View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Erasmus University Digital Repository

FULL PAPER

BJC

British Journal of Cancer (2016) 115, 579–587 | doi: 10.1038/bjc.2016.200

Keywords: DNA methylation; cervical intraepithelial neoplasia; cervicovaginal lavage; self-sampling; qMSP

FAM19A4 methylation analysis in self-samples compared with cervical scrapes for detecting cervical (pre)cancer in HPV-positive women

Roosmarijn Luttmer[1,12], Lise M A De Strooper[1,12], Maaike G Dijkstra[2], Johannes Berkhof[3], Peter J F Snijders[1], Renske D M Steenbergen[1], Folkert J van Kemenade[4], Lawrence Rozendaal[1], Theo J M Helmerhorst[5], René H M Verheijen[6], W Abraham ter Harmsel[7], W Marchien van Baal[8], Peppino G C M Graziosi[9], Wim G V Quint[10], Johan W M Spruijt[2], Dorenda K E van Dijken[11], Daniëlle A M Heideman[1] and Chris J L M Meijer*,[1]

[1]Department of Pathology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands; [2]Department of Obstetrics and Gynaecology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands; [3]Department of Epidemiology and Biostatistics, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands; [4]Department of Pathology, Erasmus Medical Center, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands; [5]Department of Obstetrics and Gynaecology, Erasmus MC University Medical Center, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands; [6]UMC Utrecht Cancer Center, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands; [7]Roosevelt Clinic, Rooseveltstraat 67, 2321 CT Leiden, The Netherlands; [8]Department of Obstetrics and Gynaecology, Flevo Hospital, Hospitaalweg 1, 1315 RA Almere, The Netherlands; [9]Department of Obstetrics and Gynaecology, Sint Antonius Hospital, Koekoekslaan 1, 3435 CM Nieuwegein, The Netherlands; [10]DDL Diagnostic Laboratory, Visseringlaan 25, 2288 ER Rijswijk, The Netherlands and [11]Department of Obstetrics and Gynaecology, Onze Lieve Vrouwe Gasthuis West, Oosterpark 9, 1091 AC Amsterdam, The Netherlands

Background: High-risk human papillomavirus (hrHPV)-positive women require triage to identify those with cervical high-grade intraepithelial neoplasia and cancer (⩾CIN3 (cervical intraepithelial neoplasia grade 3)). *FAM19A4* methylation analysis, which detects advanced CIN and cancer, is applicable to different sample types. However, studies comparing the performance of *FAM19A4* methylation analysis in hrHPV-positive self-samples and paired physician-taken scrapes are lacking.

Methods: We compared the performance of *FAM19A4* methylation analysis (and/or HPV16/18 genotyping) in self-samples and paired physician-taken scrapes for ⩾CIN3 detection in hrHPV-positive women ($n = 450$,18–66 years).

Results: Overall *FAM19A4* methylation levels between sample types were significantly correlated, with strongest correlation in women with ⩾CIN3 (Spearman's ρ 0.697, $P < 0.001$). The performance of *FAM19A4* methylation analysis and/or HPV16/18 genotyping did not differ significantly between sample types. In women ⩾30 years, ⩾CIN3 sensitivity of *FAM19A4* methylation analysis was 78.4% in self-samples and 88.2% in scrapes (ratio 0.89; CI: 0.75–1.05). In women <30 years, ⩾CIN3 sensitivities were 37.5% and 45.8%, respectively (ratio 0.82; CI: 0.55–1.21). In both groups, ⩾CIN3 specificity of *FAM19A4* methylation analysis was significantly higher in self-samples compared with scrapes.

Conclusions: *FAM19A4* methylation analysis in hrHPV-positive self-samples had a slightly lower sensitivity and a higher specificity for ⩾CIN3 compared with paired physician-taken scrapes. With a similarly good clinical performance in both sample types, combined *FAM19A4* methylation analysis and HPV16/18 genotyping provides a feasible triage strategy for hrHPV-positive women, with direct applicability on self-samples.

*Correspondence: Professor CJLM Meijer; Email: cjlm.meijer@vumc.nl

[12]These authors contributed equally to this work.

Received 17 March 2016; revised 31 May 2016; accepted 2 June 2016; published online 14 July 2016

© 2016 Cancer Research UK. All rights reserved 0007 – 0920/16

Epidemiological studies have established that a persistent infection with a high-risk type of the human papillomavirus (hrHPV) is a necessary aetiological factor for cervical cancer. Several randomised trials have demonstrated that testing for the presence of hrHPV is a more sensitive screening tool compared with cytology, providing improved detection of cervical intraepithelial neoplasia grade 3 (CIN3) and cervical cancer (Dillner, 2013; Ronco *et al*, 2014). However, the majority of hrHPV infections is transient, and only upon accumulation of genetic and epigenetic changes in the host and/or viral genomes, progression towards invasive cancer may occur (Snijders *et al*, 2006; Steenbergen *et al*, 2014). To avoid unnecessary referral to the gynaecologist of women with transient hrHPV infections, identification of women with lesions reflecting clinically relevant infections with a high risk of progression towards cancer is required. Currently, cytology is the most advocated triage tool for this purpose (Zorzi *et al*, 2013; Dijkstra *et al*, 2014). However, cytology triage requires repeat testing to ensure sufficient sensitivity (Rijkaart *et al*, 2012; Dijkstra *et al*, 2014) and has a subjective test result (Dijkstra *et al*, 2014). Moreover, prior knowledge of the hrHPV status influences cytology reading, resulting in an easier classification of abnormal cytology, thus increasing the number of false-positives (Moriarty *et al*, 2014). Although HPV16/18 genotyping might be a valuable addition to cytology by eliminating the necessity of a repeat test (Cox *et al*, 2013; Dijkstra *et al*, 2014), this combination may suffer from non-detection of (pre)cancers associated with non-HPV16/18 hrHPV types.

As gene silencing by promoter hypermethylation has been shown to contribute to cervical carcinogenesis, methylation analysis of cancer-specific genes has been suggested as a valuable, alternative or additive triage tool (Steenbergen *et al*, 2004; Overmeer *et al*, 2009; Wilting *et al*, 2010; Saavedra *et al*, 2012). Furthermore, in contrast to cytology, DNA methylation analysis detects virtually all cervical carcinomas and is assumed to detect 'advanced' CIN lesions, that is, lesions with a long duration of existence and with many chromosomal aberrations, which have a high risk of short-term progression to cancer (De Strooper *et al*, 2014; Steenbergen *et al*, 2014). Gene promoter methylation can be easily accessed by sensitive, quantitative methylation-specific PCR (qMSP) providing an objective test outcome. Previous results in hrHPV-positive cervical scrapes have been promising (Huang *et al*, 2010; Hesselink *et al*, 2011, 2014; Eijsink *et al*, 2012; Verhoef *et al*, 2014a), with sensitivities for CIN3 and cervical cancer (⩾CIN3) equalling those of cytology analysis (Hesselink *et al*, 2011; Verhoef *et al*, 2014a).

An important development in cervical cancer screening is the use of self-collected cervicovaginal samples for women who do not attend screening (Ogilvie *et al*, 2005; Snijders *et al*, 2013; Arbyn *et al*, 2014). However, as cytology cannot be reliably performed on self-sampled material (Nobbenhuis *et al*, 2002; Garcia *et al*, 2003), triage of hrHPV-positive women by cytology still requires a visit to a physician. In contrast, methylation analysis can be performed directly on the self-collected sample (Eijsink *et al*, 2011; Hesselink *et al*, 2014; Verhoef *et al*, 2014a). Indeed, a prospective trial in non-responders has shown that detection of high-grade CIN with direct *MAL/miR124-2* methylation analysis on hrHPV-positive lavage-collected self-samples is non-inferior to cytology triage on hrHPV-positive physician-taken scrapes, thereby preventing diagnostic delay and loss to follow-up (Verhoef *et al*, 2014a). Recently, methylation analysis of the *FAM19A4* gene has been shown to yield attractive ⩾CIN3 sensitivities and specificities in both hrHPV-positive brush-collected and lavage-collected self-samples (De Strooper *et al*, 2016) supporting the concept that it serves as a universal triage test for HPV-positive cervicovaginal self-samples collected by different self-collection devices.

