Comparing the performance of *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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Short title
FAM19A4 methylation, cytology and HPV16/18 genotyping in hrHPV-positive women

Keywords
DNA methylation, cervical intraepithelial neoplasia, cervical cancer, cervical scrapes, human papillomavirus, qMSP

Abbreviations

hrHPV: high-risk human papillomavirus; CIN: cervical intraepithelial neoplasia, qMSP: quantitative methylation-specific PCR; PHI: preceding HPV-infection; METc: Medical ethical committee; PCR: Polymerase chain reaction; LLETZ: large loop excision of the transformation zone; CISOE-A: composition, inflammation, squamous, other and endometrium, and endocervical cylindrical epithelium, and adequacy; ASC-US: Atypical squamous cells of undetermined significance, ASC-H: Atypical squamous cells cannot exclude HSIL; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; BMD: borderline or mild dyskaryosis; *ACTB:* β-actin; *FAM19A4*: Family with sequence similarity 19 (chemokine (C-C)-motif)-like), member A4; Cq: Quantification Cycle; 95% CI: 95% confidence interval; PPV: positive predictive value; 1-NPV: complemented negative predictive value; SCC: squamous cell carcinoma; AdCA: adenocarcinoma; UE: uterus expiration; ntd: not to determine.

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Early detection and diagnosis

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Novelty and impact of the paper

Women who test high-risk HPV-positive require follow-up testing to avoid over-diagnosis and over-treatment. This paper is the first to compare the clinical performance of *FAM19A4* methylation analysis for the detection of cervical (pre)cancer to cytology and HPV16/18 genotyping in a prospective observational multi-center cohort study in a hrHPV-positive gynecological outpatient population. Performance of methylation analysis to detect high-grade disease was dependent on age. Among hrHPV-positive women aged ≥30 years, methylation analysis appeared a valuable alternative to cytology and HPV16/18 genotyping.

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 ($p \leq 0.001$ 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[6,13]. 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, *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, *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 *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 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, the Netherlands) for hrHPV testing using GP5+/6+ PCR-enzyme immunoassay analysis (EIA kit HPV GP HR, Diassay B.V., the Netherlands)[18]. 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, 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[25], 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 to manufacturers' instructions[16], 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[26].

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[27,28]. Bisulphite-converted DNA was used as template for *FAM19A4* methylation analysis by qMSP using housekeeping gene β-actin *(ACTB)* as a reference gene[22,23]. 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: $2^{[Cq\ (ACTB)\ -\ Ct\ (FAM19A4)]}$ x100. 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.

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-infections[1] 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 uterus extirpation. 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 (AdCA)), 87 women (17.1%) had CIN3 (including 2 adenocarinoma 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 non-cervix-related complaints whereas in women ≥30 years

56% was referred with an abnormal scrape. 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.

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[33,34], *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 under the 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[23,35], in contrast to cytology[1,15,23,36]. 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[37,38]. 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[23]. 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[39].

Among many previously described methylation markers[1,40], *FAM19A4* has shown to efficiently detect virtually all cervical carcinomas and advanced CIN3 lesions[23]. In pilot studies, *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., *CADM1/MAL*, in a cross-sectional cohort[41]. 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[41]. In the current study, we found that the clinical performance of *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 *FAM19A4* methylation positivity. This finding is in line with Hansel et al.[42], 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 et al.[16] 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[34,43,44], contributing to a very low cancer incidence in this age group[45]. 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 *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[46–48].

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[49,50]. Interestingly, in hrHPV-positive women in the age category ≥25 years (Supplementary Figure 1), the performance of *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.

