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Long-term HPV-specific immune response after one versus two and three doses of bivalent HPV vaccination in Dutch girls

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ABSTRACT

Background: In view of further reduction of HPV vaccination schedules, gaining more insight into humoral and cellular immune responses after a single HPV vaccine is of great interest. Therefore, these responses were evaluated after different doses of the bivalent (2v) HPV-vaccine in girls.
Methods: Blood was collected yearly up to seven years post-vaccination with one-, two- or three-doses of the 2vHPV vaccine (N = 890). HPV-type-specific IgG and IgA-antibody levels, IgG-isotypes and avidity indexes were measured by a virus-like-particle-based multiplex-immuno-assay for two vaccine and five non-vaccine HPV types. HPV-type-specific memory B-cell numbers- and T-cell cytokine responses were determined in a subpopulation.
Results: HPV-type-specific antibody concentrations were significantly lower in one- than in two- and three-dose vaccinated girls but remained stable over seven years. The lower antibody response coincided with reduced HPV-type-specific B- and T-cell responses. There were no differences in both the IgG subtypes and the avidity of the HPV16-specific antibodies between the groups.
Conclusions: One-dose of the 2vHPV vaccine is immunogenic, but results in less B- and T-cell memory and considerable lower antibody responses when compared with more doses. Therefore, at least of some of girls receiving the one-dose of the vaccination might be at higher risk for waning immunity to HPV in the long-term.

1. Introduction

Human papillomaviruses (HPVs) are capable of infecting cutaneous or mucosal epithelium. Infection with a high-risk (hr) HPV type can be oncogenic, thereby leading to several anogenital- and oropharyngeal cancers, whereof cervical cancer is most common [1]. Nowadays it is possible to prevent cervical lesions by reducing HPV infections via vaccination. The prophylactic HPV vaccines Cervarix, Gardasil and Gardasil9 are highly efficacious against two (2vHPV), four (4vHPV), and nine (9vHPV) persistent vaccine-type HPV-infections, respectively, and against HPV induced cervical lesions [2–8]. Current recommended HPV vaccination schedules by the WHO include two-doses for girls below the age of 15 and three-doses for girls aged 15 years and above [9]. However, several studies reported robust and sustainable antibody responses in young women after only one-dose of the 2vHPV- or 4vHPV vaccine [10–12]. This response is associated with a low incidence of HPV16 and HPV18 infections up to seven years post-vaccination. These data, although determining efficacy of the one-dose schedule was not a priori study objective, suggest that a single dose of the 2vHPV- and 4vHPV vaccines provides a strong protection for at least seven years.

Vaccine-mediated immunity is often multifactorial and best protection is likely to be elicited by the combination of strong humoral and cellular immune responses [13]. A limited number of studies have assessed the induction of memory B-cells and T-cells after HPV vaccination [14–17]. Insight in memory B- and T-cell immunity after HPV vaccination can help us understand the mechanisms of immunity additive in the HPV-specific antibody response. Moreover, this is of added value in the light of reduced dosing schedules.

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This study is the first comprehensive comparison of B- and T-cell immunity following a one-, two- and three-dose 2vHPV vaccination schedule to HPV-types 16 and 18, as well as the cross-reactive types 31 and 45. We show that the magnitude of the HPV-specific humoral response is lower in one-dose vaccinated individuals in comparison to two or three injections, and this coincides with a lower memory B- and T-cell response to the vaccine.

2. Methods

2.1. Study procedures

Samples were obtained from several studies. An overview is given of all groups in Fig. 1. The Dutch national vaccination registry Praeventis was used to cross-sectionally select participants that had been vaccinated with the 2vHPV vaccine. Theone-dose 2vHPV vaccinated girls were vaccinated between 2011 and 2016 (birth cohort 1998–2003), the two-dose vaccinated girls between 2010 and 2013 (birth cohorts 1997–200) and ofthe three-dose vaccinated girls vaccinated between 2009 and 2010 (birth cohort 1993–1994). The two- and three-dose vaccinated girls were cross-sectionally recruited for blood sampling during the years obtained from ongoing vaccine monitoring studies [18,19], respectively. The one and two-dose vaccinated girls were immunized at 12 years af age, while the 3-dose vaccinated girls were immunized at 16 year of age.

Sample size calculations showed that in each dosing group of each birth cohort at least 47 girls should be included. Taken into account a response rate of 8%, 588 girls per birth cohorts and schedule were needed to participate in the study. Serum samples and PBMCs were collected cross-sectional over seven consecutive years following a one, two or three-doses of the 2vHPV vaccine (Cervarix®, GlaxoSmithKline) (Fig. 1). As a control, a group of non-vaccinated (NV) girls was included [19]. From each individual a questionnaire, including demographic characteristics and information on sexual activity was registered at T0. All participants and parents or legal guardians for those below 16 years of age signed an informed consent. All study proposals were approved by the Medical Ethics Review Committee of the VU University Medical Center (protocol number 2014/230 and 2009/022), Amsterdam, The Netherlands and was conducted in adherence to the Declaration of Helsinki.

2.2. Serological measurements

HPV-specific IgG antibodies against HPV L1 virus-like-particles (VLPs) 16, 18, 31, 33, 45, 52 and 58 were measured in 50 participants on average per yearly time-point and per dosing schedule as well as in NV girls using a VLP-based multiplex immunoassay (MIA)[20]. VLPs were kindly donated by GSK (GlaxoSmithKline, Rixensart, Belgium) and MSD (Merck & Co,Inc, Kenilworth, NJ). Sera were incubated with HPV-specific VLP-conjugated beads (Bio-Rad Laboratories, Hercules, CA). HPV-specific antibodies were detected using R-phycoerythrin (PE) conjugated goat anti-human IgG (Jackson Immunoresearch, West Grove, PA). The 'in-house' control sera and a standard (IVIG Baxter, Utrecht, the Netherlands) were used on each Multiscreen HTS filter plate (Millipore, Burlington, MA). HPV-specific antibodies were analyzed using the Bioplex-system 200 with Bioplex-software (Bio-Rad Laboratories, Hercules, CA). Sera were considered IgG seropositive at the following previously determined cut-offs 9, 13, 27, 11, 19, 14 and 31 LU/mL for HPV16, 18, 31, 33, 45, 52 and 58, respectively [21].