Studies evaluating the concordance of methylation analysis between self-collected cervicovaginal samples and physician-taken cervical scrapes are however limited (Boers *et al*, 2014; Chang *et al*, 2015). Such evaluations are crucial to reveal the relative clinical performance for ⩾CIN3 detection compared with physician-taken scrapes, as has been assessed for HPV testing (Arbyn *et al*, 2014). It is well known that each sample type has its own cellular composition with likely a different fraction of indicator cells exfoliated from ⩾CIN3, which may affect the performance of molecular reflex tests. The present study compared the clinical performance of *FAM19A4* methylation analysis with and without additional HPV16/18 genotyping, for the detection of ⩾CIN3 in a unique cohort of paired self-collected cervicovaginal lavage samples and physician-taken cervical scrapes of hrHPV-positive women.

MATERIALS AND METHODS

Study design, participants and procedures. The present study was conducted as a *post hoc* analysis of data obtained in a prospective observational multicentre cohort study (COMETH), which aimed to compare different triage strategies in hrHPV-positive women (Luttmer *et al*, 2016). From December 2010 till December 2013, women (age 18–70 years) who visited a gynaecological outpatient clinic in one of six hospitals in the Netherlands were asked to participate in the study. Women could participate in the study regardless of their reason for referral to the gynaecology outpatient clinic. After providing informed consent, participants collected cervicovaginal lavage material (using the Delphi screener; Delphi Bioscience, Scherpenzeel, The Netherlands) for hrHPV testing. Women who met the inclusion criteria and tested hrHPV-positive on the cervicovaginal lavage were invited for a cervical scrape and colposcopy. The cervical scrape was taken by a physician using a Cervex-Brush (Rovers Medical Devices BV, Oss, The Netherlands) or a Medscand Cytobrush Plus (CooperSurgical Inc., Trumbull, CT, USA). Scrape material was stored in 20 ml of Thinprep PreservCyt solution (Hologic, Marlborough, MA, USA) and was evaluated by *FAM19A4* methylation analysis and HPV16/18 genotyping (cytology results on these scrapes have been reported previously; Luttmer *et al*, 2016)). The study was approved by the Medical Ethical Committee of all participating hospitals (METc-VUmc2009/178) and registered as NTR2447. Women with a history of treatment for cervical dysplasia or cervical cancer, current cancer, pregnancy or lactation were excluded from participation (Luttmer *et al*, 2016).

For logistic reasons, from 141 (31.3%) women, cervicovaginal lavage material was collected by the physician before cervical scraping using a Delphi screener according to the protocol. The remaining 309 (68.7%) women self-collected lavage material at home. In 268 (59.5%) women, the cervical scrape was taken at a minimum of 2 weeks before colposcopy. In 182 (40.4%) women, for logistic reasons, the cervical scrape was taken immediately before colposcopy during the same visit to the gynaecologist.

As shown in Figure 1, in all women who tested both hrHPV-positive on the cervicovaginal lavage and the cervical scrape ($n = 532$), *FAM19A4* methylation analysis was performed on each sample type. All of these women had colposcopy-directed biopsy. Women with invalid test results for *FAM19A4* methylation analysis of the cervicovaginal lavage ($n = 48$; 9.0%) or cervical scrape ($n = 43$; 8.0%) were excluded from analyses. Of these, nine women had an invalid test result in both the lavage and scrape samples. The remaining women comprised the final study population ($n = 450$).

Virtually all women (65 of 66; 98.4%) with histologically confirmed CIN3 and adenocarcinoma *in situ* (AIS) in the biopsy specimen were treated by large loop excision of the transformation zone (LLETZ) or cervical conisation; one woman was followed up

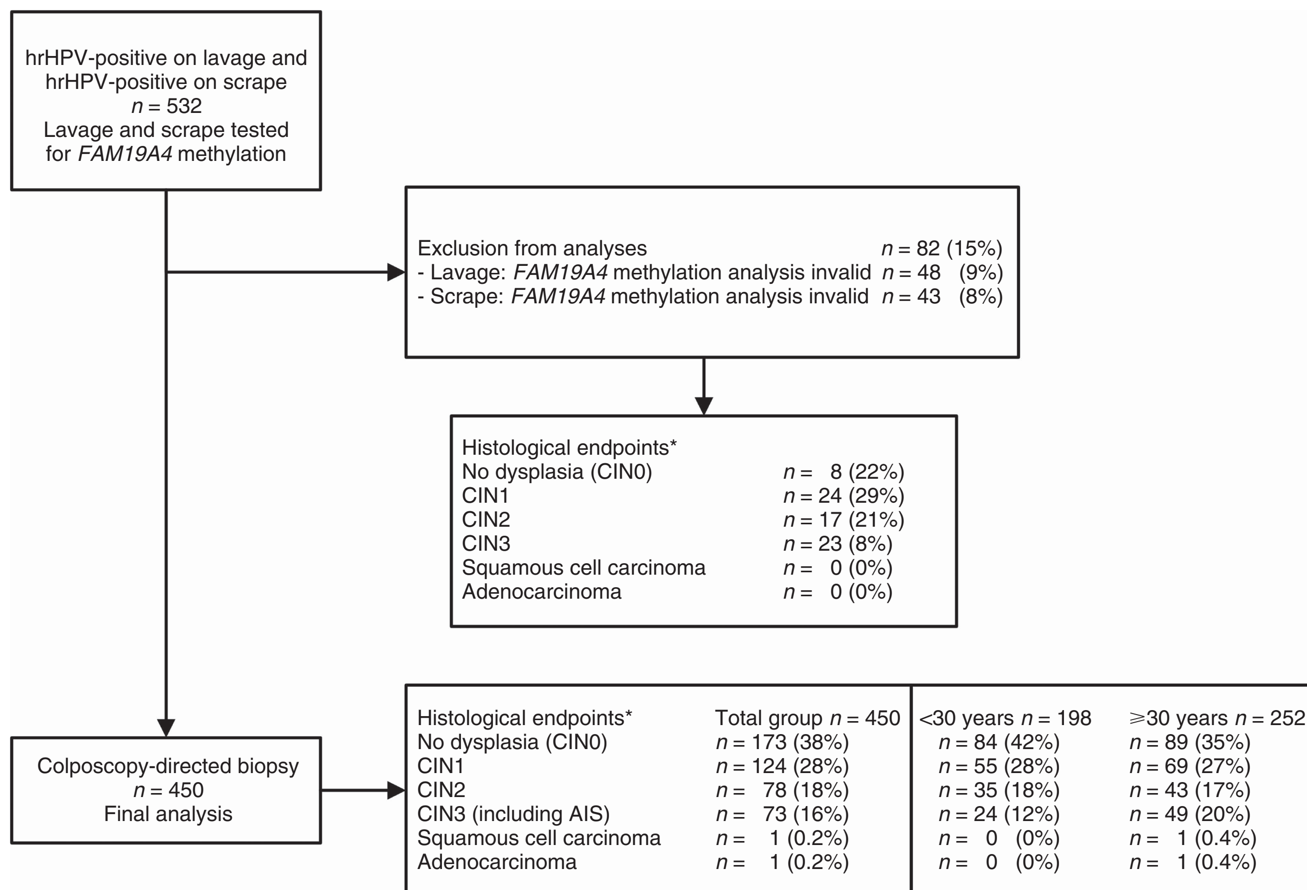

Figure 1. Overview of the study population and histological endpoints. *Histological endpoints were based on the histological outcome of the colposcopy-directed biopsy, or, if classified worse, on the histology result of the specimen excised by LLETZ, conisation or hysterectomy. AIS = adenocarcinoma *in situ*; CIN = cervical intraepithelial neoplasia; hrHPV = high-risk human papillomavirus.

without treatment. Depending on the size of the lesion, also 55 of 85 (64.7%) women with a CIN2 biopsy underwent LLETZ. Of these women, eight (14.5%) were diagnosed with CIN3 in the LLETZ tissue, and categorised accordingly. In addition, one woman with a CIN0 in the biopsy specimen had a CIN3 in the tissue, which was obtained by (diagnostic) LLETZ.

