CHAPTER 4

Comparing the performance of \textit{FAM19A4} methylation analysis, cytology and HPV16/18 genotyping for the detection of cervical (pre)cancer in high-risk HPV-positive women of a gynecologic outpatient population (COMETH study)

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ABSTRACT

Recently, DNA methylation analysis of FAM19A4 in cervical scrapes has been shown to adequately detect high-grade cervical intraepithelial neoplasia and cervical cancer (≥CIN3) in high-risk HPV (hrHPV)-positive women. Here, we compared the clinical performance of FAM19A4 methylation analysis to cytology and HPV16/18 genotyping, separately and in combination, for ≥CIN3 detection in hrHPV-positive women participating in a prospective observational multi-center cohort study. The study population comprised hrHPV-positive women aged 18-66 years, visiting a gynecological outpatient clinic. From these women, cervical scrapes and colposcopy-directed biopsies (for histological confirmation) were obtained. Cervical scrapes were analyzed for FAM19A4 gene promoter methylation, cytology and HPV16/18 genotyping. Methylation analysis was performed by quantitative methylation-specific PCR (qMSP). Sensitivities and specificities for ≥CIN3 were compared between tests. Stratified analyses were performed for variables that potentially influence marker performance. Of all 508 hrHPV-positive women, the sensitivities for ≥CIN3 of cytology, FAM19A4 methylation analysis, and cytology combined with HPV16/18 genotyping were 85.6%, 75.6% and 92.2%, respectively, with corresponding specificities of 49.8%, 71.1%, and 29.4%, respectively. Both sensitivity and specificity of FAM19A4 methylation analysis were associated with age (p≤0.001 each). In women ≥30 years (n=287), ≥CIN3 sensitivity of FAM19A4 methylation analysis was 88.3% (95%CI:80.2-96.5) which was non-inferior to that of cytology [85.5% (95%CI:76.0-94.0)], at a significantly higher specificity [62.1% (95%CI:55.8-68.4) compared to 47.6% (95%CI:41.1-54.1)]. In conclusion, among hrHPV-positive women from an outpatient population aged ≥30 years, methylation analysis of FAM19A4 is an attractive marker for the identification of women with ≥CIN3.
INTRODUCTION

An infection with high-risk human papillomavirus (hrHPV) is essential for the development of cervical cancer.\(^1,2\) hrHPV DNA testing has emerged as a more sensitive screening tool than cytology, leading to a higher protection against cervical intraepithelial neoplasia grade 3 (CIN3) and cervical cancer.\(^3-5\) However, many hrHPV infections have an indolent nature and only a fraction of hrHPV-positive women have high-grade CIN lesions with a high cancer progression risk. In order to reduce over-diagnosis and unnecessary referral, additional triage testing is required to detect the subgroup of hrHPV-positive women with clinically meaningful cervical disease. To date, various triage strategies for hrHPV-positive women have been considered including repeat cytology testing,\(^6\) HPV E7 mRNA analysis,\(^7,8\) p16/ki67 cytological dual staining,\(^9,10\) HPV16/18 genotyping,\(^11,12\) and combinations thereof.\(^5,13\) Besides these markers, epigenetic changes in the host and/or viral genome that are associated with progression towards invasive cancer\(^1,14\) are attractive targets to design objective and molecular biomarkers to detect amongst hrHPV-positive women those who have cervical (pre)cancer. DNA methylation analysis of human genes by (quantitative) methylation-specific PCR (MSP)-based methods has shown promising results on both hrHPV-positive cervical scrapes and self-collected specimens,\(^15-20\) with overall sensitivities for ≥CIN3 similar to those of cytology, and extremely high sensitivities (up to 100\%) for cervical carcinoma.\(^16,18,21\) When applied to cervical scrapes, a methylation marker recently identified by a genome-wide methylation screen, \textit{FAM19A4},\(^22\) was shown to detect all cervical carcinomas and CIN3 lesions with a long-term (i.e., ≥5 years) duration of preceding hrHPV-infection (PHI, used as a proxy of duration of lesion existence).\(^23\) The latter are considered the more advanced CIN3 lesions with a high short-term progression risk to cancer, partially explained by a high number of chromosomal alterations.\(^24\) As such, \textit{FAM19A4} can be an attractive marker for cervical disease in hrHPV-positive women. In this prospective cohort study, performed on hrHPV-positive women from six outpatient clinics, we compared the clinical performance of \textit{FAM19A4} methylation analysis to cytology and HPV16/18 genotyping, separately and in combination, for the detection of CIN3 or cervical cancer (≥CIN3).

