Natural and vaccine derived immunity against the human papillomavirus

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CHAPTER 4

PERSISTING ANTIBODY RESPONSE NINE YEARS AFTER BIVALENT HPV VACCINATION IN A COHORT OF DUTCH WOMEN: IMMUNE RESPONSE AND THE RELATION WITH GENITAL HPV INFECTIONS

Journal of Infectious Diseases · 2020
DOI: 10.1093/infdis/jiaa007

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ABSTRACT

The bivalent HPV vaccine is highly effective and induces robust serological responses. Using a Dutch prospective cohort initiated in 2009 including 744 vaccinated and 294 unvaccinated girls (1993-1994), who provide a vaginal self-swab, serum sample and questionnaire yearly, we report a high, persisting antibody response up to nine years post-vaccination for vaccine types HPV16/18. Antibodies against non-vaccine types HPV31/33/45/52/58 were lower, but still significantly higher than in unvaccinated individuals. This was also reflected in the seroprevalence. We compared participant characteristics and antibody levels between vaccinated women with and without HPV infections one-year pre-infection (204 incident and 64 persistent infections) but observed no consistent difference in type-specific antibody levels. Having a hrHPV infection was associated with sexual risk behavior and smoking one-year pre-infection. While high antibody levels are necessary for protection, our study suggests that on individual level other factors such as HPV-exposure or antibody avidity could be important.

INTRODUCTION

Human Papillomavirus (HPV) is a common, sexually transmitted virus of which some types can cause anogenital and/or oropharyngeal infections. A persistent infection with a high-risk HPV (hrHPV) type can lead to the progression of malignancies at specific anatomical sites [1]. The most frequently observed cancer type in women associated with HPV is cervical cancer. In total, 99% of all cervical cancer cases are caused by HPV infections, whereof type HPV16 and HPV18 are responsible for about 70% of the cases [2]. In order to prevent persistent HPV infections and subsequent lesions, prophylactic HPV vaccination was registered in 2006 with the ultimate goal of prevention of HPV-related cancers [3]. In 2010, the Netherlands implemented the bivalent vaccine into the National Immunization Program (NIP) as a girls-only vaccine in a three-dose schedule (0, 1, 6 months) vaccinating girls in the year they turn thirteen. Moreover, a catch-up campaign was initiated for birth cohorts 1993-1996 in 2009 (i.e. 13-16 year-olds) [4]. From 2014, the Netherlands shifted to a two-dose schedule (starting with birth cohort 2001).

Vaccine effectiveness (VE) of the bivalent vaccine against HPV16/18 infections is high, with VE estimates over 90% [5, 6]. Also, in the Dutch cohort described in this paper high VE estimates have been reported, with very few infections among vaccinated individuals [7]. Furthermore, for multiple non-vaccine types varying rates of cross protection against infections have been found [5, 8-10] and clinical trials and more recently population-based studies have demonstrated the impact of HPV16/18 vaccination on cervical intraepithelial neoplasia's (CIN) and pre-stages of invasive cancer [11-14]. In addition, HPV vaccination induces robust serological responses [12, 15, 16], which are generally high and can be a 100-fold higher compared to naturally elicited antibodies. Among vaccinated individuals the seroconversion rate is high for vaccine types (95-100%), while a measurable immune response only occurs in 40-60% of naturally infected individuals [17]. Even though high antibody levels are considered to be important for protection, a correlate of protection for HPV is lacking [18]. The observed high VE against vaccine types (HPV16/18) is impeding this search, although at infection level some breakthrough cases occur. This study aims to explore the longitudinal relation between antibody response against HPV16/18/31/33/45/52/58 and HPV-DNA infections. We first describe antibody levels against these seven hrHPV types in vaccinated and unvaccinated young women up to nine years after vaccination with the bivalent vaccine in a three-dose schedule. We then compare participant characteristics and antibody levels between vaccinated women with and without HPV-DNA infections in the next year (either vaccine-type (i.e. HPV16/18), cross-protective type (HPV31/45), or non-vaccine type (HPV33/52/58) infections) to assess whether higher antibody levels protect against infection.

METHODS

Study design

In 2009, the HPV Among Vaccinated And Non-vaccinated Adolescents (HAVANA) study was initiated as a prospective cohort study as previously described [4]. In short, 9500 girls who were eligible for the catch-up campaign were randomly invited to participate in the study in 2009. One month prior to vaccination and each consecutive year post vaccination a vaginal self-swab, a blood sample, a cervical secretion sample (CVS) using a tampon (optionally), and a questionnaire

were collected. A voucher of 25 euros was provided after each year of participation. The HAVANA study was approved by the Medical Ethics Committee of the VU University Medical Centre (2009/022) and was conducted according to the Declaration of Helsinki. Informed consent had to be collected before participants could be included.