The presence of long-term HPV16 and-18-specific IgG subclasses (IgG1, IgG2, IgG3, IgG4), IgA and IgG avidity were determined at 5 years post-vaccination in randomly selected vaccinated girls with one-(n = 20), two-(n = 16) or three-doses (n = 20). Analysis was performed as described above, by using IgG-isotype-specific mouse anti-human R-PE conjugated

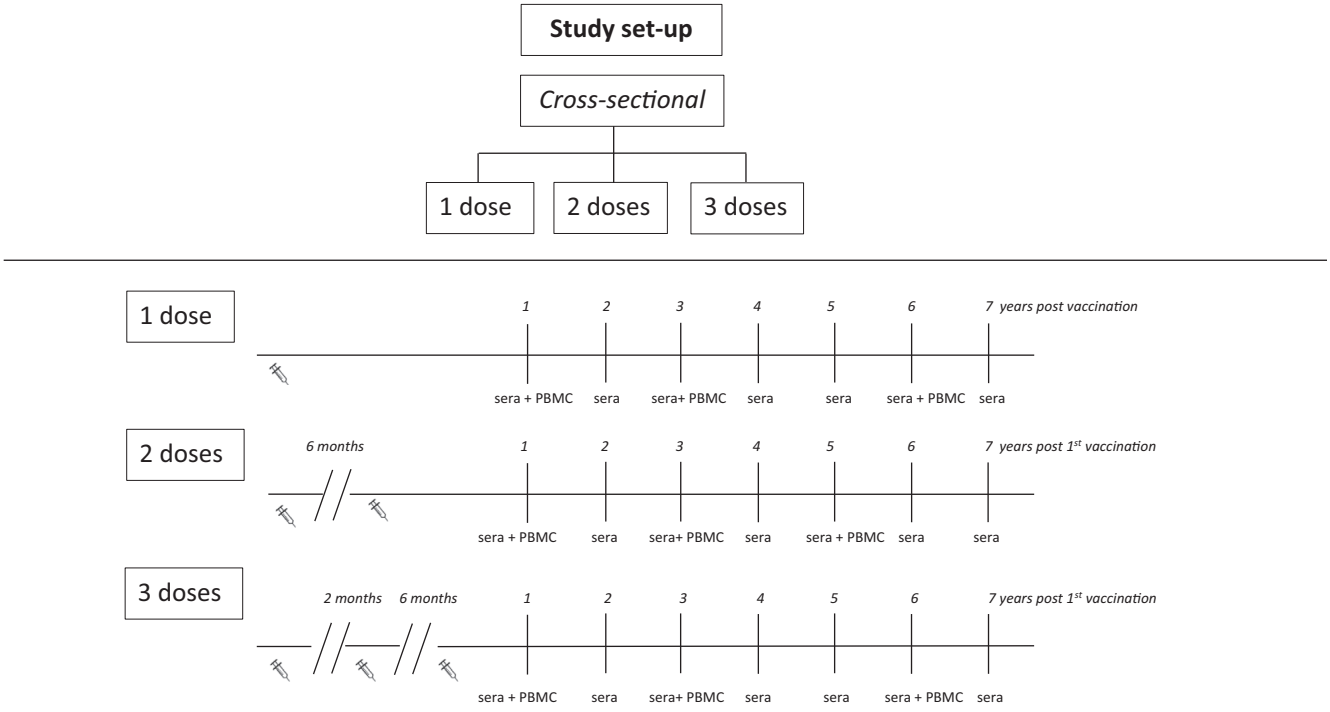

Fig. 1. Study set-up and sampling scheme. Samples were cross-sectionally obtained one until seven years post the first vaccination from one-, two- and three dose 2vHPV vaccinated girls. The one-dose 2vHPV vaccinated girls were vaccinated between 2011 and 2016 (birth cohort 1998–2003), the two dose vaccinated girls between 2010 and 2013 (birth cohort 1997–2000) and the three-dose vaccinated girls vaccinated between 2009 and 2010 (birth cohort 1993–1994). In addition, a group of non-vaccinated (NV) girls was included (not depicted in this figure).

secondary antibodies used in 1/500 dilution (IgG1), 1/100 (IgG2-4) (SouthernBiotech, Birmingham, AL) and 1/200 dilution of R-PE conjugated goat anti-human IgA (Jackson Immunoresearch, West Grove, PA). Distributions of IgG-subclasses in percentages were calculated using median fluorescent intensity (MFI) of the IgG subclasses separately in relation to the MFI of the sum of all subclasses, which was set at 100%. Semi-quantitative IgA antibody concentrations were expressed in MFIs. In the same samples as used for the subclass measurements, HPV16 and −18 IgG avidity was determined by using a modification of the above mentioned IgG-MIA as described [22]. Ammonium thiocynate (NH_4SCN, Sigma-Aldricht, St Louis, MI) was used to dissociate low-avidity antigen-to-antibody binding. After incubation of VLP-conjugated beads with serum, 2.5 mM NH_4SCN in PBS and PBS only was added for 10 min at RT. Antibodies that remain bound to the VLP-conjugated beads after treatment with NH_4SCN defines the avidity index.

2.3. Memory B cell responses

HPV-specific memory B-cells were measured in one-dose, two-dose and three-dose vaccinated participants at one year, three years and five/six years post-vaccination as well as in NV girls.

B-cells were purified from PBMCs by a CD19+ selection kit (StemCell Technologies, Vancouver, Canada) and stimulated polyclonally for five days as described previously [23]. HPV16/18/31/45-specific ELISPOT-assays were performed by coating multiscreen-IP plates (Millipore, Burlington, MA) with PBS containing 20 ug/ml HPV16, 18, 31 or 45 VLP's. A concentration of 1×10^5 B-cells was added per antigen in triplicate per participant. Tetanus toxoid, 7LF/ml in PBS, and PBS-coated wells were included as positive- and negative controls, respectively.

