



First-void urine as a non-invasive liquid biopsy source to detect vaccine-induced human papillomavirus antibodies originating from cervicovaginal secretions

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ARTICLE INFO

Keywords:

HPV
Vaccine
Antibody
Urine
Non-invasive
Self-sampling

ABSTRACT

Background: Monitoring HPV antibodies non-invasively would be a major advantage for large epidemiological studies and follow-up of vaccinees.

Objectives: This study investigated the presence of HPV-specific antibody transudates from systemic circulation in first-void urine of (un)vaccinated subjects and the agreement with paired sera.

Study design: In this case-control study, 55 paired first-void urine and serum samples were included from 19- to 26-year-old women, unvaccinated (n = 19) or vaccinated (n = 36) with the bi- or quadrivalent HPV vaccine during adolescence (NCT02714114). Human IgA, total human IgG, and HPV6/11/16/18-Ig(M/G/A) were measured in paired samples.

Results: Significant positive Spearman rank correlations (r_s) were found in HPV-specific antibody levels between paired samples (HPV6: $r_s = 0.777$; HPV11: $r_s = 0.757$; HPV16: $r_s = 0.876$; HPV18: $r_s = 0.636$ ($p < 0.001$)). In both first-void urine and serum, significantly higher HPV6/11/16/18 antibody levels were observed in vaccinated compared with unvaccinated women ($p \leq 0.017$).

Conclusions: The present study provides the first proof that vaccine-induced HPV antibodies are detectable in the first-void urine of young women. Moreover, significant positive correlations were observed between HPV6/11/16/18-antibodies in first-void urine and paired sera. Further optimization and validation are required to demonstrate its potential use in epidemiological studies and follow-up of HPV vaccination.

1. Background

Human papillomavirus (HPV), the most common sexually transmitted infection, affects nearly all sexually active people during their lifetime. Although the immune system clears over 90% of HPV infections within two years of acquisition [1] persistent infection is the main

etiological factor of cervical cancer [2]. Unlike other mucosal secretions where immunoglobulin A (IgA) predominates, cervicovaginal secretions (CVS) mainly contain IgG transudate from serum and locally produced IgG and secretory IgA (sIgA) [3,4]. Clearance of infection does not necessarily lead to immunity, and only 50–70% of individuals develop humoral antibodies against HPV (HPV-Ab) after natural

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<https://doi.org/10.1016/j.jcv.2019.05.004>

Received 11 March 2019; Received in revised form 4 May 2019; Accepted 10 May 2019

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infection [5]. Moreover, CVS antibody concentrations are influenced by oral contraceptive (OCC) use and the menstrual cycle, being lowest during ovulation [6].

Prophylactic vaccination is imperative to induce effective HPV-Abs [7–9]. Unlike natural infection, HPV vaccination induces high serum antibody titers, with high avidity, against HPV L1, conferring protection against disease among virtually all women naive to vaccine-included HPV types. Since licensure in 2006, millions of women in over 85 countries (through March 2018) have received HPV vaccinations via national immunization programmes [10].

The feasibility of using women's urine as a liquid biopsy to monitor HPV vaccination impact through viral end-points has been reported [11–13]. Monitoring HPV-Abs is also important to confirm the impact of HPV vaccines, in addition to measures of persistent HPV-infections and related disease. HPV vaccine trials mainly measure humoral immunogenicity although the samples most relevant to protective immunological activity are those taken locally at the mucosal site where infection occurs. Transudating neutralizing antibodies from systemic circulation to the infection site, particularly the cervical transformation zone, are vital to prevent HPV infections and avert cervical disease [14,15]. Any microtrauma allowing HPV to infect the mucosal surface triggers exudation of antibodies and can lead to additional HPV-Abs at the anogenital site [14]. In the absence of a correlate of protection [14,16,17], HPV-Abs detection may become an important alternative monitoring tool.

Several studies found correlations between serum and CVS HPV-Ab titers [3,6,18–22]. First-void urine (FVU) contains identifying HPV-DNA and other biomarkers [23], but we are unaware of any studies to investigate the presence of HPV-Abs in debris and exfoliated cells from the female genital organs in FVU. Non-invasive assessment of HPV-Abs in FVU from girls and women of all ages would be an important tool for epidemiological studies and follow-up of HPV-vaccination trials and programs, and may even inform investigations of the post-vaccination correlate of protection.

2. Objectives

We investigated the presence of HPV-Abs in FVU and their correlation with paired serum samples according to vaccination status in a cohort of 19–26-year-old women.

3. Study design

3.1. Study participants

We enrolled 57 healthy women, 19–26 years of age, between November and December 2015. Of which 38 had documented receipt of bivalent (2vHPV) or quadrivalent (4vHPV) prophylactic HPV-vaccination during adolescence (Fig. 1). Enrolment of 19 non-vaccinees was based on self-reporting. All enrolled volunteers provided informed consent before proceeding with any study-related procedures. All study procedures were approved by the institutional review board of Antwerp University Hospital (UZA)/UAntwerp, Belgium (B300201525584) and the trial was registered on clinicaltrials.gov (NCT02714114).

3.2. Sample collection and storage

Before the study visit women were requested beforehand not to extensively wash their genitals, nor to urinate at least one hour prior to this visit, and to schedule their appointment at least two days after the last day of menstruation. Women were asked to collect a FVU sample with the Colli-Pee® device (Novosanis, Belgium), and a paired 10 ml blood sample (two 5 ml BD Vacutainer® Plus Plastic Serum Tubes, Becton-Dickinson, Benelux) was taken. Upon collection, FVU samples

were immediately placed on ice and aliquots stored at -80°C . Blood samples were allowed to clot for 30–60 min, centrifuged at 1000 g for 10 min at 20°C , and the serum was divided in aliquots prior to storage at -80°C .

