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Tissue-based methylation testing as a predictor of clinical regression in patients with high- grade cervical intraepithelial neoplasia (CIN2/3)

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Abstract

Introduction.

High-grade cervical intraepithelial neoplasia (CIN2/3) exhibit heterogeneous clinical behavior, ranging from spontaneous regression to progression to cancer. Methylation testing of *FAM19A4/miR124-2* genes in clinician- and self-collected cervical scrapes has demonstrated to predict clinical regression in women with untreated CIN2/3 (CONCERVE study). This *post hoc* analysis of the CONCERVE study evaluated the prognostic value of *FAM19A4/miR124-2* methylation testing, immunoscore (based on the extent of $p16^{INK4a}$ and Ki-67 expression), and HPV E4 expression in CIN2/3 biopsies, to predict clinical regression.

Methods.

CIN2/3 biopsies of 89 women with known HPV genotypes were analyzed by *FAM19A4/miR124-2* methylation testing and immunohistochemistry for $p16^{INK4a}$, Ki-67 and HPV E4. Association with clinical regression was assessed using univariable and multivariable Cox regression. Regression incidences were estimated using the Kaplan-Meier method.

Results.

Women with a $p16^{INK4a}$ block-positive and methylation-negative CIN2/3 biopsy (n = 42) had significantly higher 2-year regression rates (76%; 95% CI: 57.7–86.4) than women with methylation-positive biopsy (n = 33, 44.9%; 95% CI: 23.8–60.1, $p = 0.009$). This prognostic effect was particularly evident in HPV16-positive women (n = 47), with a 65.9% (95% CI: 32.3–82.9) regression rate in methylation-negative lesions versus 29.5% (95% CI: 0-48.7, $p = 0.029$) in methylation-positive ones. $P16^{INK4a}$/Ki-67 immunoscore and HPV E4 expression were not associated with clinical regression.

Conclusions.

A negative *FAM19A4/miR124-2* methylation test on baseline, $p16^{INK4a}$ block-positive CIN2/3 biopsies is prognostic for clinical regression, supporting its auxiliary role in personalized management strategies.

KEY POINTS

• methylation testing on colposcopy-derived CIN2/3 biopsies provides prognostic value beyond conventional histology and immunohistochemistry.

• Methylation-negative CIN2/3 lesions are more likely to regress spontaneously, including HPV16-positive ones.

• Methylation testing in routine diagnostic workflows could support a wait-and-see policy in selected patients, reducing unnecessary surgical interventions.

1. INTRODUCTION

High-grade cervical intraepithelial neoplasia (CIN2/3) is the precursor lesion of cervical cancer. These lesions are caused by persistent infection with high-risk human papillomavirus (HPV) and frequently diagnosed in young women [1]. Treatment of CIN2/3 by large loop excision of the transformation zone (LLETZ) or cold-knife conization is considered an effective strategy for cervical cancer prevention [2]. However, most HPV infections are cleared by the immune system and spontaneous regression is observed in approximately 55% of CIN2 and 28% of CIN3 lesions [3]. Given that treatment of CIN2/3 lesions is associated with an increased risk of spontaneous preterm birth [4, 5], intervention should be reserved for women at higher risk of cancer development to reduce overtreatment. Previous studies have shown that active surveillance of CIN2 in young women is a safe approach [6–8]. Moreover, the latest European Society of Gynaecologic Oncology (ESGO) consensus statement on active surveillance of CIN2 reiterates the evidence supporting this approach in patients carefully selected based on a series of clinical

parameters, providing an algorithm where treatment can be deferred in the presence of regressive disease [9]. For this reason, it is key to differentiate between regressive and persistent or progressive CIN lesions. Since this distinction cannot be made based solely on routine histopathological staining, the exploration of prognostic biomarkers has become increasingly important [10].

Application of $p16^{INK4a}$ immunohistochemistry is recommended to inform the diagnosis of high-grade CIN versus low-grade CIN in morphologically equivocal cases, or to aid in the distinction of high-grade CIN from mimics [11]. Block-positive $p16^{INK4a}$ staining is a biomarker for transforming HPV infections and increases with lesion severity, as does suprabasal Ki-67 expression [12]. HPV E4 expression, on the other hand, serves as an immunohistochemical marker for productive HPV infections, as is seen in low-grade, and occasionally also in high-grade CIN lesions [13]. It is currently unknown whether in the context of histologically confirmed CIN2/3, the extent of $p16^{INK4a}$ and Ki-67 expression (their cumulative score referred to as immunoscore [14]), or HPV E4 expression, may hold prognostic value for CIN2/3 clinical outcome.

In addition to immunohistochemistry, DNA methylation of tumor suppressor genes, an essential signature of cervical carcinogenesis, provides objective biomarkers (i.e., methylation markers) for early detection of cervical cancer. Methylation testing of tumor suppressor genes, such as *FAM19A4/miR124-2*, in cervical samples detects virtually all cervical cancers and advanced CIN2/3, which pose a higher risk of progression to cancer [15]. *FAM19A4/miR124-2* methylation, especially, has been tested on HPV-positive clinician-collected cervical scrapes from European screening populations, detecting 77% of CIN3 and 47% of CIN2 [16]. Importantly, a negative *FAM19A4/mir124-2* methylation test in clinician-collected cervical scrapes has been associated to a low cervical cancer risk in HPV-positive women aged 30 and older [17]. Recently, the CONCERVE study, in which women with relatively small CIN2/3 (≤ 50% colposcopic volume) were prospectively followed for 24 months with a wait-and-see policy, has demonstrated that a negative *FAM19A4/miR124-2* methylation test in clinician- and self-collected cervical scrapes is associated with CIN2/3 regression. Specifically, lesions from women with a methylation-negative test showed significantly more regression than those from women with a methylation-positive test (74.7% versus 51.4%, $p < 0.05$) [18]. Given the above, we hypothesize that *FAM19A4/miR124-2* methylation testing on colposcopy-derived CIN2/3 biopsies may also have prognostic value for lesion regression, which is of particular relevance considering that treatment decisions rely on a histological diagnosis of high-grade CIN.

