



Human Papillomavirus neutralizing and cross-reactive antibodies induced in HIV-positive subjects after vaccination with quadrivalent and bivalent HPV vaccines



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ABSTRACT

Ninety-one HIV-infected individuals (61 men and 30 women) were randomized to vaccination either with quadrivalent (GardasilTM) or bivalent (CervarixTM) HPV vaccine. Neutralizing and specific HPV-binding serum antibodies were measured at baseline and 12 months after the first vaccine dose. Presence of neutralizing and binding antibodies had good agreement (average Kappa for HPV types 6, 11, 16, 18, 31, 33 and 45 was 0.65). At baseline, 88% of subjects had antibodies against at least one genital HPV. Following vaccination with CervarixTM, all subjects became seropositive for HPV16 and 18. After GardasilTM vaccination, 96% of subjects seroconverted for HPV16 and 73% for HPV18. Levels of HPV16-specific antibodies were <1 international unit (IU) in 87% of study subjects before vaccination but >10 IU in 85% of study subjects after vaccination. Antibodies against non-vaccine HPV types appeared after GardasilTM vaccination for >50% of vaccinated females for HPV 31, 35 and 73 and for >50% of CervarixTM-vaccinated females for HPV 31, 33, 35, 45, 56 and 58. Cross-reactivity with non-genital HPV types was also detected. In conclusion, HIV-infected subjects responded to HPV vaccination with induction of neutralizing antibodies against both vaccine and non-vaccine types.

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1. Introduction

Globally, about 10% of human cancers are caused by Human Papillomavirus (HPV) infection. The majority of HPV-related cancers occur in the anogenital tract and in the oropharynx. Both the bivalent CervarixTM and the quadrivalent GardasilTM vaccine have demonstrated efficacy up to 100% against persistent infections with HPV 16 and HPV 18 and safety [1]. These 2 viruses are responsible for causing about 70% of all HPV-related cancers. Besides these types, several other HPV types are also established as carcinogenic to human (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) with HPV 68 classified as a probable carcinogen [2]. The bivalent vaccine induces significant protection also against non-vaccine HPV types HPV 31, 33, 45, 51 and the quadrivalent vaccine against HPV 31, through

induction of cross-protective antibody responses [3]. The Distribution of carcinogenic HPV types differs between regions of the world. For example in East Asia HPV 52 and 58 are prevalent and a 9-valent HPV vaccine including HPV types 6/11/16/18/31/33/45/52/58 has the potential to prevent up to 90% of cervical cancer cases, in these region as well [4].

Vaccine immunogenicity analyses are important for several reasons. They help to determine the range and duration of responses and can be used in bridging studies to extend vaccination recommendations to groups that are difficult to evaluate specifically in efficacy trials. For example, clinical outcomes for children cannot be measured in realistic time frames. Also, because of the large amount of data on efficacy of vaccines, it is no longer ethical to use placebo groups who do not receive vaccine and could acquire oncogenic HPV infection and develop HPV-associated diseases. Therefore, induction of HPV specific antibodies has emerged as a most important outcome measure for HPV vaccine research [5]. So far, all large scale HPV vaccine efficacy trials have used

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only in-house standards and methods to measure immunogenicity [6,7], such as Enzyme-linked Immunosorbent Assays (ELISAs) that employ virus-like particles as antigen, *in vitro* neutralization assays that measure the biologically relevant subset of capsid-binding antibodies that can prevent infection or competitive Luminex Immunoassays (cLia) that measure the subset of antibodies that compete with a type-specific neutralizing monoclonal antibody for binding to an epitope of the virus capsid. WHO has been standardizing HPV serology, for both ELISA and for the *in vitro* neutralization assay [8]. To evaluate efficacy of next generation vaccines, high-throughput methods with wide HPV type coverage are necessary [9]. The neutralization assay has been automated to a high-throughput scale to simultaneously measure neutralizing antibodies to HPV 16, 18, 31, 33 and 45 [10]. Merck has developed a cLia for monitoring antibodies against the 9 HPV types included in Gardasil-9 [11]. A multiplexed serology method based on mammalian cell-derived pseudovirions (Pseudovirion-Luminex) [12] can simultaneously measure antibodies to 21 different HPV types (15 of these are sexually-transmitted anogenital HPVs). The antibodies measured with this method correlate well with natural HPV infection [13]. We wished to employ this methodology in HPV vaccine research. To ensure reliable outcome measurements, we also measured neutralizing antibodies on the same samples [14,15]. We now report the results of an HPV vaccine trial investigating the effect of vaccination with either Cervarix™ or Gardasil™ in HIV-positive subjects, as evaluated using the HPV immunogenicity endpoint.

