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INVITED REVIEW

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Assessing the risk of cervical neoplasia in the post-HPV vaccination era

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Abstract

This review is based on the recent EUROGIN scientific session: "Assessing risk of cervical cancer in the post-vaccination era," which addressed the demands of cervical intraepithelial neoplasia (CIN)/squamous intraepithelial lesion (SIL) triage now that the prevalence of vaccine-targeted oncogenic high-risk (hr) human papillomaviruses (HPVs) is decreasing. Change in the prevalence distribution of oncogenic HPV types that follows national HPV vaccination programs is setting the stage for loss of positive predictive value of conventional but possibly also new triage modalities. Understanding the contribution of the latter, most notably hypermethylation of cellular and viral genes in a new setting where most oncogenic HPV types are no longer present, requires studies on their performance in vaccinated women with CIN/SIL that are associated with nonvaccine HPV types. Lessons learned from this research may highlight the potential of cervical cells for risk prediction of all women's cancers.

KEYWORDS

cervical cancer, epigenetics, gynecological cancers, human papillomavirus, methylation

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; WID, woman's cancer risk identification.

1 | INTRODUCTION

This review is based on a recent EUROGIN main scientific session (12 April 2022) on assessing the risk of cervical cancer in the post-human papillomavirus (HPV) vaccination era. In keeping with those presentations, we wish to review the new demands and possibilities related to the management of cervical intraepithelial neoplasia (CIN): screening and triage of high-grade squamous intraepithelial lesion (HSIL)/cervical adenocarcinoma in situ (AIS) in HPV vaccinated and unvaccinated women.

The prevalence of vaccine-targeted oncogenic, high-risk (hr) HPV types is rapidly decreasing in countries with effective national vaccination programs.[1–5] Although the prevalence of nontargeted HPV types has not significantly changed the vaccination has led to changes in their relative proportions and in the overall ecological diversity of mucosal HPV types (Figure 1).[5,9–11] Test performance, most importantly positive predictive value (PPV) of conventional screening tests (Pap-smear, HPV-tests) now faces new demands of the decreasing background of the HPV types with large oncogenic potential as the majority of positive findings threaten to be false positive findings as previously illustrated[12] and most recently demonstrated.[13,14]

Increased understanding of the epigenetic changes (methylation) of both cellular and viral genes is now offering a new roadmap for cervical neoplasia triage of unvaccinated women[15–17] who have the majority of severe cervical lesions that require triage and treatment. In fact, early identification of a number of gynecological cancers is emerging via assessment of cervical cells' methylation status.[18] Fortunately, the performance of the new risk-assessment measures can now be evaluated in women, who had been vaccinated against HPV 15 years ago as early adolescents. Even if among these women the necessary causes of cervical cancer HPV types 16/18 are abolished HSIL remains found (Figure 2), and validation of methylation markers here and now is pivotal to the future use of the new epigenetic measures.

2 | EVOLUTIONARY REPERCUSSION OF HPV VACCINATION ON DEFINING THE RISK OF CERVICAL NEOPLASIA

Papillomaviruses are one of the most oncogenic viruses infecting humans with a high viral diversity and a remarkably sustained common evolutionary human-pathogen interaction history.[19,20] HPV vaccination and its current global implementation underline a quintessential need to systematically assess the likely changes in this deep evolutionary virus-host interaction. For the first time in postvaccinated populations a sizeable proportion of adolescent and early adults mostly women have developed a sustained strongly protective vaccine-induced immune response against the vaccine-targeted oncogenic hrHPVs. Moreover, with a readily achieved community-level