For detection of antibody-producing cells as spots, alkaline-phosphatase conjugated goat anti-human IgG was added in combination with BCIP/NBT substrate (Sigma Aldrich, Saint Louis, MI). Spots were analysed using an Immunospot reader and software (CTL Immunospot S6 Ultra-V Analyzer, Bonn, Germany). Geometric mean (GM) of spot numbers in the PBS-coated wells per participant were subtracted from all antigen-specific spot numbers per participant. GM numbers of HPV-type-specific memory B-cells were expressed per 10^5 B-cells. When no HPV-specific spots were detected in any of the wells, values were $<0.2/10^5$ B-cells and set at a value of 0.1.

2.4. Memory T-cell responses

HPV-specific IFN-γ producing cells were used as a measure for T-cells, and were determined in groups as described for the B-cell ELISPOT. PBMCs were stimulated with VLPs: 4 μg/mL(HPV16-31–45) and 2 μg/mL(HPV18), in triplicate, in 3×10^5 cells/well in AIMV medium (Gibco, Waltham, MA) containing 10% human AB-serum (Harlan, Indianapolis, IN), for 4 days at 37 °C and 5% CO_2. Culture supernatants were collected and stored at −80 °C until used for cytokine analyses. Unstimulated and lectin-stimulated cells served as negative and positive controls, respectively. Subsequently, the numbers of IFN-γ-producing cells specific for HPV16,-18,-31 and −45 were measured using ELISPOT-assays as described [24,25]. Spots were counted using an Immunospot reader (version V3.0) and software (version V6.1) (A.EL.VIS GmbH, Hannover, Germany). Geometric mean (GM) spot numbers of unstimulated cells per participant were subtracted from the HPV-type-specific spot numbers per participant. GM numbers of HPV-type-specific IFN-γ producing cells were expressed per 3×10^5 PBMCs. When no spots were detected the value <1 per 3×10^5 was set at 0.5.

2.5. Cytokines

Supernatants from the HPV-type-specific stimulated cells were tested for 13 cytokines: IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IFN-γ, TNF-α, IL-17A, IL-17F, IL-4, IL-21, IL-22 using multiplex-kits (human Th-cytokine panel, BD, San Diego, CA) following manufacturers' protocol. In brief, supernatants were mixed on a V-bottom plate, with 25 μl of beadmix per well. After incubation for 2 h and washing steps, 25 μl of detection antibodies was added. After a second incubation and washing steps beads were resuspended in PBS and read on a flow-cytometer (BD LSRFortessa™, BD, San Diego, CA). Values of unstimulated cells per participant were subtracted from all HPV-type-specific cytokine levels per participant and expressed in pg/ml.

2.6. Statistical analysis

Analyses were stratified by cohorts, defined by time since the first dose of vaccination. Socio-demographic characteristics of girls who have received one, two or three-doses of vaccine were compared using a Fisher's Exact test; for the differences in ages and time since vaccination a two sample median test was used. The IgG geometric mean concentrations (GMCs), corrected for age, for HPV type-specific antibodies with corresponding 95% confidence interval (CI) for the one- versus two- and three-doses were calculated. Data analysis was performed using SAS software package 9.3 (SAS Institute INC., Cary, NC).

Differences in the number of HPV-specific- memory B-cells, IFN-γ producing cells and cytokine responses were compared using the Kruskal-Wallis test with a Dunn's-method post-hoc analysis.

The normalized z-scores were displayed on a color scale in heat maps and are a representative of the deviation from the highest responder. For these analyses Graphpad Prism V7 were used.

To determine the relationships between different immune markers, we used Spearman-rank correlation for continuous values. These statistical analyses were performed using Graphpad Prism 7.0 software.

3. Results

3.1. Socio-demographics of study participants

A total of 890 girls, between 13 and 21 years of age at time of sampling, were included in this study; 239 girls received one-dose, 222 girls received two-doses and 378 girls received three-doses of the 2vHPV vaccine and 51 girls did not receive any HPV vaccine dose. Participants who received one-dose did differ significantly from those who vaccinated with two- or three-doses in age, oral anti-contraceptive use and sexual behavior at most time-points of sample collection. The required sample size was not reached at all time-points for two-dose vaccinated girls, as these birth cohorts had limited numbers of qualifying girls. The sociodemographic characteristics of the participants per time-point are presented in Table 1.

3.2. One 2vHPV-vaccine dose results in less seropositivity and lower antibody levels than two- or three-doses

The levels of the HPV16 and −18 specific antibodies in one-dose vaccinated girls were significantly lower than those in two-dose and three-dose vaccinated girls at all time points. As expected, the levels in the NV group were significantly lower for the vaccine types HPV16 and −18 compared to vaccinated girls, at all time points and irrespective of number of doses (Fig. 2A,B). Importantly,

Table 1
Sociodemographic Characteristics of Participants stratified by Dosing Schedule.

