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RESEARCH ARTICLE

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HPV-specific antibodies in female genital tract secretions captured via first-void urine retain their neutralizing capacity

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ABSTRACT

Human papillomavirus (HPV) vaccines, primarily relying on neutralizing antibodies, have proven highly effective. Recently, HPV-specific antibodies have been detected in the female genital tract secretions captured by first-void urine (FVU), offering a minimally invasive diagnostic approach. In this study, we investigated whether HPV16-specific antibodies present in FVU samples retain their neutralizing capacity by using pseudovirion-based neutralization assays. Paired FVU and serum samples (vaccinated $n = 25$, unvaccinated $n = 25$, aged 18–25) were analyzed using two orthogonal pseudovirion-based neutralization assays, one using fluorescence microscopy and the other using luminescence-based spectrophotometry. Results were compared with HPV16-specific IgG concentrations and correlations between neutralizing antibodies in FVU and serum were explored. The study demonstrated the presence of neutralizing antibodies in FVU using both pseudovirion-based neutralization assays, with the luminescence-based assay showing higher sensitivity for FVU samples, while the fluorescence microscopy-based assay exhibited better specificity for serum and overall higher reproducibility. High Spearman correlation values were calculated between HPV16-IgG and HPV16-neutralizing antibodies for both protocols (r_s: 0.54–0.94, $p < .001$). Significant Spearman correlations between FVU and serum concentrations were also established for all assays (r_s: 0.44–0.91, $p < .01$). This study demonstrates the continued neutralizing ability of antibodies captured with FVU, supporting the hypothesis that HPV vaccination may reduce autoinoculation and transmission risk to the sexual partner. Although further protocol optimizations are warranted, these findings provide a foundation for future research and larger cohort studies that could have implications for the optimal design, evaluation, and implementation of HPV vaccination programs.

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Introduction

Human papillomavirus (HPV) is a highly prevalent sexually transmitted virus causing diseases like genital warts and cervical cancer.[1–3] Current HPV vaccines are highly effective in preventing HPV infections and associated diseases.[4–7] The important role of neutralizing antibodies (nAbs) for protection against HPV infection and disease is well-established.[8–10] However, only limited data is available on the neutralizing capacity of HPV-specific nAbs in the genital tract, the primary site of HPV infection.

First-void urine (FVU) has been proposed as a noninvasive sample for assessing HPV-specific antibodies originating from the genital tract.[11–13] Current methods for measuring HPV-specific nAbs involve blood draws, which can be uncomfortable for patients and may not accurately reflect the neutralizing capacity of Abs at the site of infection. With FVU, the initial part of the urine stream is collected, serving as a rinsing liquid to capture the female genital tract secretions that have accumulated around the labia minora.[14] FVU collection is a simple and convenient procedure that individuals can perform in their homes and does not cause any trauma at the site of infection.[15–17]

To date, HPV vaccination programs only target young individuals, and although studies indicate some benefits for people living with HPV-associated disease, vaccinating at an older age is not routinely considered.[6–7-22] While current prophylactic vaccines are not expected to provide therapeutic effects, the induced nAbs could potentially interact with newly produced virions, preventing the spread of HPV within the genital tract and transmission to the sexual partner.[23] With female genital tract sampling using FVU, antibodies at the site of infection are captured, making it an ideal source to investigate the virion-antibody interaction.

Pseudovirion (PsV)-based neutralization assays (PBNA) measure nAbs against HPV using cell lines that express a reporter gene in response to HPV PsV infection.[24] While widely used for testing serum samples and in some cases for cervical samples, its application in FVU samples is

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unexplored.[25–28] Due to lower concentrations of HPV-specific antibodies in FVU, enrichment is likely needed.[11,29] As FVU contains a concentrated amount of proteins, DNA, viral particles, bacteria, and (debris of) exfoliating cells, compared to the subsequent urine fractions, the odds of contaminating cell growth or interference with PsV infection within the PBNA is expected to be high. Hence, purification of the samples will also be required.

Most of the HPV PBNA protocols use Gaussia luciferase (Gluc), nanoluciferase (NLuc), or secreted alkaline phosphatase (SEAP) due to their simplicity and compatibility with 96-well or 384-well plate formats.[24–30–33] Gluc PBNAs yield comparable results to SEAP while significantly reducing hands-on and detection time. NLuc, as a reporter, offers additional advantages over Gluc, including a longer lifetime, smaller size, and thermal stability.[32,34,35] Another explored option is utilizing enhanced green fluorescent protein (EGFP).[24–32–36–38] However, current methods using PsV with an EGFP reporter in PBNA require collection and/or lysis of the infected cells for measurement using a flow cytometer, making it a less commonly used method due to its time- and labor-intensive nature. Earlier comparisons of the different readouts showed good significant correlations between the different PBNA readout methods, with EGFP providing the least nAb positive samples.[32]

In this study, we are the first that investigated the neutralizing capacity of HPV16-specific antibodies in non-invasively collected FVU. This was done using two different PBNA methods: a widefield microscopy-based PBNA (based on EGFP) and luminescence-based PBNA (based on Nluc), and the results were compared to HPV16-specific IgG concentrations. We analyzed FVU samples from both HPV-vaccinated and unvaccinated women, aiming to identify the most sensitive and reliable PBNA method for measuring HPV-specific nAbs in FVU. Additionally, we evaluated the correlation between FVU and serum nAb concentrations quantified by both PBNA methods. Our study contributed to understanding HPV infection protection mechanisms by exploring the neutralizing capacity of FVU antibodies, providing insights into preventing HPV infection and disease at the infection site.

Materials and methods

Study population

Fifty healthy females aged 18–25, comprising 25 unvaccinated and 25 vaccinated with an HPV vaccine, provided paired FVU and serum samples between May and July 2020 (clinicaltrials.gov ID: NCT04391647). The institutional review board of the Biobank and the ethical committee of Antwerp University Hospital (UZA)/UAntwerp, Belgium (B300201734258) approved all study procedures, and we obtained informed consent from all women before sample collection.

Sample collection and storage

Women were notified about the study via e-mail or social media and interested participants registered online through the Centre for the Evaluation of Vaccination (CEV) webpage. Using the R package tidyverse, the study randomly selected 25 vaccinated and 25 unvaccinated women with a normal age distribution, who received detailed study information via e-mail, including an information brochure and an informed consent form. The women were then asked to plan their appointment at the CEV, where the study team explained the study in detail. To ensure consistent collection of FVU samples, women were instructed not to wash their genitals thoroughly, use a tampon, or urinate at least two hours before FVU sample collection. All FVU samples were collected using the Colli-Pee® 20 ml FV-5020 device (Novosanis, Belgium) prefilled with a urine conservation medium (UCM). Samples were immediately placed in the refrigerator (up to 4 hours post collection), aliquoted, and stored at −80°C (Biobank Antwerpen, Antwerp, Belgium; ID: BE 71,030,031,000) before further analysis. Hemastix® reagent strips were used to assess erythrocyte presence (Siemens Healthcare Diagnostics Inc., Belgium). Blood samples were collected using 10 ml BD Vacutainer® Serum tubes without anticoagulant (Becton-Dickinson, Benelux) and allowed to clot for 30–60 min. After clotting, blood samples were centrifuged at 1000 × g for 10 min at 20°C, and serum was divided into aliquots before storage at − 80°C. All volunteers were asked to complete a questionnaire including information on the menstruation cycle and contraception, demographics, and their sexual status.