Histological endpoints. In concordance with earlier work (Luttmer *et al*, 2016), we used histologically confirmed ⩾CIN3 as primary study end point and ⩾CIN2 as a secondary study end point. Unless otherwise specified, histological end points were based on the histological outcome of the colposcopy-directed biopsy, or, if classified worse, on the histology result of the specimen excised by LLETZ, conisation or hysterectomy. In addition, we attempted to provide a rough estimation of the volume of CIN lesions. Therefore, histology results of biopsy specimens and those of the corresponding LLETZ (or conisation/hysterectomy) specimens are presented (Table 1). The presence of ⩾CIN3 in the LLETZ (or conisation/hysterectomy) specimen, irrespective of the histological result of the biopsy, was considered indicative of a high-volume ⩾CIN3 lesion. The presence of CIN3 in the biopsy specimen, combined with ⩽CIN2 in the corresponding LLETZ specimen, was assumed to represent a low-volume CIN3 lesion. Similarly, the presence of CIN2 in the LLETZ specimen combined with ⩽CIN2 in the corresponding biopsy specimen was considered to indicate a high-volume CIN2 lesion. The presence of CIN2 in the biopsy with ⩽CIN1 in the LLETZ specimen was assumed to represent a low-volume CIN2 lesion. In women who were diagnosed with high-grade CIN on the biopsy specimen and who did not receive LLETZ (according to physician's advice), lesions were classified as low volume.

Table 1. Histology results of biopsy specimens and corresponding LLETZ specimens

	Histology LLETZ (or conisation or hysterectomy)			
Histology biopsy	**⩾CIN3**	**CIN2**	**⩽CIN1**	**No LLETZ**
⩾CIN3	37^{a*}	17^{b}	11^{b}	1^{b}
CIN2	8^{a}	25^{c}	22^{d}	30^{d}
⩽CIN1	1^{a}	1^{c}	3^{e}	294^{e}

Abbreviations: CIN = cervical intraepithelial neoplasia; LLETZ = large loop excision of the transformation zone.
[a]High-volume ⩾CIN3 lesion (*including two carcinomas).
[b]Low-volume CIN3 lesion.
[c]High-volume CIN2 lesion.
[d]Low-volume CIN2 lesion.
[e]⩽CIN1.

HPV genotyping. DNA was isolated from one-fifth of cervicovaginal lavage specimens and one-tenth of cervical scrape material using the Nucleo-Mag 96 Tissue Kit (Macherey-Nagel, Düren, Germany) and a Microlab Star robotic system (Hamilton, Planegg, Germany) according to the manufacturer's instructions (Hesselink *et al*, 2011). Isolated DNA was subjected to GP5+/6+ PCR-enzyme immunoassay analysis (EIA Kit HPV GP HR; Diassay BV, Rijswijk, The Netherlands) (Jacobs *et al*, 1997). 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 xMAP, Luminex Corp, Austin, TX, USA) as described previously (Schmitt *et al*, 2006).

qMSP analysis. Extracted DNA from hrHPV-positive cervicovaginal lavage specimens and cervical scrapes was treated with bisulphite using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) as described previously (Overmeer *et al*, 2008, 2009). Bisulphite-converted DNA was used as template for *FAM19A4* methylation analysis by qMSP using housekeeping gene β-actin (*ACTB*) as a reference (Steenbergen *et al*, 2013; De Strooper *et al*, 2014). Quantitative methylation-specific PCR analysis was carried out using an ABI 7500 Real-Time PCR-System (Applied Biosystems, Waltham, MA, USA). For each target, quantification cycle (Cq) values were measured at a fixed fluorescence threshold. To assure sample quality, all samples included in the study had a Cq value for *ACTB* <30. In case of poor DNA concentration and an invalid test result, DNA isolation, bisulphite treatment and qMSP were repeated with double sample input if sufficient material was available for analysis. Cq ratios were calculated for each sample using the following formula: $2^{(\mathrm{Cq}\ (ACTB)-\mathrm{Cq}\ (FAM19A4))}\times 100$. For both cervicovaginal lavage specimens and cervical scrapes, assay thresholds, which gave rise to a $\geqslant$CIN3 specificity of 70% using a training-validation set approach (De Strooper *et al*, 2014), were chosen to consider a specimen positive for *FAM19A4* methylation.

Statistical analysis. The sample size was set such that 90% power was achieved for demonstrating non-inferiority of *FAM19A4* methylation analysis in cervicovaginal lavages to *FAM19A4* methylation analysis in cervical scrapes using a matched-sample score test (Tang *et al*, 2003; Meijer *et al*, 2009). A minimum of 300 hrHPV-positive women needed to be included at rejection rate α of 0.05. Finally, 450 hrHPV-positive women were included with results for all markers.

For assessing overall genotype concordance, results were either scored as concordant (sample types yielded completely identical genotype results), compatible (one or more of the same genotypes were detected) or discordant (no genotype similarities detected). Paired evaluation of *FAM19A4* Cq ratios in cervicovaginal lavages and in cervical scrapes was carried out by Spearman's rank analysis.

The relation of the classification combining histological severity and lesion volume with the percentage of *FAM19A4* methylation-positive and/or HPV16/18-positive samples was assessed using Fisher's exact test.

For both cervicovaginal lavages and cervical scrapes, clinical performance of *FAM19A4* methylation analysis and the combined use of *FAM19A4* methylation analysis and HPV16/18 genotyping was evaluated. Clinical performance indicators were sensitivity, specificity, positive predictive value (PPV) and complemented NPV (1-negative predictive value, a measure of disease risk after a negative result) for $\geqslant$CIN3 and $\geqslant$CIN2, and referral rate (based on % marker positivity) were calculated. To enable comparisons, relative sensitivity (ratio of the sensitivity of a marker in one sample type to its sensitivity in the other sample type) and relative specificity (ratio of the specificity of a marker in one sample type to its specificity in the other sample type) were calculated with 95% CIs. If the 95% CI of the relative sensitivity or specificity was entirely below or above one, the difference in sensitivity or specificity was considered statistically significant. In case of non-significant differences in sensitivity or specificity, an additional test was performed to evaluate non-inferiority. Non-inferiority was defined as a relative sensitivity or specificity of at least 90% using a matched-sample score test (Tang *et al*, 2003; Meijer *et al*, 2009).

Using logistic regression, we analysed the influence of several factors on the sensitivity and specificity of *FAM19A4* methylation analysis in this study population: the age of the participants (<30 years and $\geqslant$30 years), the reason for referral to the gynaecologist (abnormal cytology result or other, non-cervix-related, gynaecological complaints), the sampling method of the cervicovaginal lavage (collected by the participant at home or by the physician) and the sampling moment of the cervical scrape (during a separate visit 2–3 weeks before colposcopy or combined with the colposcopy procedure). After finding a factor that significantly influenced the performance of the different markers, data were stratified for this factor. *P*-values <0.05 were considered statistically significant. All statistical analyses and computations of graphs were performed in IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA), STATA 11.0 (StataCorp LP, College Station, TX, USA) and Excel.

RESULTS

Study population. The study flowchart and histological end points are shown in Figure 1. The final analysis comprised 450 women (age 18–66 years) who tested hrHPV-positive on both the cervicovaginal lavage material and the cervical scrape, had valid results for *FAM19A4* methylation in both sample types and a histological end point. *FAM19A4* methylation scored positive in 26.4% (119 of 450) of the cervicovaginal lavage specimens and in 35.8% (161 of 450) of the cervical scrape specimens. Human papillomavirus16 and/or HPV18 was present in 46.4% (209 of 450) of the cervicovaginal lavage specimens and 47.3% (213 of 450) of the cervical scrape specimens. Two women (0.4%) had invasive cervical carcinoma (i.e., one squamous cell carcinoma (SCC) and one adenocarcinoma), 73 women (16.2%) had CIN3 (including one AIS), 78 women (17.3%) had CIN2, 124 (27.6%) had CIN1 and 173 (38.4%) had no CIN. In Table 1, histological results of biopsy specimens and corresponding LLETZ results are presented.

