Classification of high-grade cervical intraepithelial neoplasia by p16\textsuperscript{ink4a}, Ki-67, HPV E4 and FAM19A4/miR124-2 methylation status demonstrates considerable heterogeneity with potential consequences for management

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Abstract

High-grade cervical intraepithelial neoplasia (CIN2 and CIN3) represents a heterogeneous disease with varying cancer progression risks. Biomarkers indicative for a productive human papillomavirus (HPV) infection (HPV E4) and a transforming HPV infection (p16\textsuperscript{ink4a}, Ki-67 and host-cell DNA methylation) could provide guidance for clinical management in women with high-grade CIN. This study evaluates the cumulative score of immunohistochemical expression of p16\textsuperscript{ink4a} (Scores 0-3) and Ki-67 (Scores 0-3) referred to as the “immunoscore” (IS), in 262 CIN2 and 235 CIN3 lesions derived from five European cohorts in relation to immunohistochemical HPV E4 expression and FAM19A4/miR124-2 methylation in the corresponding cervical scrape. The immunoscore classification resulted in 30 lesions within IS group 0-2 (6.0%), 151 lesions within IS group 3-4 (30.4%) and 316 lesions within IS group 5-6 (63.6%). E4 expression decreased significantly from CIN2 to CIN3 (P < .001) and with increasing immunoscore group (P\textsubscript{trend} < .001). Methylation positivity increased significantly from CIN2 to CIN3 (P < .001) and with increasing immunoscore group (P\textsubscript{trend} < .001). E4 expression was present in 9.8% of CIN3 (23/235) and in 12.0% of IS group 5-6 (38/316). Notably, in a minority (43/497, 8.7%) of high-grade lesions, characteristics of both transforming HPV infection (DNA hypermethylation) and...
Cervical screening programmes aim to prevent cervical cancer by detection and treatment of the precursor lesion cervical intraepithelial neoplasia (CIN). Women with abnormal cervical scrapes are referred to the gynaecologist for colposcopy and, if indicated, a guided biopsy is taken from the most suspicious area for high-grade CIN. Pathologists classify these CIN lesions with increasing CIN grade into grades 1 to 3 based on the degree of replacement of the normal epithelium by dysplastic epithelium and the severity of atypia of the abnormal cells in haematoxylin and eosin (H&E)-stained sections.1 However, classical histological grading of CIN remains subjective and is associated with a considerable interobserver and intraobserver variation and a moderate reproducibility.2-6 

Accurate assessment of high-grade CIN (ie, CIN2 and CIN3) is essential since treatment depends predominantly on lesion grade. In current practice, CIN3 and the majority of CIN2 lesions are treated by large-loop excision of the transformation zone (LLETZ) or conisation in order to prevent progression to cervical cancer. However, spontaneous regression of CIN2 (40-50%) and CIN3 lesions (1-30%) frequently occurs,7-9 indicating that the current diagnostic process is associated with substantial overtreatment. Particularly women within the reproductive age would benefit from a more conservative approach to prevent obstetric complications like preterm birth due to excisional treatment.10 There is thus a need for a classification system that can help to prevent overtreatment of high-grade CIN.

Cervical cancer and CIN lesions are caused by a persistent infection with the human papillomavirus (HPV). High-grade CIN lesions represent a heterogeneous group consisting of a subset of productive CIN lesions, characterised by the production of new viral particles, and a subset of transforming CIN lesions, characterised by HPV E6 and E7 deregulation.11 Biomarkers could help to differentiate CIN lesions associated with a productive HPV infection and high regression rates from CIN lesions associated with a transforming HPV infection and a presumed high short-term progression risk to cervical cancer.11 An important biomarker in the detection of transforming HPV infections is p16ink4a, which is used as a surrogate marker for transforming activity of HPV E7.12 Immunohistochemical staining of p16ink4a in clinical practice is currently only recommended by the LAST criteria in certain contexts, including cases where there is morphological doubt between low-grade and high-grade CIN.13 Another potential biomarker for CIN classification is Ki-67, a marker for cell cycle activity.14 Coexpression of p16ink4a and Ki-67, within the same cell, reflects cellular dysfunction and is only seen in HPV-transformed dysplastic cells.15,16 Recently, a classification system based on the cumulative score of p16ink4a and Ki-67 expression has shown to be more accurate and reproducible for CIN grading compared with haematoxylin and eosin (H&E) assessment alone. The cumulative score also reduces the diagnosis of CIN2 lesions for which clinical management is not uniform.17 This cumulative score of p16ink4a and Ki-67 is further referred to as the “immunoscore” (IS). Another immunohistochemical marker that could improve the accuracy of CIN diagnosis is the panHPV E4 protein (E4).18 E4 accumulates in cervical cells supporting HPV viral genome amplification. Therefore, it could be used as a biomarker for the identification of low-grade lesions and productive subsets of high-grade lesions.19,20