	NV	0–1 years				1–2 years				2–3 years				3–4 years			
		1 Dose	2 Doses	3 Doses	p-value	1 Dose	2 Doses	3 Doses	p-value	1 Dose	2 Doses	3 Doses	p-value	1 Dose	2 Doses	3 Doses	p-value
N	**51**	**4**	**57**	**55**		**48**	**51**	**51**		**40**	**40**	**51**		**45**	**52**	**50**	
Age					<0.001				<0.001				<0.001				<0.001
Median (range)	16 (15–19)	17,5 (14–19)	13 (13–18)	16 (15–18)		14 (13–18)	14 (13–18)	17 (16–18)		15 (14–18)	15 (15–18)	18 (17–19)		16 (15–19)	16 (16–19)	19 (18–20)	
Current educational level					0.039				0.428				0.011				0.195
Low	10	1	5	8		5	7	5		9	4	1		4	4	0	
Middle	13	0	19	17		10	19	20		10	15	25		19	18	8	
High	28	0	29	27		26	24	22		20	20	20		21	28	22	
Unknown	0	3	4	3		7	1	4		1	1	3		1	2	20	
No	0	0	0	0		0	0	0		0	0	2		0	0	0	
Oral anticonceptive use					<0.001				<0.001				<0.001				0.002
Current user	26	1	3	26		7	7	27		10	14	36		17	22	23	
Past user	4	0	1	3		1	2	2		2	2	2		4	7	0	
No	21	1	51	24		35	41	18		27	23	11		24	21	5	
Unknown	0	2	2	2		5	1	4		1	1	2		0	2	22	
Ever had sex					<0.001				<0.001				<0.001				<0.001
Yes	19	1	0	21		2	0	25		6	6	30		18	18	25	
Never	32	1	55	32		41	50	22		33	33	19		27	32	0	
Unknown	0	2	2	2		5	1	4		1	1	2		0	2	25	

		4–5 years				5–6 years				6–7 years			
		1 Dose	**2 Doses**	**3 Doses**	**p-value**	**1 Dose**	**2 Doses**	**3 Doses**	**p-value**	**1 Dose**	**2 Doses**	**3 Doses**	**p-value**
N		**35**	**7**	**48**		**39**	**12**	**60**		**28**	**3**	**63**	
Age													
	Median (range)				<0.001				0.012				0.504
		17 (16–18)	18 (17–18)	20 (18–21)		18 (17–19)	18 (17–19)	18 (14–18)		19 (18–19)	19 (18–19)	19 (18–19)	
Current educational level*													
	Low				0.185				0.695				0.628
	Middle	2	0	0		1	1	3		0	0	0	
	High	14	1	11		14	6	19		6	1	13	
	Unknown	15	5	25		14	5	33		15	2	45	
	No	4	1	12		10	0	5		7	0	5	
		0	0	0		0	0	0		0	0	0	
Oral anticonceptive use													
	Current user				0.039				0.93				0.36
	Past user	19	4	34		19	6	34		13	3	37	
	No	4	0	0		4	1	8		4	0	15	
	Unknown	9	3	7		9	5	17		7	0	8	
		3	0	7		7	0	1		4	0	3	
Ever had sex													
	Yes				0.12				0.447				0.497
	Never	19	3	7		22	7	30		14	3	39	
	Unknown	12	4	0		10	5	28		10	0	21	
		4	0	41		7	0	2		4	0	3	

* Low = primary or lower general vocational secondary education; Middle = intermediate vocational secondary education; High = higher vocational/general secondary education, (pre) university education.

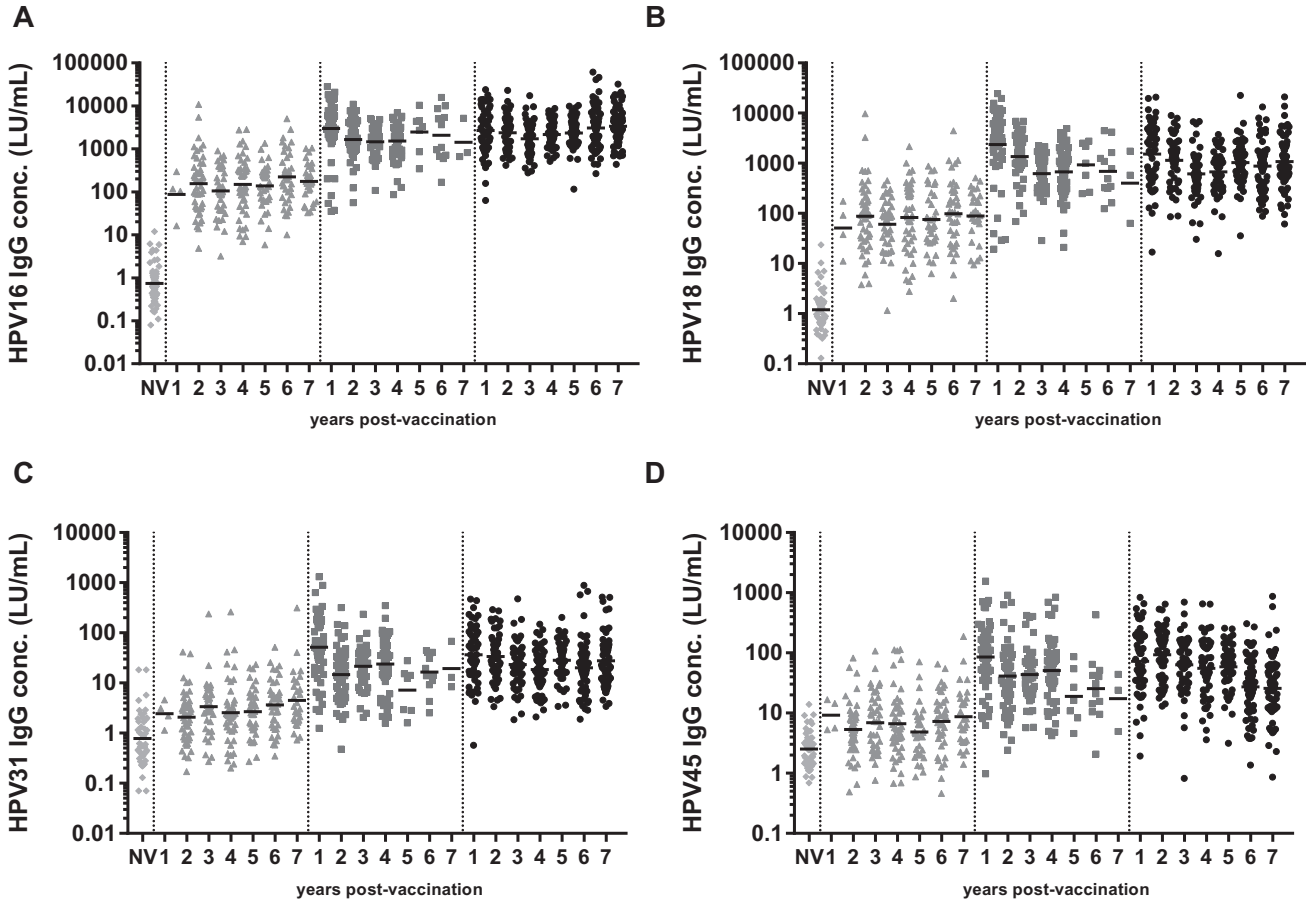

Fig. 2. HPV16 (A), HPV18(B), HPV31 (C) and HPV45 (D) specific IgG antibody concentrations (LU/ml) of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls from one till seven years post-vaccination. The lines indicate the geometric mean concentration (n = 50 per group).

