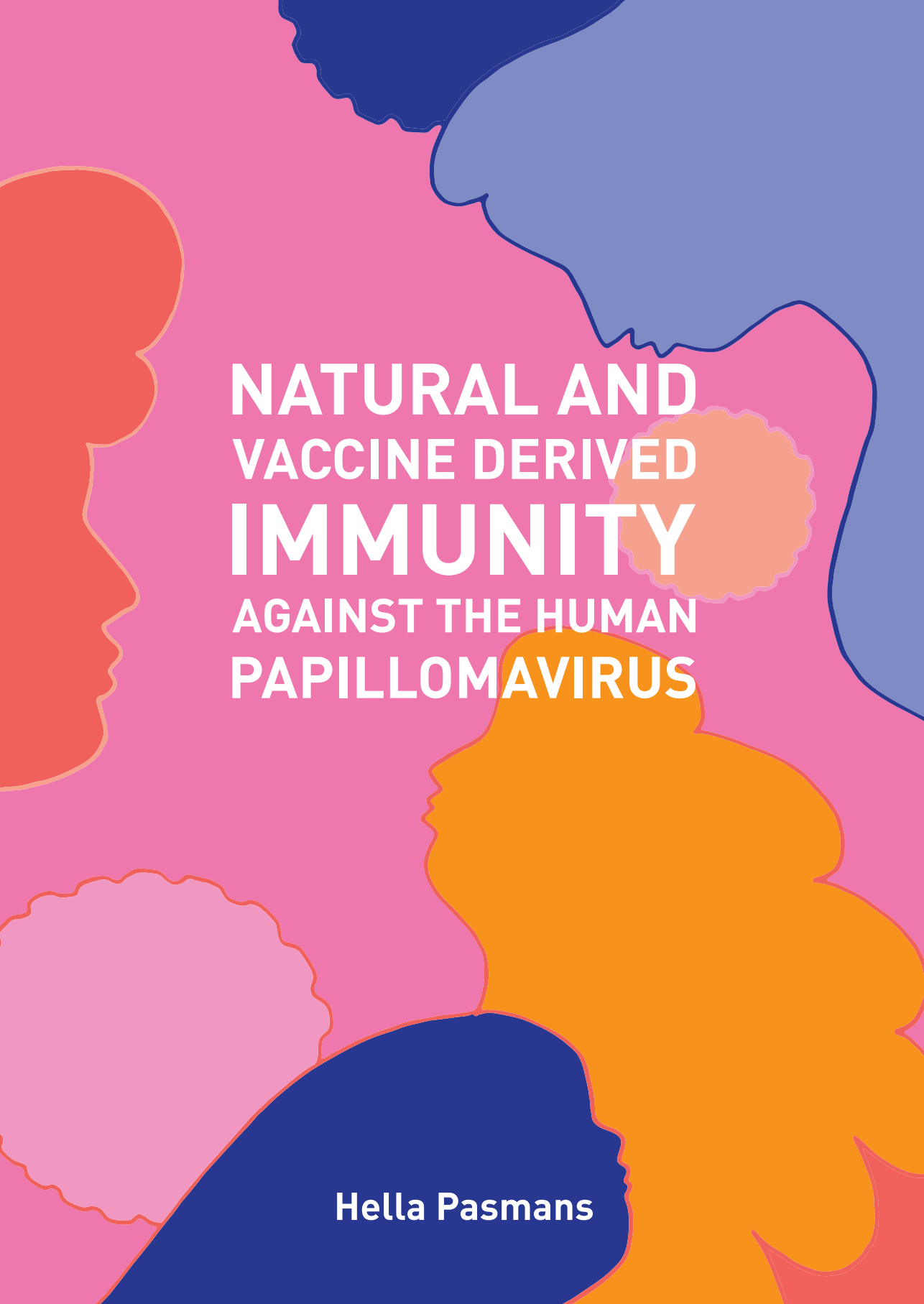
NATURAL AND
VACCINE DERIVED
IMMUNITY
AGAINST THE HUMAN
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Hella Pasmans

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Promotor:	Prof. dr. S.H. van der Burg	
Copromotoren:	dr. A.M. Buisman	RIVM
	dr. F.R.M. van der Klis	RIVM
Leden promotiecommissie:	Prof. dr. L.G. Visser	
	Prof. dr. A. Geluk	
	dr. M.I.E. van Poelgeest	
	Prof. dr. D. van Baarle	UMCG
	Prof. dr. M.F. Schim van der Loeff	GGD Amsterdam

Voor papa en mama

CONTENT

CHAPTER 1

General Introduction and scope of this thesis

Papillomavirus (family) properties/genome

Human papillomaviruses (HPV), are small, non-enveloped, double-stranded DNA viruses of approximately 8000 base pairs and belong to the Papillomaviridae family [1]. The HPV capsid is composed out two late proteins, L1 and L2, in an icosahedral structure. Its genome has the capacity to code these two capsid proteins and for at least six early proteins, E1, E2, E4-E7 (Figure 1). The early proteins are essential for the replication of the viral DNA and the assembly of newly produced virus particles for instance within the infected cells [2, 3]. E6 and E7 are the so called oncoproteins due to their capacity to inhibit tumor suppressor genes like p53 and RB[4]. At present, over 200 different HPV types have been described to be genetically different, which are subdivided into different genera, whereof the alpha genera is the most studied one [5-7]. This genera, containing around 40 HPV types are common in infecting the anogenital epithelium. These HPV types are further subdivided into high-risk (hr) and low-risk (lr) HPV types according to their oncogenic potential. Currently, thirteen HPV types are considered as group 1 carcinogens, and therefore hr-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66) [8, 9]. HPV16 and HPV18 are the most predominant oncogenic types and are responsible for 70% of all cervical cancer cases [10], therefore being most commonly targeted in research and vaccination strategies. An infection with an lr-HPV type can cause benign lesions of the anogenital areas known as genital warts (condylomata acuminate), as well as low-grade squamous intraepithelial lesions of the cervix.