DNA methylation of promoter regions in tumour suppressor genes is a key event during cervical carcinogenesis.11 Several studies have shown the applicability of methylation markers for the

1 INTRODUCTION

What's new?

Treating all high-grade cervical intraepithelial neoplasia (CIN2/3) with excisional therapy leads to overtreatment, as these lesions have varying cancer progression risks. Here, the authors evaluated expression patterns of p16ink4a, Ki-67 and the HPV E4 protein, and methylation of FAM19A4/miR124-2 in high-grade CIN. The biomarker expression patterns revealed the high degree of heterogeneity among CIN2/3 lesions. Biomarker profiles based on the presumed cancer progression risks were established and could guide clinicians in choosing whether to treat immediately or wait and see.
identification of transforming CIN lesions, as well as the potential to predict regression and/or progression of CIN lesions.\textsuperscript{21,22} Furthermore, methylation analysis has demonstrated that CIN2/3 lesions associated with a persistent HPV infection of at least 5 years had cancer-like methylation patterns, suggesting a high short-term progression risk to cervical cancer.\textsuperscript{23,24} Methylation analysis in combination with immunohistochemical staining of p16\textsuperscript{INK4a}, Ki-67 and E4 might provide biomarker profiles that could improve the clinical guidance in women with high-grade CIN to prevent overtreatment of regressive lesions and potential obstetric complications.

The current study evaluates the cumulative p16\textsuperscript{INK4a} and Ki-67 immunoscore in a large series of high-grade CIN lesions derived from five European cervical referral or screening cohorts in relation with immunohistochemical HPV E4 expression patterns and \textit{FAM19A4/miR124-2} methylation analysis in the corresponding cervical scrape. All high-grade CIN lesions were stratified according to the p16\textsuperscript{INK4a} and Ki-67 immunoscore, E4 expression and \textit{FAM19A4/miR124-2} methylation status into biomarker profiles in order to assess heterogeneity within high-grade CIN lesions and to obtain biomarker profiles that could help to personalise treatment of high-grade CIN lesions based on the presumed cancer progression risk.

2  |  METHODS

2.1  |  Study population and sample collection

An international, multicentre, post hoc study was designed within five European prospective referral or HPV-based screening cohorts to assess biomarker patterns in a large series of high-risk HPV-positive high-grade cervical lesions. CIN2/3 lesions were selected based on local pathology diagnosis and availability of corresponding cervical scrapes. CIN lesions were included in the study population when diagnosis of CIN2/3 was confirmed after revision by an expert pathologist based on H&E staining. Formalin-fixed paraffin-embedded (FFPE) cervical biopsies or LLETZ specimen were used to assess p16\textsuperscript{INK4a}, Ki-67 and E4 expression. Data on HPV status and \textit{FAM19A4/miR124-2} methylation analysis in the corresponding cervical scrape were obtained from the participating institutes.\textsuperscript{25} Supplementary Table 1 shows detailed information on included samples from all participating institutes.

2.2  |  HPV testing and methylation analysis

HPV status and \textit{FAM19A4/miR124-2} methylation analysis were evaluated in cervical scrapes corresponding to the selected tissue blocks. CIN lesions were only included in case the corresponding cervical scrape was high-risk HPV positive. Participating institutes used clinically validated high-risk HPV DNA assays to determine HPV status\textsuperscript{26} and used the QIAsure Methylation Test (QIAGEN, Hilden, Germany) on the Rotorgene PCR-platform (QIAGEN, Hilden, Germany) to assess \textit{FAM19A4/miR124-2} methylation status as described previously.\textsuperscript{25,27} The housekeeping gene $\beta$-actin ($ACTB$) was used as a reference gene. Methylation levels were calculated as $\Delta\Delta C_t$ values by comparing the target $C_t$ values to the $C_t$ values of $ACTB$ relative to that of the calibrator. Methylation status was labelled positive if the QIAsure Methylation Test result exceeded the preset $\Delta\Delta C_t$ value threshold for methylation positivity for \textit{FAM19A4} and/or \textit{miR124-2} according to manufacturer’s instructions.

