



Evaluation of systemic and mucosal anti-HPV16 and anti-HPV18 antibody responses from vaccinated women

Troy J. Kemp^a, Alfonso García-Piñeres^{a,1}, Roni T. Falk^b, Sylviane Poncelet^c, Francis Dessy^c, Sandra L. Giannini^c, Ana Cecilia Rodriguez^d, Carolina Porras^d, Rolando Herrero^d, Allan Hildesheim^b, Ligia A. Pinto^{a,*},
for the Costa Rica Vaccine Trial (CVT) Group²

^a HPV Immunology Laboratory, SAIC-Frederick Inc., NCI-Frederick, Building 469, Room 120, Frederick, MD 21702, USA

^b Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

^c Research and Development, GlaxoSmithKline Biologicals, Rue de l'Institut 89, 1330 Rixensart, Belgium

^d Proyecto Epidemiológico Guanacaste, San José, Costa Rica

ARTICLE INFO

Article history:

Received 12 February 2008

Received in revised form 21 April 2008

Accepted 29 April 2008

Available online 19 May 2008

Keywords:

Human papillomavirus

Neutralization assay

Cervical secretions

ABSTRACT

Ideal methods to monitor HPV neutralizing antibodies induced by vaccination have not been established yet. Here, we evaluated systemic and cervical antibody levels induced by HPV16/18 AS04-adjuvanted vaccine (GlaxoSmithKline Biologicals) using a secreted alkaline phosphatase neutralization assay (SEAP-NA) and enzyme-linked immunosorbent assay (ELISA). Serum and cervical secretions from 50 vaccinated women were used to assess (1) overall assay reproducibility; (2) inter-assay and inter-specimen correlation; (3) correlations between month 1 and month 12 titers. Strong correlations between SEAP-NA and ELISA were observed (serum anti-HPV16/18, $\rho = 0.91/0.85$; cervix anti-HPV16/18, $\rho = 0.84/0.89$). Systemic and cervical antibody measures also correlated well (ρ range: 0.64–0.75); except at mid-cycle (ρ range: 0.28–0.65). Correlations between antibody levels at 1 and 12 months following the start of vaccination were poor (ρ range: 0.16–0.38). In conclusion, HPV16/18 VLP-based ELISA is a reliable and valid method to monitor anti-HPV16/18 neutralizing potential for the first year following vaccination; however, additional studies will be required to better define the effects of the time on cycle and patterns of antibody response over time following vaccination.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Of the 40 genital tract associated human papillomaviruses, approximately 15 have been classified as being 'high risk' for cervical oncogenesis [1,2]. In particular, HPV16 and HPV18 are associated with over 70% of all cervical cancer cases worldwide [1,3–5]. Due to the high prevalence of HPV16 and HPV18 in cervical cancer, HPV vaccine research has led to the development of prophylactic vaccines against HPV16 and HPV18. HPV L1 virus-like particle (VLP) vaccines have demonstrated excellent safety, immunogenicity and protection against infection by homologous HPV types [6–13]. Based on several pre-clinical models, neutralizing antibodies are expected to be the primary immune mechanism for protection

against persistent HPV16/18 infection in women vaccinated with HPV L1 VLP-based vaccine [14,15]. However, ideal methods to measure neutralizing antibodies, either systemic or local, have not been established yet.

Several assays have been developed to monitor antibody responses to HPVs, including competitive radioimmunoassay (cRIA) [16], competitive luminex immunoassay (CLIA) [17], secreted alkaline phosphatase neutralization assay (SEAP-NA) [18] and enzyme-linked immunosorbent assay (ELISA) [19]. With a few exceptions [18,20], these assays have not been directly compared. L1 VLP-based ELISA has been extensively used in studies of HPV epidemiology and vaccinology [6,19,21–24]. Generally, ELISAs are a fast and highly reproducible method for quantifying humoral responses against specific viral antigens, but the quantitation is independent of neutralizing activity. Previous studies examined the correlation between anti-HPV16 ELISA titers and neutralizing potential using the traditional focal transformation assay and found a good correlation ($\rho = 0.85$) between the two assays in a small group of vaccine recipients ($n = 9$), suggesting that the ELISA titers might be a

* Corresponding author. Tel.: +1 301 846 1766; fax: +1 301 846 6954.

E-mail address: lpinto@ncifcrf.gov (L.A. Pinto).

¹ Current address: Escuela de Química, Universidad de Costa Rica.

² See Appendix A.

surrogate for measuring neutralization titers [6]. The SEAP-NA was developed as a scalable and sensitive method of monitoring neutralization titers following vaccine and natural infection with HPV [18]. In contrast to the L1 VLP-based ELISA, which measures levels of HPV type-specific immunoglobulins (Ig), IgG or IgA, the SEAP-NA detects total neutralization activity, which may prove to be a more relevant measure of responses in prophylactic vaccine studies.

Immunogenicity evaluation in HPV vaccine trials have relied largely on serological samples, yet the samples most likely to be relevant to protective immunological activity are cervical samples, where infection occurs. A study performed by Nardelli-Haeffliger et al. examined levels of anti-HPV16 antibody at the cervix following vaccination and found that cervical HPV16 type-specific antibodies range from 0.5 to 50% of systemic levels [25]. It is currently unclear whether systemic measures of post-vaccination immunity can serve as a good surrogate for local levels of immune protection at the cervix.

Serum and cervical secretions collected from a subgroup of women, who are part of a large community-based trial of an HPV16/18 AS04-adjuvanted vaccine (GlaxoSmithKline Biologicals (GSK Bio) vaccine candidate) in young women in Costa Rica were used to evaluate the reproducibility of the ELISA and SEAP-NA. We also evaluated the correlations between these two assays and between specimen types (serum and cervical secretions). Finally, we determined whether anti-HPV16 and anti-HPV18 antibody titers induced by vaccination were predictive of each other, and whether responses observed following a single vaccine dose was predictive of levels observed following the full three-dose series.

2. Materials and methods

2.1. Participants

7466 women participated in the double blind, controlled, randomized, phase III study of the efficacy of an HPV16/18 VLP vaccine in Costa Rica. Details of recruitment and study design have been published [26]. Briefly, participants were randomized to receive either an HPV16/18 AS04-adjuvanted vaccine (GSK Bio, Rixensart, Belgium) or a control hepatitis A vaccine (GSK Bio) at 0- (enrollment), 1- and 6-month schedule. In addition, a follow-up visit occurred 12 months following enrollment. Pelvic evaluation was performed for all sexually experienced women at enrollment and 12-month visit, at which time cervical secretions were collected. Blood was also collected from participants at each visit. All participants provided informed consent, and the trial was approved by human subjects review committees at the National Cancer Institute (NCI) and Costa Rica.