all girls vaccinated according to a two- and three-dose schedule were seropositive for HPV16 and −18, while the one-dose vaccinated girls showed 98.3% and 87.6% seropositivity, respectively. In addition, HPV16- and 18 specific antibody concentrations in one-dose vaccinated girls stayed above an arbitrary level of 100LU/ml for 64.4.% and 46.7%, respectively.

The antibody levels to the non-vaccine types (31, 45) were significantly lower in one-dose vaccinated girls compared with two- and three-dose vaccinated girls (Fig. 2C,D), reaching the levels observed in the NV group. This is also seen for HPV33, 52 and 58 (data not shown). All HPV-type-specific GMCs, corrected for age, and seroprevalences are presented in Supplementary Table 1.

In addition to the HPV-specific IgG response the HPV-specific-IgA response was also significantly lower in the one-dose compared to the two- and three-dose vaccinated girls for HPV16 and −18 (Supplementary Fig. 2A,B). For the non-vaccine types HPV31, −33 and −45, the one-dose group was only lower in comparison to the three-dose vaccinated group (Supplementary Fig. 2C–E). No differences were observed for HPV52 and −58 specific IgA between the groups (Supplementary Fig. 2F,G).

3.3. One dose of 2vHPV leads to a qualitatively similar HPV-specific IgG response

The IgG-avidity index for HPV16 did not differ between one-, two- or three-dose vaccinated girls five years post-vaccination, whereas that for HPV18 was higher in one-dose vaccinated girls compared with two- or three-dose vaccinated girls. Moreover, the HPV16 avidity index appeared to be higher than for HPV18 (Supplementary Fig. 4).

The most abundant IgG subclass induced after 2vHPV vaccination was IgG1 (70–79.8%), followed by IgG3 (19.7–28.5%). Very small amounts of IgG2 and IgG4 were found, (0.2–1.9%, 0.1–0.8%, respectively). Similar IgG-isotype distributions were observed after all three dosing schedules at five years post-vaccination (data not shown).

3.4. Quantitatively lower cellular responses to HPV in individuals that received only one 2vHPV-dose compared with two and three-doses

We observed for all HPV serotypes that HPV-specific- memory B-cells and IFN-γ producing cells in the two- and three-dose vaccinated girls were higher, although not significant, compared to the one-dose group and the NV-group. Notably, there was no difference in the magnitude of these responses to all types of HPV between the NV and the one-dose schedule group. In general, the numbers of HPV-specific B- (Fig. 3) and IFN-γ producing cells were amplified by increasing doses of vaccines (Fig. 4). After depletion of CD56+ NK-cells the IFN-γ producing cell numbers were similar (data not shown).

3.5. Limited production of HPV-specific Th1 and Th2 cytokines in VLP stimulated PBMC after one-dose

The HPV16-specific cytokine responses in the groups receiving no, one-, two- or a three-dose schedules are summarized in