2.3  |  Immunohistochemistry

Serial sections of 3 $\mu$m were cut from all selected tissue blocks. The “sandwich” technique was used to enable histopathologic review of the H&E stained tissue sections flanking the sections subjected to immunohistochemical staining. In between sections were immunostained with mouse monoclonal antibodies (mAb) against Ki-67 antigen (Clone MIB-1, DAKO, Agilent Technologies, Santa Clara, CA), or p16\textsuperscript{INK4a} antigen (Clone E6H4, CINtec, Roche, Basel, Switzerland) by the automated IHC Ventana staining machine (Ventana Medical Systems, Roche, Oro Valley, AZ). The mAb panHPV E4 staining (developed in the laboratory of J. Doorbar, Cambridge, England, available through Labo Bio-medical Products B.V., Rijswijk, The Netherlands) was performed as described previously.\textsuperscript{20,28} In case of multiple available FFPE blocks per patient, the tissue block including the worst histology outcome was selected using H&E sections or original histology reports. Lesions with uninterpretable immunohistochemical staining were excluded ($n = 3$).

2.4  |  Scoring of Ki-67, p16\textsuperscript{INK4a} and E4

Two expert pathologists scored Ki-67, p16\textsuperscript{INK4a} and E4, independent of HPV and methylation results, as described previously.\textsuperscript{17,20} In short, nuclear Ki-67 staining in cells of the squamous epithelium was scored positive. Score 0 was considered a normal staining pattern (ie, scattered staining of nuclei in the basal layers). Scores 1, 2 and 3 were defined as increased nuclear staining predominantly found in the lower one-third, lower two-third and more than two-thirds of the epithelium, respectively. For p16\textsuperscript{INK4a} scoring, diffuse or “block” staining of the cell cytoplasm and/or nucleus in squamous epithelium was considered positive. Score 0 was defined as either no p16\textsuperscript{INK4a} positivity or focally scattered positive cells or small cell clusters (ie, patchy staining). Scores 1, 2 and 3 were defined as diffuse positive staining restricted to the lower one-third of the epithelium, diffuse positive staining restricted to the lower two-thirds of the epithelium and diffuse positive staining involving the full thickness of the epithelium, respectively. The cumulative score of Ki-67 and p16\textsuperscript{INK4a} (ranging from 0-6) was referred to as the “immunoscore” (IS).\textsuperscript{17} IS group 5-6 (high immunoscore) are considered CIN3-like lesions, IS group 3-4 (intermediate immunoscore) are considered CIN2-like lesions and IS group 0-2 (low immunoscore) are considered CIN1-like lesions. Membranous and/or cytoplasmic E4 staining was scored as either negative (Score 0), focally positive (ie, limited staining of some cells restricted to the superficial layer of the epithelium, Score 1) or extensively positive (ie,
widespread positive staining in the superficial layers of the epithelium extending to half of the epithelial width, Score 2).\textsuperscript{29} Examples of the scoring of E4 staining are shown in Supplementary Figure 2.

2.5 | Statistical analysis

Methylation status and HPV E4 expression were associated with CIN grade and immunoscore groups (0-2, 3-4 and 5-6). \( \chi^2 \) tests and \( \chi^2 \) tests for trend were conducted to assess E4 expression and methylation positivity with increasing CIN grade and immunoscore. Adjusted for CIN grade or immunoscore group, the association between age and methylation status or HPV E4 expression was examined using a Mantel-Haenszel analysis. Extensive E4 staining was considered as E4 positive. Differences in DNA methylation levels between groups were assessed using pairwise Mann-Whitney U-tests. A \( P \) value of .05 was considered as statistically significant with Bonferroni adjustment for multiple testing. All \( P \) values are considered two-sided. All statistical analyses were performed using SPSS (version 26.0, IBM Corp, Armonk, NY).