In this *post hoc* analysis of the CONCERVE study, we evaluated whether *FAM19A4/miR124-2* methylation testing, the extent of $p16^{INK4a}$ and Ki-67 expression (immunoscore), and HPV E4 expression in CIN2/3 biopsies could predict clinical regression.

2. METHODS

2.1 Patients and sample collection

This *post hoc* analysis was performed on the study population of the CONCERVE study (Trial registration ID: NTR6069), which was previously published and described in detail, including all clinical characteristics [18, 19]. The laboratory analyses, as presented in the current study, are newly performed on archived tissue samples from this cohort. Briefly, the CONCERVE study was a multicenter, longitudinal clinical cohort study including 114 women aged 18–55 years, who were diagnosed with CIN2 (n = 80) or CIN3 (n = 34) lesions covering ≤ 50% of the cervix, and may benefit from close surveillance because of assumed lower risk of progression to cancer. Participants were prospectively monitored every six months for a period of 24 months. At baseline and during each follow-up visit, clinician and self-collected cervical samples were collected, the first tested for cytology. Cytology results were classified according to the CISOE-A classification and reported following the Bethesda system [20]. Each visit also included colposcopic examination where cervical biopsies were taken in case of CIN2 or worse (CIN2+) clinical impression, unless cytology showed no abnormalities at 12 and/or 18 months, in which case colposcopic examination could be omitted. If clinical progression occurred throughout the study, or CIN2 + was diagnosed at the final study visit, surgical treatment was recommended following national guidelines. In the present study, only women with an available baseline CIN2/3 biopsy with sufficient material for further molecular and immunohistochemical characterization (n = 89) were included.

2.2 Tissue preparation and histopathological review

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from CIN2/3 baseline biopsies were sectioned using the sandwich method. The first and last sections were stained with haematoxylin-eosin (H&E), and histopathologically reviewed by an expert-

pathologist to confirm the presence of the cervical lesion. In case of insufficient residual material or absence of lesional tissue in the last H&E slide, patients were excluded. In between sections were used for DNA isolation and immunostaining. Immunohistochemistry was performed using mouse monoclonal antibodies (mAb) targeting $p16^{INK4a}$ (Clone E6H4, CINtec, Roche, Basel, Switzerland) and Ki-67 (Clone MIB-1, DAKO, Agilent Technologies, Santa Clara, CA) by the automated Ventana staining machine (Ventana Medical Systems, Roche, Oro Valley, AZ, USA), and a mouse mAb targeting panHPV E4 (developed in the laboratory of J. Doorbar, Cambridge, England, available through Labo Bio-medical Products B.V., Rijswijk, The Netherlands) as previously described [14].

2.2.1 Immunohistochemical scoring

Immunohistochemical staining for $p16^{INK4a}$, Ki-67 and HPV E4 were scored as previously described [14]. Cytoplasmic and/or nuclear $p16^{INK4a}$ staining was scored 0–3 and considered as negative or "patchy" staining (0), or block-positive staining in the lower one-third (1), up to the lower two-third (2) and more than two thirds (3) of the epithelium. Similarly, nuclear Ki-67 staining of squamous epithelial cells was scored 0–3 and considered as normal staining pattern of the basal layers (0) or increased positive staining in the lower one-third (1), up to the lower two-third (2) and more than two thirds (3) of the epithelium The cumulative score of $p16^{INK4a}$ and Ki-67 (0-6) was referred to as the "immunoscore" (IS) [14]. IS groups 0–2 were categorized as low immunoscore, IS groups 2–4 as intermediate, and IS groups 5–6 as high. Membranous and/or cytoplasmic HPV E4 staining was scored as negative (0), focally positive (1) or extensively positive (2).

2.3 DNA methylation and HPV testing

DNA was isolated using the QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The QIAsure Methylation Test (Qiagen) was used for *FAM19A4/miR124-2* methylation testing on 50 ng of bisulfite-converted DNA (EZ DNA methylation kit, Zymo Research, Irvine, California, USA) following manufacturers' instructions. *FAM19A4/miR124-2* methylation levels were quantified as ΔΔCt ratios using the comparative Ct method ($2^{-\Delta\Delta Ct}$x100), and predetermined thresholds defined according to a <CIN2 specificity of 70% were used to categorise samples into methylation-positive or methylation-negative [21]. The HPV status of the cervical biopsies was assumed to be reflected by that of the paired clinician-collected cervical scrapes, for which data on high-risk HPV testing were available [18]. In eight women HPV testing was performed using the QIAscreen HPV PCR Test (Qiagen) on the baseline biopsy, as no HPV genotyping results on clinician-collected cervical scrapes were available.

2.4 Statistical analyses

General characteristics of the women included in this *post hoc* analysis are described by counts and percentages, or by median and interquartile range (IQR). Clinical endpoints of the study were i) clinical regression, ii) persistence and iii) clinical progression. Clinical regression was defined as histologically confirmed absence of CIN2 + or a high-risk HPV-negative cervical scrape with normal cytology if histology was not available; clinical progression as increased colposcopic volume of the lesion covering more than 50% of the cervix, histologic progression from CIN2 to CIN3, and/or histologic diagnosis of AIS; and persistence as no evidence of either clinical regression or progression.