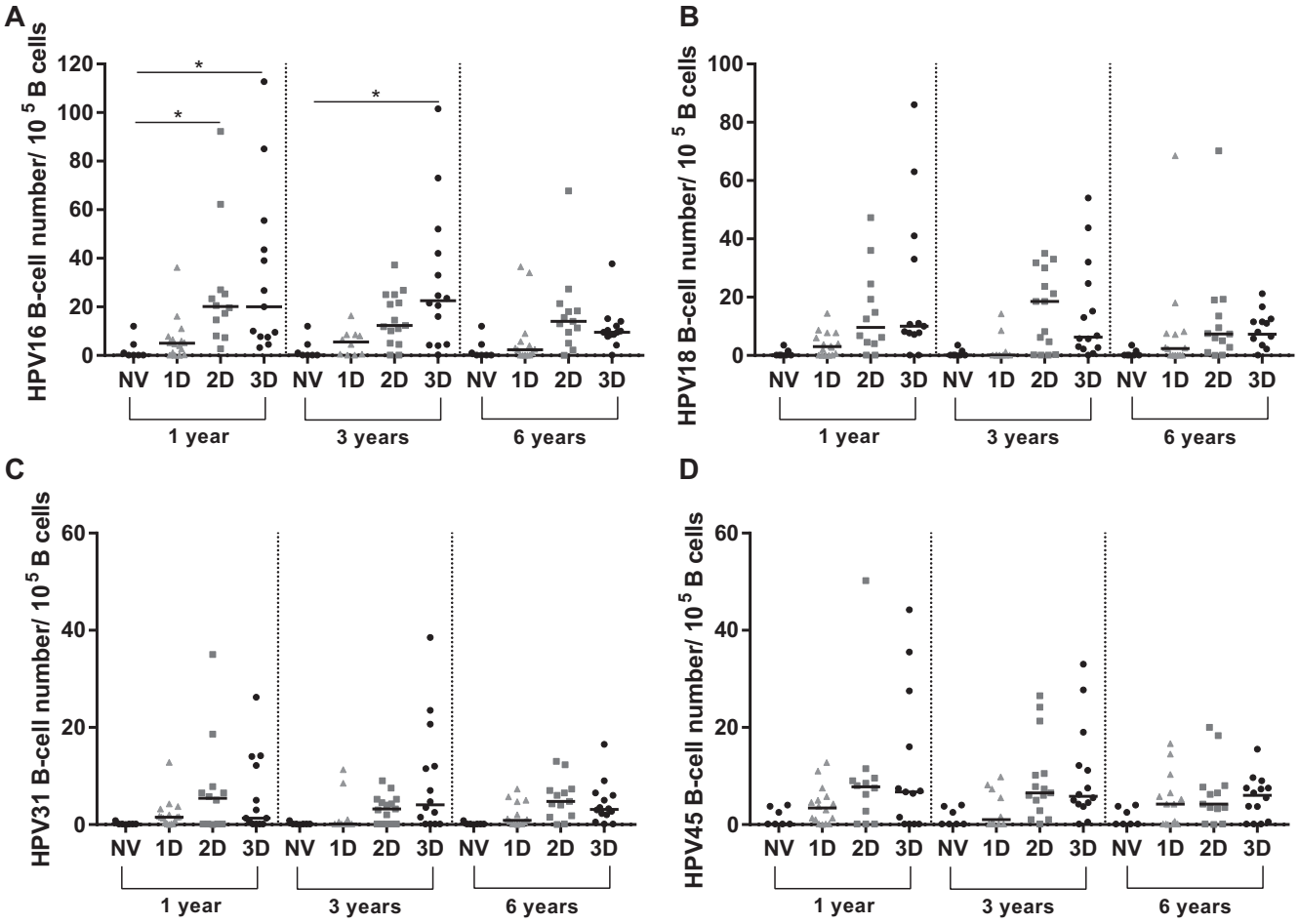

Fig. 3. HPV16 (A), HPV18(B), HPV31 (C) and HPV45 (D) specific numbers of memory B-cells/ 10^5B-cells of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls at one, three and six years post-vaccination. The lines indicate the median (n = 10–15 per group). *p < 0.01.

Fig. 5. Overall, there is an increase in the levels of Th1 and Th2-cytokines with increasing number of doses of the vaccine. Indeed, the one-dose group showed significant lower levels of the Th2 cytokines IL-13 and IL-5 (Supplementary Fig. 4AB) as well as of the Th1 cytokines IFN-γ and TNF-α (Supplementary Fig. 4CD), compared with the two- and three-dose group. IL-17F was higher in the three-dose vaccinated group compared to the one-dose group (Supplementary Fig. 5B). For IL-17A, IL-2 and IL-10 no differences were observed between the different dosing schedules (Supplementary Fig. 5A,C,D). HPV18, −31 and −45-specific cytokine responses showed a similar trend as observed for HPV16, with the exception that no differences were observed for IL-10 and TNF-α (Supplementary Fig. 3). There were no differences between the Th1/Th2 ratios of the one-, two- and three-dose vaccinated girls (data not shown).

3.6. Th2 responses correlate with the humoral HPV-response

To determine the relationship between cellular and antibody responses HPV16-specific cellular responses were correlated to the corresponding HPV16-specific IgG concentrations ($R = 0.55$, $p < 0.001$). This revealed a relationship of the numbers of HPV16-specific memory-B-cells and IgG-concentrations one year post-vaccination. Moreover, specifically the levels of Th2-cell produced IL-13 correlated with the levels of HPV16-specific IgG ($R = 0.66$, $p < 0.001$), suggesting a relation between the magnitude of the Th2-response and the production of HPV-specific-antibodies.

4. Discussion

We showed that one-dose of the 2vHPVvaccine is immunogenic in girls by inducing long-term antibody responses and HPV-type specific T- and B-cell memory cells up to seven years post vaccination. However, HPV-specific B- and T-cell responses appeared to be less pronounced although not being significant and HPV-specific antibody concentrations were significantly lower in one-dose vaccinated girls compared with two- and three-dose vaccinated girls. Despite these differences, the quality of the HPV-specific antibodies is similar between the different dosing schedules measured by the avidity index. Altogether, although a correlate of protection for HPV is still lacking, this implies that a one-dose schedule is less immunogenic when compared with a two- and three-dose schedule.

Reduced dose HPV-vaccination schedules are of great interest in respect to global health HPV burden. It has been shown that a single-dose of the HPV vaccine is effective in preventing HPV infections and is capable of inducing antibody concentrations that last for at least seven years [10–12]. This suggests that a single-dose may provide durable protection, which is in contrast to the current thought that protein vaccines must be administered in a prime/boost regimen to be able to induce protective antibody levels. We indeed found the one-dose of the 2vHPV vaccine being immunogenic in girls by inducing long-term HPV-specific antibody responses. However, in addition to the observed lower antibody levels in the one-dose group in comparison to the other dosing

