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# *CADM1*, *MAL* and *miR124-2* methylation analysis in cervical scrapes to detect cervical and endometrial cancer

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# ABSTRACT

**Aims** Gene promoter hypermethylation is recognized as an essential early step in carcinogenesis, indicating important application areas for DNA methylation analysis in early cancer detection. The current study was set out to assess the performance of *CADM1*, *MAL* and *miR124*-2 methylation analysis in cervical scrapes for detection of cervical and endometrial cancer.

**Methods** A series of cervical scrapes of women with cervical (n=79) or endometrial (n=21) cancer, CIN3 (n=16) or CIN2 (n= 32), and women without evidence of CIN2 or worse (n=120), was assessed for methylation of *CADM1*, *MAL* and *miR124-2*. Methylation analysis was done by the PreCursor-M assay, a multiplex quantitative methylation-specific PCR.

**Results** All samples of women with cervical cancer (79/79, 100%), independent of the histotype, and 76% (16/21; 95% CI:58.0-94.4) of women with endometrial cancer scored positive for DNA methylation of at least one of the three genes. In women without cancer, methylation frequencies increased significantly to severity of disease, from 19.2% (23/120; 95% CI:12.1-26.2) in women without CIN2 or worse to 37.5% (12/32; 95% CI: 20.7-54.3) and 68.8% (11/16; 95% CI:46.0-91.5) in women with CIN2 and CIN3, respectively. Not only overall methylation positivity, but also the number of methylated genes increased proportionally to the lesion severity.

**Conclusions** DNA methylation analysis of *CADM1*, *MAL* and *miR124-2* in cervical scrapes consistently detects cervical cancer and the majority of CIN3 lesions, and has the capacity to broaden its use on cervical scrapes through the detection of a substantial subset of endometrial carcinomas.

# INTRODUCTION

Cervical cancer is the third most common cancer in women worldwide. In 2008, cervical cancer accounted for 9% (529,800) of all new cancer cases and 8% (275,100) of all cancer-related deaths.[1] The introduction of cytology-based screening programs, either organized or opportunistic, has markedly reduced the incidence and mortality rates of invasive cervical cancer in developed countries. Additionally, different studies have described detection of other gynaecological pathologies such as endometrial carcinoma by the cytology-based cervical screening program.[2–6] However, the effects of cytology-based screening have levelled off. This is mainly due to the suboptimal sensitivity of the screening tool (i.e., cytology) for cervical intraepithelial neoplasia (CIN) grades 2 or 3 (CIN2/3) and cancer (CIN2/3+), as well a substantial number of women not attending cervical screening. Therefore, efforts to improve screening have focused on alternative screening tools to overcome the limitations of cytology, and attracting more women into the screening program.

