committee of the VU University Medical Center in Amsterdam, the Netherlands (nr 2006/169).

Isolation of nucleic acids and HPV testing and typing

For DNA isolation all sections were cut according to the sandwich method, in which the histology of sections used for DNA isolation (cut at 10 µM) was checked by H&E staining of the first and last section (cut at 4 µM). All slides were seen and scored by at least two pathologists. Of all tissue specimens and cervical scrapings, DNA was extracted by proteinase K digestion, followed by purification using a High Pure PCR Template Preparation kit (Roche Diagnostics, Almere, the Netherlands) according to standard procedures. Detailed information on sample preparation and PCR procedures is also described by de Wilde *et al.* [20]. HPV detection and genotyping was performed using the GP5+/6+-PCR with an enzyme immunoassay (EIA) readout [21], followed by reverse line blot analysis of EIA-positive cases [22]. A sample was assigned as containing HPV X when the pooled hrHPV EIA analysis was positive, but the subsequent reverse line blot genotyping remained negative. This suggests the presence of variant(s), subtype(s) or related type(s) not reacting with the type-specific oligo-probes in the line blot assay.

DNA modification and qMSP analysis

Bisulfite treatment of genomic DNA was performed using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA). MAL qMSP assays for two distinct regions (referred to as MAL-M1 and MAL-M2) were performed using primers and TaqMan probes as described previously (in addition, see Table 1) [6]. Quantitative MSP assays targeting two regions within the CADM1 promoter (from nucleotide position -192 to -102 and from position -67 to +23, relative to the translation start site; referred to as CADM1-M12 and CADM1-M18, respectively) were newly designed using primer sets, probes and annealing temperatures described in Table 1.

Gene (Promoter region)	Forward 5'-3' primer	6-FAM 5'-3'-TAMRA probe	Reverse 5'-3' primer	Tm [a] (^{o}C)	Amplicon Size (bp)
CADM1 M12	GCGTCGTCGAACGT TAGCGT	CCTCCCCCACCCCGCCC CCT	AACCAATCACAACG CCGCG	60	90
CADM1 M18	ATTTTATTAGTTGTT GGTTCGGGT	ACCTACCTCAAACTAACG ACGTTAACTACCTCCGA	CTCGACAACACTAC ACTCGCC	60	90
MAL M1	GCGTAGTATTAAGT AGAGAGGTTCG	ACTAAACCGACGCTAATT CGACGACGCT	AATAAAAAATAAAAC CGACCGC	59	107
MAL M2	TTAGGTTATTGGGT TTCGCG	TCCGCGCAAACCTCTCG CTAAC	GTACTAACGTCGAC CTTAAAACGA	59	86
β-actin	TGGTGATGGAGGA GGTTTAGTAAGT	ACCACCACCCAACACACA ATAACAAACACA	AACCAATAAAACCT ACTCCTCCCTTAA	60	133

[a] Tm = annealing temperature

TABLE 1. Primer and probe sequences used for quantitative MSP.

These qMSPs target CADM1 promoter regions closely related to those previously analysed by conventional MSP assays [15]. For reasons of primer, probe and amplicon size restrictions the region targeted by the CADM1-M12 qMSP is located 60 bp 3' of the conventional CADM1-M5 MSP, whereas the CADM1-M18 amplicon largely overlaps with the amplicon of the conventional CADM1-M9 MSP. The reason for conversion to qMSP formats was that for clinical application of methylation markers qMSP is preferred over a binary readout using conventional MSP [23].

The CADM1 qMSPs were validated on methylation-positive (i.e. SiHa) and -negative (i.e. primary human keratinocyte) cell cultures and showed a detection limit of 0.1% methylated DNA in a background of unmethylated DNA. In a pilot experiment performed on a set of 31 cervical carcinomas, the newly developed CADM1 qMSP assays showed high concordance with conventional MSP methods targeting the related promoter regions. A 84% concordance was found for conventional CADM1-M5 MSP versus CADM1-M12 qMSP and a 90% concordance for the conventional CADM1-M9 MSP versus the CADM1-M18 qMSP.