3.3. Immunoassays

3.3.1. Pre-analytical processing of first-void urine samples

Blood in FVU was measured using 10 μl samples on Hemastix® reagent strips for urinalysis (Siemens Healthcare Diagnostics Inc., Belgium). To avoid possible bias due to antibody-containing blood contamination, samples with 200 or more erythrocytes/ μl were excluded (Fig. 1) [19].

We previously found that FVU samples could be enhanced to provide more reproducible results in HPV-Ab analysis with the glutathione S-transferase-L1-fusion protein multiplex serology assay (German Cancer Research Center (DKFZ), Germany) by using an optimized formulation of buffered urine conservation medium (UCM, UAntwerp, Belgium) and including an Amicon filtration step (*data not shown*). Accordingly, after 5–6 months storage FVU samples were thawed and two volumes of FVU with one volume of UCM, with a total volume of 4 mL, were centrifuged at 3820 g for 10 min at 21°C in an Amicon Ultra-4 50K filter device (Merck Millipore, Belgium). Concentrate retained on the filter was diluted with DPBS (Dulbecco's phosphate buffered saline, Gibco, United Kingdom) to a final volume of 500 μl . FVU concentrates were then stored at -80°C .

3.3.2. Human IgA and total human IgG isotyping

Human IgA and total human IgG were measured in paired FVU and serum samples using the BioPlex Pro™ Human Isotyping Assay for human IgA and total human IgG (Bio-Rad, USA), respectively, according to the manufacturer's instructions. Briefly, 50 μl of pre-processed, diluted FVU (1:128; 1:256; 1:512) or serum (1:40,000) was mixed with captured antibodies coupled to fluorescent-labelled, magnetic polystyrene beads, detecting either human IgA or total human IgG in a single well using the LX200 platform (Luminex, Austin, Texas, USA). Using a five-parameter logistic regression, antibody concentrations ($\mu\text{g}/\text{ml}$) were quantitated from the median fluorescence intensity (MFI) values, within a working range of 0.130×10^{-3} –0.532 and 0.003–30.27 $\mu\text{g}/\text{ml}$ for human IgA and total human IgG, respectively. Human IgA and total human IgG in FVU were reported as the average of three dilution-corrected concentrations.

3.3.3. Glutathione S-transferase HPV-L1-fusion protein multiplex serology

We used a bead-based multiplex serology assay for HPV-Ab detection, based on glutathione S-transferase (GST) HPV-L1-fusion protein antigens [24,25]. Briefly, viral antigens specific for HPV6/11/16/18, bacterially expressed as GST fusion proteins, were coupled to fluorescent-labelled polystyrene beads. A previously described pre-incubation buffer [25], optimized for use with FVU, was used to dilute the pre-processed samples (1:4 FVU; 1:100 serum) to a volume of 50 μl and mixed with the bead sets detecting HPV6, 11, 16, 18, and the GST-tag (SeroMap, Luminex, Austin, Texas, USA). HPV-Ab (IgM/G/A) are reported as MFI values from at least 100 beads analysed per bead set for each antigen.

3.4. Statistical analysis

This case-control study was designed to investigate the presence of HPV-Ab in FVU and its correlation with paired sera according to vaccination status. With a continuous outcome variable (HPV-Ab concentration), power of 0.80, significance level of 0.05, and standardized difference set at 0.80 (a large effect size expected due to high seroconversion rates after vaccination), this study had an estimated