Over the last years, the importance of primary screening by detection of human papillomavirus (HPV) DNA in cervical samples in the prevention of cervical cancer has become clear. A persistent infection with a high-risk type of HPV (hrHPV) is necessary for the development of cervical cancer and its high-grade precursors.[7] HPV DNA testing appeared substantially more sensitive in detecting CIN2/3+ lesions than cytology, providing 50% greater protection against cervical carcinoma and its high-grade precursor lesions.[8,9] In addition, HPV DNA testing can also be applied to self-collected (cervico-)vaginal specimens, the latter being important to increase the compliance rate in cervical screening.[10–12] Yet, HPV DNA testing shows a reduced specificity compared to cytology, since many infected women harbour transient viral infections that are not associated with clinically meaningful disease. Therefore, it is important to triage hrHPV-positive women for colposcopy to identify those women with the highest risk for CIN2/3+ thereby reducing over-referral and overtreatment.[7,13,14] DNA methylation analysis of cancer-related genes by quantitative methylation specific PCR (qMSP) has emerged as a promising and objective triage tool for early detection of cervical neoplasia.[15] Hypermethylation of CpG islands in the promoter regions of tumour suppressor genes leads to gene silencing, and is recognized as an essential step in cancer development.[16–20] Methylation of cancer-related genes has been described in a variety of gynaecological carcinomas, including cervical cancer[15,16,18,21] and endometrial cancer.[22–25] We previously showed that methylation-mediated silencing of *CADM1* (cell adhesion molecule 1), *MAL* (T-lymphocyte maturation-associated protein) and *miR124*-2 (micro-RNA124-2) is functionally involved in cervical carcinogenesis[17,19,20], and a frequent event detectable in tissue biopsies from CIN3 lesions and cervical carcinoma.[18,26] Methylation analysis of the promoter regions of these genes by quantitative methylation-specific PCR (qMSP) is valuable in colposcopy triage of hrHPV-positive women, both when using cervical scrapes[27] and self-collected specimens.[28,29] The bi-marker panel *CADM1*/*MAL* has been validated on hrHPV-positive cervical scrapes collected in a population-based screening setting, and showed to be equally discriminatory for CIN3+ as cytology at the same specificity level.[27] In a recent prospective randomized trial among non-responders of the regular cervical screening program, the bi-marker panel *MAL*/*miR124-2* on hrHPV-positive self-samples was non-inferior to cytology triage via a physician-taken cervical scrape in detecting CIN2+.[29] Of note, methylation levels of these three genes have shown to be related to the severity and duration of cervical disease, and are exceptionally increased in cervical cancer.[18,30] As a consequence, methylation analysis could be particularly effective in detecting advanced precursor lesions (with likely high short-term progression risk) and cervical cancers[15], and could serve as complementary tool to cytology triage of hrHPV-positive women to gain a higher reassurance of not missing advanced lesions and cervical cancer.[31] These data suggest that cancers are unlikely to remain undetected by DNA methylation analysis of cervical scrapes. Yet, no large series of cervical scrapes from women with cervical cancer have been evaluated so far, as these are not merely encountered in large numbers in a screening setting given the rarity of cervical cancer in the screening population.

In the current study, we evaluated *CADM1*, *MAL* and *miR124*-2 methylation in a large series of cervical scrapes from women with various underlying disease grades, including 79 women with cervical cancer and 21 with endometrial cancer. We assessed the performance of a multiplex qMSP kit

(PreCursor-M, Self-screen B.V., The Netherlands) that allows the combined detection of *CADM1*, *MAL* and *miR124*-2 methylation in cervical scrapes.

# METHODS

## Study population

For this study, cervical scrapes of 268 women who participated in population-based cervical screening or attended a gynaecological outpatient clinic, were used. Details on the number of cervical scrapes in relation to underlying disease category and age of the women are listed in Table 1.Cervical scrapes were retrieved from the pathology archive and in case no slides were available, cytological preparations were made from diagnostic left over specimen. In this way were able to obtain from 235/268 (88%) cytology results. Cytology was reported according to the standard classification in the Netherlands, i.e., CISOE-A classification, that can be easily translated into Bethesda classification[32], in which borderline or mild dyskaryosis (BMD) equals ASC-US/ASC-H/LSIL. Presence of hrHPV-DNA was determined by GP5+/6+-PCR.[33] For this study, ethical approval was waved, since diagnostic left-over specimens were used that had been anonymized according to the Dutch regulations.[34]

**Table 1: Overview of cervical scrapes used in this study.**

| Disease category | Number of scrapes | Median age of women (years) |
|---|---|---|
| Cervical carcinoma* | 79 | 48 (range: 23-85) |
| Endometrial carcinoma | 21 | 60 (range: 44-87) |
| CIN3 | 16 | 39 (range: 28-49) |
| CIN2 | 32 | 40.5 (range: 33-53) |
| No evidence of CIN2+ | 120 | 37 (range:18-64) |

*comprising squamous cell carcinoma (SCC; n=62); adenocarcinoma (AdCa; n=12); adenosquamous carcinoma (n=2); and undifferentiated carcinoma (n=3).