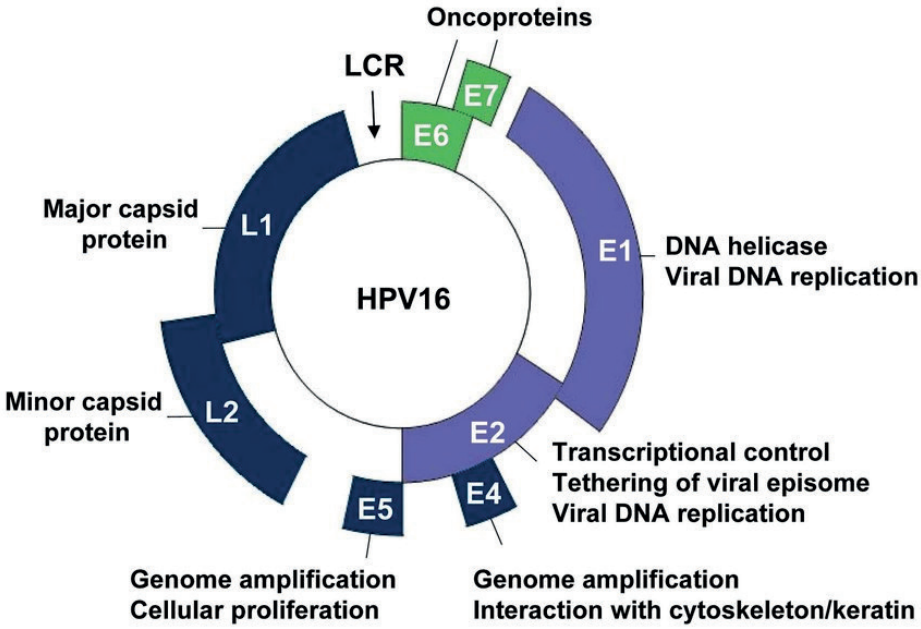

Figure 1 A schematic representation of the genomic organization of the HPV genome. Adapted from D'Abramo et al. [11]

Human papillomavirus associated diseases and burden

HPV is the most common sexually transmitted infection worldwide. Consequently, at some point in their life approximately 80% of the sexually active population will be infected with a hr-HPV [12]. For cervix uteri cancer, HPV has been identified as a necessary cause, and is the most common HPV-associated cancer [13, 14]. Besides cervical cancer, HPV has also been associated with other anogenital cancers, i.e. anus, vulva and penis. More recent also various cancers in the head and neck region have been associated with a hr-HPV infection, resulting in that approximately 5% of all cancers worldwide are associated with HPV [15].

Cervical carcinogenesis

The progression of cervical cancer starts with a persistent hr-HPV infection of the cervix epithelium and is marked by pre-cancerous states called squamous intraepithelial lesions (SIL). These infections, potentially caused by each individual hr-HPV type, can independently develop into an associated SIL, which can be graded as a low-grade SIL (LSIL) or a high-grade SIL (HSIL)(Figure 2). These lesions are formerly known as cervical intraepithelial lesions (CIN), grading from CIN1 to CIN3. The transition towards a HSIL after infection can occur within three to five years [16]. Persistent infections can progress, remain stable, regress and be cleared or become latent. SILs can also regress at any stage. The potentially HPV clearance and regression of SIL is diminished with increased severity of the SIL. However, the progression of a hr-HPV infection into cancer is a long process, which can last for decades [17]. Therefore treatment of SILs is efficacious, thereby preventing cancers.

Cancer of the cervix uteri is the fourth leading cancer among women worldwide [19]. The majority of cases (85%) occur in low-income countries. Globally, around 800 women die of cervical