MATERIALS AND METHODS

Study design, participants, and procedures

From December 2010 till December 2013, women between 18 and 70 years were recruited for participation in a prospective observational multi-center cohort study among women visiting a gynecological outpatient clinic in one of six hospitals in the Netherlands: VU University Medical Center (VUmc), Sint Lucas Andreas Hospital, University Medical Center Utrecht, Reinier de Graaf Groep, Sint Antonius Hospital and Flevo Hospital. The study was approved by the Medical Ethical Committee of all participating hospitals (METc-VUmc2009/178) and registered as NTR2447. Women were eligible for participation in the study regardless of their reason for visiting the
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gynecologist. Consequently, also women who had been referred because of a recent abnormal cervical scrape could participate. Exclusion criteria included any history of treatment for cervical dysplasia or cervical cancer, current cancer, pregnancy or lactation. As shown in Figure 1, in total 2970 women gave informed consent and participated in the study. These women were offered self-sampling of cervico-vaginal lavage material using the Delphi screener (Delphi Bioscience B.V., the Netherlands – currently produced by Rover Medical Devices, the Netherlands) for hrHPV testing using GP5+/6+ PCR-enzyme immunoassay analysis (EIA kit HPV GP HR, Diassay B.V., the Netherlands). All self-sample analyses were performed at the department of Pathology at VUmc. From 717 women who tested hrHPV-positive on self-collected material, 78 (11%) had to be excluded as they did not meet the inclusion criteria. From the remaining 639 women, a cervical scrape was taken by the gynecologist using a Cervex-Brush (Rovers Medical Devices B.V., the Netherlands) or a Medscand Cytobrush Plus (CooperSurgical Inc., USA). Material was stored in 20 ml of Thinprep PreservCyt solution (Hologic Inc., USA). Cervical scrapes that tested hrHPV-positive (n=556) were subsequently tested for three markers: (liquid based) cytology, FAM19A4 methylation and HPV16/18 genotyping. Women with valid test results in all three assays (n=508) comprised the final study population and all underwent colposcopy-examination. In 289 (57%) women, the cervical scrape was taken at a separate visit, at a minimum of two weeks prior to colposcopy. In 219 (43%) women, for logistic reasons, the cervical scrape was done immediately prior to colposcopy.

At colposcopy, cervical biopsies were taken from every visible lesion for histological assessment and classified as normal (CIN0), CIN1, CIN2, CIN3, or invasive cancer, according to international criteria. In case no lesions were visible, it was mandatory to take two random biopsies (6 and 12 o’clock). In case the squamocolumnar junction could not be brought into view, endocervical curettage was performed. All women were treated according to national guidelines for CIN and cervical cancer. All women with histologically confirmed CIN3 underwent large loop excision of the transformation zone (LLETZ) or cervical conisation. Depending on the size of the lesion, also 65% (i.e., 65/100) of women with CIN2 underwent LLETZ. Of these, 10 (15%) were diagnosed with CIN3 in the LLETZ tissue, and categorized accordingly.

Cytology
Liquid based cytology preparations were cytologically classified according to the CISOE-A classification (reporting on composition, inflammation, squamous, other and endometrium, and endocervical cylindrical epithelium, and adequacy) used in the Netherlands. The results can be translated into the Bethesda classification, in which borderline or mild dyskaryosis (BMD) equals ASC-US/ASC-H/LSIL, and >BMD equals high-grade squamous intraepithelial lesion (HSIL). Cytotechnicians were aware of the hrHPV-positive status of the cervical scrapes.

HPV genotyping
DNA was isolated from 1/10th of cervical scrape material using the Nucleo-Spin 96 Tissue kit (Macherey-Nagel, Germany) and a Microlab Star robotic system (Hamilton, Germany) according
FAM19A4 methylation, cytology and HPV16/18 genotyping

Figure 1. Overview of study population. hrHPV: high-risk human papillomavirus, LLETZ: large loop excision of the transformation zone, CIN: cervical intraepithelial neoplasia, AIS: adenocarcinoma in situ

to manufacturers’ instructions, and subjected to GP5+/6+PCR-EIA. Subsequent genotyping for the high-risk HPV types 16/18/31/33/35/39/45/51/52/56/58/59/66/68 was performed using a microsphere bead-based assay (Luminex) as previously described.

qMSP analysis

Extracted DNA from hrHPV-positive cervical scrapes was subjected to bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research, USA) as described previously. Bisulphite-converted DNA was used as template for FAM19A4 methylation analysis by qMSP using housekeeping gene β-actin (ACTB) as a reference gene. qMSP analysis was performed on an ABI 7500 real-time PCR-system (Applied Biosystems, USA). For each target, Quantification Cycle (Cq) values were measured at a fixed fluorescence threshold. All samples included in the study had a Cq value for ACTB <32 to assure sample quality. For each sample Cq ratios were calculated using the following formula: $2^{(\text{Cq}_\text{ACTB} - \text{Cq}_\text{FAM19A4})} \times 100$. The threshold value (0.415) that gave rise to
a ≥CIN3 specificity of 70%, as determined and validated in De Strooper et al.,23 was chosen to consider a specimen positive for FAM19A4 methylation analysis.