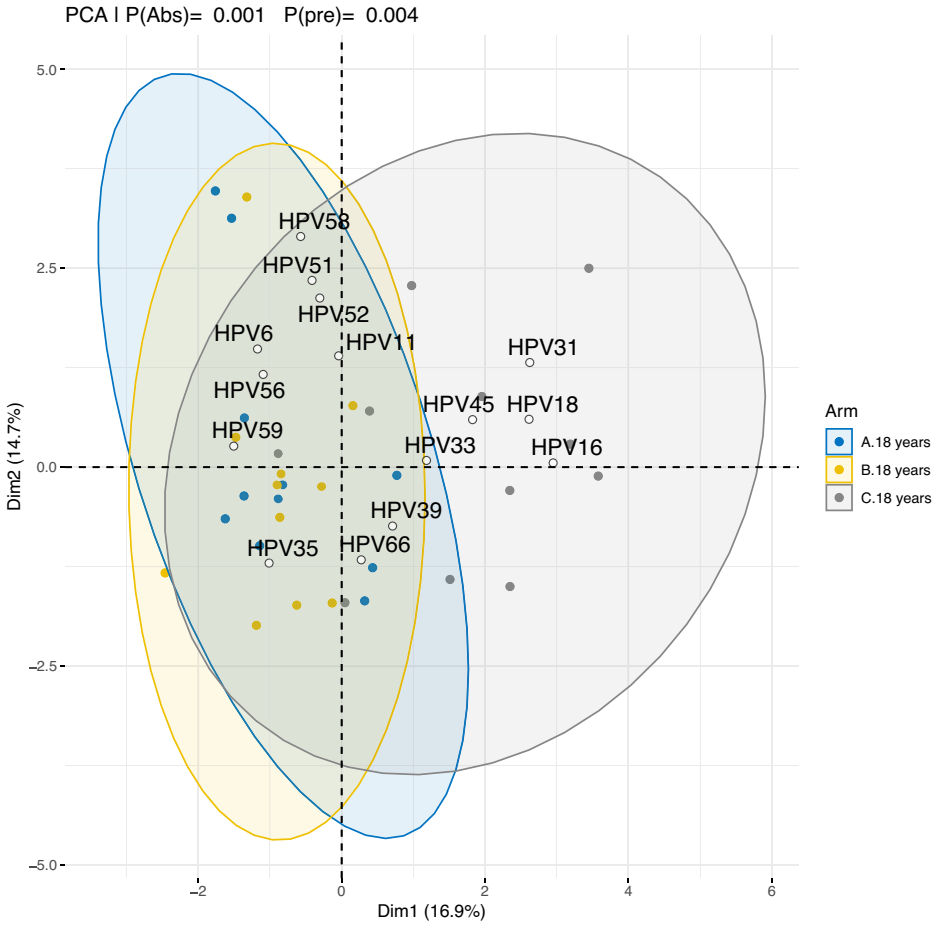

FIGURE 1 Community-level human papillomavirus (HPV) prevalence distribution visualized using ecological β-diversity analysis[6] among young 18-year-old women 4 years after community-randomized gender-neutral (A) or girls-only (B) HPV vaccination, and control communities where hepatitis B-virus vaccination was implemented (C). Arm A/B communities cluster separately from the control arm C communities mostly due to depletion of vaccine-targeted HPV types 16/18/31/45 in the intervention A and B communities but also due to differential clustering driven by the not vaccine-targeted HPV types 51/58/59. White dots represent HPV types community-level prevalence distribution in two dimensions of the dissimilarity matrix with the blue (A), yellow (C) and gray (C) dots representing each of the 11 communities in each trial arm. The elliptic circles represent the overall diversity among the gender-neutral (A) or girls-only (B) HPV vaccinated and control (C) communities, respectively. Original HPVs prevalence data has been previously described by Gray et al[7] and Louvanto et al[8]