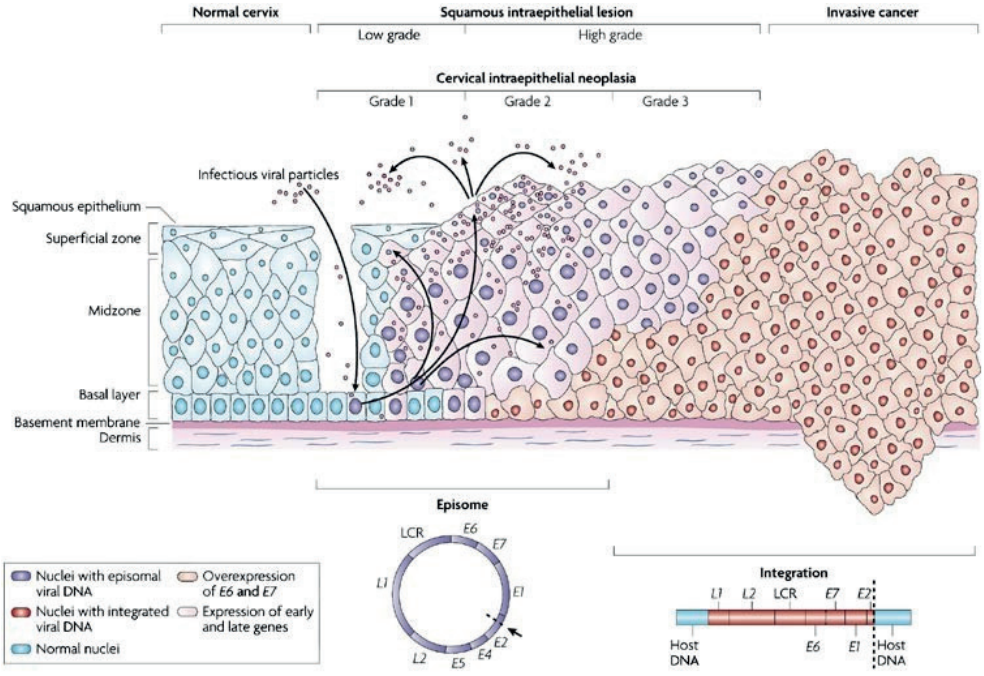

Figure 2 Schematic representation of HPV infection, progression and carcinogenesis. The different stages of a high-risk HPV infection are displayed, along with the transformations occurring in the cellular tissue. Adapted from Woodman *et al.* [18]

cancer every day [15]. In the Netherlands, annually 5300 women are diagnosed with high-grade squamous intra-epithelial neoplasia (HSIL), and 800 with cervical cancer resulting in approximately 200 deaths [20].

Non-cervical disease and its precursor lesions

Also for other HPV-related cancers precursor lesions have been described. Anal intraepithelial neoplasia (AIN), penile intraepithelial neoplasia (PIN or PeIN), vulvar SIL and vaginal SIL have been described as relevant precursors for their respective cancers. Lesions can be low or high-grade, similar to those at the cervix. The proportion of these cancers attributed by HPV is dependent on the different cancer sites. Anal cancers are considered to be 88% attributable to HPV, and for vaginal and vulvar cancers this is respectively 70% and 43%. For penile cancers over half is attributed to HPV. HPV has also shown to be present in oropharyngeal squamous cell carcinoma (OPSCC) (including tonsils and base of the tongue), where it causes 30 to 50% of the cases [15, 19]. These HPV positive OPSCC have a different molecular profile and respond much better to therapy when compared to HPV negative OPSCC. No precursor lesions for OPSCC have been identified yet, making an early detection of these carcinomas not feasible.
Worldwide numbers of cancer cases attributable to HPV are summarized in Table 1. The incidence and mortality rate of the majority of them have been quite stable in the Netherlands in the past years, although the prevalence of HPV-attributable oropharyngeal cancers have increased. Also, a higher rate for anal cancer is found for men who have sex with men.

Besides cancers also other diseases are attributable to HPV, over 90% of all anogenital warts are caused by lr-HPV types HPV6 and 11. Pre-vaccine data showed an annual incidence of both these infections of 0.1-0.2% in developed countries, peaking in teenagers and young adults, with high recurrence rates [19]. A rare syndrome 'recurrent respiratory papillomatosis' (RRP) is also caused by HPV6 or HPV11. It mostly affects children and young adults, having immunodeficiency and related infections as important risk factors [21].
Overall, HPV showed the highest burden of disease within the vaccine preventable infectious diseases in the Netherlands [22], being expressed in an average of 10,600 disability adjusted life years (DALY) in female and 3346 DALY in males. Although the disease burden is highest in females, this is decreasing while the burden in males is increasing over time [23].

Route of HPV infections

The most vulnerable sites for tumorigenesis due to HPV are the cervix and anus. The cervix connects the vagina and uterus, and is divided in the ectocervix, which covers the surface of the vagina and the endocervix, bordering the endocervical canal of the uterus. The ectocervix is lined with squamous epithelium, whereas the endocervix has columnar epithelium. The ecto- and endocervix meet at the so-called squamo-columnar junction, which shifts during puberty from ectocervix to endocervix and glandular epithelium being replaced by metaplastic epithelial, also known as the transformation zone. This zone is highly susceptible to HPV infection, and almost all HPV-associated cervical lesions originate at this place [18]. The anus also has a transformation zone [24] and at the oropharynx it is the reticulated epithelium which provides an optimal site for a HPV infection [25].
The HPV life cycle starts with the infection of the basal layer of the epithelium through microtraumas compromising the epithelial barrier, commonly caused during sexual intercourse. The trans-

Table 1 Worldwide number of cancer cases attributable to HPV and corresponding attributable fraction (AF), by cancer site, sex and age. Adapted from de Martel *et al.* [15] and supplemented with numbers of Globocan 2018 where available.