Statistical analysis

We used histologically confirmed ≥CIN3 as primary study endpoint. ≥CIN2 was used as secondary study endpoint, as the category of CIN2 reflects heterogeneous disease, of which a substantial portion represent productive hrHPV-infections1 that will regress spontaneously.29,30 Study endpoint was assessed based on the histological outcome of the colposcopy-directed biopsy, or, if classified worse, on the histology result of the specimen excised by LLETZ, conisation or hysterectomy. The sample size was set such that 90% power was achieved for demonstrating non-inferiority of FAM19A4 methylation analysis or HPV16/18 genotyping compared to cytology using a matched-sample score test.31,32 A minimum of 300 hrHPV-positive women needed to be included at rejection rate α of 0.05. Finally, 508 hrHPV-positive women were included with results for all markers. For FAM19A4 methylation analysis, cytology, HPV16/18 genotyping and combinations (i.e., FAM19A4 and cytology, FAM19A4 and HPV16/18 genotyping, HPV16/18 genotyping and cytology), sensitivity, specificity, positive predictive value (PPV), 1-negative predictive value (complemented NPV, a measure of disease risk after a negative result) for detection of ≥CIN3 and ≥CIN2 and referral rate (based on % marker positivity) were calculated with 95% confidence intervals (95% CI). Relative sensitivities (ratios of the sensitivity of one test to the sensitivity of another test) and relative specificities (ratios of the specificity of one test to the specificity of another test) were calculated with 95% CIs to enable comparisons. A difference in sensitivity or specificity was considered significant if the 95%CI of the relative sensitivity or specificity was entirely below or above one. Forest plots of relative sensitivities and specificities of the different tests were made using cytology as reference test. In case of non-significant differences in sensitivity, an additional non-inferiority test was performed. Non-inferiority was defined as a relative sensitivity of at least 90% using a matched-sample score test.31,32 We considered the influence of three factors when estimating the sensitivity and specificity of the different markers. First, the age of the participants (aged ≥30 years (cervical screening target in the Netherlands) versus <30 years); secondly, the reason of referral to the gynecologist (non-cervix-related gynecological complaints versus a recent abnormal cytology result in cervical screening); and thirdly, the sampling method of the cervical scrape (whether the collection of the cervical scrape was done during a separate visit prior to colposcopy versus cervical scrape combined with the colposcopy procedure in one visit). The influence of the factors was studied using logistic regression. After determining the factors that significantly influenced the performance of the different markers (significance: p<0.05), data were stratified for these factors. All statistical analyses and computation of graphs were performed in IBM SPSS Statistics 20, STATA 11.0 and Excel.
RESULTS

Patients and histological outcomes
The study flowchart is shown in Figure 1. Final analysis comprised 508 women who had valid results for all three markers and who underwent colposcopy-examination. Histology revealed that three women (0.6%) had invasive cervical carcinoma (i.e., one squamous cell carcinoma (SCC), one adenosquamous carcinoma and one adenocarcinoma), 87 women (17.1%) had CIN3 (including 2 adenocarcinoma in situ), 90 women (17.7%) had CIN2, 138 (27.2%) had CIN1 and 190 (37.4%) had no CIN. Of the corresponding 508 hrHPV-positive cervical scrapes, 37% (189/508) scored positive for FAM19A4 methylation, 56% (287/508) had abnormal cytology (≥BMD) and 48% (243/508) tested positive for HPV16 and/or HPV18 (HPV16/18). All three women diagnosed with cervical cancer tested positive for both FAM19A4 methylation and cytology. Two of them scored HPV16 positive, and one woman with SCC had a single infection with HPV39.

Performance of markers
Test specifications of the investigated markers, and combinations thereof, for detection of ≥CIN3 in the total study population are shown in Table 1 (upper panel). Relative sensitivities and specificities for ≥CIN3 of FAM19A4 methylation analysis, HPV16/18 genotyping, and various marker combinations compared to cytology (which was used as reference), are shown in Figure 2 (upper panel). The ≥CIN3 sensitivity of FAM19A4 methylation analysis (75.6%) and cytology (85.6%) did not differ significantly. As a statistical difference between the sensitivities of both markers could not be established, subsequent matched sample score testing was performed to evaluate non-inferiority. As the relative sensitivity of FAM19A4 methylation analysis was lower than 90%, the non-inferiority threshold was not met (p=0.61). The ≥CIN3 specificity (71.1%) of FAM19A4 methylation analysis was significantly higher than that of cytology (49.8%). For ≥CIN2 outcome (Table 2 and Figure 3, upper panel), a significantly lower sensitivity of FAM19A4 methylation analysis (57.8%) compared to cytology (82.8%) was observed at a significantly higher specificity (74.1% versus 57.9%). In addition, the ≥CIN3 sensitivity of FAM19A4 methylation analysis was significantly lower than that of cytology combined with HPV16/18 genotyping (75.6% versus 92.2%; ratio 0.82; 95%CI:0.72-0.93), at significantly higher specificity (71.1% versus 29.4%; ratio 2.41; 95%CI:2.07-2.82). Similar results were obtained for ≥CIN2 detection (FAM19A4 methylation analysis versus cytology and HPV16/18 testing: sensitivity 57.8% versus 89.4%; ratio 0.65; 95%CI:0.57-0.74; specificity 74.1% versus 33.8%; ratio 2.19; 95%CI:1.86-2.57; Table 2). The ≥CIN3 sensitivity of FAM19A4 methylation analysis combined with cytology was significantly higher than that of cytology alone (94.4% versus 85.6%), but had a significantly lower specificity (37.8% versus 49.8%; Table 1; Figure 2). The ≥CIN3 sensitivity of combined FAM19A4 methylation analysis and HPV16/18 genotyping was non-inferior to cytology (92.2% versus 85.6%; p<0.001) but had a significantly lower specificity (42.3% versus 49.8%; Table 1; Figure 2).
Factors influencing marker performance

Three factors were evaluated for a potential influence on marker performance: 1) age of the participants, i.e., women ≥30 years (n=287) or <30 years (n=221); 2) reason of referral, i.e., because of a recent abnormal cytological scrape (n=213) or non-cervix-related gynecological complaints (n=295), 3) moment of taking cervical scrape, i.e., at a separate visit 2-3 weeks prior to the colposcopy visit (n=289) or at the same visit as colposcopy (n=219). The performance of FAM19A4 methylation analysis was significantly influenced by age of the participants (Table 3A), whereas the performance of cytology was significantly correlated to the referral reason to the gynecologist, both in univariate and multivariate analyses (Table 3B). The performance of HPV16/18 genotyping was not influenced by any of these variables (data not shown). The moment of taking the cervical scrape did not influence the performance of the investigated markers.