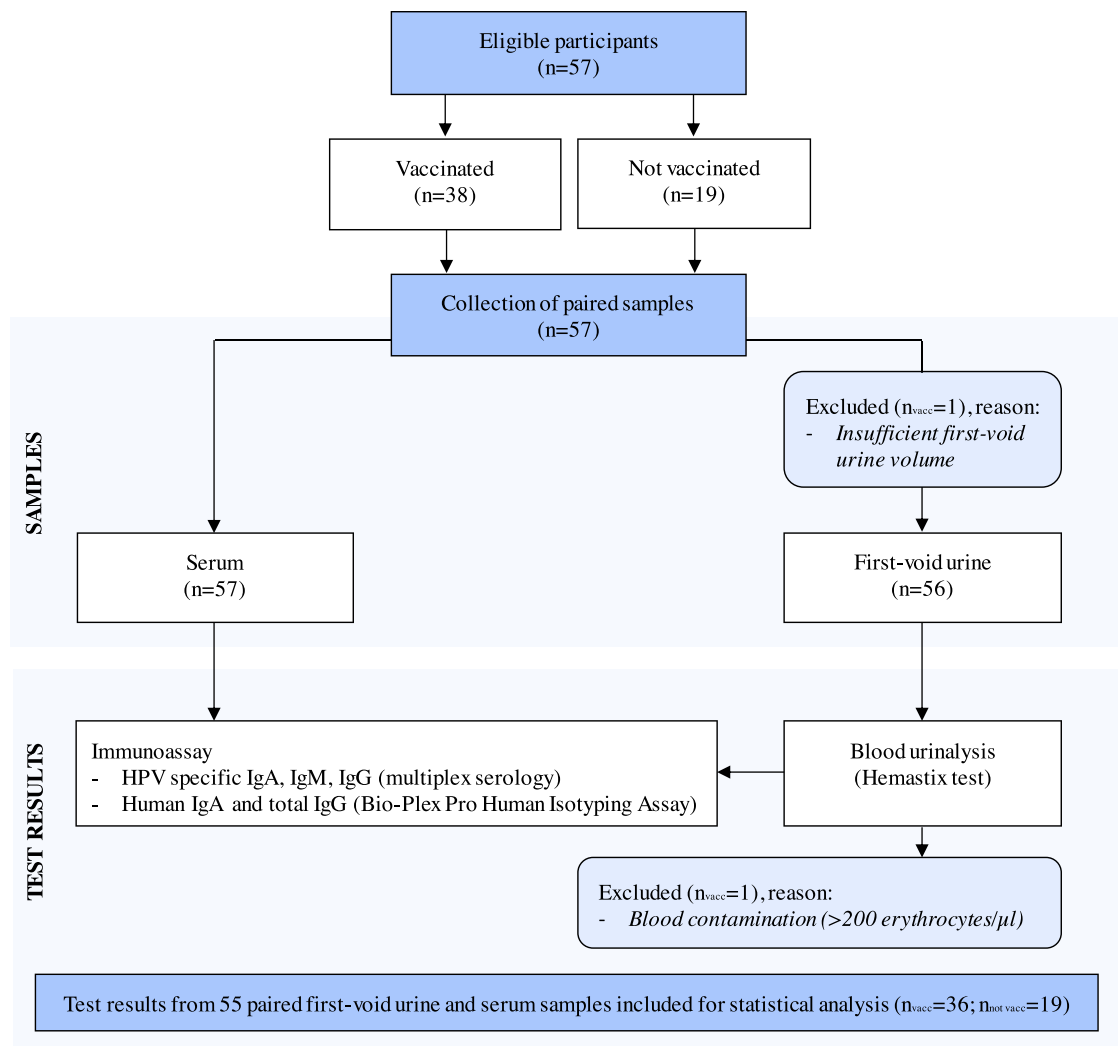


Fig. 1. Flow diagram for the inclusion of study participants, samples, and tests performed.

sample size (N) of 50 according to the Altman nomogram [26]. Assuming unequal groups with twice as many cases (vaccinated) than controls (unvaccinated women) ($k = n_1/n_2 = 2$), a modified sample size $N' = N(1 + k)^2 / 4k = 50(1 + 2)^2 / (4 \times 2) = 56.25$, i.e., 57, was obtained (N' vaccinated = 38; N' unvaccinated = 19). Participants were included on four different days, and all FVU and sera were randomized before pre-analytical processing and antibody detection.

Fisher exact and Chi square tests were used to assess the association of the study population characteristics between vaccinated and unvaccinated cohorts and between FVU and serum for a given HPV-Ab. Mann Whitney *U* test and unpaired Student's *t*-test were used for differences in continuous measures and within levels of categorical measures. Antibody correlations were calculated using the Spearman rank test. Cut-off values to discriminate between (un)vaccinated women were calculated for each HPV-Ab and sample type based on the mean plus three standard deviations of the unvaccinated group. Cohen's kappa (κ) was calculated to assess the HPV-Ab agreement between FVU and serum and was judged as follows: $\kappa \leq 0.20$, poor; $0.21 \leq \kappa \leq 0.40$, fair; $0.41 \leq \kappa \leq 0.60$, moderate; $0.61 \leq \kappa \leq 0.80$, good; and $\kappa \geq 0.81$, very good agreement [26]. Human IgA, total human IgG, and HPV-Ab levels were $\log_{10}(x)$ transformed for the Spearman rank test and boxplots (Mann Whitney *U* test), respectively. For correlation plots according to Spearman rank tests, HPV-Ab levels were $\log_{10}(x+1)$ transformed. Statistical analyses were performed at a significance level of 5% using IBM SPSS Statistics Version 23 and JMP Pro 13 software.

4. Results

4.1. Study population characteristics

After two exclusions, data from 55 19–26 year-old women were included for statistical analysis, 36 vaccinated and 19 unvaccinated (Fig. 1). Study population characteristics are detailed in Table 1. All vaccinees had received three doses, most the quadrivalent vaccine ($n = 31/36$; 86%), the remainder bivalent vaccine ($n = 4/36$; 11%) or a combination of both ($n = 1/36$; 3%). Mean ages at first and last vaccinations were 15.1 and 15.7 years, respectively. There was no difference in age distribution between vaccinated and unvaccinated women, most were non-smokers, and all had obtained or were studying for a higher educational degree (college or university). Significant associations according to vaccination status were only found for OCC and age at first sexual activity, OCC being higher and debut of sexual activity being later among vaccinees (Table 1).

4.2. Antibody levels in paired first-void urine and serum samples

A median estimated volume of 19 ml FVU (IQR: 18–20 ml) was collected within 2 h and 26 min (median; IQR: 1:45–3:26) of the previous urination. After collection, FVU and serum aliquots were stored at -80°C within a median time span of 7 (IQR: 0:05–0:29) and 58 min (IQR: 0:50–1:07), respectively.

Table 1
Study population characteristics according to vaccination status.

Characteristic	Total	According to vaccination status		
		Vaccinated	Not vaccinated	P-value ^b
Total (n, column %)	55 (100)	36 (100)	19 (100)	–
Median age in years (median, IQR)	22.0 (20.0–24.0)	22.0 (20.25–23.0)	24.0 (20.0–24.0)	0.316
Age at first vaccine dose, years (mean ± SE)	15.1 ± 0.28	15.1 ± 0.28	–	–
Age at completion of 3-dose vaccination schedule, years (mean ± SE)	15.7 ± 0.29	15.7 ± 0.29	–	–
History of sexual activity (n, column %) ^a	49 (89.1)	34 (94.4)	15 (79.0)	0.167
Age of first sexual activity, years (median, IQR)	17.0 (16.0–19.0)	18.0 (16.0–19.0)	16.0 (15.0–18.0)	0.045*
Smoking (n, column %)	4 (7.3)	1 (2.78)	3 (15.8)	0.114
Oral contraceptive use (n, column %)	40 (72.7)	30 (83.3)	10 (52.6)	0.017*
History of pap smear (n, column %)	30 (54.6)	23 (63.9)	7 (36.8)	0.055
Age at first pap smear, years (mean ± SE) ^a	19.3 ± 0.55	18.8 ± 0.61	21.1 ± 1.10	0.071

n: number; IQR: interquartile range; SE: standard error.