**DNA isolation, bisulphite treatment and qMSP methylation analysis**

DNA from cervical scrapes was isolated using the Nucleo-Spin 96 Tissue kit (Macherey-Nagel) and a Microlab Star robotic system (Hamilton, Germany) according to manufacturers' protocol or by standard salt-chloroform extraction and isopropanol precipitation.[21,27] Extracted DNA was subjected to bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research, USA) as described previously.[19,20] DNA methylation analysis of *CADM1*, *MAL* and *miR124-2* was performed by a commercial multiplex qMSP (PreCursor-M, Self-Screen B.V., The Netherlands). PreCursor-M uses primers and probes specific for methylated DNA of *CADM1*, *MAL* and *miR-124-2*, and methylation-independent β-actin as sample quality control. The multiplex format enables simultaneous amplification and detection of the four targets within one reaction.[35] Analyses were performed on an ABI 7500 real-time PCR-system (Applied Biosystems, USA). All samples had a Cq (Quantification Cycle) value for β-actin <29 to assure sample quality. The target methylation result relative to that of a calibrator was calculated as ΔΔCq ratio for each marker gene (i.e., *CADM1*, *MAL*, or *miR124-2*), being a measure for hypermethylation using the $2^{-\Delta\Delta Cq}$ method.[36] Cervical scrapes were scored positive based on preset ΔΔCq ratio thresholds according to manufacturers' instructions (i.e., validated thresholds that on a validation set of cervical scrapes of hrHPV-positive women gave rise to a maximum CIN3+ sensitivity at 70% specificity).

**Data and statistical analysis**

For calculations, a sample was considered methylation-positive for a specific target if the ΔΔCq ratio was above the preset threshold of the respective target and overall methylation-positive when at least one target was above its preset threshold. The threshold for cytology positivity was BMD. 95% Wald confidence intervals (95% CIs) were determined for the proportions of positive samples. The proportions of overall methylation-positive samples per disease category (i.e., without evidence of CIN2+, CIN2, CIN3 or cervical cancer) were compared using Chi-square analysis. Calculations were performed in Microsoft Excel (2010) and SPSS (version 20).

# RESULTS

**Methylation analysis of cervical scrapes**

A series of 268 cervical scrapes was evaluated (Table 2). DNA methylation of at least one of the three loci (*CADM1, MAL,* and/or *miR124-2)* was detected in all cervical scrapes of women with cervical cancer, independent of the histotype. This percentage was 76.2% for women with endometrial cancer, 68.8% for CIN3 and 37.5% for CIN2. By comparison, 19.2% of women without evidence of CIN2+ had a methylation-positive cervical scrape, comprising 24.7% (19/77;95%CI:15.1-34.3) of the hrHPV-positive women and 9.3% (4/43;95%CI:6.2-18.0) of the hrHPV-negative women. The frequency of methylation positivity increased significantly with the severity of the cervical lesion (Figure 1).

**Table 2: *CADM1/ MAL/miR124-2* methylation, cytology and hrHPV data of cervical scrapes in relation to underlying disease category**

| | **Positive by** | | | | | |
|---|---|---|---|---|---|---|
| | **Methylation** | | **Cytology** | | **hrHPV** | |
| **Disease category** | **n/N** | **% (95%CI)** | **n/N** | **% (95%CI)** | **n/N** | **% (95%CI)** |
| **No evidence of CIN2+** | 23/120 | 19.2 (12.1-26.2) | 29/120 | 24.2 (16.5-31.8) | 77/120 | 64.2 (55.6-72.8) |
| **CIN2** | 12/32 | 37.5 (20.7-54.3) | 23/32 | 71.9 (56.3-87.5) | 32/32 | 100 (100-100) |
| **CIN3** | 11/16 | 68.8 (46.0-91.5) | 14/16 | 87.5 (71.3-100) | 16/16 | 100 (100-100) |
| **Cervical carcinoma** | 79/79 | 100 (100-100) | 39/47[#] | 83.0 (72.2-93.7) | 73/79 | 92.4 (86.6-98.3) |
| *SCC* | *62/62* | *100 (100-100)* | *32/38[#]* | *84.2 (72.6-95.8)* | *60/62* | *96.8 (92.4-100)* |
| *AdCa* | *12/12* | *100 (100-100)* | *5/6[#]* | *83.3 (53.5-100)* | *9/12* | *75.0 (50.5-99.5)* |
| *Other** | *5/5* | *100 (100-100)* | *2/3[#]* | *66.7 (13.3-100)* | *4/5* | *80.0 (44.9-100)* |
| **Endometrial carcinoma** | 16/21 | 76.2 (58.0-94.4) | 9/20[$] | 45.0 ( 23.2-66.8) | 4/21 | 19.0 (2.3-35.8) |