Concordance of HPV genotypes and *FAM19A4* methylation analysis. Genotype agreement between the cervical scrape and cervicovaginal lavage was concordant (sample types yielded completely identical genotype results) in 60.7% (273 of 450) and compatible (one or more of the same genotypes were detected) in 28.9% (130 of 450) of cases. In the remaining cases (47 of 450; 10.4%), genotypes were discordant between both sample types. As shown in Table 2, for the majority of hrHPV types, genotype concordance between cervical scrapes and cervicovaginal lavages was substantial to almost perfect (Landis and Koch, 1977), both in the total group and in the strata of histological severity. *FAM19A4* Cq ratios in lavage samples were significantly correlated to those in cervical scrapes in the total study population (Spearman's ρ 0.394, $P<0.001$). In the strata of histological severity, this correlation was found to be strongest in women with $\geqslant$CIN3 (Spearman's ρ 0.697, $P<0.001$), followed by CIN2 (Spearman's ρ 0.255, $P=0.024$) and $\leqslant$CIN1 (Spearman's ρ 0.257, $P<0.001$). The correlation between methylation Cq ratio in cervical scrapes and lavage samples in the strata of histological severity is presented in Supplementary Figure 1. Both women diagnosed with cervical cancer tested positive for *FAM19A4* methylation analysis in both sample types. The woman with an adenocarcinoma tested HPV16-positive, whereas the woman with an SCC harboured HPV39.

Performance of *FAM19A4* methylation analysis in total study population. Test specifications of *FAM19A4* methylation analysis in cervical scrapes (which were described previously; Luttmer *et al*, 2016) and in cervicovaginal lavages for detection of $\geqslant$CIN3 and $\geqslant$CIN2 in the total study population are shown in Table 3 (upper panel). Although the sensitivity of *FAM19A4* methylation analysis for the detection of $\geqslant$CIN3 was lower in cervicovaginal lavage material (65.3%) compared with that in cervical scrapes (74.7%), this difference was not statistically significant (ratio 0.88; CI: 0.75–1.02). However, statistical non-inferiority (of lavages relative to scrapes) could not be demonstrated ($P=0.64$). The $\geqslant$CIN3 specificity of *FAM19A4* methylation analysis was significantly

Table 2. HPV genotype concordance in cervical scrapes and cervicovaginal lavages

	Scrapes		Lavages						
HPV genotype	Positive *n*[a]	Percentage (%)[a]	Positive *n*[a]	Percentage (%)[a]	Discordant pairs *n*[b]	Concordance total group *κ*	⩾CIN3 *κ*	⩾CIN2 *κ*	⩽CIN1 *κ*
HPV16	175	39	175	39	38	0.822	0.871	0.856	0.789
HPV31	58	13	64	14	32	0.697	0.721	0.655	0.717
HPV56	56	12	51	11	27	0.714	0.654	0.542	0.755
HPV18	52	12	52	12	22	0.761	0.802	0.762	0.760
HPV51	51	11	50	11	19	0.788	0.793	0.843	0.766
HPV66	49	11	64	14	19	0.808	0.517	0.802	0.811
HPV52	46	10	48	11	28	0.667	0.686	0.694	0.653
HPV45	41	9	41	9	24	0.678	0.737	0.765	0.642
HPV59	35	8	38	8	23	0.657	0.661	0.564	0.685
HPV58	25	6	25	6	20	0.576	0.551	0.719	0.490
HPV33	23	5	28	6	11	0.771	0.661	0.759	0.775
HPV35	21	5	31	7	18	0.633	0.786	0.745	0.559
HPV39	20	4	14	3	10	0.695	0.793	0.601	0.753

Abbreviations: CIN = cervical intraepithelial neoplasia; hrHPV = high-risk human papillomavirus; κ = kappa.
[a]Frequencies and percentages indicated here include the presence of types both in single and multiple infections; total *n* = 450 hrHPV-positive women; HPV68 was excluded owing to low frequency (positive *n* = 4 in scrapes and *n* = 2 in lavages).
[b]Number of discordant samples between scrape and lavage per single HPV type.

Table 3. Test specifications of *FAM19A4* methylation analysis in cervical scrapes and cervicovaginal lavages for detection of ⩾CIN3 and ⩾CIN2

	Triage marker	*n1/N1*	Sensitivity (95% CI)	*n2/N2*	Specificity (95% CI)	PPV (95% CI)	1-NPV (95% CI)	Referral rate (%)
Total group (*n* = 450)								
⩾CIN3	*FAM19A4* methylation scrape	56/75	74.7% (64.8–84.5)	270/375	72.0% (67.5–76.5)	34.8% (27.4–42.1)	6.6% (3.7–9.4)	35.8
	FAM19A4 methylation lavage	49/75	65.3%[a] (54.6–76.1)	305/375	81.3%[b] (77.4–85.3)	41.2% (32.3–50.0)	7.9% (5.0–10.8)	26.4
⩾CIN2	*FAM19A4* methylation scrape	87/153	56.9% (49.0–64.7)	223/297	75.1% (70.2–80.0)	54.0% (46.3–61.7)	22.8% (18.0–27.7)	35.8
	FAM19A4 methylation lavage	68/153	44.4%[b] (36.6–52.3)	246/297	82.8%[b] (78.5–87.1)	57.1% (48.3–66.0)	25.7% (21.0–30.4)	26.4
Subgroup: women ⩾30 years (*n* = 252)								
⩾CIN3	*FAM19A4* methylation scrape	45/51	88.2% (79.4–97.1)	127/201	63.2% (56.5–69.9)	37.8% (29.1–46.5)	4.5% (1.0–8.0)	47.2
	FAM19A4 methylation lavage	40/51	78.4%[a] (67.1–89.7)	147/201	73.1%[b] (67.0–79.3)	42.6% (32.6–52.5)	7.0% (3.0–10.9)	37.3
⩾CIN2	*FAM19A4* methylation scrape	66/94	70.2% (61.0–79.5)	105/158	66.5% (59.1–73.8)	55.5% (46.5–64.4)	21.1% (14.1–28.0)	47.2
	FAM19A4 methylation lavage	55/94	58.5%[b] (48.6–68.5)	119/158	75.3%[b] (68.6–82.0)	58.5% (48.6–68.5)	24.7% (18.0–31.4)	37.3
Subgroup: women <30 years (*n* = 198)								
⩾CIN3	*FAM19A4* methylation scrape	11/24	45.8% (25.9–65.8)	143/174	82.2% (76.5–87.9)	26.2% (12.9–39.5)	8.3% (4.0–12.7)	21.2
	FAM19A4 methylation lavage	9/24	37.5%[a] (18.1–56.9)	158/174	90.8%[b] (86.5–95.1)	36.0% (17.2–54.8)	8.7% (4.5–12.9)	12.6
⩾CIN2	*FAM19A4* methylation scrape	21/59	35.6% (23.4–47.8)	118/139	84.9% (78.9–90.8)	50.0% (34.9–65.1)	24.4% (17.6–31.1)	21.2
	FAM19A4 methylation lavage	13/59	22.0%[b] (11.5–32.6)	127/139	91.4%[c] (86.7–96.0)	52.0% (32.4–71.6)	26.6% (20.0–33.2)	12.6

Abbreviations: CI = confidence interval; CIN = cervical intraepithelial neoplasia (grade 2 or 3 or higher); HPV = human papillomavirus; *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; 1-NPV = complemented negative predictive value; PPV = positive predictive value.
[a]No significant difference with *FAM19A4* methylation in cervical scrape (in the corresponding category); non-inferiority test not significant.
[b]Significant difference with *FAM19A4* methylation in cervical scrape (in the corresponding category).
[c]Non-inferior to *FAM19A4* methylation in cervical scrape (in the corresponding category).

higher in cervicovaginal lavages (81.3%) compared with that in cervical scrapes (72.0%; ratio 1.13; CI: 1.05–1.21). For ⩾CIN2 outcome in the total study population (Table 3; upper panel), *FAM19A4* methylation analysis had a significantly lower sensitivity in cervicovaginal lavages (44.4%) compared with that in cervical scrapes (56.9%), whereas its specificity in lavages (82.8%) was significantly higher compared with that in scrapes (75.1%; relative specificity 1.10; CI: 1.03–1.18).