Violin plots with a standard notation were computed to display *FAM19A4* and *miR124-2* methylation levels (ΔΔCt ratios) between HPV E4-positive (score 2) and -negative (score 0–1) biopsies. Pairwise comparisons were done using the Mann–Whitney U test. Conditional scatterplots were designed to visualize *FAM19A4* and *miR124-2* methylation levels (ΔΔCt ratios) in paired biopsies and clinician-collected cervical scrapes, and differences between sample types were assessed using the Mann–Whitney U test.

The association between CIN2/3 clinical regression and the biopsies' methylation status (positive, negative), $p16^{INK4a}$/Ki-67 immunoscore (low-to-intermediate if 2–4, high if 5–6), although intended to support accurate CIN grading, and HPV E4 expression (positive if scored 2, otherwise negative), was estimated using univariable and multivariable Cox regression, and hazard ratios (HRs) were used to estimate the level of association. Potential confounders included in the multivariable analysis were identified among those found to be associated with clinical regression in our previous study [18] by designing directed acyclic graphs (DAG). Potential confounders were patient age (continuous) and HPV genotype (HPV16, non-HPV16 types) (Supplementary Fig. 1).

Cumulative incidence of clinical regression was estimated using the Kaplan-Meier method, indicating time to event as the number of months between baseline histology and the date of clinical regression. Women were censored when lost-to-follow-up, or when surgical treatment was provided. If the endpoint was not reached and indication for surgical treatment was not reported, censoring occurred at the last study visit. Separate Kaplan-Meier curves were stratified by i) *FAM19A4/miR124-2* methylation (positive, negative), and in line with our previous study [18] ii) HPV genotyping (HPV16, or non-HPV16 types) combined with *FAM19A4/miR124-2* methylation. Additionally, cumulative regression incidences were calculated by stratifying women based on the *FAM19A4/miR124-2* methylation status of paired clinician collected-cervical scrapes. All Kaplan Meier curves were compared using the log-rank test. Combined HPV genotyping and methylation cumulative regression incidences were presented as risk plots.

To evaluate the impact of a more stringent definition of clinical regression on our analyses, univariable and multivariable Cox regression analyses were performed defining clinical regression as absence of CIN grade 1 (CIN1) + or a high-risk HPV-negative cervical scrape with normal cytology, and Kaplan Meier curves stratified by *FAM19A4/miR124-2* methylation were estimated accordingly.

Statistical analyses and data visualization were performed in R (version 4.2, R, Vienna, Austria), using the packages dagitty (version 0.3-4), survival (version 3.5-7), survminer (version 0.4.9) and ggplot2 (version 3.5.0).

3. RESULTS

3.1 Study population

Of 114 women participating in the CONCERVE study, 89 (78%) were included in the following analyses, as shown in Fig. 1. Baseline biopsies from these women included 62 (69.7%) CIN2 and 27 (30.3%) CIN3. Their median (IQR) age was 30 (28–35) years. Fifty women (56.2%) had a baseline cytology result of atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL), and 39 (43.8%) of high-grade squamous intraepithelial lesion (HSIL). All 89 baseline biopsies (100%) had a block-positive staining pattern for $p16^{INK4a}$ and showed increased Ki-67 staining (Supplementary Table 1). Of these, 61 (68.5%) were collected from lesions with a baseline colposcopic volume of < 25%, while 22 (24.8%) between 25–50%. In three women (3.4%) no lesion was visible during baseline colposcopy, and for the remaining three (3.4%) information on baseline colposcopic impression was not available. Clinical regression occurred in 51 (57.3%) women, 15 (16.9%) had persistent CIN2/3 lesions and progression (i.e., increased colposcopic volume of the lesion > 50% of the cervix, histologic progression from CIN2 to CIN3, and/or histologic diagnosis of AIS) occurred in 23 (25.8%; none progressed to cancer) (Fig. 2).

3.2 Immunohistochemical (IHC) and molecular analysis of CIN2/3 biopsies

Figure 2 provides an overview of immunohistochemical and molecular findings from the biopsies. Out of all 89 biopsies with block-positive $p16^{INK4a}$ staining, the extent of $p16^{INK4a}$ and Ki-67 expression, measured as their cumulative score and referred to as immunoscore (IS), was high in 40 (44.9%), intermediate in 48 (53.9%) and low in 1 (1.1%). HPV E4 expression was negative in 49 (55.1%), focally positive in 8 (9%) and extensively positive in 29 (32.6%). HPV E4 staining could not be performed in three biopsies (3.4%). HPV genotyping results were available for all 89 women, with 47 (52.8%) testing positive for HPV16, 41 (46.1%) for non-HPV16 types and one (1.1%) testing HPV-negative. Of 89 biopsies tested for methylation, 75 (84.3%) had a valid *FAM19A4/miR124-2* methylation test result, with 33 methylation-positive (33/75, 44%) including 14 (42.4%) regressing and 19 (57.6%) non-regressing cases (i.e. persistent and progressing), and 42 methylation-negative (42/75, 56%) including 30 regressing (71.4%) and 12 (28.6%) non-regressing cases. These results stratified by original histology are presented in Supplementary Table 1.

Analysis of the relationship between methylation levels and HPV E4 expression showed no difference in methylation levels between HPV E4-positive and HPV E4-negative biopsies (Supplementary Fig. 2).

