



Evidence for Missing Positive Results for Human Papilloma Virus 45 (HPV-45) and HPV-59 with the SPF₁₀-DEIA-LiPA₂₅ (Version 1) Platform Compared to Type-Specific Real-Time Quantitative PCR Assays and Impact on Vaccine Effectiveness Estimates

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ABSTRACT Human papillomavirus (HPV) epidemiological and vaccine studies require highly sensitive HPV detection systems. The widely used broad-spectrum SPF₁₀-DEIA-LiPA₂₅ (SPF₁₀ method) has reduced sensitivity toward HPV-45 and -59. Therefore, anogenital samples from the PASSYON study were retrospectively analyzed with type-specific (TS) HPV-45 and -59 real-time quantitative PCR (qPCR) assays. The SPF₁₀ method missed 51.1% of HPV-45 and 76.1% of HPV-59 infections that were detected by the TS qPCR assays. The viral copy number (VCn) of SPF₁₀-missed HPV-45 and -59 was significantly lower than SPF₁₀-detected HPV-45 and -59 ($P < 0.0001$ for both HPV types). Sanger sequencing showed no phylogenetic distinction between SPF₁₀-missed and SPF₁₀-detected HPV-59 variants, but variants bearing the A6562G single-nucleotide polymorphism (SNP) in the SPF₁₀ target region were more likely to be missed ($P = 0.0392$). HPV cooccurrence slightly influenced the detection probability of HPV-45 and -59 with the SPF₁₀ method. Moreover, HPV-59 detection with the SPF₁₀ method was hampered more in nonvaccinated women than vaccinated women, likely due to a stronger masking effect by increased HPV cooccurrence in the former group. Consequently, the SPF₁₀ method led to a strong negative vaccine effectiveness (VE) of -84.6% against HPV-59, while the VE based on TS qPCR was 3.1%. For HPV-45, the relative increase in detection in nonvaccinated women compared vaccinated women was more similar, resulting in comparable VE estimates. In conclusion, this study shows that HPV-45 and -59 detection with the SPF₁₀ method is dependent on factors including VCn, HPV cooccurrence, and vaccination, thereby showing that knowledge of the limitations of the HPV detection method used is of great importance.

KEYWORDS human papillomavirus, SPF₁₀-DEIA-LiPA₂₅, unmasking, TS qPCR assay, vaccination

Persistent infection with high-risk human papillomavirus (hrHPV) is associated with multiple types of cancer, including penile, anal, vaginal/vulvar, cervical, and oropharyngeal cancer. Overall, hrHPV was responsible for 690,000 new cancer cases in 2018 (1). Cervical cancer is the most common HPV-related cancer worldwide, and approximately 70% is associated with HPV-16 and -18 (1). An additional 13 types (i.e., HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82) are labeled as hrHPV, and HPV-26, -53, and -66 are considered potentially oncogenic (2).

Prophylactic vaccination offers the best opportunity to reduce the burden of cervical

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and other HPV-related cancers (3). All currently available HPV vaccines target at least HPV-16 and -18 (4). Nevertheless, accurate monitoring of the other hrHPV types is relevant in postvaccine surveillance, as the remaining hrHPV types determine the residual cancer risk in vaccinated individuals. For example, the cumulative attribution of HPV-45 and -59 to cervical cancer globally compared to other (potential) hrHPV is 5.9% and 1.1%, respectively (5).

The broad-spectrum L1-based SPF₁₀-LiPA₂₅ version 1 method (DDL Diagnostics, the Netherlands), here called the SPF₁₀ method, is widely used in epidemiological HPV monitoring and vaccine effectiveness studies. This test utilizes a cocktail of primers to detect many of the essential hrHPV types, such as HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and -59. Furthermore, the use of generalized primers to target a broad range of HPV types reduces the total number of primers needed (6, 7).

One limitation of the SPF₁₀ method is the various HPV detection sensitivities. The limit of detection (LOD) for the included hrHPV types, as determined by the manufacturer, is lowest for HPV-18 and -33 with one DNA copy per reaction. Conversely, the LOD is highest for HPV-59 with 44 DNA copies per reaction, followed by HPV-45 with 9 DNA copies per reaction.

Recent observational studies using the SPF₁₀ method in Dutch sexual health center visitors reported high vaccine effectiveness (VE) against HPV-16 and -18 and significant cross-protection against HPV-31, -35, -45, and -52. Conversely, remarkably strong negative VE against HPV-59 was also estimated (8, 9). In light of these observations, the highly negative VE against HPV-59 was the motivation behind the in-depth analysis of HPV-59-specific detection sensitivity of the SPF₁₀ method. Furthermore, the HPV-45-specific LOD of the SPF₁₀ method is relatively high. Therefore, SPF₁₀ detection rates of these two types and the reliability of associated VE estimates require further investigation.

Here, we compared the detection rate of HPV-45 and -59 with the SPF₁₀ method with two separate HPV-45 and -59 type-specific (TS) quantitative PCR (qPCR) assays in anogenital swabs of Dutch sexual health center visitors, previously described in Woestenbergh et al. (8). We also examined the effect of several virological factors influencing HPV-45 and -59 detection with the SPF₁₀ method. Additionally, the HPV-45- and HPV-59-specific VE estimates were calculated based on the detection rates of the SPF₁₀ method and the TS qPCR assays.

MATERIALS AND METHODS

Clinical samples. For the current research, genital and anal swab samples from men and women who participated in 2009, 2015, and 2017, were selected from the biennial cross-sectional PASSYON (PApillomavirus Surveillance among STI clinic YOungsters in the Netherlands) study. The study design is described in detail elsewhere (10). Briefly, the PASSYON study started in 2009, before the introduction of the Dutch HPV vaccination program. Male and female sexual health center visitors, 16 to 24 years of age, were asked to fill in a questionnaire including vaccination status and to provide a self-collected genital swab for HPV testing. A subset of the participants also provided an anal swab. This study was approved by the Medical Ethical Committee of the University of Utrecht, the Netherlands (protocol number 08/397).

Sample preparation and SPF₁₀-based HPV genotyping. Anal and genital swabs were stored at -20°C until analysis. Then, a 200- μ l specimen was spiked with Phocine herpesvirus-1 (PhHV-1) prior to DNA extraction using the MagNA Pure 96 (MP96) platform (total nucleic acid isolation kit, Roche Diagnostics, Rotkreuz, Switzerland). The DNA was eluted in 100 μ l elution buffer. All DNA samples were tested for HPV types using the HPV SPF₁₀ LiPA₂₅ version 1 method according to the manufacturer's protocol (DDL Diagnostic Laboratory, the Netherlands). Briefly, HPV DNA was amplified using the broad-range SPF₁₀ primers. Subsequently, a DNA enzyme immunoassay (DEIA) was used for the detection of HPV DNA in all samples. In order to determine HPV genotypes, amplicons of all HPV DNA-positive samples were analyzed in the line probe assay (LiPA), which is able to identify 25 HPV genotypes, including hrHPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68 and potential hrHPV-66. The reproducibility of the SPF₁₀ method was analyzed and met the criteria of >90% identical or compatible HPV types. Validation of the SPF₁₀ method with DNA isolated with the MP96 compared to the MagNA Pure LC 2.0 was successfully performed with >92% identical genotyping results.

