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Low methylation marker levels among human papillomavirus-vaccinated women with cervical high-grade squamous intraepithelial lesions

Karolina Louvanto[1,2] | Lisanne Verhoef[3,4] | Ville Pimenoff[5] | Tiina Eriksson[1] | Siiri Leppälä[1] | Camilla Lagheden[5] | Penelope Gray[5] | Dorota Scibior-Bentkowska[6] | Elizabeth Sumiec[6] | Pekka Nieminen[7] | Joakim Dillner[5] | Johannes Berkhof[8] | Chris J. L. M. Meijer[3,4] | Matti Lehtinen[1,5] | Belinda Nedjai[6] | Daniëlle A. M. Heideman[3,4]

[1]Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland

[2]Department of Obstetrics and Gynecology, Tampere University Hospital, Tampere, Finland

[3]Pathology, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

[4]Imaging and Biomarkers, Cancer Center Amsterdam, Amsterdam, The Netherlands

[5]Unit of Cervical Cancer Elimination, Department of Clinical Science, Intervention and Technology, CLINTEC, Karolinska Institute, Stockholm, Sweden

[6]Centre for Cancer Screening, Prevention and Early Detection (SPED), Wolfson Institute of Population Health, Queen Mary University of London, London, UK

[7]Department of Obstetrics and Gynecology, Helsinki University Hospital, Helsinki, Finland

[8]Epidemiology and Data Science, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

Correspondence
Karolina Louvanto, Department of Obstetrics and Gynecology, Faculty of Medicine and Health Technology, Tampere University Hospital and Tampere University, Arvo Ylpön katu 34, PO Box 100, Tampere 33014, Finland.
Email: karolina.louvanto@tuni.fi

Present address
Daniëlle A. M. Heideman, Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

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Abstract

Cervical cancer screening programs, including triage tests, need redesigning as human papillomavirus (HPV)-vaccinated women are entering the programs. Methylation markers offer a potential solution to reduce false-positive rates by identifying clinically relevant cervical lesions with progressive potential. In a nested case–control study, 9242 women who received the three-dose HPV16/18-vaccine at ages 12–15 or 18 in a community-randomized trial were included. Subsequently, they were re-randomized for either frequent or infrequent cervical cancer screening trials. Over a 15-year post-vaccination follow-up until 2022, 17 high-grade squamous intraepithelial lesion (HSIL) and 15 low-grade (LSIL) cases were identified at the 25-year screening round, alongside 371 age and community-matched HPV16/18-vaccinated controls. Methylation analyses were performed on cervical samples collected at age 25, preceding histologically confirmed LSIL or HSIL diagnoses. DNA methylation of viral (HPV16/18/31/33)

Karolina Louvanto and Lisanne Verhoef equally contributed as first authors.

Belinda Nedjai and Daniëlle A. M. Heideman equally contributed as last authors.

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and host-cell genes (*EPB41L3*, *FAM19A4*, and *miR124-2)* was measured, along with HPV-genotyping. No HPV16/18 HSIL cases were observed. The predominant HPV-genotypes were HPV52 (29.4%), HPV59/HPV51/HPV58 (each 23.5%), and HPV33 (17.7%). Methylation levels were generally low, with no significant differences in mean methylation levels of viral or host-cell genes between the LSIL/HSIL and controls. However, a significant difference in methylation levels was found between HSIL cases and controls when considering a combination of viral genes and *EPB41L3* (p value = .0001). HPV-vaccinated women with HSIL had HPV infections with uncommon HPV types that very rarely cause cancer and displayed low methylation levels. Further investigation is warranted to understand the likely regressive nature of HSIL among HPV-vaccinated women and its implications for management.

KEYWORDS
human papillomavirus, high-grade squamous intraepithelial lesion, methylation, screening, vaccination

What's new?

Cervical cancer screening programs, including triage tests, need to be redesigned as human papillomavirus (HPV)-vaccinated women now enter the programs. In this nested case–control study, HPV-vaccinated women with cervical high-grade squamous intraepithelial lesions had infections with HPV genotypes that very rarely cause cancer in this age group. Moreover, they displayed low methylation levels similar with those in HPV-vaccinated women with low-grade squamous intraepithelial lesions and HPV-vaccinated controls. The low methylation levels and potential for lesion regression suggest that HPV-vaccinated women would benefit from active surveillance of their high-grade squamous intraepithelial lesions rather than immediate treatment.

1 | INTRODUCTION

Human papillomavirus (HPV) vaccinated birth cohorts of women are now reaching the age for cervical cancer screening programs. Over the past years, many countries have transitioned their cervical cancer screening programs to HPV primary screening, with pap cytology used as the triage test.[1,2] HPV vaccination has led to a reduction in the prevalence of many HPV genotypes targeted by cervical cancer screening, resulting in a lower prevalence of high-risk (hr) HPV and associated cervical lesions among HPV-vaccinated women.[3–5] This reduction is expected to significantly decrease the positive predictive value of current cervical cancer screening tests. Therefore, it is crucial for cervical cancer screening programs to carefully consider adaptations for HPV-vaccinated women to balance the potential risks and benefits.[6,7] Furthermore, there is an urgent need to explore alternative screening tests that can effectively identify HPV-vaccinated women with a substantial risk of cervical cancer.