Stratified analysis of marker performance

Subsequently, marker analysis was performed after stratification for age (for FAM19A4 methylation analysis) and referral reason (for cytology). Of note, in women <30 years, 31% was referred based on an abnormal scrape, whereas in women ≥30 years, 56% was referred for this reason. The performance of the markers in the subpopulation of women ≥30 years for the detection of ≥CIN3 (n=287; 60 ≥CIN3, 51 CIN2 and 176 ≤CIN1), the age group targeted in Dutch national screening, are shown in Table 1 and Figure 2 (lower panel). In this subpopulation, the ≥CIN3 sensitivity of FAM19A4 methylation analysis (88.3%) was non-inferior (p=0.024) to that of cytology (85.0%), whereas its specificity was significantly higher (62.1% versus 47.6%). Results for ≥CIN2 are presented in Figure 3 and Table 2. In the subgroup of women <30 years (n=221; 30 ≥CIN3, 39 CIN2 and 152 ≤CIN1), the ≥CIN3 sensitivity of FAM19A4 methylation analysis was significantly lower compared to that of cytology (50.0% versus 86.7%; ratio 0.58; 95%CI:0.40-0.83; Table 1), whereas its specificity was significantly higher (81.7% versus 52.4%; ratio 1.56; 95%CI:1.35-1.80; Table 1). In the subpopulation of women referred to the gynecologist because of non-cervix-related gynecological complaints (n=295; 26 ≥CIN3, 43 CIN2 and 226 ≤CIN1), the ≥CIN3 sensitivity of FAM19A4 methylation analysis did not differ significantly from cytology (61.5% versus 80.8%; ratio 0.76; 95%CI:0.55-1.05). Subsequent matched sample score testing was performed to evaluate non-inferiority, which could not be established (p=0.85). The specificity of FAM19A4 methylation analysis was significantly higher than that of cytology (73.2% versus 63.6%; ratio 1.15; 95%CI:1.03-1.28). Results for ≥CIN2 are presented in Table 2.
Figure 2. Sensitivities and specificities of different markers for ≥CIN3 detection in hrHPV-positive women. Forest plots showing the relative sensitivity and specificity of the different markers compared to cytology are presented in the total study population and the subgroup of women aged ≥30 years.
Table 1. Test specifications of cytology, FAM19A4 methylation, HPV16/18 genotyping and combinations of markers for detection of eCIN3 in hr HPV-positive women in the total study population, and stratified by age and referral reason

CIN: cervical intraepithelial neoplasia (grade 3 or higher); CI: confidence interval; PPV: positive predictive value; 1-NPV: complemented negative predictive value; n1: number of test positive disease cases; N1: total number of disease cases; n2: number of test negative non-disease cases; N2: total number of non-disease cases; *based on % marker positivity

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<th>Specificity (95% CI)</th>
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</table>
Table 2. Test specifications of cytology, FAM19A4 methylation, HPV16/18 genotyping and combinations of markers for detection of ≥CIN2 in hrHPV-positive women in the total study population, and stratified by age and referral reason

CIN: cervical intraepithelial neoplasia (grade 2 or higher); CI: confidence interval; PPV: positive predictive value; 1-NPV: complemented negative predictive value; n1: number of test positive disease cases; n2: number of test negative non-disease cases; N: total number of non-disease cases; *based on % marker positivity

