Cross-protective vaccine efficacy of the bivalent HPV vaccine against HPV31 is associated with humoral immune responses

Results from the Costa Rica vaccine trial

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Background: We investigated the role of antibody responses as potential mechanism for the cross-protective vaccine-efficacies (VE) observed from randomized clinical trials of the HPV16/18 bivalent vaccine.

Results: HPV31 cases had lower HPV16 antibody levels than controls (OR_{4th quartile compared with 1st quartile} = 0.63; 95% CI: 0.36–1.08; p-trend = 0.03). HPV31 cases were also less likely to have detectable HPV31 neutralization, and HPV16 avidity than controls. No statistically significant differences by HPV18 antibody or HPV45 neutralization were observed among HPV45 cases and controls. Protection against HPV58 was not associated with any of the markers, confirming the specificity of our findings.

Methods: Samples are from three-dose HPV vaccine recipients from the Costa Rica HPV16/18 vaccine trial. Women with a new HPV31, HPV45, or HPV58 infections over four years of follow-up were compared with randomly selected control women—with no new infection with HPV31/45/58—with respect to HPV16 and HPV18 antibody, HPV31, HPV45, and HPV58 neutralization, and HPV16 avidity.

Conclusions: High HPV16 levels and avidity, and the ability to neutralize HPV31 were associated with protection against newly detected HPV31 infections, suggesting that the partial VE demonstrated for HPV31 is likely to be mediated at least in part through antibodies induced by HPV16/18 vaccination.

Vaccines against oncogenic human papillomaviruses (HPV) types 16 and 18 are highly immunogenic and effective at preventing infection with homologous HPV types and partially protective against infection with some heterologous HPV types phylogenetically related to HPV16/18 (1–4). Although correlates of protective immunity against infection with HPV have not yet been identified, neutralizing antibodies elicited by vaccination are postulated to be the main mediators of protection against vaccine and related HPV types.^{5–7} In addition, the role of antibody avidity i.e., antibody-antigen binding property, has not been investigated.

Because protection observed against HPV16 and HPV18 (the HPV types in the vaccine formulation) is near complete, only few vaccine failures against these homologous HPV types have been observed within randomized trials in the first few years after vaccination, making the evaluation of the mechanisms and identification of correlates of protection against HPV16 and HPV18

difficult. In contrast, efficacy observed against phylogenetically related HPV types (mainly HPV types 31, 33 and 45) has been partial and thus, failures have been observed in the first four years following vaccination. Comparing vaccinated individuals who develop an infection with the cross-related HPV types for which there is evidence of partial cross-protection to those who do not, presents an opportunity to study the immune mechanisms and correlates of protection against phylogenetically related types, and could provide insights into the mechanisms of protection against HPV16/18 afforded by this vaccine.

Here we evaluated whether postulated markers of protection against cross-related HPV types, particularly antibody levels, neutralization titers, and avidity of HPV L1 antibodies are associated with efficacy in a case-control study nested within the Costa Rica HPV16/18 (bivalent) Vaccine Trial (CVT). Specifically we compared antibody responses among vaccine failures, defined as vaccinated women with breakthrough newly detected infections

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Table 1. Comparison of selected enrollment characteristics by case-control status

	Control	31 cases	45 cases	58 cases
	n(%)	n(%)	n(%)	n(%)
Age				
18–21	62 (51.67)	45 (58.44)	23 (44.23)	58 (58.00)
22–25	58 (48.33)	32 (41.56)	29 (55.77)	42 (42.00)
Lifetime number of partners				
2-Jan	85 (70.83)	49 (63.64)	23 (44.23)	62 (62.00)
3+	35 (29.17)	28 (36.36)	29 (55.77)	38 (38.00)
New partners over follow-up period				
None	78 (65)	41 (53.95)	39 (75)	54 (54)
More than 1	42 (35)	35 (46.05)	13 (25)	46 (46)
Enrollment cervical HPV DNA infection				
Negative	85 (70.83)	36 (46.75)	20 (38.46)	50 (50.00)
16 or 18 positive	13 (10.83)	16 (20.78)	8 (15.38)	10 (10.00)
Other Carcinogenic*	14 (11.67)	21 (27.27)	20 (38.46)	32 (32.00)
Non-carcinogenic	8 (6.67)	4 (5.19)	4 (7.69)	8 (8.00)
Enrollment 16/18 Serological status				
Negative	74 (64.35)	39 (53.42)	20 (40.00)	54 (56.25)
16+ only	12 (10.43)	14 (19.18)	9 (18.00)	15 (15.63)
18+ only	10 (8.70)	5 (6.85)	5 (10.00)	11 (11.46)
16+ or 18+	19 (16.52)	15 (20.55)	16 (32.00)	16 (16.67)
Vaccination window				
			<i>Dose 1 to dose 2 (days)</i>	
Median (IQR)	38 (33 – 50)	35 (32 – 45)	40 (34 – 58)	37 (33 – 50)
			<i>Dose 2 to dose 3 (days)</i>	
Median (IQR)	133 (121 – 153)	139 (126 – 162)	134 (121 – 150)	137 (120 – 156)