Lab procedures – Serology

Blood was drawn using a serum tube (VACUETTE®, Greiner Bio-one) and participants who were not able to visit a blood drawing session were offered a self-sample set to draw finger-prick blood at home resulting in dry blood spot samples (DBS) (Whatman 903 Protein Saver Card) [19]. A virus like particle (VLP)-based multiplex immunoassay (MIA) was used to quantify type specific HPV antibodies to HPV16, 18, 31, 33, 45, 52, and 58 both for serum and DBS samples. For the analysis of antibodies in the first 7 years of follow-up we used HPV VLP's produced by GSK (GlaxoSmithKline Biologicals, Rixensart, Belgium) and for the subsequent years we used VLP's donated by MSD (Merck Sharp & Dohme Corp., Kenilworth, NJ). VLPs were linked to seven distinct color-coded fluorescent microspheres and the MIA was performed as described elsewhere [19-21]. The HPV specific antibodies were analyzed with a Bioplex system 200 with Bioplex software (Bio-Rad Laboratories, Hercules, CA). For each analyte, median fluorescent intensity (MFI) was converted to Luminex Units/ml (LU/ml). We assumed samples to be seropositive at different type specific cut-off levels determined previously [21] of 9, 13, 27, 11, 19, 14, and 31 LU/ml for HPV16, 18, 31, 33, 45, 52, and 58, respectively.

Lab procedures – HPV-DNA detection and genotyping

Vaginal self-samples were collected through a vaginal Viba brush (Rovers). After collection, samples were stored in 1 mL of phosphate-buffered saline at −20°C. 200 µL of the sample was used for DNA extraction with the MagNA Pure 96 DNA and Viral NA small volume Kit (Roche, Mannheim, Germany). The DNA was then eluted in 100 µL elution buffer. A sample of 10 µL DNA extract was used for HPV amplification, making use of the sensitive SPF10 primer sets [22]. To detect the amplified HPV-DNA, a DNA enzyme-linked immunoassay (HPV-DEIA; DDL Diagnostics Laboratory, Rijswijk, the Netherlands) was applied. Amplicons that were HPV-DEIA positive were then analyzed with a reverse line blot assay (HPV-LiPA25; DDL Diagnostics Laboratory) in order to determine the genotype. Twenty-five HPV genotypes could be detected, including the following hrHPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Other HPV types that could be detected were HPV6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 68, 70, 73 and 74 (of which HPV 53, 66, 68, 70, and 73 are classified as possibly oncogenic) [23]. All vaginal self-samples collected in 2009 (n=1152) were subjected to a quality check by testing for beta-actin as a marker for the presence of human DNA. Since 99.3 % were positive for beta-actin this control was not routinely performed for the remaining years of follow-up.

Statistical analysis

To be included for analyses, participants had to be either non-vaccinated or fully-vaccinated according to the three-dose schedule, and not been vaccinated before the baseline measurement was performed or after the first follow-up moment (i.e. participants who decided to get vaccinated after year one of follow-up). Participants were allowed to miss follow-up moments (not censored). Differences between vaccinated and unvaccinated participants in type-specific seroprevalence based on IgG were explored per year using a Chi-squared test. In addition, we

calculated geometric mean concentrations (GMC) of serum antibodies. Differences between vaccination status groups were assessed per year by a t-test on the log transformed data and trends over time within vaccination status group were studied with a linear mixed model.

We examined the association between demographic or sexual behavior characteristics one year prior to infection and HPV infection (irrespective of persistence) among vaccinated participants. To be included, participants needed to be HPV-DNA negative at the baseline measurement (pre-vaccination) for the seven included hrHPV types. Using univariate Generalized Estimation Equation (GEE) logistics regression models with exchangeable correlation structure we combined data of participants over time. The uninfected comprised individuals who were negative for all high risk types as determined per round and were compared to either HPV16/18/31/45 (vaccine type and cross protective types as defined by a significant type-specific VE in the current cohort [7]) infected participants one year pre-infection, or HPV33/52/58 (non-vaccine types) infected participants one year pre-infection. Year of follow-up was added to the model to adjust for the fluctuation related to time. Baseline measurements were used to determine HPV-DNA status but were not included in these analyses as participants were not vaccinated at baseline yet. The association between log-transformed type-specific serum IgG and infection status in the subsequent year was assessed as well, for incident and persistent infections in multilevel linear models with unstructured covariance matrix. Participants had to be baseline HPV-DNA negative for the respective type. An incident HPV infection was defined as being HPV-DNA negative in the previous year and being HPV-DNA positive in the current year. A persistent infection was defined as being HPV-DNA positive in at least two consecutive years. Random-intercept at participant-level was added to the model. Again, year of follow-up was added to the model. Participants added to the uninfected group in years they were HPV-negative for the respective type. The outcome was expressed as the GMC ratio with 95% confidence interval of antibody levels pre-infection comparing vaccinated participants without an infection to vaccinated participants with an infection. All analyses were conducted in SAS (version 9.4).