<table>
<thead>
<tr>
<th>Triage marker</th>
<th>n1/N1</th>
<th>Sensitivity (95% CI)</th>
<th>n2/N2</th>
<th>Specificity (95% CI)</th>
<th>PPV (95% CI)</th>
<th>1-NPV (95% CI)</th>
<th>Referral rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total study population (n=508)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>149/180</td>
<td>82.8% (77.3-88.3%)</td>
<td>190/328</td>
<td>57.9% (52.6-63.3%)</td>
<td>51.9% (46.1-57.7%)</td>
<td>14.0% (9.4-18.6%)</td>
<td>56.5%</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>104/180</td>
<td>57.8% (50.6-65.0%)</td>
<td>243/328</td>
<td>74.1% (69.3-78.8%)</td>
<td>55.0% (47.9-62.1%)</td>
<td>23.8% (19.1-28.5%)</td>
<td>37.2%</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>106/180</td>
<td>58.9% (51.7-66.1%)</td>
<td>191/328</td>
<td>58.2% (52.9-63.6%)</td>
<td>43.6% (37.4-49.9%)</td>
<td>27.9% (22.5-33.3%)</td>
<td>47.8%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>162/180</td>
<td>90.0% (85.6-94.4%)</td>
<td>145/328</td>
<td>44.2% (38.8-49.6%)</td>
<td>47.0% (41.7-52.2%)</td>
<td>11.0% (6.2-15.9%)</td>
<td>67.9%</td>
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<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>141/180</td>
<td>78.3% (72.3-84.4%)</td>
<td>145/328</td>
<td>44.2% (38.8-49.6%)</td>
<td>43.5% (38.1-48.9%)</td>
<td>21.2% (15.3-27.1%)</td>
<td>63.8%</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>161/180</td>
<td>89.4% (85.0-93.9%)</td>
<td>111/328</td>
<td>33.8% (28.7-39.0%)</td>
<td>42.6% (37.6-47.6%)</td>
<td>14.6% (8.5-20.7%)</td>
<td>74.4%</td>
</tr>
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<td><strong>Subgroup: women &lt;30 years (n=221)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>53/69</td>
<td>76.8% (66.9-86.8%)</td>
<td>88/152</td>
<td>57.9% (50.0-65.7%)</td>
<td>45.3% (36.3-54.3%)</td>
<td>15.4% (8.5-22.3%)</td>
<td>52.9%</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>25/69</td>
<td>36.2% (24.9-47.6%)</td>
<td>127/152</td>
<td>83.6% (77.7-89.4%)</td>
<td>50.0% (36.1-63.9%)</td>
<td>25.7% (19.2-32.3%)</td>
<td>22.6%</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>42/69</td>
<td>60.9% (49.4-72.4%)</td>
<td>89/152</td>
<td>58.6% (50.7-66.4%)</td>
<td>40.0% (30.6-49.4%)</td>
<td>23.3% (15.6-31.0%)</td>
<td>47.5%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>56/69</td>
<td>81.2% (71.9-90.4%)</td>
<td>75/152</td>
<td>49.3% (41.4-57.3%)</td>
<td>42.1% (33.7-50.5%)</td>
<td>14.8% (7.4-22.2%)</td>
<td>60.2%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>49/69</td>
<td>71.0% (60.3-81.8%)</td>
<td>76/152</td>
<td>50.0% (42.1-57.9%)</td>
<td>39.2% (30.6-47.8%)</td>
<td>20.8% (12.7-29.0%)</td>
<td>56.6%</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>60/69</td>
<td>87.0% (79.0-94.9%)</td>
<td>51/152</td>
<td>33.6% (26.0-41.1%)</td>
<td>37.3% (29.8-44.7%)</td>
<td>15.0% (6.0-24.0%)</td>
<td>72.9%</td>
</tr>
<tr>
<td><strong>Subgroup: women ≥30 years (n=287)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>96/111</td>
<td>86.5% (80.1-92.8%)</td>
<td>102/176</td>
<td>58.0% (50.7-65.2%)</td>
<td>56.3% (49.0-63.9%)</td>
<td>12.8% (6.8-18.9%)</td>
<td>59.2%</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>79/111</td>
<td>71.2% (62.7-79.6%)</td>
<td>116/176</td>
<td>65.9% (58.9-72.9%)</td>
<td>56.5% (48.6-65.1%)</td>
<td>21.6% (15.0-28.3%)</td>
<td>48.4%</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>64/111</td>
<td>57.7% (48.5-66.6%)</td>
<td>102/176</td>
<td>58.0% (50.7-65.2%)</td>
<td>46.4% (38.1-54.7%)</td>
<td>31.5% (24.1-61.9%)</td>
<td>48.1%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>106/111</td>
<td>95.5% (91.6-99.4%)</td>
<td>70/176</td>
<td>39.8% (32.5-47.0%)</td>
<td>50.6% (43.3-56.7%)</td>
<td>6.7% (1.0-12.3%)</td>
<td>73.9%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>92/111</td>
<td>82.9% (75.9-89.9%)</td>
<td>69/176</td>
<td>39.2% (32.0-46.4%)</td>
<td>46.3% (39.3-53.2%)</td>
<td>21.6% (13.0-30.2%)</td>
<td>69.3%</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>101/111</td>
<td>91.0% (85.7-96.3%)</td>
<td>60/176</td>
<td>34.1% (27.1-41.1%)</td>
<td>46.3% (39.9-53.2%)</td>
<td>14.3% (6.1-22.5%)</td>
<td>75.6%</td>
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<tr>
<td><strong>Subgroup: women referred to gynecologist for non-cervix-related complaints (n=295)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>49/69</td>
<td>71.0% (60.3-81.7%)</td>
<td>156/226</td>
<td>69.0% (63.0-75.1%)</td>
<td>41.2% (32.3-50.0%)</td>
<td>11.4% (6.7-16.1%)</td>
<td>40.3%</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>35/69</td>
<td>50.7% (38.9-62.5%)</td>
<td>173/226</td>
<td>76.5% (71.0-82.1%)</td>
<td>39.8% (29.5-50.0%)</td>
<td>16.4% (11.4-21.5%)</td>
<td>29.8%</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>44/69</td>
<td>63.8% (52.4-75.1%)</td>
<td>135/226</td>
<td>59.7% (53.3-66.1%)</td>
<td>32.6% (24.7-40.5%)</td>
<td>15.6% (10.0-21.3%)</td>
<td>45.8%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>55/69</td>
<td>79.7% (70.2-89.2%)</td>
<td>122/226</td>
<td>54.0% (47.5-60.5%)</td>
<td>34.6% (27.2-42.0%)</td>
<td>10.3% (5.2-15.4%)</td>
<td>53.9%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>54/69</td>
<td>78.3% (68.5-88.0%)</td>
<td>108/226</td>
<td>47.8% (41.3-54.3%)</td>
<td>31.4% (24.5-38.3%)</td>
<td>12.2% (6.4-18.0%)</td>
<td>58.3%</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>58/69</td>
<td>84.1% (75.4-92.7%)</td>
<td>92/226</td>
<td>40.7% (34.3-47.1%)</td>
<td>30.2% (23.7-36.7%)</td>
<td>10.7% (4.7-16.6%)</td>
<td>65.1%</td>
</tr>
</tbody>
</table>
Figure 3. Sensitivities and specificities of different markers for ≥CIN2 detection in hr HPV-positive women. Forest plots showing the relative sensitivity and specificity of the different markers compared to cytology are presented in the total study population and the subgroup of women aged ≥30 years.