*By definition does not include 31/45/58 for each of the respective cases.

over the four year follow-up period with HPV31 and HPV45 which are phylogenetically related to HPV16 and HPV18, respectively, and for which partial vaccine efficacy has been reported^{1,4} to presumably protected women i.e., vaccinated women who did not develop breakthrough infections with these HPV types over the four years follow-up period of CVT. We also include a second control group consisting of HPV58 cases. We chose HPV58 because it is phylogenetically related to HPV16 but vaccine efficacy against it has not been observed; we reasoned that its analysis would ensure that observed associations with HPV31 and HPV45 are specific to HPV types for which evidence for protection are documented.

Results

Comparison of cases and controls with respect to selected factors at enrollment are provided in Table 1. As expected, at enrollment, cases reported more lifetime number of sexual partners than controls ($p = 0.01$), and were less likely to be HPV-negative at enrollment ($p = 0.001$). There were no differences among the cases and controls by vaccination window. Figure S1 shows seroprevalence for the measured humoral immune response markers at different visits by case-control status.

Table 2 presents the results of the univariate and multivariate GEE models among HPV31 cases and controls (median levels are presented in Table S2). All multivariate models were adjusted for age, number of lifetime sexual partners and enrollment HPV16 DNA and seropositivity. In multivariate models, compared with controls, HPV31 cases had lower HPV16 antibody levels (OR 0.63; 95% CI: 0.36–1.08 comparing the 4th quartile to the lowest quartile; p -trend = 0.03). HPV31 cases were also less likely than controls to develop detectable HPV31 neutralization titers (OR = 0.68; 95% CI: 0.46–1.06). Lastly, HPV31 cases had lower HPV16 avidity levels compared with controls (OR = 0.72; 95% CI: 0.54–0.97). To account for assay variability, we also examined the association between seropositivity by all three markers and case-control status. HPV31 cases were significantly less likely to have had an immune response to all three biomarkers measured than controls (OR_{positive by all 3 markers} = 0.51, 95% CI = 0.32–0.81; p -trend = 0.002).

Table 3 presents the results of the univariate and multivariate GEE model among HPV45 cases and controls. All multivariate models were adjusted for age, number of lifetime sexual partners and enrollment HPV18 DNA and seropositivity. While we observed that HPV45 cases were also less likely to have higher HPV18 antibodies and HPV45 neutralization than controls,

Table 2. Univariate and multivariate association between markers measured and HPV31 cases and controls

HPV16 ELISA				
Titers (range within quartile)	Controls n(%)	HPV31 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Qtl 1 (1–353.67)	79 (26)	57 (30)	1	1
Qtl 2 (≥ 353.67 –969.05)	72 (23)	53 (27)	1.02 (0.63–1.65)	0.99 (0.61–1.61)
Qtl 3 (≥ 969.05 –467.08)	80 (26)	40 (21)	0.69 (0.43–1.12)	0.61 (0.38–0.97)
Qtl 4 ≥ 2467.08 –106,096.04	77 (25)	43 (22)	0.77 (0.46–1.31)	0.63 (0.36–1.08)
				p trend 0.03
HPV31 Neutralization				
	Controls n(%)	HPV31 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Seroconvert: No	138 (45)	100 (52)	1	1
Seroconvert: Yes	170 (55)	93 (48)	0.75 (0.50–1.15)	0.68 (0.46–1.06)
Positive: Below median < 77.41	95 (31)	35 (18)	0.51 (0.31–0.84)	0.52 (0.31–0.87)
Positive: Above median ≥ 77.41	75 (24)	58 (30)	1.07 (0.62–1.83)	0.89 (0.49–1.60)
HPV16 Avidity				
	Controls n(%)	HPV31 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Below median: < 1.68	141 (46)	106 (55)	1	1
Above median: ≥ 1.68	167 (54)	87 (45)	0.69 (0.52–0.93)	0.72 (0.54–0.97)
Number of markers present**				
Positive # of markers	Controls n(%)	HPV31 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
None	74 (24)	62 (32)	1	1
One marker	67 (22)	43 (22)	0.77 (0.46–1.27)	0.73 (0.44–1.20)
Two markers	74 (24)	44 (22)	0.71 (0.44–1.15)	0.59 (0.36–0.96)
Three markers	93 (30)	44 (22)	0.37 (0.48–0.87)	0.51 (0.33–0.80)
				p trend 0.002