Factors influencing the performance of *FAM19A4* methylation analysis: age. The performance of *FAM19A4* methylation analysis was significantly influenced by the age of the participants in both sample types (Supplementary Tables 1A and B). Neither the reason for referral of the participant (abnormal cytology result or other, non-cervix-related, gynaecologic complaints) nor the sampling method of the lavage (self-collected by the participant at home or physician-collected) or scrape (during a separate visit 2–3 weeks before colposcopy or combined with the colposcopy procedure) influenced the performance of *FAM19A4* methylation analysis significantly (Supplementary Tables 1A and B). Given the influence of age, the performance of *FAM19A4* methylation analysis was assessed in subgroups of women 18–30 years (referred to as <30years) and women 30–66 years (referred to as ⩾30 years). Sensitivity, specificity, PPV and complemented NPV of *FAM19A4* methylation analysis in both sample types and in both age strata are shown in Table 3.

Table 4. Test specifications of *FAM19A4* methylation analysis combined with HPV16/18 genotyping in cervical scrapes and cervicovaginal lavages for detection of ⩾CIN3 and ⩾CIN2

	Triage marker	n1/N1	Sensitivity (95% CI)	n2/N2	Specificity (95% CI)	PPV (95% CI)	1-NPV (95% CI)	Referral rate (%)
Total group (*n* = 450)								
⩾CIN3	*FAM19A4* methylation and/or HPV16/18 genotyping scrape	69/75	92.0% (85.9–98.1)	165/375	44.0% (39.0–49.0)	24.7% (19.7–29.8)	3.5% (0.8–6.3)	62.0
	FAM19A4 methylation and/or HPV16/18 genotyping lavage	67/75	89.3%[a] (82.3–96.3)	181/375	48.3%[a] (43.2–53.3)	25.7% (20.4–31.0)	4.2% (1.4–7.1)	58.0
⩾CIN2	*FAM19A4* methylation and/or HPV16/18 genotyping scrape	117/153	76.5% (69.7–83.2)	135/297	45.5% (39.8–51.1)	41.9% (36.1–47.7%)	21.1% (14.9–27.2)	62.0
	FAM19A4 methylation and/or HPV16/18 genotyping lavage	110/153	71.9%[b] (64.8–79.0)	146/297	49.2%[a] (43.5–54.8)	42.1% (36.2–48.1)	22.8% (16.8–28.7)	58.0
Subgroup: women ⩾30 years (*n* = 252)								
⩾CIN3	*FAM19A4* methylation and/or HPV16/18 genotyping scrape	48/51	94.1% (87.7–100.0)	77/201	38.3% (31.6–45.0)	27.9% (21.2–34.6)	3.8% (0.0–7.9)	68.3
	FAM19A4 methylation and/or HPV16/18 genotyping lavage	47/51	92.2%[b] (84.8–99.5)	88/201	43.8%[a] (36.9–50.6)	29.4% (22.3–36.4)	4.3% (0.2–8.5)	63.5
⩾CIN2	*FAM19A4* methylation and/or HPV16/18 genotyping scrape	77/94	81.9% (74.1–89.7)	63/158	39.9% (32.2–47.5)	44.8% (37.3–52.2)	21.3% (12.3–30.2)	68.3
	FAM19A4 methylation and/or HPV16/18 genotyping lavage	73/94	77.7%[b] (69.2–86.1)	71/158	44.9%[b] (37.2–52.7)	45.6% (37.9–53.3)	22.8% (14.2–31.4)	63.5
Subgroup: women <30 years (*n* = 198)								
⩾CIN3	*FAM19A4* methylation and/ or HPV16/18 genotyping scrape	21/24	87.5% (74.3–100.0)	88/174	50.6% (43.1–58.0)	19.6% (12.1–27.2)	3.3% (0.0–7.0%)	54.0
	FAM19A4 methylation and/or HPV16/18 genotyping lavage	20/24	83.3%[b] (68.4–98.2)	93/174	53.4%[b] (46.0–60.9)	19.8% (12.0–27.6%)	4.1% (0.2–8.1)	51.0
⩾CIN2	*FAM19A4* methylation and/or HPV16/18 genotyping scrape	40/59	67.8% (55.9–79.7)	72/139	51.8% (43.5–60.1)	37.4% (28.2–46.6)	20.9% (12.5–29.2)	54.0
	FAM19A4 methylation and/or HPV16/18 genotyping lavage	37/59	62.7%[b] (50.4–75.1)	75/139	54.0%[b] (45.7–62.2)	36.6% (27.2–46.0%)	22.7% (14.3–31.0)	51.0

Abbreviations: CI = confidence interval; CIN = cervical intraepithelial neoplasia (grade 2 or 3 or higher); HPV = human papillomavirus; *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; 1-NPV = complemented negative predictive value; PPV = positive predictive value.

[a]Non-inferior to *FAM19A4* methylation and/or HPV16/18 genotyping in cervical scrape (in the corresponding category).

[b]No significant difference with *FAM19A4* methylation and/or HPV16/18 genotyping in cervical scrape (in the corresponding category); non-inferiority test not significant.

In women ⩾30 years of age ($n = 252$), 51 ⩾CIN3, 43 CIN2 and 158 ⩽CIN1 were present (Table 3; middle panel). In this sub-population, the sensitivity of *FAM19A4* methylation analysis for ⩾CIN3 was lower in cervicovaginal lavages (78.4%) compared with that in cervical scrapes (88.2%). Although this difference was not statistically significant (ratio 0.89; CI: 0.75–1.05), statistical non-inferiority could not be demonstrated ($P = 0.56$). The specificity of *FAM19A4* methylation analysis for ⩾CIN3 was significantly higher in lavages (73.1%) compared with that in scrapes (63.2%; ratio 1.16; CI: 1.03–1.30). For detection of ⩾CIN2 in women aged ⩾30 years, the sensitivity of *FAM19A4* methylation analysis in lavages (58.5%) was lower compared with that in scrapes (70.2%; ratio 0.83; CI: 0.70–0.999), and its specificity in lavages (75.3%) was higher compared with that in scrapes (66.5%; ratio 1.13; CI: 1.01–1.28).

In the subgroup of women <30 years of age ($n = 198$), 24 ⩾CIN3, 35 CIN2 and 139 ⩽CIN1 were present (Table 3, lower panel). The sensitivity of *FAM19A4* methylation analysis for ⩾CIN3 in lavages did not differ significantly from its sensitivity in scrapes (37.5% *vs* 45.8%; ratio 0.82; CI: 0.55–1.21), but statistical non-inferiority could not be established ($P = 0.69$). The specificity of *FAM19A4* methylation analysis for ⩾CIN3 in lavages (90.8%) was significantly higher compared with that in scrapes (82.2%; ratio 1.10; CI: 1.02–1.20). The sensitivity of *FAM19A4* methylation for ⩾CIN2 in lavages was significantly lower in lavages (22.0%) compared with that in scrapes (35.6%; ratio 0.62; CI: 0.40–0.96), whereas the ⩾CIN2 specificity of this marker in lavages (91.4%) was non-inferior to its specificity in scrapes (84.9%; ratio 1.08; CI: 0.99–1.17; non-inferiority test: $P = 0.01$).

The performance of *FAM19A4* methylation analysis combined with HPV16/18 genotyping. Table 4 presents the clinical performance of *FAM19A4* methylation analysis combined with HPV16/18 genotyping for ⩾CIN3 and ⩾CIN2 in both sample types. Compared with *FAM19A4* methylation analysis alone (Table 3), the combined marker panel (Table 4) reached significantly higher ⩾CIN3 and ⩾CIN2 sensitivities, at significantly lower specificities in both sample types and in both age categories. In contrast to *FAM19A4* methylation analysis alone, the performance of combined *FAM19A4* methylation analysis and HPV16/18 genotyping did not differ significantly between cervicovaginal lavages and cervical scrapes (Table 4).