Type-specific real-time qPCR (TS qPCR). The viral copy number (VCn) of all DNA samples, previously isolated with the MP96, was quantified with separate HPV-45 and HPV-59 type-specific (TS) real-time quantitative PCR (qPCR) assays. TS qPCR assays for HPV-45 and -59 were developed in previous research (11, 12). Each TS qPCR assay targets a region on the capsid-encoding L1 gene and is adjusted

TABLE 1 Overview of the HPV-45 and -59 positivity rate as determined by the SPF₁₀ method and TS qPCR assays^a

| HPV | SPF ₁₀ result | No. of TS qPCR + (%) | No. of TS qPCR – (%) | Total (%) | Kappa (95% CI) ^b | McNemar's <i>P</i> |
|-----|--------------------------|----------------------|----------------------|--------------|-----------------------------|--------------------|
| 45 | SPF ₁₀ + | 104 (1.8) | 9 (0.2) | 113 (2.0) | 0.629 (0.567–0.691) | <0.0001 |
| | SPF ₁₀ – | 109 (1.7) | 5,722 (96.3) | 5,831 (98.0) | | |
| | Total | 213 (3.5) | 5,731 (96.5) | 5,944 (100) | | |
| 59 | SPF ₁₀ + | 188 (3.2) | 2 (0.0) | 190 (3.2) | 0.352 (0.314–0.389) | <0.0001 |
| | SPF ₁₀ – | 598 (10.1) | 5,156 (86.7) | 5,754 (96.8) | | |
| | Total | 786 (13.3) | 5,158 (86.7) | 5,944 (100) | | |

^a+, positive; –, negative.

^bKappa values are stated with a 95% confidence interval.

for specificity from Seaman et al. (13) and is optimized to exceed sensitivity levels of the SPF₁₀ method. For HPV-45 and -59, primers and probes were designed to target a 107-bp and a 116-bp region within the L1 gene of HPV-45 and -59, respectively (12). Briefly, 5 μ l DNA was added to 15 μ l master mix containing LightCycler 480 Probes Master (Roche), 400 nM forward primer, 400 nM reverse primer, and 100 nM probe. The TS qPCR assay was performed on the Roche LightCycler 480 platform (Roche) with cycling conditions of 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s. The specificity and sensitivity of the TS qPCRs for HPV-45 and -59 were determined previously as described by van der Weele et al. (12). Table S1 in the supplemental material shows the differences between the SPF₁₀ method and the HPV-45 and -59 TS qPCR assays.

Phylogenetic and SNP analyses of HPV-59 L1 sequences. Sanger sequencing was performed on the L1 gene of a subset of HPV-59 SPF₁₀ and/or TS qPCR positive samples. Prior to sequencing, the complete L1 gene was PCR amplified in three overlapping fragments using the primers listed in Table S2. PCR amplification and Sanger sequencing were performed similarly as described for the HPV16/18 L1 gene (14). Sequence assembly was carried out against the reference HPV-59 isolate (GenBank accession number [X77858](https://pave.niaid.nih.gov/)) retrieved from the Papilloma Virus Episteme (PAVE) database (15; <https://pave.niaid.nih.gov/>). Phylogenetic and single nucleotide polymorphism (SNP) analyses were performed with BioNumerics 7.6.3. Additionally, nine HPV-59-positive samples detected by the TS qPCR only, with a VCn above the SPF₁₀ detection limit for HPV-59, were confirmed by an external lab with the SPF₁₀ method (20 μ l SPF₁₀ product taken for LIPA instead of the standard 10 μ l), an alternative highly sensitive MPTS2 PCR Luminex assay (16), and next-generation sequencing (NGS). Three, seven, and six samples were found to be HPV-59 positive after SPF₁₀ genotyping, the PCR Luminex assay, and NGS, respectively.

Study population to measure VE estimates. In order to evaluate the potential change in HPV-45- and HPV-59-specific VE estimates, HPV-45 and -59 detection rates were analyzed with the SPF₁₀ method and the TS qPCR assays in vaccinated and nonvaccinated women. For this analysis, we selected genital samples from women eligible for vaccination (born in 1993 or later) and with a known self-reported vaccination status.

Statistical analyses. Statistical analysis was performed with R Studio 3.6.0 and GraphPad Prism 8. Cohen's κ statistic was used to assess the degree of agreement between the SPF₁₀ method and the TS qPCR assays. κ agreement levels are stated according to reference 17. In addition, McNemar's test was used for pairwise comparison of the HPV-45 and -59 positivity rates with the SPF₁₀ method or the TS qPCR assays. The Mann-Whitney U (MWU) test was used to compare VCn measurements between SPF₁₀-detected and SPF₁₀-missed HPV-45 and -59 given in copies (c) per reaction (rxn). Only VCn-positive measurements were included. Fisher's exact test was used to analyze potential differences in the presence of SNPs in SPF₁₀-missed and SPF₁₀-detected HPV-59 variants. Only the SNPs occurring in more than one sample were taken along in the analysis. The effect of cooccurring HPV types on the HPV-45 and -59 SPF₁₀ detection rate within TS qPCR positive samples was analyzed with a logistic regression model and corrected for VCn. VE was estimated as 1 – adjusted odds ratio (OR) \times 100% according to Woestenbergh et al. (8) and corrected for known demographics and sexual behavior with a logistic mixed model. A *P* value smaller than 0.05 was considered statistically significant.

Data availability. The sequences obtained during this study have been submitted to GenBank (accession numbers [MT934137](https://www.ncbi.nlm.nih.gov/nuclot/MT934137) to [MT934364](https://www.ncbi.nlm.nih.gov/nuclot/MT934364)).