RESULTS

Study population

Characteristics of the participants are described in Table 1. In total, 1038 participants with baseline measurement (of which 71.7% was vaccinated) were included in the current analyses. Due to loss to follow up the number of participants decreased to 514 in the ninth-year post vaccination (of which 76.7% was vaccinated). Among vaccinated participants we observed a total of 204 incident and 64 persistent infections for HPV 16/18/31/33/45/52/58 which were included in the type-specific analyses.

Immunogenicity

In all years post-vaccination, a significant difference in seroprevalence was observed between vaccinated and unvaccinated participants for all HPV types ($p<.0001$, Table 2). Seropositivity mounted to 100% among vaccinated girls for vaccine types HPV16/18 directly after vaccination and remained 100% up to nine years post-vaccination. Among unvaccinated this was only 9.7% and 4.8% in the first year of follow-up increasing to 20.8% and 9.3% in the last year for HPV16 and HPV18, respectively. Also, for other HPV types (HPV31/33/45/52/58), a remarkably higher

Table 1 Characteristics of study participants over time.
*:p<.0001.

Sociodemographic characteristics	**Baseline** N (%)	**Round 1** N (%)	**Round 2** N (%)	**Round 3** N (%)	**Round 4** N (%)	**Round 5** N (%)	**Round 6** N (%)	**Round 7** N (%)	**Round 8** N (%)	**Round 9** N (%)
Total number of participants	1038	797	765	722	624	641	569	589	579	514
Fully vaccinated	744 (71.7)	590 (74.0)	562 (73.5)	533 (73.8)	475 (76.1)	467 (72.9)	407 (71.5)	426 (72.3)	427 (73.7)	394 (76.7)
Age in years, mean (range)	15 (14-16)	16 (15-17)	17 (16-18)	18 (17-19)	19 (18-20)	20 (19-21)	21 (20-22)	22 (21-23)	23 (22-24)	24 (23-25)
Urbanization - high	933 (89.9)	724 (90.8)	686 (89.7)	590 (81.7)	553 (88.6)	575 (89.7)	518 (91.0)	498 (84.6)	485 (83.8)	436 (84.8)
Ethnicity - Dutch	898 (86.5)	699 (87.7)	670 (87.6)	644 (89.2)	557 (89.3)	573 (89.4)	518 (91.0)	535 (90.8)	526 (90.8)	467 (90.9)
Education - high	572 (55.1)	498 (62.5)	491 (64.2)	476 (65.9)	417 (66.8)	460 (71.8)	422 (74.2)	430 (73.0)	439 (75.8)	380 (73.9)
Ever smoked - yes	345 (33.2)	330 (41.4)	300 (39.2)	368 (51.0)	344 (55.1)	367 (57.3)	335 (58.9)	339 (57.6)	345 (59.6)	317 (61.7)
Current smoker - yes	137 (13.2)	245 (30.7)	252 (32.9)	272 (37.7)	238 (38.1)	158 (24.6)	205 (36.0)	215 (36.5)	192 (33.2)	166 (32.3)
Ever used contraception - yes	408 (39.3)	489 (61.4)	580 (75.8)	623 (86.3)	577 (92.5)	602 (93.9)	537 (94.4)	570 (96.8)	559 (96.5)	503 (97.9)
Ever had sex - yes	239 (23.0)	341 (42.8)	445 (58.2)	506 (70.1)	509 (81.6)	552 (86.1)	503 (88.4)	521 (88.5)	531 (91.7)	478 (93.0)
Sexual behavior characteristics among sexually active										
Age sexual debut <15 years - yes	119 (49.8)	84 (24.6)	80 (18.0)	84 (16.6)	78 (15.3)	76 (13.8)	82 (16.3)	75 (14.4)	75 (14.1)	73 (15.3)
Lifetime number sexual partners, mean (range)	1.7 (1-16)	2.0 (1-15)	2.6 (1-20)	2.8 (1-11)	3.4 (1-20)	4.3 (1-50)	5.0 (1-50)	5.4 (1-45)	6.5 (1-50)	6.8 (1-40)
New number of partners 12 past months, mean (range)	-	1.2 (0-7)	1.1 (0-12)	1.0 (0-7)	1.0 (0-11)	1.2 (0-31)	0.9 (0-14)	1.7 (0-10)	1.7 (0-12)	1.8 (0-8)
Current steady partner - yes	156 (65.3)	212 (62.2)	299 (67.2)	356 (70.4)	374 (73.5)	396 (71.7)	402 (79.9)	421 (80.8)	430 (81.0)	393 (82.2)
Diagnosed with STI previous 12 months - yes	2 (0.8)	5 (1.5)	9 (2.0)	20 (4.0)	17 (3.3)	26 (4.7)	28 (5.6)	33 (6.3)	28 (5.3)	16 (3.3)

Round means: study year. After the baseline measurement, participants are followed over study years/rounds/years of follow up (post vaccination, in case of vaccinated individuals). High urbanization was defined as: very to moderately urban (as opposed to low urban and country side). High educational level was defined as: higher general secondary education, pre-university education, university of applied sciences and university (as compared to Low/middle educational level which included all other levels of education).
Abbreviation: STI, sexually transmitted infection. aData represent no. (%) of participants unless otherwise specified.