3.3 Assessment of CIN2/3 clinical regression

The association between CIN2/3 clinical regression and *FAM19A4/miR124-2* methylation, $p16^{INK4a}$/Ki-67 immunoscore, and HPV E4 expression was evaluated through univariable Cox regression and presented in Table 1. Methylation status was a significant predictor of clinical regression ($p = 0.011$). No significance was found for $p16^{INK4a}$/Ki-67 immunoscore and HPV E4 expression. Age- and HPV genotype-adjusted multivariable Cox regression analysis showed that methylation status was independently associated with clinical regression ($p = 0.017$), with a hazard ratio (HR) of 2.27 (95%CI: 1.61–4.45, Table 1). Comparable results were obtained when using a more stringent definition of clinical regression, defined as absence of CIN1 + or a high-risk HPV-negative cervical scrape with normal cytology (Supplementary Table 2).

Women with a methylation-negative CIN2/3 biopsy showed a higher rate of clinical regression (76%; 95%CI: 57.7–86.4) compared to women with a methylation-positive CIN2/3 biopsy (44.9%; 95%CI: 23.8–60.1, $p = 0.009$; Fig. 3A). When using a more stringent definition of clinical regression, as previously described, regression incidences were generally lower, although higher in women with a methylation-negative biopsy (55%; 95%CI: 36.4–68.2) compared to those with a methylation-positive one (29%; 95%CI: 10.8–43.6; $p = 0.029$; Fig. 3B).

Cumulative regression incidences based on high-risk HPV16 genotyping, followed by methylation testing on baseline biopsies, are shown in Fig. 4. In HPV16-positive women, clinical regression was higher after a methylation-negative test (65.9%; 95%CI: 32.3–82.9) than a methylation-positive test (29.5%; 95%CI: 0-48.7, $p = 0.029$). In women positive for non-HPV16 types, relatively high clinical regression rates were observed following both methylation-negative and methylation-positive test results (81.8%; 95%CI: 55.8–92.5, and 66.7%; 95%CI: 27.9–84.6, respectively).

Finally, the regression incidences obtained from testing baseline biopsies were compared to those obtained from testing paired, baseline clinician-collected cervical scrapes. Of 75 women with a valid methylation test result on their biopsy, cervical scrapes from 3 (4%) were not collected at baseline, six (8%) could not be tested because of insufficient DNA quality, and one (1.3%) yielded an invalid methylation test result. Consequently, methylation results on cervical scrapes from the remaining 65 (86.7%) women showed 79.9% (95%CI: 58.2–90.4) regression incidences if methylation-negative and 44.9% (95%CI: 23.8–60.1, $p = 0.008$) if methylation-positive (Supplementary Fig. 4). *FAM19A4* methylation levels in paired baseline biopsies and cervical scrapes did not significantly differ ($p = 0.483$), while *miR124-2* methylation levels were lower in cervical scrapes ($p < 0.001$) (Supplementary Fig. 5).

4. DISCUSSION

This study demonstrates that a *FAM19A4/miR124-2* methylation-negative test on a CIN2/3 biopsy is prognostic for clinical regression of $p16^{INK4a}$ block-positive lesions with < 50% colposcopic volume. Methylation-negative CIN2/3 showed a higher cumulative incidence of clinical regression at two-year follow-up (76%; 95%CI: 57.7–86.4) compared to methylation-positive cases (44.9%; 95%CI: 23.8–60.1). The difference in regression incidences between methylation-positive and methylation-negative CIN2/3 biopsies was most pronounced in HPV16-positive women (29.5%; 95%CI: 0-48.7 versus 65.9%; 95%CI: 32.3–82.9, respectively). In cases where a non-HPV16 type was present, both groups showed relatively high regression rates (66.7%; 95%CI: 27.9–84.6 in methylation-positive and 81.8%; 95%CI: 55.8–92.5 in methylation-negative cases). While methylation testing provides prognostic value in $p16^{INK4a}$ block-positive CIN2/3, the cumulative score of $p16^{INK4a}$ and Ki-67 expression (immunoscore) did not show prognostic potential, nor did HPV E4 expression.

FAM19A4/miR124-2 methylation testing on clinician and self-collected cervical scrapes has been proposed as a triage test for CIN3 + detection in high-risk HPV-positive women [16, 17, 22–24]. Recently, the CONCERVE study showed its valuable prognostic performance in distinguishing regressive from non-regressive CIN2/3 when tested on clinician and self-collected cervical scrapes [18]. In this *post hoc* analysis, we demonstrate a similar prognostic value of *FAM19A4/miR124-2* methylation, when determined on biopsies. Direct comparison of methylation findings between these sample types is limited by differences in cellular composition and by the proportion of lesional cells [25]. Nonetheless, similar regression incidences were obtained when testing biopsies and clinician-collected scrapes from the same women (respectively, 76% and 79.9% if methylation-negative; and both 44.9% if methylation-positive). Considering that a biopsy is collected as part of routine care for histological CIN2/3 diagnosis, these results indicate that, with respect to prognostic utility, tissue-based methylation testing can be performed on readily available material providing a practical alternative when cytology samples are unavailable or unsuitable.