RESULTS

HPV-45 and -59 detection with the SPF₁₀ method and TS qPCR assays. For this study, 5,944 genital and anal swab specimens were previously tested with the SPF₁₀ method. In total, 2.0% ($n = 113$) and 3.2% ($n = 190$) of the specimens were HPV-45 and HPV-59 positive, respectively. Reanalysis of all samples with the TS qPCR assays identified 1.7% ($n = 109$) and 10.1% ($n = 598$) additional swab specimens as HPV-45 and -59 positive, respectively. Sporadically, the SPF₁₀ method detected HPV-45 (0.2%, $n = 9$) and HPV-59 (0.03%, $n = 2$) positivity not detected by the TS qPCR assays. The agreement between the SPF₁₀ method and the TS qPCR assay was “substantial” (κ , 0.63; 95% confidence interval [CI], 0.57 to 0.69) for HPV-45 but only “fair” (κ , 0.35; 95% CI, 0.31 to 0.39) for HPV-59 (Table 1). Additionally, the results indicate a significant difference in

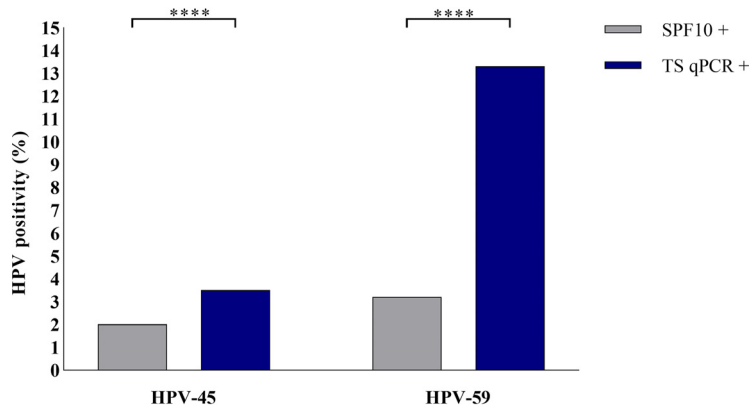


FIG 1 HPV-45 and -59 positivity rate as determined by the SPF₁₀ method and the TS qPCR assays. ****, $P < 0.0001$.

HPV-45 ($P < 0.0001$) and HPV-59 ($P < 0.0001$) detection rate between the TS qPCR assays and the SPF₁₀ method on sample level (Fig. 1).

HPV-45 and -59 VCn and SPF₁₀ detection rate. Since the LOD of the SPF₁₀ method influences the HPV detection sensitivity, HPV-45 and -59 VCn was compared between samples detected or missed by the SPF₁₀ method (Fig. 2, left and right, respectively). The median VCn of detected HPV-45 (947.5 c/rxn, $n = 104$) was significantly higher ($P < 0.0001$) than the median VCn of missed HPV-45 (4.64 c/rxn, $n = 109$). Similarly, the median VCn of detected HPV-59 (42,700 c/rxn, $n = 188$) was significantly higher ($P < 0.0001$) than the median VCn of missed HPV-59 (116 c/rxn, $n = 598$). Thus, SPF₁₀-detected HPV-45 and -59 generally have a higher VCn than SPF₁₀-missed HPV-45 and -59, although (non)detection did not always coincide with the LOD as determined by the manufacturer.

Sequencing of HPV-59 and L1 variant analysis. The difference between the detection rates of the TS qPCR assays and the SPF₁₀ method was stronger for HPV-59 than HPV-45 and served as the incentive to further analyze the authenticity of the HPV-59-positive samples. L1 sequencing was performed on a subset of HPV-59-positive samples ($n = 228$), which confirmed the presence of HPV-59. Of this subset, 43% ($n = 99$) were determined HPV-59 positive with the SPF₁₀ method and TS qPCR, and 57% ($n = 129$) were determined HPV-59 positive with only the TS qPCR. All sequenced samples had an HPV-59 VCn above the LOD of 44 c/rxn, ranging from 51.4 to 175×10^6 c/rxn, with the exception of one sample with a VCn of 33.7 c/rxn. Furthermore, the sequences were dichotomized into variant lineages A or B, based on Chen et al. (18). Lineage B (82%, $n = 187$) was more abundant than lineage A (18%, $n = 41$), although

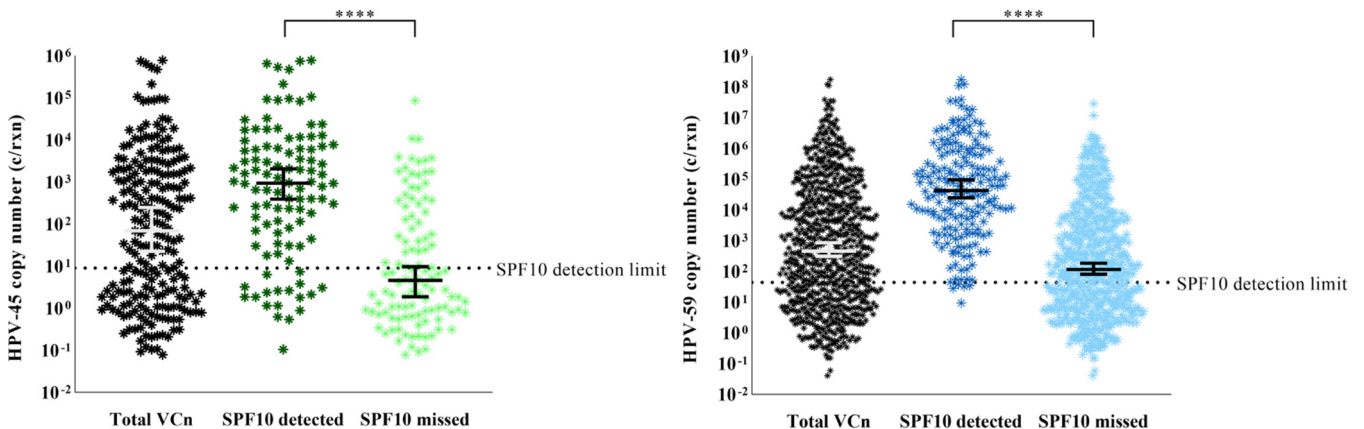


FIG 2 HPV-45 (left) and -59 (right) log VCn measurements of detected and missed infections with the SPF₁₀ method. ****, $P < 0.0001$.

