Performance of CADM1/MAL-methylation analysis for monitoring of women treated for high-grade CIN

M.H. Uijterwaal[1,a,b], M. van Zummeren[1,a], M. Kocken[a], R. Luttmer[a], J. Berkhof[c], B.I. Witte[c], W.M. van Baal[b], G.C.M. Graziosi[d], R.H.M. Verheijen[e], T.J.M. Helmerhorst[f], D.K.E. van Dijken[g], J.W.M. Spruijt[h], F.J. van Kemenade[i], N. Fransen-Daalmeijer[a], M. Bekker-Lettink[a], D.A.M. Heideman[a], P.J.F. Snijders[a], R.D.M. Steenbergen[a], C.J.L.M. Meijer[a]

[1] Both authors contributed equally to this work

[a] Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

[b] Department of Obstetrics and Gynecology, Flevo Hospital, Almere, The Netherlands

[c] Department of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands

[d] Department of Obstetrics and Gynecology, Sint Antonius Hospital, Nieuwegein, The Netherlands

[e] Department of Gynecological Oncology, UMCU Utrecht Cancer Center, The Netherlands

[f] Department of Obstetrics and Gynecology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

[g] Department of Obstetrics and Gynecology, Onze Lieve Vrouwen Gasthuis West, Amsterdam, The Netherlands

[h] Department of Obstetrics and Gynecology, VU University Medical Center, Amsterdam, The Netherlands

[i] Department of Pathology, Erasmus University Medical Center, Rotterdam, The Netherlands

Corresponding author:

Chris J.L.M. Meijer, VU University Medical Center, Department of Pathology, P.O. Box 7057, 1007 MB Amsterdam, the Netherlands, telephone: +31 20 444 4070, e-mail: cjlm.meijer@vumc.nl

Key words:

Post-Treatment; Human Papillomavirus DNA Test; Cytology; DNA Methylation; Recurrent High-Grade Cervical Intraepithelial Neoplasia; Re-LLETZ

Highlights:

- First study to show the performance of methylation marker analysis for detecting recurrent high-grade CIN lesions (rCIN2/3) in post-treatment monitoring
- CADM1/MAL-methylation is associated with the severity of recurrent disease
- Post-treatment monitoring by CADM1/MAL-methylation analysis identifies women with an increased risk of rCIN2/3

This article is published in Gynecologic Oncology *(2016)* and available online 16th of July 2016:

doi:10.1016/j.ygyno.2016.07.089

Abstract

Introduction: Recent studies have shown that CADM1/MAL-methylation testing detects high-grade CIN lesions with a high short-term progression risk for cervical cancer. Women treated for CIN2/3 are at risk of post-treatment disease, representing either persistent (incompletely treated) or incident (early onset) lesions. Here, we evaluated CADM1/MAL-methylation analysis as potential tool for detecting recurrent high-grade CIN lesions (rCIN2/3).

Methods and materials: A multicenter prospective clinical cohort study was conducted among 364 women treated for CIN2/3. Cervical scrapes were taken prior to treatment, and six and 12 months post-treatment and tested for cytology, hrHPV (plus genotype) and CADM1/MAL-methylation. When at six months either of these tests was positive, a colposcopy-directed biopsy was obtained. At 12 months, all women underwent an exit-colposcopy with biopsy. In case of rCIN2/3, re-treatment was done.

Results: We found 28 rCIN2 (7.7%) and 14 rCIN3 (3.8%), resulting in a total recurrence rate of 11.5%. All 14 women with rCIN3 and 15/28 (54%) with rCIN2 showed hrHPV type-persistence. Of these, 9/14 (64%) rCIN3 and 8/15 (53%) rCIN2 were CADM1/MAL-methylation positive. All incident rCIN2, characterized by hrHPV genotype-switch, were CADM1/MAL-methylation negative. All three carcinomas found after re-treatment were CADM1/MAL-methylation positive. CADM1/MAL-methylation positivity at both baseline and follow-up significantly increased the risk of ≥rCIN3 (from 0.7% to 18.4%), and ≥rCIN2 (from 8.2% to 36.8%), compared to a consistently CADM1/MAL-methylation negative result (p-value: <0.001).

Conclusion: Post-treatment monitoring by CADM1/MAL-methylation analysis identifies women with an increased risk of rCIN2/3. Our results confirm previous data indicating that CADM1/MAL-methylation analysis provides a high reassurance against cancer.

1. Introduction

Long-term follow-up studies have indicated that women who undergo treatment for cervical intra-epithelial neoplasia grade 2 or 3 (CIN2/3) have an increased long term risk of recurrent CIN2 lesions or worse (≥rCIN2) compared to the general population.[1,2] Because of this long-term risk, gynecologists in many countries use surveillance strategies in order to detect recurrent disease. These surveillance strategies vary greatly in content and length. In many Western countries, women are monitored by cervical cytology at six, 12 and 24 months after treatment, and are referred to the routine screening program after three subsequent negative scrapes.[3] Recently, it has been shown that the addition of high-risk human papillomavirus (hrHPV) DNA testing to cytology at six months after treatment dramatically increases the sensitivity for ≥rCIN2.[2,4] When at six months both test results are negative, testing at 12 months can be omitted without an increased ≥rCIN2 risk.[2]

Recurrent CIN2/3 lesions are known to represent a group of heterogeneous diseases, comprising persistent lesions resulting from residual (i.e. incompletely treated) disease with persistence of the same HPV genotype, and incident (i.e. early onset) lesions. Incident lesions can either result from an infection with a hrHPV type different from the original type in the resected CIN2/3 lesion (incident rCIN), or a re-infection with the same hrHPV type as the original one.[5] Morphologically, persistent rCIN2/3 lesions cannot be distinguished from incident counterparts, and all rCIN2/3 are therefore treated. This may lead to overtreatment, which is (especially) harmful for women in their reproductive age since it may lead to adverse pregnancy outcomes.[6] For a more tailored management of women diagnosed with recurrent disease, studies on biomarkers that can detect women in immediate need of re-treatment are warranted. Ideally these biomarkers should distinguish persistent rCIN2/3 lesions, with likely a high short-progression risk to cancer (so-called advanced lesions) and therefore in need of immediate treatment, from incident, so-called early rCIN2/3 lesions, with a likely low short-term progression risk to cancer and for which a more conservative approach is acceptable.

Potential biomarker tests that can discriminate between early and advanced stages of cervical disease involve tests assessing DNA promoter methylation of certain host cell tumor suppressor genes involved in cervical carcinogenesis.[7–10] Cell adhesion molecule 1 (*CADM1*) and myelin and lymphocyte (*MAL*) belong to the most frequently methylated genes in cervical carcinoma.[8,11] Silencing of these genes by DNA promoter methylation is a common and functionally relevant event in cervical cancer development.[12–14] We have previously shown that the extent of DNA promoter methylation of *CADM1* and *MAL* genes increases with the severity of cervical disease and that these epigenetic changes are considered to reflect the presence of a more advanced high-grade CIN lesion with a longer duration of existence.[9,11,15] This is supported by the finding that levels of *CADM1* and *MAL* promoter methylation are extremely high in cervical cancer and significantly increased in CIN3 lesions of women with a hrHPV infection that has persisted over a long time period (≥5 years) compared to early onset CIN3 lesions resulting from a recently acquired hrHPV infection.[7]

In this study, we have evaluated the performance of CADM1/MAL-methylation analysis as a potential tool to monitor women treated for CIN2/3 for recurrent disease.