2. Methods

2.1. Study population

The study design is described in detail by Toft et al. [14]. Briefly, it was a double-blind clinical trial (NCT01386164) in which HIV-infected adults were randomized to vaccination with either Cervarix™ or Gardasil™. The study was conducted at the Department of Infectious Diseases, Aarhus University Hospital, Denmark. The adult volunteers received 3 doses of HPV vaccine at days 0, 45, and 180 and were followed up to months 7 and 12 post receipt of the first vaccine dose. In the present study, samples from the baseline (day 0) and the final visit (12 months after first vaccine dose) were analyzed. Altogether 91 individuals (61 men and 30 women, average age 46 years, 82% Caucasians) were vaccinated and tested for HPV DNA and HPV serum antibodies.

2.2. HPV DNA testing

The HPV DNA testing has been described by Bonde et al. [16] and Toft et al. [14]. Briefly, anal and cervical swabs were collected at baseline and at month 7. HPV DNA was purified using MagNA Pure Nucleic Acid Isolation Kit (Roche Diagnostics, Switzerland). HPV genotyping was done at Aarhus University, Denmark, using the Genomica CLART HPV2 Genotyping micro array (Genomica), which detects 35 different types of HPV.

2.3. Pseudovirion Luminex

Detection of HPV specific IgG serum antibodies was performed as described [13]. We produced pseudovirions for 17 HPV types belonging to species alpha (HPV 3, 6, 11, 16, 18, 31, 32, 33, 35, 39, 45, 52, 56, 58, 59, 68, 73) and for 4 HPV types belonging to species beta (HPV 5, 15, 38, 76) as well as pseudovirions for a control virus (Merkel cell polyomavirus (MCV)). Pseudovirions were generated by transfection of 293TT cells, as described [17]. VLPs from another control virus (JC polyomavirus (JCV)) were a kindly provided from Dr. K. Sasnauskas. Serum samples from baseline and final visit

(12 months after the first vaccine dose) were analyzed in dilution 1:150. Cut-off values to define seropositivity were calculated independently for each HPV type by analysing the mean fluorescence intensity unit (MFI) values obtained from 106 children's sera (≤ 12 years old). The cut-off algorithm was as recommended by the global HPV LabNet (mean MFI value of a negative control serum panel plus 3 standard deviations) [18]. If this cut-off value was unreasonably low (less than 400 MFI) we used 400 MFI as cut-off to have sensitivity and specificity similar to classical ELISA [12]. HPV 16 specific antibody levels were calculated into international units (IU) using International Standard Serum for HPV 16 (10IU) and wPLLmodel [19].

2.4. Neutralization assay for HPV 6 and 11

A conventional neutralization assay was performed using pseudovirions (PsV) carrying secreted alkaline phosphatase (SEAP) reporter gene, generated by transfection of 293TT cells as described [17]. For HPV 6 and 11, the neutralization assay protocol by Pastrana et al. [20] was followed using a serum dilution of 1:500. Chemoluminescence was read for 0.2 s per well using a Wallac Victor 1420 Multilabel counter. A serum was considered neutralizing, if the secreted alkaline phosphatase signal was reduced by more than 50%.

2.5. High-throughput pseudovirion-based neutralization assay (HT-PBNA) for HPV 16, 18, 31, 33, 45

An automated pseudovirion-based neutralization assay was performed as described [10,14,15]. Bovine Papillomavirus type1 (BPV 1) was used as a control. The mean ED₅₀ value plus 3 standard deviations of the serum samples reacting with the negative control (BPV 1) was used as a cut-off (ED₅₀ = 160).

