Clinical Infectious Diseases
MAJOR ARTICLE

FAM19A4/miR124-2 Methylation Testing and Human Papillomavirus (HPV) 16/18 Genotyping in HPV-Positive Women Under the Age of 30 Years

Frederique J. Vink,[1,2] Chris J. L. M. Meijer,[1,2] Albertus T. Hesselink,[3] Arno N. Floore,[3] Birgit I. Lissenberg-Witte,[4] Jesper H. Bonde,[5] Helle Pedersen,[5] Kate Cuschieri,[6,7] Ramya Bhatia,[6,7] Mario Poljak,[8] Anja Oštrbenk Valenčak,[8] Peter Hillemanns,[9] Wim G. V. Quint,[10] Marta del Pino,[11] Gemma G. Kenter,[12] Renske D. M. Steenbergen,[1,2] Daniëlle A. M. Heideman,[1,2] and Maaike C. G. Bleeker[1,2]

[1]Amsterdam University Medical Center, Vrije Universiteit Amsterdam, Pathology, Amsterdam, the Netherlands; [2]Imaging and Biomarkers, Cancer Center Amsterdam, Amsterdam, The Netherlands; [3]Self-screen B.V., Amsterdam, The Netherlands; [4]Amsterdam University Medical Center, Vrije Universiteit Amsterdam, Epidemiology and Data Science, Amsterdam, the Netherlands; [5]Molecular Pathology Laboratory, Department of Pathology, Copenhagen University Hospital, Hvidovre Hospital, Hvidovre, Denmark; [6]Scottish HPV Reference Laboratory, Royal Infirmary of Edinburgh, NHS Lothian, Edinburgh, Scotland; [7]Centre for Reproductive Health, University of Edinburgh, Edinburgh, Scotland; [8]Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [9]Department of Obstetrics and Gynaecology, Hannover Medical School, Hannover, Germany; [10]DDL Diagnostic Laboratory, Rijswijk, The Netherlands; [11]Institut Clinic of Gynecology, Obstetrics and Neonatology, Gynecology Oncology Unit, Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer, Faculty of Medicine, University of Barcelona, Barcelona, Spain; and [12]Center for Gynecologic Oncology Amsterdam, Amsterdam University Medical Center and Netherlands Cancer Institute, Amsterdam, The Netherlands

Background. High-grade squamous intraepithelial lesions (HSIL) or cervical intraepithelial neoplasia (CIN) grade 2/3 lesions in human papillomavirus (HPV)–positive women <30 years of age have high spontaneous regression rates. To reduce overtreatment, biomarkers are needed to delineate advanced CIN lesions that require treatment. We analyzed the *FAM19A4/miR124-2* methylation test and HPV16/18 genotyping in HPV-positive women aged <30 years, aiming to identify CIN2/3 lesions in need of treatment.

Methods. A European multicenter retrospective study was designed evaluating the *FAM19A4/miR124-2* methylation test and HPV16/18 genotyping in cervical scrapes of 1061 HPV-positive women aged 15–29 years (690 ≤CIN1, 166 CIN2, and 205 CIN3+). A subset of 62 CIN2 and 103 CIN3 were immunohistochemically characterized by HPV E4 expression, a marker for a productive HPV infection, and $p16^{ink4a}$ and Ki-67, markers indicative for a transforming infection. CIN2/3 lesions with low HPV E4 expression and high $p16^{ink4a}$/Ki-67 expression were considered as nonproductive, transforming CIN, compatible with advanced CIN2/3 lesions in need of treatment.

Results. *FAM19A4/miR124-2* methylation positivity increased significantly with CIN grade and age groups (<25, 25–29, and ≥30 years), while HPV16/18 positivity was comparable across age groups. *FAM19A4/miR124-2* methylation positivity was HPV type independent. Methylation-positive CIN2/3 lesions had higher $p16^{ink4a}$/Ki-67-immunoscores ($P = .003$) and expressed less HPV E4 ($P = .033$) compared with methylation-negative CIN2/3 lesions. These differences in HPV E4 and $p16^{ink4a}$/Ki-67 expression were not found between HPV16/18–positive and non-16/18 HPV–positive lesions.

Conclusions. Compared with HPV16/18 genotyping, the *FAM19A4/miR124-2* methylation test detects nonproductive, transforming CIN2/3 lesions with high specificity in women aged <30 years, providing clinicians supportive information about the need for treatment of CIN2/3 in young HPV-positive women.

Keywords. host cell DNA methylation; cervical cancer; human papillomavirus; cervical intraepithelial neoplasia; HPV E4; $p16^{ink4a}$/Ki-67 immunoscore.

Human papillomavirus (HPV) infections are very common in young women aged <30 years [1–4]. Although HPV infections are often transient and many associated cervical intraepithelial neoplasia (CIN) lesions resolve spontaneously, surgical treatment of women with CIN2/3 or high-grade squamous intraepithelial lesions (HSIL) is very common [5–7]. The regression rate of CIN2 lesions is estimated up to 50%, while data in young women aged <30 years show even higher rates of regression [8, 9]. Consequently, cytology and/or HPV screening in young women often results in the detection of regressive CIN2/3 cervical lesions and unnecessary treatments [10]. On the other hand, recent data show how young women would benefit from HPV screening in terms of better cancer protection [11]. Improved triaging biomarkers are therefore needed to identify HPV-positive young women with advanced CIN lesions in need of treatment.