Methylation of both host-cell and viral genes has shown promising results in identifying unvaccinated women with high-grade lesions and cervical cancer. Methylation levels tend to increase with lesion severity.[8–10] Two well-studied marker panels, the QIAsure Methylation Test, including host-cell methylation markers *FAM19A4* and *miR124-2*[11–13] and the S5-classifier, including host-cell methylation marker *EPB41L3*, and viral genes *HPV16-L1*, *HPV16-L2*, *HPV18-L2*, *HPV31-L1*, and *HPV33-L2*,[9] have demonstrated accurate detection of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) and grade 3 or worse (CIN3+), as well as malignancy in unvaccinated women across various geographic contexts and settings (Asia, Europe, Africa, and the United States).[14,15] Both panels have proven effective in identifying progressive high-grade squamous intraepithelial (HSIL) cervical lesions in unvaccinated women.[16,17] Importantly, implementation of predictive cervical cancer screening or triage tests could reduce the number of colposcopy referrals and the need for further follow-up testing among both unvaccinated and vaccinated women with regressive cervical lesions. Additionally, these panels have shown improved triage performance in cervical cancer screening compared to hrHPV genotyping, pap cytology or a combination of the two in unvaccinated women.[13,18,19]

Overall, methylation markers have demonstrated good performance among unvaccinated women but require thorough evaluation among HPV-vaccinated women. A recent study reported surgically treated low-grade squamous intraepithelial (LSIL) and HSIL cervical lesions among HPV-vaccinated women.[20] In this study, we aimed to

evaluate established methylation markers in cervical LSIL and HSIL lesions among HPV-vaccinated women.

2 | MATERIALS AND METHODS

2.1 | Study enrolment, follow-up, and sampling

A community-randomized, phase IV trial (NCT 00534639) comparing the impact of gender-neutral versus girls-only HPV vaccination strategies was conducted in Finnish junior high-schools and municipal study sites. The trial spanned from 2007 to 2010 (vaccination phase),[21] and from 2010 to 2014 for the follow-up phase.[22] A total of 80,000 early adolescents, aged 12–15 year, born between 1992 and 1995, were invited to participate. Among them 32,175 (20,514 girls and 11,661 boys) attended the trial. Of the girls, 12,402 received three doses of the bivalent HPV16/18 vaccine, while 8112 girls received the hepatitis B-virus (HBV) control vaccine during the vaccination phase (2007–2010). During the follow-up phase (2010–2014), 18-year-old female study participants attended the municipal study sites for pelvic examination, during which cervical cytological samples were obtained. At this phase, 2284 participants who originally received the HBV-vaccine were administered three doses of the bivalent HPV16/18 vaccine, as previously described.[20,22]

In 2014, all 14,686 HPV16/18 vaccine recipients were individually re-randomized and invited to participate in a trial (NCT02149030) comparing the accuracy of frequent versus infrequent screening of HPV-vaccinated women at ages 22, 25, and 28.[3,20] A total of 6958 females consented to participate in the screening trial and extended follow-up. Samples for this study were obtained at the third follow-up visit, at age 25, prior to histological confirmation of LSIL or HSIL cervical lesions among HPV-vaccinated women. Pap cytology was collected simultaneously with the cervical sample used for HPV genotyping and DNA methylation testing.

2.2 | Identification of cervical lesions and selection of HPV16/18 vaccinated controls

By the end of 2021, a total of 46 cases with cervical lesions had been identified through extended follow-up, including, 26 cases of LSIL and 20 cases of HSIL, with 32 histo-pathologically confirmed from cone biopsies after age 25. For this nested case–control study, eight 25-year-old controls with no cervical lesion record were matched for each of the 46 cervical LSIL/HSIL cases. Controls were matched based on place of residence and birth cohort from the HPV16/18 vaccinated females participating in the HPV-vaccinated women's screening trial (NCT02149030). The larger control group selection was done to have the opportunity to stratify possible vaccine and non-vaccine hrHPV types separately within the cases and controls, and to provide more robust comparisons and help mitigate potential biases or confounding factors. The control women had previously undergone pelvic examination with cervical sampling at age 18 during the community-randomized trial (NCT00534639).[22]

2.3 | HPV DNA analyses

Cervical samples collected during the third follow-up visit at age 25 were subjected to HPV DNA analysis using the modified general primer polymerase chain reaction (PCR), followed by Luminex, detecting 12 carcinogenic HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59); 13 probably carcinogenic HPV types (HPV26, 30, 34, 53, 66, 67, 68, 69, 70, 73, 82, 85, and 97); and 18 non-carcinogenic HPV types (HPV6, 11, 40, 42, 43, 54, 61, 62, 71, 72, 74, 81, 83, 86, 87, 89, 90, and 91).