2. **Methods and Materials**

2.1. Study population

This study was designed as a multicenter prospective clinical cohort study conducted in six outpatient clinics in The Netherlands. The study flowchart is depicted in Figure 1. The participating centers were VU University Medical Center, Erasmus MC University Medical Center Rotterdam, University Medical Center Utrecht, Flevo Hospital Almere, Sint Antonius Hospital Nieuwegein and Onze Lieve Vrouwen Gasthuis West Amsterdam. Between April 2010 and June 2012, all women aged 18 years and older, who were scheduled for treatment of a biopsy confirmed CIN2/3 lesion by Large Loop Excision of the Transformation Zone (LLETZ), were asked to participate in this study. Also women who were treated directly without prior biopsy and in whom the presence of a CIN2/3 or an adenocarcinoma *in situ* (AIS) lesion was confirmed in the LLETZ specimen (see-and-treat), were enrolled. The study was approved by the Medical Ethical Committee (METC) of the VU medical center (METC-VUmc 2009/285) and endorsed by all other participating clinics and registered in The Netherlands Trial Register (NTR1964). All women gave written informed consent prior to any study procedure.

2.2. Study procedures

Prior to treatment, a cervical scrape was taken with a Cervex brush® (Rovers medical devices B.V., Oss, the Netherlands). Scrapes were stored in Thinprep (Hologic, Malborough MA, USA) and used for hrHPV detection and CADM1/MAL-methylation analysis. At six months post-treatment, two cervical scrapes were obtained from each study participant. The first specimen was collected for cytology according to local protocols (conventional slide or stored in Thinprep) of the participating hospital. The second specimen was collected in Thinprep medium for hrHPV and CADM1/MAL-methylation analysis. HrHPV and methylation tests were performed in a reference laboratory (Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands). Women were referred for colposcopy when at least one of the three tests was positive, i.e., borderline or mild dyskaryosis or worse cytology (≥BMD; comparable with ≥ASC-US; see classification details below) and/or hrHPV positive and/or positive for CADM1/MAL-methylation. At 12 months post-treatment, one cervical scrape was collected in Thinprep medium and

analyzed in the reference laboratory for cytology, hrHPV presence and methylation status. After taking the cervical scrape, an exit-colposcopy with mandatory biopsy was also performed at this 12 month visit. Primary outcome measure was ≥rCIN3 and ≥rCIN2.

2.3. Cytology reading

The Dutch CISOE-A classification was used to report the cytological results. The results can easily be translated into the Bethesda 2001 classification.[16] Cytological results were grouped as normal, BMD or ≥BMD. Cytology results classified in CISOE-A as ≥S2, E3 or O3, comparable to ≥BMD were considered abnormal.

2.4. DNA isolation and hrHPV DNA testing

DNA was isolated from cervical scrapes using the Nucleo-Spin 96 Tissue kit (Macherey-Nagel, Germany) and Microlab Star robotic system (Hamilton, Germany), according to the recommendations of the manufacturer. Detection and genotyping of HPV was performed using the clinically validated GP5+/6+-PCR with an enzyme-immuno assay (EIA) readout followed by reverse line blot analysis of EIA positive cases to identify the hrHPV genotype (i.e. HPV 16,18,31,33,35,39,45,51,52,56,58,59,66 and/or 68).[17,18] In case of a positive hrHPV EIA test but a negative reverse line blot genotyping result, the sample was considered to harbor an unknown HPV (sub) type or variant denoted as 'HPV X'.

2.5. Quantitative methylation-specific PCR (qMSP)

Isolated DNA was modified by bisulfite treatment using the EZ DNA Modification Kit (Zymo Research, Baseclear, Leiden, The Netherlands) as described previously.[13] Quantitative Methylation Specific PCR (qMSP) analysis for CADM1 and MAL was performed on an ABI 7500 real-time PCR-system (Applied Biosystems, USA) as described previously, using β-actin (ACTB) as a reference.[19] All qMSP assays were run in separate reactions. Cycle threshold (Ct) values were measured at a fixed fluorescence threshold (i.e., 0.01). Ct ratios between the Ct values of the β-actin and target were used to quantify the level of methylation, as calculated by the following formula: 2[Ct (β-actin) − Ct (target)] × 100. Ct signals of more than 40 were considered to represent a negative test result, whereas samples with Ct values for β-actin that were higher than 32 were considered invalid and therefore excluded from analysis because of an

indication of poor DNA quality or poor recovery after bisulfite treatment.[19] The CADM1 and MAL qMSP Ct ratio thresholds that were used to score the CADM1/MAL-methylation test positive were those that in a previous study gave rise to a ≥CIN3 specificity of ≥70%.[19] The CADM1/MAL-methylation test is considered positive if either one or both markers score positive.

2.6. Colposcopic examination

During colposcopic assessment, biopsies were taken from all suspicious areas. In case no suspicious lesion was seen, or if the transformation zone could not be fully visualized (in which the colposcopy was considered 'unsatisfactory'), a biopsy from a random location and/or endocervical curettage was performed. An exit-colposcopy was performed at the last study visit in all subjects to exclude any histological abnormalities. Histological specimens were graded as CIN grade 0 (no dysplasia), 1,2,3, or invasive cancer.[20] For the purpose of this study AIS was counted as CIN3.

2.7. Study outcomes

Women reached the primary study-endpoint when they had ≥rCIN2 in the biopsy specimen taken during colposcopy at six months post-treatment or at the exit-colposcopy at 12 months. Women who developed recurrent disease (rCIN2/3) were re-treated by re-LLETZ or conisation and followed-up according to present guidelines.[3] In January 2014, we verified follow-up data of all women with post-treatment disease from local hospital databases. When histology results from re-treatment (either re-LLETZ or conisation) were available, these were taken as secondary endpoint.

2.8. Statistical analysis

The sample size was set at 360 assuming that 10% of treated women would present with ≥rCIN2, and taking 5% drop out into account.

2.8.1. Cross-sectional analysis

The association between hrHPV status, CADM1/MAL-methylation status, and histology groups (LLETZ result at baseline) was assessed using the χ^2-test. Also for assessing the association between cytology, hrHPV, CADM1/MAL-methylation, and the risk for ≥rCIN3 and ≥rCIN2, the χ^2-test was used.

2.8.2. Longitudinal analysis

For comparisons between the CADM1/MAL-methylation results at baseline and histological outcome of recurrent disease, we used the study endpoint of ≥rCIN2 at six or 12 months. For evaluation of the methylation status over six and 12 months in relation to the study endpoint we used the results of the scrape taken directly prior to the detected rCIN. For comparison of CADM1/MAL-methylation results between incident and persistent hrHPV infections in rCIN2/3, a Fisher's exact test was used. A hrHPV infection was considered indisputably incident when a genotype-switch had occurred between baseline treatment and time of recurrent disease (time frame six or 12 months). A hrHPV infection was scored as persistent when the hrHPV type detected at baseline and at time of recurrent disease (time frame six or 12 months) were the same.