Sample processing

FVU samples were purified using an in-house enrichment and purification method. Briefly, we centrifuged a 4 ml aliquot at 4000 × g for 20 min at 21°C in an Amicon Ultra-4 50 K filter device (Merck Millipore, Belgium). Subsequently, the concentrate (>50 µl) was collected with sterile 1X Dulbecco's phosphate buffered saline (dPBS, Thermo Fisher Scientific) to reach a final volume of 500 µl, followed by a second centrifugation at 1000 × g for 10 minutes at 21°C. The resulting supernatant was stored at −20°C in clean 1.5 ml Axygen® MAXYMum Recovery® microtubes (Corning, Inc., U.S.A.) until further analysis. We performed no purification step for serum samples.

DELFIA assay for HPV16-IgG

We measured HPV16-specific IgG concentrations in all FVU and serum samples using an in-house developed HPV16 immunoassay based on time-resolved fluorescence (DELFIA). Dilution series determined optimal sample dilutions and lower limit of quantification (LLOQ). Samples were then tested using a serial 2-fold dilution starting at 1:2 for enriched FVU and 1:800 for serum. A minimum of four dilutions were tested for each sample. Plates were coated with 100 µl of 0.5 µg/ml HPV16 PsV, incubated overnight at 4°C, and washed with 300 µl wash solution (Revvity, Lier, Belgium) before blocking with dPBS (Thermo Fisher Scientific) containing 1% DTPA-purified Bovine Serum Albumin (BSA) (Revvity, Lier, Belgium). After a 1-hour incubation, 100 µl of the sample was added to the plate and left to incubate for 2 h at ambient temperature and with shaking at 300 rpm. Following incubation, plates were washed three times, and 100 µl of 200

ng/ml Eu-anti-human antibody was added to each well for a 1-hour incubation at ambient temperature with shaking at 300 rpm. Plates were washed six times to remove all unbound Eu-labeled reagents, and 200 µl of enhancement solution (Revvity, Lier, Belgium) was added to each well. After 30 min incubation at ambient temperature and shaking at 300 rpm, plates were read using the Viktor Nivo multimode plate reader (Revvity, Lier, Belgium). On each plate, we added a negative and positive control and four dilutions of a serum sample with a known international units (IU)/ml. The parallel line method (PLL) was used to calculate HPV16-specific IgG concentrations for each sample.[39]

Pseudovirion based neutralisation assay (PBNA)

Since we performed a PBNA using two different reporters, we produced two PsV stocks, one with an enhanced green fluorescent protein (EGFP) reporter and the other with a Nanoluciferase (NLuc) reporter. All HPV16 PsV were produced using the protocol by Buck et al.[40] with minor adaptations. Briefly, 293TT HEK cells were cotransfected with the HPV16 L1L2 genes contained in the p16sheLL plasmid and either reporter plasmid phsNuc for NLuc or pCIneoEGFP for EGFP. pCIneoEGFP and p16sheLL were gifts from John Schiller and phsNuc was a gift from Christopher Buck.[41,42] The PsV maturation time for the EGFP PsV was 24 h and 48 h for NLuc PsV. After production, the PsV were titrated and specificity for HPV16 was confirmed with H16.V5 monoclonal antibodies kindly gifted by Dr. Neil Christensen.[43]

For the PBNA, 293TT HEK cells were plated in a 96-well plate in 100 µl neutralization buffer (DMEM w/o phenol red, 10% heat-inactivated Fetal Bovine Serum (FBS), 1% glutamax, 1% nonessential amino acids, 1% Antibiotic-Antimycotic) approximately 4 hours before infection. The amount of preplated cells was 20,000 cells/well in a Cellvis black polystyrene glass bottom plate (Cellvis, California, U.S.A.) for EGFP PBNA and 30,000 cells/well in a tissue culture-treated flat bottom plate (Corning-Costar, New York, U.S.A) for NLuc PBNA. Outer wells were excluded to avoid evaporation and edge effects. Serum samples were 2-fold diluted starting at dilution 1:40 and enriched FVU samples were diluted 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6 using neutralization buffer. Optiprep purified EGFP PsV stocks were diluted 1:480 to reach a minimum of 30% EGFP infection and NLuc PsV stocks were diluted 1:48000 to fall between the linear range of the multimode plate reader. 96 µl of diluted PsV stocks were combined with 24 µl of diluted samples and incubated on ice for 1 h. Additionally, positive neutralization controls (serum from a vaccinated volunteer and heparin (1 mg/ml)) and a negative control (serum from an unvaccinated volunteer that had no measurable HPV16 antibodies) were added to each plate. After incubation, the PsV antibody mixtures were incubated 5 more minutes at room temperature (RT) and 100 µl was added to the preplated cells. The plates were incubated at 37°C and 5% CO_2 for 68-72 h.

High-throughput automated widefield microscopy – EGFP method

For EGFP measurement, 100 µl of supernatant was removed, and the cells were fixated for 20 minutes with 100 µl 4% paraformaldehyde (PFA) before immunofluorescent staining. Cells were washed twice with 200 µl 1X dPBS, permeabilized for 8 minutes using 100 µl of 0.5% Tergitol and washed once with 200 µl 1X dPBS. They were then incubated for 30 minutes at RT with 100 µl 0.02% cyanine5 (Cy5) in a 50% HI-FBS and 50% 1X dPBS mixture. After incubation, cells were washed twice with 200 µl 1X dPBS and incubated for 15 minutes at RT with 100 µl of 1 µg/ml 4′, 6-diamidino-2′-phenylindole, dihydrochloride (DAPI) in 1X dPBS. Cells were washed once more with 200 µl 1X dPBS and 200 µl PBS-NaN_3 was added before storing the plates at 4°C until imaging. Imaging, was performed on a fully automated Nikon Ti Eclipse inverted widefield fluorescence microscope, equipped with a Perfect Focus System and LED-based illumination source. For each well, 20 regions were scanned using a 20×/0.75 Plan Fluor dry lens. The illumination was fine-tuned to ensure minimal intensity fluctuation (<5%) across the entire field of view. For excitation of DAPI, EGFP, and Cy5, we employed 395/25 nm, 470/24 nm, and 640/30 nm LED illumination (Lumencor), respectively. Detection was accomplished using a quadruple dichroic in conjunction with 435/26 nm, 510/40 and 705/72 nm bandpass filters, respectively, employing a DS-Qi2 CMOS camera.

FIJI image analysis freeware version 2.9.0, with the De Vos lab image analysis pipeline (https://github.com/DeVosLab/CellBlocks) was used for image analysis.[44–46] Infected cells were identified by applying a user-defined intensity threshold to the raw EGFP intensity or the EGFP intensity normalized to the average of control cells per independent experimental replicate. Based on this, the ratio of infected cells to the total number of cells was calculated using R statistical software version 4.2.2 (packages dplyr, car, tidyverse). The percentage of infected cells was used to calculate the 50% neutralization activity (effective concentration, EC50) using the 4-parameter curve fit for serum and linear fit for FVU in GraphPad Prism version 9.5.1. Samples were given a concentration if R^2 was above 0.85. Serum samples were considered positive if the HPV type-specific neutralization titer was ≥ 100, as described previously.[33] For FVU samples, only the curve fit and R^2 were used since no cutoff has been established yet.