HPV-related cancer site (ICD-10 code[f])	Number of incident cases	Number attributable to HPV	AF%	Number attributable to HPV by sexe		Number attributable to HPV by age group		
				Males	Females	<50 years	50-69 years	70+ years
Cervix uteri[b] (C53)	570,000	570,000	100	0	570,000	250,000	250,000	71,000
Anus[e] (C21)	40,000	35,000	88	17,000	18,000	6,600	17,000	12,000
Vulva[b] (C51)	44,000	11,000	24.9	0	11,000	2,100	4,000	5,000
Vagina[b] (C52)	18,000	14,000	78	0	14,000	2,800	6,200	4,700
Penis[b] (C60)	34,000	17,000	50	17,000	0	2,000	8,300	5,900
Oropharynx[e] (C01, C09-10)	96,000	29,000	30.8	24,000	5,500	5,400	18,000	6,000
Oral cavity[e] (C02-06)	200,000	4,400	2.2	2,900	1,500	890	2,300	1,200
Larynx[b] (C32)	180,000	4,300	2.4	3,700	540	490	2,500	1,200
Total HPV-related sites	1,200,000	680,000		65,000	620,000	270,000	310,000	110,000

[a] Source: Globocan 2012
[b] Source Globocan 2018
[c] Numbers are rounded to two significant digits.
[d] Attributable fractions according de Martel, 2012 were used for Globocan 2018 data
[e] These cancer sites were not directly available in Globocan 2012; therefore data from the Cancer Incidence in Five Continents (CI5-X) database were used to estimate the corresponding numbers of cases.
[f] ICD-10: International Statistical Classification of Diseases and Related Health Problems 10th Revision, 2019

mission of the virus is also possible via skin-to-skin contact, like intimate contacts of genital or other mucosal surfaces [26]. HPV does not directly bind to cells, but requires contact via the basement membrane. Here it can bind via heparan sulphate proteoglycans, where a series of conformational changes of the virus occur, beginning with furin-mediated cleavage of the minor capsid protein L2, thereby exposing the receptor-binding sites on L1. This results in the binding of the virus to a cell surface receptor, which is still unknown, and infection of basal epithelial keratinocytes [27] (Figure 3). At first the HPV genome is maintained at a low copy number in the infected host basal cells, but upon differentiation of epithelial cells, the virus replicates to a higher copy number.

HPV infections can be either transient, being defined as clearing within 12-18 months depending on HPV type, persistent or latent [28]. About 80% of the HPV infections are estimated to be transient, while the remaining 20% persists within the host, whereof just a small part (1-3.5%) can eventually cause premalignant lesions and finally HPV related cancers [12, 29]. For persistence of an infection, HPV requires to infect basal epithelial cells that show stem cell like features thereby still being able to proliferate. The precise mechanism of infection is HPV-type dependent, however, a common feature of all infections is the slow infection kinetics, making the virus susceptible to neutralizing antibodies.

HPV interaction with the host immune system

The immune system can broadly be divided into the innate and adaptive components, with an intensive cross-talk between them (Figure 4). The host immune response to HPV involves both of these components [31]. HPV has developed several mechanisms to evade and/or suppress the host's immune response, evidenced by their persistence despite viral activity in keratinocytes. One of the factors contributing to HPV persistence is the non-lytic nature and the exclusive intraepithelial residence of the infection, away from dermal immune cells. Thus spontaneous contact of the immune system with HPV is limited to cells at the basal membrane. Consequently, HPV-specific immunity develops quite late during persistent infections or in an early stage thereby preventing a persistent infection [32].

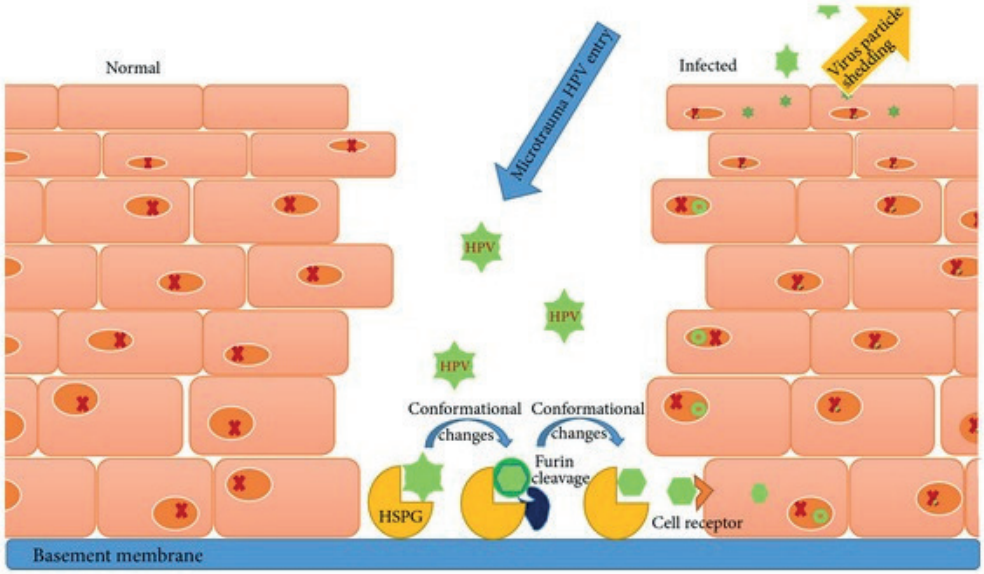

Figure 3 Schematic representation of the HPV infection into the basement membrane. Adapted from Deligeoroglou *et al.* [30].

Innate immunity

The nonspecific part of the immune system, the innate immune response, is the first line of the defense against infection and is mediated by the epithelial barrier, intracellular signaling pathways, the complement system and various innate cell subsets like granulocytes (basophils, eosinophils and neutrophils), mast cells, monocytes/ macrophages, dendritic cells (DC) and natural killer (NK) cells, having a variety of functions, i.e. killing, phagocytosing- or antigen presentation. Pattern-recognition receptors (PRR), like toll-like receptors (TLRs), expressed by innate immune cells and keratinocytes, recognize both endo- and exogenous threats by pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). Hr-HPVs can interfere with the signaling of these pathogen receptors in keratinocytes, thereby suppressing the accompanied cytokine production responsible for attracting and activating the immune cells. The protein ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is partially responsible for this [33], which is thought to function via suppression of RIG-I, TLR3 and TLR9 [34, 35]. This might limit the chance that hr-HPV infected cells initiates direct anti-viral responses and send out stress signals to alert the adaptive immune system.