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Conflict of interest

CJLMM has been on the sponsored speakers bureau of GlaxoSmithKline, Qiagen, Merck, Roche, Menarini and Segeene, and served occasionally on the scientific advisory board of GlaxoSmithKline, Qiagen, Merck, and Roche. CJLMM has occasionally been consultant for Qiagen and Genticel and is a minority shareholder of Diassay B.V. Formerly, CJLMM was a minority shareholder of Delphi Biosciences. PJFS has been on the speakers bureau of Roche, Qiagen, Abbott, Gen-Probe and Seegene. PJFS is consultant for Crucell Holland B.V. JB has played an advisory role for Merck and Roche, has been on the speakers bureau of Qiagen and has received a travel reimbursement from DDL Diagnostic Laboratory. WAH, RV and TJMH have been primary investigators of a GlaxoSmithKline sponsored study. WGVQ is a minority shareholder of Diassay B.V. and obtained grants from GlaxoSmithKline. CJLMM, PJFS, RDMS, DAMH have minority stake in Self-screen B.V., a spin-off company of VU University Medical Center Amsterdam.

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TABLES

Table 1. Test specifications of cytology, *FAM19A4* methylation analysis, HPV16/18 genotyping and combinations of markers for detection of ≥CIN3 in hrHPV-positive women in the total study population, and stratified by age and referral reason.