DNA methylation as a biomarker for CIN2/3 outcome has also been evaluated by others. A prospective study by Louvanto *et al.* showed that the S5 methylation classifier, targeting viral and host genes, was able to distinguish between regressive and progressive CIN2, both alone and combined with cytology and/or HPV genotyping [26]. Another recent study on women aged 20–29 years with CIN2/3, showed slightly higher 12-month regression rates after a baseline methylation-negative cervical scrape (GynTect® assay, targeting six host genes) than a methylation-positive one (respectively, 60% and 50% regression rates) [27]. Collectively, these studies support the implementation of methylation testing for decisions regarding patient management.

HPV16 has been evaluated in relation to CIN regression by other studies, generally demonstrating an inverse correlation between HPV16 positivity and regression [28, 29]. In our cohort, HPV16 genotyping and methylation testing alone showed comparable prognostic performance for CIN2/3 clinical regression (regression rates were 76% *vs* 45% for methylation-negative *vs* -positive; 74% *vs* 51% for non-HPV16 *vs* HPV16-positive). Additionally, when combining HPV genotyping with biopsy methylation testing we could further stratify the estimated regression rates. While generally high clinical regression rates were observed in non-HPV16-positive women, both for those with a methylation-negative (81%) and a methylation-positive (66.7%) test, HPV16-positive women with a methylation-positive test showed less clinical regression (29.5%), markedly reduced compared to HPV16-positive women with a methylation-negative test (65.9%). Similar trends were noted for clinician-collected cervical scrapes of the CONCERVE study [18]. In line with this, a recent prospective study reported that HPV status together with *FAM19A4/miR124-2* methylation testing improved prediction of CIN2 regression [30]. These data combined support a strategy where CIN2/3 lesions are immediately treated when methylation and HPV16 genotyping results are positive, while close surveillance may be considered otherwise. A wait-and-see policy would be most beneficial for women of reproductive age to prevent the risk of obstetric complications. At the same time, while the long-term risk of cancer development remains low, it has been reported as higher after active surveillance rather than immediate treatment [31], underscoring the need for informed decision-making and careful follow-up. In the Dutch cervical cancer screening program, at present, all HPV-positive women with HSIL and HPV16/18-positive women with abnormal cytology are referred for colposcopy [32]. If lower volume CIN2/3 lesions are identified in colposcopy-derived biopsies, active surveillance may be considered also based on methylation testing results and HPV genotype. This study demonstrates that *FAM19A4/miR124-2* methylation testing on CIN2/3 biopsies provides additional value, as lesions with a negative methylation test and a non-HPV16 infection have a high likelihood of regression. This allows for a more objective justification of the decision to opt for active surveillance in women with CIN2/3.

FAM19A4/miR124-2 methylation has been previously evaluated in relation to immunohistochemical biomarkers $p16^{INK4a}$, Ki-67 and HPV E4. In these studies, methylation positivity in clinician-collected cervical scrapes appeared to increase with CIN grade, and was associated with increased $p16^{INK4a}$ and Ki-67 expression, as well as reduced HPV E4 expression in paired cervical biopsies [14, 33]. While DNA methylation markers have additionally demonstrated prognostic value for CIN clinical outcome, immunohistochemical $p16^{INK4a}$ and Ki-67 expression has been mostly shown to improve accuracy and reproducibility of CIN grading [12]. On the other hand, data on a prognostic role of these immunomarkers is controversial, with studies reporting both association and lack thereof between $p16^{INK4a}$ status and CIN clinical outcome [34–36]. Of note, $p16^{INK4a}$ staining is recommended for use in clinical practice to confirm a morphologically ambiguous high-grade CIN diagnosis [11], implying that a $p16^{INK4a}$-negative status at baseline is already indicative of a low-grade lesion. In our study, neither combined $p16^{INK4a}$/Ki-67 immunoscoring nor HPV E4, a biomarker for productive infections [14], could significantly predict CIN2/3 regression.

Strengths of this study include a unique cohort of 89 women with CIN2/3 undergoing a 2-year period of watchful waiting. Additionally, a criterion for inclusion in the CONCERVE study was the diagnosis of relatively small CIN2/3 lesions (colposcopic volume ≤ 50% of the visible cervix), which reflects a real-life situation, particularly in some European countries, where a wait-and-see policy would be considered. To align with clinical practice, analyses in our study were based on the original CIN2/3 diagnosis obtained from different pathology laboratories, even though CIN grading is prone to interobserver variability [37]. A limitation is that biopsies were taken throughout the study, which might have had a curative effect in women, thereby overestimating the number of true natural regressions. Nonetheless, our previous analysis has demonstrated that censoring data after a follow-up biopsy only had limited impact on the regression incidences obtained in the methylation-negative and methylation-positive groups [18]. A further limitation might be that this *post hoc* analysis was carried out on a subgroup of women of the CONCERVE study, based on the availability of their CIN2/3 biopsies for additional molecular and immunohistochemical analysis, which limited our analysis to a smaller cohort.

In conclusion, this study demonstrates the association between a *FAM19A4/miR124-2* methylation-negative test on $p16^{INK4a}$ block-positive CIN2/3 biopsies and clinical regression of the lesion. Consistent with our previous data on *FAM19A4/miR124-2* methylation testing on clinician- and self-collected cervical scrapes, testing on biopsies performed just as well in predicting clinical regression. This study supports the implementation of methylation testing to the diagnostic workup of women with CIN2/3 to guide a wait-and-see policy, thereby reducing overtreatment of CIN lesions and associated cervical morbidity.

Declarations

Conflicts of interest.