To compare the baseline and follow-up CADM1/MAL-methylation results, the absolute risk of ≥rCIN3 and ≥rCIN2 in baseline and follow-up in CADM1/MAL-methylation negative and positive groups was calculated by using a t-test. The methylation status at follow-up, either at six or 12 months indicated as 'last test negative/positive' was determined at the moment of rCIN detection.

Adjusted endpoints were used in the cross-sectional analyses at six months and 12 months post-treatment. When no histology was available at these time points, women who were cytology negative and hrHPV negative were scored as adjusted endpoint ≤CIN1. When either cytology or hrHPV DNA testing was positive and no histology was available, these results were scored as 'no endpoint'. Concerning the re-treatment results, when no re-LLETZ histology was available, a double normal cytology result or a normal cytology result in combination with HPV negativity in the follow-up of the rCIN was considered adjusted endpoint ≤CIN1. All analysis were done in IBM SPSS version 20 (International Business Machines Corp., Armonk, New York, USA).

3. Results

3.1. Study population

Between March 1st, 2010 and April 1st, 2012, 387 women treated by a LLETZ-procedure were recruited as presented in the study flowchart in Figure 1. The mean age at baseline (moment of treatment) was 36.2 years (range 19-59 years). None of the women received prophylactic HPV vaccination prior to treatment or during follow-up. Of the treated women, 339 women (87.6%) had a CIN2/3 diagnosed on colposcopy directed biopsy and 48 women (12.4%) were treated without prior biopsy with CIN2/3 or AIS confirmed in LLETZ. After the LLETZ-procedure, 23 women (5.9%) were excluded. Exclusion was based on the presence of squamous cell carcinoma (SCC) in the LLETZ-tissue (n=3/23; 13.0%) or non-evaluable LLETZ-tissue (n=2/23; 8.7%). Furthermore, 16/23 women (69.6%) were lost to follow-up and another 2/23 women (8.7%) underwent a hysterectomy at their own request within two months after the LLETZ-procedure. This resulted in a study cohort of 364 women. The histology results from the LLETZ tissue specimens showed 38 CIN0, 42 CIN1, 122 CIN2, 160 CIN3 and two AIS lesions.

3.2. Cross-sectional analysis

3.2.1. Baseline results

From eight of 364 women no cervical scrape prior to treatment was available, resulting in 356 women with a hrHPV and/or CADM1/MAL-methylation marker test result at baseline (Table 1). Of those, 321 women (90.2%) tested positive for hrHPV. Among these women, 148 (46.1%) were diagnosed on LLETZ histology with a ≥CIN3 lesion and 262 (81.6%) with ≥CIN2. Of the hrHPV positive women, 182 (56.7%) had hrHPV genotype 16 or 18, and 139 (43.3%) were positive for other (non16/18) hrHPV type(s). One-hundred-sixty women (44.9%) had a positive CADM1/MAL-methylation test at baseline, of whom 94 (58.8%) were diagnosed on LLETZ histology with a ≥CIN3 and 133 (83.1%) with ≥CIN2. There was a significant relation between increasing hrHPV positivity rate and increasing severity of disease: hrHPV positivity ranged from 75.6% (59/78) in women with ≤CIN1, to 95.8% (114/119) in women with CIN2, and 93.1% (148/159) in women with CIN3 (p-value <0.001). Also CADM1/MAL-methylation was significantly

associated with severity of disease (≤CIN1: 27/78; 35.0%, CIN2: 39/119; 32.8%, and CIN3: 94/159; 59.1%) (p-value <0.001).

3.2.2. Six months post-treatment

Six months post-treatment, 364 women were supposed to have their first follow-up visit. Nine women did not show up and five women were pregnant, resulting in 350 women having their first follow-up visit. Among those, 160 women (45.7%) tested negative for all markers and were re-invited at 12 months. The remaining 190 women (54.3%) had at least one positive test result (i.e., cytology, hrHPV and/or CADM1/MAL-methylation positive). According to study protocol, all but 11 women underwent colposcopy-directed biopsy, resulting in the following histology outcome: 157 ≤CIN1, 15 CIN2 and seven CIN3. Thus, 22 of these women reached the study endpoint. All women with ≤CIN1 and those who had no biopsy taken, were followed up at 12 months.

Of the 11 women without histology, one was scored as adjusted ≤CIN1 endpoint given a cytology-negative and hrHPV-negative cervical scrape, and 10 as 'no endpoint', resulting in 340 women for 6-month cross-sectional analysis (Table 1). Among these 340 women, 79 women (23.2%) had a positive cytology result; six (7.6%) of them had ≥rCIN3 and 17 (21.5%) had ≥rCIN2. HrHPV positivity was found in 114 women (33.5%), including seven women (6.1%) with ≥rCIN3 and 20 women (17.5%) with ≥rCIN2 (p-value <0.001). HrHPV genotype 16 or 18 was found in 54 women (47.4%) and a non-16/18 hrHPV genotype in 58 women (50.9%). A total of 82 from 340 women (24.1%) tested positive for CADM1/MAL-methylation. Among them, four (4.9%) were diagnosed with ≥rCIN3 on biopsy and 11 women (13.4%) with ≥rCIN2. The prevalence of ≥rCIN2 was higher in the CADM1/MAL-methylation positive group compared to the CADM1/MAL-methylation negative group (p-value 0.003). A positive test result for cytology, hrHPV DNA and CADM1/MAL-methylation was associated with a significantly higher risk of ≥rCIN2 compared to a negative test result (p-values <0.05). Detailed information per women can be found in the Supplementary Table 1.

3.2.3. Twelve months post-treatment

At six months post-treatment 22 women reached study ≥rCIN2 endpoint, leaving 342 women who were supposed to have their second follow-up visit at 12 months post-treatment. Nineteen of them did not show up at 12 months, five were pregnant, two emigrated, one deceased and three underwent a hysterectomy. Of the resulting 312 evaluable women, all but ten women received colposcopy including an exit biopsy. Histology revealed 282 ≤CIN1, 13 CIN2 and seven CIN3. Of the ten women without histology, seven were scored as adjusted ≤CIN1 endpoint given a cytology-negative and hrHPV-negative cervical scrape, and three as 'no endpoint'. Moreover, one woman with rCIN2 had a protocol violation: only an exit biopsy was taken without a cervical scrape. Valid test results (either cytology, hrHPV and/or methylation marker testing) were available from 308 women.

At 12 months post-treatment a positive test result for either cytology, hrHPV or CADM1/MAL-methylation was associated with a significantly increased risk of ≥rCIN2 in the exit-biopsy (Table 1). For cytology, 95 women (30.9%) tested positive of whom seven (7.4%) had ≥rCIN3 and 17 (17.9%) ≥rCIN2 in the exit-biopsy. Seventy-four women (24.2%) tested positive for hrHPV, of whom seven (9.5%) were diagnosed with ≥rCIN3 and 15 (20.3%) with ≥rCIN2. Of the 74 hrHPV positive women, 30 women (41.7%) tested positive for hrHPV genotypes 16 and/or 18 and 42 women (58.3%) tested positive for hrHPV genotypes non 16 and/or 18. Methylation positivity was found in 60 women (19.6%), of whom five (8.3%) were diagnosed with ≥rCIN3 on exit-biopsy and 11 (18.3%) with ≥rCIN2.