For the present study, a subgroup of women ($n=50$), who were scheduled to receive three doses of the HPV16/18 AS04-adjuvanted vaccine, were randomly selected based on the following criteria: ≥ 12 months of follow-up since enrollment, successful collection of serum at months 0, 1 and 12, and successful collection of cervical secretions at months 0 and 12. Although specimens were collected on all 50 women, four women did not receive the scheduled three doses of the vaccine (1 dose, $n=1$; 2 doses, $n=3$). Data about oral contraceptive (OC) use and time since participant's last menstrual period (LMP) were collected. All testing was performed blinded and data was sent to the trial Data Management Center (Information Management Services, IMS; Silver Spring, MD) for statistical analysis, under the direction of the study statistician (RF).

2.2. Serum

Testing was performed on serum collected at months 1 and 12 from the 50 women selected for this analysis (Table 1). In addition, pre-vaccination specimens from 5 of these 50 women were also tested (month 0). Serum from each woman at each visit (month 0 ($n=5$), month 1 ($n=50$) and month 12 ($n=50$)) was aliquoted and distributed to both laboratories (NCI and GSK Bio). Serum samples were randomly batched in order to allow for testing of each woman's visit by two different technicians on 2 different days. For the NCI SEAP-NA assay, the laboratory measured each sample in triplicate; for the GSK Bio ELISA, samples were tested in monoplicate because intra-assay variability was previously estimated during assay validation. Results from previously performed validation studies at GSK Bio to assess intra-assay variability of the GSK Bio ELISA among a panel of 16 sera tested by two technicians on 4 different days indicated that the intra-assay (within plate) coefficients of variation (CV) were 5.9 and 11.9% for HPV16 and HPV18 ELISA, respectively; whereas the inter-assay (different days, different technicians) CV were 13.9 and 16.5% for anti-HPV16 and anti-HPV18 ELISA, respectively.

2.3. Cervical secretions

Polyvinyl acetate-based Merocel™ (Medtronic Ophthalmics, Jacksonville, FL) ophthalmic sponges were used in the collection of cervical material. At pelvic exam, the clinician rested the sponge within the os of the cervix for 30 s; the sponge was removed and placed into a 10 ml tube for storage in liquid nitrogen. After shipment to the USA, the samples were stored at -70°C . Sponge weights were recorded, then extracted in a buffer containing PBS (Invitrogen, Grand Island, NY), 256 mM NaCl (Sigma-Aldrich, St. Louis, MO) and 100 $\mu\text{g/ml}$ Aprotinin (Sigma-Aldrich, St. Louis, MO). 300 μl of cervical extraction buffer was slowly added to the top of the sponge. Following 30 min incubation at 4°C , the sponges were centrifuged at $13,000 \times g$ for 15 min at 4°C . An additional 300 μl of extraction buffer was added to each Merocel™ sponge and immediately centrifuged at $13,000 \times g$ for 15 min at 4°C . Then, 5 μl of extract was tested for hemoglobin using Hemastix™ (Bayer Corporation, Elkhart, IN) prior to the addition of 4 μl of FBS. The extracts were aliquoted and frozen at -70°C until further testing.

Testing was performed on cervical secretions collected at month 12 ($n=50$) as well as a subset of five women at month 0 (Table 1). Cervical secretions were tested in a similar manner as described above for the serum testing and as outlined in Table 1. A dilution factor was used for normalization as described previously [27]. Eight samples were excluded from final analyses due to a lower weight than that of control sponges. In order to account for the variation of the IgG levels in the cervical secretions during the menstrual cycle, total IgG was measured by ELISA (Cygnus Technologies Inc., Southport, NC) in the cervical secretions and serum. Results reported herein were not normalized to total IgG because such normalization was found not to alter findings (data not shown).

2.4. Anti-HPV16 and anti-HPV18 ELISA

Quantitation of anti-HPV16/18 VLP IgG antibodies in serum or in cervical secretion extracts was performed by GSK Bio as described previously with slight modifications [10]. Briefly, HPV16 and HPV18 L1 VLPs were measured separately by adsorbing either HPV16 (270 ng/100 μl) or HPV18 (270 ng/100 μl) L1 VLPs as coating antigens at 4°C onto 96-well microtiter plates (Maxisorp, Nunc). Next, the plates were blocked with PBS containing 4% non-fat dry

Table 1
Summary of testing format by laboratory, assay and specimen type

Lab	Assay	HPV type	Specimen type	Specimen collection (months)	Total number of specimens (per time point)	Study design (number of technicians × number of days)	Number of replicates
NCI	SEAP-NA	HPV16	Serum	0/1/12	105 (5/50/50)	2 × 2	Triplicate
	SEAP-NA	HPV18	Serum	0/1/12	105 (5/50/50)	2 × 2	Triplicate
	SEAP-NA	HPV16	Cervical secretions	0/12	55 (5/50 ^a)	2 × 2	Triplicate
	SEAP-NA	HPV18	Cervical secretions	0/12	55 (5/50 ^a)	2 × 2	Triplicate
GSK Bio	SEAP-NA	HPV16	Cervical secretions	0/12	55 (5/50)	1 × 1	Singlet
	SEAP-NA	HPV18	Cervical secretions	0/12	55 (5/50)	1 × 1	Singlet
	ELISA	HPV16	Serum	0/1/12	105 (5/50/50)	2 × 2	Singlet
	ELISA	HPV18	Serum	0/1/12	105 (5/50/50)	2 × 2	Singlet
	ELISA	HPV16	Cervical secretions	0/12	55 (5/50 ^a)	2 × 2	Singlet
	ELISA	HPV18	Cervical secretions	0/12	55 (5/50 ^a)	2 × 2	Singlet

^a Eight cervical secretion samples at month 12 were excluded from final analyses due to sponge weights lower than control sponges.

milk and 0.05% Tween 20. Serum samples were serially diluted in the blocking solution starting at 1/100 in 2-fold increments up to 12,800 and cervical secretion samples were serially diluted starting 1/10 followed by seven 2-fold dilutions in the blocking solution. Following a washing step, serial dilutions of samples, standard, and controls were added to the microtiter plates. After washing the plates, horseradish peroxidase-conjugated goat anti-human IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added. Following another wash step, TMB (tetramethylbenzidine) was added to each of the wells. 0.36 N H₂SO₄ was used to stop the reaction and read at 450/620 nm. ELISA titers were calculated from a reference curve, derived from a reference pool of serum samples from human vaccinees, using a four parameter equation from Soft-Max Pro (Molecular Devices, Sunnyvale, CA, USA) and expressed as EU/ml.

2.5. HPV16 and HPV18 SEAP-NA

2.5.1. Cell culture

293TT cells were expanded and cultured as previously described [18].