^a From women's self-reporting history of sexual activity and Pap smear, respectively.

^b P-value (Chi square test) indicated by an asterisk means that the null hypothesis (H0) is rejected ($p \leq 0.05$) and that there is an association between the two categorical variables. When 20% of cells have an expected count less than five, the 2-tailed Fisher's exact test was used. P-value (Mann Whitney *U* test/unpaired Student's *t*-test, equal variances assumed) indicated by an asterisk means that the H0 is rejected and that the median/mean of two sets of continuous variables are not equal.

Table 2
Median antibody levels in first-void urine and serum according to vaccination status.

Antibody type	Total			According to vaccination status				P-value ^b FVU	P-value ^b Serum
	FVU median (IQR)	Serum median (IQR)	Ratio FVU/Serum % (IQR) ^a	Vaccinated		Unvaccinated			
				FVU median (IQR)	Serum median (IQR)	FVU median (IQR)	Serum median (IQR)		
Human IgA	1.29 (0.82–2.76)	1,060 (841.6–1,248)	0.15 (0.08–0.26)	1.28 (0.81–2.73)	1,093 (855.2–1,367)	1.31 (0.82–2.84)	902.1 (831.5–1,154)	0.894	0.240
Human IgG	36.31 (22.20–73.54)	10,137 (8,181–12,578)	0.35 (0.21–0.68)	40.19 (26.64–76.55)	10,850 (9,042–12,761)	30.29 (15.64–68.42)	9,447 (7,939–12,212)	0.159	0.142
HPV6-Ig ^c	0.16 (0.04–0.42)	37.88 (8.43–110.2)	0.34 (0.17–0.87)	0.26 (0.16–0.87)	100.0 (68.45–136.9)	0.02 (0.00–0.13)	7.63 (3.48–15.38)	< 0.001*	< 0.001*
HPV11-Ig ^c	0.10 (0.05–0.27)	27.08 (4.38–70.83)	0.57 (0.27–1.38)	0.23 (0.09–0.57)	60.10 (34.75–100.2)	0.05 (0.01–0.10)	4.23 (1.83–5.68)	< 0.001*	< 0.001*
HPV16-Ig	0.14 (0.03–0.46)	54.78 (4.58–114.8)	0.39 (0.20–1.03)	0.26 (0.14–1.05)	96.90 (55.06–161.4)	0.02 (0.0–0.05)	1.68 (0.63–6.28)	< 0.001*	< 0.001*
HPV18-Ig	0.07 (0.03–0.13)	8.18 (0.23–24.03)	0.66 (0.45–2.04)	0.10 (0.04–0.15)	18.33 (4.30–28.31)	0.06 (0.03–0.08)	0.0 (0.00–1.98)	0.017*	< 0.001*

Median human IgA/total human IgG ($\mu\text{g}/\text{mL}$) and HPV antibody (median fluorescence intensity, 10^3 MFI), plus interquartile ranges (IQR: Q25–Q75), are displayed for the total population ($n = 55$), vaccinated ($n = 36/55$), and unvaccinated ($n = 19/55$) women.

^a Antibody levels in first-void urine (FVU) divided by serum levels, denoted as the median %, plus IQR.

^b P-value (Mann Whitney *U* test) indicated by an asterisk indicates a significant difference between median antibody yield between vaccinated and unvaccinated women for (i) FVU or (ii) serum.

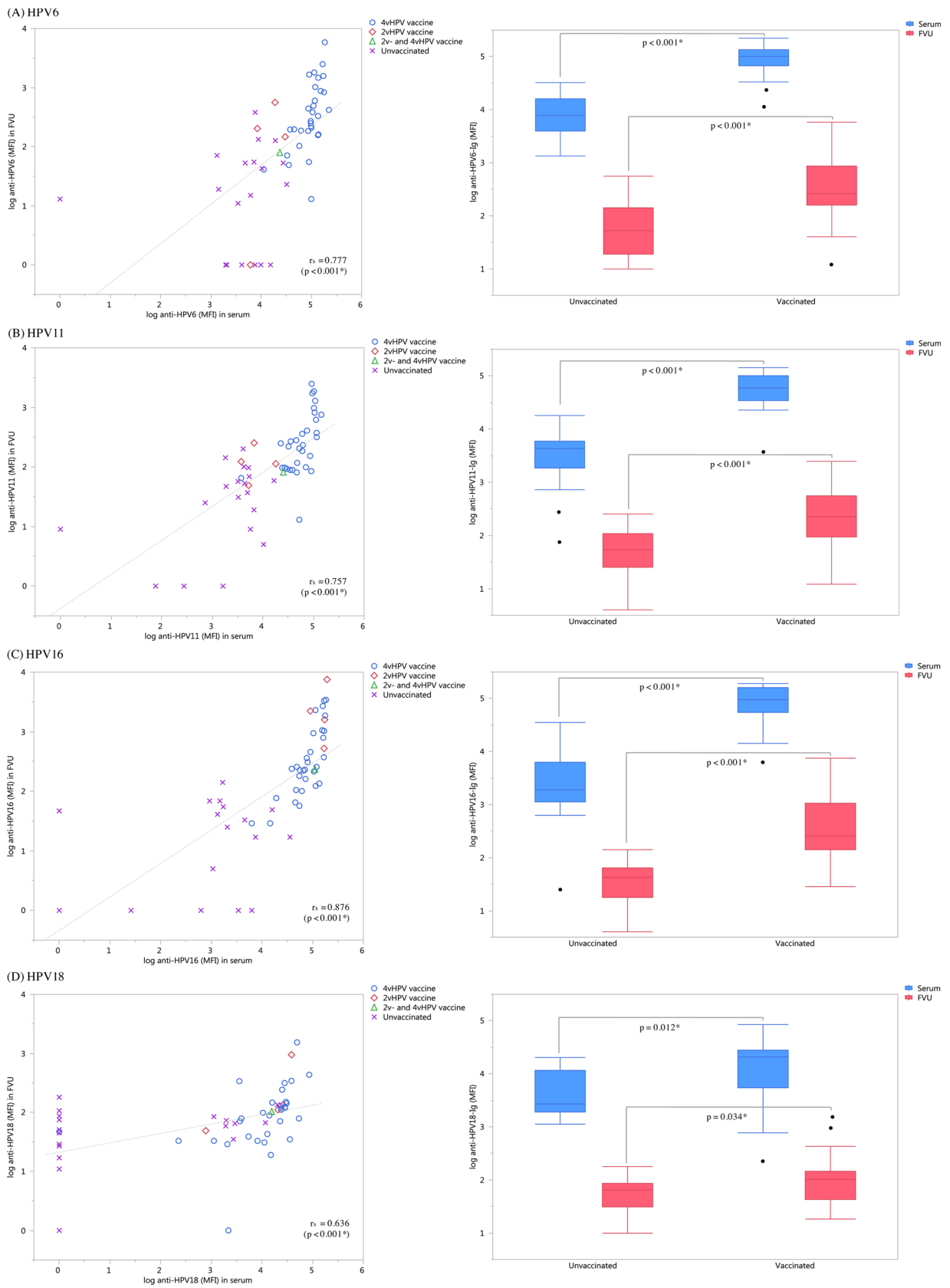
^c For HPV6/11 antibodies; women previously vaccinated with the bivalent vaccine were considered unvaccinated ($n = 32/55$ vaccinated; $n = 23/55$ unvaccinated).

