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Nonavalent HPV vaccination induces robust systemic and mucosal HPV16-neutralizing antibody responses in previously infected women

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Research Article

Keywords: HPV vaccination, Pseudovirion-based neutralization assay, Neutralizing antibodies, Mucosal immunity

Posted Date: July 23rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10318491/v1

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Additional Declarations: Competing interest reported. Cancer Epidemiology Research Program from Catalan Institute of Oncology (ICO) – Bellvitge Biomedical Research Institute (IDIBELL) has received sponsorship/grants from MSD, Roche, GSK, IDT, Hologic and Seegene which are not related to the present study.

Abstract

Background

Neutralizing antibody responses to human papillomavirus (HPV) vaccines are typically assessed in serum, while mucosal immunity at sites of infection remains less well characterized, particularly in HPV-positive individuals. We evaluated systemic and mucosal HPV16-neutralizing antibody responses following nonavalent HPV vaccination in women with a recent HPV16/18-positive cervical test.

Methods

In this longitudinal RIFT-HPV study, 39 previously unvaccinated adult women with an HPV16/18-positive cervical test done in the previous 12 months received a three-dose schedule of the nonavalent HPV vaccine (Gardasil-9). Paired serum, cervical, and oral samples were collected before and during vaccination. Neutralizing activity against HPV16 pseudovirions was measured using an enhanced green fluorescent protein pseudovirion-based neutralization assay (EGFP-PBNA).

Results

Vaccination significantly increased HPV16-neutralizing antibody responses in serum, cervical, and oral samples, irrespective of baseline HPV16 positivity. Serum EC_{50} detection increased from 33% at baseline to 100% after three doses, while cervical and oral detection increased from 8% and 10% after one dose, to 100% and 80% after three doses, respectively. With assay conditions exceeding physiological viral exposure levels, cervical HPV16 pseudovirion neutralization progressively increased from 36% before vaccination to 61%, 68% and 78% after one, two and three vaccine doses, respectively. Systemic and mucosal neutralizing responses were positively correlated. The EGFP-PBNA demonstrated high reproducibility (ICC = 0.98) and good concordance with the reference method.

Conclusions

Nonavalent HPV vaccination induced strong systemic and mucosal HPV16-neutralizing antibody responses, regardless of current HPV16 positivity. These findings demonstrate enhanced mucosal HPV16-neutralizing immunity following nonavalent vaccination in previously infected women and support further investigation of its potential role in reducing viral infectivity and transmission.

Trial registration:

ClinicalTrials.gov, NCT05334706. Registered on 19 April 2022.

Background

Annually, over 920,000 cancer cases worldwide are attributed to human papillomavirus (HPV), including 99% of cervical, 90% of anal, 70% of oropharyngeal, 75% of vaginal, 69% of vulvar, and 63% of penile cancers (1–4). Among HPV genotypes, HPV16 is the most carcinogenic type, and together with HPV18 accounts for over 70% of cervical cancers (5, 6). HPV L1 virus-like particle (HPV L1-VLP) vaccines, such as the nonavalent HPV vaccine (Gardasil-9®), are highly safe, immunogenic, and effective in preventing high-risk HPV infections and associated malignancies(7). The nonavalent HPV vaccine comprises highly purified virus-like particles formed by the recombinant major capsid (L1) proteins of HPV Types 6, 11, 16, 18, 31, 33, 45, 52, and 58 (7). Vaccine-induced neutralizing antibodies (nAbs) play a crucial role in blocking virion attachment to epithelial cells, thereby preventing the establishment of new infections (8, 9). These antibodies may also promote virion clearance via proteasomal degradation, further enhancing immune protection (10).

However, HPV L1-VLP vaccines are not therapeutic. When HPV infection is acquired prior to vaccination, nAbs are unlikely to eliminate already infected epithelial cells, allowing continued viral shedding and ongoing transmission to sexual partners (11, 12). Consequently, in line with the World Health Organization's global cervical cancer elimination strategy, vaccination programs have primarily targeted adolescents, especially females, prior to their first sexual contact (3, 11). Multiple countries have expanded vaccination programs to include young males, aiming to improve coverage and promote equitable prevention strategies (13). Although HPV vaccination in mid-adult populations is less cost-effective than vaccination at younger ages, transmission-dynamic models suggest that targeting high-risk adult women may improve cost-effectiveness (14). In settings with established female-only programs and high uptake, further increasing coverage among women is predicted to yield greater population-level benefits than extending vaccination to boys (14). Notably, most existing transmission models focus on preventing acquisition of new infections (inward transmission) and do not explicitly account for potential reductions in infectivity among HPV-positive individuals, who remain sources of onward transmission (12, 15).

Pseudovirion (PsV)-based neutralization assays (PBNAs) are widely used to quantify anti-L1 antibody activity in serum samples by measuring reporter gene expression following infection (16–21). Several reporter genes have been described for this assay, including Gaussia luciferase (Gluc), nanoluciferase (NLuc), and secreted alkaline phosphatase (SEAP), with the latter considered the gold standard (16–21). These systems often require costly reagents and specialized equipment. Enhanced green fluorescent protein (EGFP) has been explored as an alternative reporter; however, fluorescence-activated cell sorting (FACS)-based methods are labor-intensive and less widely adopted (17, 19). Recent microscopy-based EGFP-PBNA approaches have demonstrated high specificity and reproducibility for detecting nAbs in serum (16). Integration with high-throughput automated microscopy could reduce labor and costs while allowing parallel evaluation of infection conditions, thereby minimizing culture-related bias.

Standard PBNAs have largely relied on serum samples, with comparatively limited data from clinically relevant mucosal compartments. Nevertheless, anti-L1 nAbs have been detected in cervical, oral and first-void urine samples, highlighting the importance of assessing mucosal immunity (16, 22–33). Given that HPV vaccination leads to higher antibody responses compared to natural infection, it may enhance virion neutralization even in previously infected individuals (12, 15, 34). Observational studies suggest that vaccination of HPV-positive individuals is associated with reduced viral load and increased clearance of vaccine-covered genotypes, supporting a role for vaccine-induced antibodies in limiting viral infectivity and potentially autoinoculation (35–39). However, evidence regarding transmission effects remains limited. The HITCH cohort study in heterosexual couples reported lower HPV transmission from vaccinated women to unvaccinated men compared to unvaccinated women (40, 41). Additionally, vaccinated young women infected with HPV types 6/11/16/18 showed lower viral loads than unvaccinated women (40, 41). However, subsequent evaluation in the TRAP-HPV controlled trial did not provide conclusive evidence of this vaccination effect in transmission (42, 43). Interpretation of these findings is complicated by the difficulty of determining the timing of transmission events relative to vaccination or study enrollment (42, 43). Therefore, resolving whether vaccination can reduce outward transmission, and enabling incorporation of such effects into transmission models, requires controlled studies in adult women combined with assays that quantify antibody-mediated neutralization and the presence of infectious viral particles in mucosal compartments.

In this study, we assess HPV16-neutralizing antibody levels in systemic and mucosal samples from HPV16/18-positive individuals before and after nonavalent vaccination, using samples from the RIFT-HPV clinical study (NCT05334706) (44). As an initial step toward evaluating reductions in viral infectivity, we characterize antibody responses across multiple HPV-susceptible mucosal sites and assess their neutralizing capacity using an EGFP-based PBNA.

Methods

Study cohort and procedures

Serum, cervical, oral and anal samples analyzed in this study were obtained from the longitudinal RIFT-HPV trial (ClinicalTrials.gov NCT05334706; registered April 19, 2022), which aims to assess if vaccination with a 3-dose regimen of Gardasil-9® could neutralize HPV virions and reduce the infectivity of body fluids from HPV16/18-positive women (44). RIFT-HPV1 cohort included 39 unvaccinated adult cisgender women (≥ 18 years) with an HPV16/18-positive cervical test within the 12 months prior to recruitment and either no detectable lesions or cervical intraepithelial neoplasia (CIN) 1/2 lesions, corresponding to low-grade/high-grade squamous intraepithelial lesions (LSIL/HSIL) eligible for conservative treatment. Women completed a total of four visits, at months 0, 2, 6 and 7, respectively, following the standard three-dose vaccination schedule. Intramuscular Gardasil-9® vaccine was administered at visits 1 to 3 by a qualified health professional following manufacturer's protocol. Serum, cervical, oral and anal samples were collected at visits 1 to 3, prior to vaccine administration, and at visit 4 (Fig. 1). All subjects provided written informed consent and completed a 10-item questionnaire at each

of the four visits, covering previous and current health status, HPV infection and vaccination, oral contraception use, and sexual activity. Recruitment and study procedures were performed at a single study site (Department of Gynecology at Bellvitge University Hospital). Additional satellite centers (Cervical Cancer Screening Technical Office from the Cancer Epidemiology Research Programme at the Catalan Institute of Oncology, Department of Obstetrics and Gynecology at MAR Health Park, HIV and STD Unit at Bellvitge University Hospital and Sexual and Reproductive Health Care Center ASSIR Delta del Llobregat) contributed to participant identification based on inclusion criteria, with eligible candidates referred to and ultimately recruited at the study site.

