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Original article

FAM19A4/miR124-2 methylation analysis as a triage test for HPV-positive women: cross-sectional and longitudinal data from a Dutch screening cohort

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ABSTRACT

Objectives: The aim was to evaluate the cross-sectional and long-term triage performance of *FAM19A4/miR124-2* methylation analysis in human papillomavirus (HPV)-based cervical screening.
Methods: We conducted a *post hoc* analysis within a Dutch population-based HPV-positive study cohort of women aged 30–60 years (n = 979). Cross-sectional cervical intraepithelial neoplasia (CIN) 3+ sensitivity, specificity, positive predictive value and negative predictive value as well as cumulative CIN3+ or cervical cancer risks after 9 and 14 years were compared for three baseline triage strategies: (1) cytology, (2) *FAM19A4/miR124-2* methylation analysis and (3) combined *FAM19A4/miR124-2* methylation with cytology.
Results: CIN3+ sensitivity of *FAM19A4/miR124-2* methylation analysis was similar to that of cytology (71.3% vs 76.0%, ratio 0.94, 95% CI 0.84 to 1.05), at a lower specificity (78.3% vs 87.0%, ratio 0.90, 95% CI 0.86 to 0.94). Combining *FAM19A4/miR124-2* methylation analysis with cytology resulted in a CIN3+ sensitivity of 84.6% (95% CI 78.3 to 90.8) at a specificity of 69.6% (95% CI 66.5 to 72.7). Similar 9- and 14-year CIN3+ risks for baseline cytology-negative women and baseline *FAM19A4/miR124-2* methylation-negative women were observed, with risk differences of −0.42% (95% CI −2.1 to 1.4) and −0.07% (95% CI −1.9 to 1.9), respectively. The 14-year cumulative cervical cancer incidence was significantly lower for methylation-negative women compared to cytology-negative women (risk difference 0.98%, 95% CI 0.26 to 2.0).
Discussion: FAM19A4/miR124-2 methylation analysis has a good triage performance on baseline screening samples, with a cross-sectional CIN3+ sensitivity and long-term triage-negative CIN3+ risk equalling cytology triage. Therefore, *FAM19A4/miR124-2* methylation analysis appears to be a good and objective alternative to cytology in triage scenarios in HPV-based cervical screening. **F.J. Vink, Clin Microbiol Infect 2020;▪:1**

Introduction

A persistent infection with a high-risk type of human papillomavirus (HPV) is an essential step in the development of cervical cancer and its precursor lesions (cervical intraepithelial neoplasia; CIN) [1]. This insight has led to conversion of the cervical screening programme from primary cytology to primary HPV testing in several European countries and Australia, and other countries are considering this transition as well [2]. Benefits of primary HPV-based screening include the high sensitivity and negative predictive value (NPV) for the detection of high-grade CIN and cervical cancer [3,4], and the compatibility with self-sampling [5]. Furthermore, HPV testing provides better protection against

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cervical cancer and high-grade CIN than cytology [3,4]. However, HPV testing also detects transient, clinically irrelevant HPV infections, resulting in a lower specificity than cytology. Triage testing of HPV-positive women is needed to identify the women with clinically relevant disease, in order to reduce unnecessary referral for colposcopy [6].

Different triage strategies for HPV-positive women have been evaluated in recent years, including microscopy-based strategies like cytology and p16/Ki-67 dual stained cytology, and molecular triage tests such as HPV genotyping and viral or host cell DNA methylation analysis [7,8]. Currently, cytology is the most commonly used triage test for HPV-positive women [9,10]. However, cytology relies on a subjective interpretation, and is highly dependent upon experienced cytologists for correct evaluation. Furthermore, it has been reported that cytological screening performed with knowledge of HPV status can result in a loss in specificity [11]. These drawbacks underline the need for an alternative, reproducible and objective triage test. Host cell DNA methylation analysis is increasingly being studied as a potential triage test with these characteristics, and demonstrated promise for the detection of high-grade CIN and cervical cancer in triage setting for HPV-positive women [12].