Luminescence – Nluc method

For the luminescence method, after the 68-72 h plate incubation, 25 µl of the supernatant was used for further analysis using the Nano-Glo® Luciferase Assay kit (Promega, Madison,WI) and results were read on the Viktor Nivo multimode plate reader (Revvity, Lier, Belgium). The antibody concentrations showing 50% neutralization activity were calculated using the same method as described for the EGFP method.

HPV DNA detection

FVU samples were subjected to HPV DNA testing. HPV DNA testing was performed on 1 ml UCM buffered FVU using the Cobas 6800 (Roche Molecular System, Pleasanton, CF, USA) and the clinical cutoff for cervical samples at the Centre for Medical Analysis (CMA, Herentals, Belgium). If the Cobas 6800 test showed HR-HPV DNA positivity, they were genotyped using the Riatol qPCR HPV genotyping assay.[47]

Statistical analysis

We used R statistical software version 4.2.2 to analyze the data. The data was first checked for their normality using Shapiro – Wilk test. If the data were normally distributed, significant differences between parameters were examined using paired t-tests. Otherwise, non-parametric Wilcoxon signed-rank testing was used. Statistical significance was defined as p-adjusted <0.05 (using Holm – Bonferroni method for p-value adjustment). Interactions between demographic parameters and test outcomes (HPV DNA, HPV16-specific Abs, and HPV16-specific nAbs) were calculated using the 'lm' function. Receiver operating characteristic (ROC) analyses were done to determine the lowest DELFIA concentration expected to provide a nAb titer (if present) using the specific PBNA. Spearman rank correlation coefficients were calculated between different assays and sample types.

Results

Impact of patient characteristics on virological and immunological endpoints

We collected paired FVU and serum samples from 50 healthy female volunteers, 25 vaccinated with an HPV vaccine and 25 unvaccinated (Figure 1). The overall median age was 23 (IQR 23–24). Participants received their first vaccine dose at a median age of 14 (IQR 12–14), and the median time between the first vaccine dose and sample collection was 10 (IQR 9–11) years. No interaction was observed between the time since vaccination and the antibody detection ($p \geq .81$). Among the vaccinated volunteers, 12% (3/25) received the 2vHPV vaccine, 84% (21/25) the 4vHPV vaccine, and 4% (1/25) the 9vHPV vaccine, with no significant differences in antibody concentrations based on vaccine type ($p \geq .07$).

Of all participants, 6% (3/50) were current smokers, 20% (10/50) had a smoking history, and 74% (37/50) had never smoked. No interaction was observed between smoking history and antibody concentrations ($p \geq .43$), but a positive association between smoking history and HPV DNA presence was found ($p = .01$). Overall, 64% (32/50) used oral contraceptives, 2% (1/50) used condoms, 18% (9/50) used a hormonal intrauterine device (IUD), 4% (2/50) used a local IUD, and 12% (6/50) used no contraceptive method. No significant interactions were observed between contraceptive use and HPV DNA or antibody presence ($p \geq .23$). Among participants, 62% (31/50) had undergone cervical sample collection, starting at a mean age of 18 (SD 0.96) years. All women (100%) reported being sexually active but had not given birth before inclusion. The

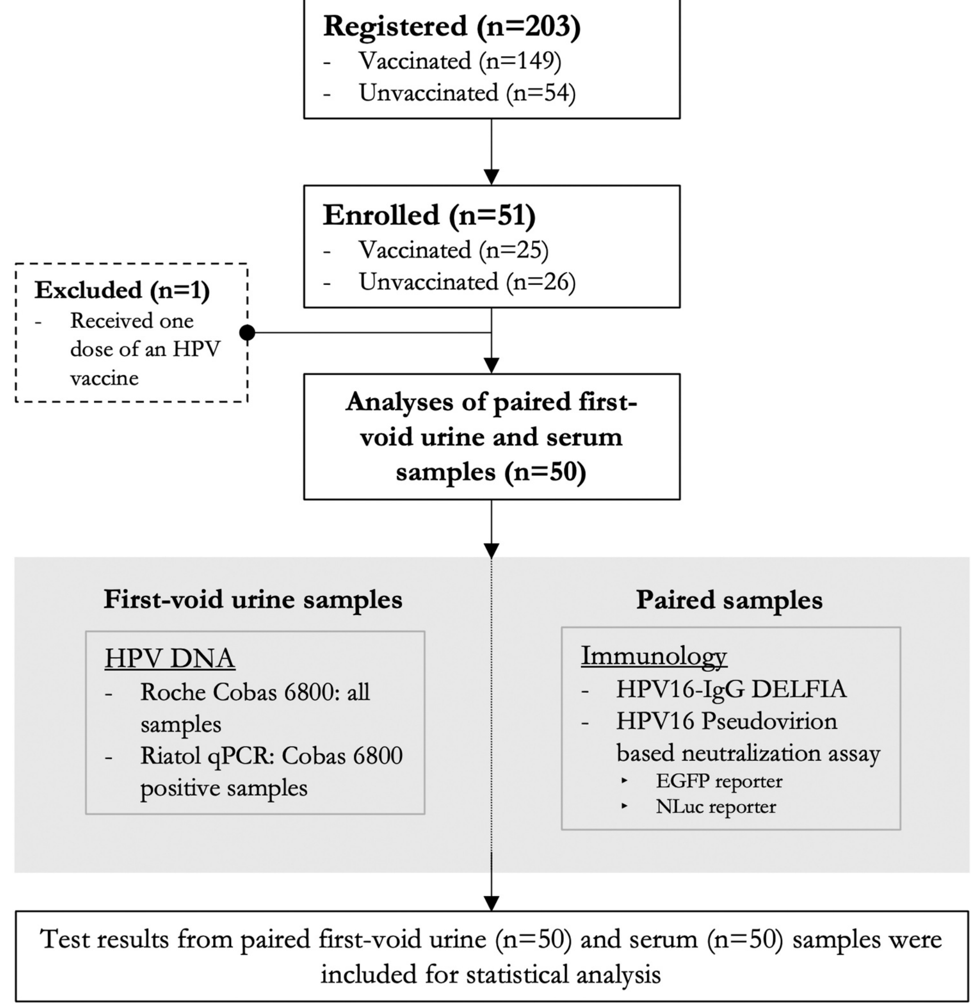

Figure 1. Flow diagram of the study. Test results from 50 paired first-void urine and serum samples were included.

Table 1. Study population characteristics according to vaccination status.