2.5.2. SEAP-NA

The SEAP-NA was performed by NCI and GSK Bio as previously described with a few modifications [18]. Briefly, 293TT cells were seeded in 96-well flat bottom plates at a cell density of 3×10^4 cells/well and incubated at 37 °C and 5% CO₂ for ≥2 h prior to the addition of controls and diluted samples. 25 μl of serially diluted serum or cervical secretion (diluted 1/10 in fourfold increments up to 1/163,840 and tested in triplicate) was incubated with 100 μl of HPV16/18 pseudovirion at 4 °C for 1 h. Next, the samples were transferred to the 293TT cells and incubated at 37 °C for 72 h. Following incubation, supernatants were clarified by centrifugation, then transferred to 96-well plates and frozen at –70 °C until further testing. The Great EscAPe SEAP assay kit was used according to the manufacturer's protocol (BD Biosciences-Clontech Laboratories Inc., Mountain View, CA). Serum and cervical secretion neutralization titers were calculated by linear interpolation and defined as the reciprocal of the dilution that caused 50% reduction in SEAP activity compared to control wells. SEAP-NA was tested at a second lab (GSK Bio: in singlet by one technician on 1 day, Table 1) to assess inter-laboratory results using cervical secretions. The inter-laboratory correlations for anti-HPV16 and anti-HPV18 antibody titers were $\rho = 0.95$ and 0.96, respectively. Due to the significant correlations between laboratories, SEAP-NA results from NCI laboratory were used for all analyses.

2.6. Statistical analysis

Results were analyzed on the logarithmic scale using a nested analysis of variance model. The components of the models were modified to reflect the difference in study design between the laboratories (i.e. triplicate (SEAP) vs. singlet (ELISA) measurements of each sample).

For the NCI SEAP-NA the model was as follows:

$$\log(Y_{ijkl}) = \mu + a(i) + bj(i) + ck(i) + \varepsilon l(ij),$$

where Y_{ijkl} denotes the antibody measurement for woman i on analysis day $j(i)$ ($j = 1, 2$), by technician $k(i)$ ($k = 1, 2$) and replicate $l(ijk)$ ($l = 1, 2, 3$); each are independent variables with variances: $\sigma^2 a$ (woman); $\sigma^2 b$ (day); $\sigma^2 c$ (technician); σ^2 (error).

For the GSK Bio ELISA, with assays run in singlet, the following model was used:

$$\log(Y_{ijk}) = \mu + a(i) + bj(i) + \varepsilon(ij),$$

where $a(i)$ corresponds to woman, $bj(i)$ to day and $\varepsilon(ij)$ to technician with variances $\sigma^2 a$ (woman), $\sigma^2 b$ (day) and $\sigma^2 e$ (technician). Restricted maximum likelihood estimates of the variance components were obtained using the SAS procedure PROC VARCOMP [28] and were used to derive the CV and the intraclass correlation coefficient (ICC) coefficient. The CV is the population standard deviation of a measurement divided by its mean. An application of the delta method demonstrates that the sum of the variance components associated with day, technician and replicate is a good estimate of the square of the overall CV [29,30]. The ICC was estimated by comparing the variance component associated with women to the sum of all components ($ICC = (\sigma^2 a / (\sigma^2 a + \sigma^2 b + \sigma^2 c + \sigma^2))$).

For comparisons across assays, specimen types, HPV type-specific antibody measures, or time, the mean of the woman's assay values was used to compute Spearman's correlation coefficients.

3. Results

3.1. ELISA and SEAP-NA variability

Assay performance was initially assessed by analyzing the components of variability for each assay. For the HPV16/18 VLP-based ELISA, the overall CVs were 11/12% for serum and 22/18% for cervical secretions (Table 2) while the ICCs were >97% for both specimens. For the SEAP-NA, the overall CVs for anti-HPV16/18 titers were 40/34% for serum and 31/29% for cervical secretions, and the ICCs were >92% for both specimens (Table 2). The magnitude of the overall CV results for the SEAP-NA is largely due to variability of replicate

Table 2
CVs and ICC by laboratory and HPV type

Lab	Specimen type	HPV type	Assay	CV (%)	ICC (%)
NCI	Serum	HPV16	SEAP-NA	39.7	96.5
	Serum	HPV18	SEAP-NA	33.9	97.0
	Cervical secretions	HPV16	SEAP-NA	30.8	93.3
	Cervical secretions	HPV18	SEAP-NA	29.5	92.9
GSK Bio	Serum	HPV16	ELISA	11.4	99.4
	Serum	HPV18	ELISA	12.5	99.2
	Cervical secretions	HPV16	ELISA	22.2	97.6
	Cervical secretions	HPV18	ELISA	18.2	98.6

samples tested in the same day (data not shown) and secondarily to operator variability. The ICCs were greater than 92% using either assay with both specimens, so the amount of variability seen between women was far greater than that observed by either assay. In addition, we observed comparable ICCs in analyses stratified by time (months 1 and 12; data not shown).

3.2. Correlations between ELISA and SEAP-NA with serum and cervical secretions

Analyses were performed to assess correlations between assays, and to evaluate whether ELISA measurements of anti-HPV16 and anti-HPV18 IgG type-specific antibodies reflect the neutralizing

activity in sera and cervical secretions of vaccine recipients. The Spearman correlation between anti-HPV16 ELISA antibody titers and anti-HPV16 SEAP-NA in serum was 0.91 ($p < .0001$; Fig. 1A), while the correlation in cervical secretions was 0.84 ($p < .0001$; Fig. 1C). The correlations between both assays for anti-HPV18 titers in serum and cervical secretions were 0.85 ($p < .0001$; Fig. 1B) and 0.89 ($p < .0001$; Fig. 1D), respectively.

3.3. Correlations between serum and cervical secretion measures

In order to assess whether serum measures are good surrogates of antibody levels at the mucosa, we compared serum anti-HPV16/18 antibody levels with the cervical secretion antibody levels. As depicted in Fig. 2A and B, the correlation was $\rho = 0.73$ ($p < .0001$) for anti-HPV16 and $\rho = 0.75$ ($p < .0001$) for anti-HPV18 ELISA. For the SEAP-NA, correlations were $\rho = 0.74$ ($p < .0001$) and $\rho = 0.64$ ($p < .0001$) for anti-HPV16 and anti-HPV18, respectively (Fig. 2C and D).

Additional analyses were performed to examine the influence of OC-use on the correlation between serum and cervical secretion measures. Similar correlations were observed within OC-use strata for ELISA anti-HPV16 and anti-HPV18 antibody measures (OC vs. non-OC users; $\rho = 0.74$ vs. $\rho = 0.70$, anti-HPV16 and $\rho = 0.74$ vs. $\rho = 0.69$, anti-HPV18). There were comparable findings for the SEAP-NA (data not shown).