Host cell DNA methylation is a frequently observed epigenetic phenomenon in cervical carcinogenesis following a persistent HPV infection [1]. The hypermethylation of CpG-rich gene promoter regions of human genes, often tumour suppressor genes, can lead to gene silencing, which contributes to cancer development. Methylation analysis of *FAM19A4* (family with sequence similarity 19 (chemokine (C−C)-motif)-like), member A4) (also known as *TAFA4* (TAFA chemokine like family member 4)) and *miR124-2* (microRNA 124-2) genes has shown good reproducibility [13], a high sensitivity for CIN3+ [14,15] and detects nearly all cervical carcinomas [16]. Initial longitudinal studies illustrated that triage of HPV-positive women with *FAM19A4/miR124-2* methylation analysis provides at least similar long-term risk stratification for cervical cancer and CIN3 as cytology over 14 years [17,18], substantiating the triage capability of the *FAM19A4/miR124-2* marker panel.

Here, we present a *post hoc* analysis within a large HPV-positive population-based Dutch screening cohort to assess the cross-sectional performance and long-term triage-negative CIN3+ and cervical cancer risks of baseline *FAM19A4/miR124-2* methylation analysis. Data were compared with cytology triage testing following a HPV-positive test (threshold borderline dyskaryosis or atypical squamous cells of undetermined significance) and to a combined (i.e. methylation with cytology) triage test strategy.

Materials and methods

Study population

This study is a *post hoc* analysis of baseline *FAM19A4/miR124-2* methylation analysis (QIAsure Methylation Test®, QIAGEN, Hilden, Germany) within the VUSA-Screen cohort, a population-based cervical screening cohort study carried out in the setting of the Dutch screening programme between October 2003 and August 2005. The design has been described previously [19]. For this study, we selected all baseline high-risk HPV-positive women ($n = 1303$). All participants provided written informed consent. The VUSA-Screen study was approved by the Ministry of Public Heath (2002/02-WBO; ISBN10:90-5549-452-6) and registered in the trial register (NTR215, ISRCTN64621295). Histopathological and cytopathological follow-up data of women participating in the VUSA-Screen study were collected through the nationwide network and registry of histopathology and cytopathology (PALGA) [20]. Results were retrieved until 12 December 2017, resulting in up to 14 years of follow-up. Detailed study procedures within the VUSA-Screen study and details on methylation analysis are described in the supplementary material.

Data and statistical analysis

The endpoint was CIN grade 3 or worse (CIN3+). Study endpoints were assessed based on the histological outcome of the colposcopy-directed biopsy, or, if classified worse, on the histology result of the specimen excised by large loop excision of the transformation zone (LLETZ), conization or hysterectomy. Three-year follow-up results were used as cross-sectional study endpoint, as previously described [19]. For the long-term CIN3 and cervical cancer risks we used 9- and 14-year follow-up results, equally defined as in long-term evaluations of the Dutch POBASCAM study [21].

We considered the following three baseline triage strategies for HPV-positive women: (1) cytology, (2) *FAM19A4/miR124-2* methylation analysis and (3) combined *FAM19A4/miR124-2* methylation analysis with cytology. Strategy 1 was labelled positive if the result was borderline or mild dyskaryosis or worse. Strategy 2 was labelled positive if the QIAsure Methylation Test® result was methylation-positive. Strategy 3 was labelled positive in case the QIAsure Methylation Test® result was methylation-positive and/or cytology was borderline or mild dyskaryosis or worse. Proportions of methylation test positivity within baseline cytology results were compared using chi-square tests.

Cross-sectional analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the detection of CIN3+ were calculated with 95% confidence intervals (95% CI). Loss to follow-up among baseline cytology-negative women was accounted for by imputing observed proportions of histological outcomes from women with complete cross-sectional follow-up into women with incomplete cross-sectional follow-up (please see supplementary material). Differences between baseline triage strategies were calculated using the relative sensitivity and relative specificity (i.e. ratio of the sensitivity or specificity of one test to the sensitivity or specificity of another test, respectively). A difference was considered significant if the 95% CI of the relative sensitivity or specificity was entirely below or above 1.

Longitudinal analysis

Follow-up data until 14 years after inclusion were retrieved. Cumulative 9- and 14-year histology outcomes were categorized using worst histology outcome. Details on censoring and exclusion rules are described in thesupplementary material. Cumulative incidences of CIN3+ and cervical cancer, stratified by different triage strategies at baseline, were estimated using the Kaplan−Meier method and were compared by calculating risk differences after 9 and 14 years of follow-up. We constructed 95% CIs for the risk differences via Bootstrap in R (version 3.6.1, Vienna, Austria). If the 95% CI did not contain the value 0, the difference was considered significant. All other analyses were carried out with IBM SPSS (version 24.0, IBM Corp, Armonk, NY, USA), STATA (version 14.1, Texas, USA) and Excel.