Triage marker	n1 / N1	Sensitivity	(95%CI)	n2 / N2	Specificity	(95%CI)	PPV	(95% CI)	1-NPV	(95%CI)	Referral rate*
Total study population (n=508)											
Cytology	77 / 90	85.6%	(78.3% - 92.8%)	208 / 418	49.8%	(45.0% - 54.6%)	26.8%	(21.7% - 32.0%)	5.9%	(2.8% - 9.0%)	56.5%
FAM19A4 methylation	68 / 90	75.6%	(66.7% - 84.4%)	297 / 418	71.1%	(66.7% - 75.4%)	36.0%	(29.1% - 42.8%)	6.9%	(4.1% - 9.7%)	37.2%
HPV16/18 genotyping	65 / 90	72.2%	(63.0% - 81.5%)	307 / 418	57.4%	(52.7% - 62.2%)	26.7%	(21.2% - 32.3%)	9.4%	(5.9% - 13.0%)	47.8%
FAM19A4 methylation and/or cytology	85 / 90	94.4%	(89.7% - 99.2%)	158 / 418	37.8%	(33.2% - 42.4%)	24.6%	(20.1% - 29.2%)	3.1%	(0.4% - 5.7%)	67.9%
FAM19A4 methylation and/or HPV16/18 genotyping	83 / 90	92.2%	(86.7% - 97.8%)	177 / 418	42.3%	(37.6% - 47.1%)	25.6%	(20.9% - 30.4%)	3.8%	(1.0% - 6.6%)	63.8%
Cytology and/or HPV16/18 genotyping	83 / 90	92.2%	(86.7% - 97.8%)	123 / 418	29.4%	(25.1% - 33.8%)	22.0%	(17.8% - 26.1%)	5.4%	(1.5% - 9.3%)	74.4%
Subgroup: women <30 years (n=221)											
Cytology	26 / 30	86.7%	(74.5% - 98.8%)	100 / 191	52.4%	(45.3% - 59.4%)	22.2%	(14.7% - 29.8%)	3.8%	(0.2% - 7.5%)	52.9%
FAM19A4 methylation	15 / 30	50.0%	(32.1% - 67.9%)	156 / 191	81.7%	(76.2% - 87.2%)	30.0%	(17.3% - 42.7%)	8.8%	(4.5% - 13.0%)	22.6%
HPV16/18 genotyping	23 / 30	76.7%	(61.5% - 91.8%)	109 / 191	57.1%	(50.0% - 64.1%)	21.9%	(14.0% - 29.8%)	6.0%	(1.7% - 10.4%)	47.5%
FAM19A4 methylation and/or cytology	27 / 30	90.0%	(79.3% - 100.0%)	85 / 191	44.5%	(37.5% - 51.6%)	20.3%	(13.5% - 27.1%)	3.4%	(0.0% - 7.2%)	60.2%
FAM19A4 methylation and/or HPV16/18 genotyping	27 / 30	90.0%	(79.3% - 100.0%)	93 / 191	48.7%	(41.6% - 55.8%)	21.6%	(14.4% - 28.8%)	3.1%	(0.0% - 6.6%)	56.6%
Cytology and/or HPV16/18 genotyping	29 / 30	96.7%	(90.2% - 100.0%)	59 / 191	30.9%	(24.3% - 37.4%)	18.0%	(12.1% - 23.9%)	1.7%	(0.0% - 4.9%)	72.9%
Subgroup: women ≥30 years (n=287)											
Cytology	51 / 60	85.0%	(76.0% - 94.0%)	108 / 227	47.6%	(41.1% - 54.1%)	30.0%	(23.1% - 36.9%)	7.7%	(2.9% - 12.5%)	59.2%
FAM19A4 methylation	53 / 60	88.3%	(80.2% - 96.5%)	141 / 227	62.1%	(55.8% - 68.4%)	38.1%	(30.1% - 46.2%)	4.7%	(1.3% - 8.1%)	48.4%
HPV16/18 genotyping	42 / 60	70.0%	(58.4% - 81.6%)	131 / 227	57.7%	(51.3% - 64.1%)	30.4%	(22.8% - 38.1%)	12.1%	(6.8% - 12.1%)	48.1%
FAM19A4 methylation and/or cytology	58 / 60	96.7%	(92.1% - 100.0%)	73 / 227	32.2%	(26.1% - 38.2%)	27.4%	(21.4% - 33.4%)	2.7%	(0.0% - 6.3%)	73.9%
FAM19A4 methylation and/or HPV16/18 genotyping	56 / 60	93.3%	(87.0% - 99.6%)	84 / 227	37.0%	(30.7% - 43.3%)	28.1%	(21.9% - 34.4%)	4.5%	(0.2% - 8.9%)	69.3%
Cytology and/or HPV16/18 genotyping	54 / 60	90.0%	(82.4% - 97.6%)	64 / 227	28.2%	(22.3% - 34.0%)	24.9%	(19.1% - 30.6%)	8.6%	(2.0% - 15.1%)	75.6%
Subgroup: women referred to gynecologist for non-cervix related complaints (n=295)											
Cytology	21 / 26	80.8%	(65.6% - 95.9%)	171 / 269	63.6%	(57.8% - 69.3%)	17.6%	(10.8% - 24.5%)	2.8%	(0.4% - 5.3%)	40.3%
FAM19A4 methylation	16 / 26	61.5%	(42.8% - 80.2%)	197 / 269	73.2%	(67.9% - 78.5%)	18.2%	(10.1% - 26.2%)	4.8%	(1.9% - 7.8%)	29.8%
HPV16/18 genotyping	20 / 26	76.9%	(60.7% - 93.1%)	154 / 269	57.2%	(51.3% - 63.2%)	14.8%	(8.8% - 20.8%)	3.8%	(0.8% - 6.7%)	45.8%
FAM19A4 methylation and/or cytology	23 / 26	88.5%	(76.2% - 100.0%)	133 / 269	49.4%	(43.5% - 55.4%)	14.5%	(9.0% - 19.9%)	2.2%	(0.0% - 4.7%)	53.9%
FAM19A4 methylation and/or HPV16/18 genotyping	23 / 26	88.5%	(76.2% - 100.0%)	120 / 269	44.6%	(38.7% - 50.6%)	13.4%	(8.3% - 19.9%)	2.4%	(0.0% - 5.2%)	58.3%
Cytology and/or HPV16/18 genotyping	24 / 26	92.3%	(82.1% - 100.0%)	101 / 269	37.5%	(31.8% - 43.3%)	12.5%	(7.8% - 17.2%)	1.9%	(0.0% - 4.6%)	65.1%

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 2. Test specifications of cytology, *FAM19A4* methylation analysis, 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.