3 | RESULTS

3.1 | Study population

The study population consisted of 497 high-grade CIN lesions: 262 CIN2 (52.7%) and 235 CIN3 (47.3%). Supplementary Figure 1 shows the study flowchart of five European prospective referral or HPV-based screening cohorts from which the high-grade CIN lesions were derived. Supplementary Table 1 shows detailed cohort information per participating centre.\textsuperscript{30-34} Classification of these high-grade CIN lesions into immunoscore (IS) groups resulted in 30 CIN lesions within IS group 0-2 (6.0%), 151 CIN lesions within IS group 3-4 (30.4%) and 316 CIN lesions within IS group 5-6 (63.6%). Table 1 shows the association between H&E CIN diagnosis and immunoscore groups.

3.2 | E4 expression and methylation marker status in CIN grades and immunoscore groups

Positive E4 staining was present in 17.3% (86/497) of all CIN lesions. E4 positivity was significantly higher in CIN2 lesions than CIN3 lesions (Table 2A; \( P < .001 \)) and decreased significantly with increasing immunoscore group (Table 2B; \( P_{\text{trend}} < .001 \)). Women <29 years showed 26.3% (25/95) positive E4 staining compared with 15.2% (61/402) positive E4 staining in women \( \geq 29 \) years (\( P = .010 \)). Adjusted for CIN grade and IS group, E4 positivity was significantly higher in women <29 years than women \( \geq 29 \) years (\( P = .020 \) and \( P = .004 \), respectively).

\textit{FAM19A4/miR124-2} methylation in corresponding cervical scrapes was positive in 70.6% (351/497) (Table 2). Methylation positivity increased significantly with severity of CIN grade (Table 2A; \( P < .001 \)) and with increasing immunoscore group (Table 2B; \( P_{\text{trend}} < .001 \)). Women <29 years were methylation positive in 57.9% (55/95) compared with 73.6% (296/402) in women \( \geq 29 \) years (\( P = .002 \)). Adjusted for CIN grade and IS group, methylation positivity was significantly lower in women <29 years compared with women \( \geq 29 \) years (\( P = .005 \) and \( P = .001 \), respectively).

3.3 | Methylation levels in E4 positive and negative CIN lesions

Overall, methylation positivity was higher in E4-negative lesions than E4-positive lesions (308/411, 74.9% vs 43/86, 50.0%; \( P < .001 \)). Figure 1 shows methylation levels of \textit{FAM19A4} and \textit{miR124-2} in E4-positive and E4-negative lesions stratified by CIN grade (A,C) and IS group (B,D). A reference population of 230 squamous cell carcinomas,\textsuperscript{35} of which the corresponding cervical scrape was tested for \textit{FAM19A4/miR124-2} methylation, was added to this figure to enable comparison of methylation levels between CIN2/3 lesions and cervical cancer. For every CIN grade and IS group, E4-negative lesions showed higher methylation levels than E4-positive lesions, although not statistically significant for some comparisons (Figure 1). E4 expression was present in 9.8% (23/235) of CIN3 and in 12.0% (38/316) of IS group 5-6. These E4-positive CIN3 and IS 5-6 lesions showed methylation positivity in 60.9% (14/23) and 65.8% (25/38) of corresponding scrapes, respectively (Figure 1E). Similar findings were found in CIN2 and IS group 3-4.

3.4 | Productive and transforming characteristics in a small subset of CIN 2/3 lesions

In Table 3, we stratified all high-grade CIN lesions according to immunoscore, methylation status and E4 positivity. The various combinations of biomarker expression patterns confirm the heterogeneity within high-grade CIN lesions. These findings also indicate that CIN lesions can exhibit both productive (E4 positivity) and transforming characteristics (high p16\textsuperscript{ink4a} and Ki-67 scores and methylation positivity) simultaneously. Figure 2 shows examples of CIN2 and CIN3 lesions with combined productive and transforming characteristics.