qMSP reactions were carried out in a 12 µL reaction volume containing 50 ng of bisulfite-treated DNA, 417 nM of each primer, 208 nM probe and 1x QuantiTect Probe PCR Kit master mix (Qiagen, Westburg, Leusden, the Netherlands). The amplification and real-time measurements were performed using the 7500Fast ABI system (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). In each assay H_2O, unmodified genomic DNA (SiHa) and bisulfite-treated unmethylated DNA (primary keratinocytes) were included as negative controls, and as a positive control bisulfite-treated SiHa DNA was taken along. The housekeeping gene β-actin (ACTB) was chosen as an internal reference [24]. All samples analysed in the study showed an ACTB CT value that was equal to or below 31 (CT≤31). To calculate the quantity of amplification products using the SDS software package of Applied Biosystems, the fluorescence threshold was set at 0.01, which was always in the exponential phase of the amplification curves. For quantification, a standard curve of a serial dilution of bisulfite-treated SiHa DNA (50, 5, 2.5, 0.5, 0.25 and 0.05 ng) was included in each qMSP run. qMSP values of target genes were adjusted for DNA input by expressing the results as ratios between the absolute target measurement and the ACTB measurement (quantity of methylated DNA/DNA quantity for β-actin * 1000), as described previously [6,7,25,26]. Samples were scored positive for methylation when the target gene/ACTB ratio was above a calculated cut-off value. For each gene the cut-off was based on the 99% confidence interval of the mean ratio of normal control samples (i.e. mean ratio multiplied by 2.58 * standard deviation). When qMSPs were combined, positivity for at least one of the qMSPs in a given combination was taken into account for the final score. For tissue specimens and cervical scrapings separate cut-off values were calculated.

RESULTS

Methylation status of CADM1 and MAL promoter regions in cervical tissue specimens

qMSP assays for two CADM1 promoter regions (i.e. CADM1-M12 and CADM1-M18) were applied to 19 normal cervices, 64 CIN1 lesions, 59 CIN3 lesions, 94 SCCs and 25 AdCAs that previously had been analysed by qMSP for two promoter regions of MAL (i.e. MAL-M1 and MAL-M2 [6]).

CADM1 and MAL qMSP data and corresponding hrHPV results for each specimen in the different histomorphological groups are displayed in Figure 1. As expected on the basis of previous findings [6,15], all qMSP markers showed increased methylation frequencies with increasing severity of the cervical lesion. Amongst the single methylation markers, MAL-M1 and MAL-M2 revealed the highest positivity rates in carcinomas (94% and 96%, respectively, for SCCs, and 100% and 96%, respectively, for AdCAs). Most frequent methylation in CIN3 lesions was seen for markers CADM1-M12 and MAL-M2 (78% and 83%, respectively). By scoring for hrHPV presence plus positivity for a two qMSP combination, the percentage of test-positive SCCs increased to 99% (combinations CADM1-M12 and/or MAL-M2, and MAL-M1 and/or MAL-M2; Figure 1 and Table 2).

The increase in positivity rate achieved by a combination of two qMSP assays was most pronounced (>10%) for CIN3 lesions. The combination of CADM1-M12 and MAL-M2 marker yielded the highest percentage of positive CIN3 lesions (i.e. 97%). Adding other qMSPs to this combination did not further increase the detection rate of CIN3 lesions. The difference in methylation frequency for the combination CADM1-M12/MAL-M2 between normal samples and CIN1 on one hand and CIN3 on the other hand was highly significant ($p<0.001$).

Taken together, these data indicate that almost all CIN3 lesions and cervical carcinomas have methylated MAL and/or CADM1 promoter regions that can be detected with two qMSP assays.

