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Long-term CIN3+ risk of HPV positive women after triage with *FAM19A4/miR124-2* methylation analysis

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HIGHLIGHTS

- A negative *FAM19A4/miR124-2* methylation result provides a similar safety in HPV positive women compared to cytology.
- CIN3+ risk following a positive methylation test justifies immediate colposcopy referral.
- Triage with methylation analysis enables full molecular screening which is also applicable on self-collected material.

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ABSTRACT

Objective. This study evaluates the long-term risk for cervical intraepithelial neoplasia grade 3 or worse (CIN3+) among HPV positive women triaged with *FAM19A4/miR124-2* methylation analysis.

Methods. In a post hoc analysis, data on *FAM19A4/miR124-2* methylation, cytology, and HPV16/18 genotyping of HPV positive women (n = 1025) from a large population-based screening cohort with 14-year follow-up were evaluated. Cumulative CIN3+ incidences over 3 screening rounds (5-year intervals) of 4 triage strategies were compared: *FAM19A4/miR124-2* methylation analysis, cytology, HPV16/18 genotyping with *FAM19A4/miR124-2* methylation, and HPV16/18 genotyping with cytology.

Results. Kaplan-Meier estimates of 14-year cumulative CIN3+ incidence of HPV positive women with a negative methylation and a negative cytology triage test were comparable (16.3% and 15.6%, respectively). The cumulative CIN3+ incidence of methylation positive and cytology positive women were 39.8% and 46.5%, respectively. HPV16/18 genotyping with methylation and HPV16/18 genotyping with cytology resulted in the lowest 14-year cumulative CIN3+ incidence among triage negative women (10.7% and 10.0%, respectively), but cumulative CIN3+ incidence among triage positive women was lower (33.4% and 35.7%, respectively) compared with triage by methylation alone and cytology alone.

Conclusions. Among HPV positive women of 30 years and older, a negative *FAM19A4/miR124-2* methylation triage test provides a similar long-term CIN3+ risk compared with a negative cytology triage test. Because of their high CIN3+ risk, women with a positive methylation triage test could be referred for colposcopy. Therefore, *FAM19A4/miR124-2* methylation analysis is a promising alternative to cytology for triage of HPV positive women.

1. Background

Since human papillomavirus (HPV) testing provides better protection against cervical cancer compared with cytology, new cervical cancer screening guidelines recommend high-risk HPV testing as a primary screening tool [1–5]. To prevent unnecessary colposcopy referral and associated harms for women with transient HPV infections, effective triage testing of HPV positive women is critical [6,7]. Currently used triage tests include cytology, either or not combined with HPV16/18 genotyping, and $p16^{INK4A}$/Ki-67 dual-stained cytology [8–10]. However, these triage tests are all based on cytology, which depends on subjective interpretation, and cannot be conducted on self-collected material [11]. Molecular triage markers can bypass these issues and could be used to further optimize cervical cancer screening.

DNA methylation analysis of viral and/or host cell genes has been identified as a promising molecular tool to triage HPV positive women and has the advantage of a quantitative and objective outcome

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[8,12–14]. Previous studies have shown that methylation analysis of host cell genes *FAM19A4* and *miR124-2* has good sensitivity and specificity for detection of cervical intraepithelial neoplasia grade 3 or worse (CIN3+) in HPV positive women [8,15–18]. Methylation analysis of *FAM19A4*/*miR124-2* is particularly sensitive for the detection of cervical cancer (Vink et al., submitted) and advanced CIN2/3 [17], which are lesions with a persistent HPV infection of at least 5 years, characterized by a 'cancer-like' genetic and epigenetic profile [17,19]. The high cross-sectional sensitivity translates into a 14-year cervical cancer risk of 1.7% in HPV positive women with a negative *FAM19A4*/*miR124-2* methylation result, which is lower compared with HPV positive women with a negative cytology result (2.4%) [20]. Further longitudinal evaluations are needed to determine the long-term CIN3+ risk among HPV positive women following triage testing with *FAM19A4*/*miR124-2* methylation analysis and to direct the development of management guidelines for HPV positive women.

In this study, we conducted a post hoc analysis in an HPV positive screening cohort to assess the CIN3+ risk among women triaged by *FAM19A4*/*miR124-2* methylation analysis, cytology, HPV16/18 genotyping with *FAM19A4*/*miR124-2* methylation analysis, and HPV16/18 genotyping with cytology up to 14 years of follow-up.