Results

Study cohort and baseline findings

The study flowchart is shown in Fig. 1. Of the 1303 HPV-positive women, 236 were excluded due to insufficient leftover material for methylation analysis and 61 were excluded because no longitudinal

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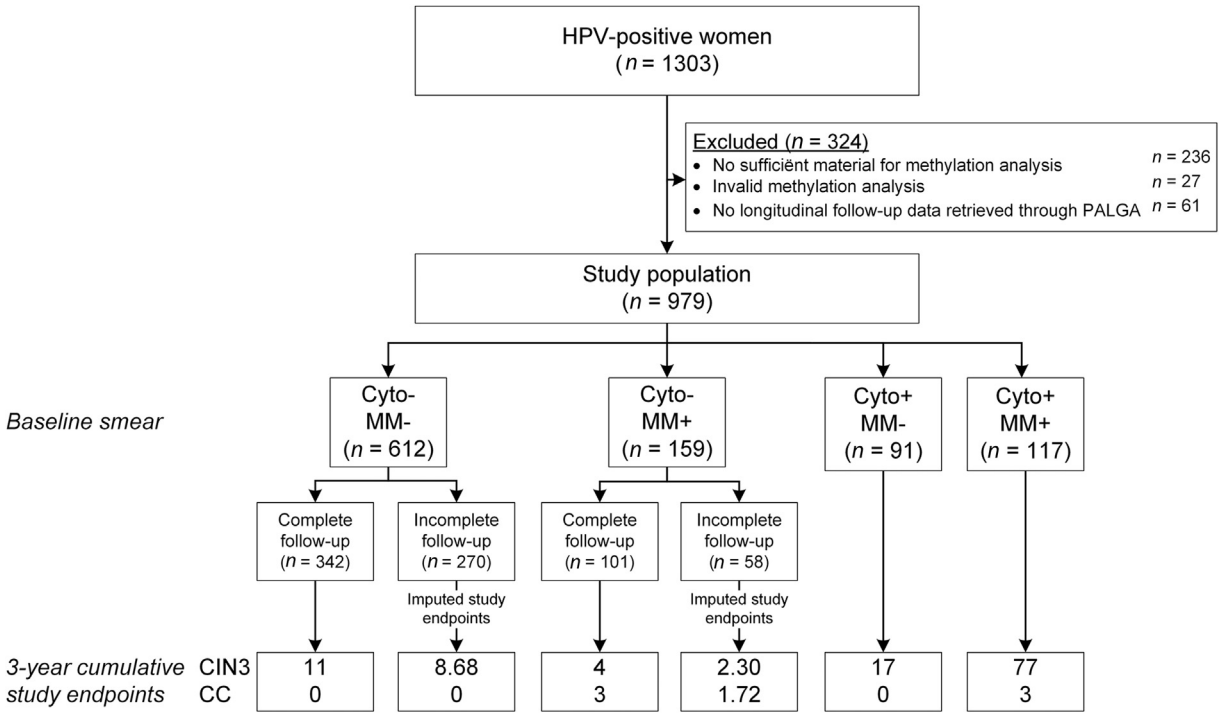

Fig. 1. Study flowchart showing baseline cytology and *FAM19A4/miR124-2* methylation analysis results and cross-sectional 3-year cumulative histology endpoints with imputed study endpoints to account for incomplete follow-up during the cross-sectional study period. HPV, human papillomavirus; Cyto-, cytology-negative; Cyto+, cytology-positive; MM-, methylation-negative; MM+, methylation-positive; CIN3, cervical intraepithelial neoplasia grade 3; CC, cervical cancer. Adenocarcinoma *in situ* was counted as CIN3.

follow-up data were retrieved through PALGA. Another 27 women were excluded due to an invalid *FAM19A4/miR124-2* methylation test result, leaving 979 HPV-positive women (median age of 35 years; range 29–61 years) in the final analysis. No differences were found in baseline characteristics (age, cytology results, CIN3+ proportions) between the study population used for final analysis ($n = 979$ women) and the excluded population ($n = 324$ women).

Of the 979 HPV-positive women, 276 (28.2%) tested methylation positive in their baseline cervical smear. Baseline cytology results were normal in 771 (78.8%), borderline or mild dyskaryosis in 123 (12.6%) and moderate dyskaryosis or worse in 85 (8.7%) women. The proportion of women with a methylation-positive test was 20.6% (159/771) in women with normal cytology, 38.2% (47/123) in women with borderline or mild dyskaryosis and 82.4% (70/85) in women with moderate dyskaryosis or worse ($p < 0.0001$).