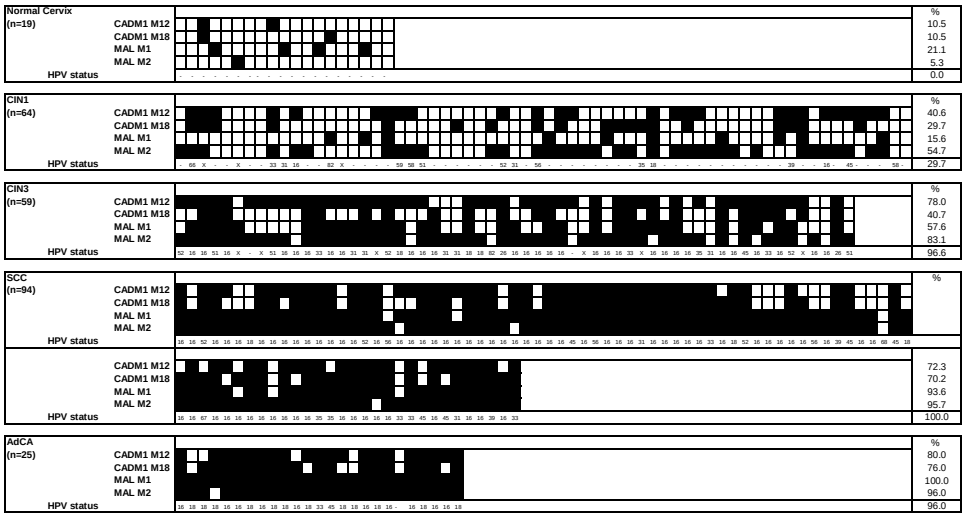

FIGURE 1. Summary of MAL and CADM1 qMSP results on cervical tissue specimens. DNA methylation is depicted in black, white boxes indicate unmethylated DNA; HPV status of specimens is depicted as specific hrHPV type present or negative (-). The percentages of positivity for each sole methylation marker amongst the various histological categories are indicated in the panels on the right.

Combined CADM1 and MAL promoter methylation detection in cervical scrapings is highly predictive for underlying high-grade lesions

To further assess the potential value of CADM1 and MAL methylation analysis as a triage tool for hrHPV-positive women in cervical screening, we subsequently analysed 70 cervical scrapings of hrHPV-positive women who participated in the intervention group (hrHPV plus cytology screening) of the POBASCAM population-based screening trial [1].

Marker(s)	Normal (n=19) hrHPV+ (n=0)		CIN1 (n=64) hrHPV+ (n=20)		CIN3 (n=59) hrHPV+ (n=57)		SCC (n=94) hrHPV+ (n=94)		AdCA (n=25) hrHPV+ (n=24)	
	n	%	n	%	n	%	n	%	n	%
CM12	0	0%	12	19%	44	75%	68	72%	20	80%
CM18	0	0%	7	11%	24	41%	66	70%	19	76%
MM1	0	0%	2	3%	34	58%	88	94%	24	96%
MM2	0	0%	12	19%	48	81%	90	96%	23	92%
CM12/CM18	0	0%	13	20%	45	76%	75	80%	22	88%
MM1/MM2	0	0%	13	20%	50	85%	93	99%	24	96%
MM1/CM12	0	0%	13	20%	48	81%	89	95%	24	96%
MM1/CM18	0	0%	8	13%	37	63%	89	95%	24	96%
MM2/CM12	0	0%	13	20%	55	93%	93	99%	23	92%
MM2/CM18	0	0%	14	22%	51	86%	92	98%	24	96%

TABLE 2. Positivity rates for sequential hrHPV testing and CADM1 and MAL methylation detection in cervical tissue specimens. Methylation frequencies are based on individual qMSPs assays and all various combinations of qMSP assays, thereby taking the sum of samples positive for each assay into account (i.e. and/or scoring). CM12: CADM1 M12; CM18: CADM1 M18; MM1: MAL M1; MM2: MAL M2.

These scrapings represented two groups of randomly selected women. The first group comprises 40 hrHPV-positive women with normal cytology without evidence of CIN disease up to the next screening round after 5 years. The second group consists of 30 hrHPV-positive women having abnormal cytology (≥moderate dyskaryosis, equalling HSIL) with a CIN3 diagnosis within 18 months of follow-up. For the individual markers, positivity rates varied from 5% to 8% for women without CIN and 53% to 87% for women with CIN3 (Table 3). The test outcomes obtained after considering various marker combinations, are also depicted in Table 3. In light of the methylation markers potentially being implemented as a triage tool in future hrHPV-based screening programs, priority was given to markers displaying the highest positivity in CIN3 cases, with lowest positivity in controls being secondary. Amongst the combinations giving rise to the highest positivity rates for CIN3 (i.e. 90%), the CADM1-M18/MAL-M1

combination revealed the lowest positivity rate in the control scrapings. The difference in CADM1-M18 and/or MAL-M1 methylation positivity between the CIN3 group (90%) and hrHPV-positive normal cytology samples (12.5%) was highly significant (p<0.001).