Table 2 Seroprevalence per vaccination status group over time.

Seroprevalence#	Baseline	Round 1	Round 2	Round 3	Round 4	Round 5	Round 6	Round 7	Round 8	Round 9
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Unvaccinated										
HPV16	10 (3.4)	20 (9.7)*	19 (9.4)*	24 (12.7)*	29 (19.5)*	27 (15.5)*	27 (16.7)*	30 (18.40)*	27 (20.0)*	25 (20.8)*
HPV18	5 (1.7)	10 (4.8)*	8 (3.9)*	9 (4.8)*	15 (10.4)*	6 (3.5)*	10 (6.2)*	17 (10.4)*	16 (11.9)*	11 (9.3)*
HPV31	2 (0.7)	0 (0.0)*	3 (1.5)*	2 (1.1)*	4 (2.9)*	3 (1.7)*	5 (3.1)*	6 (3.7)*	9 (6.7)*	9 (7.5)*
HPV33	8 (2.7)	10 (4.8)*	16 (7.9)*	6 (3.2)*	7 (5.1)*	14 (8.1)*	11 (6.8)*	22 (13.5)*	14 (10.5)*	11 (9.2)*
HPV45	7 (2.4)	11 (5.3)*	24 (11.8)*	7 (3.7)*	14 (9.7)*	12 (6.9)*	19 (11.7)*	20 (12.3)*	22 (16.4)*	14 (11.7)*
HPV52	15 (5.1)	11 (5.3)*	9 (4.4)*	6 (3.2)*	7 (5.0)*	11 (6.3)*	13 (8.0)*	17 (10.4)*	12 (8.9)*	12 (10.0)*
HPV58	5 (1.7)	3 (1.5)*	5 (2.5)*	5 (2.7)*	3 (2.2)*	7 (4.0)*	6 (3.7)*	10 (6.1)*	13 (9.6)*	13 (10.8)*
Vaccinated										
HPV16	18 (2.4)	590 (100.0)*	561 (99.8)*	531 (99.6)*	475 (100.0)*	466 (99.8)*	406 (99.8)*	426 (100.0)*	427 (100)*	393 (99.8)*
HPV18	20 (2.7)	590 (100.0)*	560 (99.8)*	531 (99.6)*	472 (99.4)*	466 (99.8)*	404 (99.2)*	426 (100.0)*	426 (100)*	393 (99.8)*
HPV31	8 (1.1)	383 (64.9)*	336 (59.8)*	252 (47.3)*	196 (42.9)*	225 (48.2)*	206 (50.6)*	154 (36.2)*	195 (45.7)*	189 (48.1)*
HPV33	21 (2.8)	314 (53.2)*	384 (68.3)*	312 (58.5)*	227 (52.4)*	279 (59.7)*	232 (57.0)*	227 (53.3)*	209 (49.0)*	203 (51.7)*
HPV45	16 (2.2)	529 (89.7)*	522 (92.9)*	460 (86.3)*	377 (80.2)*	386 (82.7)*	291 (71.5)*	329 (77.2)*	320 (75.0)*	286 (72.8)*
HPV52	38 (5.1)	368 (62.4)*	325 (57.8)*	236 (44.3)*	219 (49.9)*	246 (52.7)*	218 (53.6)*	266 (62.4)*	215 (50.4)*	212 (53.9)*
HPV58	14 (1.9)	237 (40.2)*	251 (44.7)*	248 (46.5)*	141 (31.5)*	228 (48.8)*	214 (52.6)*	219 (51.4)*	154 (36.1)*	146 (37.2)*

#Cut-offs for seropositivity were defined as follows: 9, 13, 27, 11, 19, 14, and 31 LU/ml for HPV16, 18, 31, 33, 45, 52, and 58, respectively. *:p<.0001.