2.4 | DNA methylation analyses

The same cervical samples that were HPV genotyped were analyzed for DNA methylation. Extracted DNA underwent bisulfite conversion to convert unmethylated cytosines to uracils using the EZ DNA methylation kit (Zymo Research, Irvine, USA). DNA methylation of CpG islands from *EPB41L3* and viral late genes (L1 and L2) of HPV16, HPV18, HPV31, and HPV33 were then amplified and pyrosequenced on a PyroMark Q96ID (Qiagen, Hilden, Germany) as previously described.[23] Methylation levels of *FAM19A4 and miR124-2* were determined using the QIAsure Methylation Test (QIAGEN, Hilden, Germany) via quantitative methylation-specific PCR (qMSP) according to the instructions of the manufacturer.

2.5 | Statistical analyses

Only cases with histologically confirmed LSIL or HSIL following the 25-year-old follow-up visit were included in the analyses. This allows us to evaluate the methylations tests' ability to identify women with HSIL cervical lesions. The final study population consisted of 15 LSIL and 17 HSIL cases, along with 371 controls. Groups were compared for differences in baseline characteristics, HPV genotypes and DNA methylation results using Kruskal-Wallis comparison. Fisher's exact test was used if the number of observations was lower than five, with post hoc testing using Bonferroni-corrected test. Methylation levels of HPV-vaccinated women were compared with those of unvaccinated women, stratified by disease category. The methylation data of the unvaccinated group were obtained from previously published data.[11,12,23] All statistical tests performed were two-sided and considered significant at the p value <.05 level. Analyses were done with STATA/SE 16.1 (Stata Corp., College Station, TX, USA).

3 | RESULTS

The study population consisted of 403 HPV-vaccinated women born between 1992 and 1995, who participated in Finnish community and individually randomized trial cohorts, representing a population-based, country-wide HPV vaccination and cervical cancer screening trial established in 2007.[20,22] Among them, 15 had histology confirmed LSIL and 17 had HSIL. The mean time from cervical sample collection to

TABLE 1 Characteristic of 403 women included in the nested case–control study at the age of 25-year-old follow-up visit from the Finnish community and individually randomized trial cohorts.

		HPV-vaccinated cervical lesions	
	Controls ($n = 371$)	LSIL ($n = 15$) n (%)	HSIL ($n = 17$)
Year of birth			
1992	89 (24.1)	3 (20.0)	7 (41.2)
1993	45 (12.2)	3 (20.0)	3 (17.7)
1994	151 (40.9)	5 (33.3)	7 (41.2)
1995	84 (22.8)	4 (26.7)	–
Vaccination age (Mean [±SD])	**14.6 (±0.7)**[a]	14.7 (±0.2)	**15.0 (±0.1)**[a]
HPV			
HPV negative	**230 (62.0)**[a]	**2 (13.3)**[a]	**2 (11.7)**[a]
Any HPV+	**141 (38.0)**[a]	**13 (86.7)**[a]	**15 (88.2)**[a]
hrHPV+	**98 (26.4)**[a]	**12 (80.0)**[a]	**15 (88.2)**[a]
lrHPV+	72 (19.4)	5 (33.3)	4 (23.5)
Single HPV	81 (21.8)	4 (26.7)	6 (35.3)
Multiple HPV 2+	**60 (16.2)**[a]	**8 (53.3)**[a]	**9 (52.6)**[a]
Multiple HPV			
2	49 (13.2)	4 (26.7)	5 (29.4)
3	8 (2.2)	3 (20.0)	3 (17.7)
4	2 (0.5)	2 (13.3)	1 (5.9)
5	1 (0.3)	–	–
HPV genotypes by groups[b]			
Group 1 – Carcinogenic			
HPV16	1 (0.3)	–	–
HPV33	**4 (1.1)**[a]	–	**3 (17.7)**[a]
HPV35	2 (0.5)	–	1 (5.9)
HPV39	4 (1.1)	2 (13.3)	1 (5.9)
HPV45	1 (0.3)	–	–
HPV51	**15 (4.0)**[a]	**7 (46.7)**[a]	**4 (23.5)**[a]
HPV52	**23 (6.2)**[a]	1 (6.7)	**5 (29.4)**[a]
HPV56	14 (3.8)	1 (6.7)	1 (5.9)
HPV58	**6 (1.6)**[a]	1 (6.7)	**4 (23.5)**[a]
HPV59	**12 (3.2)**[a]	**5 (33.3)**[a]	**4 (23.5)**[a]
Group 2a and 2b – Probably carcinogenic			
HPV30	5 (1.4)	–	–
HPV53	3 (0.8)	1 (6.7)	–
HPV66	12 (3.2)	2 (13.3)	1 (5.9)
HPV67	6 (1.6)	1 (6.7)	–
HPV68	8 (2.2)	–	–
HPV70	**5 (1.4)**[a]	**2 (13.3)**[a]	1 (5.9)
HPV73	4 (1.1)	–	–
HPV82	1 (0.3)	–	–
Group 3 – Not carcinogenic			
HPV6	8 (2.2)	–	2 (11.8)
HPV11	2 (0.5)	1 (6.7)	–
HPV40	4 (1.1)	–	–
HPV42	10 (2.7)	–	–