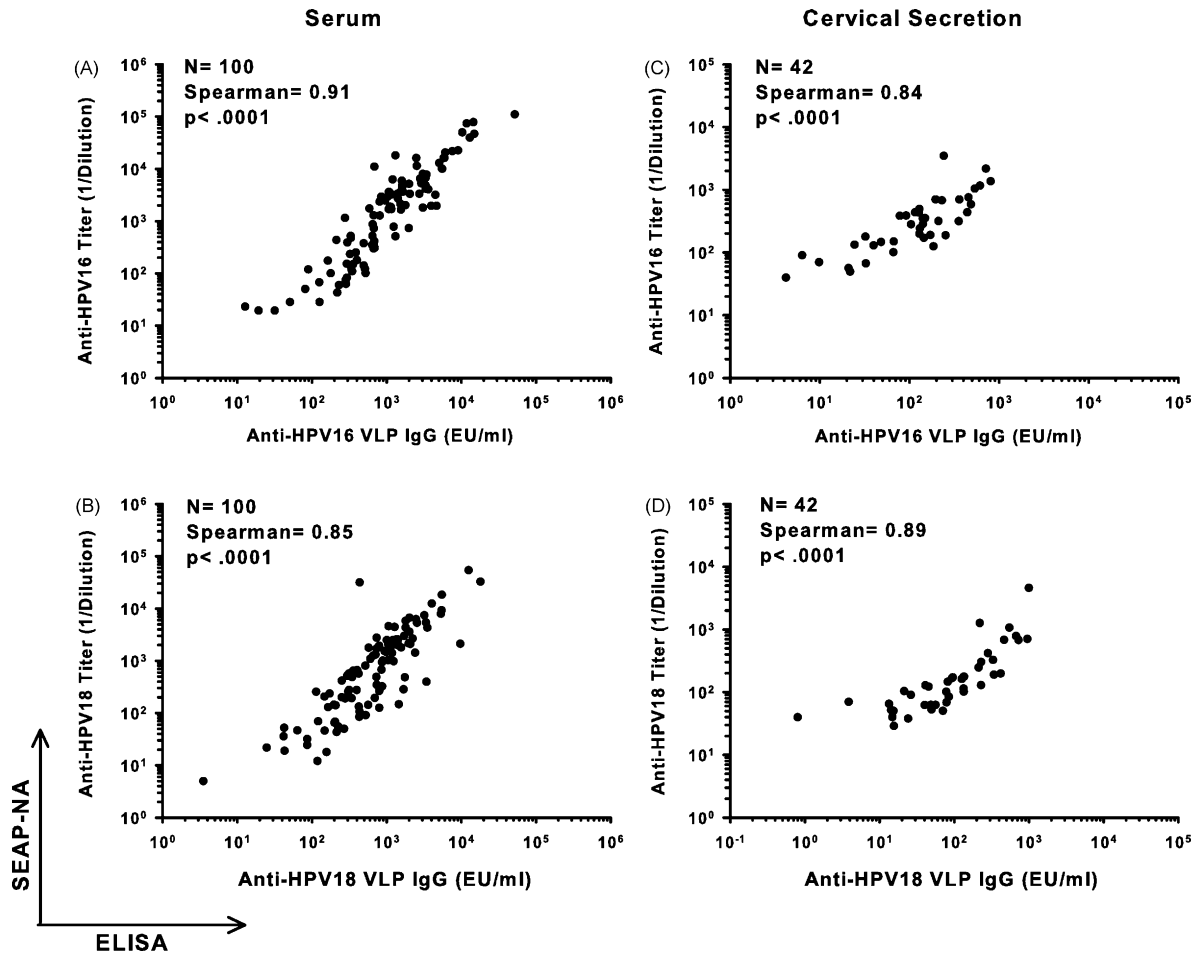


Fig. 1. SEAP-NA and ELISA anti-HPV16 and anti-HPV18 titers were correlated with each other among serum and cervical secretions. (A) HPV16 SEAP-NA correlated with HPV16 ELISA using serum samples. (B) HPV18 SEAP-NA correlated with HPV18 ELISA using serum samples. (C) HPV16 SEAP-NA correlated with HPV16 ELISA using cervical secretions. (D) HPV18 SEAP-NA correlated with HPV18 ELISA using cervical secretions. Within each graph the total number of samples used in the analysis is represented by 'N', and the Spearman rank correlation coefficient and p-value is also indicated on each graph.

To determine the influence of the menstrual cycle phase on agreement between serum and cervical measures at the time of specimen collection, correlations were stratified by days since last menstrual period (follicular: 0–11 days (OC, $n=10$; non-OC, $n=3$), peri-ovulatory/mid-cycle: 12–16 days (OC, $n=9$; non-OC, $n=5$) and luteal: 17+ days (OC, $n=8$; non-OC, $n=7$)). Correlations derived from the ELISA between serum and cervical secretions were reduced when specimens were collected mid-cycle ($\rho=0.28$, anti-HPV16; $\rho=0.65$, anti-HPV18) relative to those observed when specimens were collected in the follicular ($\rho=0.85$, anti-HPV16; $\rho=0.88$, anti-HPV18) or luteal ($\rho=0.85$, anti-HPV16; $\rho=0.84$, anti-HPV18) phases of the cycle. All correlations presented for the LMP strata were derived from the ELISA; however, the SEAP-NA had similar correlations for each stratum (data not shown). Although numbers were limiting, findings from analyses stratified by OC usage and phase of the cycle indicated that reductions in the correlation between serum and cervical measures mid-cycle were evident among OC users and non-OC users (data not shown).

We also examined whether blood levels detected in cervical secretions affected the degree of agreement between serum and cervical measures. Blood levels were measured by Hemastix™ and stratified into three groups: no/trace levels ($n=10$), low (+)/moderate (++) levels ($n=14$) and high (+++) levels ($n=18$). Although numbers were limiting, we observed good correlations derived from the ELISA between serum and cervical secretions for spec-

imens with no/trace blood levels ($\rho=0.75$, anti-HPV16; $\rho=0.81$, anti-HPV18) and those with high blood levels ($\rho=0.83$, anti-HPV16; $\rho=0.76$, anti-HPV18). The correlations observed between serum and cervical secretions for specimens with intermediate blood levels were reduced ($\rho=0.26$, anti-HPV16; $\rho=0.62$, anti-HPV18). The reduction may be explained by the fact that 5 of the 14 (36%) specimens with intermediate blood levels were collected during mid-cycle, whereas only 1 of the 10 (10%) and 2 of the 18 (11%) specimens with no/trace and high blood levels, respectively, were collected during mid-cycle. All correlations presented for the blood levels were derived from the ELISA; however, the SEAP-NA had similar correlations for each stratum (data not shown).