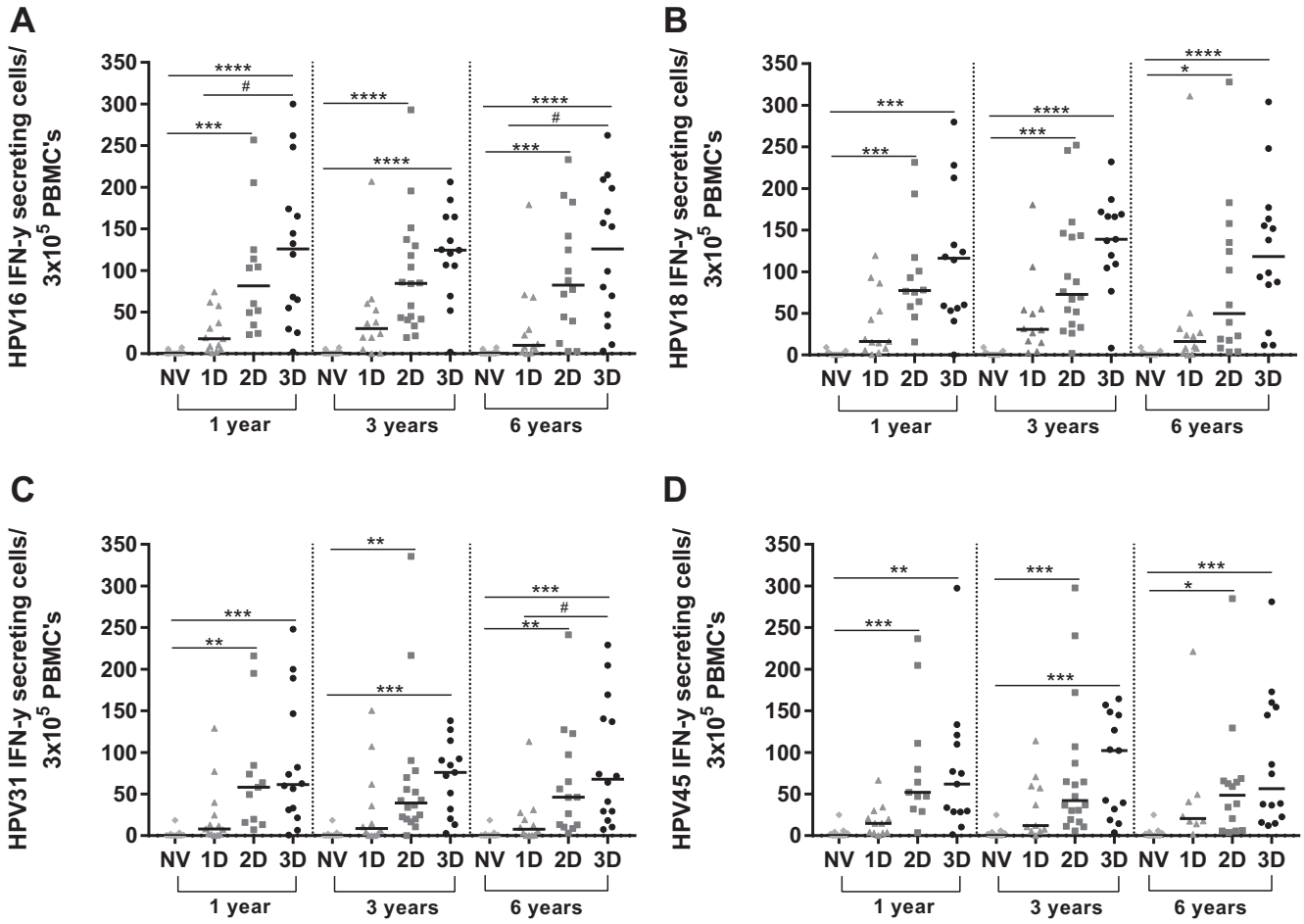

Fig. 4. Numbers of IFN-γ producing cells for HPV16 (A), HPV18(B), HPV31 (C) and HPV45 (D) of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls at one, three and six years post-vaccination. The lines indicate the median (n = 10–15 per group).

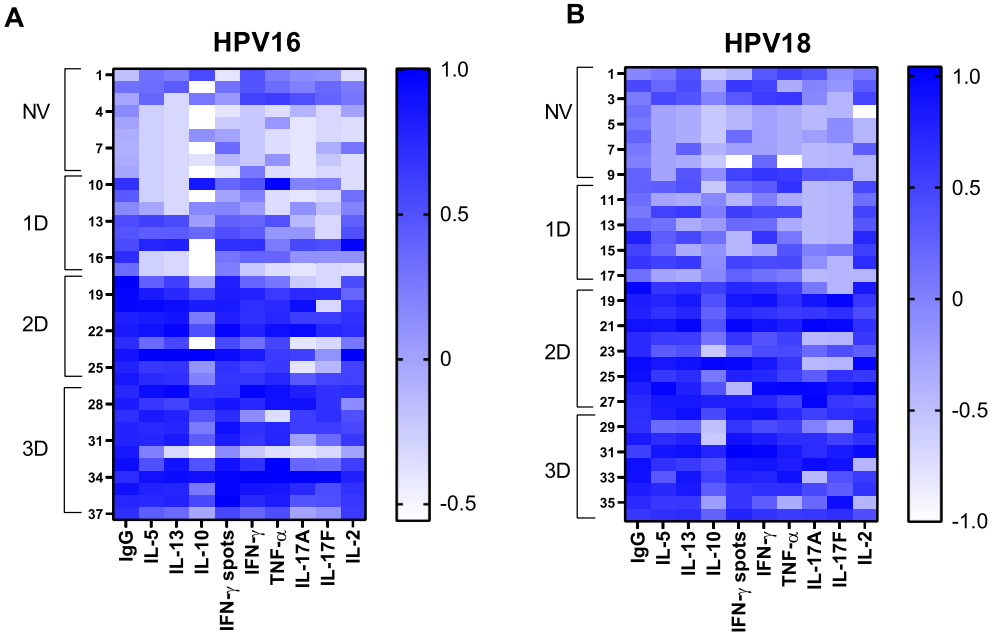

Fig. 5. Heat map comparing IFN-γ producing cell numbers and cytokines in supernatants of PBMCs stimulated with HPV16 (A) or HPV18(B), as well as HPV16- or HPV18-specific IgG levels between non-vaccinated, one-, two- and three-dose 2vHPV vaccinated girls at one-year post vaccination. The normalized z-scores are displayed on a color scale, ranging from light to dark. The color darkness is representative of the deviation from the highest responder (n = 7–10 per group).

groups, we surprisingly observed that in one-third and more than half of the one-dose vaccinated girls respectively HPV16- and HPV18-specific antibody concentrations stay below an arbitrary level of 100 LU/mL, which was rarely seen in the two- or three dose group. This suggests that, although protective cut-off levels are unknown, at least part of the one-dose vaccinated girls might be at higher risk for waning immunity to HPV on the long-term during life.