Generally, type I interferons (IFN) and pro-inflammatory cytokines are produced upon PRR ligation via signaling of interferon regulatory factor (IRF), and nuclear factor of kappa-light-chain-enhancer of activated B-cells (NKkB) activating pathways. Type I IFN stimulate cells to induce genes expressing an anti-viral state and stimulate DCs to act as a bridge between innate and adaptive immunity. Hr-HPV interferes with the production of type I IFN at several points in the signaling cascade [36-38]. This not only results in impaired anti-viral activity but also results in a lack of release of cytokines and an impaired recruitment and activation of antigen-presenting cells, such as Langerhans cells, which are immature DCs, and effector cells of the immune system [39, 40]. Moreover, the transformation zone is associated with a significant lower number of Langerhans cells compared to the ectocervix [41] and the immunosuppressive interleukin 10 (IL-10) is more commonly expressed in the transformation zone than in the ectocervix [42].

Finally, macrophages, being derived from monocytes and situated in tissue, have a phagocytic role. Various proteins, such as monocyte chemotactic protein-1 and macrophage inflammatory protein are needed for the attraction of macrophages. Both of these proteins appear to be down-regulated by HPV, in a direct or indirect manner [43, 44].

Adaptive immunity

Following the initial wave of innate immunity, the specific part of the immune system becomes activated. This so-called adaptive immune response is important in viral clearance, host recovery and establishment of immunological memory, consisting out of B and T cells. B cells are responsible for the humoral immune response, especially the production of virus-specific antibodies. T cells, which are divided in helper-, cytotoxic- and regulatory T cells, have a variety of functions. The enhanced prevalence of HPV infections and HPV-related disease in immunocompromised subjects, such as HIV patients and organ transplant patients, suggest that the cellular immune system plays an important role in the control of HPV infections [45, 46].

T cells

T helper (Th) cells, also known as CD4+ T cells due to their CD4 surface protein, determine the direction of the immune response due to their cytokine production. After activation by APCs in the lymph nodes, a naïve CD4+ T cell may differentiate into one of several distinctive T-cell

lineages, of which Th1, Th2, Th17 and Treg are the most known, each with their own effector function and cytokine secretion profiles (Figure 5). The main effector functions of Th1 cells is clearance of intracellular pathogens, of Th2 cells the clearance of extracellular pathogens, and of Th17 the clearance of mucosal extracellular pathogens. Tregs are important for suppressing the immune response in order to prevent immune pathology and for the induction of peripheral immune tolerance [48, 49].

Hr-HPV has evolved mechanisms to resist this attack by Th1 cells [50]. Th1 cells produce IFN-γ and TNF-α as well and can interact with keratinocytes via CD40L-CD40 interaction [51], but HPV interferes with this cascade, in a similar way as with PRR-induced NFkB signaling, by its E proteins and endogenous proteins. Additionally, HPV interferes with the immune-mediated block on proliferation and the induction of apoptosis and necroptosis [52, 53].

Virus-infected cells are effectively attacked by T cells by a specific mechanism to prevent production and spread of virus particles. Viral protein derived peptides are presented by APCs in the context of major histocompatibility complex (MHC) type 1 molecules which can be recognized by cytotoxic T cells (CTLs), characterized by their surface protein CD8. Keratinocytes can be excellent candidates for presentation to antigen-specific CTLs. Hr-HPV, however, evades CTL-lysis by its expression of E5 and E7, which reduce MHC-I surface expression [40, 54, 55], and as such leads to a reduced presentation of HPV's antigen and consequently immune escape [56, 57].

Importantly, spontaneous regression of HPV-induced genital lesions is associated with the infiltration and circulation of both HPV-specific CD4+ Th cells, comprising Th1 and Th2 responses, and HPV-specific CD8+ T cells. These cells are especially reactive to a broad array of epitopes within the early antigens [58-65]. Chronic vulvar infected patients whom mount an HPV-specific Th1 response display a better clinical outcome, which can even lead to complete regression when treated with immune stimulators, such as TLR7 agonist imiquimod [64].

Tregs responding to HPV antigens have been found in patients with high grade disease and cervical cancer. During a HPV infection Tregs have been reported for the increased production of