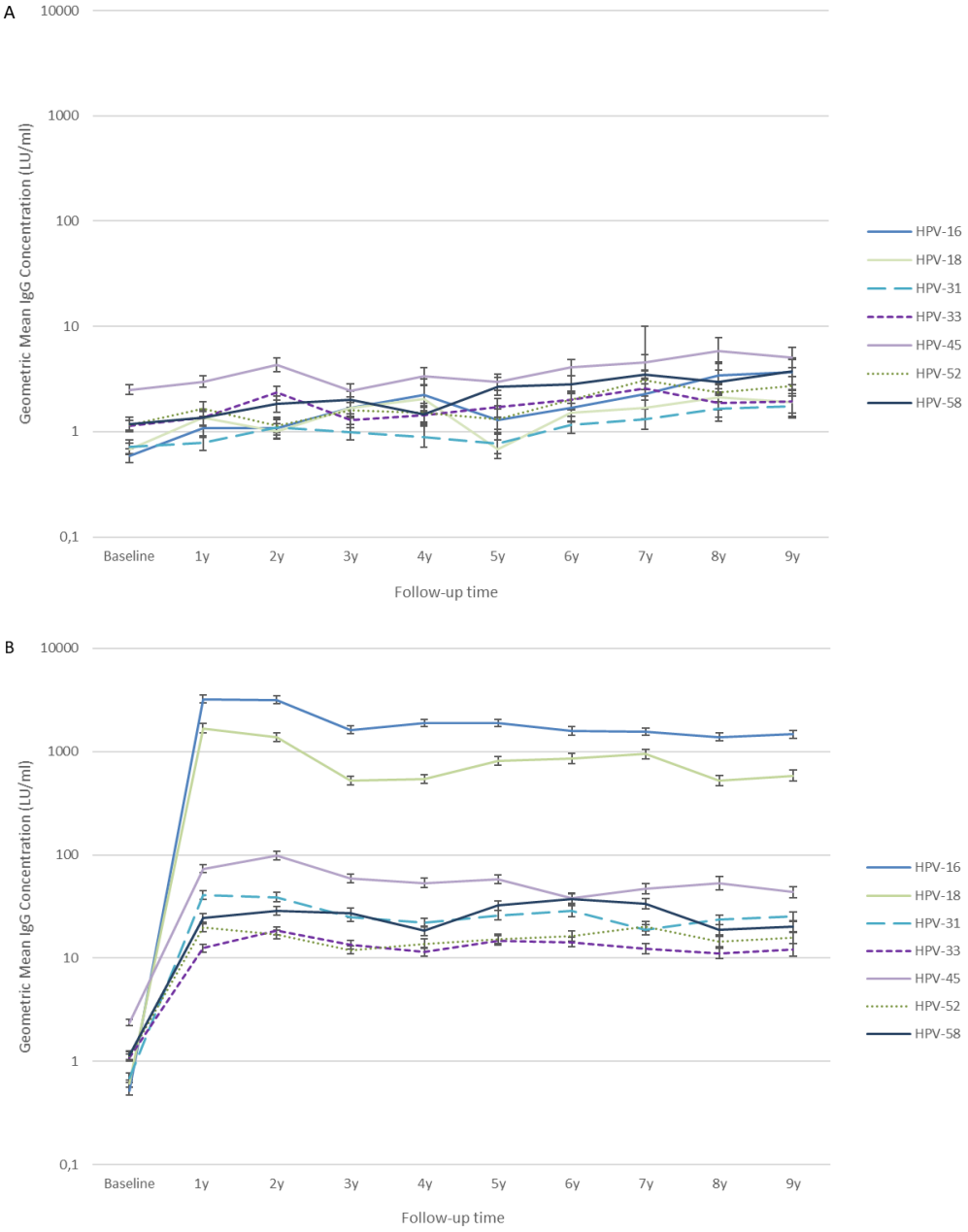

Figure 1 Geometric Mean antibody Concentrations of IgG among unvaccinated (A) and fully vaccinated (B) participants against seven different HPV types

Table 3 Risk factors for contracting an HPV infection in the subsequent year among vaccinated women. Stratified analyses for vaccine or cross protective type and non-vaccine type infection.

	One year pre-HPV16/18/31/45 infection OR + 95% CI	**One year pre-HPV33/52/58 infection OR + 95% CI**
Urbanization		
High	Ref	Ref
Low	1.8 (0.4-7.9)	0.8 (0.4-1.5)
Ethnicity		
Dutch	Ref	Ref
Other	0.7 (0.3-1.8)	1.4 (0.8- 2.3)
Education		
High	Ref	Ref
Low	1.2 (0.6-2.2)	1.0 (0.7-1.6)
Ever smoked		
No	Ref	Ref
Yes	**3.9 (2.0-7.5)**	**2.1 (1.4-3.0)**
Current smoker		
No	Ref	Ref
Yes	**2.6 (1.4- 4.7)**	**1.6 (1.1-2.4)**
Ever used contraception		
No	Ref	Ref
Yes	2.6 (0.9-7.3)	**4.8 (1.8-13.2)**
Ever had sex		
No	ref	Ref
Yes	**6.4 (2.1-19.3)**	**4.6 (2.5-8.6)**
Age sexual debut		
≥15 years	Ref	Ref
<15 years	0.8 (0.3-2.0)	**1.8 (1.1-2.9)**
Lifetime number sexual partners		
0	Ref	Ref
1	**3.6 (1.0-12.7)**	**2.4 (1.2-5.0)**
≥2	**8.0 (2.6- 24.7)**	**5.5 (2.9-10.7)**
Number of partners 12 past months		
0	Ref	Ref
1	**3.7 (1.9-7.5)**	**2.3 (1.5-3.7)**
≥2	**3.4 (1.5-8.1)**	**3.0 (1.8-5.1)**
Current steady partner		
No	Ref	Ref
Yes	**0.5 (0.3-0.9)**	**0.4 (0.3- 0.6)**
Diagnosed with STI previous 12 months		
No	Ref	Ref
Yes	**5.8 (2.5- 13.2)**	1.8 (0.8-4.4)