	hrHPV-positive without CIN lesion (n = 40)		**hrHPV-positive with CIN3 lesion (n = 30)**	
	n	%	n	%
CM12	2	5.0%	20	66.7%
CM18	2	5.0%	26	86.7%
MM1	3	7.5%	25	83.3%
MM2	2	5.0%	16	53.3%
CM12/CM18	3	7.5%	26	86.7%
MM1/MM2	5	12.5%	25	83.3%
MM1/CM12	5	12.5%	26	86.7%
MM1/CM18	5	12.5%	27	90.0%
MM2/CM12	3	7.5%	23	76.7%
MM2/CM18	3	7.5%	26	86.7%
MM1/CM12/CM18	6	15.0%	27	90.0%
MM2/CM12/CM18	4	10.0%	26	86.7%
MM1/MM2/CM12	6	15.0%	26	86.7%
MM1/MM2/CM18	6	15.0%	27	90.0%
MM1/MM2/CM12/CM18	7	17.5%	27	90.0%

TABLE 3. Frequencies of CADM1 and MAL methylation detection in hrHPV-positive cervical scrapings of the POBASCAM study. Methylation frequencies are based on individual qMSPs assays and all various combinations of qMSP assays, thereby taking the sum of samples positive for each assay into account (i.e. and/or scoring). CM12: CADM1 M12; CM18: CADM1 M18; MM1: MAL M1; MM2: MAL M2.

Collectively, these results show that also for cervical scrapings a combination of two of the four qMSP assays suffices to optimally detect CIN3+. Moreover, a panel comprising one CADM1 marker and one MAL marker, particularly CADM1-M18/MAL-M1, can provide a promising triage test for further cross-sectional evaluation in hrHPV-positive women.

Cross-sectional evaluation of the CADM1/MAL methylation marker panel

Subsequently, a pilot study was performed on women referred to the Gynaecologic outpatient clinic for colposcopy because of previous abnormal cytology to evaluate the diagnostic value for CIN3+ of hrHPV testing and triage of hrHPV-positive women by CADM1-M18/MAL-M1 methylation analysis (in comparison to triage by liquid-based cytology). Prior to colposcopy, a cervical scraping was taken and firstly used for hrHPV testing by GP5+/6+-PCR. In case of a positive hrHPV test, CADM1-M18/MAL-M1 methylation analysis was performed and when sufficient sample material was left also liquid-based cytology was performed for comparison.

Twenty four women had no CIN lesion, 15 were diagnosed with CIN1, 10 with CIN2, 28 with CIN3 and two with AdCA. An overview of hrHPV testing and methylation results per disease category/diagnosis is given in Table 4.

Diagnosis	hrHPV+	hrHPV+/methylation+	hrHPV+/LBC+ *
CIN0	58%(14/24)	13% (3/24)	10% (2/20)
CIN1	80%(12/15)	27% (4/15)	7% (1/14)
CIN2	90% (9/10)	30% (3/10)	30% (3/10)
CIN3	96% (27/28)	68% (19/28)	57% (12/21)
Cancer	100% (2/2)	100% (2/2)	0% (0/2)
Total	81% (64/79)	39% (31/79)	27% (18/67)

*LBC was only performed on hrHPV-positive samples with sufficient material available

TABLE 4. Summary of hrHPV testing results combined with CADM1/MAL methylation analysis and liquid-based cytology (LBC) on women visiting the gynaecologic outpatient clinic for colposcopy.