Sample collection

Blood samples were extracted in a red-top vacutainer tube by standard venipuncture procedures and mixed by gently inverting the tube 5 times. Blood tubes were left for one hour at room temperature for coagulation, after which they were centrifuged at 1500 x g for 10 minutes to separate serum from blood cells. Cervical samples were collected using a Cervex-Brush® (Cat# 380100311, Rovers Medical Devices B.V., North Brabant, Netherlands), which was immediately immersed in a sterile collection tube containing 10 mL of phosphate-buffered saline (PBS). Oral samples were collected by a 15-second rinse and 15-second gargle with 10 mL of PBS. Anal samples were collected using an Endocervical Brush (Cat# 94321, Bexen Medical, Gipuzkoa, Spain) and immediately immersed in 5 mL of PBS. All samples were aliquoted in 1.5 mL tubes and stored at -75°C, except for the aliquots for short-term usage which were stored at -20°C (HPV DNA testing and PBNA).

HPV DNA testing

HPV DNA detection and genotyping were performed using the Allplex HPV28 kit (Cat#HP10372X, Seegene, Inc., Seoul, South Korea), identifying up to 28 HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73 and 82), including the vaccine-preventable ones (6, 11, 16, 18, 31, 33, 45, 52, and 58). Tested samples included cervical and anal samples in all visits, and oral samples at visit 1. Samples DNA was extracted using the automated Maxwell® 16 LEV Blood DNA Kit (Cat#AS1290, Promega Corporation, Wisconsin, USA) according to manufacturer instructions.

Production of HPV16 pseudovirions (PsVs)

HPV16 PsVs were generated as previously described (45). Briefly, 293TT cells were transfected using Lipofectamine 3000 (Cat#L3000001, Thermo Fisher Scientific Inc., Massachusetts, U.S.) with an HPV16 shell plasmid (p16sheLL) encoding HPV16 L1 and L2 proteins, and a reporter plasmid (either pfwB or pYSEAP), encoding green fluorescent protein (EGFP) or secreted alkaline phosphatase (SEAP), respectively. p16sheLL, pfwB and pYSEAP were gifts from John Schiller (Addgene plasmids #37320, #37329 and #37326 from http://n2t.net/addgene, respectively) (17, 46, 47). After 48 hours, cells were harvested and lysed following protocol specifications. The resulting lysate was incubated for 24 hours at

37°C to allow capsids to mature, after which it was chilled on ice and centrifuged at 5,000 × g for 10 minutes at 4∘C. The resulting clarified supernatant was stored at − 75°C in 20 µL aliquots.

Viral reporter genomic equivalents

Measurement of encapsidated plasmids was performed to standardize our EGFP-HPV16 PsV stock concentration for PBNAs. Protocols for reporter plasmid extraction and quantitative polymerase chain reaction (qPCR) analysis were adapted from previous studies (48, 49). Reporter genome extraction was performed using the PureLink Viral RNA/DNA extraction kit (Cat#12280050, Thermo Fisher Scientific Inc., Massachusetts, U.S.), using 10 µL of purified EGFP-HPV16 PsV stock. qPCR was performed according to manufacturer's instructions using TaqMan Real-Time PCR Assays with EGFP forward primer 5'-CGGCATCAAGGTGAACTTCA-3' (500nM), reverse primer 5'-ACCATGTGATCGCGCTTCTC-3' (500nM), and probe 5'-CCACTACCAGCAGAACA-3' (500nM) with 6'-carboxyfluorescein (6FAM) as dye and 3' Iowa Black® FQ as quencher (Integrated DNA Technologies, Inc., Iowa, U.S.). To determine viral genomic equivalents (VGE), known amounts of pfwB plasmid ranging from 10^9–10^5 copies were used as standards (46).

EGFP pseudovirion-based neutralization assay (EGFP-PBNA)

EGFP-PBNA protocol was adapted from previously described papillomavirus neutralization assay methods (50). 293TT cells were pre-plated 2–4 hours before PsV addition in 96-well cell culture plates (Cat#167008, Thermo Fisher Scientific Inc., Massachusetts, U.S.) at a cell density of 3×10^4 cells/well in a total of 100 µL of neutralizing buffer (NB). NB consists of DMEM with HEPES and without phenol red (Cat#21063029, Thermo Fisher Scientific Inc., Massachusetts, U.S.), 10% fetal bovine serum (FBS) (Cat#10270106, Thermo Fisher Scientific Inc., Massachusetts, U.S.), 1% MEM non-essential amino acids (Cat#11140050, Thermo Fisher Scientific Inc., Massachusetts, U.S.), 1% L-glutamine (200 mM) (Cat#25030024, Thermo Fisher Scientific Inc., Massachusetts, U.S.), and 1% Antibiotic-antimycotic (100X) (Cat#A5955, MilliporeSigma, Massachusetts, U.S.). EGFP-HPV16 PsV stocks were diluted 1:7500 to reach a minimum of 40% PsV-infection. 80 µL of diluted EGFP PsV stocks (1:7500) were mixed with 20 µL of sample and incubated on ice for one hour. Negative control wells contained 100 µL of NB, whilst positive control wells contained 20 µL of NB and 80 µL of diluted EGFP-HPV16 PsV stock (1:7500). After incubation, the PsV-antibody mixtures were added to the corresponding wells of the previously seeded 293TT cells plates (final EGFP-HPV16 PsV dilution of 1:18750). Plates were incubated at 37°C and 5% CO_2 for 72 hours (Fig. 2). Before measurement, Hoechst nucleic acid stain (Cat#62249, Thermo Fisher Scientific Inc., Massachusetts, U.S.) was added on each well at a final dilution of 1µg/mL for cell counting. EGFP measurement was measured using a Zeiss Axio Observer Z1 + Apotome inverted fluorescence microscope that allowed us to automatically acquire 5 images/well. These images were then analyzed with a custom macro for FIJI/ImageJ image processing software, obtaining the number of total cells and EGFP-expressing cells on each image (https://doi.org/10.34810/DATA3460) (51). PsV

neutralization percentages for EGFP-PBNAs were defined as $\left[100\ x\left(1-\frac{(\%\ EGFP{+}cells\ sample\ -\ \ Mean\ \%\ EGFP{+}cells\ negative\ control)}{Mean\ \%\ EGFP{+}cells\ in\ positive\ control}\right)\right]$, as previously described (17). A mean value > 35% EGFP-positive cells in positive control wells was required for assay acceptance. Using this assay workflow, serum, cervical, oral and anal samples were tested to evaluate 50% effective concentration (EC_{50}) values across study visits. Samples were serially diluted in sterile polystyrene plates (Cat#268200, Thermo Fisher Scientific Inc., Massachusetts, U.S.), with a final volume of 20 µL. To avoid cell culture contamination, oral and anal samples were previously centrifuged at 5000 x g for 10 minutes, using only the supernatant. Serum samples were 4-fold diluted starting at 1:40 up to 1:40960, to avoid the nonspecific inhibition of PsV activity that has been reported in lower dilutions (1:10 – 1:40)(17). Cervical samples were 2-fold diluted starting at 1:1 up to 1:32, and oral and anal samples were 1.5-fold diluted starting at 1:1 up to 1:7.6. EC_{50} values were defined as (1 / final dilution needed to obtain 50% PsV-neutralization). EC_{50} values were calculated using the four-parameter sigmoidal curve fit for serum samples and three-parameter logistic curve fit for cervical and oral samples in GraphPad Prism 10.4.1. Samples with less than 50% PsV neutralization at 1:1 or dilution curves with a coefficient of determination (R^2) < 0.85 were excluded from the analysis. Additionally, following the same assay structure as previously described, 20 µL of undiluted cervical samples from all participants were tested in triplicate to further evaluate differences in PsV neutralization across study visits.