***FAM19A4* methylation analysis in relation to histological severity.** In an attempt to evaluate *FAM19A4* methylation analysis in relation to lesion severity and volume, participants were stratified on the basis of a combination of histological severity and volume of the CIN lesion (Table 1). *FAM19A4* methylation positivity rates in cervicovaginal lavages and cervical scrapes, in relation to this combined classification, are presented in Table 5. In both cervicovaginal lavage samples and cervical scrapes, the percentage of *FAM19A4* methylation-positive cases increased with the lesion severity and volume ($P < 0.001$ for both sample types; Table 5). A similar increase in positivity rate was observed when combining *FAM19A4* methylation analysis and HPV16/18 genotyping, yet at overall higher positivity rates for each volume category (Table 5).

DISCUSSION

This study compared the performance of *FAM19A4* methylation analysis in large series of paired self-collected cervicovaginal lavage samples and physician-taken cervical scrapes for the detection of

Table 5. *FAM19A4* methylation results in cervicovaginal lavages and cervical scrapes in relation to histological results of the biopsy and LLETZ specimen

	FAM19A4 methylation analysis				*FAM19A4* methylation analysis and/or HPV16/18 genotyping			
	Lavage		Scrape		Lavage		Scrape	
Histology and lesion volume	Positive *n*/ total *n*	Percentage	Positive *n*/ total *n*	Percentage	Positive *n*/ total *n*	Percentage	Positive *n*/ total *n*	Percentage
High-volume ⩾CIN3[a]	35/46	76.1	41/46	89.1	45/46	97.8%	45/46	97.8
Low-volume CIN3[b]	14/29	48.3	15/29	51.7	22/29	75.9	24/29	82.8
High-volume CIN2[c]	8/26	30.8	12/26	46.2	15/26	57.7	17/26	65.4
Low-volume CIN2[d]	11/52	21.2	19/52	36.5	28/52	53.8	31/52	59.6
⩽CIN1[e]	51/297	17.2	74/297	24.9	151/297	50.8	162/297	54.5

Abbreviations: CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; LLETZ = large loop excision of the transformation zone. Fisher's exact test $P<0.001$ for both *FAM19A4* methylation analysis and the combination of *FAM19A4* methylation analysis and HPV16/18 genotyping, in both lavages and scrapes.
[a]LLETZ (or conisation/hysterectomy) specimen ⩾CIN3 (including two carcinomas).
[b]Biopsy specimen CIN3, LLETZ specimen ⩽CIN2.
[c]Biopsy specimen ⩽CIN2, LLETZ specimen CIN2.
[d]Biopsy specimen CIN2, LLETZ specimen ⩽CIN1.
[e]Both biopsy specimen and LLETZ specimen (if available) ⩽CIN1.

⩾CIN3 in hrHPV-positive women from a gynaecologic outpatient population. *FAM19A4* methylation analysis on self-collected lavage material had a slightly lower sensitivity and a significantly higher specificity compared with *FAM19A4* methylation analysis on physician-taken scrapes.

This study is the first large prospective multicentre cohort study comparing the performance of methylation marker analysis in hrHPV-positive cervicovaginal lavage samples to cervical scrapes from the same women. The required number of participants with and without ⩾CIN3 lesions in this study was calculated in advance to allow comparison of sensitivity and specificity in both sample types. Until now, limited data comparing methylation marker performance in paired samples were available. The methylation markers studied by Boers *et al* (2014) (*C13ORF18*, *EPB41L3*, *JAM3* and *TERT*) (Boers *et al*, 2014) and Chang *et al* (2015) (*PAX1*, *SOX1* and *ZNF582*) have shown moderate to good concordance in self-samples and cervical scrapes, yet these studies were performed on very small study populations with limited controls and require confirmation from other prospective studies.

In the current study, the differences in clinical performance between the two sample types can most likely be attributed to a different cellular composition and proportion of hypermethylation-positive cervical indicator cells. During cervical scrape collection, a physician will target the transformation zone of the cervix, contributing to a likely large number of cervical indicator cells. In case of self-collection by a lavage device, a more random distribution of vaginal and cervical indicator cells can be expected. Consequently, the lower fraction of hypermethylated indicator cells in self-collected lavages might explain the slightly lower sensitivity and higher specificity of *FAM19A4* methylation analysis.

Our previous studies have shown that *FAM19A4* methylation analysis and cytology on HPV-positive cervical scrapes have a similar ⩾CIN3 sensitivity (De Strooper *et al*, 2014; Luttmer *et al*, 2016). However, *FAM19A4* methylation analysis has proven to be more sensitive for the detection of CIN3 lesions with a longer duration of existence (the so-called advanced lesions) and cervical carcinoma (Luttmer *et al*, 2016). An advantage of the present study is the availability of both biopsy and LLETZ histological results from all participants with a high-grade lesion enabling a semiquantitative assessment of lesion volume. We showed that in both hrHPV-positive cervicovaginal lavages and cervical scrapes, *FAM19A4* methylation-positivity increases with the volume of high-grade CIN lesions. These results support the concept that *FAM19A4* methylation analysis has a preference for detecting the larger, probably more advanced CIN lesions (De Strooper *et al*, 2014). Of note, combined *FAM19A4* methylation analysis and HPV16/18 genotyping detected all but one high-volume ⩾CIN3 lesions in both cervical scrapes and cervicovaginal lavages.

As expected, and found previously (Verhoef *et al*, 2014b; De Strooper *et al*, 2016), the addition of HPV16/18 genotyping to *FAM19A4* methylation analysis yielded a significant increase in sensitivity at the cost of a marked decrease in specificity. Of interest, whereas *FAM19A4* methylation analysis alone was found to be slightly less sensitive and more specific in the cervicovaginal lavages compared with the cervical scrapes, no significant differences were found for the marker combining *FAM19A4* methylation analysis and HPV16/18 genotyping. Thus, the combination of *FAM19A4* methylation analysis with HPV16/18 genotyping might be a safe alternative triage strategy that performs similarly on both sample types.

In line with earlier studies (Luttmer *et al*, 2016), our data showed a significant influence of age on *FAM19A4* methylation positivity in both cervical scrapes and cervicovaginal lavage material. Accordingly, in young women, *FAM19A4* methylation analysis results in quite low ⩾CIN2/3 sensitivities of only 37.5–45.8%, at relatively high specificities of 82.2–90.0%. In young women, hrHPV prevalence is known to be high, but the majority of infections are transient and most lesions, also a substantial part of CIN3 lesions, tend to regress spontaneously (Winer *et al*, 2003; Insinga *et al*, 2010; Jaisamrarn *et al*, 2013), contributing to a very low cancer incidence in this age category (Benard *et al*, 2012). As *FAM19A4* methylation analysis has previously shown to preferably detect advanced CIN lesions and cervical cancer (De Strooper *et al*, 2014; Steenbergen *et al*, 2014), this lower sensitivity has the clinical benefit that it could protect young women from overtreatment. This is particularly relevant for women in their reproductive age as treatment may lead to adverse pregnancy outcome.

Owing to the selection of an outpatient population, the translation of our results into screening settings should be handled with care. Further confirmation in population-based screening trials is required as the percentage of HPV-positive women with ⩾CIN3 lesions in a screening population is lower compared with that in a gynaecologic referral population. Another limitation of our study is the relatively large number of samples with an invalid test result for *FAM19A4* methylation analysis of 8% in the cervical scrapes and 9% in the cervicovaginal lavage samples. These invalid test results on the cervical scrapes might result from cautious scraping during colposcopy to prevent bleeding and thus poor

visualisation, leading to low DNA concentrations (Overmeer *et al*, 2011). Indeed, 88.4% of the invalid results were found in scrapes that were collected directly before colposcopy, a procedure that is not likely to be applied in a routine cervical screening setting. Also in self-samples, the number of cells can be limited or the sample can be of poor quality, for example, by collection or storage failures. This problem can be circumvented by using a larger fraction of the scrape or self-sample in the DNA isolation procedure, or by decreasing the elution volume during isolation. Given the study setting aiming to evaluate different triage strategies, leftover sample material was not always sufficient to perform repeat methylation analysis. In routine setting, this limitation will likely not be applicable, as also supported by the limited occurrence of methylation-invalid samples in a randomised controlled trial in screening setting (Verhoef *et al*, 2014a).