Received 24 February 2022; editorial decision 20 May 2022; published online 10 June 2022

Correspondence: M. C. G. Bleeker, Amsterdam UMC, Vrije Universiteit Amsterdam, Pathology, Postbus 7057, 1007 MB, Amsterdam, The Netherlands (mcg.bleeker@amsterdamumc.nl).

Clinical Infectious Diseases® **2023;76(3):e827–e34**

© The Author(s) 2022. Published by Oxford University Press on behalf of the Infectious Diseases Society of America.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (https://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

https://doi.org/10.1093/cid/ciac433

Hypermethylation of tumor suppressor genes is seen as a hallmark of cervical carcinogenesis. Recent clinical studies showed that the CE-In Vitro Diagnostic marked and standardized *FAM19A4/miR124-2* methylation test detects virtually all cervical cancers (>98%) [12] and reliably detects advanced CIN lesions [13]. Advanced CIN lesions were earlier defined as CIN2/3 lesions with a cancer-like methylation profile that are associated with a longstanding HPV infection and are presumed to have a high short-term cancer progression risk [12, 13]. In women aged ≥30 years, this test showed a 77% sensitivity for CIN3 detection [14]. Finally, in a prospective clinical cohort study, absence of *FAM19A4/miR124-2* methylation is associated with a high regression rate of CIN2/3 lesions [15, 16]. HPV16/18 genotyping is a currently recommended triage strategy based on the understanding that carcinogenic potential differs between different HPV types [17, 18].

An earlier study reported that *FAM19A4* methylation testing resulted in a relatively low positivity rate for CIN2/3 in young HPV-positive women aged <30 years compared with women ≥30 years, while the positivity rate of HPV16/18 genotyping remained similar [19]. From these data we hypothesized that in this young age group the *FAM19A4/miR124-2* methylation test could better (ie, with a higher specificity) reassure against the above-described advanced CIN lesions in need of treatment in comparison to HPV16/18 genotyping. In this study, we evaluate the *FAM19A4/miR124-2* methylation test and HPV16/18 genotyping in a large European multicenter cohort of HPV-positive women aged <30 years, and relate findings to underlying histology.

METHODS

Study Design and Cohort

A multicenter retrospective study was designed within the VALID-SCREEN (European Union Horizon 2020) framework to evaluate the *FAM19A4/miR124-2* methylation test in cervical scrapes from HPV-positive women aged <30 years and relate findings to histologic outcome [12, 14, 20–22]. Samples were derived from cervical screening and referral settings from Scotland, Slovenia, Denmark, Germany, Spain, and the Netherlands. Samples were selected ensuring inclusion of a sufficient amount of CIN2/3 lesions (for details, see the Supplementary Materials). Data on cytology, HPV status, and local pathology diagnoses were provided by the parent institutes. Sample collection media, HPV screening assay, and DNA extraction for methylation testing are summarized in Supplementary Table 1.

Inclusion criteria were (1) HPV-positive cervical scrapes from women <30 years of age derived from a screening or outpatient population; (2) adequate baseline cytology and HPV test results obtained with a clinically validated test; and (3) containing sufficient cytology material for methylation analysis.

Molecular Analysis

All partners used clinically validated HPV DNA assays to determine HPV status and partial HPV genotyping [23]. *FAM19A4/miR124-2* methylation analysis and sample preprocessing (ie, DNA extraction and bisulphite conversion) was performed locally with cytology specimens as described previously [20]. The EZ DNA Methylation Kit was used for bisulphite conversion according to the manufacturer's specifications (Zymo Research, Irvine, California) with a standard DNA input of 250 ng. For samples with insufficient DNA yield to accomplish an input of 250 ng, a minimal input of 100 ng was used. Bisulphite-converted DNA was subsequently used as input for quantitative methylation-specific polymerase chain reaction analysis of the *FAM19A4* and *miR124-2* genes using the QIAsure Methylation Test (Qiagen, Hilden, Germany). The QIAsure Methylation Test has been evaluated in several European study cohorts and no indication of influence of ethnicity on performance of the assay was found [14, 24–26]. In all laboratories, the assay was performed on a Rotor-Gene Q mdx 5plex HRM instrument (Qiagen). The housekeeping gene β-actin (*ACTB*) was used as a reference to assure successful bisulphite conversion, sample quality, and normalization. Methylation status was labeled positive if the QIAsure Methylation Test result exceeded the preset ΔΔ cycle threshold (Ct) value threshold for methylation positivity for *FAM19A4* and/or *miR124-2* according to the manufacturer's instructions.

Immunohistochemistry

For a subset of 175 women diagnosed with CIN2/3 lesions (62 CIN2, 113 CIN3), formalin-fixed, paraffin-embedded (FFPE) tissue blocks were available for further characterization by $p16^{ink4a}$, Ki-67, and HPV E4 immunohistochemistry. HPV E4 is a marker for a productive HPV infection that may give rise to mild or moderate cellular abnormalities usually regressing spontaneously within 1–2 years [27, 28]. $P16^{ink4a}$ is a marker for a transforming HPV infection and Ki-67 is a cell cycle activity marker [29], both widely used to guide CIN grading by pathologists.