2. Materials and methods

2.1. Study population

This study is a post hoc analysis within the POBASCAM trial (Trial registration ID: NTR218; ISRCTN20781131), including all women from the control group with a positive HPV result and valid results for genotyping, cytology and *FAM19A4*/*miR124-2* methylation analysis at baseline (n = 1025) [20]. The POBASCAM trial was approved by the Medical Ethics Committee of the VU University Medical Center (no 96/103A) and by the Ministry of Public Health (VWS no 328 650). All participating women provided written informed consent.

2.2. Study procedures within the control group of the POBASCAM trial

A detailed description of the POBASCAM trial has been published before [1,21,22]. Between January 1999 and September 2002, a total of 44,938 women, aged 29–61 years, who were invited within the national cervical screening program were enrolled, of whom 22,518 were randomized to the control group.

Cervical scrapes were classified according to the CISOE-A classification, the standard classification system for cytology in the Netherlands, which can be translated into the Pap classification or the Bethesda system [23]. Testing for the presence of HPV DNA was performed using the GP5+/6+ PCR-EIA followed by reverse line blot assay for genotyping [24]. Women in the control group, with blinded HPV testing, were managed based on cytology only at the first screening round. Management was based on HPV and cytology co-testing at the second screening round after 5 years, and based on cytology only at the third screening round after 10 years.

For management based on cytology at the first and third round, (i) women with normal cytology were referred to routine screening (screening interval 5 years); (ii) women with borderline or mild dyskaryosis (BMD, comparable with atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL) in the Bethesda classification) were retested with cytology at 6 and 18 months and referred for colposcopy in case of BMD or worse; and (iii) women with moderate dyskaryosis or worse (>BMD, comparable with high-grade squamous intraepithelial lesion (HSIL) or worse) were directly referred for colposcopy.

For management based on HPV and cytology co-testing at the second round, (i) women who tested HPV negative and had normal cytology were referred to routine screening (screening interval 5 years); (ii) HPV positive women with normal or BMD cytology were retested with co-testing at 6 and 18 months and referred for colposcopy in case of a positive HPV result or cytology showing BMD or worse; and (iii) women with >BMD were directly referred for colposcopy.

Histological follow-up data were retrieved from the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA) up to July 2013, at which moment women had the opportunity to attend three screening rounds [25]. Histology results were classified as no dysplasia, CIN grade 1, 2 or 3, or cervical cancer. Adenocarcinoma in situ was classified as CIN3.

2.3. FAM19A4/miR124-2 methylation results

FAM19A4/*miR124-2* methylation analysis was performed previously, blinded for cytology and histology outcomes, by quantitative methylation specific PCR (qMSP) on bisulphite converted DNA from cervical scrapes collected at baseline using a prototype version of the QIAsure Methylation Test® (Qiagen, Hilden, Germany) [20].

2.4. Statistical analysis

The cumulative CIN3+ incidence over two screening rounds did not differ between the control group and the intervention group of the POBASCAM trial [1], and therefore the control group is considered valid for long-term risk evaluations. The Kaplan-Meier curve was used to estimate the cumulative CIN3+ incidence (CIN3+ risk) up to 14 years of follow-up. Separate estimates were obtained for 4 different triage strategies: (I) cytology; (II) *FAM19A4*/*miR124-2* methylation analysis; (III) HPV16/18 genotyping with cytology; and (IV) HPV16/18 genotyping with *FAM19A4*/*miR124-2* methylation analysis. Strategy I was labelled positive if the result was BMD or worse, and labelled negative otherwise. Strategy II was labelled positive if the methylation result was above the predefined threshold of the QIAsure Methylation Test®, and labelled negative otherwise. Strategy III was labelled positive in case HPV16/18 was present or in case the cytology result was BMD or worse, and labelled negative if both HPV16/18 and cytology were negative. Strategy IV was labelled positive in case HPV16/18 was present or in case the methylation result was above the predefined threshold, and labelled negative if both HPV16/18 and methylation analysis were negative. Endpoint was reached in case someone developed CIN3+. Time to event was defined as the number of years between the baseline cervical scrape and the time of diagnosis. In case of non-attendance to follow-up screening rounds, time to event was censored after the last attending screening round (i.e., after 4 or 9 years). Hysterectomy or CIN2+ excision were censored at the date of the interrupting event [3]. Women without event were censored after 14 years. The histological diagnosis of CIN2 contains a very heterogeneous disease category and therefore CIN2 or worse was not evaluated as study endpoint [13].