3.3. Longitudinal analysis

3.3.1. Recurrent CIN lesions

Over a period of in total 12 months follow-up, 28 CIN2 lesions (7.7%) and 14 CIN3 lesions (3.8%) were detected in this post-treatment cohort, amounting to a CIN2/3 recurrence rate of 11.5%. Of the 28 women with rCIN2 in their biopsies, the re-LLETZ specimen contained ≤CIN1 in 18 women (64%), CIN2 in two (7%), CIN3 in four (14%). Four women (14%) did not undergo a re-LLETZ but had a cervical scrape read as BMD at follow-up (no endpoint). Of the 14 women with rCIN3 biopsies, the re-LLETZ specimen contained, ≤CIN1 in five women (36%), CIN2 in three (21%), CIN3 in three (21%) and three women had SCC of the cervix (21%). The relation between the methylation status on the scrapes taken prior to biopsy

and histology results is shown in Table 2. CADM1/MAL-methylation negative scrapes often yielded a ≤CIN1 lesion (75%) in the re-LLETZ tissue. Furthermore, all three cervical carcinomas found in the re-LLETZ were preceded by a CADM1/MAL-methylation positive scrape.

3.3.2. Incident versus persistent infections

In Table 3 the methylation marker status in rCIN2, rCIN3 and in the overall rCIN group, stratified according to HPV genotype-switch in follow-up to define incident or persistent infection is shown. Of all rCIN2 lesions, five showed an incident infection with type switch and 15 revealed a persistent infection. All rCIN3 lesions showed type persistence. Of women with persistent infections, 9/14 rCIN3 (64%) and 8/15 rCIN2 (53%) were CADM1/MAL-methylation positive. All five recurrent lesions with incident infections were CADM1/MAL-methylation negative. The relationship between hrHPV type in rCIN2/3 and the CADM1/MAL-methylation status is depicted in Supplementary Table 2.

3.3.3. Pre- and post-treatment methylation marker status

For longitudinal analysis, the methylation status at baseline was combined with methylation status at follow-up and related to the risk of ≥rCIN3 and ≥rCIN2 (Table 4). Compared to methylation marker negative test results at both baseline and follow-up, methylation positivity at both baseline and follow-up significantly increased the risk of ≥rCIN3 from 0.7% to 18.4%, and for ≥rCIN2 from 8.2% to 36.8% (p-values <0.001). Furthermore, within the group of women with a positive CADM1/MAL-methylation test at baseline, the ≥rCIN3 risk was significantly higher for those that were CADM1/MAL-methylation positive at follow-up (18.4%) compared to those that were CADM1/MAL-methylation negative at follow-up (3.7%, p-value <0.007). The same figures were seen for ≥rCIN2 (i.e., 36.8% versus 6.4%, p-value <0.001).

4. Discussion

Various studies have analyzed the use of methylation markers as triage tool for HPV-positive women in cervical cancer screening.[8–10] However, to the best of our knowledge the use of a methylation marker analysis for post-treatment monitoring has not been evaluated before.

In this study, we evaluated the performance of CADM1/MAL-methylation analysis for monitoring women treated for high-grade CIN. We assessed the cross-sectional and longitudinal performance, and the relationship with persistence of recurrent disease using persistent HPV infections as a surrogate marker. The rCIN2/3 percentage of 11.5% after 12 months follow-up is in agreement with previous studies.[1,2] All 14 rCIN3 were associated with a persistent hrHPV infection, and nine thereof (64%) were CADM1/MAL-methylation positive. Of 28 rCIN2 lesions, at least five were incident lesions as evidenced by a hrHPV type-switch, and these were all negative for CADM1/MAL-methylation. Of the 15 rCIN2 with a persistent hrHPV infection, eight (53%) were positive for CADM1/MAL-methylation. CADM1/ MAL-methylation was found significantly associated with severity of disease in this cohort of women treated for CIN2/3. Moreover, cross-sectionally, a positive CADM1/MAL-methylation test result at baseline, six months or 12 months post-treatment was associated with an increased risk of ≥rCIN3 and ≥rCIN2. In the longitudinal analysis, a positive CADM1/MAL-methylation test at baseline and in follow-up significantly increased the risk of ≥rCIN3 from 0.7% to 18.4%, and from 8.2% to 36.8% for ≥rCIN2. Cases of early carcinoma (FIGO stage IAI) (n=3) were detected upon re-treatment (at six months) only within the CADM1/MAL-methylation positive group. Of notice, one of these carcinomas was negative by cytology. Upon re-treatment, CADM1/MAL-methylation negative women were often associated with a ≤CIN1 lesion (75%) in the re-LLETZ tissue. Altogether, post-treatment monitoring by CADM1/MAL-methylation analysis identifies women at an increased risk of rCIN2/3.

Post-treatment disease consists of a heterogeneous group of rCIN lesions. We differentiated on the basis of the type of hrHPV infection (type-switch or type-persistence) between recurrent incident lesions and persistent lesions, and found that all five incident rCIN lesions were CADM1/MAL-methylation negative. On the other hand, not all persistent rCIN2/3 lesions scored positive for CADM1/MAL-methylation.

However, it should be noted that some rCIN2/3 lesions that had be classified as persistent in fact might have been incident lesions caused by reinfection of the cervical epithelium with the same hrHPV genotype that was present in the primary lesion. This subset may well be associated with a negative CADM1/MAL-methylation test result. Alternatively an infection with a variant of the same HPV genotype may have been present.

At present three times cytology at six, 12, and 24 months is the standard follow-up procedure for rCIN2/3 monitoring.[3] Recently it has been shown that by adding hrHPV testing to cytology the 12 months follow-up visit can be omitted in case of a negative co-test at six months.[2] Our results show that hrHPV and/or cytology co-testing detects more rCIN2/3 than a CADM1/MAL-methylation test (Table 1). However, based on our findings the CADM1/MAL-methylation test can be of value in preventing overtreatment of women with a rCIN lesion. Women with a CADM1/MAL-methylation negative scrape had a very low risk for ≥rCIN3. As reported in earlier work.[15] and observed in small numbers in this study, a negative CADM1/MAL-methylation test result reassures against cervical cancer. Collectively, these data support a less aggressive management in clinical practice for women with a negative CADM1/MAL-methylation test. Preservation of the cervix on guidance of a negative CADM1/MAL-methylation test result may especially be important for women with a child wish. An additional advantage of a methylation test lies in its ability to be applied on self-sampled cervico-vaginal specimens, which can simplify the follow-up procedure.[9,21,22]

A limitation of our study is the length of the follow-up period of 12 months. To evaluate the use of methylation markers for long-term post-treatment surveillance, further studies with a longer follow-up period are warranted. At the stage of design of this study CADM1 and MAL were the most promising methylation markers for the detection of cervical (pre)cancer in hrHPV-positive scrapes. In retrospect, it would be very interesting to test recently discovered methylation markers on left-over material of this study population.