<table>
<thead>
<tr>
<th>Triage marker</th>
<th>Relative sensitivity ≥CIN2 compared to cytology (95% CI)</th>
<th>Relative specificity ≥CIN2 compared to cytology (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Group (n=508)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>1 (reference)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>0.70 (0.61 - 0.80)</td>
<td>1.28 (1.15 - 1.43)</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>0.71 (0.63 - 0.81)</td>
<td>1.01 (0.88 - 1.14)</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>1.09 (1.04 - 1.14)</td>
<td>0.76 (0.71 - 0.83)</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>0.95 (0.86 - 1.04)</td>
<td>0.76 (0.66 - 0.89)</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>1.08 (1.03 - 1.13)</td>
<td>0.58 (0.52 - 0.66)</td>
</tr>
<tr>
<td>Subgroup women aged ≥ 30 years (n=287)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology</td>
<td>1 (reference)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>0.82 (0.72 - 0.94)</td>
<td>1.14 (0.97 - 1.33)</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>0.67 (0.57 - 0.78)</td>
<td>1.00 (0.84 - 1.19)</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>1.10 (1.04 - 1.17)</td>
<td>0.69 (0.60 - 0.78)</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>0.96 (0.86 - 1.07)</td>
<td>0.68 (0.54 - 0.84)</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>1.05 (1.01 - 1.10)</td>
<td>0.59 (0.50 - 0.69)</td>
</tr>
</tbody>
</table>
FAM19A4 methylation, cytology and HPV16/18 genotyping

### Table 3A. Age as a covariate of the performance of FAM19A4 methylation analysis
The odds ratios represent the odds of a positive FAM19A4 result in women aged ≥30 relative to women aged <30 (stratified by histological endpoint). *Adjusted for referral reason of patient and sampling method of cervical scrape; # p-value obtained by logistic regression; CIN: cervical intraepithelial neoplasia (grade 2 or 3 or higher); CI: confidence interval

<table>
<thead>
<tr>
<th>Inclusion criterion</th>
<th>Response variable: FAM19A4 methylation analysis (0= negative, 1= positive)</th>
<th>Covariate: age (0=&lt; 30 years, 1= ≥ 30 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted analysis</td>
<td>Adjusted analysis*</td>
</tr>
<tr>
<td></td>
<td>odds ratio (95% CI)</td>
<td>p#</td>
</tr>
<tr>
<td>≥CIN3</td>
<td>7.571 (2.611-21.956)</td>
<td>0.000</td>
</tr>
<tr>
<td>&lt;CIN3</td>
<td>0.368 (0.234-0.579)</td>
<td>0.000</td>
</tr>
<tr>
<td>≥CIN2</td>
<td>4.345 (2.291-8.240)</td>
<td>0.000</td>
</tr>
<tr>
<td>&lt;CIN2</td>
<td>0.381 (0.224-0.647)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

### Table 3B. Referral reason as a covariate of the performance of cytology
The odds ratios represent the odds of a positive cytology result in women referred to the gynecologist because of abnormal cytology relative to women referred for non-cervix-related complaints (stratified by histological endpoint). *Adjusted for age of patient and sampling method of cervical scrape; # p-value obtained by logistic regression; CIN: cervical intraepithelial neoplasia (grade 2 or 3 or higher); CI: confidence interval

<table>
<thead>
<tr>
<th>Inclusion criterion</th>
<th>Response variable: cytology (0= negative, 1= positive)</th>
<th>Covariate: referral reason to gynecologist (0= non-cervix-related complaints, 1= abnormal cytology)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted analysis</td>
<td>Adjusted analysis*</td>
</tr>
<tr>
<td></td>
<td>odds ratio (95% CI)</td>
<td>p#</td>
</tr>
<tr>
<td>≥CIN3</td>
<td>1.667 (0.490-5.672)</td>
<td>0.414</td>
</tr>
<tr>
<td>&lt;CIN3</td>
<td>5.291 (3.378-8.264)</td>
<td>0.000</td>
</tr>
<tr>
<td>≥CIN2</td>
<td>3.711 (1.649-8.351)</td>
<td>0.002</td>
</tr>
<tr>
<td>&lt;CIN2</td>
<td>4.464 (2.703-7.353)</td>
<td>0.000</td>
</tr>
</tbody>
</table>
DISCUSSION

This prospective multi-center cohort study shows that molecular analysis of FAM19A4 methylation is non-inferior to cytology with respect to sensitivity for ≥CIN3 (88.3% versus 85.0%) in hrHPV-positive women from an outpatient population, aged ≥30 years, at a significantly higher specificity (62.1% versus 47.6%). In women <30 years, an age category known to often harbor transient hrHPV infections, FAM19A4 methylation analysis had a poor ≥CIN3 sensitivity compared to cytology (50% versus 86.7%), but had a significantly higher specificity (81.7% versus 52.4%).

To our knowledge, this study is the first to compare the clinical features of FAM19A4 methylation analysis to those of other commonly used tests for detecting cervical disease in a large cohort of women with hrHPV-positive cervical scrapes. Even in a setting of potential cytology bias, given that a part of women were included with a previously abnormal cytology test, the sensitivity of FAM19A4 methylation analysis reached non-inferiority to cytology in hrHPV-positive women aged ≥30 years. In notice of a cytology bias, we included in our study a subgroup analysis of women who visited the outpatient clinic for non-cervix-related complaints, in which ≥CIN3 sensitivity of FAM19A4 methylation analysis also did not differ significantly from cytology (61.5% versus 80.8%; ratio 0.76; 95%CI:0.55-1.05), although non-inferiority could not be established.