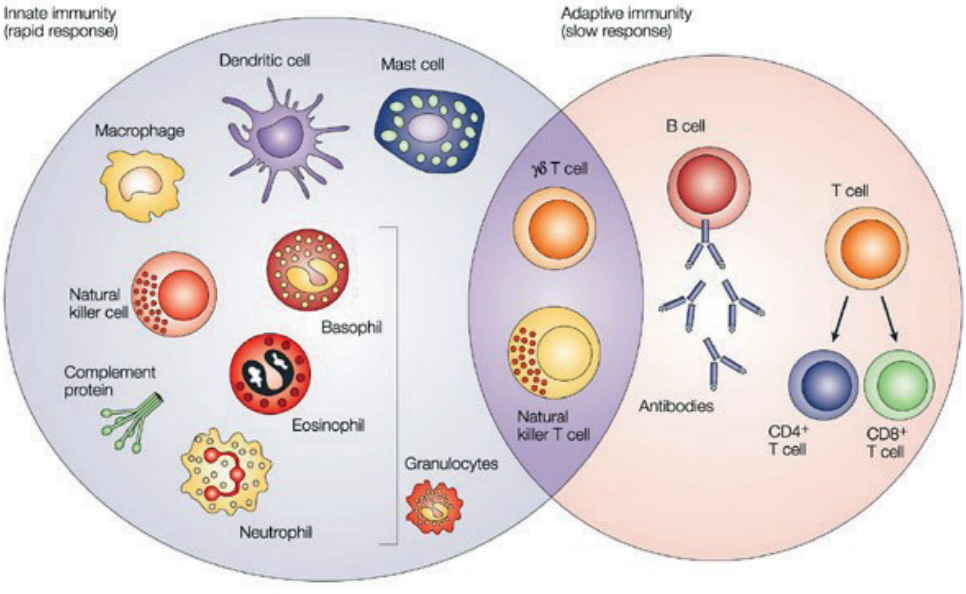

Figure 4 The innate and adaptive immune response. Adapted from Dranoff [47].

TGF-β1 and TGF-β2 in invasive cervical cancer whereas that of the classic Th1 cytokines IL-12 and TNF-α levels had decreased [66-68]. Additionally, patients who do not properly respond to therapeutic HPV vaccination show increased numbers of HPV-specific T regs [69].

B cells and HPV-specific antibody production

B cells are stimulated by APCs and Th cells which assist B cells to mature and produce antibodies against a specific epitope. After B cells maturation in the bone marrow, they migrate through the blood through secondary lymphoid organs (SLO), like the lymph nodes or spleen. Upon stimulation of the B cell receptor, B cells proliferate into plasma cells or memory B cells in germinal center reactions. Short-lived plasma cells, mostly making IgM, are generated upon a primary response. B cells can also be formed into follicles thereby forming germinal centers, here memory B cells and long-lived plasma cells are formed, and home back to the bone marrow. Long-lived plasma cells will predominantly make switched isotype antibodies (Figure 6)[71, 72].

In a natural HPV infection, antibodies target against conformational epitopes in the variable regions of the major viral coat protein, L1 [73], although low serum levels of antibodies against E2, E6, E7 and L2 also have been described. Antibodies against the oncoproteins E6 and E7 reflect productive HPV infections, and are potential clinically significant determinants of disease status in HPV-positive oropharyngeal cancers [74]. Serological studies mostly focus on IgG levels, which are antibodies most common found in serum. There are four subclasses of IgG whereof IgG1 is most abundant, followed by IgG2, IgG3 and IgG4. IgA antibodies are predominantly found in mucosal tissues [75]. IgG1 and IgA are isotypes being most abundantly found after a natural

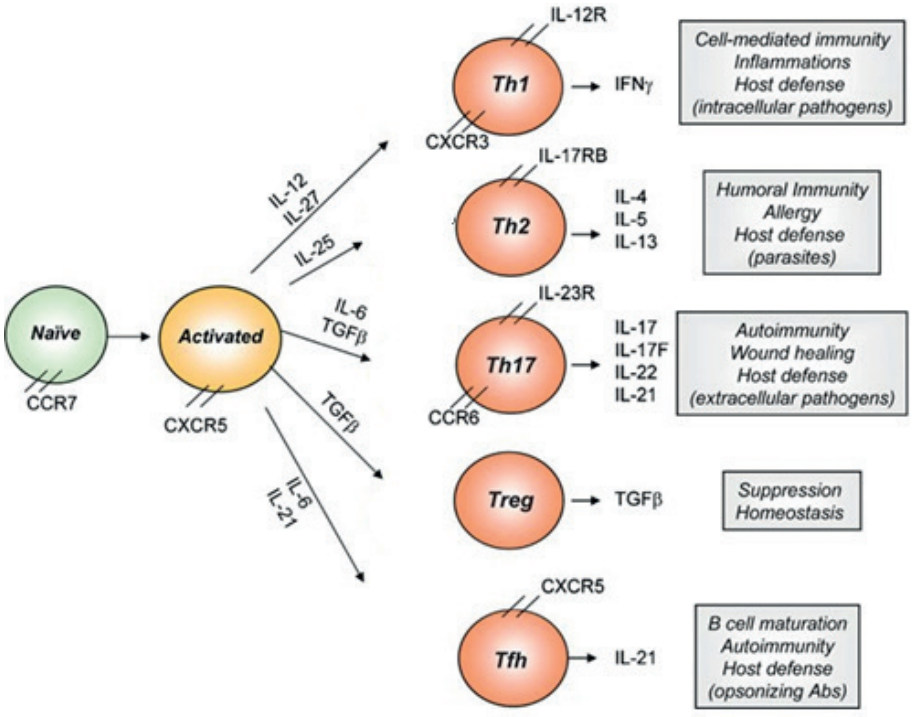

Figure 5 Differentiation of T helper cells, upon encountering antigen presented by professional APCs, naïve CD4+ T cells differentiate into different subsets which corresponding cytokine production, expression of transcription factors and chemokine receptors and immune regulatory functions. Adapted and modified from Nurieva *et al.*[70]