SEAP pseudovirion-based neutralization assay (SEAP-PBNA)

SEAP-PBNA assays were performed as previously described (17, 50). Briefly, 293TT cells were pre-plated 2–4 hours before PsV addition in 96-well cell culture plates (Cat#167008, Thermo Fisher Scientific Inc., Massachusetts, U.S.) at a cell density of $3{\times}10^4$ cells/well in a total of 100 µL of NB. Samples were processed and serially diluted as previously described. 80 µL of diluted SEAP-HPV16 PsV stocks (1:4000) were mixed with 20 µL of diluted samples and incubated on ice for one hour. Negative control wells contained 100 µL of NB, positive control wells contained 20 µL of NB and 80 µL of diluted SEAP-HPV16 PsV stocks (1:4000), and positive neutralization controls contained 20 µL of HPV.16V5 antibody (1:50000) (Cat#MABF3220, MilliporeSigma, Massachusetts, U.S.) and 80 µL of diluted SEAP-HPV16 PsV stocks (1:4000). After incubation, the PsV-antibody mixtures were added to the corresponding wells of the previously seeded 293TT cells plates (final SEAP-HPV16 PsV dilution of 1:10000). Plates were incubated at 37°C and 5% CO_2 for 72 hours. Plates were gently mixed by hand and 50 µL of medium from each well were transferred to a new 96-well round-bottom microplates (Cat#268200, Thermo Fisher Scientific Inc., Massachusetts, U.S.). SEAP development was performed using the Great Escape SEAP Chemiluminescence Detection Kit 2 (Cat#631738, Takara Bio USA, Inc., California, U.S.) and 96-well flat-bottom opaques white plates (Cat#6055290, Revvity Inc., Massachusetts, U.S.). Microplates were read on a luminometer (POLARstar Omega, BMG Labtech, Ortenberg, Germany) with end-point positioning delay at 0.2, measurement interval time at 0.2, optic at top and gain at 1500. PsV neutralization

percentages for SEAP-PBNAs were defined as $\left[100\ x\left(1-\frac{Mean\ RLU\ measured\ in\ sample}{Mean\ RLU\ in\ SEAP\ PsV\ positive\ control}\right)\right]$. EC_{50} values were calculated as previously described to study inter-assay concordance.

Bicinchoninic Acid (BCA) protein assay

Total protein concentration in mucosal samples was quantified using the Pierce™ Dilution-Free™ Rapid Gold BCA Protein Assay (Thermo Fisher Scientific) according to the manufacturer's instructions. Absorbance was measured using a microplate reader at 480 nm, and protein concentrations were calculated based on a standard curve generated for each assay plate. Measurements for each sample were performed in triplicate. Although EC_{50} values are relative and cannot be normalized, total protein quantification was performed to assess inter-visit variability and rule out any significant differences in protein content that could impact PBNAs.

Statistical analysis

Baseline associations between abnormal cervical findings and established risk factors (smoking and number of sexual partners) were evaluated using Fisher's exact test for the smoking and Cochran-Armitage trend test for the number of sexual partners. For the Cochran-Armitage test, the null hypothesis was the hypothesis of no trend, which means that the binomial proportion is the same for all levels of the explanatory variable. Pairwise comparisons using the Games–Howell test were performed to evaluate the association between HPV status and the time elapsed from the previous HPV diagnostic test and first study visit. The longitudinal profile of HPV status in cervical and anal samples across visits was presented using Sankey diagrams. EC_{50} values were transformed using the natural logarithm (ln) for statistical analysis and presented as geometric means (GM) with 95% confidence intervals (CI). Differences in EC_{50} detection rates, GM EC_{50} values, and neutralization percentages across study visits were assessed using mixed-effects linear regression models with individual random effects (glmmTMB, R). Distributional form of the response in mixed models was as assessed through the descdist() function of the fitdistrplus R package and p-values were adjusted using the Holm method. Potential covariates influencing antibody concentrations, including HPV16 positivity and menopausal status (for cervical models), were evaluated, selecting the most parsimonious model according to the Bayesian Information Criterion (BIC). Oral contraceptive use, although previously reported to influence cervical immunity, was not included in the model due to the low number of cases in the cohort (n = 3), which precluded reliable estimation. Pairwise comparisons using Games–Howell were performed to assess differences in total protein concentrations from cervical and oral samples across visits. Correlations between systemic and mucosal ln (EC_{50}) were assessed in paired samples, using multilevel Spearman correlation with visit and subject specified as random effects (52). Intraclass correlation coefficients (ICCs) were estimated using a variance-components model (random effects for subject–sample–visit combinations), equivalent to Lin's concordance correlation coefficient (CCC), to assess assay reproducibility. Correlation between EGFP-PBNA and SEAP-PBNA ln (EC_{50}) was evaluated in paired data using multilevel Spearman

correlation with visit, subject, and sample type included as random effects. No unilateral hypothesis for the hypothesis tests was considered.

Results

RIFT-HPV1 study population characteristics

A total of 39 women enrolled in the RIFT-HPV clinical trial (NCT05334706) were included in the study, all with a previous HPV16 (72%) or HPV18 (28%) positive cervical test performed in the last 12 months (Fig. 1). All participants received the standard three-dose nonavalent vaccine schedule at visits 1 to 3 and completed a follow up visit at month 7 (visit 4). The overall median age was 44 years (IQR 38–50) (Table 1). Participants were referred from four centers, with the majority coming from the Cervical Cancer Screening Technical Office from the Cancer Epidemiology Research Programme at Catalan Institute of Oncology (59%, 23/39), followed by the Sexual and Reproductive Health Care Center - ASSIR Delta del Llobregat (23%, 9/39), Bellvitge University Hospital (10%, 4/39), and Hospital del Mar - MAR Health Park (8%, 3/39). Regarding sociodemographic factors, 33% (13/39) of participants reported current tobacco use, while 67% (26/39) were non-smokers. Alcohol consumption was less frequent, with 18% (7/39) reporting alcohol use and 82% (32/39) not consuming. According to previous gynecologic records, 41% (16/39) of participants had normal cytological findings, 21% (8/39) had atypical squamous cells of undetermined significance (ASC-US), and 38% (15/39) had CIN1/LSIL lesions. In terms of menopausal status, 67% (26/39) were pre-menopausal, 15% (6/39) peri-menopausal, and 18% (7/39) post-menopausal. Excluding post-menopausal participants, 9.4% (3/32) of participants reported the use of oral contraceptives. Regarding sexual behavior, 15% (6/39) reported not having sexual relationships in the last 6 months, 69% (27/39) reported having one sexual partner, and 15% (6/39) reported two or more sexual partners. A positive association was observed between abnormal cytology and/or cervical lesions (ASC-US and CIN1/LSIL) and smoking ($p < 0.001$). Abnormal findings were also positively associated with having had ≥ 2 sexual partners in the last 6 months, but the association was not statistically significant ($p = 0.078$).

HPV-positivity in cervical and anal samples throughout visits

Upon re-testing of cervical samples at visit 1, 41% participants (16/39) were HPV16-positive, 13% (5/39) were HPV18-positive and 46% (18/39) had cleared the previous HPV16/18 infection (Table 1). Time elapsed between the previous HPV diagnostic test and visit 1 (ranging from 0 to 12 months according to the inclusion criteria) was not associated with infection clearance outcomes ($p > 0.09$) (Additional file 1). Among those 21 participants (54%) HPV16/18-positive at visit 1, 19 participants (91%) maintained positivity through visit 4. For anal samples, 20 participants (51%) were HPV16/18-positive at visit 1, with 8 participants (40%) maintaining the positivity through visit 4, and one sample yielding invalid results (Fig. 2). Oral samples were tested at visit 1, with two participants being HPV16-positive (5%).