TABLE 1 (Continued)

	Controls ($n = 371$)	HPV-vaccinated cervical lesions	
		LSIL ($n = 15$) *n* (%)	HSIL ($n = 17$)
HPV43	–	1 (6.7)	–
HPV61	7 (1.9)	–	1 (5.9)
HPV74	8 (2.2)	–	–
HPV81	6 (1.6)	–	–
HPV83	2 (0.5)	–	–
HPV86	1 (0.3)	–	–
HPV87	9 (2.4)	1 (6.7)	1 (5.9)
HPV89	12 (3.2)	1 (6.7)	–
HPV90	17 (4.6)	1 (6.7)	–
HPV91	3 (0.8)	–	–

Abbreviations: HPV, human papillomavirus; hr, high-risk; HSIL, high-grade intraepithelial lesion; lr, low-risk; LSIL, low-grade intraepithelial lesion; SD, standard deviation.

[a]Comparisons in bold, *p* values <.05.

[b]Part of single or multiple infection. Classification according to IARC; those HPV genotypes that were negative in all groups are not shown (Group 1: HPV18, 31; Group 2: HPV26, 34, 69, 85, 97; and Group 3: HPV54, 62, 71, and 72).

TABLE 2 Individual HPV genotyping results from the earlier screening rounds of the Finnish community and individually randomized trial cohorts of the 17 HPV-vaccinated high-grade intraepithelial lesion (HSIL) biopsy confirmed cases.

		HPV genotypes by follow-up visits		
Case		18 years	22 years	25 years
1	HSIL	Neg	Neg	33, 59
2	HSIL	Neg	**33**	**33**, 59, 66
3	HSIL	Neg	39, 68	58
4	HSIL	–	51, 56, 66	52, 59, 61, 70
5	HSIL	6, **52**	**52**, 56, 58	**52**, 58
6	HSIL	Neg	**52**	**52**
7	HSIL	Neg	66	6, 51
8	HSIL	–	**59**	39, **59**
9	HSIL	Neg	**51**	**51**
10	HSIL	–	**33**, 39, 51	6, **33**, 52
11	HSIL	Neg	Neg	51
12	HSIL	66	Neg	59
13	HSIL	Neg	Neg	Neg
14	HSIL	Neg	11, **35**, 51	**35**
15	HSIL	Neg	Neg	Neg
16	HSIL	Neg	**51**, 52, 66	**51**, 56
17	HSIL	Neg	66	58, 87

Note: Persistent HPV infections are shown in bold.

Abbreviations: HPV, human papillomavirus; HSIL, high-grade intraepithelial lesion; neg, negative.

colposcopy for LSIL cases was 12.9 months (range 0.8–44.3 months) and for HSIL cases was 8.3 months (range 1.0–41.3 months).

Among the vaccinated HSIL cases, nine had HSIL Pap cytology recorded, with two cases of atypical glandular cells, not otherwise specified (AGC-NOS), and three atypical squamous cells, cannot rule out HSIL (ASC-H) pap cytology. All vaccinated LSIL/HSIL cases had negative for intraepithelial lesion or malignancy (NILM) or atypical squamous cells of undetermined significance (ASC-US) pap cytology recorded at the earlier follow-up visit at the age of 22.

The vaccination age varied slightly between vaccinated HSIL cases and controls, with a mean age of 15.0 years compared to 14.6 years (Table 1). In the vaccinated HSIL cases, the majority of women were from the 1992 or 1994 birth cohorts (both 41.2%). Regarding HPV status, all vaccinated LSIL/HSIL cases had a higher overall HPV prevalence (86.7% and 88.2%, respectively) compared to controls (38.0%). Significant observations were seen among any hrHPV genotypes (88.2% and 80.0%) compared to controls (26.4%). The most common HPV genotype among vaccinated HSIL cases was HPV52 ($n = 5$, 29.4%), followed by HPV59, HPV51 and HPV58 (all $n = 4$, 23.5%), and HPV33 ($n = 3$, 17.7%). No HPV16/18 genotypes were observed. Multiple HPV infections were significantly more prominent among all HPV-vaccinated HSIL/LSIL cases (52.6–53.3%) compared to controls (16.2%). Eight (47.1%) women had a persistent type-specific HPV infection (HPV33, 35, 51, 52, or 59) recorded prior to colposcopy referral at the age of 18 and/or 22 years (Table 2).

DNA methylation was evaluated among all 403 women for viral (HPV16, 18, 31, and 33) and host-cell genes (*EPB41L3*, *FAM19A4*, and *miR124-2*) (Figure 1). None of these separate DNA methylation markers showed significant differences between vaccinated controls and vaccinated LSIL/HSIL cases. For the viral DNA methylation sites, only a few individuals had methylation of HPV33 L2 gene detected among vaccinated HSIL and control groups, 18.06 (SD ±11.37) and 14.15 (SD ±23.73), respectively. For the remaining HPV types (16, 18, and 31), no viral methylation was recorded.