Qtl, quartile. *All models adjusted for age, number of lifetime partners, and enrollment HPV16 DNA and serostatus. **The markers were: HPV16 antibody levels (measured by ELISA, dichotomized on the median), HPV31 neutralization potential (measured by SEAP, positive vs. negative), and HPV16 avidity (dichotomized on the median levels).

none of the effects were statistically significant and there was no evidence of dose response. Comparisons of combined HPV18 antibody levels and HPV45 neutralization also did not reveal any trends for positivity by the two markers measured.

In addition, protection against HPV58 was not associated with HPV16 or HPV18 antibody titers, HPV16 avidity or HPV58 specific neutralization, or combination of markers (Table 4).

Lastly, we examined the association between HPV16 antibody levels and HPV31 neutralization titers among HPV31 cases and controls stratified by enrollment HPV16 DNA and serostatus to investigate the presence of anamnestic response among those that were DNA-positive and seropositive at study entry. The effects we observed were similar among those who were HPV16 DNA-positive and seropositives at enrollment to those HPV16 DNA-negative and seronegatives at enrollment (data not shown), suggesting no evidence of cross-type anamnestic response during the follow-up period.

Discussion

We observed that high HPV16 antibody levels and HPV31 neutralization were associated with protection against newly

detected HPV31 infections, suggesting that the partial vaccine efficacy demonstrated for HPV31 may be in part mediated through neutralizing antibodies. In addition, to provide a more complete view of the quality and function of systemic antibodies induced by vaccination we also measured HPV16 avidity. While the contribution of affinity maturation to protection against viral infections is not well defined and controversial,^{8–10} this is the first study that reports the association of avidity with protection against infection with HPV and efficacy provided by a vaccine, suggesting a role for involvement of antibody avidity in protection afforded by this vaccine for cross-related HPV types.

Furthermore, we reasoned that since each assay may be imperfect at identifying individuals who are true responders, evaluating those who are positive for all the markers measured rather than looking at individual markers may result at better defining the subset of individuals who are the true robust responders. Specific to HPV31 cases and controls, we showed that cases were half as likely as controls to develop an immune response to all the three markers evaluated suggesting the possibility that because each of the assays has some degree of variability, particularly at lower levels of detection, evaluating all three markers in this manner made the associations we observed stronger.

Table 3. Univariate and multivariate association between markers measured and HPV45 cases and controls

HPV18 ELISA				
Titers (range within quartile)	Controls n(%)	HPV45 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Qtl 1 (1–175.11)	71 (23)	51 (22)	1	1
Qtl 2 (≥ 175.11 –486.26)	82 (27)	24 (22)	0.83 (0.44–1.57)	0.76 (0.39–1.49)
Qtl 3 (≥ 486.26 –1214.90)	81 (26)	30 (28)	1.10 (0.58–2.06)	0.80 (0.43–1.47)
Qtl 4 ≥ 1214.90 –46,734.13	74 (24)	30 (28)	1.20 (0.61–2.35)	0.91 (0.47–1.77)
				p trend 0.88
HPV45 neutralization				
	Controls n(%)	HPV45 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Seroconvert: No	152 (49)	57 (53)	1	1
Seroconvert: Yes	156 (51)	50 (47)	0.85 (0.50–1.47)	0.79 (0.44–1.39)
Positive: Below median < 28.55	78 (25)	27 (25)	0.51 (0.31–0.84)	0.92 (0.50–1.69)
Positive: Above median ≥ 28.55	78 (25)	23 (22)	1.07 (0.62–1.83)	0.79 (0.38–1.64)
Number of markers present**				
Positive # of markers	Controls n(%)	HPV45 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
None	98 (32)	32 (30)	1	1
One marker	109 (35)	40 (37)	1.12 (0.64–1.97)	1.10 (0.62–1.94)
Two markers [†]	101 (33)	35 (33)	1.06 (0.57–1.97)	0.82 (0.44–1.52)
				p trend 0.50