In FVU, median human IgA and total human IgG concentrations were 1.29 and 36.31 $\mu\text{g}/\text{mL}$, respectively, whereas median serum concentrations were 1060 and 10,137 $\mu\text{g}/\text{mL}$. FVU antibody concentrations were approximately 0.15% human IgA and 0.35% total human IgG of those in serum. No significant differences were found between the median human IgA and total human IgG concentrations in FVU or serum according to vaccination status (Table 2). Likewise, no significant correlations were found for human IgA or total human IgG in the FVU versus serum (*data not shown*). Human IgA significantly correlated with total human IgG in FVU ($r_s = 0.830$; $p < 0.001$), but not in serum ($r_s = 0.110$; $p = 0.426$).

HPV-specific antibody levels (MFI) in FVU were approximately 0.34%, 0.57%, 0.39%, and 0.66% of HPV6, 11, 16, and 18 MFI values in serum, respectively. Significant differences were found between MFI values according to vaccination status in both FVU and serum for all four HPV genotypes (Table 2).

4.3. Correlation between HPV antibody levels in paired first-void urine and serum samples

Significant positive HPV-Ab correlations between paired samples were observed for all genotypes targeted with the bivalent and quadrivalent vaccines (Fig. 2). The strongest correlation was observed for HPV16 ($r_s = 0.876$, $p < 0.001$), followed by HPV6 ($r_s = 0.777$, $p < 0.001$), HPV11 ($r_s = 0.757$, $p < 0.001$), and HPV18 ($r_s = 0.636$, $p < 0.001$). Significant correlations were also observed when normalising HPV-Ab levels over total human IgG to correct for fluctuations due to OCC or time during the menstrual cycle (*data not shown*). Median log HPV-Ab levels for serum compared with paired FVU were higher ($p < 0.001$); 2.31–2.59 (vaccinees) and 1.63–2.16 (unvaccinated women). However, significantly different median HPV-Ab levels were observed for all four genotypes in vaccinees versus unvaccinated women, with 0.20–0.78 and 0.88–1.71 log higher levels for vaccinated participants in FVU and serum, respectively (Fig. 2).



(caption on next page)

Fig. 2. HPV-specific antibody levels according to sample type and vaccination status.

Correlation plots of HPV6 (A), 11 (B), 16 (C), and 18 (D) antibodies (anti-HPV) are visualized in the panels on the left. $\log_{10}(x+1)$ transformed HPV antibody levels (median fluorescence intensity, MFI) for first-void urine (FVU) and serum are plotted on the y- and x-axis, respectively. Markers are used to visualize women vaccinated with the quadrivalent (4vHPV; blue circles), bivalent (2vHPV; red squares), a combination of both vaccines (2v- and 4vHPV; green triangle), and unvaccinated women (purple crosses). In the panels on the right, median $\log_{10}(x)$ transformed HPV6 (A), 11 (B), 16 (C), and 18 (D) antibody levels (MFI) are visualized according to vaccination status (x-axis) for serum (blue boxplots) and FVU (red boxplots). For HPV6/11 antibodies, women previously vaccinated with the bivalent vaccine were considered unvaccinated ($n = 32/55$ vaccinated; $n = 23/55$ unvaccinated). P-values indicated by an asterisk indicate (i) significant relationships (Spearman rank correlation coefficients; r_s) between antibodies in paired FVU and serum samples (left panels; Spearman rank correlation), and (ii) a significant difference between median antibody levels between vaccinated and unvaccinated women (right panels; Mann Whitney U test).

Table 3

Agreement between vaccination status and HPV antibody levels in first-void urine or serum.