Serial sections of 3 µm were cut from all available tissue blocks. Sections were stained with mouse monoclonal antibodies (mAbs) against Ki-67 antigen (Clone MIB-1, DAKO, Agilent Technologies, Santa Clara, California), or $p16^{ink4a}$ antigen (Clone E6H4, CINtec, Roche, Basel, Switzerland) by the automated IHC Ventana staining machine (Ventana Medical Systems, Roche, Oro Valley, Arizona). Sections were also stained with the mAb panHPVE4 (developed in the laboratory of J. Doorbar, Cambridge, England, available through Labo Bio-medical Products B.V., Rijswijk, The Netherlands), as described previously [30, 31].

Two expert pathologists who were blinded to HPV genotyping and methylation results independently rendered a $p16^{ink4a}$ score (0–3), Ki-67 score (0–3), and HPV E4 score, as described

previously [21, 30]. The cumulative score of Ki-67 and p16^{ink4a} (ranging from 0 to 6) was referred to as the "immunoscore." Immunoscore 0–4 is considered a low to intermediate score, and immunoscore 5–6 is considered a high score. Membranous and/or cytoplasmic HPV E4 staining was scored as either negative (score 0), focally positive (ie, limited staining of some cells restricted to the superficial layer of the epithelium, score 1), or extensively positive (ie, widespread positive staining in the superficial layers of the epithelium extending to half of the epithelial width, score 2). For dichotomous scoring, only extensive HPV E4 staining was considered HPV E4 positive.

Study Endpoints

Cytological specimens were classified according to the Bethesda classification as no evidence of intraepithelial lesion or malignancy (NILM), atypical squamous cells with undetermined significance or cannot exclude high-grade lesion (ASC-US/ASC-H), low-grade squamous intraepithelial lesion (LSIL), and HSIL [32]. All histological specimens were classified as no dysplasia, CIN1, CIN2, CIN3, or cancer according to international criteria by local pathology departments [33]. To study the age trends of *FAM19A4/miR124-2* methylation test and HPV16/18 genotyping in cervical scrapes and immunohistochemical p16^{ink4a}, Ki-67, and E4 expression, a reference population of 2264 women aged ≥30 years of the same European multicenter study was used (reference population 1) [14, 21]. Moreover, 12 cervical squamous cell carcinomas or adenocarcinomas from women aged 25–29 years, of which the corresponding cervical scrape was tested for *FAM19A4/miR124-2* methylation [12], was used to enable comparison of methylation levels between CIN3 and cervical cancer (reference population 2).

Data and Statistical Analysis

Log_{10}-transformed Ct ratios were visualized in boxplots. Trends in *FAM19A4/miR124-2* methylation and HPV16/18 genotyping positivity rates among age groups were evaluated with a χ^2 test for trend, separately for different histological outcomes. Methylation and HPV16/18 genotyping positivity rates among disease categories were compared using McNemar test. The Kruskal-Wallis omnibus test was performed on each methylated gene to assess differences in methylation levels among disease categories. Following a significant result from the omnibus test, post hoc testing was performed using Mann-Whitney *U* test. Bonferroni correction was used to correct *P* values for multiple testing. The associations between HPV16/18 genotyping, methylation, and the p16^{ink4a}/Ki-67 immunoscore and E4 immunohistochemistry were corrected for CIN grade using a Mantel-Haenszel analysis. A *P* value of .05 was considered statistically significant. All statistical analyses were performed using SPSS version 26.0 software (IBM, Armonk, New York).

RESULTS

Study Population

In total, 1061 cervical scrapes from HPV-positive women were evaluated in this study (Supplementary Table 1). Mean age of women was 25.3 years (range, 15–29 years). Cytology data were the following: 211 women with NILM, 184 women with ASC-US, 74 women with ASC-H, 256 women with LSIL, and 336 women with HSIL. In total, 385 women had no histology endpoint, 163 women had no CIN, 142 women had CIN1, 166 women had CIN2, 204 women had CIN3, and 1 woman had cervical squamous cell carcinoma.

Methylation Analysis and HPV16/18 Genotyping

Table 1 shows *FAM19A4/miR124-2* methylation test and HPV16/18 positivity rates stratified for histology and age. Reference population 1 (women ≥30 years) is added to this table to compare age-related trends. While *FAM19A4/miR124-2* methylation test positivity increased significantly from women <25 years, to women 25–29 years and women ≥30 years in both CIN2 and CIN3 (both $P < .001$), no significant differences in HPV16/18 positivity rates between women <25 years, 25–29 years, and ≥30 years were found in either CIN2 or CIN3. Supplementary Figure 1 shows methylation levels of *FAM19A4* and *miR124-2* genes, stratified for age groups of women <25 years, women 25–29 years, and the reference populations 1 (women ≥30 years) and 2 (women 25–29 years with cervical cancer). Although not significant for every age category, a trend is seen that for CIN2 and CIN3, methylation levels increase with age.

Table 2 shows the correlation between HPV16/18 genotyping and methylation analysis. Stratified for histological disease category, methylation positivity rates are similar in cervical scrapes of women being HPV16/18–positive vs non-16/18 HPV–positive ($P = .253$), indicating that methylation positivity in women <30 years is HPV type independent.