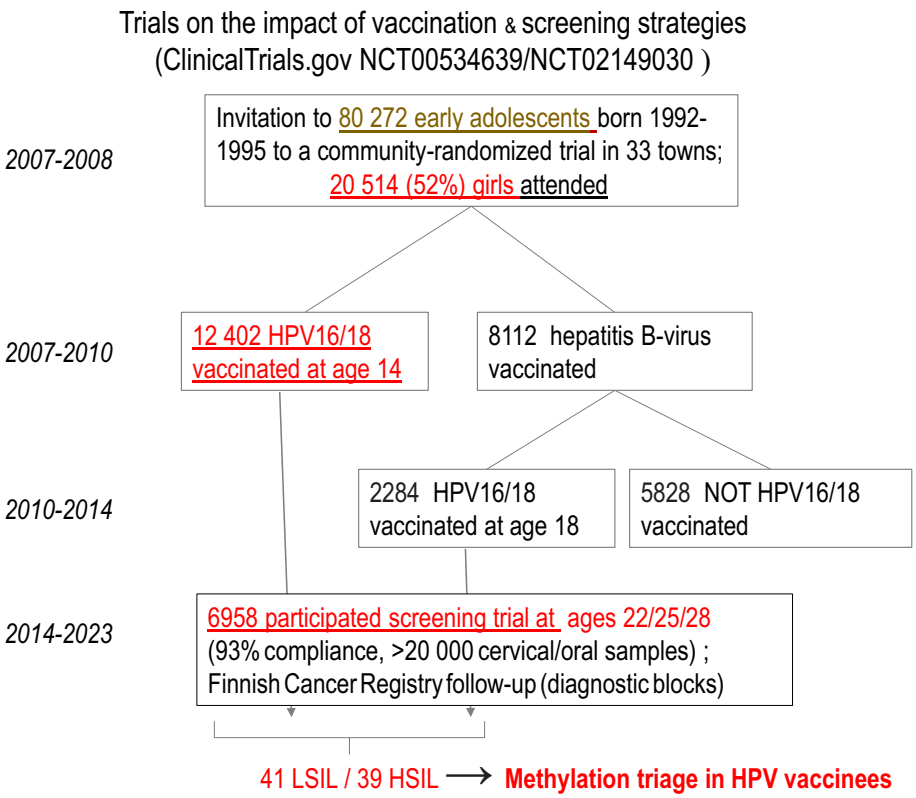

FIGURE 2 Finnish community and individually-randomized trial cohorts with population-based, country-wide human papillomavirus (HPV) vaccination and cervical screening of 1992 to 1995 birth cohorts since 2007

coverage of gender-neutral HPV vaccination the unvaccinated women and men have thus far been up to 15 years under herd protection against the targeted oncogenic HPVs.[2,3,21] This direct and indirect protection gained from gender-neutral HPV vaccination has profoundly changed the community-level diversity distribution of vaccine-targeted and nonvaccine targeted HPV types (Figure 1).

Our recent work exploiting the population-based community-randomized HPV vaccination trial data from the vaccinated Finnish birth cohorts is demonstrating the powerful population-level effects of both gender-neutral and girls-only HPV vaccination on HPV type distribution (Figure 1).[2,3,7,11,22–24] A subsequent question is: what will be the viral evolutionary response to the HPV vaccination? Rapid viral evolutionary responses have been observed most notoriously with SARS-CoV-2RNA-virus showing the emergence of new viral variants with escape mutants and higher transmissibility after vaccination. However, for DNA viruses with a slower rate of evolution and better proof-reading mechanisms such evolutionary responses are less likely and will require much more time.[25]

The theory is that host immune recognition postvaccination will favor the selection of particular virus lineages. Proportional increase of immune individuals by vaccination enhances such evolutionary selection pressures.[26] Another fundament is that such evolutionary processes depend upon genetic diversity, which is high even for the most oncogenic hrHPVs both at species and strain level.[11,19] Therefore, it has been important to systematically examine the available community-randomized HPV vaccine trial data for possible clearance patterns of vaccine-targeted HPVs ecological niche[3] and search signs of evolutionary responses of the nonvaccine targeted lower oncogenicity hrHPV types such as type replacement.[7,23]

In the postvaccination era, it will be important to explore both the ecological and epigenetic variation in infection outcome at large for HPVs. Comprehensive understanding of the changes in virus-host interaction leading to differential lesion severity and cervical HPV types in vaccinated and unvaccinated women will likely pave the way for improved methods for future screening of cervical cancer.