3.4. Correlations between anti-HPV16 and anti-HPV18 antibody titers

In Fig. 3A–D, we examined the correlations between anti-HPV16 and anti-HPV18 antibody measures using both assays to determine whether an individual's response to one HPV antigen is predictive of a response to another HPV antigen contained in the vaccine. The correlations observed between anti-HPV16 and anti-HPV18 antibody levels was $\rho=0.75$ for serum ELISA ($p<0.0001$; Fig. 3A), $\rho=0.72$ for serum SEAP-NA ($p<0.0001$; Fig. 3B), $\rho=0.81$ for cervical secretion ELISA ($p<0.0001$; Fig. 3C) and $\rho=0.65$ for cervical secretion SEAP-NA ($p<0.0001$; Fig. 3D).

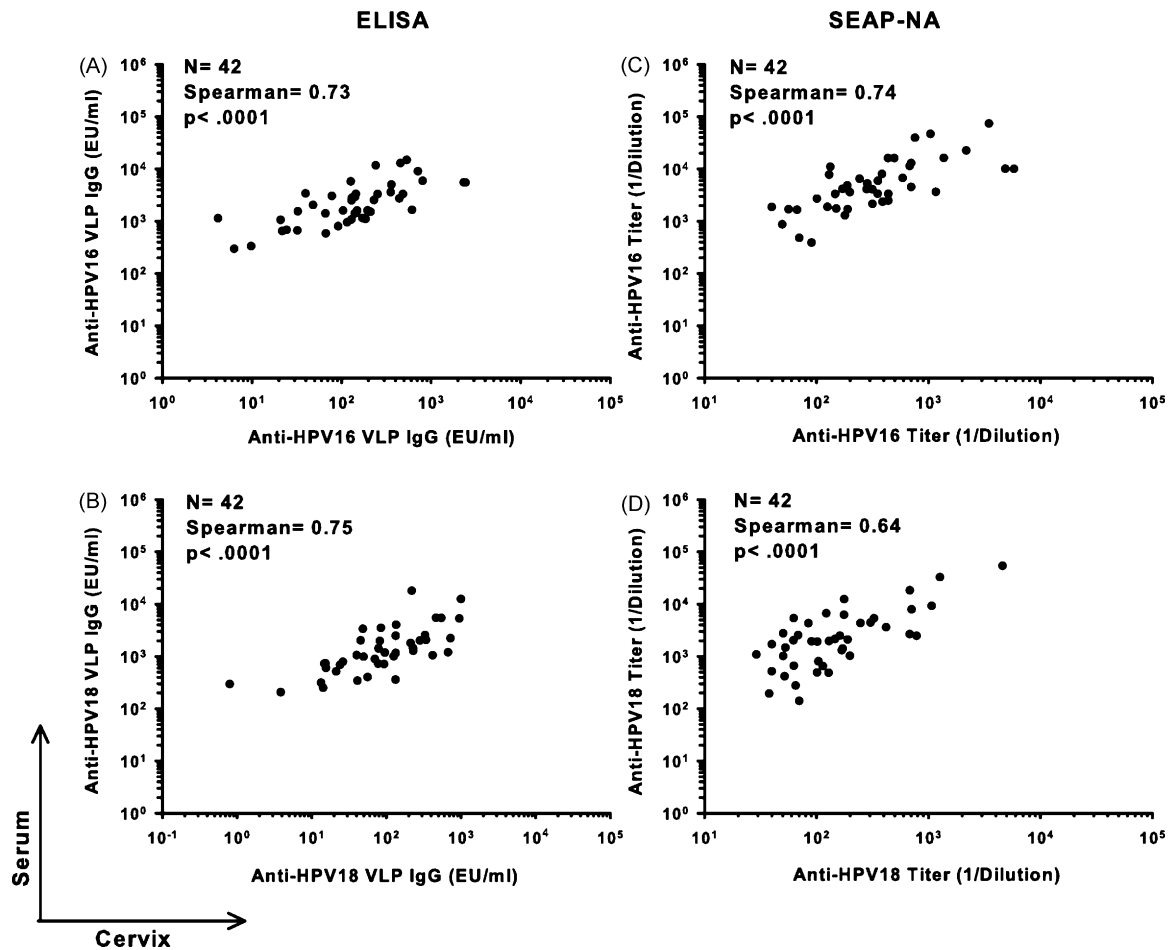


Fig. 2. Correlations calculated between serum anti-HPV16 and anti-HPV18 antibody responses and cervical secretion anti-HPV16 and anti-HPV18 antibody responses. (A) Serum anti-HPV16 antibody titers correlated with cervical secretion anti-HPV16 antibody titers using the HPV16 L1 VLP-based ELISA. (B) Serum anti-HPV18 antibody titers correlated with cervical secretion anti-HPV18 antibody titers using the HPV18 L1 VLP-based ELISA. (C) Serum anti-HPV16 antibody titers correlated with cervical secretion anti-HPV16 antibody titers using the SEAP-NA. (D) Serum anti-HPV18 antibody titers correlated with cervical secretion anti-HPV18 antibody titers using the SEAP-NA. Within each graph the total number of samples used in the analysis is represented by 'N', and the Spearman rank correlation coefficient and p -value is also indicated on each graph.