Immunohistochemistry

Table 3 shows cumulative p16^{ink4a}/Ki-67 immunoscores stratified for methylation status or HPV16/18 status. Corrected for disease category, methylation-negative CIN2 and CIN3 lesions had significantly lower p16^{ink4a}/Ki-67 immunoscores compared with methylation-positive CIN2 and CIN3 lesions ($P = .003$). No difference in p16^{ink4a}/Ki-67 immunoscores in HPV16/18–positive CIN2 and CIN3 lesions vs non-16/18 HPV–positive CIN2 and CIN3 lesions was found ($P = .822$). Similarly, Table 4 shows that stratified for CIN grade, methylation-negative CIN2 and CIN3 lesions were significantly more often E4-positive compared with methylation-positive

Table 1. *FAM19A4/miR124-2* Methylation and Human Papillomavirus 16/18 Detection Rates per Histology Category, Stratified by Age Group

	Age Group												
	<25 y			25–29 y			Overall <30 y			Reference Population 1 ≥30 y[a]			
Histology	No.	(%)	Total	No.	(%)	Total	No.	(%)	Total	No.	(%)	Total	*P* Value[b]
FAM19A4/miR124-2 methylation positivity rate													
≤CIN1	60	(18)	330	86	(24)	360	146	(21)	690	418	(22)	1884	.236
CIN2	6	(12)	51	40	(35)	115	46	(28)	166	57	(48)	120	.000
CIN3	14	(40)	35	112	(66)	169	126	(62)	204	171	(77)	222	.000
Carcinoma	0	(0)	0	1	(100)	1	1	(100)	1	19	(95)	20	NA
HPV16/18 positivity rate													
≤CIN1	47	(14)	330	104	(29)	360	151	(22)	690	542	(29)	1884	.000
CIN2	22	(43)	51	56	(49)	115	78	(47)	166	60	(50)	120	.449
CIN3	20	(57)	35	101	(60)	169	121	(59)	204	143	(64)	222	.268
Carcinoma	0	(0)	0	1	(100)	1	1	(100)	1	18	(90)	20	NA

The ≤CIN1 category consists of the no histology, CIN0, and CIN1 groups.

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; NA, not applicable.

[a]Reference population 1 of women aged ≥30 years of the same European multicenter study was included [14, 27].

[b]Trends in *FAM19A4/miR124-2* methylation and HPV16/18 genotyping positivity rates among age groups (<25 years, 25–29 years, and ≥30 years) were evaluated with a χ^2 test for trend.

CIN2 and CIN3 lesions, respectively ($P = .033$). No difference in E4 expression in HPV16/18–positive CIN2 and CIN3 lesions vs non-16/18 HPV–positive CIN2 and CIN3 lesions was found ($P = .588$).

Figure 1 presents proportions of women based on their *FAM19A4/miR124-2* methylation test results and p16[ink4a]/Ki-67 immunoscores and their *FAM19A4/miR124-2* methylation test and E4 results, stratified for histology. Women from reference population 1 (women aged ≥30 years) for whom immunohistochemical staining was available were added to this figure (n = 402). Figure 1 illustrates that both CIN2 and CIN3 of women aged <30 years are less often methylation positive, have lower p16[ink4a]/Ki-67 immunoscores, and show more often E4 staining compared with women aged ≥30 years.

Table 2. *FAM19A4/miR124-2* Methylation Positivity in Non-16/18 Human Papillomavirus (HPV)–Positive and HPV16/18–Positive Cervical Lesions in Women <30 Years of Age

		Methylation Negative		Methylation Positive			
Histology	HPV16/18 Genotyping	No.	(%)	No.	(%)	Total	*P* Value[a]
≤CIN1	Non-16/18 positive	431	(80)	108	(20)	539	.253
	16/18 positive	113	(75)	38	(25)	151	
CIN2	Non-16/18 positive	63	(72)	25	(28)	88	
	16/18 positive	57	(73)	21	(27)	78	
CIN3	Non-16/18 positive	34	(41)	49	(59)	83	
	16/18 positive	44	(36)	77	(64)	121	
Carcinoma	Non-16/18 positive	0	(0)	0	(0)	0	
	16/18 positive	0	(0)	1	(100)	1	

The ≤CIN1 category consists of the no histology, CIN0, and CIN1 groups.

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

[a]The association between HPV16/18 genotyping and methylation was corrected for CIN grade using a Mantel-Haenszel analysis. Stratified for histological disease category, methylation positivity rates are similar in cervical scrapes of women being non-16/18 HPV positive vs HPV16/18 positive ($P = .253$).

DISCUSSION

This study showed that the *FAM19A4/miR124-2* methylation test positivity rates increased with age (<25 vs 25–29 vs ≥30 years) and CIN grade, while HPV16/18 genotyping positivity rates were similar among age groups. In addition, *FAM19A4/miR124-2* methylation positivity rates were independent of HPV type. *FAM19A4/miR124-2* methylation-positive CIN2/3 lesions appeared to be associated with low HPV E4 and high p16[ink4a]/Ki-67 expression in young women (Figure 1). These nonproductive, transforming immunohistochemical features are compatible with earlier defined advanced CIN lesions associated with a persistent HPV infection [13, 29]. Consistently, in a recent prospective clinical cohort study of women with untreated CIN2/3, higher regression rates were observed in women with a negative *FAM19A4/miR124-2* test than in women with a positive methylation test [15] (Kremer et al.). Taken together, the *FAM19A4/miR124-2* methylation test result reflects the nature (ie, high or low short-term cancer progression risk) of the underlying CIN, thereby providing guidance to the treatment policy in women with CIN2/3. Particularly in young women in whom a high proportion of methylation-negative CIN2/3 is found, avoidance of overtreatment will be beneficial.