Table 1
Baseline characteristics of the RIFT-HPV1 study population

Characteristic	Overall N = 39[1]	HPV16 N = 16[1]	HPV18 N = 5[1]	Other N = 18[1]
Age	44 (38, 50)	49 (43, 58)	47 (43, 48)	39 (36, 44)
Ethnicity				
Caucasic white	31 (79%)	16 (100%)	3 (60%)	12 (67%)
Latin American	8 (21%)	0 (0%)	2 (40%)	6 (33%)
Center				
Catalan Institute of Oncology	23 (59%)	12 (75%)	2 (40%)	9 (50%)
ASSIR Delta del Llobregat	9 (23%)	2 (13%)	3 (60%)	4 (22%)
Bellvitge University Hospital	4 (10%)	1 (6.3%)	0 (0%)	3 (17%)
MAR Health Park	3 (8%)	1 (6.3%)	0 (0%)	2 (11%)
Tobacco				
Yes	13 (33%)	8 (50%)	1 (20%)	4 (22%)
No	26 (67%)	8 (50%)	4 (80%)	14 (78%)
Alcohol				
Yes	7 (18%)	3 (19%)	1 (20%)	3 (17%)
No	32 (82%)	13 (81%)	4 (80%)	15 (83%)
Abnormal cytology and/or cervical lesion				
None	16 (41%)	6 (38%)	3 (60%)	7 (39%)
ASC-US	8 (21%)	4 (25%)	1 (20%)	3 (17%)
CIN1/LSIL	15 (38%)	6 (38%)	1 (20%)	8 (44%)
Previous HPV test				
HPV16	28 (72%)	16 (100%)	0 (0%)	12 (67%)
HPV18	11 (28%)	0 (0%)	5 (100%)	6 (33%)
Weeks from HPV previous test to visit 1	19 (15, 27)	17 (15, 21)	15 (13, 18)	24 (18, 34)
Oral contraceptives				
Yes	3 (7.7%)	2 (13%)	0 (0%)	1 (5.6%)
No	36 (92%)	14 (88%)	5 (100%)	17 (94%)

Characteristic	Overall N = 39[1]	HPV16 N = 16[1]	HPV18 N = 5[1]	Other N = 18[1]
Menopause				
Pre-menopause	26 (67%)	7 (44%)	3 (60%)	16 (89%)
Peri-menopause	6 (15%)	3 (19%)	2 (40%)	1 (5.6%)
Post-menopause	7 (18%)	6 (38%)	0 (0%)	1 (5.6%)
Sexual partners in the last 6 months				
0	6 (15%)	3 (19%)	1 (20%)	2 (11%)
1	27 (69%)	12 (75%)	4 (80%)	11 (61%)
2+	6 (15%)	1 (6.3%)	0 (0%)	5 (28%)

[1] Median (Q1, Q3); n (%). Abbreviations: ASC-US = Atypical Squamous Cells of Undetermined Significance, CIN1 = Cervical Intraepithelial Neoplasia grade 1, LSIL = Low-grade Squamous Intraepithelial Lesion.

Neutralization levels in serum, cervical and oral samples before and after vaccination

Using the EGFP-PBNA method, neutralization curves and EC_{50} values were extracted from serum, cervical, oral, and anal samples across study visits. The utilized HPV16-EGFP PsV stock was quantified at 1.5×10^{11} EGFP plasmid copies/μL, which under assay conditions (30,000 cells per well and infection with 80 μL of 1:7,500 diluted stock) corresponded to a multiplicity of infection (MOI) of approximately 50,000 VGE per cell. After initial evaluation, anal samples were excluded from further analyses as most samples did not meet assay thresholds. Mean R^2 values for the neutralization curves of serum, cervical and oral samples were 0.99, 0.97, and 0.95, respectively. Four samples with $R^2 < 0.85$ (two cervical and two oral) were excluded from further analyses. For serum samples, neutralizing EC_{50} titers were detected in 33% (13/39) participants before vaccination, significantly increasing to 97% (38/39), 100% (39/39) and 100% (39/39) after one, two, and three doses, respectively ($p < 0.001$). GM EC_{50} values in those detected participants significantly increased from 712 (95% CI: 476–1,066) to 7,110 (3,915–12,913), 5,188 (3,140–8,573), and 19,194 (13,670–26,950), respectively; with three-dose responses being significantly higher than one- and two-dose responses ($p < 0.001$). For cervical samples, neutralizing EC_{50} titers were only detected in 8% (3/39) participants before vaccination, significantly increasing to 69% (27/39), 49% (19/39) and 100% (39/39) after one, two, and three doses, respectively ($p < 0.001$). GM EC_{50} values in those detected participants were 11.6 (95% CI: 3.34–40.2), 11.10 (8.33–14.80), 9.97 (7.39–13.50), and 10.70 (8.38–13.80), respectively. For oral samples, neutralizing EC_{50} titers were only detected in 10% (4/39) participants before vaccination, significantly increasing to 77% (30/39), 31%

(12/39), and 80% (31/39) after one, two, and three doses, respectively ($p \leq 0.01$). GM EC_{50} values in those detected participants were 10.30 (95% CI: 6.98–15.10), 9.32 (7.77–11.20), 10.10 (95% CI: 7.47–13.80), and 8.68 (7.32–10.30), respectively (Table 2). Inclusion of HPV16 positivity as a covariate did not improve EC_{50} model fit and was not significantly associated with EC_{50} values across any sample type ($p > 0.169$). Pairing systemic EC_{50} values with mucosal (cervical and oral) EC_{50} values for each participant revealed a moderate but limited positive association in both cervical ($r_s = 0.34$, $p = 0.015$) and oral samples ($r_s = 0.37$, $p = 0.011$) (Fig. 3). Total protein concentration in mucosal samples was evaluated in all participants across study visits. No significant differences were observed across visits in either cervical ($p > 0.168$) or oral samples ($p > 0.739$) (Additional file 3).

Cervical samples were further analyzed by testing the starting dilution (1:5) in triplicate for all participants across the four study visits. Mean HPV16-PsV neutralization percentages in cervical samples significantly increased with each dose, from 36.4% (95% CI: 29.0–43.7) before vaccination to 60.8% (95% CI: 53.4–68.2) after one dose, 67.9% (95% CI: 60.5–75.2) after two doses, and 77.9% (95% CI: 70.5–85.2) after three doses ($p \leq 0.001$) (Fig. 4). Inclusion of HPV16 status and menopausal status as covariates did not improve the neutralization model fit, and neither variable was significantly associated with cervical neutralization (HPV16 positivity: $p = 0.281$; Menopause: $p = 0.381$). Given the primary focus on cervical neutralization, serum and oral samples were not assessed in triplicate; however, the starting dilution values of their respective neutralization curves were compiled for comparative purposes (Fig. 5). HPV16-PsV neutralization levels in serum samples at the starting dilution (1:200) were 43.1% (95% CI: 36.1–50.3), 94.1% (89.5–96.8), 93.4% (89.0–96.1), and 98.8% (97.0–99.5) for visits 1 to 4, respectively. Consistent with the EC_{50} findings, responses after three doses were significantly higher than after one or two doses ($p < 0.006$), with no significant differences between one- and two-dose responses ($p = 0.750$). For oral samples, mean neutralization levels at the starting dilution (1:5) were 27.7% (95% CI: 23.5–32.4), 70.2% (65.5–74.6), 42.3% (37.4–47.4), and 69.5% (64.9–73.8) for visits 1 to 4, respectively. Vaccination significantly increased baseline oral neutralization levels ($p < 0.014$), with two-dose responses being significantly lower than responses after one or three doses ($p < 0.001$). See Additional file 2 for an example of PsV neutralization across visits in different sample types from the same participant.

Table 2
EC_{50} responses by sample type before and after one, two and three doses

Sample type	Vaccination	EC_{50} detection N = 39[1]	GM EC_{50}	95% CI
Serum	Before	13 (33%)	712	476–1066
	One dose	38 (97%)	7,110	3,915–12,913
	Two doses	39 (100%)	5,188	3,140–8,573
	Three doses	39 (100%)	19,194	13,670–26,950
Cervical	Before	3 (8%)	11.6	3.34–40.20
	One dose	27 (69%)	11.10	8.33–14.80
	Two doses	19 (49%)	9.97	7.39–13.50
	Three doses	39 (100%)	10.70	8.38–13.80
Oral	Before	4 (10%)	10.30	6.98–15.10
	One dose	30 (77%)	9.32	7.77–11.20
	Two doses	12 (31%)	10.10	7.47–13.80
	Three doses	31 (80%)	8.68	7.32–10.30

[1]n (%), EC_{50}: Effective concentration 50%, GM: Geometric Mean, CI: Confidence Interval

EGFP-PBNA assay performance

Assay reproducibility was assessed in a subset of samples from 4 participants at visit 1 (only for serum samples) and visits 2 and 4 (for serum, cervical and oral samples) (n = 28). Final set after exclusion of undetectable EC_{50} values included 10 serum samples, 7 cervical samples and 3 oral samples (n = 20). Each sample was analyzed in triplicate across three separate plates. Ln-transformed EC_{50} values showed high repeatability overall (ICC = 0.98, 95% CI = 0.96–0.99), obtaining sample-specific ICC values of 0.93 (95% CI = 0.81–0.98) in serum samples, 0.97 (95% CI = 0.88–0.99) in cervical samples and 0.91 (95% CI = 0.38–0.99) in oral samples (Table 3). Correlation between the EGFP-PBNA assay and the gold-standard SEAP-PBNA was evaluated in the same subset of samples (n = 28). Final paired set after exclusion of undetectable EC_{50} values included 9 serum samples, 6 cervical samples, and 6 oral samples (n = 21). Multilevel Spearman correlation showed a strong association between the EGFP-PBNA and SEAP-PBNA assays ($r_s = 0.71$, $p < 0.001$) (Fig. 6).