In conclusion, these data support the concept that CADM1 and MAL methylation marker testing identifies cervical lesions with a longer duration of existence in need of direct treatment. In a clinical setting, rCIN2/3 monitoring by three times cytology and/or HPV testing is the standard follow-up procedure, but results in overtreatment of early onset rCIN2/3 lesions, which is cumbersome for young women at child bearing age. In those women the use of a positive CADM1/MAL-methylation test can help to identify which women have an increased risk for rCIN2/3 in need of treatment, whereas a negative test would support a surveillance (wait and see) policy.

Acknowledgments

We gratefully acknowledge all women participating in the SIMONATH trial. Furthermore, we would like to thank the research staff and (cyto-)technicians of the unit of Molecular Pathology VUmc. We are grateful for the active cooperation of the teams of the outpatient clinics of the department of Obstetrics and Gynaecology of the Erasmus MC University Medical Center Rotterdam, University Medical Center Utrecht, VU University Medical Center Amsterdam, Flevo hospital Almere and the Sint Antonius hospital Nieuwegein, Onze Lieve Vrouwen Gasthuis West Amsterdam.

Author contributions

CJLMM is principal investigator of the SIMONATH study. MHU, MK, WMVB, GCMG, RHMV, TJMH, DKED, JWMS and CJLMM have set up the trial. MHU, MVZ, MK, RL, WMVB, GCMG, RHMV, TJMH, DKED, JWMS, FVJK, NFD, MBZ, DAMH, PJFS, RDMS and CJLMM were involved in data collection. JB and BIW performed the statistical analysis and MHU, MVZ and BW managed the database. MHU, MVZ, PJFS, RS and CJLMM drafted the manuscript. All authors critically reviewed the manuscript and approved the final version. All authors had full access to all of the data in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis. CJLMM affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

Conflicts of interest

All authors have completed the ICMJE uniform disclosure at www.icmje.org/coi_disclosure.pdf and declare that: (1) JB has received consultancy fees from Roche, DDL Diagnostic Laboratory, GlaxoSmithKline and Merck/SPMSD, all JB's fees were collected by his employer; (2) TJMH and RHMV have been principle investigators of a GlaxoSmithKline sponsored study (3) DAMH has been on the speaker's bureau of Hologic/Gen-Probe and serves occasionally on the scientific advisory boards of AMGEN and Pfizer; (4) PJFS, RDMS and CJLMM received research support via their institution by the Dutch Cancer Foundation and CJLMM received research support via his institution by the ERC advanced

grant (5) PJFS has been on the speakers bureau of Roche diagnostics, Gen-Probe, Abbott, Qiagen and Seegene and has been a consultant for Crucell B.V.; (6) PJFS, DAMH, RDMS and CJLMM are minority shareholders of Self-Screen B.V., a spin-off company of VUmc; (7) CJLMM has received speakers fee from GSK, Qiagen, SPMSD/Merck, Roche diagnostics, Menarini and Seegene, served occasionally on the scientific advisory board (expert meeting) of GSK, Qiagen, SPMSD/Merck., Roche and Genticel and has been by occasion consultant for Qiagen and Genticel; (8) CJLMM is minority stock holder of Diassay B.V., a spin-off company of VUmc and until 2014 he held a small number of certificates of shares in Delphi Biosciences, which went into receivership in 2014; (9) Self-Screen B.V. has patents related to the work (i.e., high-risk HPV test and methylation markers for cervical screening); (10) MHU, MVZ, MK, RL, BIW, WMVB, GCMG, DKED, JWS, FJVK, NFD and MBZ do not have any conflicts of interest to disclose.

Trial register

The Netherlands trial register, NTR 1964.

Funding

This study was supported by a grant from The Netherlands Cancer Society (KWF VU 2009-4413) and the European Research Council (ERC advanced 2012-AdG, proposal 322986 Mass-care). These funding sources had no involvement in study design, the collection, analysis and interpretation of data, the writing of the report and the decision to submit the paper for publication.

References

1. Soutter, W. P., Sasieni, P. & Panoskaltsis, T. Long-term risk of invasive cervical cancer after treatment of squamous cervical intraepithelial neoplasia. *Int. J. Cancer* **118,** 2048–2055 (2006).

2. Kocken, M. *et al.* Risk of recurrent high-grade cervical intraepithelial neoplasia after successful treatment: A long-term multi-cohort study. *Lancet Oncol.* **12,** 441–450 (2011).

3. NVOG. Inhoudsopgave. *Natl. Guidel. 'Cervical Intraepithelial Neoplasia'.* (2004). http://oncoline.nl/richtlijn/item/pagina.php?richtlijn_id=220

4. Kocken, M. *et al.* High-risk human papillomavirus testing versus cytology in predicting post-treatment disease in women treated for high-grade cervical disease: A systematic review and meta-analysis. *Gynecol. Oncol.* **125,** 500–507 (2012).

5. Bleeker, M. C. G., Meijer, C. J. L. M. & Berkhof, J. Follow-up after treatment for cervical intraepithelial neoplasia. *BMJ* **345,** 7186–7186 (2012).

6. Kyrgiou, M. *et al.* Fertility and early pregnancy outcomes after treatment for cervical intraepithelial neoplasia : systematic review and meta-analysis. *BMJ* **6192,** 1–17 (2014).

7. Bierkens, M. *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* **133,** 1293–1299 (2013).

8. Wentzensen, N., Sherman, M. E., Schiffman, M. & Wang, S. S. Utility of methylation markers in cervical cancer early detection: Appraisal of the state-of-the-science. *Gynecol. Oncol.* **112,** 293–299 (2009).

9. Steenbergen, R. D. M., Snijders, P. J. F., Heideman, D. A. M. & Meijer, C. J. L. M. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat. Rev. Cancer* **14,** 395–405 (2014).

10. Tornesello, M. L., Buonaguro, L., Giorgi-Rossi, P. & Buonaguro, F. M. Viral and cellular biomarkers in the diagnosis of cervical intraepithelial neoplasia and cancer. *Biomed Res Int* **2013,** 519619 (2013).

11. Overmeer, R. M. *et al.* Combined CADM1 and MAL promoter methylation analysis to detect (pre-)malignant cervical lesions in high-risk HPV-positive women. *Int. J. Cancer* **129,** 2218–2225 (2011).

12. Steenbergen, R. D. M. *et al.* TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. *J. Natl. Cancer Inst.* **96,** 294–305 (2004).

13. Overmeer, R. M. *et al.* Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J. Pathol.* **215,** 388–397 (2008).

14. Overmeer, R. M. *et al.* Repression of MAL tumour suppressor activity by promoter methylation during cervical carcinogenesis. *J. Pathol.* **219,** 327–336 (2009).

15. De Strooper, L. M. A. *et al.* CADM1, MAL and miR124-2 methylation analysis in cervical scrapes to detect cervical and endometrial cancer. *J. Clin. Pathol.* **0,** 1–5 (2014).