D.A.M. Heideman, C.J.L.M. Meijer and R.D.M. Steenbergen are minority shareholders of Self-screen B.V. which develops, manufactures and licenses high-risk HPV and methylation marker assays and holds patents on these tests. C.J.L.M. Meijer is part-time CEO of Self-Screen and served occasionally on the scientific advisory board and/or the speakers bureau of Qiagen. R.D.M. Steenbergen also declares consultancy fees from AstraZeneca paid to her institution. All the other authors declare that they have no competing interests.

Ethics approval.

The CONCERVE study was approved by the Medical Ethics Committee of Amsterdam UMC, VU University Medical Center (2016/471), and registered as NTR6069/NL5794 in the Netherlands National Trial Registry. All patients included in this *post hoc* study provided written consent for further use of residual cervical material for research purposes.

Consent (participation & publication)

Not applicable.

Competing Interests

D.A.M. Heideman, C.J.L.M. Meijer and R.D.M. Steenbergen are minority shareholders of Self-screen B.V. which develops, manufactures and licenses high-risk HPV and methylation marker assays and holds patents on these tests. C.J.L.M. Meijer is part-time CEO of Self-Screen and served occasionally on the scientific advisory board and/or the speakers bureau of Qiagen. R.D.M. Steenbergen also declares consultancy fees from AstraZeneca paid to her institution. All the other authors declare that they have no competing interests.

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Author Contribution

FR: Formal analysis, Interpretation, Investigation, Visualization, Writing – original draft, Writing – review and editing. MS: Formal analysis, Interpretation, Visualization, Writing – review and editing. SD: Interpretation, Investigation, Writing – review and editing. WWK: Investigation, Writing – review and editing. DAMH: Conceptualization, Interpretation, Writing – review and editing. JB: Conceptualization, Interpretation, Writing – review and editing. CJLMM: Conceptualization, Interpretation, Writing – review and editing. MCGB: Conceptualization, Interpretation, Supervision, Writing – review and editing. RDMS: Conceptualization, Interpretation, Supervision, Writing – review and editing.

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Data Availability

Data are available upon reasonable request.

Code availability:

Not applicable.

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Tables

Table 1. Association between *FAM19A4/miR124-2* methylation, $p16^{INK4a}$/Ki-67 immunoscore and HPV E4 expression, and CIN2/3 clinical regression. Clinical regression was defined as absence of CIN2+ or a high-risk HPV-negative cervical scrape with normal cytology.

Statistical significance is presented in bold.

Abbreviations: CI, confidence interval; HPV, human papillomavirus; HR, hazard ratio; IS, immunoscore.

		Univariable analysis			Multivariable analysis					
					Adjusted for age			Adjusted for age, HPV genotype		
	n	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value
Methylation test										
Positive	33	1.0								
Negative	42	**2.29**	**(1.21-4.33)**	**0.011**	**2.58**	**(1.34-4.99)**	**0.005**	**2.27**	**(1.61-4.45)**	**0.017**
$p16^{INK4a}$/Ki-67 IS										
5-6	40	1.0								
2-4	49	1.63	(0.92-2.9)	0.095	1.61	(0.91-2.87)	0.103	1.56	(0.87-2.81)	0.133
HPV E4										
Negative	57	1.0								
Positive	29	0.89	(0.49-1.62)	0.698	0.95	(0.52-1.76)	0.879	0.93	(0.5-1.72)	0.822

Figures

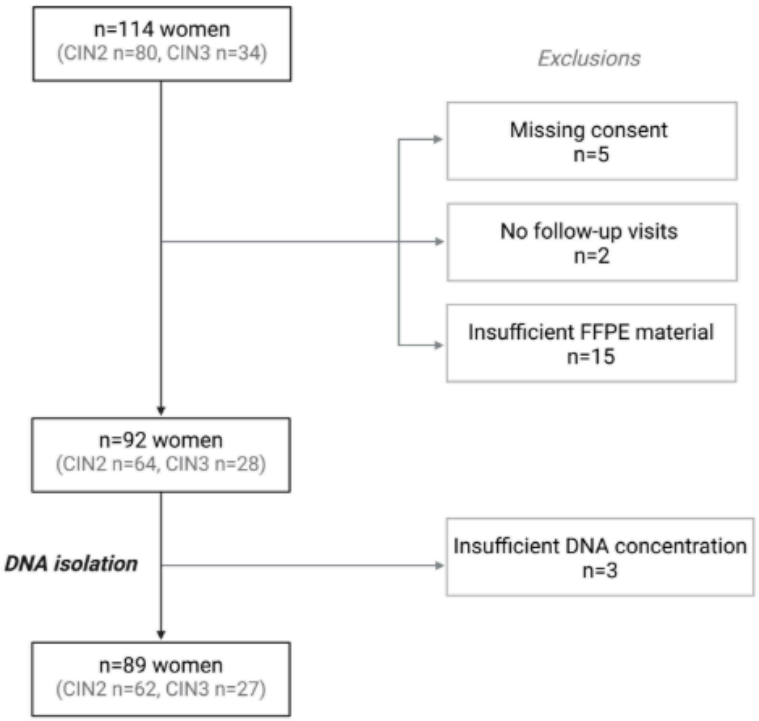


Figure 1

Study population and workflow

CIN grade is reported according to the original diagnosis.

Abbreviations: CIN, cervical intraepithelial neoplasia; FFPE, formalin-fixed paraffin-embedded.