Characteristic	Total	Vaccinated	Unvaccinated
N (%)	50 (100)	25 (100)	25 (100)
Age (median, IQR)	23 (23–24)	23 (23–24)	24 (23–25)
Age of 1st vaccination (median, IQR)	13.5 (12.2–14.2)	13.5 (12.2–14.2)	
Vaccine type (n, %)			
2v HPV	3 (6)	3 (12)	
4v HPV	21 (42)	21 (84)	
9v HPV	1 (2)	1 (4)	
Ever smoked (n, %)			
Current yes	3 (6)	1 (4)	2 (8)
Past yes	10 (20)	5 (20)	5 (20)
No	37 (74)	19 (76)	18 (72)
Contraceptive use (n, %)			
Oral	32 (64)	18 (72)	14 (56)
condom	1 (2)	0 (0)	1 (4)
Hormonal IUD	9 (18)	4 (16)	5 (20)
Local IUD	2 (4)	1 (4)	1 (4)
None	6 (12)	2 (8)	4 (16)
Ever had a cervical smear (n, %)	31 (62)	17 (68)	14 (56)
Age at first cervical smear (mean, SD)	18 (0.96)	18 (0.71)	17 (1.41)
Number of sexual partners (median, IQR)	3 (1–6)	2 (2–6)	3 (1–6)
Cobas 6800 HPV DNA pos (n, %)	11 (22)	6 (24)	5 (20)
Riatol HPV DNA pos (n, %)	9 (18)	5 (20)	4 (16)
Detected HPV types	51, 56, 58, 59, 66, 67, 68	51, 59, 66, 67, 68	51, 56, 58, 67

mean number of lifetime sexual partners was 3 (IQR 1–6), with a highly significant interaction seen with HPV DNA presence ($p \leq .0001$), while no interaction was observed with HPV16-specific antibodies ($p \geq .63$).

The Cobas 6800 assay identified high-risk HPV (HR-HPV) infection in 22% (11/50) of women, including 24% (6/25) of vaccinated and 20% (5/25) of unvaccinated women. Subsequent genotyping using the Riatol qPCR revealed a 18% (9/50) HR-HPV infection rate, including 20% (5/25) of vaccinated and 16% (4/25) of unvaccinated women. Detected HR-HPV types were HPV51, 56, 58, 59, 66, 67, and 68 (Table 1), with no infections from HPV types included in the 2vHPV or 4vHPV vaccines.

HPV16-specific antibody titers in FVU correlate well with those in serum

We used an in-house HPV16 DELFIA assay to quantify HPV16-specific antibody concentrations. Establishing the LLOQ involved a dilution series (Supplementary Figure S1). The FVU LLOQ, set at 0.00803 IU/ml, was determined based on the lowest concentration with coefficient of variation (% CV) of counts < 15%, and accuracy (measured as concordance between expected and PLL-calculated concentration) within the acceptable 80 to 120% range. All Amicon-filtered FVU samples were tested with 1:2 to 1:16 dilutions to precisely detect low antibody levels without excessive background interference. For serum, dilutions of 1:200 to 1:3200 were employed. The serum LLOQ, calculated using the formula $10*SD_{Intercept}$/slope of the standard calibration curve, was 0.0325 IU/ml.[48]

Out of all FVU samples, 62% (31/50) showed detectable HPV16 antibody concentrations (Table 2). Among the unvaccinated cohort, 24% (6/25) were antibody positive, while 100% (25/25) of vaccinated women demonstrated antibody positivity. The median (IQR) FVU HPV16 antibody concentration post-HPV vaccination was 0.047 (0.025–0.104) IU/ml, and the geometric mean titer (GMT) for all positive samples was 0.14 (95% CI 0.03–0.24) (Figure 2). For serum samples, 58% (29/50) had detectable antibody concentrations, with 16% (4/25) of unvaccinated and 100% (25/25) of vaccinated women testing positive. The median (IQR) concentration of HPV16 antibodies in serum post-HPV vaccination was 86.5 (47.2–167.9) IU/ml, and the GMT for all positive samples was 174.04 (95% CI 31.50–317.00). Median concentrations and GMT observed in FVU were approximately 0.07% and 0.08% of those in serum, respectively.

Excellent correlations were established between HPV16-IgG concentration in serum and FVU for the overall cohort ($r_s = 0.91$) and the vaccinated cohort ($r_s = 0.81$). Furthermore,

Table 2. Results for HPV16-IgG using the DELFIA assay and HPV16-nAbs using the EGFP- and Nluc-based PBNAs for both FVU and serum samples. The percentage of samples containing detectable concentrations and the median (IQR) concentration are presented.

Assay	Outcome	Total FVU	Serum	Ratio FVU/serum % (IQR)	Unvaccinated FVU	Serum	Vaccinated FVU	Serum
DELFIA	Positive (%)	31/50 (62%)	29/50 (58%)		6/25 (24%)	4/25 (16%)	25/25 (100%)	25/25 (100%)
	HPV16-IgG (IU/ml)	0.012 (0.000–0.046)	19.6 (0.0–86.1)	0.07 (0.04–0.12)	0.000 (0.000–0.000)	0.0 (0.0–0.0)	0.047 (0.025–0.104)	86.45 (47.2–167.9)
EGFP PBNA	Positive (%)	10/50 (20%)	27/50 (54%)		2/25 (8%)	2/25 (8%)	8/25 (32%)	25/25 (100%)
	HPV16-nAbs EC50	0.00 (0.00–0.00)	197 (0.00–1457)	0.22 (0.09–0.33)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–6.04)	1573 (774–4887)
Nluc PBNA	Positive (%)	19/50 (38%)	28/50 (56%)		1/25 (4%)	3/25 (12%)	18/25 (72%)	25/25 (100%)
	HPV16-Abs EC50	0.00 (0.00–6.65)	211 (0.00–2030)	0.34 (0.15–0.51)	0.00 (0.00–0.00)	0.000 (0.00–0.00)	5.69 (0.00–18.69)	1616 (520–6981)

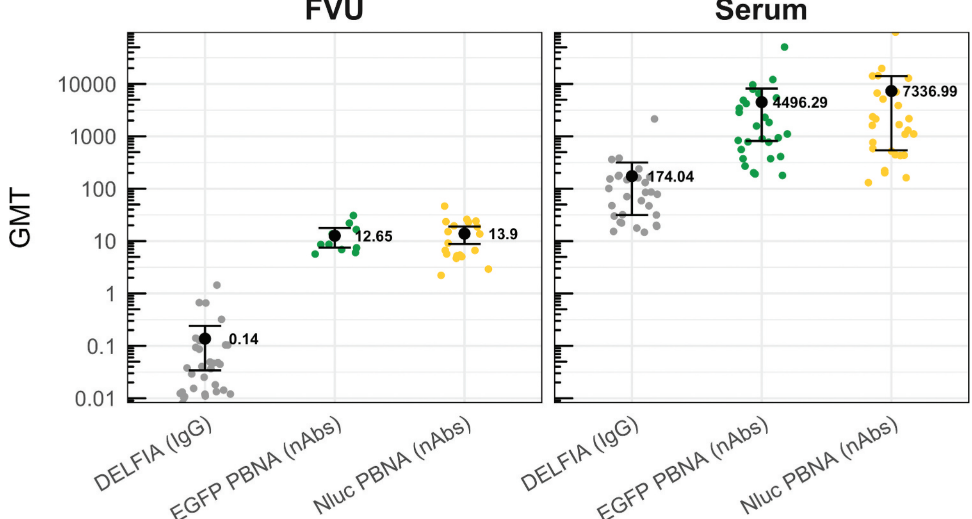

Figure 2. HPV16 IgG (IU/ml) and nAb (EC50) geometric mean titers (GMT) and confidence intervals for only antibody positive samples are presented. Colored dots represent the concentration of one sample.