Cross-sectional analysis

During 3 years of follow-up, 109 CIN3 and six cervical carcinomas were detected (Fig. 1). *FAM19A4/miR124-2* methylation positivity proportion in the baseline HPV-positive cervical smears increased significantly from 21.9% (189/864) in controls to 74.3% (81/109) in women with CIN3 and 100% (6/6) in women with cervical cancer ($p < 0.0001$). Table 1 shows sensitivities, specificities, PPVs and NPVs as well as referral rates for CIN3+, calculated for the three baseline triage strategies. The sensitivity of *FAM19A4/miR124-2* methylation analysis (Strategy 2) was similar to that of cytology (Strategy 1) (71.3% vs 76.0%, ratio 0.94, 95% CI 0.84 to 1.05), at a lower specificity (78.3% vs 87.0%, ratio 0.90, 95% CI 0.86 to 0.94). Combining *FAM19A4/miR124-2* methylation analysis with cytology (Strategy 3) showed a CIN3+ sensitivity of 84.6% (95% CI 78.3 to 90.8) at a specificity of 69.6% (95% CI 66.5 to 72.7).

Longitudinal analysis

Fig. 2 shows Kaplan–Meier curves with the cumulative incidence for CIN3+ stratified per baseline negative triage strategy. Cytology-negative women and methylation-negative women had equal cumulative CIN3+ incidences at 9 years after baseline (9.4% vs 9.8%, risk difference −0.42%, 95% CI −2.1 to 1.4) and at 14 years after baseline (12.8% vs 12.8%, risk difference −0.07%, 95% CI −1.9 to 1.9). Combining *FAM19A4/miR124-2* methylation analysis with cytology screening resulted for triage-negative women in a cumulative CIN3+ incidence of 7.7% (95% CI 5.5 to 9.9) at 9 years and 10.9% (95% CI 8.0 to 13.7) at 14 years after baseline. The 14-year cumulative cervical cancer incidence was 2.0% for cytology-

Table 1
Sensitivity, specificity, PPV, NPV and colposcopy referral rate for CIN3+, of three baseline triage strategies for HPV-positive women

		Triage algorithm	Sensitivity %	95% CI	Specificity %	95% CI	PPV %	95% CI	NPV %	95% CI	Colposcopy referral rate %	95% CI
≥CIN3	1	Cytology	76.0	68.5–83.4	87.0	84.7–89.2	46.6	39.9–53.4	96.0	94.6–97.4	21.2	8.7–23.8
	2	*FAM19A4/miR124-2* methylation	71.3	63.4–79.1	78.3	75.5–81.0	33.0	27.4–38.5	94.8	93.1–96.4	28.2	25.4–31.0
	3	*FAM19A4/miR124-2* methylation and/or cytology	84.6	78.3–90.8	69.6	66.5–72.7	29.4	24.8–34.1	96.8	95.4–98.2	37.5	34.5–40.5

The colposcopy referral rate was calculated as the proportion of HPV-positive women who had a positive triage test result. Adenocarcinoma *in situ* was counted as CIN3. PPV, positive predictive value; NPV, negative predictive value; 95% CI, 95% confidence interval.

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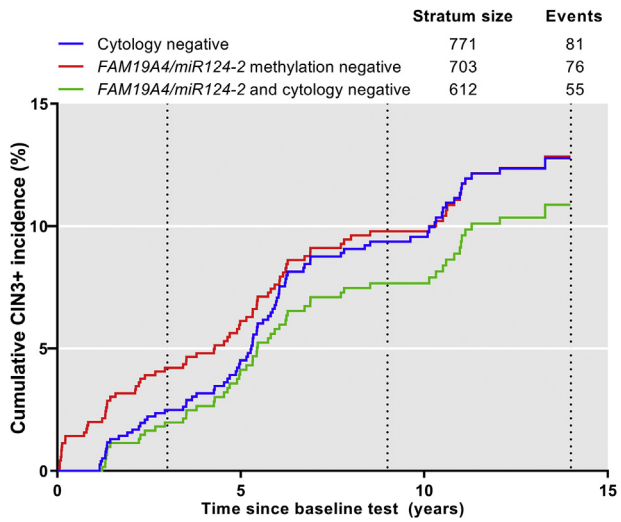

Fig. 2. Cumulative incidence for CIN3+ stratified per triage strategy. CIN3+, cervical intraepithelial neoplasia grade 3 or worse.

negative women and 1.1% for methylation-negative women (risk difference 0.98%, 95% CI 0.26 to 2.0, Fig. 3).