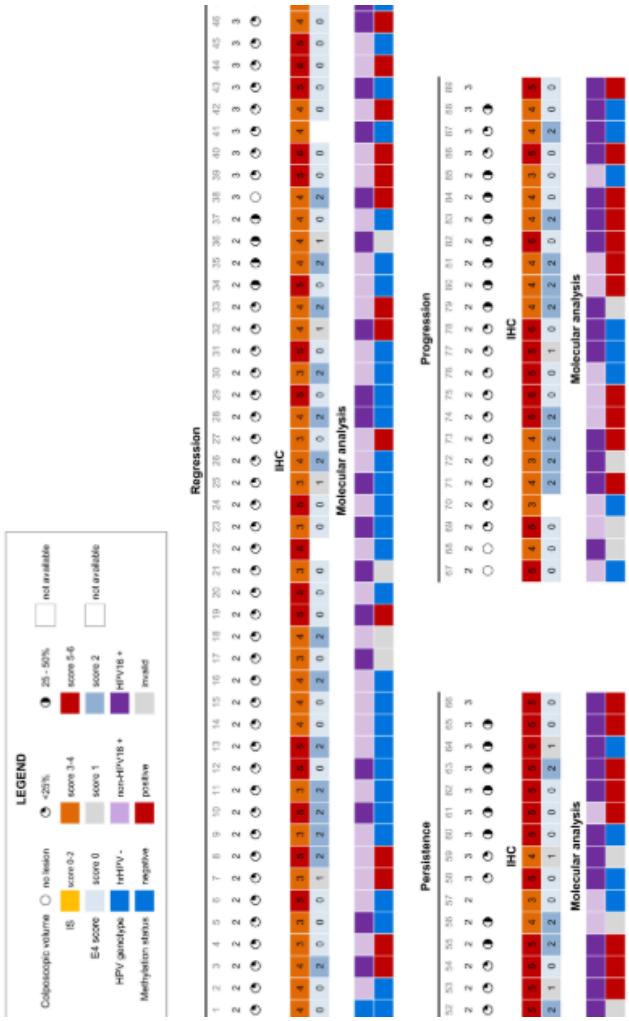


Figure 2

Overview of the original histopathological grade, colposcopic impression, immunohistochemical and molecular characterization of CIN2/3 biopsies

Original histopathological CIN grade; colposcopic volume of the lesion (no lesion, <25%, 25-50% of the visible cervix); IHC staining scores for markers $p16^{INK4a}$/Ki-67 (IS) [2-6] and HPV E4 [0-2]; HPV genotyping (HPV16 or non-HPV16); QIAsure methylation test result (positive, negative). Patients are grouped according to their clinical endpoint: regression, defined as absence of CIN2+ or a high-risk HPV-negative cervical scrape with normal cytology; progression, as increased colposcopic volume of the lesion covering more than 50% of the cervix, histologic progression from CIN2 to CIN3, and/or histologic diagnosis of AIS; persistence, as no evidence of either clinical regression or progression. In each subgroup, patients are ordered low-to-high based on their histopathological CIN diagnosis and colposcopic volume of their lesion.

Abbreviations: CIN, cervical intraepithelial neoplasia; IHC, immunohistochemistry; IS, immunoscore; HPV, human papillomavirus.