16. Bulk, S., Van Kemenade, F. J., Rozendaal, L. & Meijer, C. J. L. M. The Dutch CISOE-A framework for cytology reporting increases efficacy of screening upon standardisation since 1996. *J. Clin. Pathol.* **57,** 388–393 (2004).

17. Van Den Brule, A. J. C. *et al.* GP5+/6+ PCR followed by Reverse Line Blot Analysis Enables Rapid and High-Throughput Identification of Human Papillomavirus Genotypes. *J. Clin. Microbiol.* **40,** 779–787 (2002).

18. Snijders, P. J. F., van den Brule, A. J. C., Jacobs, M. V, Pol, R. P. & Meijer, C. J. L. M. HPV DNA detection and typing in cervical scrapes. *Methods Mol. Med.* **119,** 101–114 (2005).

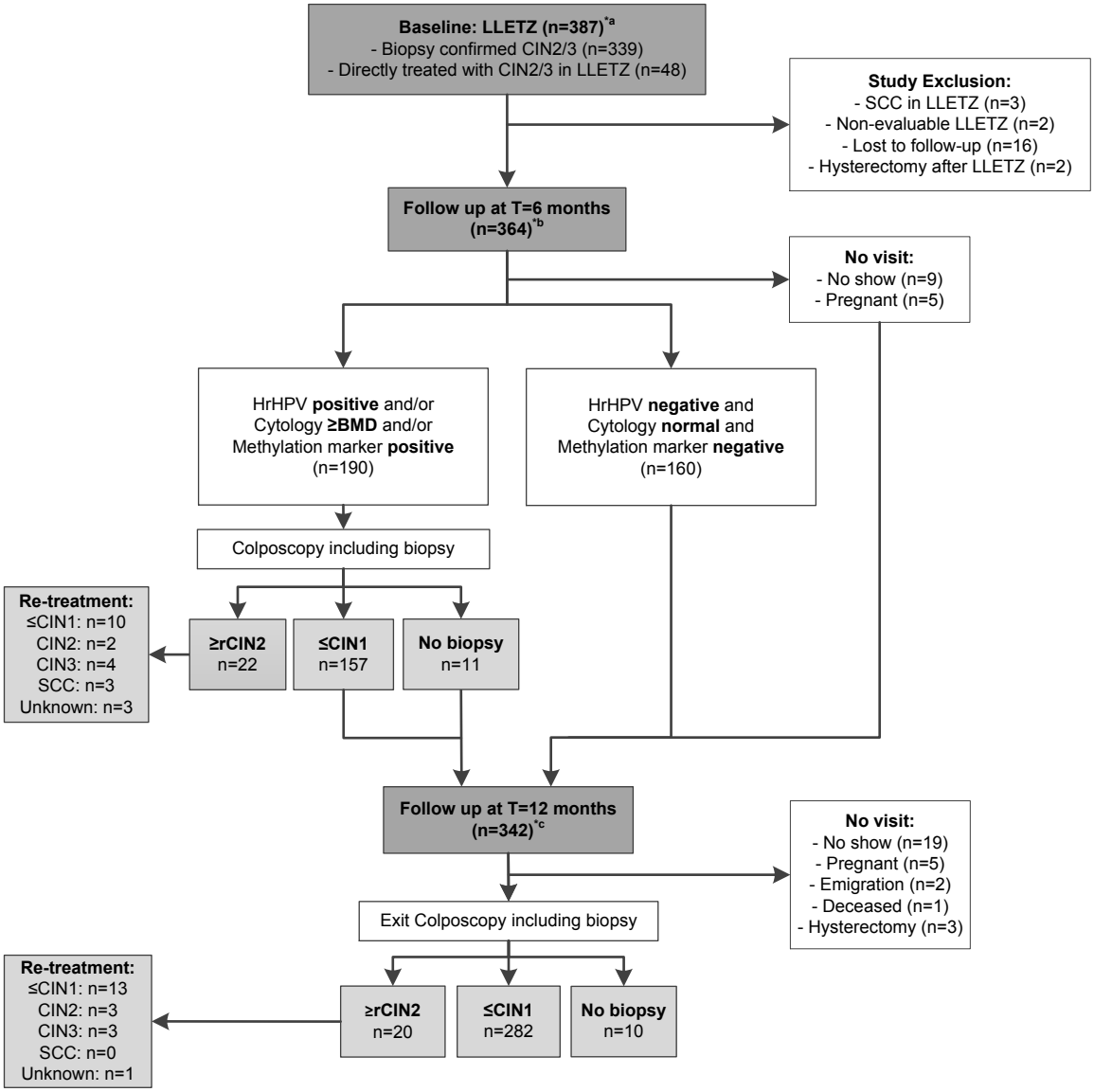
19. Hesselink, A. T. *et al.* Combined promoter methylation analysis of CADM1 and MAL: An objective triage tool for high-risk human papillomavirus DNA-positive women. *Clin. Cancer Res.* **17,** 2459–2465 (2011).

20. Wright, T. C., Kurman, R. & Ferenczy, A. *Precancerous lesions of the Cervix*. *Blaustein's Pathology of the Female Genital Tract* (2002). doi:10.1097/00004347-198809000-00010

21. Bosgraaf, R. P. *et al.* The current position and the future perspectives of cervical cancer screening. *Expert Rev. Anticancer Ther.* **14,** 75–92 (2014).

22. Verhoef, V. M. J. *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.* **15,** 315–322 (2014).

Figure 1: Study flowchart.



*a: At baseline, a cervical scrape was taken previous to the LLETZ for hrHPV and methylation marker testing.
*b: At six months post-treatment, a cervical scrape was taken previous to the biospy for cytology, hrHPV and methylation marker testing.
*c: At 12 months post-treatment, a cervical scrape was taken previous to the exit-biopsy for cytology, hrHPV and methylation marker testing.

CIN, Cervical Intraepithelial Neoplasia; (r)CIN2/3, (recurrent) CIN2 or CIN3; LLETZ, Large Loop Excision Transformation Zone; hrHPV, high risk Human Papilloma Virus; SCC, Squamous Cell Carcinoma

Table 1: Cross-sectional study results.

a) At baseline (n=356)[a]

			LLETZ histology results					LLETZ histology results				
			≤CIN1°		≥rCIN2			≤CIN2°		≥rCIN3		
Cervical scrape results		**Total[#]**	**n**	**%**	**n**	**%**	**p-value**	**n**	**%**	**n**	**%**	**p-value**
HrHPV DNA	Negative	35	19	54.3%	16	45.7%	<0.001	24	68.6%	11	31.4%	0.097
	Positive	321	59	18.4%	262	81.6%		173	53.9%	148	46.1%	
Genotyping	16/18	182	24	13.2%	158	86.8%	0.006	79	43.4%	103	56.6%	<0.001
	Non 16/18	139	35	25.2%	104	74.8%		94	67.6%	45	32.4%	
Methylation	Negative	196	51	26.0%	145	74.0%	0.038	131	66.8%	65	33.2%	<0.001
marker	Positive	160	27	16.9%	133	83.1%		66	41.3%	94	58.8%	

b) At six months post-treatment (n=340)[b]