Cumulative CIN3+ incidences were compared between different strategies by calculating risk differences after 4 years (i.e., 1 screening round), after 9 years (i.e., 2 screening rounds) and after 14 years (i.e., 3 screening rounds). Cumulative CIN3+ incidences of triage negative women could not be estimated after 4 years, because management in the first screening round was based on cytology only. This verification bias would lead to an underestimation of the CIN3+ risk as cytology had a moderate sensitivity (approximately 65% [26]). We constructed 95% confidence intervals (95% CI) for the risk differences via Bootstrap in R (version 3.2.5, Vienna, Austria). If the 95% CI did not contain the value 0, the difference was considered significant. All other statistical analysis were performed with SPSS Statistics (version 22, IBM Corp, Armonk, NY, USA).

Table 1
Histology results after 4, 9, and 14 years of follow-up (i.e. first, second, and third round) stratified by baseline cytology, methylation and HPV16/18 genotyping results.

	Cyt− MM− HPV16/18− (n = 331)	Cyt+ MM− HPV16/18− (n = 68)	Cyt− MM+ HPV16/18− (n = 105)	Cyt− MM− HPV16/18+ (n = 209)	Cyt+ MM+ HPV16/18− (n = 66)	Cyt+ MM− HPV16/18+ (n = 54)	Cyt− MM+ HPV16/18+ (n = 72)	Cyt+ MM+ HPV16/18+ (n = 120)	Total (n = 1025)
Histology first round									
CIN2	1	10	0	4	12	9	1	13	50
CIN3	4	8	1	1	20	9	2	71[a]	116
ICC	0	0	1	0	0	0	1	1	3
Histology second round									
CIN2	13	1	2	3	3	1	1	1	25
CIN3	14	2	6	22	0	6	8	5	63
ICC	0	0	0	4	0	0	5	2	11
Histology third round									
CIN2	4	0	1	1	0	0	2	0	8
CIN3	2	0	1	6	0	2	2	0	13
ICC	2	0	0	2	0	0	1	0	5

Abbreviations: CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer; Cyt−, cytology negative; Cyt+, cytology positive; MM−, methylation negative; MM+, methylation positive; HPV16/18−, HPV16/18 negative; HPV16/18+, HPV16/18 positive.
[a] One woman with CIN3 was diagnosed with cervical cancer in the third round.