TABLE 2 Overview of the SNPs in the SPF₁₀ target region of the HPV 59 L1 gene (5,606–7,132 bp) from reference strain X77858

| SNP | SPF ₁₀ missed (%) | SPF ₁₀ detected (%) | Fisher's exact <i>P</i> value |
|-----------|------------------------------|--------------------------------|-------------------------------|
| Total | 129 (100) | 99 (100) | |
| Any SNP | 28 (21.7) | 7 (7.1) | 0.0027 |
| Wild type | 101 (78.3) | 92 (92.9) | |
| T6553C | 14 (10.9) | 5 (5.1) | 0.1487 |
| Wild type | 115 (89.1) | 94 (94.9) | |
| A6562G | 14 (10.9) | 3 (3.0) | 0.0392 |
| Wild-type | 115 (89.1) | 96 (97.0) | |

no phylogenetic distinction between the SPF₁₀-detected and SPF₁₀-missed HPV-59 variants was found (Fig. S1). However, HPV-59 variants harboring any SNPs within the SPF₁₀ primer target region of L1 were significantly more likely to be missed ($P = 0.0027$) by the SPF₁₀ method compared to wild-type HPV-59 variants (100% similarity to the SPF₁₀ target region) (Table 2). Specifically, HPV-59 variants bearing the A6562G SNP were significantly more likely to be missed by the SPF₁₀ method ($P = 0.0392$). Overall, the results indicate that detected and missed HPV-59 variants are phylogenetically similar, but missed HPV-59 variants more often carry the A6562G SNP than do detected HPV-59 variants.

The effect of HPV cooccurrence on HPV-45 and -59 detection. Cooccurrence of HPV types may hamper HPV-45 and -59 detection with the SPF₁₀ method. After correcting for the effect of VCn, HPV-45 detection probability increased with additional HPV types, although not consistently with increasing number of cooccurring HPV types (Table 3). Nevertheless, the odds of HPV-45 detection significantly increased in a coinfection with one additional HPV type relative to single infections (OR, 3.87; 95% CI, 1.24 to 12.1). After pooling the samples with cooccurring HPV types, the odds of HPV-45 detection relative to single HPV-45 were 1.61 (95% CI, 0.67 to 3.88). Conversely, the probability of detecting HPV-59 consistently decreased with additional HPV types (Table 3), although this decrease was only significant with three additional HPV types (OR, 0.32; 95% CI, 0.15 to 0.70). After pooling the samples with cooccurring HPV types, the odds of HPV-59 detection relative to single HPV-59 were 0.60 (95% CI, 0.32 to 1.11). Overall, cooccurring HPV types slightly increased HPV-45 detection probability and slightly decreased HPV-59 detection probability, although this effect was not always statistically significant.

Impact of the detection method on vaccine effectiveness estimates. HPV-45 and -59 detection rates among women eligible for vaccination ($n = 1,820$) were analyzed with the SPF₁₀ method and the TS qPCR assays (Table 4). Of the eligible women, 63.4% ($n = 1,153$) were vaccinated and 36.6% ($n = 667$) were nonvaccinated. The SPF₁₀ method and the TS qPCR detected significantly more HPV-45 in nonvaccinated women (3.1% [$n = 21$] and 7.0% [$n = 47$], respectively) than vaccinated women (0.5% [$n = 6$] and 1.3% [$n = 16$], respectively) ($P < 0.0001$ for both tests). The adjusted SPF₁₀-based VE estimate against HPV-45 of 86.7% (95% CI, 64.3 to 95.1) was comparable to the TS

TABLE 3 Numbers of SPF₁₀ detected (+) and SPF₁₀ missed (–) HPV-45 and -59 in single infections (0), with 1 to ≥ 5 cooccurring HPV types and pooled samples with cooccurring HPV types (pooled)^a

| HPV type | HPV-45 | | | HPV-59 | | |
|----------|-------------------------|-------------------------|------------------|-------------------------|-------------------------|------------------|
| | SPF ₁₀ – (%) | SPF ₁₀ + (%) | OR (95% CI) | SPF ₁₀ – (%) | SPF ₁₀ + (%) | OR (95% CI) |
| 0 | 21 (19.3) | 12 (11.5) | Reference | 79 (13.2) | 22 (11.7) | Reference |
| 1 | 14 (12.8) | 24 (23.1) | 3.87 (1.24–12.1) | 129 (21.6) | 44 (23.4) | 0.70 (0.34–1.42) |
| 2 | 24 (22) | 20 (19.2) | 0.95 (0.32–2.83) | 127 (21.2) | 49 (26.1) | 0.89 (0.44–1.79) |
| 3 | 23 (21.1) | 17 (16.4) | 1.32 (0.44–3.97) | 127 (21.2) | 24 (12.8) | 0.32 (0.15–0.70) |
| 4 | 15 (13.8) | 14 (13.5) | 1.23 (0.37–4.06) | 70 (11.7) | 24 (12.8) | 0.56 (0.25–1.26) |
| ≥ 5 | 12 (11) | 17 (16.3) | 1.97 (0.59–6.57) | 66 (11) | 25 (13.3) | 0.45 (0.20–1.03) |
| Pooled | 88 (80.7) | 92 (88.5) | 1.61 (0.67–3.88) | 519 (86.8) | 166 (88.3) | 0.60 (0.32–1.11) |

^aOdds ratios (ORs) state the odds of HPV-45 or -59 detection in samples with cooccurring HPV types relative to single infections, corrected for VCn.

TABLE 4 HPV-45 and -59 positivity rate with the SPF₁₀ method and the TS qPCR assays among vaccinated and nonvaccinated women

| HPV type ^a | SPF ₁₀ | | P value ^b | TS qPCR | | P value ^b |
|-----------------------|-------------------|--------------------|----------------------|----------------|--------------------|----------------------|
| | Vaccinated (%) | Not vaccinated (%) | | Vaccinated (%) | Not vaccinated (%) | |
| 45 + | 6 (0.5) | 21 (3.1) | <0.0001 | 16 (1.3) | 47 (7.0) | <0.0001 |
| 45 - | 1,147 (99.5) | 646 (96.9) | | 1,137 (98.7) | 620 (93.0) | |
| Total | 1,153 (100) | 667 (100) | | 1,153 (100) | 667 (100) | |
| 59 + | 61 (5.2) | 19 (2.8) | 0.017 | 223 (19.3) | 127 (19.0) | 0.902 |
| 59 - | 1,092 (94.8) | 648 (97.2) | | 930 (80.7) | 540 (81.0) | |
| Total | 1,153 (100) | 667 (100) | | 1,153 (100) | 667 (100) | |

^a+, positive; -, negative.

^bA P value in bold indicates a significant difference.

qPCR-based VE estimate of 82.7% (95% CI, 68.4 to 90.5). Interestingly, the SPF₁₀ method detected significantly more HPV-59 ($P = 0.017$) in vaccinated women (5.2%, $n = 61$) than in nonvaccinated women (2.8%, $n = 19$). The TS qPCR detected similar HPV-59 positivity ($P = 0.902$) in nonvaccinated women (19.0%, $n = 127$) and vaccinated women (19.3%, $n = 223$). The adjusted SPF₁₀-based VE estimate against HPV-59 was strongly negative at -84.6% (95% CI, -218.5 to -7.0), but this changed to 3.1% (95% CI, -25.9 to 25.4) with the TS qPCR (Fig. 3). To further investigate the effect of HPV cooccurrence on the VE against HPV-59 as estimated by the SPF₁₀ method, the analyses of cooccurrence on HPV-59 detection by the SPF₁₀ method in relation to vaccination status were repeated. In general, HPV-59 detection probability was increased in vaccinated compared to nonvaccinated women in the presence of HPV cooccurrence (OR, 2.38). Conversely, no big differences between the detection probability of single HPV-59 in vaccinated compared to nonvaccinated women (OR, 0.89) was observed.