To further characterize methylation-positive and methylation-negative CIN lesions in our study, histology sections of a subset of CIN2/3 were immunohistochemically stained for p16[ink4a]/Ki-67 and HPV E4, indicative for transforming and productive HPV infections, respectively. *FAM19A4/miR124*-2 methylation-negative lesions as found in

Table 3. p16[ink4a] and Ki-67 Immunoscores in Methylation-Negative and Methylation-Positive Lesions and in Non-16/18 Human Papillomavirus (HPV)–Positive and HPV16/18–Positive Lesions, in Women <30 Years of Age

Histology	Methylation	Immunoscore 0–4	(%)	5–6	(%)	Total	P Value[a]
CIN2	MM negative	27	(69)	12	(31)	39	.003
	MM positive	11	(48)	12	(52)	23	
CIN3	MM negative	21	(51)	20	(49)	41	
	MM positive	19	(26)	53	(74)	72	
Subtotal	MM negative	48	(60)	32	(40)	80	
	MM positive	30	(32)	65	(68)	95	
Total		78	(45)	97	(55)	175	

Histology	HPV Genotyping	Immunoscore 0–4	(%)	5–6	(%)	Total	P Value[a]
CIN2	Non-16/18 HPV positive	25	(63)	15	(38)	40	.822
	HPV16/18 positive	13	(59)	9	(41)	22	
CIN3	Non-16/18 HPV positive	17	(37)	29	(63)	46	
	HPV16/18 positive	23	(34)	44	(66)	67	
Subtotal	Non-16/18 HPV positive	42	(49)	44	(51)	86	
	HPV16/18 positive	36	(40)	53	(60)	89	
Total		78	(45)	97	(55)	175	

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; MM, methylation marker.

[a]The associations between methylation, HPV16/18 genotyping, and the p16[ink4a] and Ki-67 immunoscore were corrected for CIN grade using a Mantel-Haenszel analysis. Corrected for disease category, methylation-negative CIN2 and CIN3 lesions had significantly lower p16[ink4a]/Ki-67 immunoscores compared with methylation-positive CIN2 and CIN3 lesions ($P = .003$). No difference in p16[ink4a]/Ki-67 immunoscores in HPV16/18–positive CIN2 and CIN3 lesions vs non-16/18 HPV–positive CIN2 and CIN3 lesions was found ($P = .822$).

the young women of this study showed decreased p16[ink4a]/Ki-67 immunoscores and increased E4 expression compared with methylation-positive lesions, consistent with our premise that absence of host-cell methylation is associated with regressing CIN lesion.

Especially in young women (<30 years), CIN2 regression rates are high, with estimates up to 70% at 36 months [8, 9]. Consistently, in our study, we observed in particular a relatively low positivity rate of the methylation test in women with CIN2 (28%). In addition, we observed a trend that methylation positivity rates in women with CIN2/3 were even lower in women <25 years than in women aged 25–29 years. Further comparison of CIN2/3 lesions of women <30 years and women ≥30 years of age showed that lesions in women aged <30 years have fewer transforming features (ie, lower p16[ink4a] and Ki-67 immunoscores) and rather show productive characteristics (ie, higher rates for E4 positivity). This finding further supports the hypothesis that absence of *FAM19A4/miR124-2* methylation analysis is associated with CIN2/3 lesions early in development.

In this study, non-16/18 HPV–positive CIN lesions had similar p16[ink4a] and Ki-67 immunoscores and HPV E4 expression compared with HPV16/18–positive lesions. This indicates that in women aged <30 years, HPV16/18 genotyping cannot reliably differentiate between CIN2/3 lesions with a high vs low short-term cancer progression risk. Consequently, HPV16/18 genotyping seems no reliable biomarker to prevent overtreatment of regressing CIN2/3 lesions in these young women. Presently HPV16/18 genotyping is used in certain settings as a strategy to identify CIN2/3 in screening [34–36]. With the entrance of vaccinated cohorts in screening programs, numbers of HPV16/18–positive women will be substantially lower [37], and consequently HPV16/18 genotyping for CIN2/3 detection will be less informative. Given the retrospective nature of our study with samples collected between 2010 and 2017, the majority of women in our study are presumed to be unvaccinated. In contrast to HPV16/18 genotyping, *FAM19A4/miR124-2* methylation constitutes an HPV type and cytology independent biomarker for advanced CIN2/3 and cervical cancer and may therefore be a valid tool to detect CIN2/3 in HPV-vaccinated cohorts.

At present, performance data of methylation markers in women aged <30 years are very limited. To our knowledge, this is the largest cohort of young women evaluated to date showing that the *FAM19A4/miR124-2* methylation test is a compelling biomarker test for the detection of advanced CIN2/3 lesions. A limitation of the study is that FFPE samples for immunohistochemical staining and revision could only be retrieved from a subset of women. Furthermore, for some samples with abnormal cytology, the histological endpoint is missing, potentially overestimating methylation levels in this group.