3 | UNDERSTANDING TEST PERFORMANCE OF CERVICAL CANCER SCREENING IN THE POSTVACCINATION ERA

As alluded to earlier, with the high vaccination coverage, cross-protection and herd immunity, HPV transmission will ultimately be kept at a minimum so that cervical cancer screening must adapt to continue to provide benefit. Along with the postvaccination changes of viral genotypes prevalence distribution mentioned above, the impact on the epidemiology of cervical dysplasia in terms of reduction in cervical abnormalities has also been reported among HPV vaccinated women.[4,27–29]

Because of the population-level impact of HPV vaccination and the decline in the prevalence of HPV-related outcomes, the pertinent question then arises: what would be the consequence on screening performance and practices as cohorts of HPV-vaccinated girls and adolescents reach the age to be screened for cervical cancer? We have previously illustrated the impact on the PPV of a future cervical

cancer screening test following reductions in precancerous lesion prevalence post-HPV vaccination.[12] We showed that even for the most optimistic scenario of test performance (99% specificity), the PPV will be so low when lesion prevalence falls below 0.16 per 1000 women (~0.02%); such positive test results will most likely be false triggering unnecessary diagnostic activities. Under such conditions, the harms from screening may then outweigh the pursued benefits. In a retrospective analysis of national datasets from 95 876 women (born 1998-1993) who attended cervical cancer screening in Scotland within 1 year of turning 20 years old, a significant reduction in the PPV of high-grade dyskaryosis for the detection of CIN2+ was observed among HPV vaccinated compared to unvaccinated women (65.7% vs 76.6%, respectively, *P*-value = .002).[30] Another ecologic study showed that, following the implementation of the HPV vaccination program in 2017 in Australia, the PPV of high-grade cytology in predicting high-grade disease decreased over time particularly for the younger age cohorts which is likely an effect of HPV vaccination.[27] Similarly, using data linkage between the Swedish National Cervical Screening Registry and the HPV vaccination registry, an 8% reduction in the PPV of high-grade cytology for CIN2+ was reported for vaccinated compared to unvaccinated women.[14]

The reduction in HPV prevalence and reduced performance of cytology as a consequence of HPV vaccination calls for rethinking of CIN triage and for new, better screening tests to improve risk stratification to triage women who are positive on screening for hrHPV types. High-risk prediction of HPV-driven cervical carcinogenesis will assist the transition to a more rational screening and management approach for cervical cancer, especially as molecular HPV testing has replaced cytology for cervical cancer screening in most high-income countries. One promising approach for the proper triage of HPV infections and associated lesions would be to rely on viral and cellular methylation markers to identify true progression potential.

Of utmost importance is the notion of screening conditional on vaccination status and the need for separate guidelines for vaccinated and unvaccinated women. Ideally integrated surveillance systems linking HPV vaccination, screening and disease outcomes would enable assessment of the impact of intervention programs and determination of the potential benefit-harm balance of these programs.

4 | METHYLATION OF COMBINED HOST AND HPV GENES AND RISK OF CERVICAL NEOPLASIA IN VACCINATED WOMEN

DNA methylation is a reproducible physical epigenetic change involved in a variety of cellular processes and plays an important role in cancer progression. Viral DNA methylation status is dynamic in the context of the viral life cycle and has been suggested as a host defense mechanism to silence viral transcription and replication. The association between hypermethylation of viral HPV genes and cervical precancer lesions and cancer has primed the development of HPV methylation biomarkers for diagnostic and triage purposes.[31] Aberrant DNA methylation of not only HPV genes but also host-cell genes has been reported to increase along with the severity of cervical lesion progression, allowing this epigenetic event to be used as a biomarker, with the potential to predict whether HPV infection will lead to CIN2+ lesion or if the infection will resolve (Figure 3).[32]