Triage marker	n1 / N1	Sensitivity	(95%CI)	n2 / N2	Specificity	(95%CI)	PPV	(95% CI)	1-NPV	(95%CI)	Referral rate*
Total study population (n=508)											
Cytology	149 / 180	82.8%	(77.3% - 88.3%)	190 / 328	57.9%	(52.6% - 63.3%)	51.9%	(46.1% - 57.7%)	14.0%	(9.4% - 18.6%)	56.5%
FAM19A4 methylation	104 / 180	57.8%	(50.6% - 65.0%)	243 / 328	74.1%	(69.3% - 78.8%)	55.0%	(47.9% - 62.1%)	23.8%	(19.1% - 28.5%)	37.2%
HPV16/18 genotyping	106 / 180	58.9%	(51.7% - 66.1%)	191 / 328	58.2%	(52.9% - 63.6%)	43.6%	(37.4% - 49.9%)	27.9%	(22.5% - 33.3%)	47.8%
FAM19A4 methylation and/or cytology	162 / 180	90.0%	(85.6% - 94.4%)	145 / 328	44.2%	(38.8% - 49.6%)	47.0%	(41.7% - 52.2%)	11.0%	(6.2% - 15.9%)	67.9%
FAM19A4 methylation and/or HPV16/18 genotyping	141 / 180	78.3%	(72.3% - 84.4%)	145 / 328	44.2%	(38.8% - 49.6%)	43.5%	(38.1% - 48.9%)	11.0%	(15.3% - 27.1%)	63.8%
Cytology and/or HPV16/18 genotyping	161 / 180	89.4%	(85.0% - 93.9%)	111 / 328	33.8%	(28.7% - 39.0%)	42.6%	(37.6% - 47.6%)	14.6%	(8.5% - 20.7%)	74.4%
Subgroup: women <30 years (n=221)											
Cytology	53 / 69	76.8%	(66.9% - 86.8%)	88 / 152	57.9%	(50.0% - 65.7%)	45.3%	(36.3% - 54.3%)	15.4%	(8.5% - 22.3%)	52.9%
FAM19A4 methylation	25 / 69	36.2%	(24.9% - 47.6%)	127 / 152	83.6%	(77.7% - 89.4%)	50.0%	(36.1% - 63.9%)	25.7%	(19.2% - 32.3%)	22.6%
HPV16/18 genotyping	42 / 69	60.9%	(49.4% - 72.4%)	89 / 152	58.6%	(50.7% - 66.4%)	40.0%	(30.6% - 49.4%)	23.3%	(15.6% - 31.0%)	47.5%
FAM19A4 methylation and/or cytology	56 / 69	81.2%	(71.9% - 90.4%)	75 / 152	49.3%	(41.4% - 57.3%)	42.1%	(33.7% - 50.5%)	14.8%	(7.4% - 22.2%)	60.2%
FAM19A4 methylation and/or HPV16/18 genotyping	49 / 69	71.0%	(60.3% - 81.7%)	76 / 152	50.0%	(42.1% - 57.9%)	39.2%	(30.6% - 47.8%)	20.8%	(12.7% - 29.0%)	56.6%
Cytology and/or HPV16/18 genotyping	60 / 69	87.0%	(79.0% - 94.9%)	51 / 152	33.6%	(26.0% - 41.1%)	37.3%	(29.8% - 44.7%)	15.0%	(6.0% - 24.0%)	72.9%
Subgroup: women ≥30 years (n=287)											
Cytology	96 / 111	86.5%	(80.1% - 92.8%)	102 / 176	58.0%	(50.7% - 65.2%)	56.5%	(49.0% - 63.9%)	12.8%	(6.8% - 18.9%)	59.2%
FAM19A4 methylation	79 / 111	71.2%	(62.7% - 79.6%)	116 / 176	65.9%	(58.9% - 72.9%)	56.8%	(48.6% - 65.1%)	21.6%	(15.0% - 28.3%)	48.4%
HPV16/18 genotyping	64 / 111	57.7%	(48.5% - 66.8%)	102 / 176	58.0%	(50.7% - 65.2%)	46.4%	(38.1% - 38.1%)	31.5%	(24.1% - 61.9%)	48.1%
FAM19A4 methylation and/or cytology	106 / 111	95.5%	(91.6% - 99.4%)	70 / 176	39.8%	(32.5% - 47.0%)	50.0%	(43.3% - 56.7%)	6.7%	(1.0% - 12.3%)	73.9%
FAM19A4 methylation and/or HPV16/18 genotyping	92 / 111	82.9%	(75.9% - 89.9%)	69 / 176	39.2%	(32.0% - 46.4%)	46.2%	(39.3% - 53.2%)	21.6%	(13.0% - 30.2%)	69.3%
Cytology and/or HPV16/18 genotyping	101 / 111	91.0%	(85.7% - 96.3%)	60 / 176	34.1%	(27.1% - 41.1%)	46.5%	(39.9% - 53.2%)	14.3%	(6.1% - 22.5%)	75.6%
Subgroup: women referred to gynecologist for non-cervix related complaints (n=295)											
Cytology	49 / 69	71.0%	(60.3% - 81.7%)	156 / 226	69.0%	(63.0% - 75.1%)	41.2%	(32.3% - 50.0%)	11.4%	(6.7% - 16.1%)	40.3%
FAM19A4 methylation	35 / 69	50.7%	(38.9% - 62.5%)	173 / 226	76.5%	(71.0% - 82.1%)	39.8%	(29.5% - 50.0%)	16.4%	(11.4% - 21.5%)	29.8%
HPV16/18 genotyping	44 / 69	63.8%	(52.4% - 75.1%)	135 / 226	59.7%	(53.3% - 66.1%)	32.6%	(24.7% - 40.5%)	15.6%	(10.0% - 21.3%)	45.8%
FAM19A4 methylation and/or cytology	55 / 69	79.7%	(70.2% - 89.2%)	122 / 226	54.0%	(47.5% - 60.5%)	34.6%	(27.2% - 42.0%)	10.3%	(5.2% - 15.4%)	53.9%
FAM19A4 methylation and/or HPV16/18 genotyping	54 / 69	78.3%	(68.5% - 88.0%)	108 / 226	47.8%	(41.3% - 54.3%)	31.4%	(24.5% - 38.3%)	12.2%	(6.4% - 18.0%)	58.3%
Cytology and/or HPV16/18 genotyping	58 / 69	84.1%	(75.4% - 92.7%)	92 / 226	40.7%	(34.3% - 47.1%)	30.2%	(23.7% - 36.7%)	10.7%	(4.7% - 16.6%)	65.1%