In a subgroup analysis comprising HPV-positive women with normal cytology ≥30 years of age (data not shown), FAM19A4 methylation analysis reached a ≥CIN3 sensitivity of 77.8 (7/9; 95%CI: 50.6-100%) at 67.6% specificity (73/108; 95%CI: 58.8-76.4%). Despite the fact that data are based on relatively low numbers, these findings illustrate the value of FAM19A4 methylation analysis for HPV-positive women with normal cytology.

Previous research has outlined the high sensitivity of DNA methylation analysis for detecting CIN3 lesions with a long duration of existence (so-called advanced CIN3 which are expected to have a high short-term risk of progression to cancer) and cervical carcinoma, in contrast to cytology. In the present study, 50/57 (87.8%) women ≥30 years with CIN3 lesions tested positive by FAM19A4 methylation analysis and 48/57 (84.2%) by cytology. On the other hand, only 26/51 (51%) women with CIN2 were positive by FAM19A4 methylation analysis, whereas 45/54 (88.2%) of these women tested positive for cytology. These data underscore that a positive FAM19A4 methylation result is more likely to represent underlying CIN3 than CIN2, whereas cytology, in this outpatient population, has high sensitivities for both CIN2 and CIN3.

The overall high sensitivity of cytology in this cohort might be explained by the presence of cytology bias in this referral population and/or the prior knowledge of the HPV status of the scrapes at cytology reading. This probably results in easier classification of abnormal cytology. In an HPV-based screening program, only hrHPV-positive women will be triaged by cytology, so it is important to stay alert for potential over-referral. FAM19A4 methylation analysis can therefore be an adequate alternative triage method, with a negative test result providing high reassurance of absence of advanced cervical disease and cancer. Furthermore, as large
variation exists in the quality of cytological screening amongst different countries, a more objective triage strategy of hrHPV-positive women in the future is preferable. Among many previously described methylation markers, \textit{FAM19A4} has shown to efficiently detect virtually all cervical carcinomas and advanced CIN3 lesions. In pilot studies, \textit{FAM19A4} methylation analysis appeared to perform well on cervical scrapes, which had been collected immediately prior to colposcopy. This is remarkable, as this sampling procedure has been shown to compromise performance of a previously validated methylation marker panel, i.e., \textit{CADM1/MAL}, in a cross-sectional cohort. The decrease in marker performance was likely related to more cautious brushing of the cervix (to prevent bleeding) and therefore lower abnormal cell counts in the resulting cervical scrapes. In the current study, we found that the clinical performance of \textit{FAM19A4} methylation was not influenced by the sampling method of the cervical scrape (whether it was collected in a separate event or immediately prior to colposcopy).

Another important finding of our research was the significant influence of age on \textit{FAM19A4} methylation positivity. This finding is in line with Hansel \textit{et al.}, who have described the detection of only 5/14 ≥CIN3 in women <30 years versus 8/9 ≥CIN3 in women ≥30 years using a methylation five-marker panel. However, Hesselink \textit{et al.} did not find a correlation of methylation with age using a bi-marker panel. The latter might be explained by the limited number of women <30 years included in that study. Our study included 287 women <30 years, giving a more representative view on the correlation between age and DNA methylation in detection of ≥CIN2/3. Although hrHPV prevalence in young women is known to be high, most infections are transient and most lesions regress spontaneously, contributing to a very low cancer incidence in this age group. Screening these young women by cytology would lead to high ≥CIN2/3 sensitivities, yet likely at the cost of detecting many regressing CIN2 and a number of early CIN3 lesions, leading to significant over-referral and -treatment. Although testing hrHPV-positive young women by \textit{FAM19A4} methylation analysis would result in a lower sensitivity for ≥CIN2/3 than cytology, it likely reassures against advanced CIN lesions and cervical cancer at a substantially higher specificity. If validated in an independent study, this hypothesis may form the basis of an interesting management strategy for young hrHPV-positive women visiting a gynecological outpatient clinic, given possible treatment morbidity such as cervical insufficiency, and associated risk for pre-term delivery.

The broad age range of the population provides insight in the performance of this molecular marker in younger women. A limitation of our study might be that the age categorization used in this study (≥30 years versus <30 years) is based on the starting age at which women in the Netherlands are invited for cervical screening (i.e., 30 years). However, several Western countries start screening at an earlier age. In the USA, the FDA has approved an HPV test for use as a first-line primary cervical cancer screening test for women of ≥25 years. Interestingly, in hrHPV-positive women in the age category ≥25 years (Supplementary Figure 1), the performance of \textit{FAM19A4} methylation analysis was similar to the performance in women aged ≥30 years. Due to the selection of an outpatient population, one has to realize that the translation of our results into screening settings should be handled with care. Furthermore, the fact that
8% of *FAM19A4* methylation analyses yielded an invalid test result is relevant. We found that the majority (89.5%) of these invalid tests were done on cervical scrapes taken directly prior to colposcopy. As mentioned above, more cautious scraping by the physician (to ascertain adequate colposcopic imaging) may have resulted in insufficient cell numbers in these cervical scrapes, and associated low DNA concentrations, which may contribute to invalid test results. In conclusion, this study showed that promoter methylation analysis of *FAM19A4* is an objective, molecular marker that performs at least non-inferior to cytology for the detection of ≥CIN3 lesions in hrHPV-positive cervical scrapes from a gynecological outpatient population (aged ≥30 years) at a significantly higher specificity.
Supplementary Figure 1. Sensitivities and specificities of different markers for ≥CIN3 and ≥CIN2 detection in hrHPV-positive women. Forest plots showing the relative sensitivity and specificity of the different markers compared to cytology are presented in the subgroup of women aged ≥25 years.
### Supplementary Table 1. Test Specifications of Cytology, FAM19A4 Methylation, HPV16/18 Genotyping and Combinations of Markers for Detection of ≥CIN3 and ≥CIN2 in hrHPV-positive Women in the Subpopulation of Women Older than 25 Years