Table 3
EGFP-PBNA intraclass correlation coefficients by sample type

Sample type	EC_{50} detection	Ln (EC_{50}) ICC	95% CI
Serum	10/12 (83%)	0.93	0.81–0.98
Cervical	7/8 (89%)	0.97	0.88–0.99
Oral	3/8 (38%)	0.91	0.38–0.99
Overall	20/28 (71%)	0.98	0.96–0.99

EC_{50}: Effective concentration 50%, ICC: Intraclass correlation coefficient, CI: Confidence Interval

Discussion

Following completion of recruitment for the RIFT-HPV1 cohort, we present findings from the characterization of systemic and mucosal HPV16-neutralizing antibody responses following nonavalent HPV vaccination in women with a recent HPV16/18-positive cervical test. As expected, retesting of cervical samples after recruitment revealed a substantial clearance rate of HPV16/18 infection (46%) (53). This allowed comparison of nAb activity between women with an active infection and those who had cleared infection prior to vaccination (visit 1). The anal HPV16/18 positivity rate among women with prior cervical infection was 51%, consistent with previously reported findings (54). Oral HPV16 prevalence reached 5%, higher than reported in the general population (55). Consistent with previous studies, cervical abnormal findings (ASC-US and CIN1/LSIL) were associated with smoking and with having ≥ 2 sexual partners in the last 6 months, although the latter did not reach statistical significance (56–59).

Vaccination induced a substantial increase in HPV16-neutralizing antibodies across all compartments (serum, cervical, and oral), regardless of baseline HPV16 status. Serum analysis showed that 33% of women had seroconverted prior to vaccination, whereas 97% and 100% of women exhibited seroconversion after one and two/three doses, respectively. Geometric mean EC_{50} values significantly increased after vaccination, with three-dose responses being significantly higher than those observed after one or two doses. Similar trends were observed in mucosal compartments, where only 8% (cervical) and 10% (oral) of women exhibited measurable neutralizing activity above the EC_{50} threshold at baseline. These findings confirm, as previously reported, that although natural infection can induce detectable immune responses, vaccine-induced immunity is substantially stronger – even after a single dose (60). Collectively, these data highlight the strong immunogenicity of the nonavalent vaccine and suggest enhanced transudation of antibodies into mucosal tissues, increasing neutralizing capacity even in the context of active infection.

As previously described, systemic responses after vaccination were stronger than mucosal responses (23, 25, 61–63). After a single dose, detection rates remained lower in cervical (69%) and oral (77%)

samples, with cervical responses reaching full detection after three doses, while oral responses remained comparatively lower (80%). While serum GM EC_{50} values increased significantly after vaccination, mucosal GM EC_{50} values remained relatively stable across visits, likely reflecting lower antibody concentrations and limitations in assay sensitivity. Nevertheless, when assessing neutralization percentages at the starting dilution, clear increases were observed in mucosal samples following vaccination. Although moderate, the positive correlations found between serum and mucosal EC_{50} values support the concept of antibody transudation from the systemic circulation to mucosal tissues, resulting in detectable but lower antibody levels at viral entry sites (23, 25, 61–63). A decrease in EC_{50} detection rates in cervical and oral samples was observed between visit 2 (after one dose) and visit 3 (after two doses). This reduction may be explained by the longer interval between vaccination and sampling at visit 3 (four months vs. two months). In contrast, such temporal decline was not observed in serum, likely reflecting persistently high circulating antibody titers. These findings suggest that neutralizing antibody detection at mucosal sites may be more sensitive to temporal and dose-related variability that is not captured by standard serum measurements. Therefore, mucosal antibody assessment may provide a more informative indicator of the capacity to neutralize newly shed viral particles in HPV-positive individuals.

Further evaluating cervical neutralization levels across all study visits, dose-dependent increases were observed after vaccination, rising from 36.6% pre-vaccination to 60.7%, 68.0%, and 77.4% after one, two, and three doses, respectively. Notably, the multiplicity of infection (MOI) used in the assay (50,000 VGE/cell) greatly exceeds physiological exposure levels, as HPV16 viral loads in cervical epithelium are typically in the range of 1–2,000 viral copies per cell (64). Under these stringent assay conditions, the observed HPV16 antibody responses in cervical samples suggest that vaccine-induced mucosal immunity may contribute to neutralization of locally shed virions under physiological conditions, potentially reducing autoinoculation and onward transmission among HPV16-positive women.

While previous studies have detected HPV-specific antibodies in cervicovaginal and oral secretions after vaccination, most have focused on HPV-naïve populations or measured binding rather than functional neutralizing antibodies. In contrast, our study assesses HPV16-neutralizing activity in both HPV16-positive and HPV16-negative individuals using a pseudovirion-based neutralization assay, providing insight into the potential benefits of vaccination in already infected individuals. The inclusion of multiple compartments and a longitudinal design enabled within-subject comparisons across anatomical sites and time points. Furthermore, the EGFP-based PBNA showed good concordance with the SEAP-PBNA reference assay, supporting its suitability as a scalable and cost-effective alternative. Integration of automated microscopy increased throughput and provided additional qualitative information on infection conditions, which may be particularly valuable when analyzing heterogeneous clinical mucosal samples.

Several limitations should be considered. First, the absence of an unvaccinated group limits the ability to assess the possible effects that natural history might have in the immunity response. Second, cervical antibody levels are influenced by hormonal fluctuations across the menstrual cycle and contraceptive use, which were not controlled for and may have contributed to variability and moderate correlations

observed between serum and cervical responses (23, 61). Third, anal samples could not be reliably analyzed, highlighting technical challenges related to sample collection and emphasizing the need for optimized protocols. Finally, although these findings demonstrate strong immunogenicity, establishing an effect of vaccination on transmission requires complementary approaches assessing both viral presence and infectivity. Functional infectivity assays will be essential to determine whether vaccine-induced mucosal antibodies can neutralize newly shed virions and thereby limit autoinoculation and onward transmission among HPV-infected individuals (65). Such studies incorporating virological infectivity endpoints are currently underway within the RIFT-HPV trial framework.

Conclusions

Nonavalent HPV vaccination significantly increased HPV16-neutralizing antibody responses in serum, cervical, and oral compartments in women with a recent HPV16/18-positive cervical test, independent of baseline HPV16 status. The vaccine-induced antibody response observed in cervical samples suggests that mucosal immunity may contribute to the neutralization of locally shed virions under physiological conditions in HPV-positive women.