DISCUSSION

Insight into type-specific HPV prevalence and the effectiveness of HPV vaccination relies on robust, accurate, and reliable HPV detection. The SPF₁₀ method is an established standard and is commonly used in epidemiological and vaccine effectiveness studies for type-specific HPV monitoring (19–23). The assay targets a part of the HPV L1 sequences, permitting simultaneous amplification of over 50 HPV genotypes in a single test (6, 7). Here, we compared the HPV-45 and -59 detection rate of the widely used SPF₁₀ method with two type-specific qPCR assays. We showed that in anogenital samples from young Dutch visitors of sexual health centers, the SPF₁₀ method is hampered in particular for HPV-59 detection and to a lesser extent for HPV-45 detection

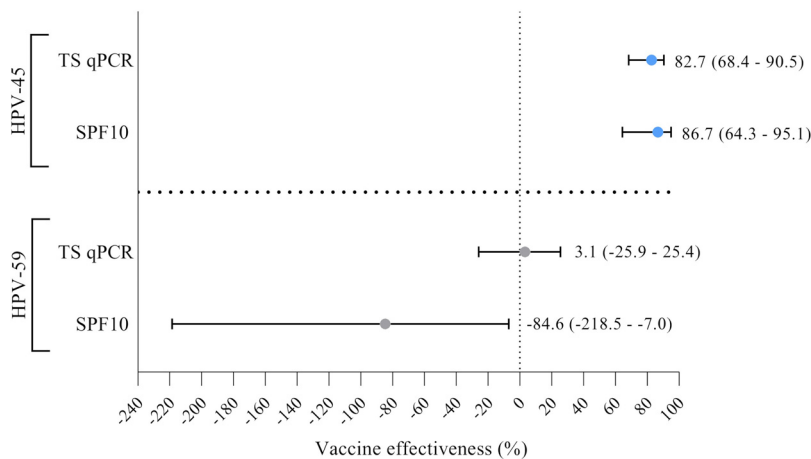


FIG 3 HPV-45 and -59 vaccine effectiveness (VE) estimates and 95% confidence intervals with the SPF₁₀ method and the TS qPCR assays after adjusting for confounders (sexual risk, behavior, and demographics).

compared to the TS qPCR assays. Consequently, the SPF₁₀ method underestimates HPV-45 and -59 prevalence, affecting HPV-45 and -59 VE estimates to various degrees. Additionally, we provided insight into the virological factors that may influence SPF₁₀ detection.

The detection sensitivity of the SPF₁₀ method was especially diminished for HPV-59 but also for HPV-45, missing 76.1% (598/786) and 51.1% (109/213) of TS qPCR assay positive results, respectively. In contrast, the TS qPCR assays missed only 8% (9/113) of SPF₁₀ HPV-45-positive results and 1.1% (2/190) of SPF₁₀ HPV-59-positive results. The lower HPV-45 and -59 detection limit of the TS qPCR compared to the SPF₁₀ method is emphasized by the significantly higher VCn of SPF₁₀-detected HPV-45 and -59 compared to the VCn of SPF₁₀-missed HPV-45 and -59. Similarly, previous research reported that HPV-16, -18, and -52 with low viral load was often missed by the clinically validated GP5+/6+ HPV detection assay, while being detected by the more sensitive SPF₁₀ method (24). Furthermore, Clifford et al. reported on the impact of low VCn on the HPV detection rate of the broad-range GP5+/6+-based PCR compared to the multiplex type-specific E7-MPG. Nevertheless, missing HPV-53, -59, and -82 was likely due to less-than-optimal PCR primer alignment (25). Contrary to Clifford et al., we observed a VCn-dependent HPV-59 detection rate with the SPF₁₀ method. Thus, differences between the sensitivity of various HPV detection tests can be caused by various issues, including virology (i.e., VCn for the SPF₁₀ method) and technical limitations (i.e., suboptimal primer alignment for the GP5+/6+-based PCR).

Interestingly, the VCn of many SPF₁₀-missed HPV-59 and several HPV-45-positive samples was above the SPF₁₀ LOD. Nonetheless, the generated HPV-59 L1 sequences (VCn above the LOD) suggest that TS qPCR-detected HPV-59 positives are genuine. Phylogenetic differences and/or the presence of SNPs might alter primer alignment efficiency, explaining the detection failure of HPV-59 with a VCn above the SPF₁₀ LOD. Although no phylogenetic distinction was found between L1 sequences of SPF₁₀-detected and -missed HPV-59 variants, more missed HPV-59 variants carried the A6562G SNP. A6562G is located 11 bases from the beginning of the SPF₁₀ target region, possibly causing less efficient SPF₁₀ primer binding. Detection failure due to primer misalignment has been observed previously for HPV-53, -59, and -82 detection with the GP5+/6+ assay (25). Likewise, GP5+/6+-impaired primer hybridization on HPV-52 was found due to a synonymous T6764C substitution (26). However, many HPV-59-positive results with a VCn above the LOD and with no variation in the SPF₁₀ target region were still missed, suggesting that additional factors influence SPF₁₀ detection performance.

Detection of HPV-45 and -59 may be hampered by cooccurring HPV types competing for PCR reagents, a process called masking. The SPF₁₀ method's use of generalized primers, which permits simultaneous amplification of multiple HPV types, increases the risk of detection failure by masking compared to the TS qPCR assays, which use type-specific primers (27–29). Our results show a trend in which SPF₁₀ detection of HPV-45 and -59 is positively and negatively impacted by HPV cooccurrence, respectively. The varying effect of HPV cooccurrence on HPV-45 and -59 detection could be partially explained by the difference in the detection sensitivity of the SPF₁₀ method. We corrected for VCn of HPV-45 and -59 but lacked information on other confounders (e.g., VCn of cooccurring HPV types). Moreover, individual factors (e.g., sexual risk behavior) or sample characteristics might explain why detection of one HPV type increases the chance of detecting HPV-45 with the SPF₁₀ method. As a result, the impact of HPV cooccurrence is different for HPV-45 and -59 detection by the SPF₁₀ method.