HPV infection. IgA seems to be induced earlier, but is not as persistent as IgG [76]. Seroconversion to HPV-specific IgG levels occurs about 6 to 18 months after the detection of HPV DNA [77]. Antibody avidity is suggested to be a marker for affinity maturation of antibodies, thereby implying that sustained germinal center reactions in the lymph nodes have occurred upon initial contact with HPV-VLPs [78]. Higher avidity levels tend to be associated with spontaneously induced neutralizing antibodies. Therefore, antibody avidity could possibly be used as a marker to distinguish between protective and non-protective HPV-specific antibodies [79]. This must however be interpreted with caution as it is suggested that avidity is a crude marker for affinity maturation [80]. The role of spontaneously induced HPV-specific antibodies in protection against HPV reinfections is still unknown [81, 82]. Protection against reinfection was more often reported in studies including seropositive younger women (aged 26-34) than in older women. This could also be attributed to waning of antibodies over time or a possible reactivation of a latent HPV infection [83]

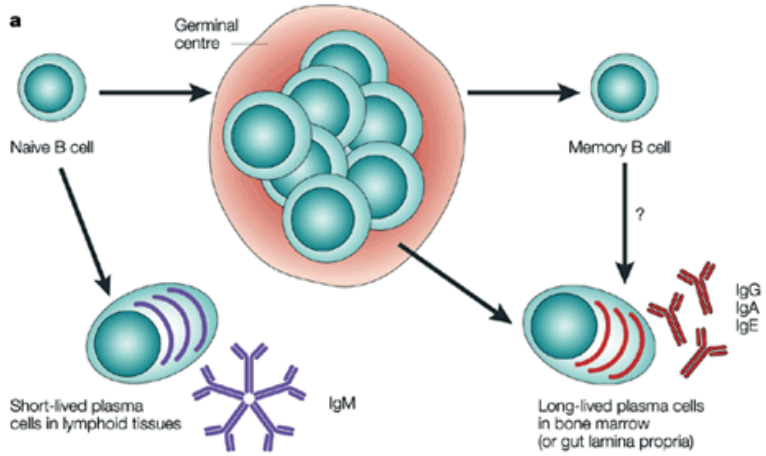

Figure 6 Development of memory B cells and plasma cells. Adapted from Gray. [71]

HPV seroprevalence

Seroprevalence studies of naturally HPV infected individuals show a rise in seropositivity soon after sexual debut [84-86]. The highest HPV seroprevalence is found among women from 20 to 40 years of age [85, 87-89]. However, hr-HPV antibodies and HPV-DNA could already be detected in sexually naïve children, albeit at very low concentrations. This shows that antibodies can also be derived from vertical or horizontal transmission. Orally acquired HPV infections could also be a reason for HPV immunity in children [90]. In older women above the age of 55, a decline in prevalence is seen, presumably due to waning of antibodies [91].

In the male population lower HPV seroprevalences were found compared to that in women [84, 85, 87, 88, 92]. These differences between males and females are assumed to lie in the immunological responses, as infections occur at different sites of entry that are accompanied by different epithelial layers [77]. Most studies found the highest seroprevalence for type HPV16 than for the other types in both males and females [87, 92-96].

Most HPV seroprevalence studies have been conducted in Western countries, with highest hr-HPV seroprevalences for any type varying between approximately 20 and 30% in the general population. Although data is scarce, show seroprevalences reaches up to 50% in other parts of the world [95, 97]. This suggests higher seroprevalence rates in the other part of the world, when compared to Western countries. Risk factors that were strongly associated with HPV seropositivity were related to age and sexual behavior, like number of lifetime partners and history of STDs [85, 87, 98, 99].

Vaccine development

Identification of HPV as a cause of cancer has made it a candidate to develop a cancer vaccine. Currently there are two types of vaccinations; therapeutic and prophylactic ones. Therapeutic

Table 2 Characteristics of the three available HPV VLP vaccines, adapted from Toh et al. [107]

	Cervarix®	Gardasil®	Gardasil9®
Manufacturer	GlaxoSmithKline Biologicals, SA	Merck Sharp & Dohme	Merck Sharp & Dohme
VLP types included	HPV16 and 18	HPV6, 11, 16 and 18	HPV6, 11, 16, 18, 31, 33, 45, 52 and 58
Dose of L1 protein	20 µg from both types	20 µg (HPV6 and 18), 40 µg (HPV11 and 16)	30 µg (HPV6), 40 µg (HPV11 and 18), 60 µg (HPV16) and 20 µg (HPV31, 33, 45, 52 and 58)
Producer cells	*Trichoplusia ni (Hi 5)* insect cell line infected with L1 recombinant	*Saccharomyces cerevisiae* expressing L1	*Saccharomyces cerevisiae* expressing L1
Adjuvant	500 µg aluminium hydroxide and 50 µg 3–0-deacylated-4′-monophosphoryl lipid A	225 µg aluminium hydroxyphosphate sulphate	500 µg aluminium hydroxyphosphate sulphate
Vaccination schedule	0, 1, and 6 months	0, 2, and 6 months	0, 2, and 6 months

vaccines are currently under development to cure existing cancers and related premalignant lesions. Several of these have already proven to be successful in curing premalignant lesions [100-104] and increasing the overall survival in patients with specific cancers [105]. Here we will further focus on prophylactic vaccines.

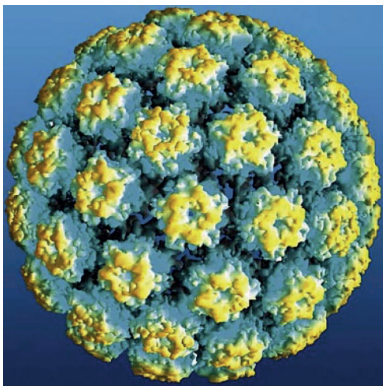

Figure 7 Atomic model of HPV16 L1 VLP, adapted from Schiller and Dowy, 2018 [78].