Abbreviations

AEMPS
Spanish Agency of Medicines and Medical Devices
ASC
US–Atypical Squamous Cells of Undetermined Significance
BCA
Bicinchoninic Acid
BIC
Bayesian Information Criterion
CCC
Concordance Correlation Coefficient
CI
Confidence Interval
CIBERESP
Biomedical Research Networking Center for Epidemiology and Public Health
CIN
Cervical Intraepithelial Neoplasia
CRE
HUB–Clinical Research Ethics Committee of Bellvitge University Hospital
DNA
Deoxyribonucleic Acid
EC_{50}

50% Effective Concentration

EGFP

Enhanced Green Fluorescent Protein

EGFP

PBNA–Enhanced Green Fluorescent Protein Pseudovirion–Based Neutralization Assay

FACS

Fluorescence–Activated Cell Sorting

GM

Geometric Mean

HPV

Human Papillomavirus

HSIL

High–Grade Squamous Intraepithelial Lesion

ICC

Intraclass Correlation Coefficient

ICH

International Council for Harmonisation

IDIBELL

Bellvitge Biomedical Research Institute

IQR

Interquartile Range

IRB

Institutional Review Board

LSIL

Low–Grade Squamous Intraepithelial Lesion

MOI

Multiplicity of Infection

nAbs

Neutralizing Antibodies

NB

Neutralization Buffer

NLuc

Nanoluciferase

PBS

Phosphate–Buffered Saline

PBNA

Pseudovirion–Based Neutralization Assay

PsV

Pseudovirion

qPCR

Quantitative Polymerase Chain Reaction
R^2
Coefficient of Determination
REec
Spanish Clinical Studies Registry
RLU
Relative Light Units
SEAP
Secreted Alkaline Phosphatase
SEAP
PBNA–Secreted Alkaline Phosphatase Pseudovirion–Based Neutralization Assay
SIN
Subject Identification Number
VGE
Viral Genomic Equivalents
WHO
World Health Organization

Declarations

Ethics approval and consent to participate

The RIFT-HPV study, EudraCT number 2021-005229-26 (posteriorly updated to European Union Clinical Trial number 2024-515228-36-00), received local Institutional Review Board (IRB) approval by CRE-HUB (AC032/21) and AEMPS (MUH/CLIN/EC) approval on March 2022, with the current protocol version 4.2 dated June 1st, 2025. The study was conducted in accordance with the principles of the Declaration of Helsinki, International Council for Harmonization (ICH) Good Clinical Practice guidelines ICH E6(R3) and local legislation (RD 1090/2015), and in full conformity with relevant regulations. Written consent was acquired from each participant upon execution of the Informed Consent Form, following which a distinct SIN was assigned to ensure anonymity. The list of study subjects' IDs and corresponding SINs was kept by the study site under strict confidentiality in a secured digital environment. Samples will not be transferred to third parties and will be used in future studies on the diagnosis and prevention of HPV infections and related diseases which have been approved by CRE-HUB. Use of samples for purposes unrelated to those specified in this section will require express reconsent from study subjects.

Consent for publication

Not applicable.

Competing Interests

Cancer Epidemiology Research Program from Catalan Institute of Oncology (ICO) – Bellvitge Biomedical Research Institute (IDIBELL) has received sponsorship/grants from MSD, Roche, GSK, IDT, Hologic and Seegene which are not related to the present study.

Funding

This study was funded by Instituto de Salud Carlos III through the projects [CIBERESP CB06/02/0073 and PI20/01819] (Co-funded by European Social Fund investing in your future / European Regional Development Fund - a way to build Europe) and MSD MISP call [IISP 59383 (RIFT1)]. This work was also supported by the Secretariat for Universities and Research of the Ministry of Business and Knowledge of the Government of Catalonia (2021SGR01354). The funders played no role in study design, data collection, analysis and interpretation of data, or the writing of this manuscript. We thank CERCA Programme / Generalitat de Catalunya for institutional support.

Author Contribution

FB and MP designed the study. VL and AA performed experiments. VL, GP and ST analyzed the data. VL and MP wrote the manuscript. ML coordinated the study. MF, CO and YP performed study interventions at the recruiting center. JS, CG, BR, MaS, MoS and PP identified candidates at satellite centers and referred them to the recruitment center. AF managed vaccine supply from the onsite pharmacy. VL, ST, GP, MF, AF, LA, FB, and MP edited manuscript. All authors reviewed and approved the final manuscript for submission.

Acknowledgement

The authors gratefully acknowledge all the women participating in this study. We thank the Clinical Research and Clinical Trials Unit (UICEC-IDIBELL) at the Bellvitge University Hospital (HUB) for their support. We acknowledge the Bioimaging Platform at the Bellvitge Biomedical Research Institute (IDIBELL) for optical microscopy training and for developing a custom FIJI/ImageJ script for automated image analysis.

Data Availability

The data supporting the conclusions of this article is included within the article and its additional files. Further de-identified data underlying the reported results are available from the corresponding author upon reasonable request. Summary study results will be reported in the Spanish Clinical Studies Registry (REec) and ClinicalTrials.gov upon study completion. The underlying code used for image analysis is available at CORA – Repositori de Dades de Recerca (RDR) and can be accessed via this link https://doi.org/10.34810/DATA3460.

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Figures

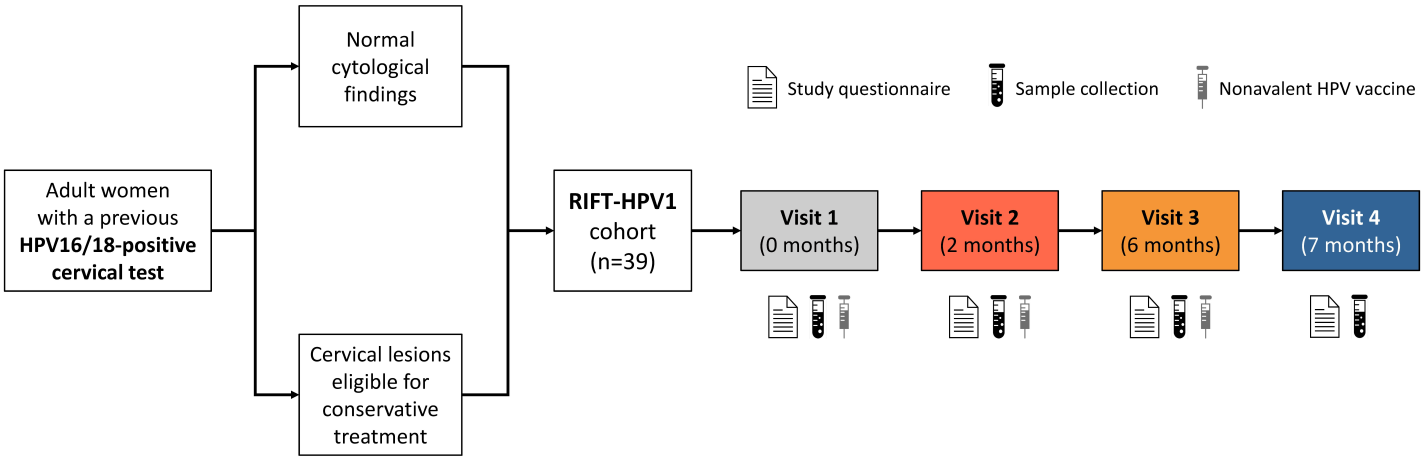


Figure 1

Study design of the RIFT HPV1 cohort. Flowchart of 39 unvaccinated adult women with cervical HPV16/18 infection completing four visits at months 0, 2, 6 and 7, including Gardasil 9 vaccination

(visits 1-3), and health questionnaire and multisite sample collection (visits 1-4).

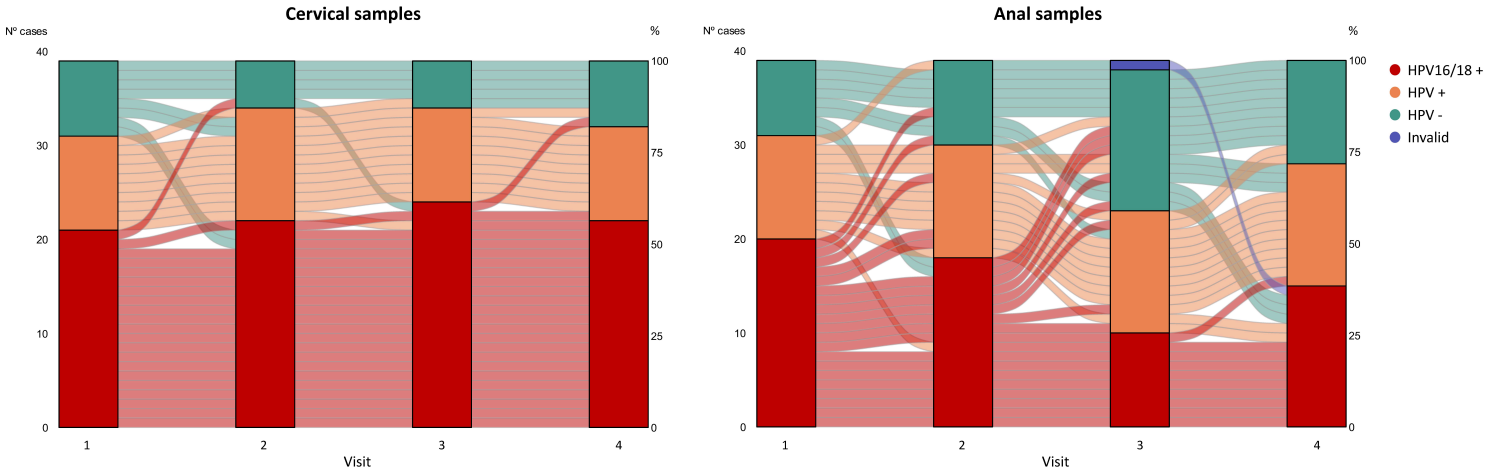


Figure 2

Longitudinal HPV status in cervical and anal samples across study visits. Sankey diagrams show HPV status across four visits (V1–V4) for cervical (left) and anal (right) samples from all participants (n=39). Colors indicate HPV16/18 positive (red), HPV positive (non-HPV16/18) (orange), HPV negative (green), and invalid (blue) test results. Flows represent individual transitions between visits.