HPV vaccination reduces the prevalence of vaccine-targeted HPV-16 and -18 but also several nonvaccine HPV types, including HPV-45 (8). Recent studies reported that HPV-45 prevalence among 16- to 24-year-old women in the UK ranged from 2.6 to 4.2% in 2008 (prevaccination) and decreased to 0.2 to 0.9% in 2016 (postvaccination) (30, 31). A Finnish cohort study reported similar HPV-59 prevalence in vaccinated women and nonvaccinated women, ranging between 1.1 and 9.9% and 0 and 9.5%, respectively

(32). The studies conducted in the UK and Finland used alternative HPV detection methods. We show that the SPF₁₀ method-based HPV-45 and -59 prevalence in vaccinated and nonvaccinated women was within the range of that observed in the Finnish and UK studies. Nevertheless, the SPF₁₀ method underestimated HPV-45 and -59 prevalence in both vaccinated and nonvaccinated women compared to the TS qPCR assays. For HPV-45, the SPF₁₀ method and the TS qPCR showed a similar relative increase in prevalence in vaccinated women and nonvaccinated women, resulting in comparable VE estimates. Interestingly, the TS qPCR assay showed a stronger relative increase in HPV-59 prevalence in nonvaccinated women compared to vaccinated women, alleviating the highly negative VE estimate obtained by the SPF₁₀ method. In fact, the TS qPCR assay found similar HPV-59 prevalence among vaccinated and nonvaccinated women, leading to a negligible VE estimate. Previous studies also reported relative increases of HPV-45, -58, and -59 persistent infections among the nonvaccinated group compared to the vaccinated group with the MPTS12 RHA detection assay instead of the SPF₁₀ method, resulting in a large increase in VE (33). Therefore, the use of alternative HPV detection methods in addition to or instead of the SPF₁₀ method to monitor postvaccine HPV prevalence and estimate VE may be preferred for HPV types with relatively high LOD values, such as HPV-45 and -59, but may also be worth investigating for other HPV types.

The discrepant HPV-59 positivity rates determined by the SPF₁₀ method and the TS qPCR assay suggest unmasking rather than type replacement. Type replacement is the genuine increase of nonvaccine HPV types due to a reduction in vaccine-targeted HPV types after vaccination. So far, no evidence for HPV type replacement has been found, possibly due to limited follow-up since the widescale implementation of HPV vaccination (32, 34, 35). Unmasking causes removal of vaccine-targeted HPV types competing for PCR reagents, leading to detection of HPV types previously less visible, especially types with low concentrations (21, 28, 33). Our findings indicate that the higher HPV-59 prevalence in vaccinated women than nonvaccinated women determined by the SPF₁₀ method is due to unmasking. Moreover, we found that HPV-59 was missed more often in nonvaccinated women than vaccinated women with the SPF₁₀ method in the presence of cooccurring HPV types. These findings suggest that HPV-59 detection is facilitated in vaccinated women due to the (cross-)protective effects of the vaccine. Vaccinated women carry fewer coexisting HPV types with possibly lower viral load compared to nonvaccinated women (12), thereby unmasking previously less visible HPV types.

Our study has several strengths and limitations. Our study population is at high risk of HPV infection, possibly generating a higher HPV-45 and -59 prevalence compared to general populations. Vaccination status was self-reported, but previous research indicated that recall bias is likely to be limited (8). HPV genotyping using the SPF₁₀ LiPA requires the hybridization of specific probe lines and can require more than one probe line to confirm the presence of certain HPV types (for example, HPV-31). Furthermore, HPV-68 and -73 cannot be discriminated from each other. Nevertheless, these complications do not apply for HPV-45 and -59, which require single probe lines. VCn measurements are not normalized for cellular content, e.g., by quantifying β -actin with a qPCR. However, the main goal of this study was to examine the detection failures of the SPF₁₀ method in a reaction. Furthermore, retrospective testing of HPV-45 and -59 with the TS qPCR assays might have been affected by the decrease in HPV-45 and -59 DNA concentrations over time. Nevertheless, this analysis emphasizes the high detection sensitivity of TS qPCR assays, as significantly more HPV-45 and -59 was detected in relatively old samples. Also, we did not perform VCn quantifications for the cooccurring HPV types. Although beyond the scope of this article, VCn quantifications will be necessary to perform in-depth analyses of the effect of HPV cooccurrence on HPV-45 and -59 detection with the SPF₁₀ method.

In summary, we demonstrate that SPF₁₀ detection performance is limited mostly for HPV-59 and to a lesser extent for HPV-45 compared with the TS qPCR assays. Suboptimal detection by the SPF₁₀ method is influenced by low VCn, intratype genetic

variation, and HPV cooccurrence. Use of the SPF₁₀ method to monitor HPV-45 and -59 may lead to inaccuracies, especially in study populations varying in HPV prevalence, vaccination coverage, and amount of cooccurring HPV types. For HPV-59, specifically, differences in detection performance of the SPF₁₀ method compared to the TS qPCR assay resulted in the change of a highly negative VE estimate to a negligible vaccination effect. Consequently, HPV-45 and -59 detection with the SPF₁₀ method alone is insufficient and should be combined with a TS qPCR assay in order to improve the accuracy. Understanding the limitations of the HPV detection method used within a specific study population is crucial for correct test usage in epidemiological studies and vaccine surveillance.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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REFERENCES

- de Martel C, Georges D, Bray F, Ferlay J, Clifford GM. 2020. Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. *Lancet Glob Health* 8:e180–e190. [https://doi.org/10.1016/S2214-109X\(19\)30488-7](https://doi.org/10.1016/S2214-109X(19)30488-7).
- Muñoz N, Bosch FX, De Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJLM, International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 348:518–527. <https://doi.org/10.1056/NEJMoa021641>.
- Lehtinen M, Baussano I, Paavonen J, Vänskä S, Dillner J. 2019. Eradication of human papillomavirus and elimination of HPV-related diseases: scientific basis for global public health policies. *Expert Rev Vaccines* 18:153–160. <https://doi.org/10.1080/14760584.2019.1568876>.
- Gupta G, Glueck R, Patel PR. 2017. HPV vaccines: global perspectives. *Hum Vaccin Immunother* 13:1–1424. <https://doi.org/10.1080/21645515.2017.1289301>.
- Arbyn M, Tommasino M, Depuydt C, Dillner J. 2014. Are 20 human papillomavirus types causing cervical cancer? *J Pathol* 234:431–435. <https://doi.org/10.1002/path.4424>.
- Kleter B, van Doorn LJ, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, ter Harmsel B, Quint W. 1998. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human