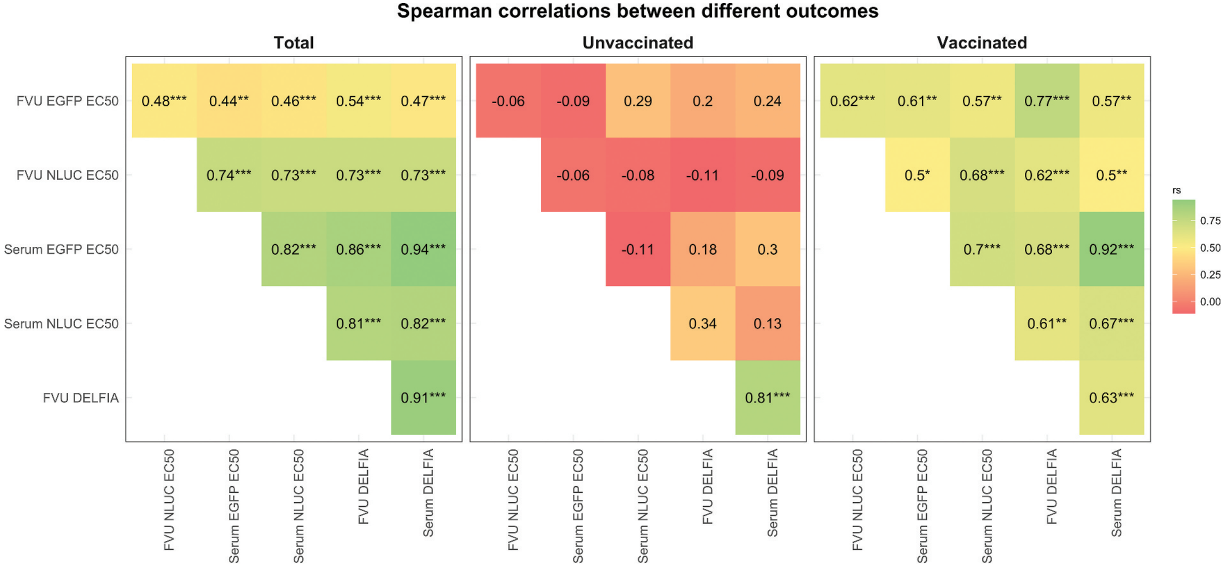

Figure 3. Spearman rank correlations (r_s) between HPV16 antibody concentrations for all different assays and sample types. Significance levels are represented in the figure by an asterisk (*$p < .05$; **$p < .01$; ***$p < .001$).

HPV16-IgG in FVU and serum remain detectable over ten years after HPV vaccination (Supplementary Figure S2). Additionally, a good correlation between serum and FVU HPV16-IgG levels was obtained for the unvaccinated cohort ($r_s = 0.63$) (Figure 3). Of the six HPV16-IgG positive FVU samples from the unvaccinated female volunteers, four also had detectable HPV16-IgG in serum and one had detectable HPV DNA for HPV51.

Both PBNA assays show good reproducibility

To measure the neutralizing capacity of the HPV16-specific antibodies, we conducted two PBNA assays: one using fluorescence microscopy (EGFP method) and the other using spectrophotometry (NLuc method). Visual inspection of the EGFP method images revealed that increasing dilutions of samples from vaccinated individuals progressively increased the number of EGFP-positive cells (Figure 4). This quantitatively translated in infection and neutralization rates. We also evaluated the differences in nuclear count over all FVU and serum plates separately, resulting in a %CV of 28.9% for FVU and 26.9% for serum. As an internal control, we calculated the average nuclear count for each sample dilution and type, resulting in a %CV of 6.7% for FVU and 14.6% for serum.

On each PBNA plate, four wells were allocated for control samples used to determine the neutralizing titers. Median %CV among the controls on individual plates was 8.5% for the EGFP method and 7.4% for Nluc (Figure 5(a)). The %CV for the average of the controls across plates was 3.5% for the EGFP method and 35.4% for the Nluc method (Figure 5(b)). Median neutralization percentage for each dilution for positive samples were plotted (Figure 5(c,d)).

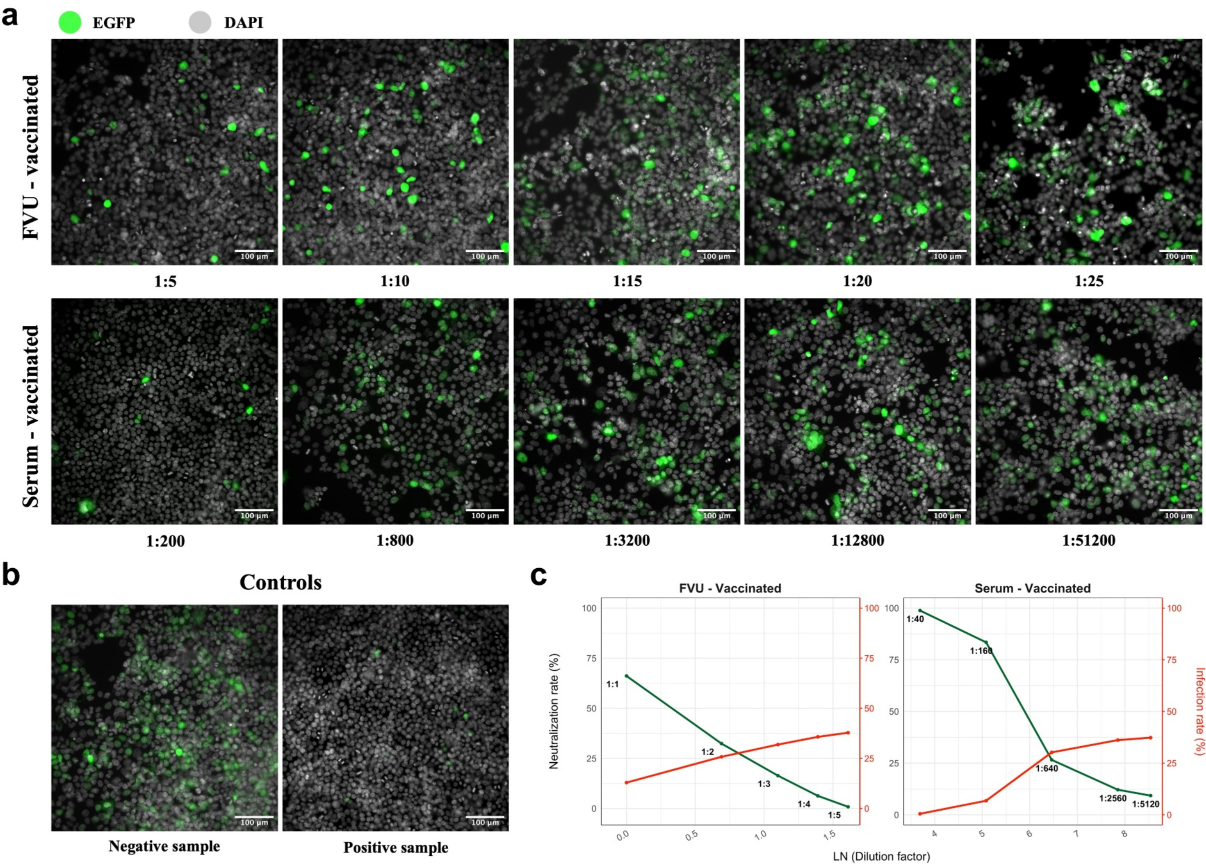

Figure 4. (a) EGFP-based PBNA microscopic images for five dilutions of a FVU and serum sample from one vaccinated female volunteer and (b) control images of a serum sample with HPV16-specific nAbs (positive sample) and without (negative samples). The overlay images show EGFP signal (green) after HPV16-PsV infection and cell nuclei (gray). (c) Neutralization rate (%) and infection rate (%) for the specific sample is presented for each dilution. Neutralization rate was calculated by comparing the infection rate to the infection rate of the positive control. Infection rate was calculated by dividing the number of cells expressing EGFP by the number of cell nuclei.