Discussion

In the present study, we evaluated the cross-sectional and longitudinal triage performance of single or combined cytology and *FAM19A4/miR124-2* methylation analysis as baseline triage strategies in a large population-based HPV-positive screening cohort.

Cross-sectional performance of *FAM19A4/miR124-2* methylation analysis was comparable to cytology, with similar CIN3+ sensitivity at a lower specificity. Both single test triage strategies (Strategy 1 and Strategy 2) showed PPV estimates appropriate for direct colposcopy referral, and NPV estimates that would require repeat testing according to accepted safety thresholds [22,23]. Combining the single test strategies (Strategy 3) raises the sensitivity with a slight decrease in specificity, but also increases the burden of screening with a relatively low PPV and markedly increased colposcopy referral rates. Strategy 3 could be visualized in either a co-testing strategy at baseline, or in a strategy with *FAM19A4/miR124-2*

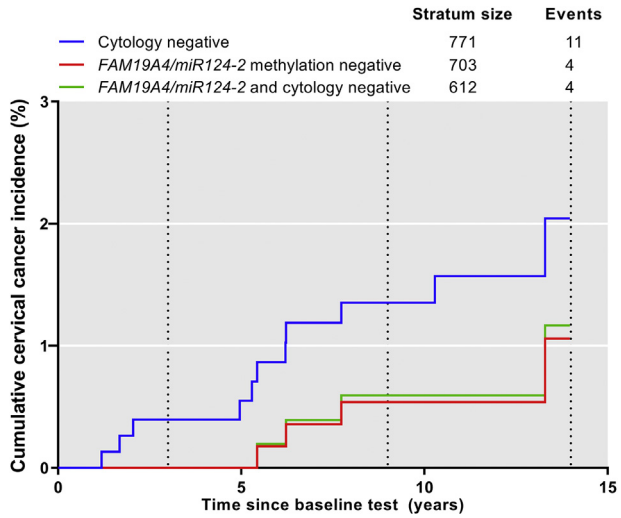

Fig. 3. Cumulative incidence for cervical cancer stratified per triage strategy.

methylation analysis at baseline and subsequent cytology testing after 6–12 months for methylation-negative women. This sequential algorithm would make baseline molecular screening feasible, with the benefit of compatibility with self-sampling, and yield a possible increase in specificity due to the expected clearance of HPV and associated regression of lesions within 6–12 months. Our longitudinal data show that women with a baseline negative *FAM19A4/miR124-2* methylation test result have equal 9- and 14-year risks for developing CIN3+ in comparison to baseline cytology-negative women. Combining these two strategies resulted in even lower 9- and 14-year cumulative incidences. Altogether, our data underline that methylation analysis may be used to build robust triage algorithms with more objective stratification of women referred for colposcopy versus re-testing compared with cytology [24,25].

Our cross-sectional data are consistent with recent evaluations of several methylation markers, with sensitivities for CIN3+ of 71.1% (95% CI 65.7 to 76.0) and a PPV of 35.0% (95% CI 28.9 to 41.6) at a set specificity of 70% [12]. The longitudinal data of our study are consistent with the results of evaluations of the 9- and 14-year CIN3+ risks for women triaged by *FAM19A4/miR124-2* methylation within the Dutch POBASCAM study [18]. In contrast to the POBASCAM study, study management in the VUSA-Screen study was based on both cytology and HPV testing, making it feasible to report cross-sectional CIN3+ risks after a negative triage test.

The *FAM19A4/miR124-2* methylation positivity proportion for cervical carcinomas diagnosed within the different screening rounds were in line with long-term evaluations of the Dutch POBASCAM study [17], though numbers in this study were limited. Consistent with the above-mentioned study, a lower 14-year cervical cancer risk among baseline methylation-negative women than cytology-negative women was found. As a high cross-sectional sensitivity and low long-term NPV for cervical cancer ensures the safety of a triage strategy, these findings are of great importance in the search for molecular triage strategies.