2.6. Statistics

Agreement of categorical values between assays was quantified using Kappa values, calculated using GraphPad QuickCalcs online calculator. R^2 was calculated using Microsoft Excel to compare continuous results from neutralization and Pseudovirion-Luminex assays. Sensitivity and specificity of Pseudovirion-Luminex assays were calculated using the neutralization assay as the golden standard. GraphPad Prism software and a Fisher's exact test were applied to evaluate differences of seroconversion by vaccine groups and gender. Mann-Whitney and Kruskal-Wallis tests were applied to estimate HPV 16 antibody level differences between groups vaccinated with Cervarix™ or Gardasil™.

3. Results

3.1. Validation of immunogenicity measurements

Categorical data obtained with the neutralization assay was compared with data from the antibody binding (Pseudovirion-Luminex) assay (Table 1). The baseline ($N=91$) and 12 months follow up (final visit) serum samples ($N=90$) were all tested with Pseudovirion-Luminex and automated neutralization assays (HT-PBNA). As the non-automated neutralization assay is highly laborious, the HPV 6 and 11 neutralization assay was performed only for a subset of samples (35 Gardasil™ and 10 Cervarix™ recipients, all 10 were HPV 6 and 11 DNA negative at baseline). The cut-off levels for the different assays have been established in previous studies. Briefly, they were based on a negative serum panel (Pseudovirion-Luminex) [13], on a bovine papillomavirus type 1 control antigen (PBNA) [10] and on the secreted alkaline

Table 1

Agreement of the Pseudovirion-Luminex (IgG) and neutralization assay (Neutr) at baseline and at the final visit (12 months from the first HPV vaccine dose). Sensitivity and specificity were calculated using neutralization assay results as the golden standard.

HPV type time point	Kappa agreement (95% CI)	IgG+ Neutr+ N	IgG– Neutr– N	IgG + Neutr– N	IgG– Neutr+ N	Total observations N	Sensitivity %	Specificity %
HPV6 baseline	0.63 Good (0.4–0.86)	14	23	5	3	45	82	82
HPV6 final visit	0.62 Good (0.29–0.95)	37	4	4	0	45	100	50
HPV11 baseline	0.55 Moderate (0.27–0.82)	8	29	3	5	45	62	91
HPV11 final visit	0.55 Moderate (0.05–0.8)	36	3	4	2	45	95	43
HPV16 baseline	0.86 Very good (0.75–0.97)	32	53	5	1	91	97	91
HPV16 final visit	0.66 Good (0.04–1.28)	88	1	1	0	90	100	50
HPV18 baseline	0.75 Good (0.59–0.9)	21	61	5	4	91	84	92
HPV18 final visit	0.68 Good (0.42–0.94)	79	6	3	2	90	98	67
HPV31 baseline	0.76 Good (0.61–0.91)	22	59	6	4	91	85	91
HPV31 final visit	0.59 Moderate (0.43–0.76)	42	30	10	8	90	84	75
HPV33 baseline	0.74 Good (0.58–0.91)	16	67	5	3	91	84	93
HPV33 final visit	0.7 Good (0.54–0.85)	28	49	8	5	90	85	86
HPV45 baseline	0.65 Good (0.46–0.85)	13	68	5	5	91	72	93
HPV45 final visit	0.37 Fair (0.19–0.55)	22	40	22	6	90	79	65

phosphatase signal strength from 293TT cells infected with HPV pseudovirions without addition of human serum (conventional neutralization assay) [20].

The agreement of the Luminex and neutralization assay was good (average Kappa 0.65), with the best agreement seen for HPV 16 at baseline (Kappa 0.86) (Table 1). The correlation coefficient for the continuous data was $R^2=0.45$ (Fig. 1). The average sensitivity of the Pseudovirion-Luminex assay compared to the neutralization assay, was 86% and specificity was 76% (Table 1).