Figure 1 illustrates the differences in methylation levels of host-cell genes *FAM19A4* and *miR124-2* (Figure 1A, B); and *EPB41L3* host-cell gene and the S5-classifier panel (Figure 1C, D). In HPV-vaccinated controls and LSIL/HSIL cases compared to the unvaccinated

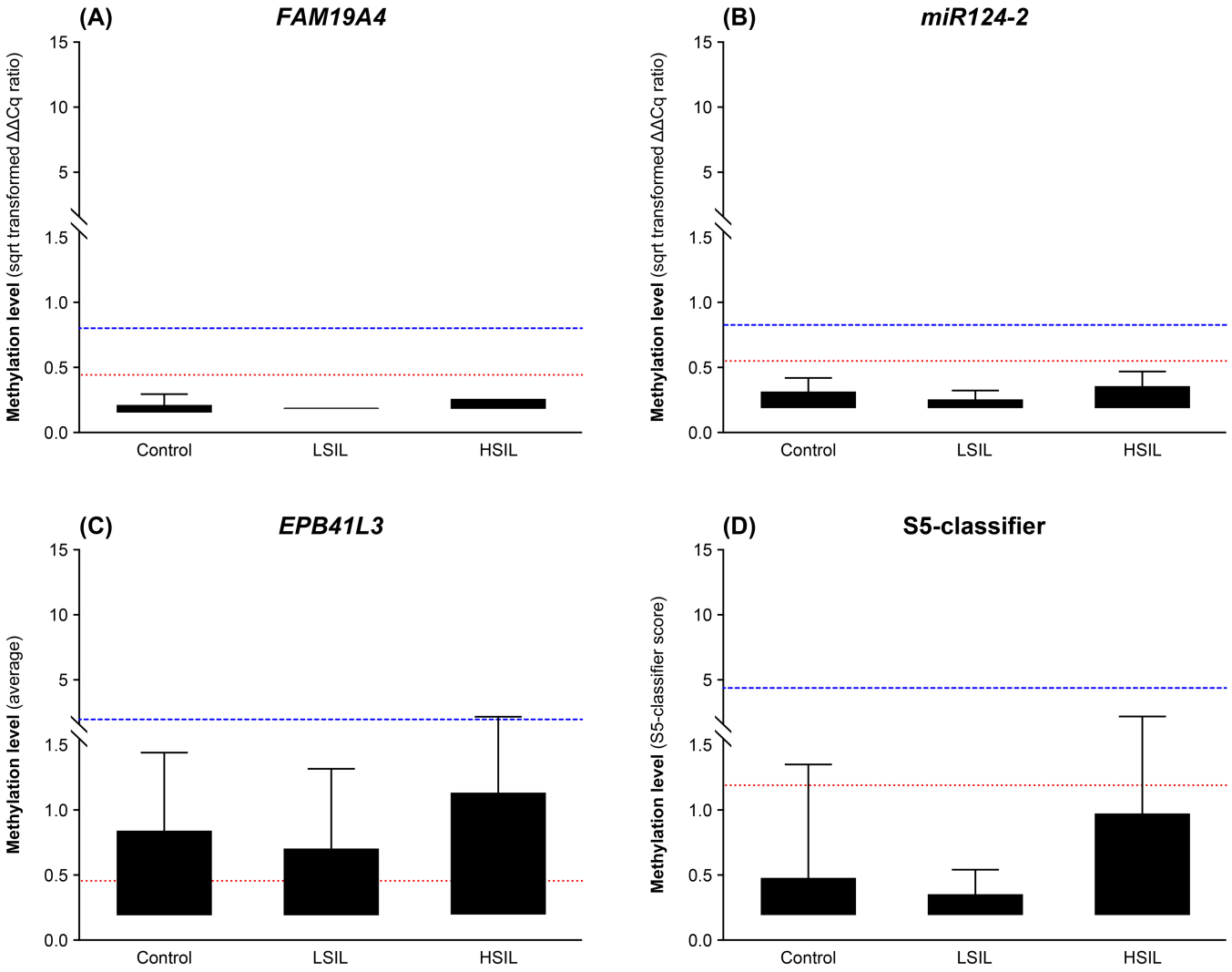

FIGURE 1 Methylation levels of *FAM19A4*, *miR124-2*, *EPB41L3*, and S5-classifier in cervical samples of HPV-vaccinated women. Methylation levels represented by boxplots of the square-root transformed ΔΔCq-ratios (*y*-axis) of (A) *FAM19A4* and (B) *miR124-2*; and (mean with standard deviation) of (C) the average *EPB41L3* methylation and (D) S5 methylation panel score (*y*-axis) in cervical samples stratified by study endpoint. The dotted lines in the figures illustrate the mean reference methylation level among unvaccinated women, red line for low-grade squamous intraepithelial lesion (LSIL) and blue line for high-grade squamous intraepithelial lesion (HSIL). Cq, quantification cycle; sqrt, square-root.