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 3.

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).

Response variable: *FAM19A4* methylation analysis (0 = negative, 1 = positive) **Covariate: age** (0 = <30 years, 1 = ≥30 years)				
Inclusion criterium	Unadjusted analysis *odds ratio (95% CI)*	*p* #	Adjusted analysis* *odds ratio (95% CI)*	*p* #
≥CIN3	7.571 (2.611 - 21.956)	**0.000**	7.061 (2.197 - 22.698)	**0.001**
<CIN3	2.717 (1.727 - 4.274)	**0.000**	2.732 (1.701 - 4.405)	**0.000**
≥CIN2	4.345 (2.291 - 8.240)	**0.000**	5.107 (2.390 - 10.915)	**0.000**
<CIN2	2.625 (1.546 - 4.464)	**0.000**	2.551 (1.477 - 4.386)	**0.001**
*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 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).

Response variable: cytology (0 = negative, 1 = positive) **Covariate: referral reason to gynecologist** (0 = non-cervix-related complaints, 1 = abnormal cytology)				
Inclusion criterium	Unadjusted analysis *odds ratio (95% CI)*	*p* #	Adjusted analysis* *odds ratio (95% CI)*	*p* #
≥CIN3	1.667 (0.490 - 5.672)	0.414	5.346 (0.533 - 53.590)	0.154
<CIN3	5.291 (3.378 - 8.264)	**0.000**	7.299 (3.356 - 15.873)	**0.000**
≥CIN2	3.711 (1.649 - 8.351)	**0.002**	5.507 (1.371 - 22.120)	**0.016**
<CIN2	4.464 (2.703 - 7.353)	**0.000**	6.024 (2.404 - 15.152)	**0.000**
* 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				

FIGURES

Figure 1. Overview of study population. hrHPV = high-risk human papillomavirus, ntd = not to determine, LLETZ = large loop excision of the transformation zone, UE = uterus expiration