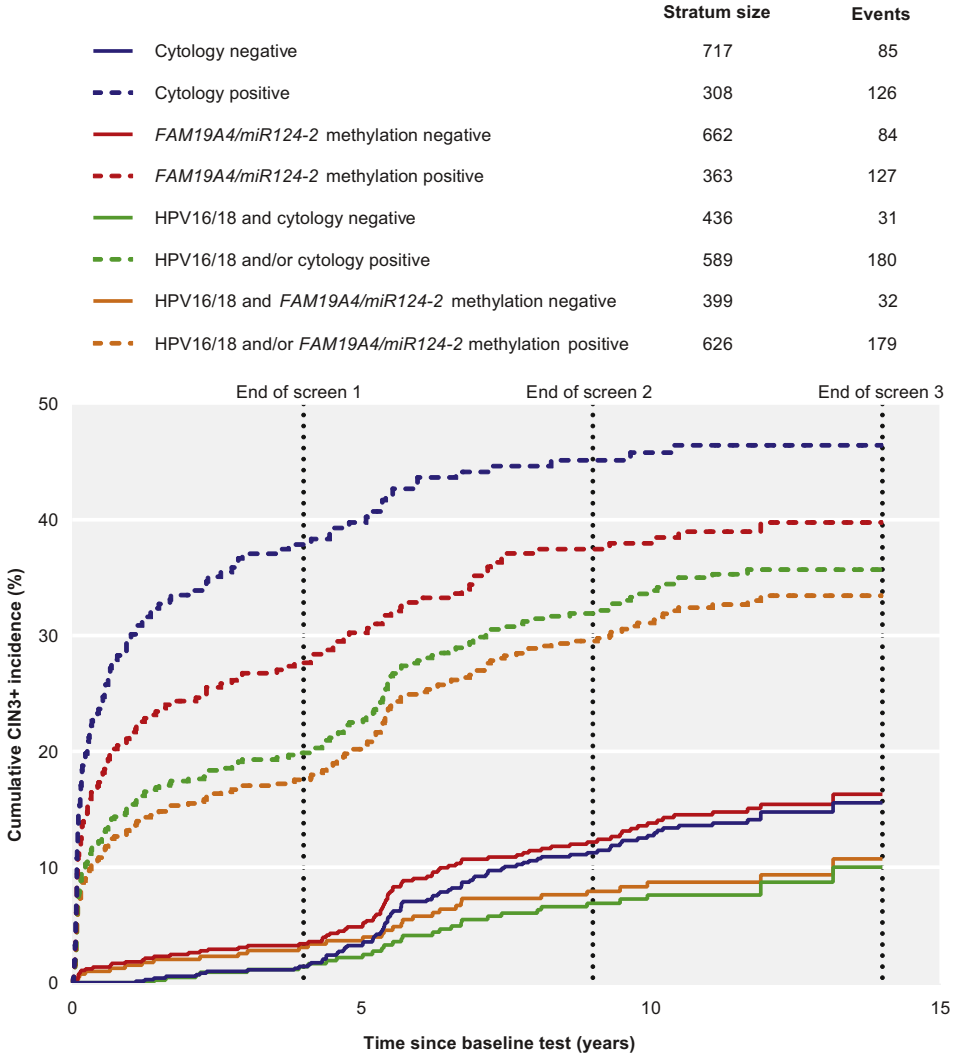

Fig. 1. Fourteen-year cumulative CIN3+ incidence among HPV positive women stratified by different triage strategies.

3. Results

3.1. Study population

Table 1 shows histology endpoints after 4, 9, and 14 years of follow-up, stratified by baseline cytology, *FAM19A4/miR124-2* methylation, and HPV16/18 genotyping results. During 14 years of follow-up, 211 of 1025 HPV positive women (20.6%) were diagnosed with CIN3+, of whom 119 (56.4%) were diagnosed within the first screening round (up to 4 years from baseline), 74 (35.1%) were diagnosed within the second screening round (between 4 and 9 years from baseline), and 18 (8.5%) were diagnosed within the third screening round (between 9 and 14 years from baseline).

Fig. 1 shows the cumulative CIN3+ incidence up to 14 years of follow-up among HPV positive women stratified by baseline results of 4 different triage strategies: (I) cytology; (II) *FAM19A4/miR124-2* methylation analysis; (III) HPV16/18 genotyping with cytology; and (IV) HPV16/18 genotyping with *FAM19A4/miR124-2* methylation analysis. The corresponding CIN3+ risk estimates after 4, 9, and 14 years following a negative or a positive triage test at baseline are reported in Table 2.

3.2. Outcomes of triage negative women

The CIN3+ risk difference between cytology negative women (strategy I) and methylation negative women (strategy II) was −0.93% (95% CI: −2.9 to 1.1%) after 9 years, and −0.73% (95% CI: −3.0 to 1.5%) after 14 years. The CIN3+ risk difference between HPV16/18 and cytology negative women (strategy III), and HPV16/18 and methylation negative women (strategy IV) was −1.0% (95% CI: −3.1 to 1.1%) after 9 years, and −0.69% (95% CI: −3.1 to1.8%) after 14 years.

3.3. Outcomes of triage positive women

The CIN3+ risk difference between cytology positive women (strategy I) and methylation positive women (strategy II) was 10.2% (95% CI: 6.4 to 14.2%) after 4 years, 7.7% (95% CI: 2.4 to 13.2%) after 9 years, and 6.7% (95% CI: 1.2 to 12.5%) after 14 years. The CIN3+ risk difference between HPV16/18 positive and/or cytology positive women (strategy III), and HPV16/18 positive and/or *FAM19A4/miR124-2* methylation positive women (strategy IV) was 2.3% (95% CI: 1.2 to 3.6%) after 4 years, 2.4% (95% CI: 0.74 to 4.2%) after 9 years and 2.3% (95% CI: 0.46 to 2.3%) after 14 years.

Table 2
Cumulative CIN3+ incidence stratified by triage strategies after 4, 9 and 14 years of follow-up.