4 | DISCUSSION

In the current study, we assessed the cumulative p16\textsuperscript{ink4a} and Ki-67 immunoscore in a large international series of high-grade CIN lesions in relation to immunohistochemical HPV E4 expression and \textit{FAM19A4/miR124-2} methylation analysis in the corresponding cervical scrape. We focused on CIN2/3 and not on CIN1, because especially in high-grade lesions the differences in biomarker results could have major consequences in clinical guidance. A considerable variation in E4 expression as indicator of a productive HPV infection was found.
in CIN2 and CIN3 lesions, and also in p16ink4a/Ki-67 expression and FAM19A4/miR124-2 methylation analysis, both markers for a transforming HPV infection. The p16ink4a and Ki-67 immunoscore grading system resulted in more CIN3-like lesions (increase of 34%) characterised by a high immunoscore (IS group 5-6) and in less CIN2-like lesions (decrease of 42%) characterised by an intermediate immunoscore (IS group 3-4). E4 expression decreased from CIN2 to CIN3 and from IS group 3-4 lesions to IS group 5-6 lesions while methylation positivity increased with severity of CIN grade and increasing immunoscore group. Stratifying all lesions according to immunoscore, methylation status and E4 expression confirmed the heterogeneity within high-grade CIN. Besides, in a small subset of lesions, combined productive and transforming characteristics were found.

We used the cumulative three-tiered immunoscore grading system with biomarkers p16ink4a and Ki-67 as an alternative grading system for CIN lesions, because it has a higher reproducibility for CIN3-like lesions (IS group 5-6) and CIN1-like lesions (IS group 0-2) and decreases the diagnosis of CIN2-like lesions (IS group 3-4) for which clinical management is not uniform and less defined.²⁷ In line with previous findings, the immunoscore grading system classified 262 CIN2 and 235 CIN3 into 316 lesions with a high immunoscore (IS group 5-6), 151 lesions with an intermediate immunoscore (IS group 3-4) and 30 lesions with a low immunoscore (IS group 0-2).

Our results showed significant increased methylation positivity with increasing CIN grade and IS group. Methylation positivity was 79.1% (186/235) in CIN3 and 78.5% (248/316) in IS group 5-6. In addition, we found a decrease in E4 expression with increasing CIN grade and IS group. E4 expression was present in 9.8% (23/235) of CIN3 and in 12.0% (38/316) of IS group 5-6. In earlier studies concerning E4 expression in CIN2/3 lesions, Griffin et al suggested largely mutually exclusive staining patterns between E4 and p16ink4a as neoplastic severity increases.²⁶ However, Leeman et al found 3% positive E4 staining in CIN3.²⁹ We showed that in a small number of CIN3 lesions (9.8%; 23/235) and IS 5-6 lesions (12.0%; 38/316), E4 positivity was found, which is in agreement with our earlier findings.²⁰ Moreover, we now extend these findings by showing that methylation was present in 60.9% (14/23) of E4-positive CIN3 and in 65.8% (25/38) of E4-positive IS 5-6 lesions. This indicates that in a minority of CIN3 and IS group 5-6, characteristics of both a productive and transforming HPV infection can be found and that production of viral particles and induction of cellular transformation is not mutually exclusive and may overlap. Until now, this phenomenon has only been described in HIV-positive women.³⁷

Previous studies have shown that CIN lesions in younger women are more likely to regress, probably because these lesions are less advanced due to a shorter duration of the associated HPV infection.³⁸ Indeed, we found that women <29 years showed more characteristics of productive HPV infections as expressed by a significant higher E4 positivity than women ≥29 years. Women <29 years also showed less methylation positivity than women ≥29 years. Although the numbers are small, in a subanalysis including only women ≤23 years (n = 13), 11 women (84.6%) showed extensive E4 expression and only 4 women (30.8%) were methylation positive. This lower methylation positivity and higher E4 expression in young women support the hypothesis that these lesions are less advanced CIN lesions.