Vaccination status (n)	HPV antibody status in FVU (n)		P-value ^b	K (95% CI) ^c	HPV antibody status in serum (n)		P-value ^b	K (95% CI) ^c
	Negative	Positive			Negative	Positive		
HPV6^a								
Unvaccinated	20	3	< 0.001*	0.601 (0.394–0.809)	23	0	< 0.001*	0.854 (0.718–0.990)
Vaccinated	8	24			4	28		
HPV11^a								
Unvaccinated	22	1	0.001*	0.449 (0.249–0.649)	21	2	< 0.001*	0.887 (0.763–1.000)
Vaccinated	15	17			1	31		
HPV16								
Unvaccinated	18	1	< 0.001*	0.770 (0.599–0.942)	17	2	< 0.01*	0.878 (0.744–1.000)
Vaccinated	5	31			1	35		
HPV18								
Unvaccinated	19	0	0.082	0.143 (0.032–0.253)	17	2	< 0.0001*	0.626 (0.422–0.830)
Vaccinated	29	7			8	28		

^a For HPV6/11 antibodies; women previously vaccinated with the bivalent vaccine were considered unvaccinated ($n = 32/55$ vaccinated; $n = 23/55$ unvaccinated).

^b P-values (Chi square test) with * mean that the null hypothesis (H_0) is rejected ($p \leq 0.05$) and that there is an association between vaccination status and the presence of HPV antibodies. When 20% of cells have an expected count of less than five, the 2-tailed Fisher's exact test was used.

^c The Cohen's kappa (κ) was judged as follows: $\kappa \leq 0.20$, poor; $0.21 \leq \kappa \leq 0.40$, fair; $0.41 \leq \kappa \leq 0.60$, moderate; $0.61 \leq \kappa \leq 0.80$, good; and $\kappa \geq 0.81$, very good agreement [26]. n: number; FVU: first-void urine; 95% CI: 95% confidence interval.

We observed a significant association between vaccination status and a positive HPV-Ab test score based on the calculated cut-offs for all genotypes in serum ($p < 0.001$). In FVU, significant associations were observed between vaccination status and HPV-Ab test score for three genotypes ($p < 0.001$ for HPV6/11/16), but not HPV18 ($p = 0.082$). Based on a two-by-two table, good and very good κ -agreements were found in sera for HPV18 and HPV6/11/16, respectively (Table 3). In FVU, this agreement was found to be good, moderate and poor for HPV16, HPV6/11, and HPV18, respectively. Similar associations and κ -agreements between vaccination status and HPV-Ab test score were obtained for HPV-Ab results normalized for IgG, with the exception of HPV16-Abs in FVU, where a moderate κ -agreement was found (*data not shown*).

5. Discussion

HPV antibodies in CVS have been reported, with good correlations between levels in paired CVS and sera [3,6,18–21]. As non-invasive assessment of HPV-Ab levels could be a major tool for epidemiological studies and follow-up of HPV-vaccination trials and programs we investigated whether HPV-Abs are detectable in FVU and correlate with paired serum samples according to vaccination status.

We believe the present study provides the first proof that HPV vaccine-induced antibodies are detectable in FVU. As with CVS [3,6,18–21], FVU HPV-Ab levels correlated with paired sera for all investigated genotypes. This supports the notion that CVS accumulated around the urethra opening are washed away with the initial urine stream and that FVU is a valuable liquid biopsy source to detect local, cervical vaccine-induced HPV-Abs that correlate with systemic levels. As previously reported [3,21,27], we did not observe alterations in the significance level of correlations between paired samples when HPV-Ab levels were normalised to total human IgG to correct for changes due to OCC or the menstrual cycle (*data not shown*), and therefore used

uncorrected data.

In contrast to HPV-Ab levels, there was no correlation between human IgA or total human IgG in FVU and serum, as previously reported [28]. This suggests that systemic human IgA and total human IgG concentrations are not reflective of cervical levels and other mechanisms including local production of human IgA and total human IgG in mucosal-associated lymphoid tissues and active/selective transport to mucosal secretions contribute to cervical antibody levels in FVU. Vaccine-induced HPV-Abs in CVS are likely due to transudation from blood rather than local production [28,29]. Which is confirmed by the observed correlation between HPV-Ab in first-void urine and serum samples.

Similar to CVS [21], there was an approximate 2-log difference in HPV-Abs levels between FVU and serum; FVU levels were 0.34–0.66% of serum levels. However, vaccinees had significantly higher antibody levels than unvaccinated women in FVU (0.20–0.78 \log_{10} difference) and serum (0.88–1.71 \log_{10} difference). The difference was largest for HPV16, followed by HPV6, 11, and 18. Vaccination status produced no significant differences for IgA or IgG.

Significant associations were observed between vaccination status and positive HPV-Ab test scores for all genotypes in serum and for all but HPV18 in FVU. Based on the same cut-off, very good (HPV6/11/16) and good (HPV18) agreements were found between serum antibody levels and vaccination status; the same trends but lower agreements were observed for FVU (good: HPV16; moderate: HPV6/11; poor: HPV18). These figures should be interpreted cautiously since this was a hypothesis-generating study without formal power calculations for sample size. Notwithstanding, these may inform more reliable sample size estimations. If a cut-off can be established from larger cohorts to differentiate women according to vaccination status, this alternative method of HPV-Ab detection might also allow determination of the correlate of protection via non-invasive FVU sampling and the durability of the presence of vaccine-induced antibodies for follow-up of

current and next-generation HPV vaccines.

Assessment of both virological (HPV DNA) and immunological (HPV-Ab) end-points in FVU could provide major logistical and financial benefits to epidemiological studies to assess vaccine uptake, vaccination impact, and epidemiological dynamics of HPV infection [30] and follow-up of HPV-vaccination. In young women who are not yet sexually active or reluctant to talk about it and cervicovaginal sampling is inappropriate, or in populations where such invasive assessments are culturally unacceptable, FVU could be used to monitor vaccine impact and effectiveness earlier in large population-based studies [31]. In addition, expansion of HPV vaccination to lower income countries, particularly with one-or two-dose schedules [32], may be facilitated by the use of such a tool to assess their impact [31].