Qtl, quartile. *All models adjusted for age, number of lifetime partners, and enrollment HPV18 DNA and serostatus. **The markers were: HPV18 antibody levels (measured by ELISA, dichotomized on the median) and HPV45 neutralization potential (measured by SEAP, positive vs. negative). [†]HPV18 antibody and HPV45 neutralization were evaluated.

None of the markers evaluated were associated with protection against HPV58 for which there is no evidence of vaccine efficacy. This suggests that the effect we observed was specific to HPV31. We note that the numbers were modest, emphasizing the need for replication to ensure findings are not due to false positivity. To our knowledge this study represents the largest effort to date to explore the associations between antibody levels, avidity and efficacy within HPV vaccinated populations comparing women who became infected compared with women who did not become infected.

None of the markers we evaluated (HPV18 antibody levels measured by ELISA and HPV45 specific neutralization measured by SEAP) were significantly associated with protections against HPV45. This was surprising considering that the vaccine has demonstrated partial protection against HPV45. One possible explanation for the different observation with HPV31 and HPV45 is that the cross-neutralizing epitopes are in different locations in L1 and so antibodies could prevent infection by different mechanisms. From the comparison of L1 and L2 neutralizations, we know that the standard neutralization assay, and presumably the ELISA, are best at detecting antibodies that bind the outer surface of the virus as it exists in solution while L2 neutralizing antibodies do not.¹¹ Perhaps HPV31 cross-neutralizing antibodies are of the type that bind the outer surface of the virus, while the HPV45 cross-neutralizing epitopes are only exposed

after virion binding haparan sulfate proteoglycans (HSPGs) and the subsequent conformational change, i.e., more like L2 neutralizing antibodies.

Interestingly, a previous report comparing Gardasil and Cervarix showed comparable HPV45 antibody titers for both vaccines,¹² however, significant cross-protective VE for HPV45 has only been observed for Cervarix.^{1,3,4} This disconnect between antibody levels and protection, led the authors to postulate a role for CD4 T-cell effector mechanisms in protection. Unlike that report, we did not measure T-cell responses, and thus cannot address whether a different mechanism such as CD4 T-cell responses may be associated with cross-protective VE observed for HPV45. Lastly, it was interesting to note that the HPV45 case group had more risk factors (56% with ≥ 3 lifetime partners compared with 29% among the controls, 36% among the HPV31 cases and 38% among the HPV58 cases), thus suggesting the possibility that differences for HPV31 and HPV45 analysis might be explained by residual negative confounding that results in a stronger attenuation of effects for HPV45 than for HPV31.

We¹³ and others^{12,14,15} have previously shown cross-reactive immune responses (both neutralization potential and antibody levels) against HPV31 (which is phylogenetically related to HPV16) and HPV45 (which is phylogenetically related to HPV18) among participants vaccinated with the bivalent HPV vaccine. While those results indicated generation of an

Table 4. Univariate and multivariate association between markers measured and HPV58 cases and controls