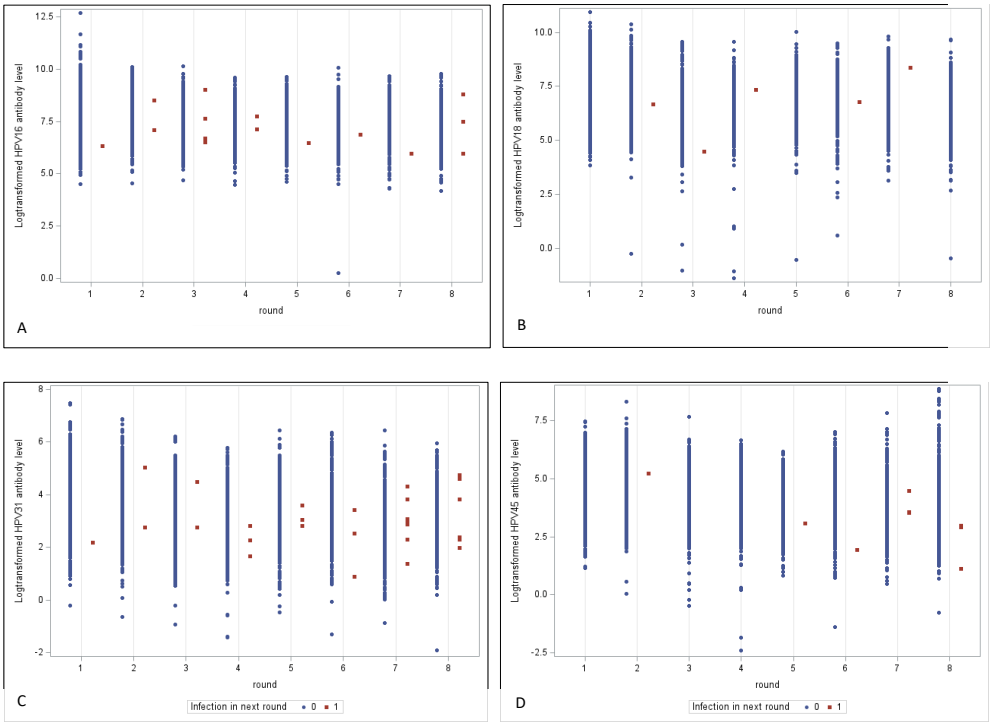

Figure 2 Antibody levels against HPV16 (A), HPV18 (B), HPV31 (C), and HPV45 (D) among vaccinated individuals with infections (red squares) and without infections (blue dots) in the subsequent year

seroprevalence was observed among vaccinated (up to 92.9% two-year post-vaccination for HPV45) compared to unvaccinated participants in the same timeframe (0.7% 11.8% for HPV45).

Pre-vaccination GMCs were comparable between vaccinated and unvaccinated participants ($p>0.05$, Figure 1). Thereafter, significant differences ($p<0.05$) were observed between vaccinated and unvaccinated participants for all types at all time points. Among vaccinated participants, antibodies against vaccine types HPV16/18 showed a peak after vaccination (GMC: 3215 LU/ml and 1680 LU/ml for HPV 16 and 18, respectively) followed by a significant decline at three-year post-vaccination (GMC: 1617 and 520 LU/ml). GMCs remained high and more or less stable up to nine years post-vaccination (GMC at 9 years: 1462 and 582 LU/ml). IgG antibody levels against other HPV types among vaccinated participants were considerably lower compared to vaccine types (range: 11-97 LU/ml), but still significantly higher than in unvaccinated participants. Antibody levels against cross protective type HPV45 displayed the highest overall concentration. In addition, after a peak following vaccination, the GMCs of other HPV types remained stable in the post-vaccination follow-up period. Among unvaccinated girls, antibody concentrations increased over time from 0.6 to 5.1 LU/ml but remained far beneath the levels observed among vaccinated participants.