PBNA protocols identify neutralizing antibody capacity with varying sensitivity and specificity

For serum samples, the EGFP method detected EC50 concentrations in 54% (27/50) of samples, while the Nluc-based assay detected concentrations in 56% (28/50) of samples (Table 2). Notably, 8% (2/25) of unvaccinated and 100% (25/25) of vaccinated women exhibited detectable titers in their serum samples using the EGFP method, and 12% (3/25) of unvaccinated and 100% (25/25) vaccinated women using the Nluc method. The median (IQR) EC50 titer after vaccination for serum samples was 1573 (774–4,887) for EGFP and 1,616 (520–6,981) for Nluc. The GMT for all positive serum samples was 4496 (95% CI 816–8177) for EGFP and 7,337 (95% CI 540–14,134) for Nluc.

Using the EGFP method, 20% (10/50) of FVU samples had EC50 titers, while the Nluc method detected titers in 38% (19/50). Among positive samples, 7 showed positivity with both methods. Specifically, 8% (2/25) of unvaccinated and 32% (8/25) of vaccinated women exhibited detectable titers in FVU samples using the EGFP method, and 4% (1/25) of unvaccinated and 72% (18/25) vaccinated women using the Nluc method. After vaccination, the median (IQR) EC50 titer for FVU samples using the EGFP method was 0.00 (0.00–6.04), and 5.69 (0.00–18.69) for the Nluc method. The GMT for all positive FVU samples was 12.65 (95% CI 7.51–17.80) for EGFP and 13.90 (95% CI 8.80–19.00) for Nluc.

Comparing FVU to serum samples, using the EGFP method, the median EC50 concentrations and GMT in FVU were around 0.22% and 0.28% of those in serum. Similarly, employing the Nluc method, the median EC50 concentrations and GMT in FVU were approximately 0.34% and 0.19% of those found in serum.

HPV16-specific neutralizing antibody titers correlate with HPV16-IgG in FVU and serum samples

Spearman rank tests were used to analyze the correlation among various assays and sample types for the entire group and separately for vaccinated and unvaccinated women (Figure 3). For serum, the highest correlation was found between EGFP HPV16 EC50 nAb and DELFIA HPV16-IgG concentrations ($r_s = 0.94$). For FVU, the highest significant Spearman rank correlation coefficient was found between Nluc HPV16 EC50 and DELFIA HPV16-IgG concentrations ($r_s = 0.73$).

To explore the feasibility of establishing a DELFIA IU/ml concentration cutoff for the PBNA assay, we conducted ROC analyses. These analyses, using the empirical rule (ER) and concordance probability method (CZ), revealed optimal cutoff

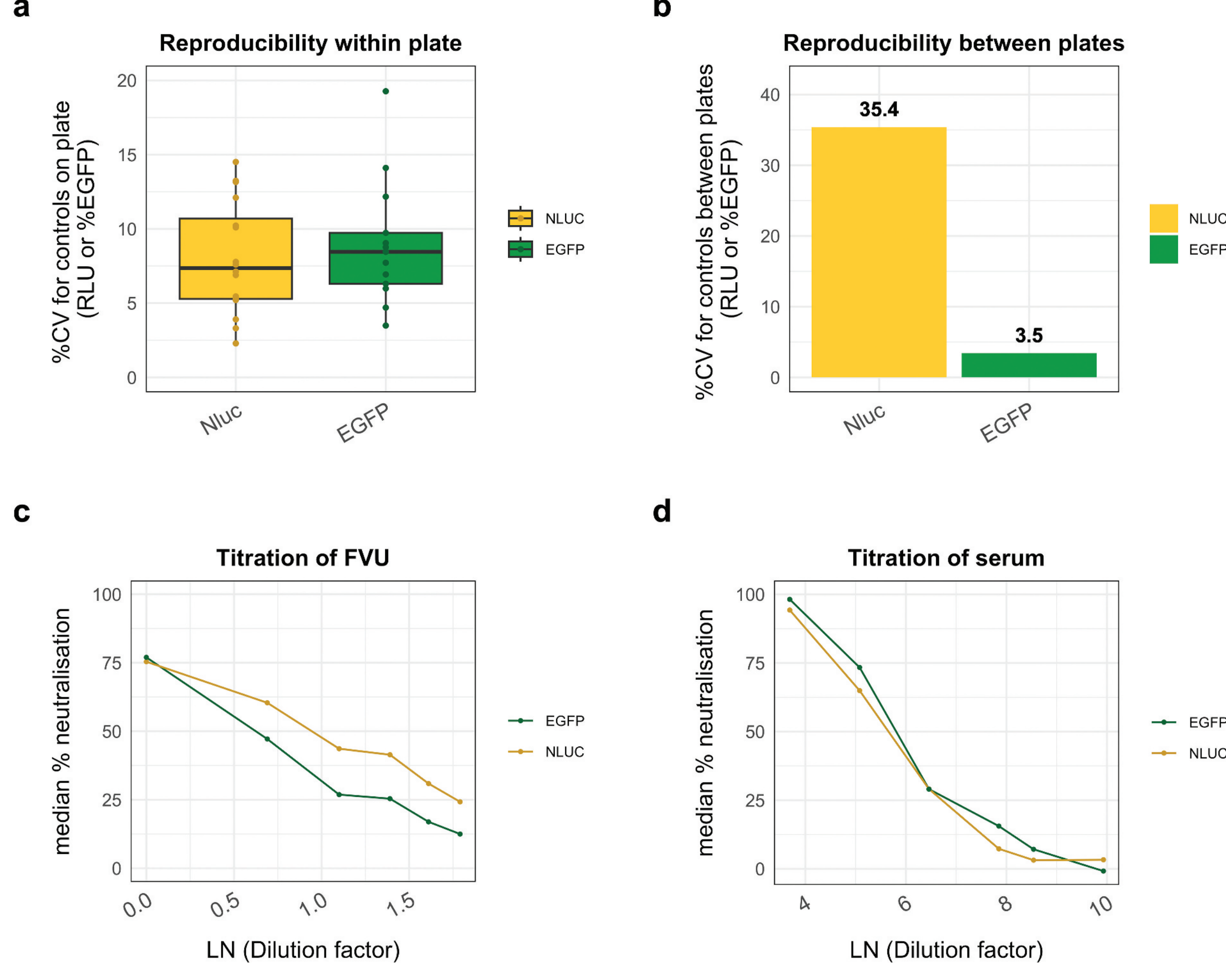

Figure 5. PBNA reproducibility and titration results for both methods. (a) Reproducibility of controls within plates and (b) between plates. The number of plates tested for using Nluc PsV was 18 and for EGFP 13 plates were analyzed. (c,d) Median values of each point on the titration curves for HPV16-nAb positive FVU and serum samples are presented for both PBNA readouts.

titers of 0.047 IU/ml for EGFP, with a sensitivity of 0.8 and specificity of 0.9, and 0.0168 IU/ml for Nluc, with a sensitivity of 0.84 and specificity of 0.87 (Supplementary Figure S3).[49] We visually represented the correlations between different PBNA assays and DELFIA assays for both FVU and serum samples (Figure 6) and provide an overview figure of all results (Supplementary Figure S4).