*including adenosquamous carcinoma (n=2) and undifferentiated carcinoma (n=3)
[#]cytology of 32 women was indeterminate (i.e., 24 SCC, 6 AdCa, 2 other)
[$]cytology of 1 woman was indeterminate

**Relative contribution of the three targets**

Not only the frequency of methylation positivity, but also the number of markers with a methylation-positive score increased with the severity of the underlying cervical lesion. Among methylation-positive cervical scrapes, those of women without evidence of CIN2+ and women with CIN2 lesions were mostly single marker positive (78.3%(18/23), and 58.3%(7/12), respectively), whereas the majority of women with CIN3 were double marker positive (54.5%;6/11) and those with cervical carcinoma triple marker positive (54.4%;43/79). Regarding the two major histotypes of cervical carcinoma, the majority of scrapes of women with SCC was triple marker positive (61.3%;38/62), whereas AdCa were predominantly double marker positive (66.7%;8/12). All double marker positive women with CIN3 and cervical carcinoma were positive for *MAL* and *miR124-2*. Among methylation-positive scrapes of women with endometrial adenocarcinoma, both double (43.8%;7/16), comprising various marker combinations) and single marker (56.3%;9/16) positivity were seen, with overall the largest contribution by *miR124-2* (87.5%;14/16).

# DISCUSSION

In this study, we explored the clinical performance of multiplex methylation analysis of *CADM1*, *MAL* and *miR124-2* promoter regions in cervical scrapes, and demonstrated a detection rate of 100% for cervical cancer, and 76% (95%CI:58.0-94.4) for endometrial cancer. Our data indicate that methylation analysis of *CADM1*, *MAL* and *miR124-2* in cervical screening would identify all cervical carcinomas and holds the detection of endometrial carcinomas as important supplement. The standardized assay detects three methylation markers in one single reaction, which requires less sample material over separate reactions as has been mainly used in previous studies.[26,28,29] As demonstrated in the present study, the three methylation markers have additive value with respect to disease detection, in line with previous findings.[26–29] The assay furthermore allows high-throughput analysis, which is advantageous in screening programs.[35]

To the best of our knowledge, this is the first study evaluating a large series of cervical scrapes from women with cervical or endometrial cancer for DNA methylation of *CADM1*, *MAL* and *miR124-2*. The figures corroborate with previous data describing a methylation positivity rate of 100% for cervical cancer in smaller sample sets using parallel singleplex assays.[26,27,30] In line with previous findings, the number of methylation markers scoring positive, which is inherent to higher methylation levels, increased with disease severity.[30] This strongly supports the concept that CIN lesions positive with this assay represent more advanced CIN lesions in need of treatment.[15] Based on our data, *MAL* and *miR124-2* are the predominant markers for the detection of high-grade cervical and endometrial lesions, at the thresholds defined for this assay, with *CADM1* adding to reach 100% detection rate of cervical cancer. Also HPV-DNA positivity rates in scrapes of women with cervical cancer were high in our study. Positivity rates for HPV-DNA in cervical adenocarcinoma tended to be lower than SCC, as reported in literature.[37] The lower HPV detection rates in scrapes of cervical adenocarcinoma versus SCC may be due to technical aspects, or misdiagnosis of endometrial carcinoma as cervical adenocarcinoma. Although comparison of the methylation test to cytology is skewed as cancer patients were mainly referred because of abnormal cytology, it was noted that scrapes with normal cytology (n=8) of women with cervical cancer were detected by the methylation assay. The detection of a substantial subset of endometrial carcinomas is an important supplement of methylation analysis of cervical scrapes. The addition of other methylation markers[25] or other molecular markers such as tumour-specific mutations[38] might improve the overall diagnostic accuracy for gynaecologic malignancies in the future.

At present, the methylation assay is proposed to be used as triage test for HPV-positive women, with clinical utility in detection of CIN2+/3+ shown for both hrHPV-positive cervical scrapes[27,31] and hrHPV-positive self-collected specimens.[28,29] Current findings also suggest the prospect of the methylation assay as primary cervical screening tool. Particularly the high sensitivity for cancer opens the way to a direct screen and treatment approach. Especially in low and middle income countries, where cervical screening is hard to implement and follow-up rates are low, this approach might be attractive.