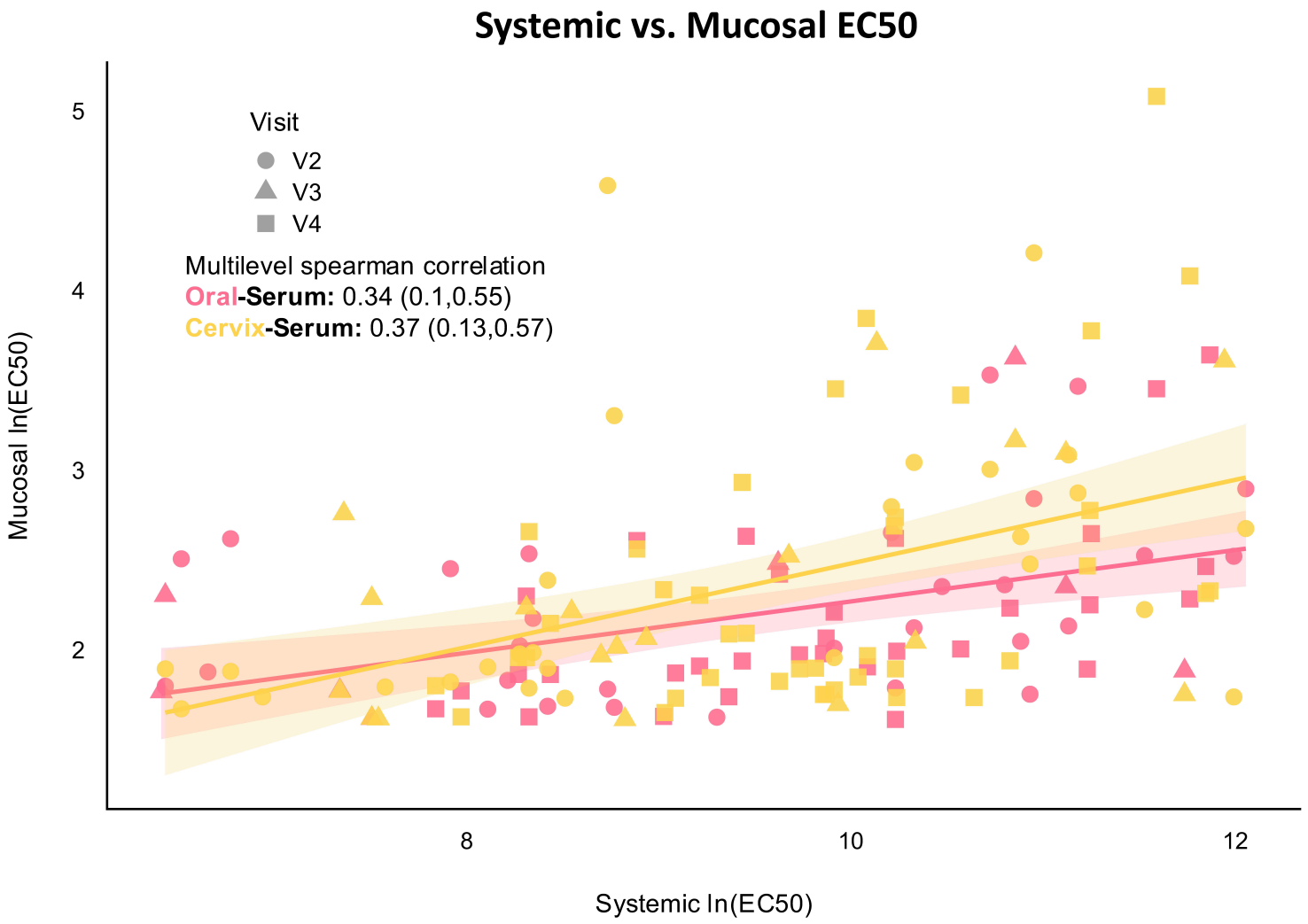


Figure 3

Correlation between systemic and mucosal EC_{50} values. EC_{50} values measured in serum (systemic) are plotted against paired mucosal EC_{50} values from oral (pink) and cervical (yellow) samples on natural logarithmic (ln) scales. Each point represents a matched serum–mucosal pair (cervical: n = 85; oral: n = 68). One-dose responses are shown as circles, two-dose responses as triangles and three-dose responses as squares. Lines represent linear fits with shaded areas indicating 95% confidence intervals.