An excellent correlation ($r_s = 0.91$) was observed for HPV16 DELFIA concentrations between FVU and serum. For EGFP and Nluc HPV16 EC50 nAb concentrations, the correlations between FVU and serum were 0.44 and 0.73, respectively. In the vaccinated cohort, similar trends as the total cohort were observed (Figure 3). Notably, in this subgroup, the correlation coefficients between FVU and serum samples for EGFP (r_s = 0.61) and Nluc ($r_s = 0.68$) HPV16 EC50 nAb concentrations showed less variability.

Discussion

The humoral immune response following HPV vaccination or infection is important, and antibody analysis is vital in understanding the protection mechanism.[4,50] Traditionally, antibody quantification has relied on blood samples, requiring minimally invasive procedures that may not fully capture local immune responses. FVU sampling, however, presents a noninvasive alternative that holds promise, particularly in the context of the female genital tract.[15] With FVU, the initial part of the urine stream is collected, acting as a rinsing liquid to capture the accumulated female genital tract secretions around the labia minora.[14] FVU also offers opportunities in settings where cultural preferences or concerns about discomfort make vaginal swabs less desirable or for sampling younger women who have not yet become sexually active.[15,51] Recent studies confirmed the presence of HPV-specific vaccine- or infection-induced antibodies in this sample type.[11–13] This study provides first evidence that antibodies non-invasively obtained from this local source have neutralizing abilities. Two orthogonal PBNA readout methods were used and compared, demonstrating good correlations between HPV16-nAb concentrations in FVU and serum, as well as with HPV16-specific IgG levels in both sample types. This breakthrough not only highlights the potential of FVU as a valuable and accessible sample for immunological studies but also has implications for diagnostics and therapeutic advancement.[23,52]

As described previously and shown in this study, HPV16-specific IgG concentrations in FVU are at least three logs lower than those detected in serum samples.[11–13] Therefore, highly sensitive immunoassays become crucial. For HPV16-IgG, we used our in-house developed DELFIA assay, able to detect

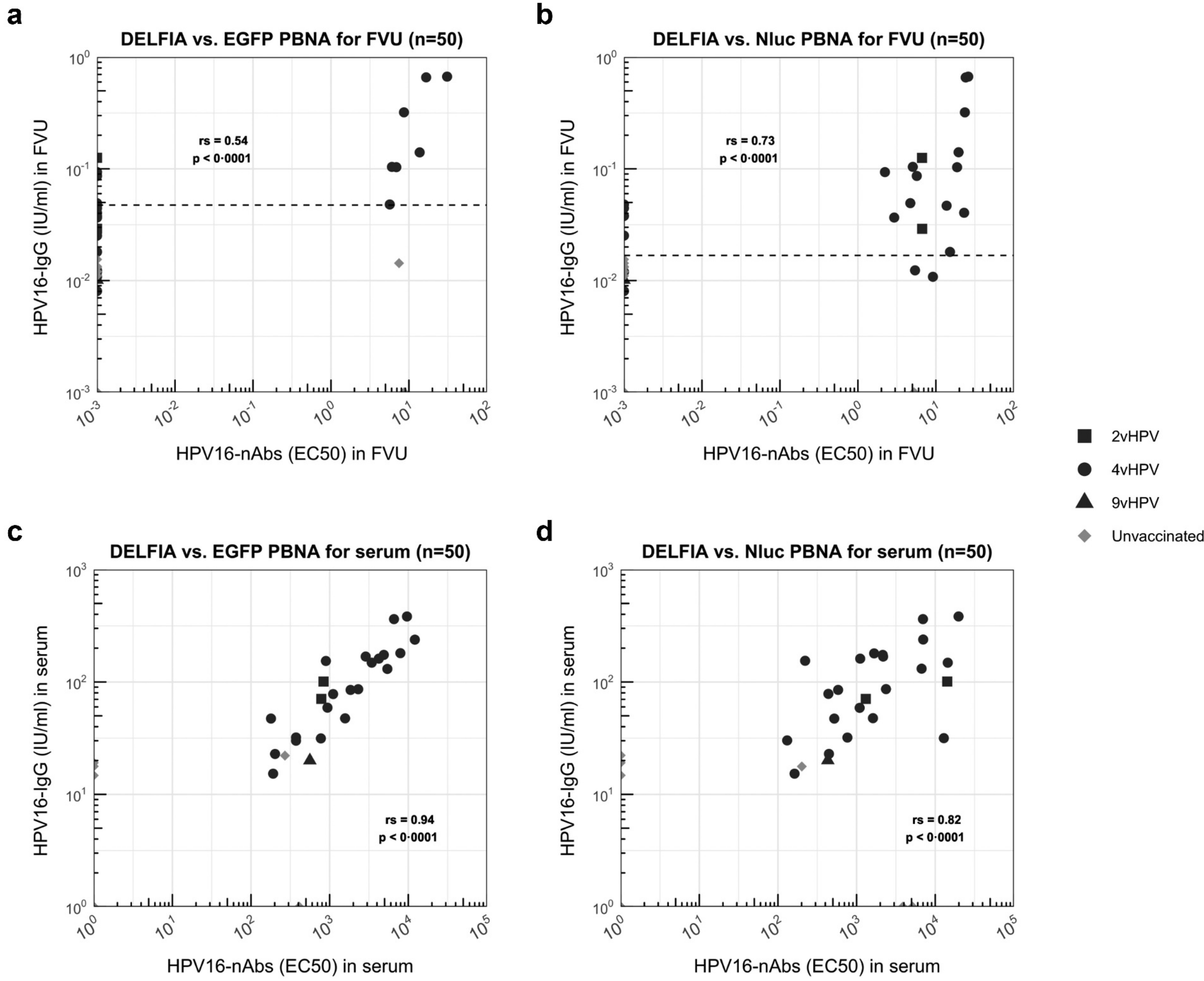

Figure 6. Correlation curves for the HPV16-IgG and HPV16-nAb concentrations quantified using the two different PBNA assays. (a, b) Correlation curves between FVU samples. The ROC analyses determined the IU/ml optimal cut point, with highest sensitivity and specificity from where nAbs in FVU are detectable using the PBNA with EGFP or Nluc method. This cutoff is presented as a dotted line. (c, d) Correlation curves between serum samples. Spearman rank correlation coefficients are presented in the figures. The color of the dots is dependent on the vaccination status and shapes are dependent on the vaccine type used.