In conclusion, multiplex DNA methylation analysis of the *CADM1*, *MAL* and *miR124-2* loci in cervical scrapes consistently identifies cervical cancer and the majority of CIN3 lesions, and has the capacity to broaden its use on cervical scrapes through the detection of a substantial subset of endometrial carcinoma. As such, it is a promising step toward a broadly applicable screening methodology and lays the foundation for a new generation of molecular screening.

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**Competing interests**
DAMH, RDMS, PJFS, and CJLMM are shareholders of Self-Screen B.V.. All other authors declare to have no conflicts of interest.

**Author contributership**
DAMH, RDMS, PJFS and CJLMM designed the study. LDS, MvZ and DAMH drafted the original manuscript. LDS, MvZ and ATH were responsible for data analyses. MCGB and GBAW provided clinical samples. All authors reviewed the manuscript and approved the final version.

**Key messages**
- Methylation analysis of *CADM1*, *MAL* and *miR124-2* in cervical scrapes consistently detects cervical cancer and the majority of CIN3 lesions.
- Overall methylation positivity and the number of methylated genes in cervical scrapes increase proportionally with underlying disease severity.
- DNA methylation analysis of *CADM1*, *MAL* and *miR124-2* in cervical scrapes has the capacity to broaden its use through the detection of a substantial subset of endometrial carcinomas.

## REFERENCES


1 Ferlay J, Shin H-R, Bray F, *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. cancer.* 2010;127:2893–917.

2 Zhou J, Tomashefski JF, Sawady J, *et al.* The diagnostic value of the ThinPrep pap test in endometrial carcinoma: a prospective study with histological follow-up. *Diagn. Cytopathol.* 2013;41:408–12.

3 Zhou J, Tomashefski JF, Khiyami A. Diagnostic value of the thin-layer, liquid-based Pap test in endometrial cancer: a retrospective study with emphasis on cytomorphologic features. *Acta Cytol.* 2007;51:735–41.

4 Guidos BJ, Selvaggi SM. Detection of endometrial adenocarcinoma with the ThinPrep Pap test. *Diagn. Cytopathol.* 2000;23:260–65.

5 Schorge JO, Hossein Saboorian M, Hynan L, *et al.* ThinPrep detection of cervical and endometrial adenocarcinoma: a retrospective cohort study. *Cancer* 2002;96:338–43.

6 Kim S-S, Suh D-S, Kim K-H, *et al.* Clinicopathological significance of atypical glandular cells on Pap smear. *Obstet. Gynecol. Sci.* 2013;56:76–83.

7 Muñoz N, Bosch FX, Castellsagué X, *et al.* Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int. J. Cancer* 2004;111:278–85.

8 Arbyn M, Ronco G, Anttila A, *et al.* Evidence Regarding Human Papillomavirus Testing in Secondary Prevention of Cervical Cancer. *Vaccine* 2013;31:6266.

9 Ronco G, Giorgi-Rossi P, Carozzi F, *et al.* Results at recruitment from a randomized controlled trial comparing human papillomavirus testing alone with conventional cytology as the primary cervical cancer screening test. *J. Natl. Cancer Inst.* 2008;100:492–501.

10 Gok M, Heideman DAM, Kemenade FJ van, *et al.* HPV testing on self collected cervicovaginal lavage specimens as screening method for women who do not attend cervical screening: cohort study. *BMJ* 2010;340:c1040.

11 Szarewski A, Cadman L, Mesher D, *et al.* HPV self-sampling as an alternative strategy in non-attenders for cervical screening - a randomised controlled trial. *Br. J. Cancer* 2011;104:915–20.

12 Giorgi Rossi P, Marsili LM, Camilloni L, *et al.* The effect of self-sampled HPV testing on participation to cervical cancer screening in Italy: a randomised controlled trial (ISRCTN96071600). *Br. J. Cancer* 2011;104:248–54.

13 Dijkstra M, Niekerk D van, Rijkaart D, *et al.* Primary hrHPV DNA testing in Cervical Cancer screening: how to manage screen positive women? A POBASCAM Trial sub study. *Cancer Epidemiol. Biomarkers Prev.* 2014;23:55–63.