3.2. Seroconversion after HPV vaccination

Most subjects were infected with at least one HPV type at baseline (Table 2A) (61% had HPV DNA of at least one HPV type). Also, 88% of subjects had specific antibodies binding to HPV for at least one sexually-transmitted HPV. Sixteen percent of subjects had antibodies to more than 10 anogenital HPV types. Also, 50% of subjects had antibodies against non-genital (cutaneous) HPV. The most prevalent HPV types in the DNA test were HPV 16 (20%), 6 (16%), and 31, 52 and 58 (all 13%) (Table 2A). The most prevalent neutralizing antibodies at baseline were neutralizing HPV 6 (38%) and 16 (37%) (Table 2B).

Seroconversion rates were derived by comparing the data from baseline and at the final visit (12 months after the first vaccine dose). Seronegative subjects who became seropositive after vaccine administration were classified as having seroconverted. After Gardasil™ administration there was a 95–100% seroconversion rate for HPV 6, 11, 16 and 73% seroconversion rate for HPV 18, while Cervarix™ induced 100% of seroconversion for both HPV 16 and HPV 18 (Table 2A and B). The differences in seroconversion rate between vaccines were statistically significant ($p < 0.05$) for HPV 6, 11, 18 and 45. The difference in response between genders was statistically significant ($p < 0.05$) for HPV 31, 35, 56 and 58 (Table 2A and B).

Cross-reactive antibodies were detected after vaccination, both in the neutralization and in the binding assay. Females presented higher rates of induction of cross-reactive antibodies than males. After vaccination with Cervarix™, there was a high rate of seroconversion for neutralizing antibodies for HPV 31 (50%) and 33 (27%). After vaccination with Gardasil™, seroconversion for neutralizing antibodies for HPV31 and 33 was observed in 29% and 18% of subjects, respectively (Table 2B). Cervarix™ did not induce any neutralizing antibodies against HPV 6, but in the binding assay seroconversion for HPV 6 binding antibodies was detected for 35% of subjects. Seroconversion for HPV-binding antibodies was detected for more >40% of female vaccine recipients for HPV 31

and 35 (both vaccines), for HPV 33, 45, 56 and 58 (Cervarix™ only) and for HPV 68 and 73 (Gardasil™ only) (Table 2A). High seroconversion rates were also seen for cutaneous HPVs as well as for the control viruses (Table 2A). More than half of the study population was HPV-negative (for both HPV DNA and HPV antibodies) for at least one vaccine-included HPV type at baseline (Table 2C).

Levels of HPV 16 specific antibodies before and after vaccination were compared to the International Standard Serum for HPV 16, the serum sample that by definition contains 10 IU of HPV16 antibodies. A majority of subjects (87%) had an antibody level below 1 IU before vaccination (Table 3) and most had >10 IU after vaccination (85%) (Table 3). Among females, Cervarix™ induced higher levels of HPV16 specific antibodies than Gardasil™, but the difference was not statistically significant (Fig. 2).

4. Discussion

The present study provides an example of how immunogenicity for multiple HPV types can be used as an endpoint in HPV vaccine research. For validation of the HPV immunogenicity measurements, we used both the neutralization assay (which measures protective antibodies) and a multiplexed pseudovirion binding assay, which detects HPV type-specific IgG antibodies. As there was good agreement between these 2 assays, it appears that valid methods for measuring HPV immunogenicity in vaccine research exist.

The seroconversions observed for the control viruses (polyomaviruses) after vaccine administration are unlikely to be induced by the HPV VLPs in the vaccines, as there is no known antigenic relatedness to these control viruses. We have two possible explanations: (i) during follow-up, infections with these polyomaviruses may have occurred (ii) adjuvants in the HPV vaccines may have boosted the immune response in an unspecific manner. This explanation may also have affected the seroconversion rates seen for the cutaneous HPV types.

The baseline HPV seroprevalences were much higher in the HIV-positive subjects studied here compared to other studies of HIV-negative populations, even though identical methodology was used [13]. Although there is thus substantial prior exposure in this population, HIV infection tremendously raises the risk for cancer [21] and at least half of the HIV-positive population was negative for one or several HPV vaccine types and could benefit from vaccination. Our finding about the differences in antibody levels after Cervarix™ administration in comparison to Gardasil™ stands in-line with previous publications [22,23].