Combining the knowledge of methylation on host-cell and viral genes, the S5 classifier involves testing the levels of DNA methylation on CpGs from the host *EPB41L3* and viral genes: HPV16-L1, HPV16-L2, HPV18-L2, HPV31-L1 and HPV33-L2.[33] The *EPB41L3* gene codes for the membrane Band 4.1-like protein 3 which acts as a tumor suppressor inhibiting cell proliferation while promoting apoptosis.[34] Hypermethylation of CpG islands on the *EPB41L3* promoter leads to a decrease in gene expression, which was associated with the

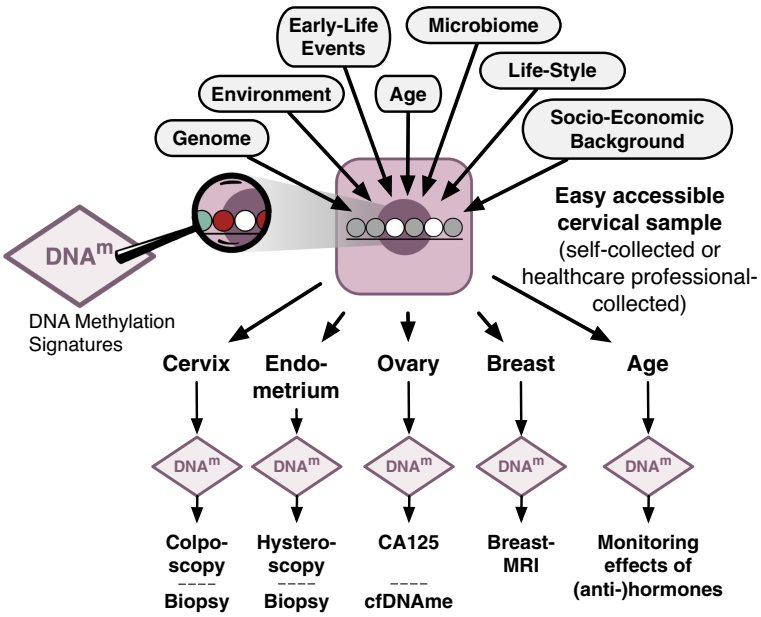

FIGURE 3 Utilizing DNA methylation signatures in easy to access epithelial cell containing cervical smear samples to predict the risk of (screen) all four women cancers

progression of multiple cancers including cervical and oropharyngeal, lung, gastric and esophageal cancer.[35–40] A recent study by Banila et al highlighted the relevance of *EPB43L1* in cancer detection as 25 out of 26 hrHPV-negative cancers (tested with multiple hrHPV-genotyping assay) were positive by S5.[17] At a cut-off of 0.80, S5 identifies more than 90% CIN3 cases and almost 100% of cervical cancers, independent of histology, FIGO stage hrHPV status.[17] In examining S5 classifier components, Banila et al[17] suggested that the relative proportion of the HPV methylation components of the S5-classifier decreased slightly with severity of lesion.[17] HPV16 methylation had the highest weight out of all viral components; however, this was 1.8 times lower than the weight of *EPB41L3* methylation in advanced cancer (CSII+) specimens.[17] This result is very important for the postvaccination era suggesting a key role for methylation analysis of host-cell genes, for example, *EPB41L3* in detecting high grade lesions and cancers. A meta-analysis on the performance of methylation assays indicated that S5 had a higher sensitivity for CIN2+ detection than considering *EPB41L3* methylation alone, without compromising specificity.[32] This indicates that the combination of host cell and viral gene targets improves the accuracy for CIN2+ detection and this will certainly hold true for vaccinated women though the value of viral genes not included in the vaccines will still need to be considered.