population, a dotted reference line was added to each figure to demonstrate mean levels of methylation among unvaccinated women, with LSIL (red) and HSIL (blue). These mean methylation levels were obtained from previously published data.[11,12,23]

FAM19A4 and *miR124-2* both showed low methylation levels in HPV-vaccinated women compared to unvaccinated women with LSIL and HSIL lesions (Figure 1A, B). With *EPB41L3* host-cell gene, methylation levels were much higher among unvaccinated women with HSIL lesions, while among LSIL cases, levels were similar between vaccinated and unvaccinated women. With the S5-classifier panel, the mean methylation levels of HPV-vaccinated women were also much lower compared to the unvaccinated HSIL S5-classifier methylation levels with a predefined cut-off of 0.8 (Figure 1D). However, significantly higher mean methylation level for S5-classifier was recorded in HSIL cases (0.81 [SD ± 1.74]) compared to HPV-vaccinated controls (0.11 [SD ± 1.04]) (p value = .0001).

4 | DISCUSSION

Our study sheds light on HPV-vaccinated women with long-term follow-up, revealing that HSIL cervical lesions can still be detected in women vaccinated in early adolescence. However, the underlying nature and carcinogenic potential of these lesions remain unclear. With these first DNA methylation results among HPV-vaccinated women, we demonstrate that methylation marker levels in nearly all HPV-vaccinated HSIL cases are similar to controls. This indicates that most cervical lesions among HPV-vaccinated women could regress without treatment.

It has been clearly demonstrated that HPV-associated disease remains a major issue, particularly in countries with a low vaccination coverage or without HPV vaccination programs.[20,24] However, screening data are emerging from women in vaccinated cohorts, showing a substantially lower cancer risk compared to unvaccinated women. Modifications to current screening tests and protocols will be necessary to reduce the harms of cervical cancer screening among HPV-vaccinated women.[6,20]

In this study, we evaluated HPV-vaccinated women with histologically confirmed LSIL or HSIL cervical lesions. The observed HPV genotypes were in line with previous publications, with HPV52, 59, 51, 58, and 33 being the most prevalent among HSIL cases.[3,20,25] These HPV genotypes are often detected in the pooled hrHPV type setting and are used for triaging women with low-grade pap cytology for repeated testing, suggesting a lower risk of cancer compared to HPV16/18, which often represents abnormal Pap cytology and requires direct referral for colposcopy.[26] A large recent systematic review, evaluating the risk of cervical HSIL for unvaccinated women, found that the risk for HPV33 was intermediate, while HPV52 and HPV58 carried only a moderate risk and HPV51 and HPV59 had the least risk.[26] Therefore, even when considering only HPV genotyping, HPV-vaccinated women with HSIL lesions have low to moderate risk for developing cervical cancer.

Natural regression of cervical HSIL lesions is common among young unvaccinated women, with almost half of them resolving spontaneously within 2 years.[27] However, in our cohort of HPV-vaccinated cases, no expectant management was employed, as they were all surgically treated with loop electrosurgical excision procedure (LEEP) according to the clinical case guidelines of Finland. LEEP can cause emotional distress and serious complications during pregnancy, highlighting the need for alternative management strategies. For instance, premature delivery, a consequence of LEEP, contributes to the challenges we face today with premature children that require extensive and costly postnatal care that sometimes becomes life-long care.[28,29]

Recent studies have shown that DNA methylation testing has the potential to distinguish unvaccinated women with progressive HSIL lesions from those likely to regress spontaneously. Among women under the age of 30 with follow-up every 6 months after CIN2 diagnoses, the S5-classifier had the highest association with women showing progressive cervical disease, with an odds ratio (OR) of 3.39.[17] Moreover, the host-cell marker panel *FAM19A4/miR124-2* reported a high regression rate in women with a CIN2/3 lesion if they had a negative baseline *FAM19A4/miR124-2* methylation test.[16] Both studies have confirmed that combining methylation testing with HPV genotyping or pap cytology could be used in clinical setting to identify women at genuine risk of progressive disease. Our study is the first to investigate DNA methylation among HPV-vaccinated women, revealing low methylation levels and suggesting a low progression potential for HSIL lesions in this population. We could only detect viral HPV33 methylation, as HPV16, 18 and 31 were not detected among the HPV-vaccinated women. No differences were recorded between the vaccinated LSIL or HSIL cases and vaccinated controls for HPV33 or any of the host-cell genes (*EPB41L3*, *FAM19A4*, and *miR124-2*), indicating low or absent methylation levels. Though methylation is known to be lower in women under the age of 30. Previous studies have shown that methylation levels increase with lesion severity among unvaccinated women, regardless of the age.[8–10,12] However, given the absence or low level of methylation in our study, it is likely that histologically confirmed HSIL cases in these young, HPV-vaccinated women have a very low progression potential. Nevertheless, with the S5-classifier, which combines results on viral genes and host-cell gene *EPB41L3*, a significant difference was detected between HPV-vaccinated HSIL cases and controls. However, the mean levels of the S5-classifier were relatively low. Altogether, our findings lead to a debate whether HPV-vaccinated women with HSIL lesions could potentially regress spontaneously without the need for LEEP and associated risks and complications.