CIN: cervical intraepithelial neoplasia (grade 2 or 3 or higher); CI: confidence interval; PPV: positive predictive value; 1-NPV: complemented negative predictive value; n1: number of test positive disease cases; N1: total number of disease cases; n2: number of test negative non-disease cases; N2: total number of non-disease cases; *based on % marker positivity

<table>
<thead>
<tr>
<th>Triage marker</th>
<th>n1/N1</th>
<th>Sensitivity (95% CI)</th>
<th>n2/N2</th>
<th>Specificity (95% CI)</th>
<th>PPV (95% CI)</th>
<th>1-NPV (95% CI)</th>
<th>Referral rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥CIN3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cytology</td>
<td>71/81</td>
<td>87.7% (80.5-94.8%)</td>
<td>165/332</td>
<td>49.7% (44.3-55.1%)</td>
<td>29.8% (24.0-35.6%)</td>
<td>5.7% (2.3-9.2%)</td>
<td>57.6%</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>66/81</td>
<td>81.5% (73.0-89.9%)</td>
<td>221/332</td>
<td>66.6% (61.5-71.6%)</td>
<td>37.3% (30.2-44.4%)</td>
<td>6.4% (3.2-9.5%)</td>
<td>42.9%</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>57/81</td>
<td>70.4% (60.4-80.3%)</td>
<td>191/332</td>
<td>57.5% (52.2-62.8%)</td>
<td>28.8% (22.5-35.1%)</td>
<td>11.2% (7.0-15.4%)</td>
<td>47.9%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>78/81</td>
<td>96.3% (92.2-100%)</td>
<td>120/332</td>
<td>36.1% (31.0-41.3%)</td>
<td>26.9% (21.8-32.0%)</td>
<td>2.4% (0.0-5.2%)</td>
<td>70.2%</td>
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<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>75/81</td>
<td>92.6% (86.9-98.3%)</td>
<td>134/332</td>
<td>40.4% (35.1-45.6%)</td>
<td>27.5% (22.2-32.8%)</td>
<td>4.3% (0.9-7.6%)</td>
<td>66.1%</td>
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<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>75/81</td>
<td>92.6% (86.9-98.3%)</td>
<td>97/332</td>
<td>29.2% (24.3-34.1%)</td>
<td>24.2% (19.4-29.0%)</td>
<td>5.8% (1.3-10.3%)</td>
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<tr>
<td>≥CIN2</td>
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<tr>
<td>Cytology</td>
<td>130/152</td>
<td>85.5% (79.9-91.1%)</td>
<td>153/261</td>
<td>58.6% (52.6-64.6%)</td>
<td>54.6% (48.3-60.9%)</td>
<td>12.6% (7.7-17.5%)</td>
<td>57.6%</td>
</tr>
<tr>
<td>FAM19A4 methylation</td>
<td>98/152</td>
<td>64.5% (56.9-72.1%)</td>
<td>182/261</td>
<td>69.7% (64.2-75.3%)</td>
<td>55.4% (48.0-62.7%)</td>
<td>22.9% (17.5-28.2%)</td>
<td>42.9%</td>
</tr>
<tr>
<td>HPV16/18 genotyping</td>
<td>89/152</td>
<td>58.6% (50.7-66.4%)</td>
<td>152/261</td>
<td>58.2% (52.3-64.2%)</td>
<td>44.9% (38.0-51.9%)</td>
<td>29.3% (23.2-35.4%)</td>
<td>47.9%</td>
</tr>
<tr>
<td>FAM19A4 methylation and/or cytology</td>
<td>141/152</td>
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<td>112/261</td>
<td>42.9% (36.9-48.9%)</td>
<td>48.6% (42.9-54.4%)</td>
<td>8.9% (3.9-14.0%)</td>
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<tr>
<td>FAM19A4 methylation and/or HPV16/18 genotyping</td>
<td>122/152</td>
<td>80.3% (73.9-86.6%)</td>
<td>110/261</td>
<td>42.1% (36.2-48.1%)</td>
<td>44.7% (38.8-50.6%)</td>
<td>21.4% (14.6-28.2%)</td>
<td>66.1%</td>
</tr>
<tr>
<td>Cytology and/or HPV16/18 genotyping</td>
<td>138/152</td>
<td>90.8% (86.2-95.4%)</td>
<td>89/261</td>
<td>34.1% (28.3-39.9%)</td>
<td>44.5% (39.0-50.0%)</td>
<td>13.6% (7.0-20.2%)</td>
<td>75.1%</td>
</tr>
</tbody>
</table>
REFERENCES