In conclusion, we have shown in a large cohort of young women <30 years, that in contrast to HPV16/18 genotyping, *FAM19A4/miR124-2* methylation test-positive CIN lesions were associated with nonproductive, transforming CIN in need of treatment. The lower fraction of *FAM19A4/miR124-2* methylation-positive CIN2/3 in young women compared

Table 4. E4 Expression in Methylation-Negative and Methylation-Positive Lesions and in Non-16/18 Human Papillomavirus (HPV)–Positive and HPV16/18–Positive Lesions

Histology	Methylation	E4 Negative	(%)	E4 Positive	(%)	Total	*P* Value[a]
CIN2	MM negative	17	(44)	22	(56)	39	.033
	MM positive	16	(70)	7	(30)	23	
CIN3	MM negative	32	(78)	9	(22)	41	
	MM positive	63	(88)	9	(13)	72	
Subtotal	MM negative	49	(61)	31	(39)	80	
	MM positive	79	(83)	16	(17)	95	
Total		128	(73)	47	(27)	175	
Histology	**HPV Genotyping**	**E4 Negative**	**(%)**	**E4 Positive**	**(%)**	**Total**	***P* Value[a]**
CIN2	Non-16/18 HPV positive	22	(55)	18	(45)	40	.588
	HPV16/18 positive	11	(50)	11	(50)	22	
CIN3	Non-16/18 HPV positive	36	(78)	10	(22)	46	
	HPV16/18 positive	59	(88)	8	(12)	67	
Subtotal	Non-16/18 HPV positive	58	(67)	28	(33)	86	
	HPV16/18 positive	70	(79)	19	(21)	89	
Total		128	(73)	47	(27)	175	

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; MM, methylation marker.

[a]The associations between HPV16/18 genotyping, methylation, and E4 immunohistochemistry were corrected for CIN grade using a Mantel-Haenszel analysis. Corrected for disease category, methylation-negative CIN2 and CIN3 lesions were significantly more often E4 positive compared with methylation-positive CIN2 and CIN3 lesions, respectively ($P = .033$). No difference in E4 expression in HPV16/18–positive CIN2 and CIN3 lesions vs non-16/18 HPV–positive CIN2 and CIN3 lesions was found ($P = .588$).

with older women is consistent with a shorter duration of the associated HPV infection, and a likely lower cancer progression risk in these women. The high specificity of *FAM19A4/miR124-2* methylation test for advanced CIN2/3 lesions in young women makes this test a promising tool to guide clinicians in management of women with CIN2/3 lesions.

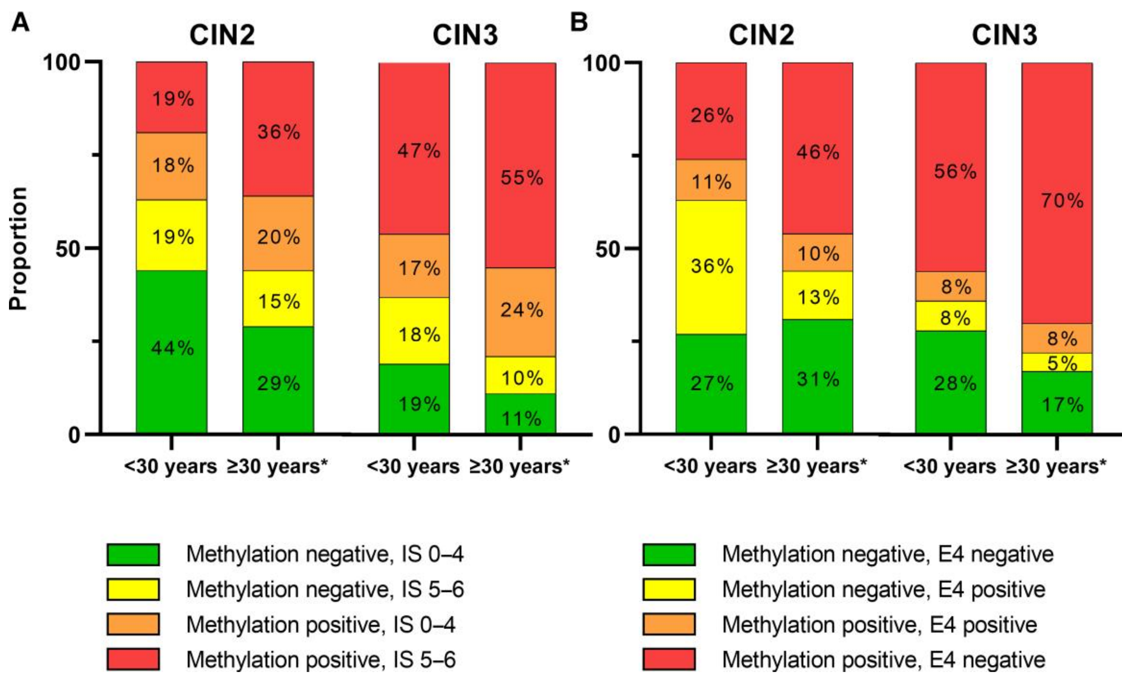

Figure 1. The proportions of women aged <30 years and ≥30 years, grouped based on their methylation and immunoscores (*A*) and methylation and E4 status (*B*), stratified for histologic outcome. *A reference population was included of women aged ≥30 years of the same European multicenter study for which immunohistochemical stainings were available [26]. Abbreviations: CIN, cervical intraepithelial neoplasia; IS, immunoscore; MM, methylation marker.

In agreement with recently observed increased regression rates in *FAM19A4/miR124-2* methylation-negative women compared with methylation-positive women [15] (Kremer et al.), a wait-and-see policy for methylation-negative CIN2/3 could be especially beneficial in young HPV-positive women.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author Contributions. Project design: A. T. H., C. J. L. M. M., M. C. G. B., D. A. M. H. Project management: F. J. V., A. T. N., A. N. F. Data collection: J. B., H. P., M. P., A. O. V., M. d. P., W. G. V. Q., K. C., B. R., P. H. Data analysis: F. J. V., B. L. W. Interpretation of data: F. J. V., A. T. H., G. G. K., R. D. M. S., M. C. G. B., D. A. M. H., C. J. L. M. M. Writing of manuscript: F. J. V., A. T. H., M. C. G. B., D. A. M. H., C. J. L. M. M. Review and final approval of the manuscript: all authors.