A triage test will be required to distinguish hrHPV-positive (nonvaccinated and vaccinated) women with clinically relevant cervical lesions from those with transient infections. The S5 has been substantially evaluated as a triage test for hrHPV-positive nonvaccinated women and has demonstrated improved triage performance compared to hrHPV genotyping or cytology alone or combined.[40–43] These observations suggest that the S5 classifier could help identify women with a high short-term risk of progression to cancer who need immediate treatment. Hernandez et al.[41] suggested that the S5 classifier could reduce colposcopy referrals by 30% to 50% without affecting sensitivity for CIN2+ and CIN3+, therefore significantly improving cost-effectiveness to allow identification of women with a true risk of cancer. In addition, S5 had the ability to distinguish between <CIN2, CIN2 and CIN3+, a finding of importance for managing CIN2, given the complexity and uncertainty associated with this diagnosis.[43]

The S5 classifier was also proven as a potential prognostic test, being able to identify women with progressive CIN2 in nonvaccinated women.[44] An improved predictive test could revolutionize the management of CIN2 as cases with progressive potential could be treated sooner and regressive cases managed expectantly. This is especially important for women in childbearing age as cervical treatments can increase the risk for preterm deliveries during pregnancies.

In the next decades, cervical cancer screening programs will have to cater for both vaccinated and nonvaccinated birth cohorts. When evaluating HPV methylation among HPV vaccinated women we need to remember the changes of HPV genotype distributions as the currently prevalent HPV genotypes among nonvaccinated women will not be detected in the future.[8] The baseline results of infrequent vs frequent cervical screening trial among women vaccinated as early adolescents, it revealed that at the age of 22-year-old, the prevalence of non HPV16/18 genotypes were extremely low (range 0.2%-2.5%) compared to the other hrHPV types with the range of 23% to 25%.[9] The role of the other hrHPV genotypes and their role in cervical carcinogenesis remain to be determined. Given the preliminary genotyping prevalence in this cohort, it is most likely that S5-score will need to be adjusted with other HPV methylation sites from genotypes that are more prevalent in HPV-vaccinated women. It is foreseeable that both vaccinated and unvaccinated women will benefit from an expansion of hrHPV methylation sites in the current S5 classifier accounting for the shift in the prevalence of HPV genotypes.

5 | METHYLATION OF CELLULAR GENES AND RISK OF CERVICAL NEOPLASIA IN VACCINATED WOMEN

An aberrant DNA methylation pattern is a hallmark of cancer cells.[45] Hypermethylation is frequently observed in transcriptional regulatory elements, such as promoters and enhancers of host-cell (tumor suppressor) genes. These host-cell DNA methylation abnormalities are necessary for the ultimate progression to cervical cancer. Methylation levels of several host-cell genes have shown to increase with increasing CIN grade and are extremely high in cervical cancer.[32,46,47]

For the well-studied host-cell methylation marker panel *FAM19A4* and *miR-124-2*, a very high methylation positivity rate was observed in cervical cancer (>98%), irrespective of histotype, FIGO stage, HPV status and geographical region of origin.[48] The high *FAM19A4/miR124-2*methylation positivity rates in cervical carcinomas were also found to be independent of hrHPV genotype,[48] suggesting that host-cell methylation analysis can similarly detect cervical cancers associated with nonvaccine targeted HPV types. Moreover, 94.7% (18/19) of hrHPV-negative cancers (as determined by multiple hrHPV assays) tested positive with the *FAM19A4/miR124-2* panel emphasizing its additional value.[48]

Within the group of high-grade CIN lesions (CIN2/3) host-cell DNA methylation patterns are heterogeneous. About half of CIN2 and three-quarters of CIN3 have a cancer-like methylation pattern.[49] It was found that CIN2/3 lesions associated with a long-term (≥5 years) HPV infection (ie, so-called advanced lesions) have significantly higher methylation levels compared to CIN2/3 lesions with a more recently acquired (<5 years) HPV infection (ie, early or incident lesions).[46,47] These findings suggest that cellular methylation positivity is characteristic of advanced cervical precursor lesions with a high short-term risk of progression to cancer.[50] This is further supported by the fact that methylation positivity of *FAM19A4* and *miR-124-2* in CIN2/3 lesions appears to be associated with increased $p16^{INK4A}$/Ki-67 immunoscores and low HPV-E4 expression[51,52] underscoring the high specificity of the *FAM19A4/miR124-2* methylation test for nonproductive, transforming CIN2/3 lesions.[52] In addition, in a prospective clinical cohort study, the absence of *FAM19A4/miR124-2* methylation was found associated with a high regression rate of CIN2/3 lesions[53] further corroborating the value of cellular methylation analysis as a biomarker that distinguishes

advanced from early lesions based on the level of epigenetic host-cell alterations. In reference to HPV vaccination, it was noted that the detection of CIN3+ by *FAM19A4/miR124-2* methylation is similar for lesions caused by HPV16/18 and those cause by other hrHPV types.[54]