In total, 81% (64/79) of women were hrHPV-positive, of which 31 (39%) were methylation-positive for CADM1-M18 and/or MAL-M1. By hrHPV testing 29 of the 30 CIN3+ lesions (97%; 95%CI 90-100) were positive compared with 35 of the 49 ≤CIN2 lesions (specificity for CIN3+: 29%; 95%CI 16-41). Additional CADM1-M18/MAL-M1 methylation analysis on hrHPV-positive women detected 21 of the 30 CIN3+ cases (70%; 95%CI 54-86), including both AdCAs. The nine undetected CIN3 lesions included one testing hrHPV-negative. Of the 49 women

with ≤CIN2, only ten were positive for both hrHPV and CADM1-M18/MAL-M1, resulting in a CIN3+ specificity increase to 78% (95%CI 66-89).
Liquid based cytology, as performed on a subset of hrHPV-positive women with sufficient sample material available, detected 52% (12/23) of CIN3+ lesions (Table 4).

DISCUSSION

In this study, we evaluated the value of methylation analysis of CADM1 and MAL as potential triage tool for hrHPV-positive women. We demonstrated that both for tissue specimens and cervical scrapings application of two of the qMSP assays targeting CADM1 and MAL each, suffices for optimal detection of CIN3+. This underlines our initial hypothesis that combining markers representing two distinct pre-cancerous events driving cervical carcinogenesis [6,9,15] enables the detection of hrHPV-positive women having an increased cancer risk. Remarkably, the marker combinations with the highest discriminative value for CIN3+ were different for tissue specimens compared to cervical scrapings. It is unlikely that the use of formalin-fixed and paraffin-embedded (FFPE) material might have influenced the performance on tissue samples, since in pilot experiments FFPE tissue samples revealed the same methylation status as corresponding frozen tissue (data not shown). A more likely explanation would be differences in the cell type composition in tissues versus scrapings, in which the different cell types may display distinct levels of background methylation at one or more of the regions analysed by qMSP. Cervical tissue samples often contain substantial amounts of non-epithelial (stromal) cells, whereas cervical scrapings are enriched with superficial epithelial cells. Indeed, the thresholds set on the basis of the 99% CI of the mean values of normal controls differed between tissue and scraping controls.

Therefore, methylation data obtained from tissue samples cannot directly be extrapolated to cervical scrapings, as was also shown previously by others [10,27]. Data from both archival and prospectively collected cervical scrapings indicate that the methylation marker panel CADM1-M18/MAL-M1 provides a promising triage assay for hrHPV-positive women.

One issue encountered in the present study requires further attention. The collection of cervical scrapings taken immediately prior to colposcopy in the cross-sectional pilot study may have led to more cautious brushing of the cervix to prevent bleeding and therefore lower abnormal cell counts in scrapings and liquid-based cytology samples. Liquid based cytology as performed on a subset of these scrapings confirmed insufficient sampling as only a minority of scrapes revealed abnormal cytology (Table 4), despite the fact that the far majority of them were referred because of abnormal screening cytology. This has probably led to an underestimation of the sensitivity of methylation markers in this cross-sectional cohort.

To the best of our knowledge, the combined CADM1 and MAL methylation analysis in hrHPV-positive women described in this study has one of the highest sensitivity and specificity figures for the detection of CIN3, SCC and AdCA, compared with other methylation-based molecular assays described before [7,28]. Amongst the methylation marker combinations published so far, HS3ST2 combined with CDH1 seemed most promising as it was positive for 100% of tumour biopsies and 83% of high-grade cervical scrapings, whereas all normal cervical tissue samples tested negative [29]. However, the high-grade specimens analysed in that study only included cytology samples without histological information, and no cytomorphologically normal specimens were included to allow proper specificity calculations.

In summary, this study has shown that combined methylation analysis for CADM1 and MAL may be a promising, objective triage tool for hrHPV-positive women. As an extent to the present study providing a first proof-of-principle for the use of methylation markers in a triage setting, further studies on the full spectrum of disease categories, including CIN2, versus all cytology outcomes

are warranted. Such a comprehensive analysis on hrHPV-positive women participating in population based screening is currently ongoing.
Finally, as yet demonstrated in this first proof-of-principle study, application of these molecular tests may also improve the detection of (pre)cancerous lesions of the columnar epithelium.

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