The main strength of our study lies in the longitudinal follow-up of women who all received a full three-dose bivalent vaccine at the age of 12–15 years old. Additionally, due to the well-established original Finnish community and individually randomized trial cohort, we were able to structure a case–control setting where both age and community at the time of vaccination were utilized. This study also represents the first evaluation of methylation markers among HSIL lesions in HPV-vaccinated women. However, our study has also several limitations. These include the small sample size of HSIL lesions and the young age of the women at time of HSIL detection. Additionally, under the current Bethesda guidelines, abnormal histology is reported based on a two-tier stratification of LSIL (formerly CIN1) or HSIL (formerly CIN2 and CIN3).[30,31] Therefore, we were unable to further stratify the HSIL lesions into CIN2 or CIN3 in this study. Our results require validation in the future with a larger number of HSIL lesions, as well as the inclusion of older HPV-vaccinated women.

As HPV-vaccinated women enter cervical cancer screening programs, a new era in screening approaches is forthcoming. To determine the need and frequency to screen HPV-vaccinated women, we must consider all potential harms and benefits of this new era. Based on these first findings, moving away from a one-size-fits-all strategy is anticipated, taking the HPV vaccination status into account, allowing for the direction of screening resources to those at highest risk and using less intensive screening for those with lower risk. Our findings suggest that active surveillance may be suitable for HPV-vaccinated women with cervical HSIL, given the potential for lesion regression. However, further validation of these results in larger cohorts of HPV-vaccinated women with longer follow-up is needed.

AUTHOR CONTRIBUTIONS

ML designed the original study. KL, VP, TE, SL, PG, CL, PN, JD, ML was involved with sample collections and analyses. KL, ML, VP, SL, LV, JB, CM, BN, DH designed the methylation study. LV, DSB, ES, BN, DH did all the methylations analyses. KL and LV wrote the first draft of the manuscript and all authors contributed to the review, writing, and editing of the manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text. All authors have seen and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

DH and CM are minority shareholders of Self-screen B.V., a spin-off company of Amsterdam UMC, location VUmc, which develops, manufactures, and licenses high-risk HPV and methylation marker assays for cervical cancer screening and holds patents on these tests. DH reports a grant from the Dutch Cancer Society during the conduct of the study (KWF 11337). CM is a part-time CEO of Self-Screen B.V. and serves occasionally on the scientific advisory boards and/or speaker bureau of GSK, Qiagen, Sanofi Pasteur, MSD/Merck, and Asuris Pharma/Famar health care VU. ML reports grants from Merck & Co. Inc. and GSK Biologicals through Tampere University for the community randomized study and long-term follow-up study on bivalent HPV vaccine effectiveness. KL has received grants for research from Research Council of Finland, Finnish Cancer Foundation, and Sigrid Juselius Foundation. None of the other authors has any conflicts of interest with respect to the contents of this manuscript.

DATA AVAILABILITY STATEMENT

Individual participant data that underlie the results reported in this article, after de-identification, will be available for researchers who provide a methodologically sound proposal with achievable aims. Proposals should be directed via e-mail to the study cohort's principal investigator (ML, matti.lehtinen@tuni.fi); data requestors will need to sign a data access agreement. Further information is available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Approval was granted by the Pirkanmaa Hospital District Ethical Review Board, Finland (Dated: June 12, 2007; February 19, 2014; February 4, 2020/No. R07113M; R13149; R191936). Informed written consent was obtained from all individual participants included in the study (NCT00534639).

ORCID

Karolina Louvanto https://orcid.org/0000-0002-1172-363X
Lisanne Verhoef https://orcid.org/0000-0001-9877-0297
Ville Pimenoff https://orcid.org/0000-0002-0813-7031
Penelope Gray https://orcid.org/0000-0001-9065-4734
Joakim Dillner https://orcid.org/0000-0001-8588-6506
Matti Lehtinen https://orcid.org/0000-0002-9481-0535