In light of the above, host-cell DNA methylation markers provide a specific molecular means to detect advanced CIN lesions in need of treatment, and may well serve the needs of cervical cancer screening in the postvaccination era (Figure 3). At present, these markers have been extensively evaluated in mainly nonvaccinated cohorts reporting on a good triage performance with a pooled methylation sensitivity for CIN3+ of 71.1% (95% CI: 65.7-76.0) at a set specificity of 70%.[16,32,55–57] Retrospective longitudinal screening studies showed that HPV-positive but *FAM19A4/miR124-2* methylation-negative women had a 14-year CIN3+ risk equal to that of negative cytology triage outcome, and notably they had a lower risk for cervical cancer.[16,55–57] Recent data show that additional risk-stratification of HPV-positive women with low-grade cytological abnormalities by *FAM19A4/miR124-2* methylation could substantially reduce direct colposcopy referral rate, while retaining high CIN3+ sensitivity.[58] Altogether, these findings support the use of cellular methylation markers as an interesting new molecular means for future cervical cancer screening, and the need to evaluate their performance in cohorts of vaccinated women. The premise is that host-cell methylation positivity is low in vaccinated screening cohorts, providing a modality to limit the false-positive rate of screening by specific detection of cervical lesions in need of treatment.

6 | UTILIZING DNA METHYLATION IN CERVICAL SAMPLES, THE FUTURE OF A HOLISTIC CANCER SCREENING APPROACH

HPV vaccination is an effective means of reducing the burden of cervical cancers in fertile-aged women as HPV infection is a necessary cause of cervical cancer.[59–61] However, even persistent HPV-infection alone is not sufficient for cervical carcinogenesis and therefore one can assume that another driver of this process would be an underlying cervical field defect that is not limited to immune surveillance of persistent HPV but includes factors intrinsic to epithelial stem/progenitor cells which serve as the cell of origin for cervical cancer. Such a field defect may, for example, be reflected by a reduced ability to induce apoptosis upon HPV persistence or a reduced ability of stem cells to differentiate. Independent cervical neoplasia risk factors like smoking,[62] chlamydia,[63] long-term oral contraceptive pill use[64] or in utero exposure to specific drugs similar to Diethylstilbestrol[65] could trigger such a field defect.

It is noteworthy that cervical cancer is among the three most frequent cancers in women <44 years of age and but globally rare in women >45 years.[66] Upon oncogenic HPV infection, women harboring the field defect may be at a greater risk of developing a cervical cancer significantly earlier than they would do otherwise as up to 85% of 45-year-old women have had a genital HPV infection.[67] Hence, reducing the burden of the most common oncogenic HPV-infections with HPV vaccination might in the worst case scenario only result in pushing back the age of cervical cancer onset but not necessarily eliminating in all the overall burden of cervical cancer, assuming that the above-mentioned field defect is essential and can drive carcinogenesis in the presence of less oncogenic HPV subtypes that are not covered by current HPV vaccination strategies. Maybe 30 to 40 years after HPV-vaccination has commenced will we be able to assess this for invasive cervical cancer.

Ideal strategies utilizing would use an easy-to-access tissue sample, such as a cervical smear, and be capable of (a) monitoring the risk for cervical carcinogenesis irrespective of the presence of highly oncogenic HPV types and not reliant on morphological assessment of cervical cells: for example, we know that cytology is less informative in HPV vaccinated birth cohorts,[29] and (b) identifying women at risk for other cancers in order to guide primary and secondary preventive measures would be ideal.