HPV16 ELISA				
Titers (range within quartile)	Controls n(%)	HPV58 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Qtl 1 (1–353.67)	79 (26)	54 (23)	1	1
Qtl 2 ($\geq$ 353.67–969.05)	72 (23)	58 (25)	1.18 (0.75–1.85)	1.20 (0.75–1.89)
Qtl 3 ($\geq$ 969.05–2467.08)	80 (26)	60 (26)	1.10 (0.71–1.70)	1.05 (0.67–1.63)
Qtl 4 ($\geq$ 2467.08–106,096.04)	77 (25)	58 (25)	1.10 (0.66–1.84)	0.99 (0.59–1.66)
				p trend 0.83
HPV18 ELISA				
	Controls n(%)	HPV58 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Qtl 1 (1–175.11)	71 (23)	61 (27)	1	1
Qtl 2 ($\geq$ 175.11–486.26)	82 (27)	50 (22)	0.71 (0.44–1.14)	0.67 (0.42–1.09)
Qtl 3 ($\geq$ 486.26–1214.90)	81 (26)	60 (26)	0.86 (0.53–1.40)	0.78 (0.47–1.29)
Qtl 4 ($\geq$ 1214.90–46,734.13)	74 (24)	59 (26)	0.93 (0.54–1.58)	0.87 (0.50–1.50)
				p trend 0.77
HPV58 Neutralization				
	Controls n(%)	HPV58 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
Seroconvert: No	209 (68)	152 (66)	1	1
Seroconvert: Yes	98 (32)	78 (34)	1.09 (0.68 - 1.78)	1.06 (0.46–1.76)
Positive: Below median < 28.74	53 (17)	43 (19)	1.12 (0.64 - 1.94)	1.05 (0.59–1.85)
Positive: Above median $\geq$ 28.74	45 (15)	35 (15)	1.07 (0.55 - 2.07)	1.09 (0.53–2.22)
Number of markers present**				
Positive # of markers	Controls n(%)	HPV58 Cases n(%)	Univariate OR (95%CI)	Multivariate* OR (95%CI)
None	72 (23)	61 (27)	1	1
One marker	83 (27)	54 (23)	0.77 (0.49–1.20)	0.73 (0.46–1.15)
Two markers	117 (38)	80 (35)	0.81 (0.59–1.17)	0.75 (0.52–1.07)
Three markers	35 (11)	35 (15)	1.18 (0.66–2.12)	1.07 (0.59–1.96)
				p trend 0.57

Qtl, quartile. *All models adjusted for age, number of lifetime partners, and enrollment HPV16 DNA and serostatus. **The markers were: HPV16 antibody levels (measured by ELISA, dichotomized on the median), HPV58 neutralization potential (measured by SEAP, positive vs. negative), and HPV16 avidity (dichotomized on the median levels).

anti-HPV31 and anti-HPV45 response among vaccinated individuals, they did not address whether these responses were associated with protection from infection. The present study extends those finding by evaluating the role of quantity (titer) and function (neutralization and avidity) of antibodies in failures i.e., cases that developed an infection, compared with controls that did not contract an infection.

Although the current study represents the largest case-control study of vaccine cross-type failures, a limitation of this study is that as with other human studies, we cannot ensure that the controls (those who did not develop a new infection) were truly exposed. To address this, in addition to statistically controlling for markers of HPV exposure in multivariate models, we examined the number of new sexual partners over the follow-up; 35% of the controls and 41% of the cases reported at least one new partner over the follow-up ($p = 0.26$). Furthermore, in an effort to account for residual confounding by sexual behavior, for the HPV31 case analysis, we further performed analysis comparing the HPV31 cases to the HPV45 and HPV58 cases combined as

an alternative comparison group. Similar to our main results, for HPV16 antibody levels, we found that HPV31 cases were less likely to have higher HPV16 antibody level compared with the HPV45/58 cases (OR 0.56; 95% CI: 0.33–0.97 comparing the 4th quartile to the lowest quartile; p -trend = 0.02).

In order to ensure that the lower marker levels observed in the cases is not due to the cases having longer follow-up visits (thus lower marker levels), we compared the follow-up time among cases and controls and confirmed that the follow-up time among controls was at least as long or longer than follow-up time in cases median 381 d (IQR 313 to 667 among controls, and 367 (IQR 217 to 571) among cases) thus ensuring that the lower responses we observed for HPV31 were not due to the longer follow-up and natural decline of the antibodies over time.

In summary, this is the first study to determine an association between antibody responses and efficacy suggesting that antibodies quantitatively and qualitatively play a role in protection against infection with cross-related types. Our approach measured and provided direct information on several immunological

markers associated with vaccination-related protection against non-vaccine types, and because of lack of failures for the HPV types included in the vaccine formulations, contributed to a better understanding of the general mechanisms of protection against HPV-16/18 that could guide future work in this area.