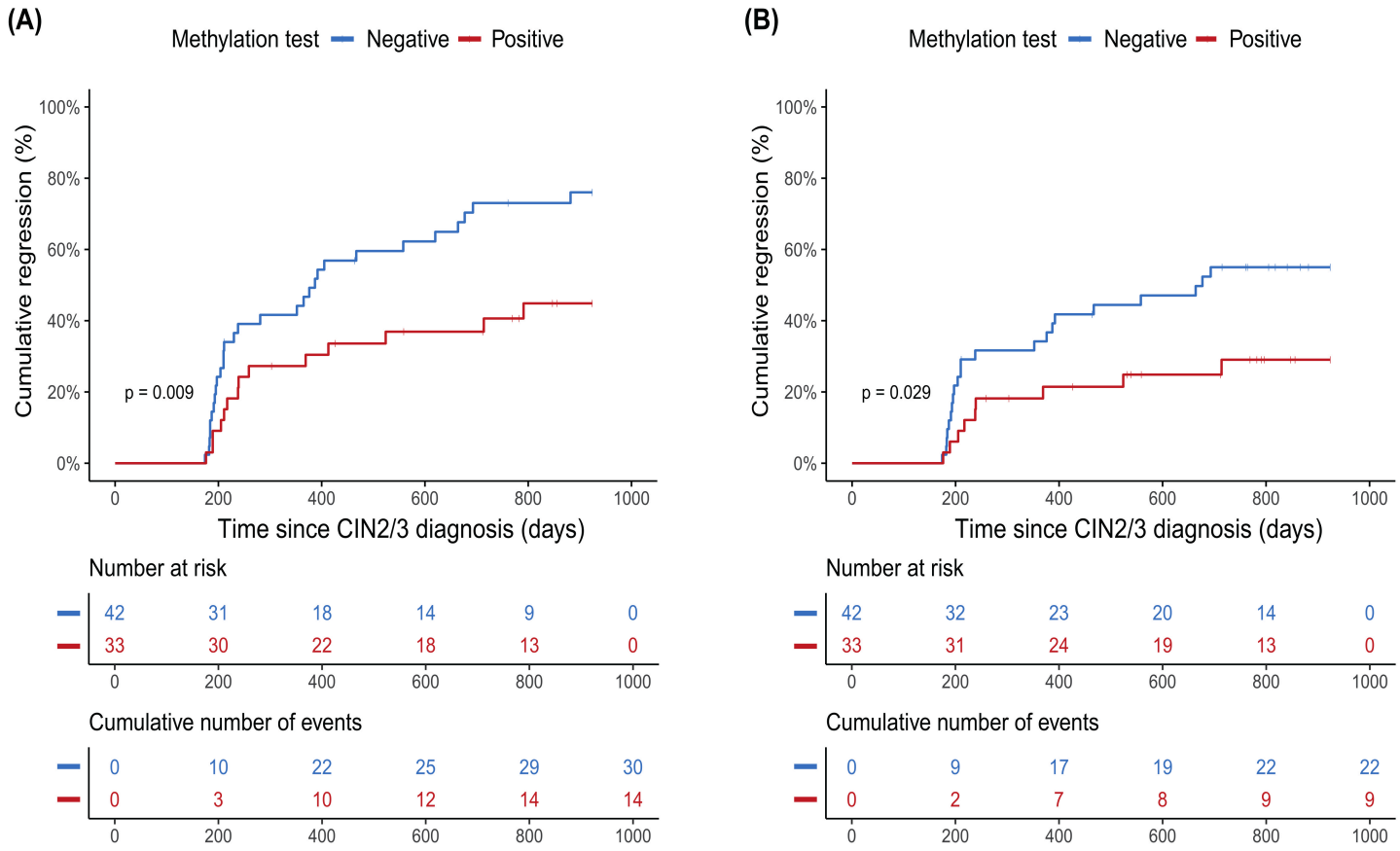


Figure 3

Cumulative regression incidences by Kaplan Meier analysis stratified by methylation results on baseline biopsies

(A) Clinical regression was defined as absence of CIN2+ or a high-risk HPV-negative cervical scrape with normal cytology. **(B)** Clinical regression was defined as absence of CIN1+ or a high-risk HPV-negative cervical scrape with normal cytology.

Abbreviations: CIN, cervical intraepithelial neoplasia.

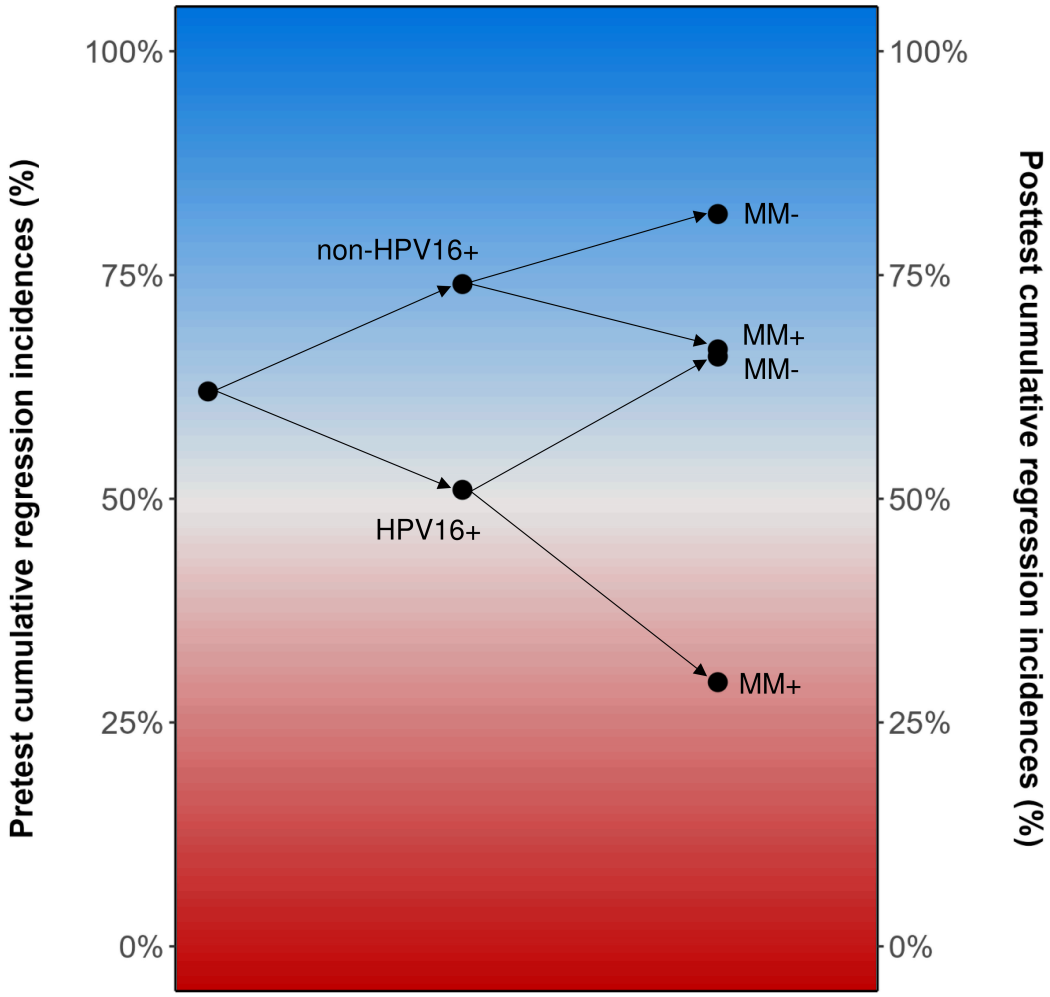


Figure 4

Cumulative regression incidences by Kaplan Meier analysis: pre-test and post-test risk plot for combined HPV genotyping and methylation testing on baseline biopsies

Clinical regression was defined as absence of CIN2+ or a high-risk HPV-negative cervical scrape with normal cytology.

Abbreviations: hrHPV, high-risk Human Papillomavirus; MM, methylation marker.

Supplementary Files

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