Acknowledgments. This work is dedicated to colleagues Prof Dr P. J. F. Snijders, who passed away on 27 May 2018, and Prof Dr K. U. Petry, who passed away on 21 April 2020, and were both active investigators in this study. The authors thank Grazyna Stanczuk for her work on the Papillomavirus Dumfries and Galloway (PaVDaG) study.

Data availability. The data that support the findings of our study are available from the corresponding author upon reasonable request.

Ethics statement. The work in this study with human-derived material was conducted under national and international rules and legislation, as well as European standards of research ethics, as it is expressed in the applicable legislation/regulations (Declaration of Helsinki; informed consent for participation of human subjects in medical and scientific research) and guidelines for Good Clinical Practice. The studies were approved by the local ethics committees where applicable. All subcontractors involved in the VALID-SCREEN project have signed a written agreement in which the obligation for obtaining an informed consent form from the women is laid down in accordance with the applicable national and European laws and regulations.

Financial support. This work was supported by the European Union Horizon 2020 program, small- and medium-sized enterprise instrument (project ID: 666800 VALID-SCREEN).

Potential conflicts of interest. C. J. L. M. M., R. D. M. S., and D. A. M. H. are minority shareholders of Self-screen B.V., a spin-off company of VU University Medical Center; Self-screen B.V. develops, manufactures and licences high-risk HPV and methylation marker assays for cervical cancer screening and holds patents on these tests. C. J. L. M. M. is part-time CEO of Self-screen B.V., had formerly a very small number of shares of MDXHealth and Qiagen, has received speaker fees from GlaxoSmithKline, Qiagen, and Sanofi Pasteur MSD/Merck and served occasionally on the scientific advisory boards (expert meetings) of these companies as well as Asieris Pharma/Ismar Healthcare NV (1-time payment); and received support for attending meetings and/or travel paid to author from Qiagen, GSK, SPMSD/Merck, and Self-screen B.V. J. B. is the principal investigator of studies funded in part by BD Diagnostics, Agena Bioscience, Genomica SAU, LifeRiver Biotech, and Qiagen and reports grants or contracts unrelated to this work from Capital Region of Denmark (public entity). He has received honoraria for lectures from BD Diagnostics, Roche Molecular Systems, Qiagen, and Genomica SAU. J. B. is an appointed member of the National Danish Cervical Screening Committee by the Danish Health Authority, and member of the regional cervical screening steering committee of the Capital Region of Denmark. A. O. V. has received reimbursement of travel expenses for attending conferences and honoraria for speaking from Abbott Molecular, Qiagen, and Seegene. M. d. P. has received personal fees for scientific advisory committee meetings and speaking fees from MSD and speaking fees from Roche. A. N. F. and A. T. H. are employed by Self-screen B.V., the legal manufacturer of the QIAsure Methylation Test. K. C. and R. B.'s institution has received research funding or consumables for free to support research from the following commercial entities in the last 3 years: Cepheid, Euroimmun, GeneFirst, Self-screen, Hiantis, Seegene, Roche, Abbott, and Hologic. K. C. also reports a position as advisor for nationally commissioned groups that support cervical screening in the United Kingdom (no personal payment but for work outside NHS Scotland time is reimbursed to employer). M. P. reports free-of-charge reagents and consumables received from Qiagen, Seegene, Abbott, and Roche to the author's institution. G. G. K. reports an unpaid role as a member of the board of a patient advocacy group for gynecological cancer. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Bosch FX, Burchell AN, Schiffman M, et al. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine **2008**; 26(Suppl 10):K1–16.
2. Smith JS, Melendy A, Rana RK, Pimenta JM. Age-specific prevalence of infection with human papillomavirus in females: a global review. J Adolesc Health **2008**; 43(4 Suppl):S5–25, S25.e1–41.
3. Moscicki AB, Shiboski S, Broering J, et al. The natural history of human papillomavirus infection as measured by repeated DNA testing in adolescent and young women. J Pediatr **1998**; 132:277–84.
4. de Sanjosé S, Diaz M, Castellsagué X, et al. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis **2007**; 7:453–9.
5. de Sanjosé S, Brotons M, Pavón MA. The natural history of human papillomavirus infection. Best Pract Res Clin Obstet Gynaecol **2018**; 47:2–13.
6. Bekos C, Schwameis R, Heinze G, et al. Influence of age on histologic outcome of cervical intraepithelial neoplasia during observational management: results from large cohort, systematic review, meta-analysis. Sci Rep **2018**; 8:6383.
7. Moscicki AB, Shiboski S, Hills NK, et al. Regression of low-grade squamous intraepithelial lesions in young women. Lancet **2004**; 364:1678–83.
8. Tainio K, Athanasiou A, Tikkinen KAO, et al. Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. BMJ **2018**; 360:k499.
9. Moscicki AB, Ma Y, Wibbelsman C, et al. Rate of and risks for regression of cervical intraepithelial neoplasia 2 in adolescents and young women. Obstet Gynecol **2010**; 116:1373–80.
10. Kyrgiou M, Athanasiou A, Paraskevaidi M, et al. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: systematic review and meta-analysis. BMJ **2016**; 354:i3633.
11. Rebolj M, Mathews CS, Pesola F, Castañon A, Kitchener H. Acceleration of cervical cancer diagnosis with human papillomavirus testing below age 30: observational study. Int J Cancer **2022**; 150:1412–21.
12. Vink FJ, Meijer C, Clifford GM, et al. *FAM19A4/miR124-2* methylation in invasive cervical cancer: a retrospective cross-sectional worldwide study. Int J Cancer **2020**; 147:1215–21.
13. De Strooper LM, Meijer CJ, Berkhof J, et al. 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) **2014**; 7:1251–7.
14. Bonde J, Floore A, Ejegod D, et al. Methylation markers *FAM19A4* and *miR124-2* as triage strategy for primary human papillomavirus screen positive women: a large European multicenter study. Int J Cancer **2021**; 148:396–405.
15. Kremer WW, Berkhof J, Bleeker MC, et al. Role of *FAM19A4/miR124-2* methylation analysis in predicting regression or non-regression of CIN2/3 lesions: a protocol of an observational longitudinal cohort study. BMJ Open **2019**; 9:e029017.
16. Kremer WW, Dick S, Heideman DAM, et al. Clinical regression of high-grade cervical intraepithelial neoplasia is associated with absence of *FAM19A4/miR124-2* DNA methylation (CONCERVE study). J Clin Oncol **2022**. doi:10.1200/JCO.21.02433.
17. Bonde JH, Sandri MT, Gary DS, Andrews JC. Clinical utility of human papillomavirus genotyping in cervical cancer screening: a systematic review. J Low Genit Tract Dis **2020**; 24:1–13.