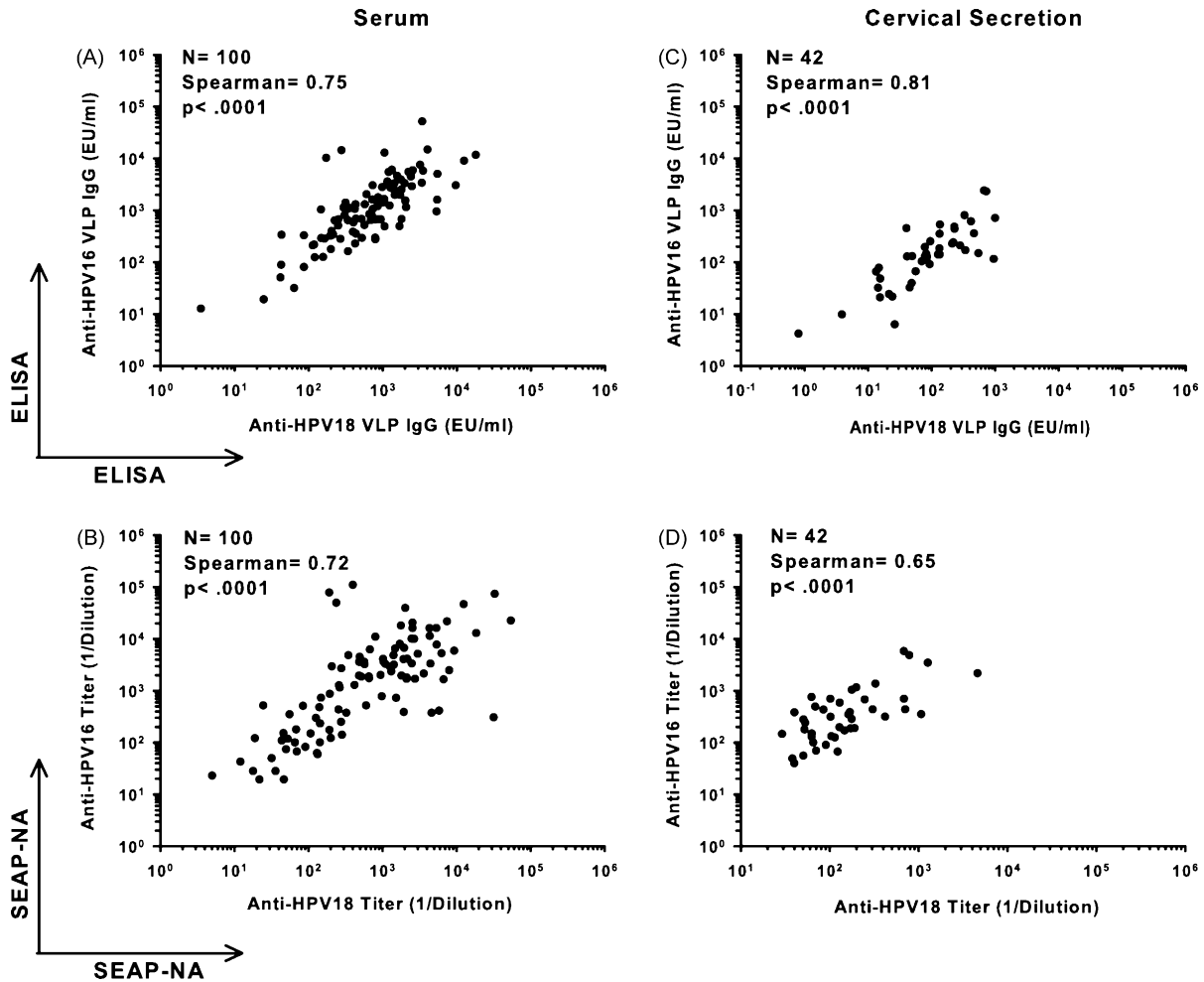


Fig. 3. Anti-HPV16 and anti-HPV18 antibody responses were correlated with each other within the same assay and specimen type. (A) Serum anti-HPV16 antibody titers were correlated with serum anti-HPV18 antibody titers using the titers calculated from the ELISA. (B) Serum anti-HPV16 antibody titers were correlated with serum anti-HPV18 antibody titers using the titers calculated from the SEAP-NA. (C) Cervical secretion anti-HPV16 antibody titers were correlated with cervical secretion anti-HPV18 antibody titers calculated from the ELISA results. (D) Cervical secretion anti-HPV16 antibody titers were correlated with cervical secretion anti-HPV18 antibody titers calculated from the SEAP-NA results. Within each graph the total number of samples used in the analysis is represented by 'N', and the Spearman rank correlation coefficient and *p*-value is also indicated on each graph.

3.5. Correlations between month 1 and month 12 serum antibody titers

The serology results from month 1 and month 12 for both assays are shown in Table 3. As expected, the anti-HPV16 and anti-HPV18 serologic geometric mean titers were higher at month 12 than at month 1 for both the ELISA (3–4-fold increase) and SEAP-NA (10-fold increase). A wide range of anti-HPV16 and anti-HPV18 titers were observed within each time point, as indicated by the broad interquartile ranges (Table 3). There was a poor correlation observed between titers measured at month 1 and those measured at month 12 ($\rho \leq 0.38$; Table 3). After excluding women who received less than three doses of the vaccine ($n = 4$) and women with month 1 titers higher than month 12 for at least one assay ($n = 14$), the correlations showed a moderate increase (ρ ranged from 0.47 to 0.58; $p < 0.01$; Table 3).

4. Discussion

Findings from pre-clinical and clinical HPV L1 VLP vaccine studies suggest that neutralizing antibodies constitute the primary mechanism of protection against infection with HPV [6,10,14,15,31].

Several assays have been developed to measure antibody responses to HPV vaccines [16–19], and in this report we have directly compared two of these assays, the HPV16/18 L1 VLP-based ELISA and SEAP-NA.

For both serum and cervical secretions, most of the variability in antibody titers observed was attributable to differences between women rather than variability associated with either of the assays ($ICC > 92\%$). A higher degree of reproducibility was noted for the ELISA (CV range: 11–22%) than for the SEAP-NA (CV range: 30–40%). This was not surprising given that the SEAP-NA is a biological assay and requires greater specimen handling/manipulation than the ELISA, increasing the potential for variable results. When components of variability were examined for the SEAP-NA, we observed that the major contributor to assay variability was observed within-plate. This is likely due to inherent variability in the growth and function of 293TT cells between assay wells, suggesting that additional improvement in assay reproducibility might prove difficult.

When the ELISA and SEAP-NA results were directly compared, a high degree of correlation was observed between the two assays for both anti-HPV16 and anti-HPV18 measures in serum and cervical secretions (ρ ranging from 0.84 to 0.91). These results, combined

Table 3
Distribution of results by assay, specimen type, HPV type and collection time

Specimen type	HPV type	Specimen collection (months)	SEAP-NA				ELISA			
			N	GMT	IQR	Spearman; <i>p</i> -value ^a	N	GMT	IQR	Spearman; <i>p</i> -value ^a
Serum	HPV16	0	5	34.3	5.7–87.5	0.25; <i>p</i> = 0.08 (0.54; <i>p</i> = 0.0009) ^b	5	9.6	4.0–11.7	0.16; <i>p</i> = 0.265 (0.47; <i>p</i> = 0.005) ^b
		1	50	373.5	100.8–1151.2		50	510.2	229.7–1240.0	
	HPV18	12	50	4694.0	2366.3–10107.0	50	2023.3	1068.7–3403.5		
		0	5	10.6	5.0–27.3	5	6.0	3.5–12.0		
		1	50	182.6	52.3–397.6	50	335.7	156.2–865.5		
		12	50	1926.9	661.1–4347.5	50	1149.1	604.0–2072.2		
Cervical secretions ^c	HPV16	0	5	ND	ND	5	ND	ND		
		12	42	326.6	147.2–679.8	42	130.0	66.1–354.2		
	HPV18	0	5	ND	ND	5	ND	ND		
		12	42	149.8	62.7–246.9	42	85.0	40.2–229.4		

GMT: geometric mean titer; IQR: interquartile range; ND: not determined. All month 0 cervical secretion samples were below the minimal detectable level for both assays (SEAP-NA and ELISA).