The antibody distribution we found of IgG1 and IgG3 and low systemic IgA-levels are in line with those to other protein vaccines given via the intramuscular route [26]. There were no differences in both IgG subtypes nor the avidity of the antibodies for HPV16 between the different dosing groups. This could indicate that one-dose of the vaccine is able to induce affinity maturation, and implies sustained germinal center reactions in the lymph nodes upon initial contact with HPV-specific-VLPs [27]. Although this must be interpreted with caution as avidity is a fairly crude measurement to determine affinity maturation [28].

Neutralizing antibodies are considered the major mechanism of protection against HPV infection. Antibody levels are maintained via the production by long-lived-plasma-cells (LLPCs), which primarily reside in the bone-marrow. For HPV, this production might be independent of additional antigen exposure. Antigen-specific LLPCs even could stay in bone marrow niches for about ten years [29,25,30]. Therefore, HPV VLPs might be potent inducers of LLPCs that is in agreement with other vaccines, like tetanus and poliovirus that induce antibody levels for decades without recurrent contact with the pathogen [29,30]. Memory B-cell responses play a role when antibody levels wane. Circulating memory B-cells are expected to reflect the total memory B-cell pool in the bone-marrow and by recirculating they will be able to react to antigen when necessary. By polyclonal stimulation via TLRs memory B-cells might be able to replenish the HPV-specific plasmacell pool that again could maintain antibody production [26]. This is confirmed by the correlation found between the HPV-specific-antibody levels and numbers of memory B-cells in our study. We showed for the first time that even one-dose of HPV vaccine is able to induce specific memory B-cells, albeit that more doses result in higher numbers of them. The 2vHPV vaccination induced memory B cell numbers also for the cross-reactive HPV serotypes. This might be helpful for the long-term replenishment of the HPV-specific plasmacell-pool and especially in protection to breakthrough infection with HPV infections during life, suggesting that the two- and three-dose vaccine may be more effective at the long term.

The functionality of the HPV-specific memory T-cells post-vaccination was studied by measuring the number of IFN-γ -producing-cells and the amount of cytokines in the supernatant. IFN-γ is the most important T-cell cytokine to combat viral infections and the numbers of HPV-specific IFN-γ -producing-cells remained relatively stable during the years post-vaccination. This is line with T-cell responses for other pathogens [31,32]. Stimulation with purified VLPs primarily results in antigen presentation via the MHC-class-II route that leads to especially $CD4^+$-T-cells producing cytokines [33]. The production of Th1 and Th2-cytokines was higher after two- and three-doses of the 2vHPV vaccine than after one-dose. This was partly in line with the data of Toh *et al.* who reported that six years after 4vHPV vaccination, HPV18-specific cytokine responses were significantly lower in the one- or two-dose recipients when compared to three-dose recipients, but similar for those specific for HPV16 [17]. Our data confirm those by Smith *et al.* showing that both Th1 and Th2 T-cells provide help for B-cell clonal expansion and antibody synthesis [34–36]. For the induction of high-affinity antibody responses, follicular T (Tfh) cells play a role by supporting the activation and differentiation of B-cells into Ig-secreting cells [37,38]. Unfortunately, we were not able to study the Tfh-subsets, since they circulate around 7–14 days after vaccination and cannot be found in the circulation year's post-vaccination [39].

Although the number of HPV-specific IFN-γ producing-cells was not significantly different between the dosing schedules, most likely due to a low sample size, there is a clear tendency to higher numbers of these cells with increasing doses of the vaccine. This is confirmed by the significantly higher concentrations of IFN-γ and TNF-α in the culture supernatants from the vaccinees who received more vaccine doses. Moreover, the amount of the Th-2 cytokines IL-13 and IL-5 was respectively higher or showed a trend with a higher number of vaccine doses for all HPV-serotypes measured. This is in accordance with previous studies investigating the cytokine response following 2vHPV vaccination, showing the greatest relative increase in Th2 cytokine responses following a booster-vaccination [40]. Since IL-13 and IL-5 are produced by the CD4 + Th2-cells and are involved in the stimulation of antibody production by B-cell activation [33], they probably play a role in the induction of HPV-specific memory B-cells and antibody responses. Indeed, we found correlations between both Th2-responses, IL-5 and IL-13, and the IgG-concentrations. This leads to the suggestion that differences in Th2-activity is, at least partly, responsible for the differences observed in antibody levels between the different dosing schedules. The heatmaps show that individuals with both a high Th1- and Th2-response also display high IgG-levels and more memory T-cells, indicating that a more coherent total immune response is amplified by an increasing number of vaccinations.

This study has some limitations; samples were collected within several studies, which resulted in a cross-sectional design and could lead to potential biases. For instance, there seems to be a skew in the age range collected which resulted median age differs between the three-dose group in comparison with the one- and two-dose group, thereby possibly affecting results. Fortunately, the one and two-dose group can be compared as no differences in sociodemographic characteristics were found between these groups. Furthermore, the sample size used in the cellular analyses is relatively small, explaining that differences did not reach significance. However, a strength of this study is that a broad array of immune responses is presented for all three-dose schedules by measuring antibody levels, subclass distribution, avidity in combination with memory B- and T cell responses on the same samples.

From a public health perspective, the expected efficacy data from ongoing randomized controlled trials will provide us with information on the clinical impact of a one-dose 2vHPV HPV vaccination schedule. To our knowledge, this is the first study to report the presence of HPV-specific memory B cells after just one-dose of the 2vHPV vaccine. We show that the levels of HPV-specific antibodies after a single dose are 10-fold lower than in two- or three dose-vaccinated girls, albeit that there are no qualitative differences of HPV-specific antibodies as measured in our assays. The lower antibody response coincided with a significantly lower production of T cell produced cytokines. Follow-up data should clarify whether this lower immune response is also of clinical relevance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

All authors contributed significantly, H.P, T.S. H.M and F.K designed the study; H.P M.B D.B. were responsible for laboratory analysis; H.P, F.K, S.B and A.B. provided input for writing of the paper and all authors contributed to the final version of the paper. We have no conflicts of interest to disclose.

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Potential conflicts of interest

All authors: No reported conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.09.066.

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