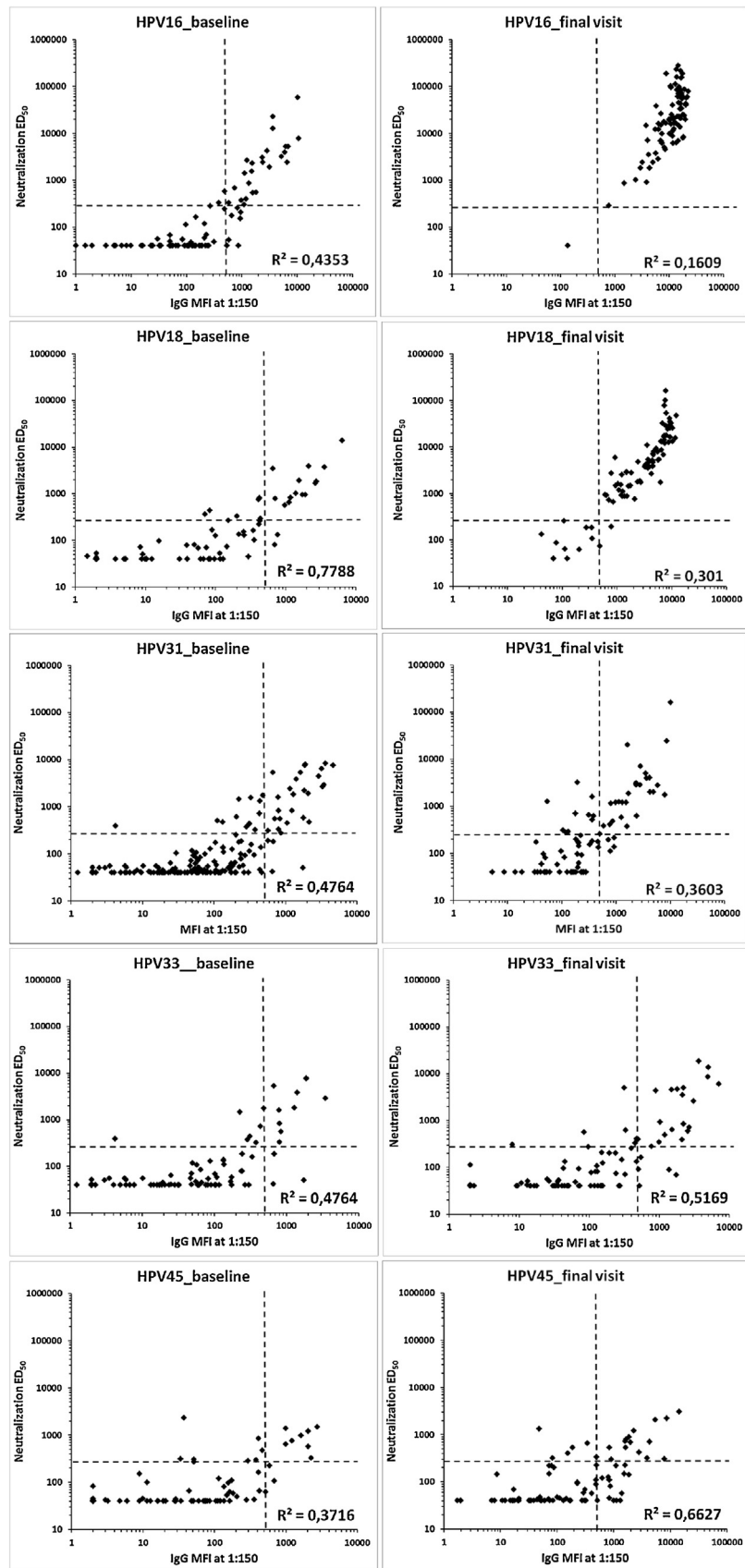


Fig. 1. Correlation of neutralization ED₅₀ and Pseudovirion-heparin Luminex (IgG) at serum dilution 1:150 at baseline and after the final visit (12 month from the first vaccine dose). Dashed line – cut-off. Each dot represents one vaccinated person.