- papillomaviruses. *Am J Clin Pathol* 153:1731–1739. [https://doi.org/10.1016/S0002-9440\(10\)65688-X](https://doi.org/10.1016/S0002-9440(10)65688-X).
7. Kleter B, van Doorn LJ, Schrauwen L, Molijn A, Sastrowijoto S, Ter Schegget J, Lindeman J, ter Harmsel B, Burger M, Quint W. 1999. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol* 37:2508–2517. <https://doi.org/10.1128/JCM.37.8.2508-2517.1999>.
 8. Woestenbergh PJ, King AJ, van Benthem BHB, Donken R, Leussink S, van der Klis FRM, de Melker HE, van der Sande MAB, Hoebe CJP, Bogaards JA, Medical Microbiological Laboratories and the Public Health Services, et al. 2018. Bivalent vaccine effectiveness against type-specific HPV positivity: evidence for cross-protection against oncogenic types among Dutch STI clinic visitors. *J Infect Dis* 217:213–222. <https://doi.org/10.1093/infdis/jix582>.
 9. Bogaards JA, Van Der Weele P, Woestenbergh PJ, Van Benthem BHB, King AJ. 2019. Bivalent human papillomavirus (HPV) vaccine effectiveness correlates with phylogenetic distance from HPV vaccine types 16 and 18. *J Infect Dis* 220:1141–1146. <https://doi.org/10.1093/infdis/jiz280>.
 10. Vriend HJ, Boot HJ, van der Sande MA, Medical Microbiological Laboratories, Municipal Health Services. 2012. Type-specific human papillomavirus infections among young heterosexual male and female STI clinic attendees. *Sex Transm Dis* 39:72–78. <https://doi.org/10.1097/OLQ.0b013e318235b3b0>.
 11. van der Weele P, van Logchem E, Wolffs P, van den Broek I, Feltkamp M, de Melker H, Meijer CJLM, Boot H, King AJ. 2016. Correlation between viral load, multiplicity of infection, and persistence of HPV16 and HPV18 infection in a Dutch cohort of young women. *J Clin Virol* 83:6–11. <https://doi.org/10.1016/j.jcv.2016.07.020>.
 12. van der Weele P, Breeuwsma M, Donken R, van Logchem E, van Marm-Wattimena N, de Melker H, Meijer CJLM, King AJ. 2019. Effect of the bivalent HPV vaccine on viral load of vaccine and non-vaccine HPV types in incident clearing and persistent infections in young Dutch females. *PLoS One* 14:e0212927. <https://doi.org/10.1371/journal.pone.0212927>.
 13. Seaman WT, Andrews E, Couch M, Kojic EM, Cu-Uvin S, Palefsky J, Deal AM, Webster-Cyriaque J. 2010. Detection and quantitation of HPV in genital and oral tissues and fluids by real time PCR. *Virol J* 7:194–117. <https://doi.org/10.1186/1743-422X-7-194>.
 14. King AJ, Sonsma JA, Vriend HJ, Van Der Sande MAB, Feltkamp MC, Boot HJ, Koopmans MPG, Medical Microbiological Laboratories and Municipal Health Services. 2016. Genetic diversity in the major capsid L1 protein of HPV-16 and HPV-18 in the Netherlands. *PLoS One* 11:e0152782. <https://doi.org/10.1371/journal.pone.0152782>.
 15. Van Doorslaer K, Tan Q, Xirasagar S, Bandaru S, Gopalan V, Mohamoud Y, Huyen Y, McBride AA. 2013. The Papillomavirus Episteme: a central resource for papillomavirus sequence data and analysis. *Nucleic Acids Res* 41:D571–D578. <https://doi.org/10.1093/nar/gks984>.
 16. van Alewijk D, Kleter B, Vent M, Delroisse J-M, de Koning M, van Doorn LJ, Quint W, Colau B. 2013. A human papilloma virus testing algorithm comprising a combination of the L1 broad-spectrum SPF₁₀ PCR assay and a novel E6 high-risk multiplex type-specific genotyping PCR assay. *J Clin Microbiol* 51:1171–1178. <https://doi.org/10.1128/JCM.02831-12>.
 17. Watson PF, Petrie A. 2010. Method agreement analysis: a review of correct methodology. *Theriogenology* 73:1167–1179. <https://doi.org/10.1016/j.theriogenology.2010.01.003>.
 18. Chen Z, Schiffman M, Herrero R, DeSalle R, Anastos K, Segondy M, Sahasrabudde VV, Gravitt PE, Hsing AW, Burk RD. 2013. Evolution and taxonomic classification of Alphapapillomavirus 7 complete genomes: HPV18, HPV39, HPV45, HPV59, HPV68 and HPV70. *PLoS One* 8:e72565. <https://doi.org/10.1371/journal.pone.0072565>.
 19. Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuid A, Zahaf T, Innis B, Naud P, De Carvalho NS, Roteli-Martins CM, Teixeira J, Blatter MM, Korn AP, Quint W, Dubin G, GlaxoSmithKline HPV Vaccine Study Group. 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomized controlled trial. *Lancet* 364:1757–1765. [https://doi.org/10.1016/S0140-6736\(04\)17398-4](https://doi.org/10.1016/S0140-6736(04)17398-4).
 20. Kreimer AR, González P, Katki HA, Porras C, Schiffman M, Rodriguez AC, Solomon D, Jiménez S, Schiller JT, Lowy DR, van Doorn LJ, Struijk L, Quint W, Chen S, Wacholder S, Hildesheim A, Herrero R, CVT Vaccine Group. 2011. Efficacy of a bivalent HPV 16/18 vaccine against anal HPV 16/18 infection among young women: a nested analysis within the Costa Rica Vaccine Trial. *Lancet Oncol* 12:862–870. [https://doi.org/10.1016/S1470-2045\(11\)70213-3](https://doi.org/10.1016/S1470-2045(11)70213-3).
 21. Wheeler CM, Castellsagué X, Garland SM, Szarewski A, Paavonen J, Naud P, Salmerón J, Chow SN, Apter D, Kitchener H, Teixeira JC, Skinner SR, Jaisamrarn U, Limson G, Romanowski B, Aoki FY, Schwarz TF, Poppe WAJ, Bosch FX, Harper DM, Huh W, Hardt K, Zahaf T, Descamps D, Struyf F, Dubin G, Lehtinen M, HPV PATRICIA Study Group. 2012. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. *Lancet Oncol* 13:100–110. [https://doi.org/10.1016/S1470-2045\(11\)70287-X](https://doi.org/10.1016/S1470-2045(11)70287-X).
 22. Geraets DT, Struijk L, Kleter B, Molijn A, van Doorn LJ, Quint WG, Colau B. 2015. The original SPF₁₀ LiPA₂₅ algorithm is more sensitive and suitable for epidemiologic HPV research than the SPF₁₀ INNO-LiPA Extra. *J Virol Methods* 215-216:22–29. <https://doi.org/10.1016/j.jviromet.2015.01.001>.
 23. Apter D, Wheeler CM, Paavonen J, Castellsagué X, Garland SM, Skinner SR, Naud P, Salmerón J, Chow SN, Kitchener HC, Teixeira JC, Jaisamrarn U, Limson G, Szarewski A, Romanowski B, Aoki FY, Schwarz TF, Poppe WA, Bosch FX, Mindel A, de Sutter P, Hardt K, Zahaf T, Descamps D, Struyf F, Lehtinen M, Dubin G, HPV PATRICIA Study Group. 2015. Efficacy of human papillomavirus 16 and 18 (HPV-16/18) AS04-adjuvanted vaccine against cervical infection and precancer in young women: final event-driven analysis of the randomized, double-blind PATRICIA trial. *Clin Vaccine Immunol* 22:361–373. <https://doi.org/10.1128/CVI.00591-14>.
 24. Hesselink AT, Van Ham MAPC, Heideman DAM, Groothuisink ZMA, Rozendaal L, Berkhof J, van Kemenade FJ, Massuger LAFG, Melchers WJG, Meijer CJLM, Snijders PJF. 2008. Comparison of GP5/6-PCR and SPF₁₀-line blot assays for detection of high-risk human papillomavirus in samples from women with normal cytology results who develop grade 3 cervical intraepithelial neoplasia. *J Clin Microbiol* 46:3215–3221. <https://doi.org/10.1128/JCM.00476-08>.
 25. Clifford GM, Vaccarella S, Franceschi S, Tenet V, Umulisa MC, Tshomo U, Dondog B, Vorsters A, Tommasino M, Heideman DA, Snijders PJ, Gheit T. 2016. Comparison of two widely used human papillomavirus detection and genotyping methods, GP5+6+-based PCR followed by reverse line blot hybridization and multiplex type-specific e7-based PCR. *J Clin Microbiol* 54:2031–2038. <https://doi.org/10.1128/JCM.00618-16>.
 26. Chan PKS, Cheung TH, Tam AO, Lo KW, Yim SF, Yu MM, To KF, Wong YF, Cheung JL, Chan DP, Hui M, Ip M. 2006. Biases in human papillomavirus genotype prevalence assessment associated with commonly used consensus primers. *Int J Cancer* 118:243–245. <https://doi.org/10.1002/ijc.21299>.
 27. van Doorn LJ, Quint W, Kleter B, Molijn A, Colau B, Martin MT, Kravang-In Torrez-Martinez N, Peyton CL, Wheeler CM. 2002. Genotyping of human papillomavirus in liquid cytology cervical specimens by the PGMV line blot assay and the SPF10 line probe assay. *J Clin Microbiol* 40:979–983. <https://doi.org/10.1128/JCM.40.3.979-983.2002>.
 28. van Doorn LJ, Molijn A, Kleter B, Quint W, Colau B. 2006. Highly effective detection of human papillomavirus 16 and 18 DNA by a testing algorithm combining broad-spectrum and type-specific PCR. *J Clin Microbiol* 44:3292–3298. <https://doi.org/10.1128/JCM.00539-06>.
 29. Tota JE, Ramanakumar AV, Villa L, Richardson H, Burchell AN, Koushik A, Mayrand M, Coutlée F, Franco EL. 2015. Evaluation of human papillomavirus type replacement postvaccination must account for diagnostic artifacts: masking of HPV52 by HPV16 in anogenital specimens. *Cancer Epidemiol Biomarkers Prev* 24:286–290. <https://doi.org/10.1158/1055-9965.EPI-14-0566>.
 30. Mesher D, Panwar K, Thomas SL, Beddows S, Soldan K. 2016. Continuing reductions in HPV 16/18 in a population with high coverage of bivalent HPV vaccination in England: an ongoing cross-sectional study. *BMJ Open* 6:e009915. <https://doi.org/10.1136/bmjopen-2015-009915>.
 31. Mesher D, Panwar K, Thomas SL, Edmundson C, Choi YH, Beddows S, Soldan K. 2018. The impact of the national HPV vaccination program in England using the bivalent HPV vaccine: surveillance of type-specific HPV in young females, 2010–2016. *J Infect Dis* 218:911–921. <https://doi.org/10.1093/infdis/jiy249>.
 32. Gray P, Luostarinen T, Vänskä S, Eriksson T, Lagheden C, Man I, Palmroth J, Pimenoff VN, Söderlund-Strand A, Dillner J, Lehtinen M. 2019. Occurrence of human papillomavirus (HPV) type replacement by sexual risk-taking behaviour group: post-hoc analysis of a community randomized clinical trial up to 9 years after vaccination (IV). *Int J Cancer* 145:785–796. <https://doi.org/10.1002/ijc.32189>.
 33. Struyf F, Colau B, Wheeler CM, Naud P, Garland S, Quint W, Chow SN, Salmerón J, Lehtinen M, Del Rosario-Raymundo MR, Paavonen J, Teixeira JC, Germar MJ, Peters K, Skinner SR, Limson G, Castellsagué X, Poppe