^a Spearman rank correlation coefficients are based on month 1 vs. month 12 for serum samples only.

^b Participants were excluded from the analyses due to receiving less than three doses of the HPV L1 VLP 16 and 18 vaccine (*n* = 4) as well as having month 1 antibody titers greater than month 12 (*n* = 14) for a single assay.

^c Cervical secretions are adjusted for sponge weight.

with the higher reproducibility and simplicity of the ELISA, suggest that the ELISA is a good surrogate measure for neutralizing potential, at least during the first year following vaccination.

Another objective of this study was to determine how serologic antibody measurements reflect cervical antibody titers. While overall correlations were good (ρ ranging from 0.64 to 0.75), we did note a menstrual cycle phase effect at the time of specimen collection on agreement levels; correlations were high in the follicular and luteal phases of the menstrual cycle (ρ ranging from 0.68 to 0.88) but lower in mid-cycle (ρ ranging from 0.28 to 0.65). However, it is important to note that these analyses were based on a small number of observations as defined in the results section, and the menstrual cycle phase was inferred, and not confirmed by hormone analysis. This suggests the importance of future studies to better understand the effects of the menstrual cycle phase on antibody measurements in the periphery and cervix. We observed that OC use was not an important modifier of the degree of correlation observed between serum and cervical secretion measures, which appears to contrast with previous data from Nardelli-Haeffliger et al. [25]. This contradiction may be due to variations in sample size and study design, such as in the Nardelli-Haeffliger et al. study participants were sampled multiple times during a 2-month period following immunization while our correlations are only based on month 12 samples for serum and cervical secretions. We also observed that the level of hemoglobin (no/trace and high) present in the cervical secretion did not effect the correlations between serum and cervical secretion measures as indicated by the significant correlations.

We observed a good correlation between anti-HPV16 and anti-HPV18 antibody levels among women (ρ = 0.75 and 0.81 for serum and cervical secretion ELISA titers, respectively). This suggests that women who respond strongly to one antigen of the bi-valent HPV16/18 AS04-adjuvanted vaccine are likely to mount a strong response to the second antigen as well.

Finally, we found antibody responses after one and all three doses to be heterogeneous between women, and there was only a modest correlation between serum antibody titers at different time points (ρ ranging from 0.16 to 0.38). Assuming that vaccine efficacy will wane over time, it will be interesting in the future to determine whether immune response patterns seen after a single or all three vaccine doses are the most predictive of long-term vaccine success or failure.

In conclusion, our study demonstrates that the HPV16/18 L1 VLP-based ELISA is a reliable and valid method for monitoring

anti-HPV16/18 antibody titers within vaccinees in the first year following vaccination. The reduced correlation observed between blood and cervical measures during mid-cycle suggests the need for additional studies to better understand the effect of the time of cycle on local levels of HPV-specific antibodies after vaccination.

Acknowledgements

This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under contract (N01-CO-12400). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

The Costa Rica HPV Vaccine Trial is a long-standing collaboration between investigators in Costa Rica and the NCI. The trial is sponsored and funded by the NCI (grant N01-CP-11005), with funding support from the National Institutes of Health Office for Research on Women's Health, and conducted with support from the Ministry of Health of Costa Rica. Vaccine was provided for our trial by GlaxoSmithKline Biologicals (GSK Bio, Rixensart, Belgium), under a Clinical Trials Agreement with the NCI. GSK Bio also provided support for aspects of the trial associated with regulatory submission needs of the company under grant FDA BB-IND 7920. Laboratory testing was performed at the NCI-sponsored SAIC-Frederick Inc., HPV Immunology Laboratory in Frederick, MD and the GSK Bio facilities in Rixensart, Belgium. The NCI and Costa Rica investigators are responsible for the design and conduct of the study; collection, management, analysis and interpretation of the data; and preparation of the manuscript. The NCI and Costa Rica investigators make final editorial decisions on this and subsequent publications; GSK Bio has the right to review and comment.

Conflict of interest statement: Troy J. Kemp, no conflict; Alfonso García-Piñeres, no conflict; Roni T. Falk, no conflict; Sylviane Poncelet is employed by GSK Biologicals, the manufacturer of the vaccine used in this trial; Francis Dessy is employed by GSK Biologicals, the manufacturer of the vaccine used in this trial; Sandra L. Giannini is employed by GSK Biologicals, the manufacturer of the vaccine used in this trial; Ana Cecilia Rodriguez, no conflict; Carolina Porras, no conflict; Rolando Herrero, no conflict; Allan Hildesheim, no conflict; Ligia A. Pinto, no conflict.

Merocel is a trademark of Medtronic Ophthalmics, a division of Medtronic Xomed Inc., Jacksonville, FL, and Hemastix is a trademark of Bayer Corporation, Elkhart, IN.

We would like to thank Information Management Services (IMS) especially Brian Befano for all statistical analysis as well as the Costa Rica Vaccine Trial (CVT) group members.

Appendix A

Names and affiliations of the Costa Rica Vaccine Trial (CVT) Group members are as follows:

Proyecto Epidemiológico Guanacaste, Fundación INCIENSA, San José, Costa Rica

Mario Alfaro (Cytologist)
Manuel Barrantes (Field Supervisor)
M. Concepcion Bratti (Co-Investigator)
Fernando Cárdenas (General Field Supervisor)
Bernal Cortés (Specimen and Repository Manager)
Albert Espinoza (Head, Coding and Data Entry)
Yenory Estrada (Pharmacist)
Paula Gonzalez (Co-Investigator)
Diego Guillén (Pathologist)
Rolando Herrero (Co-Principal Investigator)
Silvia E. Jimenez (Trial Coordinator)
Jorge Morales (Colposcopist)
Lidia Ana Morera (Head Study Nurse)
Elmer Pérez (Field Supervisor)
Carolina Porras (Co-Investigator)
Ana Cecilia Rodriguez (Co-Investigator)
Maricela Villegas (Clinic M.D.)