concentrations until 0.00803 IU/ml and of which in-house experiments showed strong correlations with established HPV-specific immunoassays like M9ELISA, M4ELISA, and GST-L1-MIA.[33,53,54] The DELFIA assay offers superior sensitivity and a broader dynamic range than traditional ELISA due to the prolonged luminescence of lanthanides.[55] In this study, we were able to demonstrate excellent correlations between HPV16-specific IgG concentration in FVU and serum (r_s = 0.91) using this HPV16 DELFIA assay. Since the golden standard for nAb detection is still a PBNA, we amended the well-described PBNA protocol from Pastrana et al. to adequately detect low concentrations of nAbs.[24] Additionally, we introduced a novel EGFP method, utilizing automated microscopy, and compared it with an Nluc-based method. The EGFP method calculated infection ratios irrespective of the variations in cell count across wells.[44] Given the observed differences in cell count among plates (%CV > 26.9) and sample dilutions (%CV > 6.7), correcting for cell count is crucial when quantifying neutralization, particularly for low antibody titers in FVU.

Both PBNA protocols confirmed the presence of HPV-specific neutralizing antibodies in both FVU and serum, with significant correlations established between the sample types. However, using the current protocols, the Nluc method provided better results for FVU samples. Using the Nluc-based PBNA, 72% of FVU samples from vaccinated women exhibited a detectable nAb titer, compared to 32% for the EGFP method. Additionally, the correlation between the FVU Nluc-based PBNA concentrations and the FVU DELFIA (r_s = 0.73) or the serum Nluc-based PBNA (r_s = 0.73) were higher than for the EGFP method (r_s ≥0.54). For both assays, the GMT of only the nAb positive FVU samples was similar. For serum samples, both PBNA readouts provided comparable results, but the EGFP method exhibited a better correlation with the HPV16-IgG assay, indicating slightly improved specificity. The EGFP method demonstrated significantly lower %CV across different plates, emphasizing enhanced reproducibility, likely attributed to the incorporation of cell count correction – a distinct and valuable advantage.

In the unvaccinated cohort, only four serum samples showed detectable naturally induced HPV16-specific antibodies. However, a higher number of FVU samples (n = 6) tested positive for HPV16-IgG before vaccination. Variations in memory B cell isotypes might explain these differences in antibody detection between sample types.[56] Memory B cell activation, crucial for producing high-quality antibodies, occurs not only after

vaccination but also to some extent after natural infection.[57,58] The isotypes of memory B cells can vary between individuals, and the locally present spectrum of B cells overlaps with, but is distinct from, systemic B-cells.[59] This makes it particularly intriguing to investigate local immune responses, detectable in first-void urine. Furthermore, our study highlights the limited neutralizing capacity of these naturally induced antibodies, aligning with existing literature. Notably, among the six HPV16-IgG positive FVU samples from unvaccinated volunteers, only one or two exhibited neutralizing antibodies, depending on the PBNA method.

While achieving the detection and quantification of HPV16-specific neutralizing antibodies in FVU is significant, opportunities for further optimization persist. Various steps in the PBNA protocol were optimized, including diverse antibody enrichment and isolation methods. Given the substantial impurities in FVU, sample purification was essential to prevent contamination of the cells in the PBNA. Methods such as protein G magnetic bead purification and melon gel purification yielded purified samples but resulted in nonspecific quantification of nAbs due to interference with HPV PsV infectivity. Desalting and buffer exchange failed to resolve the issue. Ultimately, overcoming aspecific interference with the infection was achieved through a method combining Amicon filtration with a medium-speed centrifugation step. We attempted further optimization by comparing various concentrate collection volumes following Amicon filtration. However, capturing the concentrate in volumes lower than 500 µl or utilizing a reversible Amicon® Ultra-2 device led to aspecific inhibition of infection, mirroring challenges encountered in other purification methods. In addition to the optimization of the enrichment protocol, various dilution series and PsV-sample ratios were explored. This evaluation led to the identification of a dilution series where the lowest concentrations were reliably detectable, as used in this study. Exploring an alternative to the current EC50 calculation method, requiring fewer dilutions of a certain sample, might be another avenue for quantifying lower nAb levels. While our current capabilities allow for detection and quantification of HPV16-specific neutralizing antibodies in FVU, further optimizations of purification and protocol methodologies are crucial to refine our understanding and ensure accurate detection at even lower concentrations.

While the units differ between DELFIA and PBNA assays, we compared the ratio of nAbs for both sample types by dividing the GMT of nAbs by the GMT of IgG. For FVU, this ratio was for 90 for the EGFP method and 99 for Nluc, and for serum, this was 26 for EGFP and 42 for Nluc. Additionally, the ratio of FVU/serum was higher for the HPV16-nAbs measured using the PBNAs (≥0.22) compared to the HPV16-IgG using the DELFIA (0.07). This indicates that the fraction of HPV16-specific antibodies that are neutralizing is higher in FVU compared to serum, again showing the added value of using this sample to investigate the specific local humoral immune response.

The primary limitation of this study is the relatively small sample size due to the exploratory nature of the study. Although the findings on feasibility and methodology provide valuable insights and directions for future research, there is a need for larger studies, especially including larger cohorts of samples with measurable local nAbs. The complexity of FVU samples and low antibody concentrations necessitates ongoing protocol refinement. Additionally, the lack of identical units between the DELFIA and the PBNA-derived concentrations introduces a potential source of variability in the interpretation of results. Furthermore, results are only established for HPV16-specific antibodies, and the neutralizing capacity of antibodies against other HPV types needs to be investigated.

This study reveals that HPV16-specific antibodies in noninvasive FVU samples maintain their neutralizing capacity and can be detected up to ten years after vaccination. Two PBNA methods, based on fluorescence (EGFP) and luminescence (Nluc) provided results supporting that FVU is a robust sample for immunological studies. The Nluc method shows a higher sensitivity for FVU samples, but the EGFP method showed better specificity for serum and overall higher reproducibility for detecting neutralizing antibodies. While acknowledging current achievements, future studies are essential for fully leveraging FVU's potential in advancing our understanding of HPV-specific immune responses. The findings of this study highlight the importance of future research related to this topic, including larger cohort studies.

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Disclosure statement

Alex Vorsters is a cofounder and former board member of Novosanis (Subsidiary of OraSure Technologies Inc., Wijnegem, Belgium), a spin-off company of the University of Antwerp and was a minority shareholder until January 2019. The University of Antwerp received grants from Merck, GSK, Hologic, Abbott, Roche, and Cepheid to support the HPV Prevention and Control Board. The University of Antwerp received a project grant and honoraria fee for lectures, presentations, and speaker bureaus from Merck. Other authors declare that they have no conflict of interest.

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Clinical trial registration

The study was registered on clinicaltrials.gov: NCT04391647.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author. Image analysis scripts are available on Github.

Ethics approval statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of UZA/University of Antwerp (B3002020000025, 30 April 2020).

Patient consent statement

All participants provided consent before participation.

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