		Cumulative CIN3+ incidence after a positive triage test (95% CI)		Cumulative CIN3+ incidence after a negative triage test (95% CI)	
4-Year					
I	Cytology	37.9%	(32.2–43.6%)	N/A	
II	Methylation	27.7%	(23.0–32.4%)	N/A	
III	HPV16/18 genotyping with cytology	19.9%	(16.6–23.2%)	N/A	
IV	HPV16/18 genotyping with methylation	17.6%	(14.5–20.6%)	N/A	
9-Year					
I	Cytology	45.2%	(39.1–50.4%)	11.2%	(8.7–13.7%)
II	Methylation	37.5%	(32.1–42.9%)	12.2%	(9.5–14.9%)
III	HPV16/18 genotyping with cytology	31.9%	(27.8–36.0%)	6.9%	(4.3–9.4%)
IV	HPV16/18 genotyping with methylation	29.5%	(25.7–33.4%)	7.9%	(5.1–10.7%)
14-Year					
I	Cytology	46.5%	(43.3–49.1%)	15.6%	(13.9–17.3%)
II	Methylation	39.8%	(36.9–42.7%)	16.3%	(14.5–18.1%)
III	HPV16/18 genotyping with cytology	35.7%	(31.4–40.1%)	10.0%	(6.0–14.0%)
IV	HPV16/18 genotyping with methylation	33.4%	(29.3–37.6%)	10.7%	(6.5–14.9%)

Abbreviations: CIN3+, cervical intraepithelial neoplasia grade 3 or worse; 95% CI, 95% confidence interval; N/A, not applicable.

4. Discussion

This study evaluated the long-term CIN3+ risk of different triage strategies for HPV positive women from a large cervical screening population. We demonstrated that the cumulative CIN3+ risk in HPV positive women after 14 years of follow-up following a negative methylation result was comparable with the CIN3+ risk following a negative cytology result (16.3% and 15.6%, respectively). When HPV16/18 genotyping was combined with either methylation or cytology, the CIN3+ risk of triage negative women decreased, resulting in comparable 14-year CIN3+ risks of 10.7% and 10.0%, respectively. Collectively, these results indicate for the first time that a negative *FAM19A4/miR124-2* methylation test provides a similar long-term protection against CIN3+ in HPV positive women over 30 years of age compared with a negative cytology result.

The cumulative CIN3+ risk after 14 years of follow-up following a positive methylation result was lower compared with the CIN3+ risk following a positive cytology result (39.8% and 46.5%, respectively). When HPV16/18 genotyping was combined with either methylation or cytology, CIN3+ risks of HPV16/18 genotyping with methylation positive women was close to the risk of HPV16/18 genotyping with cytology positive women (33.4% and 35.7%, respectively). However, CIN3+ risks after a positive triage test in this study should be interpreted with caution, because women in the trial were managed based on cytology in the first and third screening round, and based on HPV and cytology in the second screening round. Women were not managed based on their methylation result, causing a preferential effect in favour of cytology, i.e. a higher CIN3+ risk for cytology positive women.

Management of HPV positive women and thereby the most preferable triage strategy remains a topic of discussion. There is currently no international consensus on criteria for triage strategies. Various studies have evaluated triage strategies cross-sectionally, but more longitudinal evaluations to measure safety of triage strategies are needed. The data presented in this study demonstrate that among HPV positive women aged ≥30 years, a negative *FAM19A4/miR124-2* methylation test provides a similar safety in terms of long-term CIN3+ risk compared with a negative cytology test. However, safety should be weighed against screening-related burden, and the optimal balance may vary between coutries, as it depends on locally accepted risk thresholds and available resources. For example, in the United States a 3-year CIN3+ risk of approximately 5% is considered appropriate for direct colposcopic assessment [27], while in the Netherlands the accepted short-term CIN3+ risk threshold is 20% [9,28,29]. Although potentially underestimated because women were not managed according to their methylation result, the 4-year CIN3+ risk after a positive *FAM19A4/miR124-2* methylation test result presented here exceeds the US and the Dutch thresholds for direct colposcopy referral [28,29].

Major strengths of this study are the large sample size, the long follow-up period through the nationwide cyto- and histopathology registry, and its setting within a population-based cervical cancer screening program. This study is limited by the verification bias due to management based on cytology only at the first screening round. The number of CIN3+ lesions detected among HPV positive, cytology negative women in the first screening round is potentially underestimated, as these lesions were only identified during the second screening round [1]. Hence, CIN3+ risks after a negative triage test should not be interpreted before the end of the second screening round. At the third screening round, women were again managed based on cytology only, which was in accordance with the national guidelines at that time, and thus newly developed lesions could have been missed. Another

limitation of this study is that the histological diagnoses were performed within routine diagnostics by local pathologists, which carries the risk of misclassification. However, for CIN3+ diagnosis the inter-observer reliability has been shown to be very high (absolute agreement 0.97) [1].