Table 2
Prevalence of studied viruses and seroconversion after vaccination among the baseline seronegative subjects.

(A) Antibodies detected with Pseudovirion-Luminex (IgG)															
HPV type	DNA prevalence%			Baseline seroprevalence %			Seroconversion total%			Seroconversion Gardasil™%			Seroconversion Cervarix™%		
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
<i>Genital</i>															
6	20	7	16	72	30	58	76	67	71 [#]	100	100	100	33	36	35
11	10	0	7	36	20	31	54	67	59 [#]	91	100	94	0	27	11
16	27	7	20	43	37	41	100	95	98	100	90	96	100	100	100
18	12	10	11	33	20	29	83	96	88 [#]	63	91	73	100	100	100
31	15	10	13	31	30	31	31	67	43 [*]	25	64	37	39	70	50
33	8	7	8	20	30	23	18	38	24	14	22	16	24	50	33
35	10	7	9	16	27	20	20	59	32 [*]	15	55	26 [*]	25	64	37
39	5	0	3	41	40	41	22	39	28	11	40	25	33	38	31
45	3	7	4	23	13	20	36	38	37 [#]	23	15	21	52	62	56
52	12	17	13	15	30	20	10	14	11	11	9	10	8	20	12
56	3	3	3	26	37	30	13	37	20 [*]	12	20	14	15	56	28 [*]
58	13	13	13	38	53	43	18	50	27 [*]	24	40	27	8	56	27 [*]
59	12	7	10	33	27	31	17	32	22	16	31	22	18	33	23
68	8	10	9	20	20	20	14	21	16	16	43	19	13	12	14
73	3	0	2	41	27	36	44	64	52	70	100	81	13	27	19
<i>Non-genital</i>															
3	NT	NT	NT	7	7	7	23	25	24	23	27	24	22	23	23
5	NT	NT	NT	30	37	32	65	74	68	67	70	68	63	78	68
15	NT	NT	NT	21	40	27	48	39	45	48	50	49	48	25	41
32	NT	NT	NT	16	33	22	33	40	35	41	20	35	25	60	35
38	NT	NT	NT	7	20	11	21	29	23	23	25	24	19	33	23
76	NT	NT	NT	18	30	22	26	19	24	19	20	19	35	18	29
JCV	NT	NT	NT	79	63	74	69	91	79	75	80	77	60	100	82
MCV	NT	NT	NT	69	63	67	58	73	63	56	50	53	60	100	73
Any HPV	NT	NT	NT	87	93	89									
Any genital HPV	65	53	61	85	93	88									
Single genital HPV	15	33	21	13	17	14									
Any non- genital HPV	NT	NT	NT	44	63	51									
≥10 HPV	NT	NT	NT	16	17	16									
(B) Antibodies detected with neutralization assays															
HPV type	Baseline seroprevalence %			Seroconversion total%			Seroconversion Gardasil™%			Seroconversion Cervarix™%					
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total			
6	42	32	38	80	69	75 [#]	100	90	95	0	0	0			
11	27	32	29	84	85	84 [#]	100	100	100	0	0	0			
16	38	33	36	100	95	98	100	90	96	100	100	100			
18	28	27	27	86	86	86 [#]	71	78	73	100	92	97			
31	28	30	29	34	50	39	24	44	29	47	55	50			
33	17	30	21	18	35	23	17	22	18	18	45	27			
45	22	17	20	19	8	15	24	8	18	13	8	11			
(C) HPV DNA- and sero-negative population															
HPV type	HPV DNA and sero-negative %														
	Male	Female	Total												
16	53	57	54												
18	62	70	64												
16/18	45	47	46												
31	60	63	61												
33	73	63	70												
16/18/31/33	33	37	34												

[#] Statistically significant difference ($p < 0.05$) between vaccines in terms of seroconversion-rate.

^{*} Statistically significant difference ($p < 0.05$) between genders in terms of seroconversion-rate.