Despite the fact that similar cross-sectional sensitivities are observed, methylation analysis and cytology in part detect different CIN lesions [1,25,26]. It has been shown that *FAM19A4* methylation analysis in cervical smears tends to be more competent than cytology in detecting more advanced CIN2/3 as defined by a persistent HPV infection with a duration over 5 years [14]. A recent study reports that about three-quarters of cervical smears of women with CIN3 display a cancer-like methylation-high pattern, suggestive for a higher risk of progression to cervical cancer [27]. These results corroborate with the finding that without intervention only a subset of CIN3 will progress to cancer over a long time period. It can be reasoned that methylation analysis allows to distinguish the need of immediate treatment versus active surveillance. This could prevent overtreatment and the associated cervical morbidity, which is especially relevant for women of childbearing age. Indeed, it was recently shown that a methylation panel consisting of host cell and viral genes has the ability to identify progressive CIN2 lesions in young women [28]. Additional studies are presently ongoing to validate these findings [29].

The main strengths of our study are its large sample size, the long follow-up period of 14 years and the setting within the Dutch cervical screening programme. In addition, we used a standardized *FAM19A4/miR124-2* methylation assay. A limitation of this study might be seen in the high cross-sectional loss to follow-up among women with normal cytology at baseline. To account for this, observed proportions of histological outcomes from women with complete follow-up were imputed to women with incomplete follow-up. In the original study, management was based on HPV and cytology results. This verification bias may have led to an underestimation of the performance of *FAM19A4/miR124-2*

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methylation in this study. In addition, ascertainment bias could be present, given that cross-sectional study endpoints included follow-up up to 3 years after baseline and baseline colposcopy referral depended on the baseline cytology result. However, the median time to CIN3+ diagnosis was 77 days and only nine cross-sectional detected CIN3+ lesions were diagnosed between 2 and 3 years after baseline, indicating that this possible bias is limited.

In The Netherlands, cytology is well quality assured, and the accuracy of cytology reading is high [30], explaining the good performance of cytology triage testing in this cohort. Of note, quality of cytology varies widely among countries and can be difficult to retain, especially in less developed countries. In addition, in this study cytology was read without prior knowledge of the HPV result. The advantages afforded by DNA methylation analysis are the molecular basis, making triage testing automatable and less prone to training and interpretational errors than cytology. Methylation-based triage has recently shown to perform robust and reproducible in different laboratory contexts [13]. Another advantage of methylation analysis over cytology, is the compatibility with self-collected samples [24], which may allow for full molecular self-screening. While current methylation technologies may not yet be suitable for large-scale implementation, technological advances and ongoing development of methylation assays is expected to result in automated and user-friendly assays, suitable for high-throughput testing in laboratories with HPV testing facilities. With comparable performance to cytology, methylation-based triage testing could become a reproducible and robust triage algorithm in many countries.

The data presented in this study confirm that *FAM19A4/miR124-2* methylation analysis can be considered as an objective alternative to cytology in triage scenarios in HPV-based cervical screening. These findings highlight the potential of methylation testing to realize full molecular screening in future.

Transparency declaration

This work was supported by the SME Instrument in the Horizon 2020 Work Program of the European Commission (grant agreement ID: 666800, VALID-SCREEN). Potential conflicts of interest: (1) D.H., C.M. and R.S. are minority shareholders of Self-screen B.V., a spin-off company of VUmc. (2) Self-screen B.V. holds patents related to the work (i.e. high-risk HPV test and methylation markers for cervical screening) and has developed and manufactured the methylation assay, which is licensed to QIAGEN (QIAsure® Methylation Test). (3) D.H. has been on the speakers' bureau of QIAGEN and serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb. (4) C.M. has received speaker fees from GSK, QIAGEN, SPMSD/Merck and Roche diagnostics, and served occasionally on the scientific advisory board (expert meeting) of GSK, QIAGEN, SPMSD/Merck and Roche. (6) C.M. has a very small number of shares of QIAGEN and MDxHealth and holds minority stock in Self-Screen B.V. Until April 2016 he was minority shareholder of Diassay B.V. (7) C.M. is part-time director of Self-screen B.V. since September 2017. (8) J.B. reports personal fees from GlaxoSmithKline and Merck; and non-financial support from DDL; the fees from GlaxoSmithKline and Merck were collected by his employer. All other authors declare no conflicts of interest.

Author contributions

Project administration: F.V., M.B., D.H., C.M. Methodology: H.B., B.W. Investigation: F.V., F.v.K., C.M. Data curation: A.S. Formal analysis: F.V., B.L.W. Conceptualization: FV., B.L.W., C.M., H.B., R.S., M.B., D.H. Writing – original draft: F.V., M.B., D.H. Writing – review and editing: C.M., H.B., R.S., F.v.K., A.S., M.B., D.H. Review and final approval of the manuscript: all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.03.018.

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