14 Rijkaart DC, Berkhof J, Kemenade FJ van, *et al.* Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *Int. J. Cancer* 2012;130:602–10.

15 Steenbergen RDM, Snijders PJF, Heideman DAM, *et al.* Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat. Rev. Cancer* 2014;14:395–405.

16 Saavedra KF, Brebi PS, Roa JC. Epigenetics Alterations in Preneoplastic and Neoplastic Lesions of the Cervix. *Clin. Epigenetics* 2012;4:13.

17 Steenbergen RDM, Kramer D, Braakhuis BJM, *et al.* TSLC1 Gene Silencing in Cervical Cancer Cell Lines and Cervical Neoplasia. *J. Natl. Cancer Inst.* 2004;96:294–305.

18 Wilting SM, Boerdonk RAA van, Henken FE, *et al.* Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol. Cancer* 2010;9:167.

19 Overmeer RM, Henken FE, Snijders PJF, *et al.* Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J. Pathol.* 2008;1:388–97.

20 Overmeer RM, Henken FE, Bierkens M, *et al.* Repression of MAL tumour suppressor activity by promoter methylation during cervical carcinogenesis. *J. Pathol.* 2009;219:327–36.

21 Eijsink JJH, Lendvai Á, Deregowski V, *et al.* A four-gene methylation marker panel as triage test in high-risk human papillomavirus positive patients. *Int. J. Cancer* 2012;130:1861–69.

22 Tao MH, Freudenheim JL. DNA methylation in endometrial cancer. *Epigenetics* 2010;5:491–98.

23 Fukami T, Fukuhara H, Kuramochi M, *et al.* Promoter methylation of the TSLC1 gene in advanced lung tumors and various cancer cell lines. *Int. J. Cancer* 2003;107:53–59.

24 Balch C, Matei DE, Huang TH-M, *et al.* Role of epigenomics in ovarian and endometrial cancers. *Epigenomics* 2010;2:419–47.

25 Wentzensen N, Bakkum-Gamez JN, Killian JK, *et al.* Discovery and validation of methylation markers for endometrial cancer. *Int. J. Cancer* 2014;in press.

26 Overmeer RM, Louwers JA, Meijer CJLM, *et al.* Combined CADM1 and MAL promoter methylation analysis to detect (pre-)malignant cervical lesions in high-risk HPV-positive women. *Int. J. cancer* 2011;129:2218–25.

27 Hesselink AT, Heideman DAM, Steenbergen RDM, *et al.* Combined promoter methylation analysis of CADM1 and MAL: an objective triage tool for high-risk human papillomavirus DNA-positive women. *Clin. cancer Res.* 2011;17:2459–65.

28 Hesselink AT, Heideman DAM, Steenbergen RDM, *et al.* Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. *Int. J. Cancer* 2014;135:880–86.

29 Verhoef VMJ, Bosgraaf RP, Kemenade FJ van, *et al.* Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): a randomised controlled non-inferiority trial. *Lancet Oncol.* 2014;15:315–22.

30 Bierkens M, Hesselink AT, Meijer CJLM, *et al.* CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease. *Int. J. cancer* 2013;133:1293–99.

31 Strooper LMA De, Hesselink AT, Berkhof J, *et al.* Combined CADM1/MAL methylation and cytology testing for colposcopy triage of high-risk HPV-positive women. *Cancer Epidemiol. biomarkers Prev.* 2014;in press.

32 Bulk S, Kemenade FJ van, Rozendaal L, *et al.* The Dutch CISOE-A framework for cytology reporting increases efficacy of screening upon standardisation since 1996. *J. Clin. Pathol.* 2004;57:388–93.

33 Brule AJC Van Den, Pol R, Fransen-Daalmeijer N, *et al.* GP5+ / 6+ PCR followed by Reverse Line Blot Analysis Enables Rapid and High-Throughput Identification of Human Papillomavirus Genotypes. *J. Clin. Microbiol.* 2002;40:779–87.