Limitations other than the restraint on the cut-off do exist. First, study group allocation was based on self-reported vaccination status, and although vaccination certificates were requested due to lack of an up-to-date vaccination platform for this age-cohort this could not be verified. Second, the four women fully vaccinated with the bivalent vaccine were allocated as unvaccinated with respect to HPV6 and 11, since cross-protection against the phylogenetically distinct HPV16 and 18-genotypes is not conclusive, and we observed similar antibody levels for unvaccinated and bivalent-vaccinated women. Third, the antibody assays we used were not validated for use with (first-void) urine samples although optimization experiments were performed, using different dilutions and buffer compositions. Fourth, there was selection bias towards a high educational level since the recruitment population was students from UAntwerp and its associations. As this is a proof-of-concept study investigating the possibility of HPV-Ab detection in FVU, we do not believe that the above limitations will affect the main outcomes of this study.

6. Conclusions

We have proven the feasibility of detecting HPV-antibodies, particularly genotypes 6, 11, 16, and 18, in FVU of 19–26-year-old women vaccinated with bivalent and quadrivalent HPV vaccines during adolescence. Significant positive correlations were observed between urinary HPV-Abs and serum levels, with significantly higher HPV-Abs levels observed in both FVU and sera of vaccinated as opposed to unvaccinated women. Measuring HPV antibodies in FVU might offer an alternative, non-invasive tool to assess vaccine-induced antibodies at the cervicovaginal site. Further optimization, including options for normalization, and validation in larger cohorts, is required. Nonetheless, these results demonstrate that non-invasive FVU sampling creates novel opportunities to confirm immunogenicity of HPV vaccines in large epidemiological studies and follow-up of HPV-vaccination.

Funding

This work was supported by the Industrial Research Fund of the University of Antwerp, Belgium [PS ID 32387]. S. Van Keer is supported by a Ph.D. fellowship of the Research Foundation, Flanders (FWO), Belgium [1120816N] and received a travel grant from the FWO [K1C3718N]. J. Pattyn is supported by the Royal Belgian Academy of Medicine.

Competing interests

P. Van Damme and A. Vorsters are co-founders of Novosanis (Belgium), a spin-off company of the University of Antwerp. The institute of P. Van Damme and A. Vorsters received unrestricted education grants from Sanofi Pasteur MSD, Merck, and GSK. P. Van Damme and A. Vorsters participated in advisory board meetings of Merck. All other authors declare no potential conflicts of interest.

Ethical approval

All study procedures were approved by the institutional review board of Antwerp University Hospital (UZA)/UAntwerp, Belgium (B300201525584) and the trial was registered on clinicaltrials.gov (NCT02714114).

CRediT authorship contribution statement

Severien Van Keer: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - original draft, Writing - review & editing. **Martina Willhauck-Fleckenstein:** Methodology, Writing - review & editing. **Jade Pattyn:** Investigation, Writing - review & editing. **Julia Butt:** Writing - review & editing. **Wiebren A.A. Tjalma:** Funding acquisition, Writing - review & editing. **Xaveer Van Ostade:** Funding acquisition, Writing - review & editing. **Niel Hens:** Writing - review & editing. **Pierre Van Damme:** Funding acquisition, Resources, Writing - review & editing. **Tim Waterboer:** Methodology, Resources, Supervision, Writing - review & editing. **Alex Vorsters:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

Acknowledgements

We would like to express our gratitude to all the women who volunteered to participate in this study, and we thank the clinical trial unit from the Centre for the Evaluation of Vaccination (CEV, University of Antwerp, Belgium) for their practical assistance. We furthermore thank S. Biesmans, A. De Smet (CEV), and B. Aengeneyndt (German Cancer Research Center; DKFZ, Germany) for laboratory assistance, as well as H. Revets (CEV) and D. Viarisio (DKFZ) for the scientific support.

References

- [1] M. Plummer, M. Schiffman, P.E. Castle, D. Maucort-Boulch, C.M. Wheeler, A. Group, A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion, *J. Infect. Dis.* 195 (2007) 1582–1589.
- [2] J.M. Walboomers, M.V. Jacobs, M.M. Manos, F.X. Bosch, J.A. Kummer, K.V. Shah, et al., Human papillomavirus is a necessary cause of invasive cervical cancer worldwide, *J. Pathol.* 189 (1999) 12–19.
- [3] M. Scherpenisse, M. Mollers, R.M. Schepp, C.J. Meijer, H.E. de Melker, G.A. Berbers, et al., Detection of systemic and mucosal HPV-specific IgG and IgA antibodies in adolescent girls one and two years after HPV vaccination, *Hum. Vaccin. Immunother.* 9 (2013) 314–321.
- [4] J. Mestecky, Z. Moldoveanu, M.W. Russell, Immunologic uniqueness of the genital tract: challenge for vaccine development, *Am. J. Reprod. Immunol.* 53 (2005) 208–214.
- [5] J.J. Carter, L.A. Koutsky, J.P. Hughes, S.K. Lee, J. Kuypers, N. Kiviat, et al., Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection, *J. Infect. Dis.* 181 (2000) 1911–1919.
- [6] D. Nardelli-Haeffliger, D. Wirthner, J.T. Schiller, D.R. Lowy, A. Hildesheim, F. Ponci, et al., Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles, *J. Natl. Cancer Inst.* 95 (2003) 1128–1137.
- [7] J.M.L. Brotherton, P.N. Bloem, Population-based HPV vaccination programmes are safe and effective: 2017 update and the impetus for achieving better global coverage, *Best Pract. Res. Clin. Obstet. Gynaecol.* 47 (2018) 42–58.
- [8] Human papillomavirus vaccines: WHO position paper, May 2017-Recommendations, *Vaccine* 35 (2017) 5753–5755.
- [9] M. Arbyn, L. Xu, Efficacy and safety of prophylactic HPV vaccines. A Cochrane review of randomized trials, *Expert Rev. Vaccines* 17 (2018) 1085–1091.
- [10] L. Bruni, B. Serrano, M. Diaz, M. Brotons, C. Robles, S. De Sanjose, et al., Worldwide Female and Male HPV Vaccination Coverage up to 2018. *IPVC2018*. Sydney, (2018).
- [11] S. Franceschi, M. Chantal Umulisa, U. Tshomo, T. Gheit, I. Baussano, V. Tenet, et al., Urine testing to monitor the impact of HPV vaccination in Bhutan and Rwanda, *Int. J. Cancer* 139 (2016) 518–526.
- [12] A. Vorsters, S. Van Keer, P. Van Damme, The use of urine in the follow-up of HPV vaccine trials, *Hum. Vaccin. Immunother.* 11 (2015) 350–352.
- [13] K. Cuschieri, R. Nandwani, P. McGough, F. Cook, L. Hogg, C. Robertson, et al., Urine testing as a surveillance tool to monitor the impact of HPV immunization programs, *J. Med. Virol.* 83 (2011) 1983–1987.