			Biopsy histology results					Biopsy histology results				
			≤CIN1°		≥rCIN2			≤CIN2°		≥rCIN3		
Cervical scrape results		**Total[#]**	**n**	**%**	**n**	**%**	**p-value**	**n**	**%**	**n**	**%**	**p-value**
Cytology	Normal	261	256	98.1%	5	1.9%	<0.001	260	99.6%	1	0.4%	0.001*
	≥BMD	79	62	78.5%	17	21.5%		73	92.4%	6	7.6%	
HrHPV DNA	Negative	226	224	99.1%	2	0.9%	<0.001	226	100.0%	0	0.0%	<0.001*
	Positive	114	94	82.5%	20	17.5%		107	93.9%	7	6.1%	
Genotyping	16/18	54	41	75.9%	13	24.1%	0.053	47	87.0%	7	13.0%	0.005*
	Non 16/18	58	52	89.7%	6	10.3%		58	100.0%	0	0.0%	
Methylation	Negative	258	247	95.7%	11	4.3%	0.003	255	98.8%	3	1.2%	0.061*
marker	Positive	82	71	86.6%	11	13.4%		78	95.1%	4	4.9%	

c) At twelve months post-treatment (n=308)[c]

			Biopsy histology results					Biopsy histology results				
			≤CIN1°		≥rCIN2			≤CIN2°		≥rCIN3		
Cervical scrape results		**Total[#]**	**n**	**%**	**n**	**%**	**p-value**	**n**	**%**	**n**	**%**	**p-value**
Cytology	Normal	212	210	99.1%	2	0.9%	<0.001	212	100.0%	0	0.0%	<0.001*
	≥BMD	95	78	82.1%	17	17.9%		88	92.6%	7	7.4%	
HrHPV DNA	Negative	232	228	98.3%	4	1.7%	<0.001*	232	100.0%	0	0.0%	<0.001*
	Positive	74	59	79.9%	15	20.3%		67	90.5%	7	9.5%	
Genotyping	16/18	30	21	70.0%	9	30.0%	0.106	26	86.7%	4	13.3%	0.440*
	Non 16/18	42	36	85.7%	6	14.3%		39	92.9%	3	7.1%	
Methylation	Negative	246	238	96.7%	8	3.3%	<0.001*	244	99.2%	2	0.8%	0.004*
marker	Positive	60	49	81.7%	11	18.3%		55	91.7%	5	8.3%	

CIN, Cervical Intraepithelial Neoplasia; (r)CIN2/3, (recurrent) CIN2 or CIN3; LLETZ, Large Loop Excision Transformation Zone; hrHPV, high risk Human Papilloma Virus; ≥BMD, borderline or mild dyskaryosis or worse cytology

[#] Including incomplete cases (i.e. one or more non-evaluable test results)

° Including adjusted endpoints

* Fisher's exact test

[a] At baseline, in eight women from the 364 included, no cervical scrape was taken previous to LLETZ resulting in 356 cases available for analysis.

[b] At six months post-treatment, 14 women from the 364 included did not show up. In 11 women with one or more positive test results no biopsy was taken, of whom one had an adjusted endpoint ≤CIN1. This resulted in 340 cases available for analysis.

[c] At 12 months post-treatment, from the 342 women eligible for follow up 30 women did not show up. In ten women no exit biopsy was taken, of whom seven had adjusted endpoint ≤CIN1. Moreover, in one women, an exit-biopsy was taken, but no cervical scrape. This resulted in 308 cases available for analysis.

Table 2: The relation between the methylation marker results on cervical scrape taken previous to detection of rCIN-lesion on biopsy, and the re-LLETZ histology results.

		Re-LLETZ histology results							
		≤CIN1˚		CIN2		CIN3		SCC	
Methylation marker	**Total#**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Negative	16	12	75.0%	2	12.5%	2	12.5%	0	0.0%
Positive	21	10	47.6%	3	14.3%	5	23.8%	3	14.3%

LLETZ, Large Loop Excision Transformation Zone; CIN, Cervical Intraepithelial Neoplasia; SCC, Squamous Cell Carcinoma; rCIN2, recurrent CIN2

˚ Including adjusted endpoints

From the 38 available re-LLETZ results, in one women with rCIN2 on biopsy, no cervical scrape was taken and is not taken into this analysis.

Table 3: Methylation marker status compared to incident and persistent infections, detected on the cervical scrape taken at time of post-treatment disease.

	rCIN2						rCIN3					
		Methylation marker						Methylation marker				
		Negative		Positive				Negative		Positive		
HrHPV genotype	Total[#]	n	%	n	%	p-value	Total[#]	n	%	n	%	p-value
Incident	5	5	100.0%	0	0.0%	0.055*	0	0	0.0%	0	0.0%	-
Persistent	15	7	46.7%	8	53.3%		14	5	35.7%	9	64.3%	

CIN, Cervical Intraepithelial Neoplasia; (r)CIN2/3, (recurrent) CIN2 or CIN3; hrHPV, high risk Human Papilloma Virus

* Fisher's exact test

[#] From the 42 rCIN, six were hrHPV negative on cervical scrape at time of post-treatment disease. Moreover, two women had an unknown hrHPV status at time of post-treatment disease, resulting in 34 rCIN left for analysis.

Table 4: Longitudinal analysis of methylation marker status at baseline combined with methylation marker status at the last test result on cervical scrape taken at time of post-treatment disease (either at six or 12 months, depending on the moment of rCIN).

			Biopsy histology results FU					
			≤CIN1˚		≥rCIN2			
Methylation marker result on scrape		**Total**	**n**	**%**	**n**	**%**	**p-value**	
Baseline negative	Last test negative	146	134	91.8%	12	8.2%	0.010*	<0.001*
	Last test positive	31	23	74.2%	8	25.8%		
Baseline positive	Last test negative	109	102	93.6%	7	6.4%	<0.001	
	Last test positive	38	24	63.2%	14	36.8%		

			Biopsy histology results FU					
			≤CIN2˚		≥rCIN3			
Methylation marker result on scrape		**Total**	**n**	**%**	**n**	**%**	**p-value**	
Baseline negative	Last test negative	146	145	99.3%	1	0.7%	0.080*	<0.001*
	Last test positive	31	29	93.5%	2	6.5%		
Baseline positive	Last test negative	109	105	96.3%	4	3.7%	0.007*	
	Last test positive	38	31	81.6%	7	18.4%		

CIN, Cervical Intraepithelial Neoplasia; (r)CIN2/3, (recurrent) CIN2 or CIN3

˚ Including adjusted endpoints

* Fisher's exact test

Supplementary table 1: Details of all rCIN lesions.