18. Guan P, Howell-Jones R, Li N, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. Int J Cancer **2012**; 131:2349–59.
19. Luttmer R, De Strooper LM, Berkhof J, et al. 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 **2016**; 138:992–1002.
20. Floore A, Hesselink A, Ostrbenk A, et al. Intra- and inter-laboratory agreement of the *FAM19A4/mir124-2* methylation test: results from an international study. J Clin Lab Anal **2019**; 33:e22854.
21. Vink FJ, Dick S, Heideman DAM, et al. Classification of high-grade cervical intraepithelial neoplasia by p16(ink4a), Ki-67, HPV E4 and FAM19A4/miR124-2 methylation status demonstrates considerable heterogeneity with potential consequences for management. Int J Cancer **2021**; 149:707–16.
22. Available at: https://cordis.europa.eu/project/id/666800. Accessed 20 April 2022.
23. Arbyn M, Snijders PJ, Meijer CJ, et al. Which high-risk HPV assays fulfil criteria for use in primary cervical cancer screening? Clin Microbiol Infect **2015**; 21: 817–26.
24. Kremer WW, van Zummeren M, Breytenbach E, et al. The use of molecular markers for cervical screening of women living with HIV in South Africa. AIDS **2019**; 33:2035–42.
25. Vink FJ, Lissenberg-Witte BI, Meijer C, et al. FAM19A4/miR124-2 methylation analysis as a triage test for HPV-positive women: cross-sectional and longitudinal data from a Dutch screening cohort. Clin Microbiol Infect **2021**; 27:125.e1–e6.
26. Dippmann C, Schmitz M, Wunsch K, et al. Triage of hrHPV-positive women: comparison of two commercial methylation-specific PCR assays. Clin Epigenetics **2020**; 12:171.
27. Doorbar J. The E4 protein; structure, function and patterns of expression. Virology **2013**; 445:80–98.
28. Griffin H, Wu Z, Marnane R, et al. E4 antibodies facilitate detection and type-assignment of active HPV infection in cervical disease. PLoS One **2012**; 7:e49974.
29. Steenbergen RD, Snijders PJ, Heideman DA, Meijer CJ. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer **2014**; 14:395–405.
30. Zummeren MV, Kremer WW, Leeman A, et al. HPV E4 expression and DNA hypermethylation of *CADM1*, *MAL*, and *miR124-2* genes in cervical cancer and precursor lesions. Mod Pathol **2018**; 31:1842–50.
31. van Baars R, Griffin H, Wu Z, et al. Investigating diagnostic problems of CIN1 and CIN2 associated with high-risk HPV by combining the novel molecular biomarker PanHPVE4 with P16INK4a. Am J Surg Pathol **2015**; 39:1518–28.
32. Solomon D, Davey D, Kurman R, et al. The 2001 Bethesda system: terminology for reporting results of cervical cytology. JAMA **2002**; 287:2114–9.
33. Wright TC RB, Kurman RJ. Precancerous lesions of the cervix. In: Kurman R, Hedrick Ellenson L, Ronnet B. Blaustein's pathology of the female genital tract. 7th ed. Cham, Switzerland; Springer, **2019**:239–313.
34. Cuschieri K, Ronco G, Lorincz A, et al. Eurogin roadmap 2017: triage strategies for the management of HPV-positive women in cervical screening programs. Int J Cancer **2018**; 143:735–45.
35. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. J Clin Virol **2016**; 76(Suppl 1):S49–55.
36. Luttmer R, De Strooper LM, Steenbergen RD, et al. Management of high-risk HPV-positive women for detection of cervical (pre)cancer. Expert Rev Mol Diagn **2016**; 16:961–74.
37. Drolet M, Benard E, Pérez N, Brisson M; HPV Vaccination Impact Study Group. Population-level impact and herd effects following the introduction of human papillomavirus vaccination programmes: updated systematic review and meta-analysis. Lancet **2019**; 394:497–509.