In conclusion, *FAM19A4* methylation marker analysis in hrHPV-positive self-collected lavage samples had a slightly lower sensitivity and a higher specificity for $\geqslant$CIN3 compared with *FAM19A4* methylation analysis in paired physician-taken scrapes. Combined *FAM19A4* methylation analysis and HPV16/18 genotyping revealed a similarly good clinical performance, which was similar in both sample types. Therefore, this combination could provide a feasible triage strategy for hrHPV-positive women, with the advantage of direct applicability on self-collected material.

ACKNOWLEDGEMENTS

This study was supported by grants from the Dutch Cancer Society (KWF2014-7238 awarded to DAMH, PJFS and CJLMM), the European Research Council (ERC advanced 2012-AdG, proposal 322986 Mass-Care) awarded to CJLMM and the Seventh Framework Program of DG Research of the European Commission (CoheaHr-Health-F3-2013-603019) awarded to CJLMM and JB. We thank the women who participated in this study. In addition, we thank the technicians and research staff of the unit of Molecular Pathology of the Department of Pathology at the VU University Medical Center, especially Helma de Bruin, Marjolein Bekker-Lettink and Martijn Bogaarts. We also thank the administrative workers and information technology team of the Department of Pathology at the VU University Medical Center for the supportive work. We acknowledge the gynaecologists, nurses and administrative workers of the Gynaecology Departments of the VU University Medical Center, University Medical Center Utrecht, Onze Lieve Vrouwe Gasthuis West, Reinier de Graaf Group, Sint Antonius Hospital and Flevo Hospital for their contribution.

CONFLICT OF INTEREST

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. PJFS has been on the speakers bureau of Roche, Qiagen, Abbott, Gen-Probe and Seegene. PJFS is consultant for Crucell Holland BV. TJMH, RHMV and WAH have been principal investigators of a GlaxoSmithKline sponsored study. WGVQ is a minority shareholder of Diassay BV and obtained grants from GlaxoSmithKline. DAMH has been on the speakers bureau of Hologic/Gen-Probe and serves occasionally on the scientific advisory boards of AMGEN and Pfizer. CJLMM has been on the sponsored speakers bureau of GlaxoSmithKline, Qiagen, Merck, Roche, Menarini and Segeene, and served on the scientific advisory board of GlaxoSmithKline, Qiagen, Merck and Roche. CJLMM has been consultant for Qiagen and Genticel and is a minority shareholder of Diassay BV. Formerly, CJLMM was a minority shareholder of Delphi Biosciences. CJLMM, PJFS, RDMS and DAMH have minority stake in Self-screen BV, a spin-off company of VU University Medical Center Amsterdam, which holds patents related to the present work. RL, LMADS, MGD, FJvK, LR, WMvB, GCMG, JWMS and DKEvD do not have any conflict of interest to declare.

REFERENCES

Arbyn M, Verdoodt F, Snijders PJF, Verhoef VMJ, Suonio E, Dillner L, Minozzi S, Bellisario C, Banzi R, Zhao F-H, Hillemanns P, Anttila A (2014) Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis. *Lancet Oncol* **15**: 172–183.

Benard VB, Watson M, Castle PE, Saraiya M (2012) Cervical carcinoma rates among young females in the United States. *Obstet Gynecol* **120**: 1117–1123.

Boers A, Bosgraaf RP, van Leeuwen RW, Schuuring E, Heideman DAM, Massuger LFAG, Verhoef VMJ, Bulten J, Melchers WJG, van der Zee AGJ, Bekkers RLM, Wisman GBA (2014) DNA methylation analysis in self-sampled brush material as a triage test in hrHPV-positive women. *Br J Cancer* **111**: 1095–1101.

Chang C-C, Huang R-L, Liao Y-P, Su P-H, Hsu Y-W, Wang H-C, Tien C-Y, Yu M-H, Lin Y-W, Lai H-C (2015) Concordance analysis of methylation biomarkers detection in self-collected and physician-collected samples in cervical neoplasm. *BMC Cancer* **15**: 418.

Cox JT, Castle PE, Behrens CM, Sharma A, Wright TC, Cuzick J (2013) Comparison of cervical cancer screening strategies incorporating different combinations of cytology, HPV testing, and genotyping for HPV 16/18: results from the ATHENA HPV study. *Am J Obstet Gynecol* **208**: 184.e1–e184.e11.

De Strooper LMA, Meijer CJLM, Berkhof J, Hesselink AT, Snijders PJF, Steenbergen RDM, Heideman DAM (2014) Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. *Cancer Prev Res (Phila)* **7**: 1251–1257.

De Strooper LMA, Verhoef VMJ, Berkhof J, Hesselink AT, de Bruin HM, van Kemenade FJ, Bosgraaf RP, Bekkers RLM, Massuger LFAG, Melchers WJG, Steenbergen RDM, Snijders PJF, Meijer CJLM, Heideman DAM (2016) Validation of the FAM19A4/mir124-2 DNA methylation test for both lavage- and brush-based self-samples to detect cervical (pre)cancer in HPV-positive women. *Gynecol Oncol* **141**(2): 341–347.

Dijkstra M, van Niekerk D, Rijkaart D, van Kemenade FJ, Heideman DAM, Snijders PJF, Meijer CJ, Berkhof J (2014) Primary hrHPV DNA testing in Cervical Cancer screening: how to manage screen positive women? A POBASCAM Trial sub study. *Cancer Epidemiol Biomarkers Prev* **23**: 55–63.

Dillner J (2013) Primary human papillomavirus testing in organized cervical screening. *Curr Opin Obstet Gynecol* **25**: 11–16.

Eijsink JJH, Lendvai Á, Deregowski V, Klip HG, Verpooten G, Dehaspe L, de Bock GH, Hollema H, van Criekinge W, Schuuring E, van der Zee AG, Wisman GB (2012) A four-gene methylation marker panel as triage test in high-risk human papillomavirus positive patients. *Int J Cancer* **130**: 1861–1869.

Eijsink JJH, Yang N, Lendvai A, Klip HG, Volders HH, Buikema HJ, van Hemel BM, Voll M, Coelingh Bennink HJT, Schuuring E, Wisman GB, van der Zee AG (2011) Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study. *Gynecol Oncol* **120**: 280–283.

Garcia F, Barker B, Santos C, Brown EM, Nuño T, Giuliano A, Davis J (2003) Cross-sectional study of patient- and physician-collected cervical cytology and human papillomavirus. *Obstet Gynecol* **102**: 266–272.

Hesselink AT, Heideman DAM, Steenbergen RDM, Coupé VMH, Overmeer RM, Rijkaart D, Berkhof J, Meijer CJLM, Snijders PJF (2011) Combined promoter methylation analysis of CADM1 and MAL: an objective triage tool for high-risk human papillomavirus DNA-positive women. *Clin Cancer Res* **17**: 2459–2465.

Hesselink AT, Heideman DAM, Steenbergen RDM, Gök M, van Kemenade FJ, Wilting SM, Berkhof J, Meijer CJLM, Snijders PJF (2014) Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. *Int J Cancer* **135**: 880–886.

Huang T-H, Lai H-C, Liu H-W, Lin CJ, Wang K-H, Ding D-C, Chu T-Y (2010) Quantitative analysis of methylation status of the PAX1 gene for detection of cervical cancer. *Int J Gynecol Cancer* **20**: 513–519.