Based on the results of this study, we stratified all CIN2/3 lesions according to the immunoscore and methylation status into biomarker profiles (Table 4). E4 expression was not used in these biomarker

### Table 1: Association between H&E CIN diagnosis and immunoscore groups

| CIN grade | 0-2 | 3-4 | 5-6 | Total |
|-----------|-----|-----|-----|-------|
| CIN2      | 23  | 105 | 134 | 262  |
| CIN3      | 7   | 46  | 182 | 235  |
| Total     | 30  | 151 | 316 | 497  |

### Table 2: E4 expression and methylation status in CIN (A) and immunoscore groups (B)

#### Table 2A: E4 expression

| CIN grade | E4 score | Methylation status |
|-----------|----------|--------------------|
|           | Negative | Positive | Negative | Positive | Total |
| CIN2      | 199      | 96.0%     | 3        | 24.0%    | 262  |
| CIN3      | 212      | 90.2%     | 12       | 9.8%     | 235  |
| Total     | 411      | 82.7%     | 86       | 17.3%    | 497  |

#### Table 2B: Methylation status

| Immunoscore | E4 score | Methylation status |
|-------------|----------|--------------------|
|             | Negative | Positive | Negative | Positive | Total |
| 0-2         | 21       | 70.0%     | 9        | 30.0%    | 30   |
| 3-4         | 112      | 74.2%     | 39       | 25.8%    | 151  |
| 5-6         | 278      | 88.0%     | 38       | 12.0%    | 316  |
| Total       | 411      | 82.7%     | 86       | 17.3%    | 497  |

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The low rate of E4 expression (17%) in high-grade CIN lesions, the absence of E4 in cervical carcinomas and in a considerable amount of CIN1 lesions, and the presence of E4 in a minority of methylation-positive CIN2/3 lesions make the potential clinical significance of absence or presence of E4 expression difficult to interpret in terms of regression or progression. Methylation status was included since methylation levels increase with increasing disease severity and are extremely high in cervical carcinomas (Figure 1).

TABLE 3  Biomarker expression patterns reflect heterogeneity within high-grade CIN lesions

| Immunoscore | IS 0–2 | IS 3–4 | IS 5–6 |
|-------------|--------|--------|--------|
| MM status   | MM−    | MM+    | MM−    | MM+    |
| E4 status   | E4−    | E4+    | E4−    | E4+    |

| Group | E4 pos | E4 neg | E4 pos | E4 neg | E4 pos | E4 neg | E4 pos | E4 neg |
|-------|--------|--------|--------|--------|--------|--------|--------|--------|
| CIN2  | 29 / 63 | 46.0%  | 136 / 199 | 68.3%  | 14 / 23  | 60.9%  | 172 / 212 | 81.1%  |
| 0 - 2 | 1 / 9   | 11.1%  | 14 / 21  | 66.7%  | 17 / 39  | 43.6%  | 71 / 112 | 63.4%  |
| 3 - 4 | 25 / 38  | 65.8%  | 223 / 278 | 80.2%  |

Note: High-grade lesions stratified by immunoscore group, FAM19A4/miR124-2 methylation status and E4 status.

FIGURE 1  Methylation levels represented by the log_{10} transformed ΔΔCt ratios of FAM19A4 stratified by E4 positivity in CIN grade (A) and immunoscore groups (B) and of miR124-2 stratified by E4 positivity in CIN grade (C) and immunoscore groups (D). FAM19A4/miR124-2 methylation positivity rate for each subgroup is shown in E. Superscript 1 indicates FAM19A4/miR124-2 methylation levels of a reference population of 230 squamous cell carcinoma, in the corresponding smear originating from Reference 35. CIN, cervical intraepithelial neoplasia; pos, positive; n, number of methylation positives; N, group size; neg, negative. *P < .05, **P < .01, ***P < .001, ns: not significant.
associated with a long duration of the HPV infection (>5 years) in contrast to lesions associated with a short duration of the HPV infection (<5 years). In addition, a negative FAM19A4/miR124-2 methylation test in HPV-positive women provided a very low 14-year cervical cancer risk. In agreement with the latter finding, preliminary results of the CONCERVE study show that women with CIN2/3 with a negative FAM19A4/miR124-2 methylation test have a higher regression rate than women with a positive methylation test (Kremer, Dick, Mei- je et al., unpublished data, manuscript in preparation). Therefore, it is assumed that methylation-positive CIN2/3 lesions have a higher short-term progression risk to cervical cancer than methylation-negative CIN2/3 lesions.