Characteristics and antibody levels one-year pre-infection

Risk factors for contracting a vaccine type / cross protective type or non-vaccine type HPV infection one-year pre-infection are depicted in Table 3 and include smoking (both current smoking and ever smoking) and sexual behavior related characteristics. No substantial differences in risk

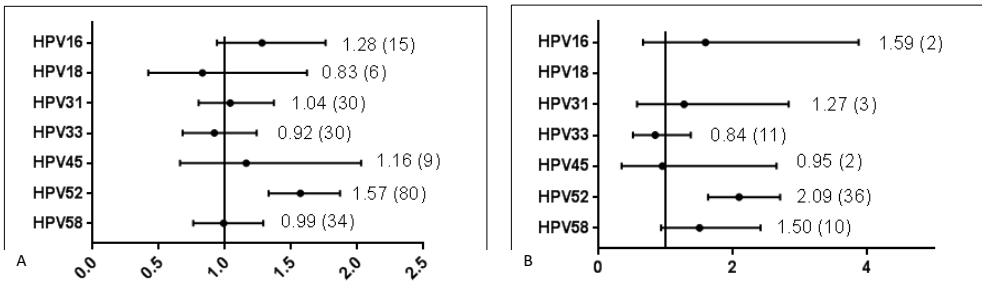

Figure 3 GMC ratio with 95% confidence interval one year pre-infection comparing non-infected vaccinated participants to infected vaccinated participants for incident infections (A) and persistent infections (B). The absolute number of type specific infections is in brackets.

factors were observed for HPV16/18/31/45 infections or HPV33/52/58 infections, although the association between smoking behavior and infection was stronger for vaccine and cross protective type infections than for other type infections. However, as multivariable analyses could not be performed due to small numbers, we could not exclude a possible confounding effect of sexual behavior in this association.

There were no consistent significant differences in IgG antibody levels one-year pre-infection between vaccinated individuals with or without an infection in the next year (Figure 2) for vaccine types or cross protective types. However, we did find significant differences for non-vaccine type HPV52 (Figure 3). A GMC ratio of 1.57 (95% CI 1.33-1.87) and 2.09 (95% CI 1.63-2.70) was observed for incident and persistent infections, respectively, showing higher antibody levels in uninfected versus infected individuals in the year before infection. GMC ratios did not show a consistent pattern across HPV types or across incident and persistent infections. In a sensitivity analysis on incidence infections, we excluded the infections that turned out to be persistent. However, this did not change the results (data not shown).

DISCUSSION

We provided an overview of the effect of the bivalent HPV vaccine on serological response against vaccine types (HPV16/18), cross protective types (HPV31/45) and non-vaccine types (HPV33/52/58) up to nine-year post-vaccination in a population-based setting. We observed high geometric mean antibody concentrations up to nine years post-vaccination against vaccine types and cross protective types. In addition, we explored the longitudinal relation between antibody response and HPV infections and showed that antibody levels among vaccinated individuals one-year pre-infection were similar for those with and without type specific HPV infections (with the exemption of HPV52). As expected only few infections occurred among vaccinated individuals. We found indications that contracting an infection in the subsequent year despite being vaccinated, was associated with smoking and sexual risk behavior.

As expected, HPV seroprevalence was high among vaccinated participants and amounted to 100% for vaccine types one month after vaccination. Our data confirm recent clinical trial results reporting seropositivity rates up to 100% 9-10 years post-vaccination [11, 24]. Among unvaccinated participants, seropositivity was considerably lower. However, rates among unvaccinated increased to 20% in the ninth year of follow-up. This is probably the result of increased exposure over time and is supported by increased self-reported sexual behavior as well as HPV-DNA prevalence (as reported previously by Donken et al [7]).

Serum IgG antibody concentrations against vaccine types remained high up to nine years post-vaccination in a population-based setting. Both clinical trials [11, 12] and recent data from the Finnish maternity cohort showed sustained antibody levels against vaccine types up to twelve years post vaccination with the bivalent vaccine [25]. Our study adds an overview of five other HPV types over time. For vaccine types HPV16 and HPV18, we observed a peak in antibody level one year post-vaccination and stable antibody levels thereafter with no sign for a significant decline in the near future. The same pattern was observed for HPV31/33/45/52/58, although at a lower level, with cross protective type HPV45 presenting the highest concentration. This is in line with the cross protection that was observed earlier in this cohort for HPV31/33/45 [7]. GMCs of vaccinated participants against all HPV types remained significantly above those from unvaccinated participants.

As a correlate of protection is lacking, it remains difficult to interpret antibody concentrations with regard to protection or effectiveness [18]. This was also shown by our further analyses, in which we studied whether vaccinated, infected individuals already have lower antibody levels before the infection is established, making them more prone to infection. An association between GMC pre-infection and infection status for vaccine types or cross protective types was not found although the number of infections was possibly too low to expect this. For HPV52 we did find an association, but as this is no vaccine type or a cross protective type, this does not explain the supposed relation between vaccine-derived antibody levels and protection. Perhaps, an association for HPV52 could be more easily detected due to more infections and a relative low antibody response compared to the other types.