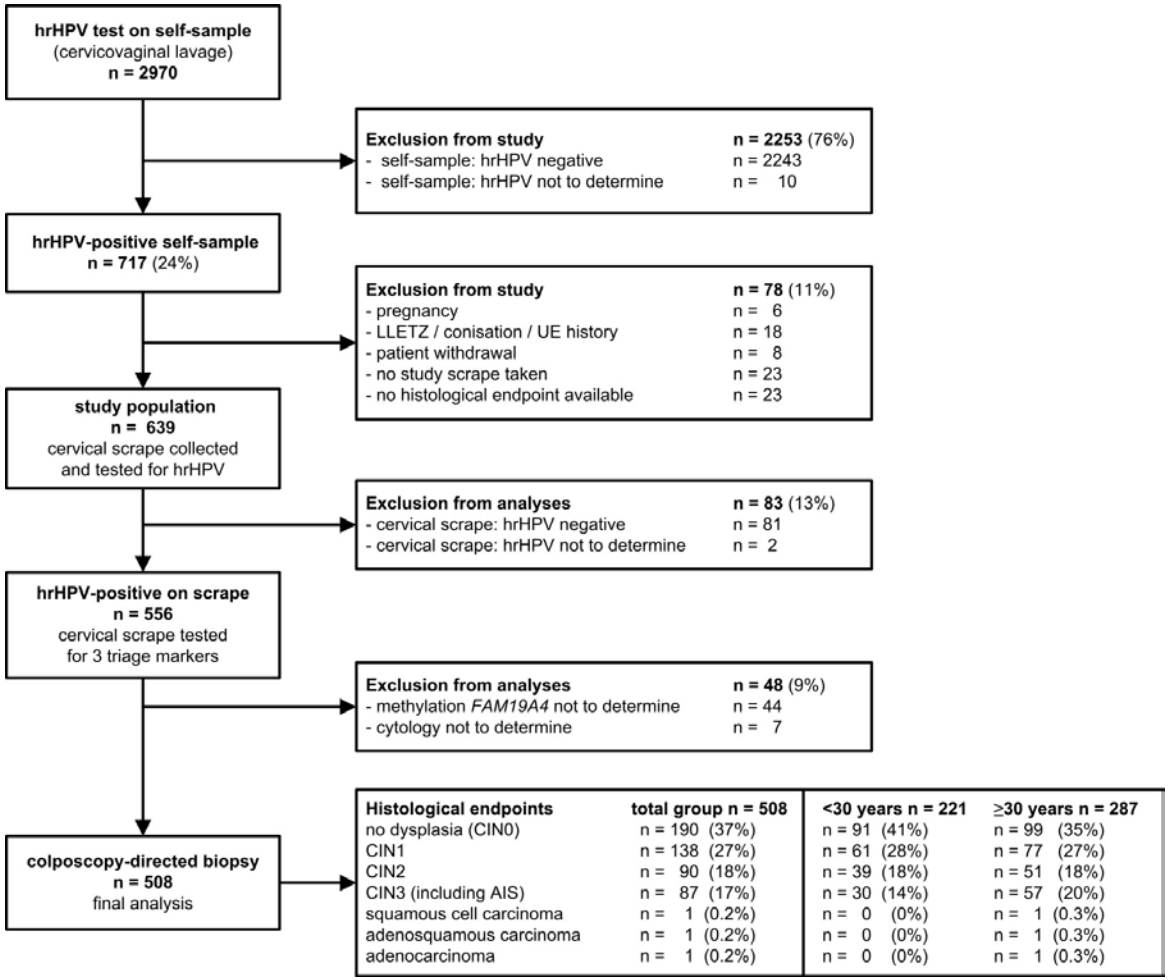


Figure 2. Sensitivities and specificities of different markers for ≥CIN3 detection in hrHPV-positive women. Forest plots showing the relative sensitivity and specificity of the different markers compared to cytology are presented in the total study population and the subgroup of women aged ≥30 years.