Table 4 shows all CIN2/3 lesions stratified according to immunoscore and methylation status into three groups. The depicted colour codes within these biomarker profiles correspond to the presumed short-term progression risk to cervical cancer based on the results of DNA methylation analysis and the immunoscore. Lesions with a presumed high short-term progression risk to cervical cancer are depicted in red and LLETZ is advised. Lesions with a presumed low short-term progression risk to cervical cancer are depicted in red.
yellow and may have a “wait and see” follow-up of 12 months, whereas the orange colour depicts the women with a presumed intermediate risk. In this proposal, no difference has been made between methylation-positive IS group 3-4 and methylation-positive IS group 5-6. Increase of p16\textsuperscript{ink4a} block stain expression has been associated with severity of CIN grade,\textsuperscript{43} but p16\textsuperscript{ink4a} expression does not predict clinical behaviour or prognosis of CIN lesions.\textsuperscript{44-46} For methylation-negative women in IS group 3-4 and IS group 5-6, we suggest a follow-up time of 6 months. Especially for women in child-bearing age, the proposed management scheme provides the physician more objective arguments for a “wait and see” strategy for some high-grade CIN lesions, thereby preventing cervical morbidity. This management proposal might be used as a clinical guidance for the physician in the treatment of high-grade CIN. Our study shows that from the 497 high-grade lesions, 15 lesions have an assumed low risk and 146 lesions have an intermediate risk. This proposal will result in the immediate treatment in 68% and in a “wait and see” policy in 32% of the high-grade CIN lesions. Since clinicians do not prefer to postpone treatment of CIN3 unless in case of pregnancy or fertility treatment or treatment, the biggest advantage may be expected in the treatment of CIN2 lesions. Table 4 shows that from the 262 CIN2 lesions, 13 lesions have a supposed low risk and 94 lesions are assumed to have an intermediate risk, which supports postponement of treatment. Of course, the presumed biological behaviour of CIN2/3 lesions with the investigated biomarker profiles needs to be further evaluated in prospective studies.

In conclusion, we found a considerable amount of heterogeneity in biomarker expression in a large series of high-grade CIN lesions evaluated with p16\textsuperscript{ink4a}, Ki-67 and E4 immunohistochemical staining and FAM19A/miR124-2 methylation. We defined biomarker profiles that might help the clinician in a more personally tailored management of women with high-grade CIN based on the presumed short-term cancer progression risk thereby preventing overtreatment, especially in young women.

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### CONFLICT OF INTERESTS

CJLMM, DAMH and RDMS are minority shareholders of Self-screen B.V., a spin-off company of VUmc; Self-screen B.V. develops, manufactures and licences the high-risk HPV assay and methylation marker assays for cervical cancer screening and holds patents on these tests. Self-screen B.V. was supported by the Valid-screen project, funded by the SME Instrument in the Horizon 2020 Work Program of the European Commission (Valid-screen 666 800). CJLMM is part-time CEO of Self-screen B.V. and has a very small number of shares of MDXHealth and previously of QIAGEN; has received speakers fees from GSK, QIAGEN and SPMSD/Merck; and served occasionally on the scientific advisory boards (expert meeting) of these companies. DAMH has been on the speakers bureau of QIAGEN and previously of QIAGEN; serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb. AOV has received reimbursement of travel expenses for attending conferences and honoraria for speaking from Abbott Molecular, QIAGEN

### TABLE 4

High-grade CIN lesions ranked according to immunoscore and FAM19A/miR124-2 methylation status with a potential management proposal based on the presumed progression risk to cervical cancer

| Immunoscore group | IS 0-2 | IS 3-4 | IS 5-6 |
|-------------------|--------|--------|--------|
| MM status         | MM−    | MM+    | MM−    | MM+    | MM−    | MM+    |
| CIN2 n            | 13     | 10     | 44     | 61     | 40     | 94     |
| CIN3 n            | 2      | 5      | 19     | 27     | 28     | 154    |
| <29 y n           | 1      | 3      | 16     | 7      | 23     | 45     |
| ≥29 y n           | 14     | 12     | 47     | 81     | 45     | 203    |
| Total n           | 15     | 15     | 63     | 88     | 68     | 248    |

Note: Yellow colour indicates low progression risk to cancer, follow-up after 12 months. Orange colour indicates intermediate progression risk to cancer, follow-up after 6 months. Red colour indicates high progression risk to cancer, LLETZ advised.

Abbreviations: CIN, cervical intraepithelial neoplasia; IS, immunoscore; MM, methylation marker; MM−, methylation marker negative; MM+, methylation marker positive; n, number of lesions.
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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