Methods

Study population. Data are from the publicly funded, community-based randomized phase III HPV16/18 vaccine trial in Costa Rica (CVT). Details of the trial have been described elsewhere.^{4,16} Briefly, the main objectives of the trial were to evaluate the efficacy of the bivalent prophylactic HPV16/18 vaccine manufactured by GlaxoSmithKline for prevention of HPV16/18 infection and related precancerous lesions compared with women receiving a control hepatitis A vaccine. A total of 7,466 women provided written, informed consent and were randomized into the trial.

At enrollment and all visits thereafter a detailed questionnaire was administered, blood drawn, and for sexually experienced women, a pelvic exam was performed and exfoliated cervical cells were collected for cytology, and HPV DNA testing. Protocols were approved by human subjects review committees of the US National Cancer Institute and Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud (INCIENSA) in Costa Rica.

For the present evaluation, we designed a nested case-control study using samples and data from participants in the HPV vaccinated arm of the CVT who received all three HPV vaccine doses. Cases were defined as participants who were HPV31 DNA-negative (at the cervix) at enrollment and developed a newly detected, incident HPV31 infection ($n = 76$), or HPV45 DNA-negative (cervical) at enrollment and developed a newly detected, incident HPV45 ($n = 52$) infections over the four year follow-up period. We chose HPV31 and HPV45 because partial vaccine efficacy has been demonstrated for these types.¹⁴ These cases were compared with a presumably protected control group, randomly selected ($n = 120$) women who did not develop an infection with the HPV types of interest during the follow-up period. In addition, we selected a group of women who were HPV58 DNA-negative (cervical) at enrollment and developed a newly detected, incident HPV58 infection over the follow-up period ($n = 100$) to compare with the same 120 controls. We chose HPV58 because it is phylogenetically related to HPV16 but vaccine efficacy against it has not been observed; we reasoned that its analysis would ensure that observed associations with HPV31 and HPV45 are specific to HPV types for which evidence for protection are documented.

For both cases and controls, we a priori chose to test samples at 3 time points, at approximately 1 mo after the initial dose, at approximately 12 mo after the initial dose and at the visit immediately preceding detection of infection among cases and comparable time-point for controls. These time-points were selected because they represent, respectively, early immune response, responses after all three vaccine doses were received and responses proximal to when infection occurred in cases.

Laboratory methods. Samples from all cases and controls and from all time-points were tested with all the assays described below.

HPV serological measurements. ELISA. Serum was used to determine HPV16 and -18 IgG serostatus using a VLP-based direct enzyme linked immunoabsorbent assay (ELISA) that measures polyclonal antibodies as described previously.¹⁷ Antibody levels, expressed as ELISA units (EU)/mL, were calculated by interpolation of OD values from the standard curve by averaging the calculated concentrations from all dilutions that fell within the working range of the standard curve. Assay properties such as positivity cutoff, range and coefficient of variation (CVs) for HPV16 and HPV18 are presented in Table S1. CVs for both HPV16 and HPV18 were under 20% (15.7% and 9.6% for HPV16 and HPV18, respectively). Spearman's rank correlations between the various markers measured are presented in Table S3.

SEAP. Neutralization titers were determined using a pseudovirion-based secreted alkaline phosphatase neutralization assay (SEAP) (for HPV31, 45 and 58) as previously described.¹⁸ Each sample was tested in duplicate, and neutralization titers were calculated by linear interpolation and defined as the reciprocal of the dilution that caused 50% reduction in the levels of secreted alkaline phosphatase compared with control wells. The reported neutralization titers reflect the mean value of duplicate testing for each sample. Neutralization titers below our lowest dilution (1/10) were arbitrarily given a value of 5. Assay properties such as positivity cutoff, range and CV for each assay are presented in Table S1. Except for HPV45 SEAP, which had a CV of 30.4%, CVs for HPV31 and HPV58 SEAP were 12.8% and 14.4%, respectively. Spearman's rank correlations between the various markers measured are presented in Table S3.