34 Federation of Biomedical Scientific Societies. Human Tissue and Medical Research: Code of conduct for responsible use (2011) http://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf (date accessed: 24 june 2014)

35 Snellenberg S, Strooper LMA De, Hesselink AT, *et al.* Development of a multiplex methylation-specific PCR as candidate triage test for women with an HPV-positive cervical scrape. *BMC Cancer* 2012;12:551.

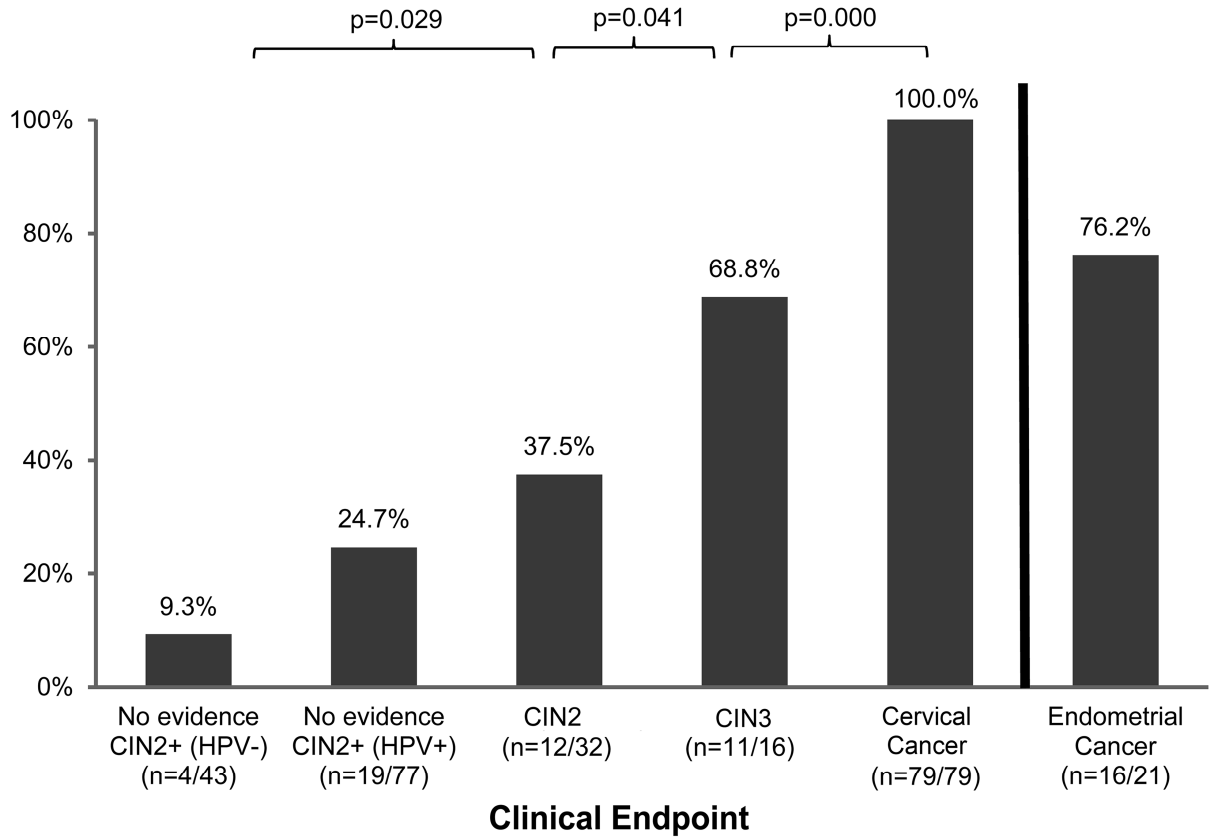
36 Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 2008;3:1101–08.

37 Li N, Franceschi S, Howell-Jones R, *et al.* Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. *Int. J. Cancer* 2011;128:927–35.

38 Kinde I, Bettegowda C, Wang Y, *et al.* Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. *Sci. Transl. Med.* 2013;5:167ra4.

# FIGURE

**Figure 1: *CADM1, MAL* and *miR124-2* methylation in cervical scrapes in relation to underlying lesion type.**




Shown is the fraction of methylation positive cases as determined by multiplex qMSP (y-axis) in relation to the lesion type (x-axis).