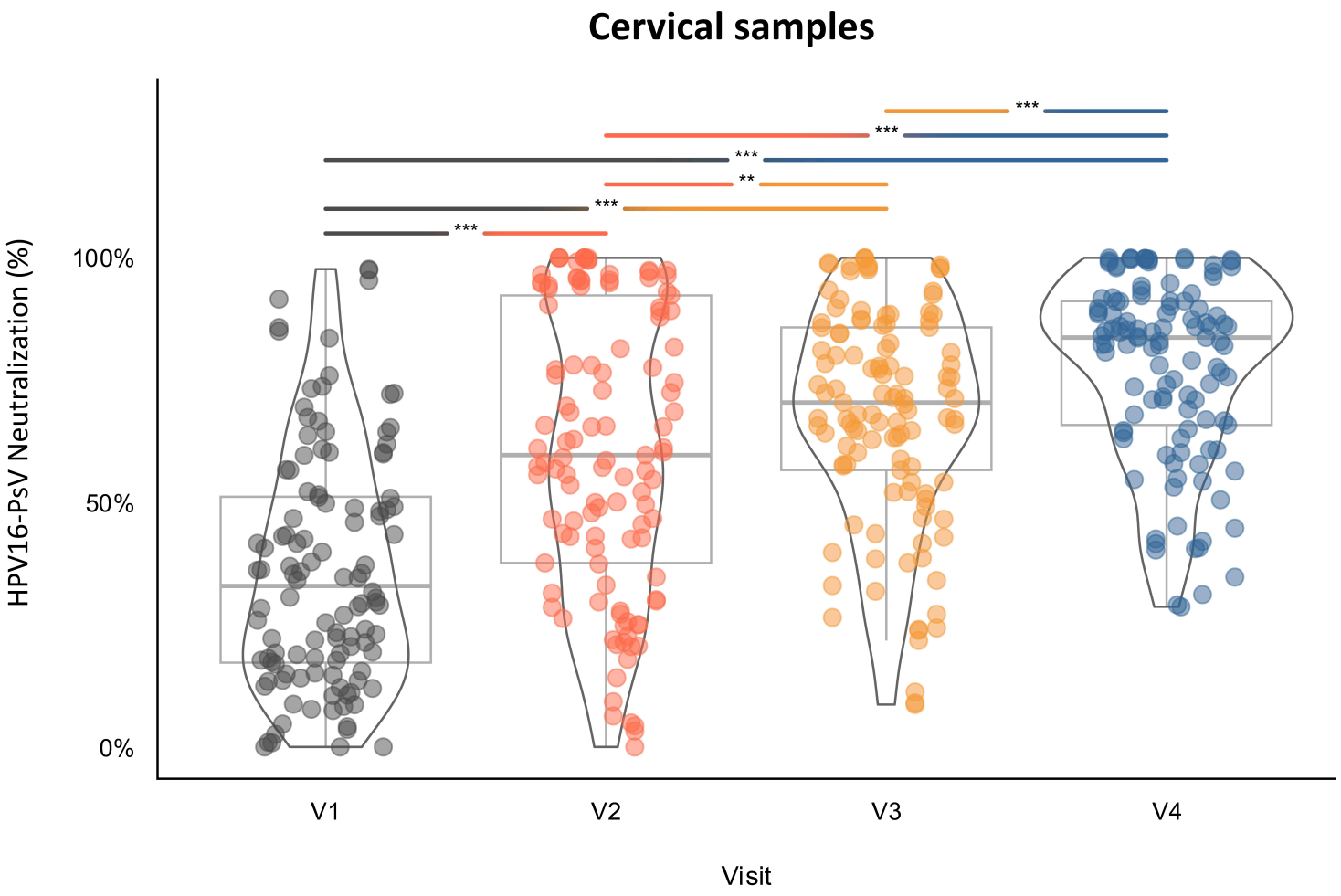


Figure 4

HPV16-PsV neutralization in cervical samples across study visits. Boxplots represent HPV16-PsV neutralization (%) in cervical samples collected before vaccination (visit 1) and after one, two and three doses (visits 2 – 4), shown in gray, coral, orange, and blue, respectively (n=39). Violin plots depict the distribution and density of values for each visit, with boxplots indicating median and interquartile range (IQR). Statistical comparisons between visits are indicated by bars above the plots (**, $p < 0.01$; ***, $p < 0.001$).

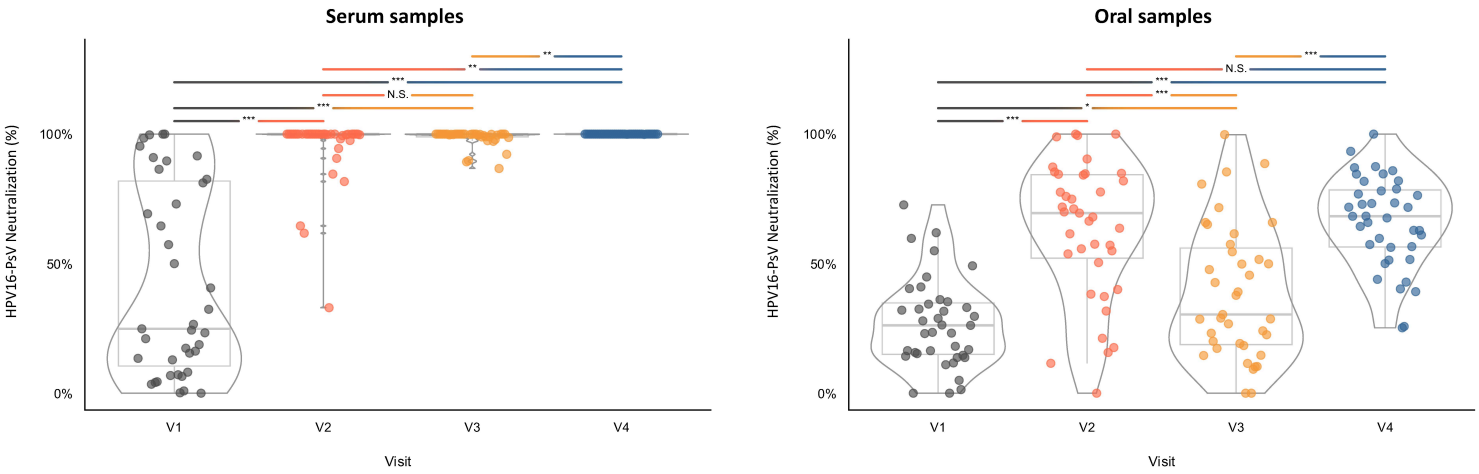


Figure 5

HPV16-PsV neutralization in serum and oral samples across study visits. Boxplots represent HPV16-PsV neutralization (%) in serum (left) and oral (right) samples before vaccination (visit 1) and after one, two and three doses (visits 2 to 4, respectively), shown in gray, coral, orange, and blue, respectively (n=39). Violin plots depict the distribution and density of values for each visit, with boxplots indicating median and interquartile range (IQR). Statistical comparisons between visits are indicated by bars above the plots (**, $p < 0.01$; ***, $p < 0.001$).

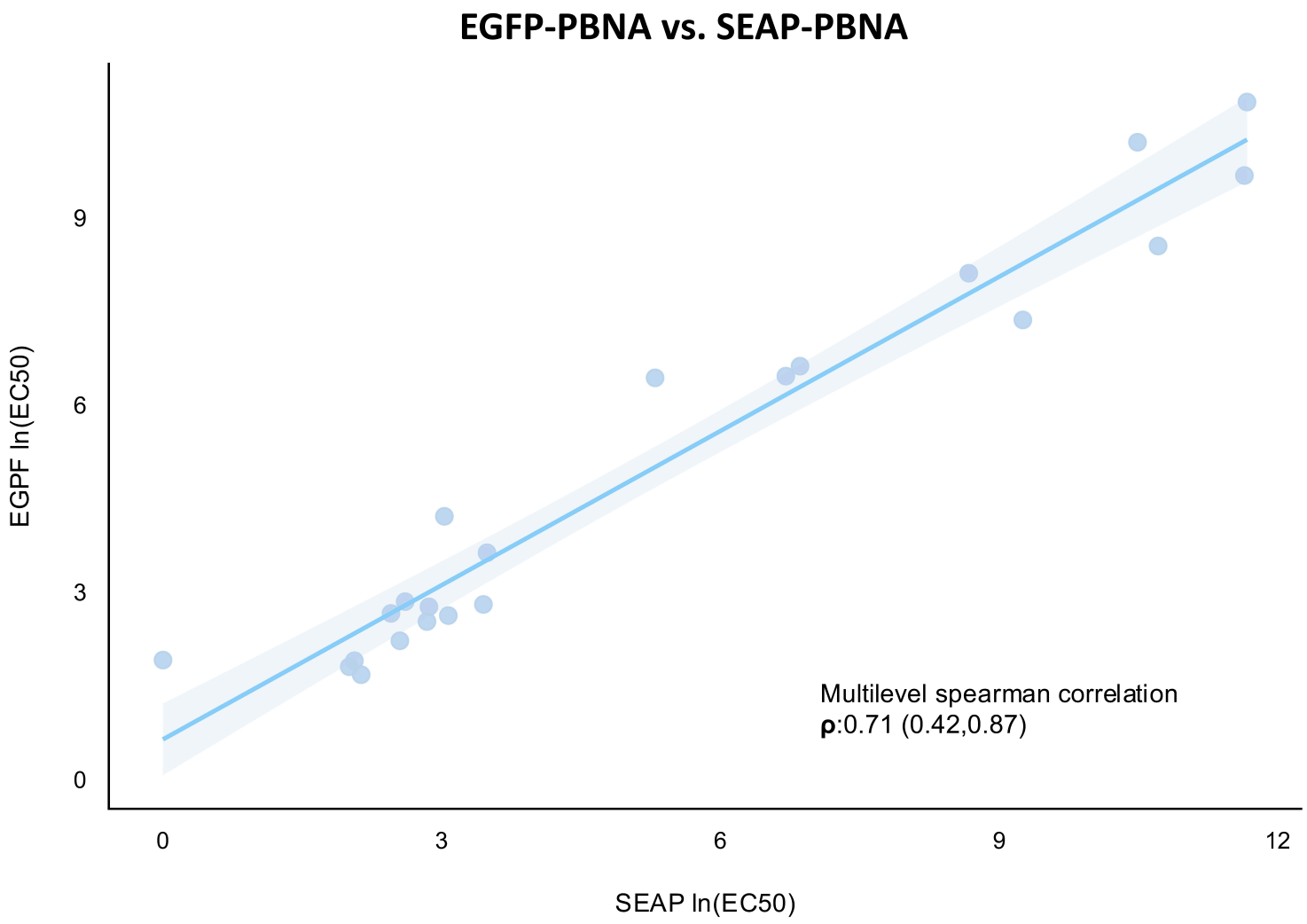


Figure 6

Association between EC_{50} values measured by EGFP-PBNA and SEAP-PBNA. Paired EC_{50} values obtained using EGFP-PBNA (y-axis) and SEAP-PBNA (x-axis) are shown on a natural logarithmic (ln) scale for a subset of samples (n = 21). Each point represents a matched measurement pair. The solid line represents a linear fit with the shaded area indicating the 95% confidence interval.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.pdf
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