Molecular tools, such as methylation analysis, offer objective triage of HPV positive women. Multiple methylation markers, including host cell and/or viral genes, have been identified as promising tools to identify HPV positive women with cervical precancer and cancer [8,12,14,15,30–36]. Most of these assays are still in research phase and their long-term reassurance against CIN3+ has not been evaluated. The *FAM19A4/miR124-2* methylation test has now reached phase 4 out of 5 phases of biomarker development for early cancer detection [37,38]. The test is available as CE-IVD marked assay (Qiasure Methylation Test ®, Qiagen, Hilden) with high intra- and inter-laboratory agreement as demonstrated in a recent international study [39]. *FAM19A4/miR124-2* methylation analysis has shown to have a very high cross-sectional detection rate of cervical cancer (Vink, et al., submitted) [17], and an earlier study has shown a 14-year cancer risk of 1.7% among HPV positive, methylation negative women, which was 0.71% lower compared with HPV positive, cytology negative women [20]. The long-term CIN3+ risk of HPV positive, triage negative women observed in this study is in line with previous evidence showing that HPV positive women with a negative triage test (either cytology, repeat HPV testing, HPV16/18 genotyping, or a combination) remain at risk for CIN3+ and require further follow-up [3,40,41]. The comparable long-term protection against CIN3+ as evaluated in this study for HPV positive, methylation negative women and HPV positive, cytology negative women, indicates that follow-up strategies of these women could be identical.

With the introduction of HPV based screening, self-sampling has become feasible. When using validated PCR assays, primary HPV testing on self-collected samples is as sensitive and specific for the detection of CIN2+ or CIN3+ compared with primary HPV testing on physician-collected samples [42,43]. An important advantage of molecular triage tests over cytology, is their applicability on self-collected cervicovaginal material. *FAM19A4/miR124-2* methylation triage testing on self-collected material bypasses the need for an additional cervical scrape after an HPV positive result, which is required for cytology triage testing, and can consequently reduce loss to follow-up and shorten the diagnostic track [30,44,45]. This advantage, combined with the objectivity and reproducibility of methylation analysis, enables full molecular screening with the potential of fully automated, high-throughput testing, and could further optimize cervical cancer screening.

5. Conclusion

This study demonstrates that among HPV positive women aged ≥30 years, a negative *FAM19A4/miR124-2* methylation test provides a similar safety in terms of long-term CIN3+ risk compared with a negative cytology test, while the CIN3+ risk following a positive methylation test justifies immediate colposcopy referral. Therefore, *FAM19A4/miR124-2* methylation analysis, either or not combined with HPV16/18 genotyping, can be considered as an objective alternative to cytology for triage testing of HPV positive women in cervical cancer screening.

Ethics approval

The POBASCAM trial was approved by the Medical Ethics Committee of the VU University Medical Center (no 96/103A) and by the Ministry of Public Health (VWS no 328 650). All participating women provided written informed consent.

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Authors' contributions

Conception and design: JB, CM, and DAMH. Development of methodology: JB, CM, and DAMH. Acquisition of data: LMAS, and SD. Data analysis: SD, WWK, and BILW. Interpretation of data: SD, WWK, RDMS, JB, CJLMM, and DAMH. Writing, review and/or revision of manuscript: SD, WWK, LMAS, BILW, RDMS, JB, CJLMM, and DAMH. Study supervision: JB, CJLMM, and DAMH.

Declaration of Competing Interest

(1) DAMH, RDMS, and CJLMM 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) DAMH has been on the speakers bureau of Qiagen and serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb; (4) JB received consultancy fees from GlaxoSmithKline, and Merck, and received travel support from DDL. All fees were collected by his employer; (5) CJLMM has received speakers fee from GSK, Qiagen, and SPMSD/Merck, and served occasionally on the scientific advisory board (expert meeting) of GSK, Qiagen, and SPMSD/Merck; (6) CJLMM has a very small number of shares of Qiagen and holds minority stock in Self-Screen B.V.; (7) CJLMM is part-time director of Self-screen B.V. since September 2017; Self-screen B.V. is supported by the Valid-screen project, funded by the SME Instrument in the Horizon 2020 Work Program of the European Commission (Valid-screen 666800). (8) LMAS is currently an employee of Bayer; (9) SD, WWK, and BILW declare no conflicts of interest.

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