Study number	time of post-disease (months)	Colposcopy outcome	Type of hrHPV infection	HrHPV			Cytology			Methylation marker			Method of follow-up	Follow-up results
				Baseline	6 months	12 months	Baseline	6 months	12 months	Baseline	6 months	12 months		
15	7	CIN3	Persistent	18, 31	18, 31		BMD	>BMD		Negative	Negative		Re-LLETZ	CIN2
402	14	CIN3	Persistent	16	16	16	BMD	>BMD	BMD	Positive	Positive	Positive	Re-LLETZ	CIN3
410	11	CIN3	Persistent	16		16	N.A.		>BMD	Positive		Negative	Re-LLETZ	CIN2
422	12	CIN3	Persistent	16	16	16	N.A.	Normal	>BMD	Positive	Negative	Positive	Re-LLETZ	≤CIN1
628	6	CIN3	Persistent	16	16		>BMD	BMD		Positive	Negative		Re-LLETZ	≤CIN1
632	12	CIN3	Persistent	31	31	31	>BMD	Normal	>BMD	Positive	Negative	Positive	Re-LLETZ	≤CIN1
638	6	CIN3	Persistent	16	16		>BMD	>BMD		Positive	Positive		Re-LLETZ	CIN3
648	6	CIN3	Persistent	16	16		>BMD	>BMD		Positive	Positive		Conisation	SCC T1A1
653	13	CIN3	Persistent	31	31	31	Normal	Normal	BMD	Positive	Positive	Negative	Re-LLETZ	CIN3
665	7	CIN3	Persistent	16	16		>BMD	Normal		Positive	Positive		Conisation	SCC T1A1
666	13	CIN3	Persistent	16, 39	16, 39	16,39,66	BMD	BMD	>BMD	Negative	Negative	Positive	Re-LLETZ	CIN2
680	6	CIN3	Persistent	16	16		BMD	>BMD		Positive	Negative		Re-LLETZ	≤CIN1
825	11	CIN3	Persistent	31	31	31	>BMD	BMD	>BMD	Negative	Positive	Positive	Re-LLETZ	≤CIN1
834	8	CIN3	Persistent	16	16		>BMD	>BMD		Positive	Positive		Conisation	SCC T1A1
4	6	CIN2	Persistent	16	16		N.A.	Normal		Positive	Positive		Re-LLETZ	≤CIN1
10	7	CIN2	Persistent	16	16		>BMD	>BMD		Positive	Positive		Re-LLETZ	CIN3
203	5	CIN2	Persistent	16	16		N.A.	>BMD		Negative	Positive		Colposcopy	CIN2
223	13	CIN2	Persistent	18	18	18	BMD	BMD	BMD	Negative	Positive	Positive	Re-LLETZ	≤CIN1
420	12	CIN2	Persistent	16, 35	16	16, 18	>BMD	>BMD	BMD	Positive	Negative	Negative	Cytology	Normal cytology 2x
669	5	CIN2	Persistent	16, 59	16		>BMD	>BMD		Negative	Negative		Cytology	BMD
672	12	CIN2	Persistent	16	16	16	>BMD	BMD	>BMD	Positive	Positive	Positive	Re-LLETZ	≤CIN1
681	6	CIN2	Persistent	16	16		>BMD	>BMD		N.T.D.	Negative		Re-LLETZ	CIN3
727	6	CIN2	Persistent	33	33		>BMD	>BMD		Positive	Positive		Conisation	CIN3
734	6	CIN2	Persistent	33	33		>BMD	BMD		Positive	Negative		Cytology	BMD
801	12	CIN2	Persistent	45	45, 52, 56, 59	45	BMD	Normal	BMD	N.T.D.	Negative	Negative	Re-LLETZ	≤CIN1
839	7	CIN2	Persistent	16, 52	31, 52		>BMD	Normal		Negative	Negative		Colposcopy	≤CIN1
872	12	CIN2	Persistent	16	16	16	>BMD	>BMD	>BMD	Positive	Positive	Positive	Re-LLETZ	CIN3
897	12	CIN2	Persistent	31, 45, 56, 59	59	31	>BMD	Normal	BMD	Negative	Negative	Positive	Cytology	Normal cytology
1008	8	CIN2	Persistent	35, 59	35,51		BMD	>BMD		Negative	Negative		Hysterectomy	≤CIN1
616	7	CIN2	Incident	16	31		N.T.D.	>BMD		Positive	Negative		Cytology	BMD
629	13	CIN2	Incident	31, 58	58	16	BMD	Normal	BMD	Negative	Negative	Negative	Cytology	Normal cytology/HPV Negative
636	11	CIN2	Incident	16, 18, 31, 51	18, 51, 52	52	N.A.	BMD	>BMD	Negative	Negative	Negative	Hysterectomy	≤CIN1
671	6	CIN2	Incident	16, 45, 51, 59	35		>BMD	BMD		Negative	Negative		Re-LLETZ	≤CIN1
719	8	CIN2	Incident	Negative	16		BMD	BMD		Negative	Negative		Cytology	Normal cytology 2x
204	12	CIN2	Negative	16, 31	31	Negative	N.A.	BMD	BMD	N.T.D.	Negative	Negative	Re-LLETZ	≤CIN1
407	11	CIN2	Negative	Negative	Negative	Negative	N.A.	BMD	BMD	Negative	Positive	Positive	Re-LLETZ	CIN2
621	12	CIN2	Negative	52	Negative	Negative	Normal	Normal	Normal	N.T.D.	Negative	Negative	Cytology	Normal cytology/HPV Negative
840	12	CIN2	Negative	16, 18, 31	Negative	Negative	>BMD	Normal	Normal	Negative	Negative	Positive	Colposcopy	≤CIN1
847	7	CIN2	Negative	52, 58	Negative		>BMD	Normal		Negative	Positive		Cytology	Normal cytology 2x
911	6	CIN2	Negative	35	Negative		BMD	Normal		Positive	Positive		Hysterectomy	≤CIN1
413	7	CIN2	Unknown	51	X		>BMD	BMD		Positive	Positive		Colposcopy	≤CIN1
624	11	CIN2	Unknown	35	31	*	>BMD	Normal	*	Negative	Negative	*	Colposcopy	≤CIN1

*, No sample taken at moment post-treatment disease; CIN, Cervical Intra-epithelial Neoplasia grade 1, 2 or 3; N.A., not applicable; N.T.D., not to determine; hrHPV, high risk Human Papilloma Virus; ≥BMD, Borderline or Mild Dyskariosis or worse; SCC T1A1, Squamous Cell Carcinoma Stage T1A1

Supplementary table 2: The relationship between hrHPV type in rCIN2/3 and the methylation marker status.

	Persistent rCIN3		Persistent rCIN2		Incident rCIN2	
	Methylation marker		Methylation marker		Methylation marker	
	Negative	Positive	Negative	Positive	Negative	Positive
Type of hrHPV	**n**	**n**	**n**	**n**	**n**	**n**
HrHPV 16	3	6	3	5	2	0
HrHPV 16 & 39*	0	1	0	0	0	0
HrHPV 18 & 31*	1	0	0	0	0	0
HrHPV 18	0	0	1	0	0	0
HrHPV 31	1	2	0	1	1	0
HrHPV 33	0	0	1	1	0	0
HrHPV 35	0	0	1	0	1	0
HrHPV 45	0	0	1	0	0	0
HrHPV 52	0	0	1	0	1	0

HrHPV, high risk Human Papilloma Virus; rCIN2/3, recurrent CIN2 or CIN3

* Persistence of multiple types