Overall, high antibody levels and especially neutralizing antibodies are considered to be indicative of protection [26]. Our assay quantifies antibodies directed against the L1 VLP in a type specific way but is not restricted to neutralizing antibodies [26, 27]. This suggests that quality of antibodies instead of quantity, could be relevant in determining the level of protectiveness; as was earlier proposed by Scherpenisse and colleagues, accumulated binding strength/affinity of antibodies (avidity) could be used as a marker for this [28]. Moreover, earlier studies also suggested that local immune responses (antibodies at the site of entry, i.e. the cervix) could be important to consider [29].Previous research showed that correlations between serum and CVS antibody levels exist [30, 31], suggesting comparable patterns could be expected. Recently, van der Weele et al. showed that HPV16/18 breakthrough infections among vaccinated in the HAVANA cohort had significantly lower viral load values compared to HPV16 /18 infections in unvaccinated young women [32]. These findings could indicate that the vaccine induced antibody response results in a reduction in viral load in breakthrough vaccine type infections. This might lead to limited capacity of the virus to cause a persistent infection, possibly via the action of neutralizing antibodies. Finally, we hypothesize that antibody concentrations rising above certain levels or physiological maxima could have no further increasing value with regard to protection or immunity [18]; if this is the case, other discriminating factors such cell-mediated immunity, or genetic host or pathogen factors might play a role in who acquires an infection despite vaccination. To study this more closely, in-depth immune cell analyses could be performed on PBMCs from infected vaccinated participants or HPV-DNA from infections could be analyzed in more detail e.g. by sequencing.

The associations between sexual risk behavior and HPV infection among vaccinated participants one year pre-infection might suggest that higher exposure to HPV results in a higher chance of hrHPV infection, including HPV16/18 and HPV31/45, despite vaccination. On the other hand, among Dutch STI clinic visitors who represent a high-risk population, high vaccine-effectiveness has been reported as well [9]. Still, the proportion of risky sexual behavior could be more equally distributed across vaccinated and unvaccinated individuals in STI clinics, resulting in high VE estimates. Furthermore, the observed association with smoking might be a proxy for more overall risky behavior resulting in higher exposure or could be related to an impaired immune response. Comparable risk factors were found between HPV16/18/31/45 and HPV33/52/58 infected individuals, although ORs for smoking were slightly higher among the vaccine type and cross protective type infected individuals. Previously, a pilot study reported that smoking did not affect GMTs after bivalent HPV vaccination but increased the risk of having low-avidity antibodies after vaccination [33]. Also among unvaccinated young women, an impaired immune response following natural HPV infection due to smoking was suggested [34].

Strengths of the current study include the long follow up time of a large population-based cohort; we did lose participants over time, but our cohort still has enough power to obtain insight into the effects of the bivalent vaccine on the Dutch female population. Despite the yearly provided incentive which could possibly lead to an included population with lower socioeconomic status, girls in this cohort were less likely to be a second-generation migrant and were higher educated compared to the general population. Therefore, we think the effect of this possible bias on our estimates of immunogenicity of the vaccine is limited [4, 35]. We do acknowledge some limitations of the current study. Firstly, the limited number of (type-specific) HPV infections

with regard to analyses. The high VE estimates are very reassuring [7], but decreased the power to detect differences one year pre-infection in our analyses. Another challenge remains in the detection of infections; it could not be determined if detected infections represent an active infection of the cervix or the transient presence of HPV-DNA in the lower genital tract. In addition to this, we did not have information on the exact timing of infection acquisition.

In conclusion, we observed high serum IgG antibody responses against vaccine types up to nine years post-vaccination, in a population-based setting among three times vaccinated girls from a catch-up campaign. While antibody concentrations remain an important monitoring tool at population level, the question remains how insightful they are at individual level as long as a cut-off for protection is lacking and infections still occur despite high antibody levels. For future studies, it remains important to monitor vaccine responses, but also failures to see how infections occur and whether they can still induce lesions. In this respect, also other factors such as antibody avidity and local antibodies at the site of infection, degree of HPV-exposure and possibly immune related factors could be interesting to take into account when evaluating HPV vaccines.

ACKNOWLEDGMENTS

We like to thank GSK (GlaxoSmithKline, Rixensart, Belgium) for providing at cost part of the VLP's and MSD (Merck& Co, Kenilworth, NJ) for kindly donating part of the VLPs that we used in our assays. GSK and MSD were provided the opportunity to review a preliminary version of this manuscript in order to ensure the protection of its proprietary information and intellectual property, but the authors are solely responsible for final content and interpretation. We are also thankful for the valuable contributions of Elske van Logchem, Alies van Lier, Madelief Mollers, and Marjan Bogaards to the logistics and laboratory analyses of the study.

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