WA, Ramjattan B, Klein TD, Schwarz TF, Chatterjee A, Tjalma WAA, Diaz-Mitoma F, Lewis DJM, Harper DM, Molijn A, van Doorn LJ, David MP, Dubin G, HPV PATRICIA Study Group. 2015. Post hoc analysis of the PATRICIA randomized trial of the efficacy of human papillomavirus type 16 (HPV-16)/HPV-18 AS04-adjuvanted vaccine against incident and persistent infection with nonvaccine oncogenic HPV types using an alternative multiplex type-specific PCR assay for HPV DNA. *Clin Vaccine Immunol* 22:235–244. <https://doi.org/10.1128/CVI.00457-14>.

34. Gray P, Palmroth J, Luostarinen T, Apter D, Dubin G, Garnett G, Eriksson T, Natunen K, Merikukka M, Pimenoff V, Söderlund-Strand A, Vänskä S, Paaavonen J, Pukkala E, Dillner J, Lehtinen M. 2018. Evaluation of HPV type-replacement in unvaccinated and vaccinated adolescent females: post-hoc analysis of a community-randomized clinical trial (II). *Int J Cancer* 142:2491–2500. <https://doi.org/10.1002/ijc.31281>.
35. Man I, Vänskä S, Lehtinen M, Bogaards JA. 2020. Human papillomavirus genotype replacement: still too early to tell? *J Infect Dis* jiaa032:1–11. <https://doi.org/10.1093/infdis/jiaa032>.