University of Costa Rica, San José, Costa Rica

Enrique Freer (Director, HPV Diagnostics Laboratory)
Jose Bonilla (Head, HPV Immunology Laboratory)
Sandra Silva (Head Microbiologist, HPV Diagnostics Laboratory)
Ivannia Atmella (Microbiologist, Immunology Laboratory)
Margarita Ramirez (Microbiologist, Immunology Laboratory)

United States National Cancer Institute, Bethesda, MD, USA

Pam Gahr (Trial Coordinator)
Allan Hildesheim (Co-Principal Investigator & NCI Co-Project Officer)
Douglas R. Lowy (HPV Virologist)
Mark Schiffman (Medical Monitor & NCI Co-Project Officer)
John T. Schiller (HPV Virologist)
Mark Sherman (QC Pathologist)
Diane Solomon (Medical Monitor & QC Pathologist)
Sholom Wacholder (Statistician)

SAIC-Frederick Inc., NCI-Frederick, Frederick, MD, USA

Ligia Pinto (Head, HPV Immunology Laboratory)
Alfonso Garcia-Pineres (Scientist, HPV Immunology Laboratory)

Womens and Infants' Hospital, Providence, RI, USA

Claire Eklund (QC Cytology)
Martha Hutchinson (QC Cytology)

DDL Diagnostic Laboratory, The Netherlands

Wim Quint (HPV DNA Testing)
Leen-Jan van Doorn (HPV DNA Testing)

References

- Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348(6):518–27.
- Cogliano V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F. Carcinogenicity of human papillomaviruses. *Lancet Oncol* 2005;6(4):204.
- Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. International biological study on cervical cancer (IBSCC) Study Group. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* 1995;87(11):796–802.
- Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004;111(2):278–85.
- Castellsague X, Diaz M, de Sanjose S, Munoz N, Herrero R, Franceschi S, et al. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. *J Natl Cancer Inst* 2006;98(5):303–15.
- Harro CD, Pang YY, Roden RB, Hildesheim A, Wang Z, Reynolds MJ, et al. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J Natl Cancer Inst* 2001;93(4):284–92.
- Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, et al. Sustained efficacy up to 4, 5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* 2006;367(9518):1247–55.
- Villa LL, Costa RL, Petta CA, Andrade RP, Ault KA, Giuliano AR, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16 and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. *Lancet Oncol* 2005;6(5):271–8.
- Villa LL, Costa RL, Petta CA, Andrade RP, Paavonen J, Iversen OE, et al. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br J Cancer* 2006;95(11):1459–66.
- Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuid A, et al. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet* 2004;364(9447):1757–65.
- The FUTURE II Study Group. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. *N Engl J Med* 2007;356(19):1915–27.
- Garland SM, Hernandez-Avila M, Wheeler CM, Perez G, Harper DM, Leodolter S, et al. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. *N Engl J Med* 2007;356(19):1928–43.
- Paavonen J, Jenkins D, Bosch FX, Naud P, Salmemon J, Wheeler CM, et al. Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. *Lancet* 2007;369(9580):2161–70.
- Breitburd F, Kirnbauer R, Hubbert NL, Nonnenmacher B, Trin-Dinh-Desmarquet C, Orth G, et al. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol* 1995;69(6):3959–63.
- Suzich JA, Ghim SJ, Palmer-Hill FJ, White WI, Tamura JK, Bell JA, et al. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. *Proc Natl Acad Sci USA* 1995;92(25):11553–7.
- Palker TJ, Monteiro JM, Martin MM, Kakareka C, Smith JF, Cook JC, et al. Antibody, cytokine and cytotoxic T lymphocyte responses in chimpanzees immunized with human papillomavirus virus-like particles. *Vaccine* 2001;19(27):3733–43.
- Dias D, Van Doren J, Schlottmann S, Kelly S, Puchalski D, Ruiz W, et al. Optimization and validation of a multiplexed luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11, 16 and 18. *Clin Diagn Lab Immunol* 2005;12(8):959–69.
- Pastrana DV, Buck CB, Pang YY, Thompson CD, Castle PE, FitzGerald PC, et al. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* 2004;321(2):205–16.
- Wideroff L, Schiffman MH, Nonnenmacher B, Hubbert N, Kirnbauer R, Greer CE, et al. Evaluation of seroreactivity to human papillomavirus type 16 virus-like particles in an incident case-control study of cervical neoplasia. *J Infect Dis* 1995;172(6):1425–30.
- Opalka D, Lachman CE, MacMullen SA, Jansen KU, Smith JF, Chirmule N, et al. Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16 and 18 by a multiplexed luminex assay. *Clin Diagn Lab Immunol* 2003;10(1):108–15.
- Chen C-J, Viscidi RP, Chuang C-H, Huang Y-C, Chiu C-H, Lin T-Y. Seroprevalence of human papillomavirus types 16 and 18 in the general population in Taiwan: implication for optimal age of human papillomavirus vaccination. *J Clin Virol* 2007;38(2):126–30.
- Nardelli-Haeffliger D, Lurati F, Wirthner D, Spertini F, Schiller JT, Lowy DR, et al. Immune responses induced by lower airway mucosal immunisation with a human papillomavirus type 16 virus-like particle vaccine. *Vaccine* 2005;23(28):3634–41.
- Wang SS, Schiffman M, Herrero R, Carreon J, Hildesheim A, Rodriguez AC, et al. Determinants of human papillomavirus 16 serological conversion and persistence in a population-based cohort of 10,000 women in Costa Rica. *Br J Cancer* 2004;91(7):1269–74.
- Viscidi RP, Schiffman M, Hildesheim A, Herrero R, Castle PE, Bratti MC, et al. Seroreactivity to human papillomavirus (HPV) types 16, 18 or 31 and risk of subsequent HPV infection: results from a population-based study in Costa Rica. *Cancer Epidemiol Biomarkers Prev* 2004;13(2):324–7.
- Nardelli-Haeffliger D, Wirthner D, Schiller JT, Lowy DR, Hildesheim A, Ponci F, et al. Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles. *J Natl Cancer Inst* 2003;95(15):1128–37.
- Hildesheim A, Herrero R, Wacholder S, Rodriguez AC, Solomon D, Bratti MC, et al. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. *JAMA* 2007;298(7):743–53.
- Castle PE, Rodriguez AC, Bowman FP, Herrero R, Schiffman M, Bratti MC, et al. Comparison of ophthalmic sponges for measurements of immune

- markers from cervical secretions. *Clin Diagn Lab Immunol* 2004;11(2):399–405.
- [28] SAS Institute I. *SAS/STAT user's guide*. 8th ed. Cary, NC: SAS Institute Inc.; 1999.
- [29] Gail MH, Fears TR, Hoover RN, Chandler DW, Donaldson JL, Hyer MB, et al. Reproducibility studies and interlaboratory concordance for assays of serum hormone levels: estrone, estradiol, estrone sulfate, and progesterone. *Cancer Epidemiol Biomarkers Prev* 1996;5(10):835–44.
- [30] Rao CR. *Linear statistical inference and its applications*. 2nd ed. New York: John Wiley; 1965.
- [31] Villa LL, Ault KA, Giuliano AR, Costa RL, Petta CA, Andrade RP, et al. Immunologic responses following administration of a vaccine targeting human papillomavirus Types 6, 11, 16 and 18. *Vaccine* 2006;24(27/28):5571–83.