Limitations of our study are that only a subset of the population was tested for HPV 6 and 11 neutralizing antibodies in the manual assay and that HPV DNA testing was based only on samples taken from the cervix and from the anus, not from the external genitalia, which might have underestimated the prevalence of HPV infections. Another limitation was the low number of patients enrolled. Especially in groups with high seroprevalence at the baseline, the number of samples used to measure seroconversion was

sometimes low and therefore results on seroconversion rates should be taken cautiously.

In conclusion, we show that immunogenicity can be used as an outcome in HPV vaccine research and that different assays were consistent. We found a high immunogenicity among the HIV-infected population. A substantial proportion of the subjects were HPV-negative, and therefore likely to benefit from vaccination. Also, the frequent induction of cross-neutralizing

Table 3
HPV16 specific antibody levels (IU) at baseline and after the final visit (12 months from the first vaccine dose).

	<1 IU (%)	1–10 IU (%)	10–100 IU (%)	100–1000 IU (%)	>1000 IU (%)
<i>Baseline</i>					
Total	86.8	6.6	5.5	1.1	0.0
Male total	85.2	8.2	4.9	1.6	0.0
Male Cervarix	86.7	10.0	0.0	3.3	0.0
Male Gardasil	83.9	6.5	9.7	0.0	0.0
Female total	90.0	3.3	6.7	0.0	0.0
Female Cervarix	86.7	6.7	6.7	0.0	0.0
Female Gardasil	93.3	0.0	6.7	0.0	0.0
Cervarix total	86.7	8.9	2.2	2.2	0.0
Gardasil total	87.0	4.3	8.7	0.0	0.0
<i>Final visit</i>					
Total	3.4	11.2	22.5	24.7	38.2
Male total	3.3	15.0	21.7	25.0	35.0
Male Cervarix	3.3	16.7	20.0	30.0	30.0
Male Gardasil	3.3	13.3	23.3	20.0	40.0
Female total	3.4	3.4	24.1	24.1	44.8
Female Cervarix	0.0	7.1	0.0	35.7	57.1
Female Gardasil	6.7	0.0	46.7	13.3	33.3
Cervarix total	2.3	13.6	13.6	31.8	38.6
Gardasil total	4.4	8.9	31.1	17.8	37.8

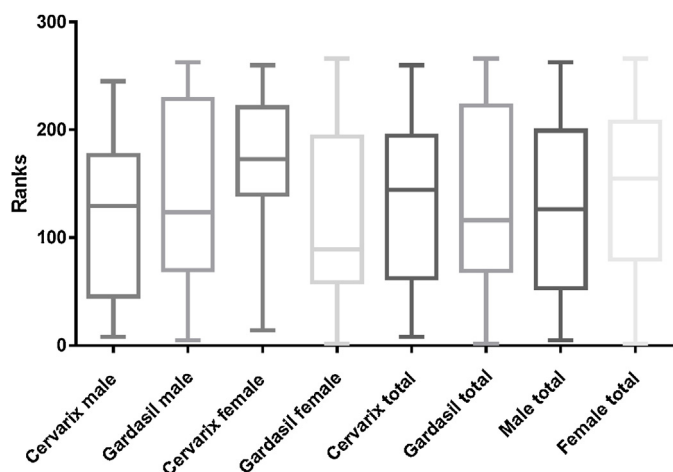


Fig. 2. HPV 16 antibody levels (IU) at final visit (12 months from first vaccine dose). Median of the ranks and range are presented on the graph. No significant difference between the groups was observed in terms of antibody level after vaccination.

antibodies argues in favor of HPV vaccination of adult HIV-positive populations.

Author contribution

All authors contributed to the discussion and writing of the manuscript. HF – performed PsV-Luminex and PBNA for HPV 6, 11, data analysis, created tables, figures and wrote the first draft of the manuscript. LT – study initiator, organized clinical trial. PS – designed and performed of automated PBNA. MM – designed of automated PBNA. JB – responsible for HPV DNA genotyping, OF – interpretation of data and critical reading of manuscript. LØ – organized clinical trial. MT – organized clinical trial. JD – supervision, writing help, finalizing and publishing the study.

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previously acted as consultant for Merck/SPMSD and has received research grants to his institution for studies on HPV vaccines.

Conflict of interest: The other authors declare no conflicts of interest.

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