- [14] S. Longet, J.T. Schiller, M. Bobst, P. Jichlinski, D. Nardelli-Haeffliger, A murine genital-challenge model is a sensitive measure of protective antibodies against human papillomavirus infection, *J. Virol.* 85 (2011) 13253–13259.
- [15] M. Stanley, L.A. Pinto, C. Trimble, Human papillomavirus vaccines—immune responses, *Vaccine*. 30 (Suppl 5) (2012) F83–7.
- [16] J. Paavonen, P. Naud, J. Salmeron, C.M. Wheeler, S.N. Chow, D. Apter, et al., Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women, *Lancet* 374 (2009) 301–314.
- [17] L.L. Villa, R.L. Costa, C.A. Petta, R.P. Andrade, J. Paavonen, O.E. Iversen, et al., High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up, *Br. J. Cancer* 95 (2006) 1459–1466.
- [18] M.H. Einstein, M.J. Levin, A. Chatterjee, N. Chakhtoura, P. Takacs, G. Catteau, et al., Comparative humoral and cellular immunogenicity and safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine and HPV-6/11/16/18 vaccine in healthy women aged 18–45 years: follow-up through Month 48 in a Phase III randomized study, *Hum. Vaccin. Immunother.* 10 (2014) 3455–3465.
- [19] T.F. Schwarz, M. Kocken, T. Petaja, M.H. Einstein, M. Spaczynski, J.A. Louwers, et al., Correlation between levels of human papillomavirus (HPV)-16 and 18 antibodies in serum and cervicovaginal secretions in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine, *Hum. Vaccine* 6 (2010) 1054–1061.
- [20] J.A. Passmore, D.J. Marais, C. Sampson, B. Allan, N. Parker, M. Milner, et al., Cervicovaginal, oral, and serum IgG and IgA responses to human papillomavirus type 16 in women with cervical intraepithelial neoplasia, *J. Med. Virol.* 79 (2007) 1375–1380.
- [21] T.J. Kemp, A. Hildesheim, A. Garcia-Pineros, M.C. Williams, G.M. Shearer, A.C. Rodriguez, et al., Elevated systemic levels of inflammatory cytokines in older women with persistent cervical human papillomavirus infection, *Cancer Epidem. Biomar.* 19 (2010) 1954–1959.
- [22] A. Hildesheim, L.M. McShane, M. Schiffman, M.C. Bratti, A.C. Rodriguez, R. Herrero, et al., Cytokine and immunoglobulin concentrations in cervical secretions: reproducibility of the Weck-cel collection instrument and correlates of immune measures, *J. Immunol. Methods* 225 (1999) 131–143.
- [23] A. Vorsters, P. Van Damme, G. Clifford, Urine testing for HPV: rationale for using first void, *Bmj.* 349 (2014) g6252.
- [24] T. Waterboer, P. Sehr, K.M. Michael, S. Franceschi, J.D. Nieland, T.O. Joos, et al., Multiplex human papillomavirus serology based on in situ-purified glutathione S-transferase fusion proteins, *Clin. Chem.* 51 (2005) 1845–1853.
- [25] T. Waterboer, P. Sehr, M. Pawlita, Suppression of non-specific binding in serological Luminex assays, *J. Immunol. Methods* 309 (2006) 200–204.
- [26] D.G. Altman, *Practical Statistics for Medical Research*, Chapman and Hall/CRC, 1991.
- [27] H.J. Bontkes, T.D. de Gruijl, J.M. Walboomers, J.T. Schiller, J. Dillner, T.J. Helmerhorst, et al., Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. II. Systemic but not local IgA responses correlate with clearance of HPV-16, *J. Gen. Virol.* 80 (Pt 2) (1999) 409–417.
- [28] M. Safaeian, T. Kemp, R.T. Falk, A.C. Rodriguez, A. Hildesheim, M. Williams, et al., Determinants and correlation of systemic and cervical concentrations of total IgA and IgG, *Cancer Epidem. Biomar.* 18 (2009) 2672–2676.
- [29] M. Stanley, D.R. Lowy, I. Frazer, Prophylactic HPV vaccines: underlying mechanisms, Chapter 12, *Vaccine* 24 (Suppl. 3) (2006) S3/106–13.
- [30] M. Mollers, J.M. Vossen, M. Scherpenisse, F.R. van der Klis, C.J. Meijer, H.E. de Melker, Review: current knowledge on the role of HPV antibodies after natural infection and vaccination: implications for monitoring an HPV vaccination programme, *J. Med. Virol.* 85 (2013) 1379–1385.
- [31] S. Franceschi, G.M. Clifford, I. Baussano, Options for design of real-world impact studies of single-dose vaccine schedules, *Vaccine* 36 (2018) 4816–4822.
- [32] M. Stanley, P. Dull, HPV single-dose vaccination: impact potential, evidence base and further evaluation, *Vaccine* 36 (2018) 4759–4760.