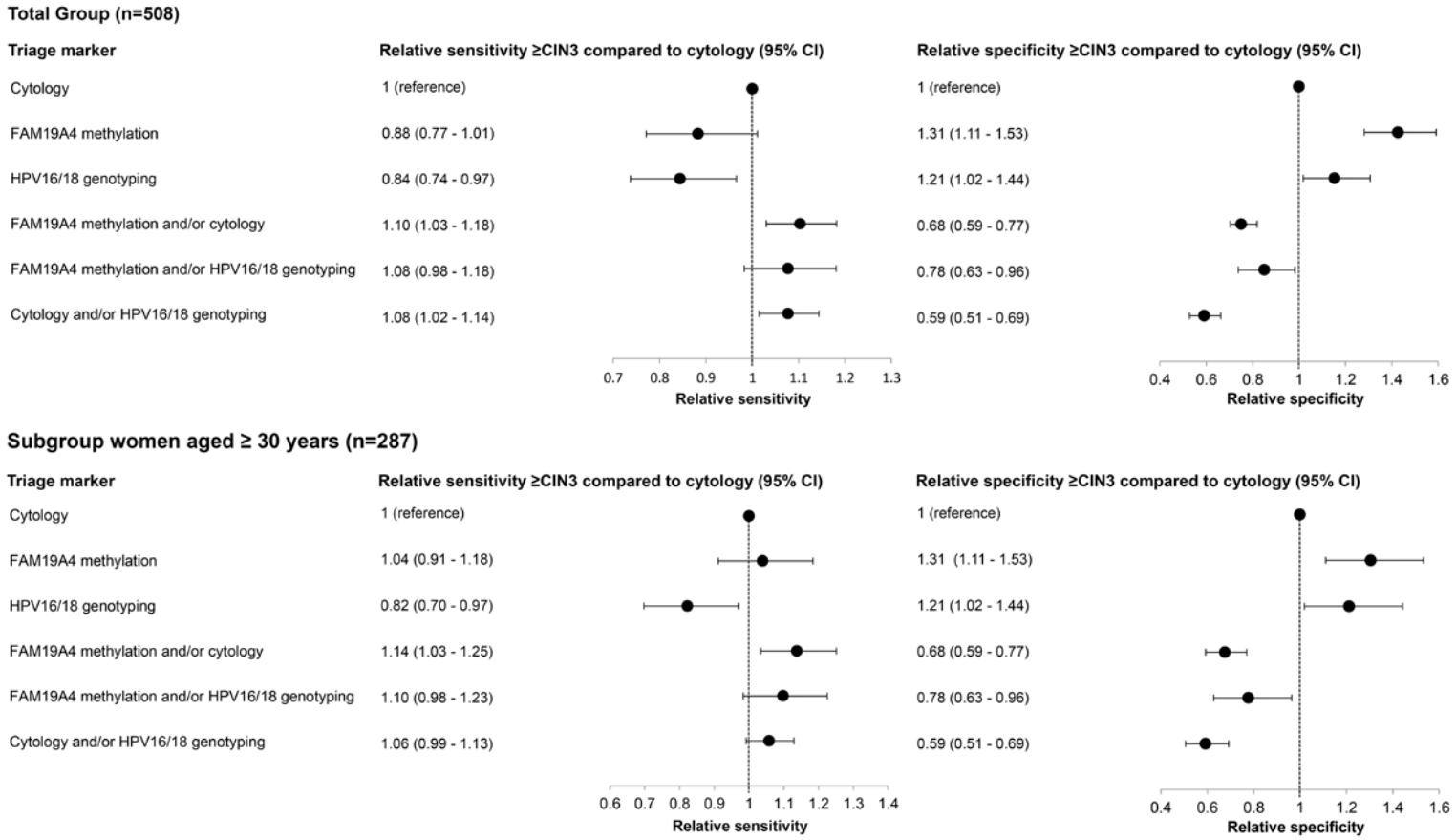
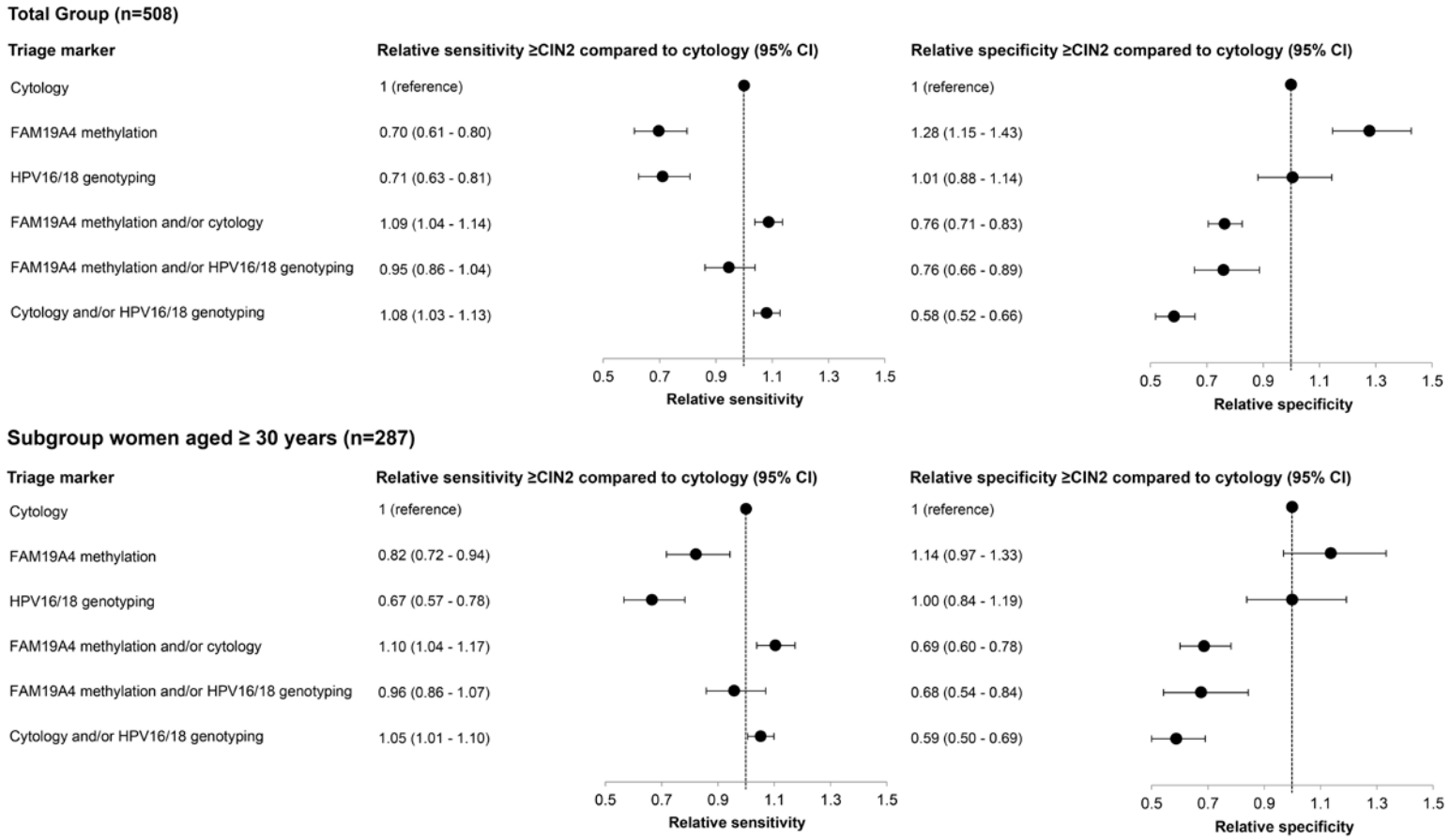


Figure 3. Sensitivities and specificities of different markers for ≥CIN2 detection in hrHPV-positive women. Forest plots showing the relative sensitivity and specificity of the different markers compared to cytology are presented in the total study population and the subgroup of women aged ≥30 years.



Supplementary Figure 1. ≥CIN3 and ≥CIN2 sensitivities and specificities of different triage tests for hrHPV-positive women aged ≥25 years. Forest plots showing the relative sensitivity and specificity of the different triage tools compared to cytology are presented.