We were the first to demonstrate that epigenetic analyses on self-samples are highly promising for cervical[68] and endometrial[69] cancer detection and have described epigenetic field defects preceding breast,[70] ovarian[71] and cervical[72,73] cancer. Very recently, we demonstrated that DNAme signatures derived in cervical smear samples are capable of detecting/predicting women with ovarian cancer, that is, the WID-OC test[74] and poor prognostic breast cancer, that is, the WID-BC test.[75] The WID-OC test was developed to identify/predict women with ovarian cancer, the majority of which arises from Müllerian Duct structures.[76] In line with the idea of an epigenetic field defect is the observation that the WID-OC test, which does not rely on the presence of tumor DNA in the sample, is able to identify endometrial cancer cases with a Receiver Operating Characteristic Area Under the Curve of 0.81 in samples with no detectable endometrial cancer DNA.[74] Finally, our yet unpublished data demonstrate that DNAme signatures can both detect and predict the future risk of cervical and endometrial cancer.

Aligned with the view that the cervical epithelial cells can capture and integrate risk factors at the level of the epigenome is the recent observation that the relative epithelial age (REA) assessed in cervical smear samples using the WID-REA test[77] allows the effects of hormones (ie, combined replacement therapy) and antihormones (ie, mifepristone) to be monitored. Modulation of the relative epithelial age is associated with the disease risk of organs distant to the cervix.

Cervical samples are likely to remain an essential component of screening in the post-HPV vaccination era. Various technologies (Figure 3) that do not rely on morphological assessments of cells, utilize self-samples and are able to identify women at risk of developing cervical as well as other prevalent or fatal cancers for which primary or secondary preventive measures are available, and can be implemented in the next 5 to 10 years.

7 | CONCLUSIONS

In the post-HPV vaccination era, the predictive values of currently used screening tests are declining as both cytology testing and broad

HPV testing will continue to test positive for lesions with nonvaccine HPV types with limited or even no oncogenic potential. Although the use of extended HPV genotyping that can focus on the most oncogenic HPV types may be helpful, DNA methylation can now provide an objective progression marker that can assist in predicting which lesions represent true precursors. This will be crucial for maintaining an acceptable balance between benefits and harms (sensitivity and specificity) of the screening. The fact that cervical cancer elimination is in sight does not imply that the cervical screening is about to be canceled. On the contrary, building on the effective, high attendance cervical screening program for assessing the risk also of additional cancer forms using methylation markers could open a new and innovative way for cancer prevention.

AUTHOR CONTRIBUTIONS

Matti Lehtinen: Introduction; Summary. **Ville N. Pimenoff:** Evolutionary repercussion. **Belinda Nedjai:** Methylation of HPV genes. **Karolina Louvanto:** Methylation of HPV genes. **Lisanne Verhoef:** Methylation of cellular genes. **Daniëlle A. M. Heideman:** Methylation of cellular genes. **Mariam El-Zein:** Understanding test performance. **Martin Widschwendter:** Utilizing DNAme in cervical samples. **Joakim Dillner:** Conclusions. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST

Matti Lehtinen, Ville N. Pimenoff, Belinda Nedjai, Karolina Louvanto, Lisanne Verhoef and Joakim Dillner have no conflicts of interest to declare. Mariam El-Zein holds a patent related to the discovery "DNA methylation markers for early detection of cervical cancer" registered at the Office of Innovation and Partnerships, McGill University, Montreal, Quebec, Canada (October 2018). Daniëlle A. M. Heideman is minority shareholder of Self-screen B.V., a spin-off company of VUmc; Self-screen B.V. develops, manufactures and licenses high-risk HPV and methylation marker assays for cervical cancer screening and hold patents of these tests. Martin Widschwendter is a shareholder of Sola Diagnostics GmbH, which holds an exclusive license to the intellectual property that protects the commercialization of the WID-tests.

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