Insinga RP, Perez G, Wheeler CM, Koutsky LA, Garland SM, Leodolter S, Joura EA, Ferris DG, Steben M, Brown DR, Elbasha EH, Paavonen J, Haupt RM (2010) Incidence, duration, and reappearance of type-specific cervical human papillomavirus infections in young women. *Cancer Epidemiol Biomarkers Prev* **19**: 1585–1594.

Jacobs MV, Snijders PJ, van den Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM (1997) A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* **35**: 791–795.

Jaisamrarn U, Castellsagué X, Garland SM, Naud P, Palmroth J, Del Rosario-Raymundo MR, Wheeler CM, Salmerón J, Chow S-N, Apter D, Teixeira JC, Skinner SR, Hedrick J, Szarewski A, Romanowski B, Aoki FY, Schwarz TF, Poppe WAJ, Bosch FX, de Carvalho NS, Germar MJ, Peters K, Paavonen J, Bozonnat M-C, Descamps D, Struyf F, Dubin GO, Rosillon D, Baril L (2013) Natural history of progression of HPV infection to cervical lesion or clearance: analysis of the control arm of the large, randomised PATRICIA study. *PLoS One* **8**: e79260.

Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. *Biometrics* **33**: 159–174.

Luttmer R, De Strooper LMA, Berkhof J, Snijders PJF, Dijkstra MG, Uijterwaal MH, Steenbergen RDM, Kemenade FJ, Van, Rozendaal L, Helmerhorst TJM, Verheijen RHM, Harmsel WA, Ter, Baal WM, Van, Heideman AM, Meijer CJLM (2016) 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). *Int J Cancer* **138**: 992–1002.

Meijer CJLM, Berkhof J, Castle PE, Hesselink AT, Franco EL, Ronco G, Arbyn M, Bosch FX, Cuzick J, Dillner J, Heideman DAM, Snijders PJF (2009) Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. *Int J Cancer* **124**: 516–520.

Moriarty AT, Nayar R, Arnold T, Gearries L, Renshaw A, Thomas N, Souers R (2014) The Tahoe Study: bias in the interpretation of Papanicolaou test results when human papillomavirus status is known. *Arch Pathol Lab Med* **138**: 1182–1185.

Nobbenhuis MAE, Helmerhorst TJM, van den Brule AJ, Rozendaal L, Jaspars LH, Voorhorst FJ, Verheijen RHM, Meijer CJLM (2002) Primary screening for high risk HPV by home obtained cervicovaginal lavage is an alternative screening tool for unscreened women. *J Clin Pathol* **55**: 435–439.

Ogilvie GS, Patrick DM, Schulzer M, Sellors JW, Petric M, Chambers K, White R, FitzGerald JM (2005) Diagnostic accuracy of self collected vaginal specimens for human papillomavirus compared to clinician collected human papillomavirus specimens: a meta-analysis. *Sex Transm Infect* **81**: 207–212.

Overmeer RM, Henken FE, Bierkens M, Wilting SM, Timmerman I, Meijer CJLM, Snijders PJF, Steenbergen RDM (2009) Repression of MAL tumour suppressor activity by promoter methylation during cervical carcinogenesis. *J Pathol* **219**: 327–336.

Overmeer RM, Henken FE, Snijders PJF, Claassen-Kramer D, Berkhof J, Helmerhorst TJM, Heideman DAM, Wilting SM, Murakami Y, Ito A, Meijer CJLM, Steenbergen RDM (2008) Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J Pathol* **215**: 388–397.

Overmeer RM, Louwers JA, Meijer CJLM, van Kemenade FJ, Hesselink AT, Daalmeijer NF, Wilting SM, Heideman DAM, Verheijen RHM, Zaal A, van Baal WM, Berkhof J, Snijders PJF, Steenbergen RDM (2011) Combined CADM1 and MAL promoter methylation analysis to detect (pre-)malignant cervical lesions in high-risk HPV-positive women. *Int J cancer* **129**: 2218–2225.

Rijkaart DC, Berkhof J, van Kemenade FJ, Coupé VMH, Hesselink AT, Rozendaal L, Heideman DAM, Verheijen RH, Bulk S, Verweij WM, Snijders PJF, Meijer CJLM (2012) Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *Int J Cancer* **130**: 602–610.

Ronco G, Dillner J, Elfström KM, Tunesi S, Snijders PJF, Arbyn M, Kitchener H, Segnan N, Gilham C, Giorgi-Rossi P, Berkhof J, Peto J, Meijer CJLM (2014) Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet* **383**: 524–532.

Saavedra KF, Brebi PS, Roa JC (2012) Epigenetics alterations in preneoplastic and neoplastic lesions of the cervix. *Clin Epigenet* **4**: 13.

Schmitt M, Bravo IG, Snijders PJF, Pawlita M, Waterboer T, Gissmann L (2006) Bead-based multiplex genotyping of human papillomaviruses bead-based multiplex genotyping of human papillomaviruses. *J Clin Microbiol* **44**: 504–512.

Snijders PJF, Steenbergen RDM, Heideman DAM, Meijer CJLM (2006) HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* **208**: 152–164.

Snijders PJF, Verhoef VMJ, Arbyn M, Ogilvie G, Minozzi S, Banzi R, van Kemenade FJ, Heideman DAM, Meijer CJLM (2013) High-risk HPV testing on self-sampled versus clinician-collected specimens: a review on the clinical accuracy and impact on population attendance in cervical cancer screening. *Int J Cancer* **132**: 2223–2236.

Steenbergen RDM, Kramer D, Braakhuis BJM, Stern PL, Verheijen RHM, Meijer CJLM, Snijders PJF (2004) TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. *J Natl Cancer Inst* **96**: 294–305.

Steenbergen RDM, Ongenaert M, Snellenberg S, Trooskens G, van der Meide WF, Pandey D, Bloushtain-Qimron N, Polyak K, CJLM Meijer, Snijders PJF, Van Criekinge W (2013) Methylation-specific digital karyotyping of HPV16E6E7 expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. *J Pathol* **231**: 53–62.

Steenbergen RDM, Snijders PJF, Heideman DAM, Meijer CJLM (2014) Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat Rev Cancer* **14**: 395–405.

Tang N-S, Tang M-L, Chan ISF (2003) On tests of equivalence via non-unity relative risk for matched-pair design. *Stat Med* **22**: 1217–1233.

Verhoef VMJ, Bosgraaf RP, van Kemenade FJ, Rozendaal L, Heideman DAM, Hesselink AT, Bekkers RLM, Steenbergen RDM, Massuger LFAG, Melchers WJG, Bulten J, Overbeek LIH, Berkhof J, Snijders PJF, Meijer CJLM (2014a) Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): a randomised controlled non-inferiority trial. *Lancet Oncol* **15**: 315–322.

Verhoef VMJ, Heideman DAM, van Kemenade FJ, Rozendaal L, Bosgraaf RP, Hesselink AT, Bekkers RLM, Massuger LFAG, Steenbergen RDM, Snijders PJF, Berkhof J, Meijer CJLM (2014b) Methylation marker analysis and HPV16/18 genotyping in high-risk HPV positive self-sampled specimens to identify women with high grade CIN or cervical cancer. *Gynecol Oncol* **135**: 58–63.

Wilting SM, van Boerdonk RAA, Henken FE, CJLM Meijer, Diosdado B, Meijer GA, le Sage C, Agami R, Snijders PJF, Steenbergen RDM (2010) Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol Cancer* **9**: 167.

Winer RL, Lee S-K, Hughes JP, Adam DE, Kiviat NB, Koutsky LA (2003) Genital human papillomavirus infection: incidence and risk factors in a cohort of female university students. *Am J Epidemiol* **157**: 218–226.

Zorzi M, Del Mistro A, Farruggio A, de'Bartolomeis L, Frayle-Salamanca H, Baboci L, Bertazzo A, Cocco P, Fedato C, Gennaro M, Marchi N, Penon MG, Cogo C, Ferro A (2013) Use of a high-risk human papillomavirus DNA test as the primary test in a cervical cancer screening programme: a population-based cohort study. *BJOG* **120**: 1260–1267; discussion 1267–1268.

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 4.0 Unported License.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)