TWITTER

Karolina Louvanto *KLouvanto*

REFERENCES

1. Veijalainen O, Kares S, Kotaniemi-Talonen L, et al. Primary HPV screening for cervical cancer: results after two screening rounds in a regional screening program in Finland. *Acta Obstet Gynecol Scand*. 2021;100(3):403-409.
2. Louvanto K, Chevarie-Davis M, Ramanakumar AV, Franco EL, Ferenczy A. HPV testing with cytology triage for cervical cancer screening in routine practice. *Am J Obstet Gynecol*. 2014;210(5):e1-e7.
3. Louvanto K, Eriksson T, Gray P, et al. Baseline findings and safety of infrequent vs. frequent screening of human papillomavirus vaccinated women. *Int J Cancer*. 2020;147(2):440-447.
4. Falcaro M, Castañon A, Ndlela B, et al. The effects of the national HPV vaccination programme in England, UK, on cervical cancer and grade 3 cervical intraepithelial neoplasia incidence: a register-based observational study. *Lancet*. 2021;398(10316):2084-2092.
5. Patel C, Brotherton JM, Pillsbury A, et al. The impact of 10 years of human papillomavirus (HPV) vaccination in Australia: what additional disease burden will a nonavalent vaccine prevent? *Euro Surveill*. 2018;23(41):1700737.
6. El-Zein M, Richardson L, Franco EL. Cervical cancer screening of HPV vaccinated populations: cytology, molecular testing, both or none. *J Clin Virol*. 2016;76:S62-S68.
7. Lei J, Ploner A, Lehtinen M, Sparan P, Dillner J, Elfstrm KM. Impact of HPV vaccination on cervical screening performance: a population-based cohort study. *Br J Cancer*. 2020;123(1):155-160.
8. Louvanto K, Franco EL, Ramanakumar AV, et al. Methylation of viral and host genes and severity of cervical lesions associated with human papillomavirus type 16. *Int J Cancer*. 2015;136(6):E638-E645.
9. Brentnall AR, Vasiljevic N, Scibior-Bentkowska D, et al. A DNA methylation classifier of cervical precancer based on human papillomavirus and human genes. *Int J Cancer*. 2014;135:1425-1432.
10. Kelly H, Benavente Y, Pavon MA, De Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (CIN2+): a systematic review and meta-analysis. *Br J Cancer*. 2019;121(11):954-965.
11. 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(4):1215-1221.
12. 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(1):e1-e6.
13. 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(2):396-405.
14. Banila C, Lorincz AT, Scibior-Bentkowska D, et al. Clinical performance of methylation as a biomarker for cervical carcinoma in situ and cancer diagnosis: a worldwide study. *Int J Cancer*. 2022;150(2):290-302.
15. Hernandez-Lopez R, Lorincz AT, Torres-Ibarra L, et al. Methylation estimates the risk of precancer in HPV-infected women with discrepant results between cytology and HPV16/18 genotyping. *Clin Epigenetics*. 2019;11(1):140.
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;40(26):3037-3046.
17. Louvanto K, Aro K, Nedjai B, et al. Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia. *Clin Infect Dis*. 2020;70(12):2582-2590.
18. Hernndez-Lapez R, Lorincz A, Torres-Ibarra L, et al. Methylation estimates the risk of precancer in HPV-infected women with discrepant results between cytology and HPV16/18 genotyping. *Clin Epigen*. 2019;11(1):140.
19. Vink FJ, Meijer C, Hesselink AT, et al. FAM19A4/miR124-2 methylation testing and human papillomavirus (HPV) 16/18 genotyping in HPV-positive women under the age of 30 years. *Clin Infect Dis*. 2023;76(3):e827-e834.

20. Lehtinen M, Pimenoff VN, Nedjai B, et al. Assessing the risk of cervical neoplasia in the post-HPV vaccination era. *Int J Cancer*. 2023; 152(6):1060-1068.
21. Lehtinen M, Apter D, Baussano I, et al. Characteristics of a cluster-randomized phase IV human papillomavirus vaccination effectiveness trial. *Vaccine*. 2015;33(10):1284-1290.
22. Lehtinen M, Soderlund-Strand A, Vanska S, et al. Impact of gender-neutral or girls-only vaccination against human papillomavirus-results of a community-randomized clinical trial (I). *Int J Cancer*. 2018;142(5): 949-958.
23. Lorincz AT, Brentnall AR, Scibior-Bentkowska D, et al. Validation of a DNA methylation HPV triage classifier in a screening sample. *Int J Cancer*. 2016;138(11):2745-2751.
24. Lehtinen M, Gray P, Louvanto K, Vänskä S. In 30 years, gender-neutral vaccination eradicates oncogenic human papillomavirus (HPV) types while screening eliminates HPV-associated cancers. *Expert Rev Vaccines*. 2022;21(6):735-738.
25. Vanska S, Luostarinen T, Baussano I, et al. Vaccination with moderate coverage eradicates oncogenic human papillomaviruses if a gender-neutral strategy is applied. *J Infect Dis*. 2020;222(6):948-956.
26. 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):1-13.
27. 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.
28. Zhuang H, Hong S, Zheng L, et al. Effects of cervical conisation on pregnancy outcome: a meta-analysis. *J Obstet Gynaecol*. 2019; 39(1):74-81.
29. McBride E, Marlow LAV, Forster AS, et al. Anxiety and distress following receipt of results from routine HPV primary testing in cervical screening: the psychological impact of primary screening (PIPS) study. *Int J Cancer*. 2020;146(8):2113-2121.
30. Darragh TM, Colgan TJ, Cox JT, et al. The lower anogenital squamous terminology standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *J Low Genit Tract Dis*. 2012;16(3):205-242.
31. Solomon D, Davey D, Kurman R, et al. The 2001 Bethesda system: terminology for reporting results of cervical cytology. *JAMA*. 2002; 287(16):2114-2119.

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