HPV16 avidity: Modified ELISA avidity assay using chaotropic elution. The method of assessing the avidity of the anti-HPV16 antibodies was previously reported.^{19,20} We only evaluated HPV16 avidity because of the availability of particles for HPV16 for ELISA assays in our laboratory. In addition, the lower neutralization titers for the cross-related HPV types, would not allow a precise estimation for the ELISA-based avidity levels. Briefly, microtiter plates were coated with HPV16 L1 VLP and each serum sample was assayed at a dilution which yielded an absorbance reading of 1.0 ± 0.5 as previously determined in and HPV16 ELISA. Guanidine-HCl (GuHCl) was added to the samples at various concentrations (0.5 to 3.5 M) to elute low affinity antibodies. The concentration of GuHCl, which reduced the optical density by 50% compared with sample wells without GuHCl treatment, defined the Avidity Index. Samples with an HPV-16 L1 VLP ELISA of ≤ 2 EU/ml were not subsequently tested with the avidity assay and were given a value of 0 as they were deemed to be negative for the antibody. Positivity cutoff, range and CV are presented in Table S1 (6.8%). Spearman's rank correlations between the various markers measured are presented in Table S3.

HPV DNA measurements. HPV DNA-SPF₁₀/DEIA/LiPA₂₅. HPV DNA detection and genotyping were performed as described previously.^{21,22} Extracted DNA was used for PCR amplification with the SPF₁₀ primer sets. The samples were run

through an HPV DNA enzyme immunoassay (DEIA) to obtain an OD reading, and categorized as HPV DNA negative, positive, or borderline. The same SPF₁₀ amplimers were used on SPF₁₀-DEIA-positive samples to identify type-specific HPV genotype by reverse hybridization on a line probe assay (LiPA) (SPF10-DEIA/HPV LiPA25, version 1; Labo Bio-Medical Products, Rijswijk, Netherlands), which detects 25 HPV genotypes.

Statistical analysis. Cases and controls were compared with respect to enrollment characteristics using Pearson's chi-square test. Seroprevalence was defined as being positive for the marker at or above the assays' positivity cut-off determined by the laboratory. Raw values for each of the markers measured were examined for distributional properties and log transformed to normalize their distribution. Each case group was separately compared with the controls. Median levels of the various markers were compared between groups using the non-parametric Kruskal-Wallis test. Because there were multiple visits per woman, we used generalized estimating equations (GEE) models²³ to determine factors associated with being a case or control. Robust methods for logistic regression with an independent correlation structure were used to provide standard errors, adjusted by multiple observations on each woman. Model coefficients were exponentiated and interpreted as the odds ratio for a given marker/exposure among cases relative to the controls. Findings were adjusted for confounding variables of interest including age at enrollment, lifetime number of sexual partners, and HPV16/18 DNA at enrollment, and serostatus at enrollment.

We first evaluated models at visits 1, 12 and the visit before infection was detected (among the cases) and comparable timepoints for controls. Because analyses stratified by timepoint yielded similar results (data not shown), we chose to present results from models that incorporate all visits to maximize study power.

We compared the following exposures between cases and controls:¹ HPV16 and HPV18 antibody levels (measured by ELISA) categorized into quartiles;² HPV16 avidity dichotomized at the median due to the narrower dynamic range of the assay;³ HPV31, HPV45, and HPV58 neutralization titers (measured by SEAP) dichotomized as positive or negative, with further dichotomization of positives based on the median levels observed.

Because HPV31 is phylogenetically closely related to HPV16, we compared results of HPV16 antibody levels, HPV16 avidity, and HPV31 neutralization titers among HPV31 cases and controls. Similarly, because of the relatedness of HPV45 and HPV18, we compared HPV18 antibody levels, and HPV45 neutralization for the HPV45 cases and controls. Lastly, we evaluated HPV16 and HPV18 antibody levels, HPV16 avidity, and HPV58 neutralization titers among the HPV58 cases and controls.

Because among the cases there were breakthrough infections at the 6 mo visit (i.e., before all vaccine doses were administered), we also performed an analysis restricting to infections that occurred at or after their month 12 visits (thus approximately 6 mo after receipt of all three vaccine doses; this resulted in data being available from 65% of the cases), using only those follow-up visits after the 12 mo visits (this represented 50% of

the visits). Because results from this sub-analysis were similar to those observed overall, we chose to present results from the overall analysis herein.

All analyses were computed using Intercooled Stata, version 10 (Stata Corp, College Station, Texas).

Conflict of Interest

D.R.L. and J.T.S. are named inventors on US government-owned HPV vaccine patents that are licensed to GlaxoSmithKline and Merck and are entitled to limited royalties as specified by federal law.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/vaccines/article/24340

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