Prophylactic vaccines

The current prophylactic HPV vaccines are designed to induce antibodies that are capable of preventing an viral infection. HPV vaccines contain the HPV capsid proteins L1 or L1 and L2, which after their production as recombinant proteins spontaneously fold themselves into so-called 'virus-like particles' (VLPs) (Figure 7). VLPs are morphologically indistinguishable from the authentic virion, but are non-infectious because of lacking any DNA. In animal models, systemic vaccination with L1 VLPs has been shown to induce highly neutralizing antibody levels as well as protection against a viral infection after challenging mice with the homologous virus.
At the time of writing, there are three prophylactic HPV vaccines licensed for the global market, i.e. a bivalent (Cervarix, GlaxoSmithKline) (GSK) that includes the hr-HPV types 16 and 18, a quadrivalent (Gardasil, Merck Sharp & Co) (MSD) that includes in addition to the hr-HPV types 16 and 18 also the lr-HPV types 6 and 11 and a nonavalent vaccine (Gardasil9, Merck Sharp & Co) that covers seven hr-HPV types (HPV16/18/31/33/45/52/58) as well as the two lr-HPV types 6 and 11. The main differences between the vaccines produced by GSK (bivalent) and MSD (quadrivalent and nonavalent), are in the dosage of VLPs, the use of different expression systems to generate the vaccines, and the use of different adjuvants (Table 2). Both vaccines contain aluminum salts as an adjuvant, to ensure a slow release of the antigen and activation of the innate immune system, resulting in T cell and B cell responses. In addition, the bivalent vaccine also contains a monophosphoryl lipid A (MPL), which is a detoxified form of lipopolysaccharide. This adjuvant, known as AS04, is claimed to activate to innate immune response through TLR-4 leading to increased antibody responses [106].

Originally, the bivalent and quadrivalent vaccines were licensed for prevention of cervical cancers, and the quadrivalent vaccine also for prevention of genital warts. Licensure has since then been extended to protection against non-cervical HPV associated disease, and for use in boys. The licensed vaccines, their composition, recommendations and schedules and are shown in Table 2.

Vaccine immunogenicity

All three vaccines generate systemic antibodies directed to L1 VLPs with levels that are 10-100 fold higher [108, 109] than those measured after natural infection. Almost 100% of vaccinated individuals seroconvert [110]. This is attributable to the direct intra-muscular delivery of the vaccines with a high antigen dose, giving a rapid and direct access to lymph nodes for initiation of adaptive immune responses. The high and sustainable/durable antibodies generated by the vaccine neutralize the virus in *in vitro* assays and protection could be passively transferred in animal challenge models [111-113]. The slow life cycle of the papillomavirus,

ensures that the HPV virions are exposed to neutralizing antibodies for an exceptionally long time. As the vaccines does not affect already established infections and the L1 protein is not expressed on the surface of infected cells, the antibodies function exclusively by preventing infection [78]. Serum antibodies are thought to either exudate, via disruption of the epithelial barrier, or transudate, via the neonatal Fc receptor, directly to the site of infection [114].
HPV antibody responses have been reported up to 10 years after vaccination for the bivalent and quadrivalent vaccine following a three-dose schedule [115-118]. Additionally, follow-up maintenance of antibody levels till 5 years is available for the nonavalent vaccine [119, 120]. The bivalent vaccine induced significantly higher antibody levels and had a higher seroconversion rate than the quadrivalent vaccine [121]. The nonavalent induces similar HPV16/18 antibody responses as the quadrivalent vaccine [120, 122]. A head to head trial between the bivalent and nonavalent vaccine, also varying the amount of doses, is still ongoing (NCT02834637).

Vaccine efficacy studies

Development from initial HPV infection to cervical cancer takes decades [17]. Therefore, the use of cancer as a disease endpoint is regarded to be unethical to assess vaccine efficacy and would be impractical. As CIN lesions are important precursor lesions for cervical cancer, CIN2 and CIN3, were established as intermediate endpoints for the vaccine efficacy in phase III clinical trials for the bivalent and quadrivalent vaccine [123, 124]. CIN2/ CIN3 lesions develop in about 2.5- 4 years after infection, which is considerably faster than cervical cancer, but still a significant amount of time. It is important that at these stages patients can still be treated, for instance by operation. Virologic endpoints, i.e. persistent infections of 6 months or longer [125] are currently chosen to determine efficacy.

The vaccine efficacy (VE) of the different HPV vaccines against infection and lesions in women above 16 years of age with no evidence of current or previous exposure to HPV are depicted in Table 3. The efficacy of all three vaccines against infections of the vaccine targeted virus types are very high, exceeding 94%. The disease endpoint of CIN2, caused by vaccine targeted HPV types are equally high for the bivalent and quadrivalent vaccine. Both these vaccines also showed cross-protection against non-vaccine types hr-HPV types [118, 126-130]. Higher vaccine efficacies are found against CIN2/CIN3 than against persistent infections. The endpoint CIN3 caused by any HPV type provides 93% protection by the bivalent vaccine, that is significantly higher than that of the quadrivalent vaccine. No data have been reported yet about this endpoint after use of the nonavalent vaccine [127]. For evaluation of the efficacy in individuals younger than 16 years of age, the immune-bridging principle is used. This means that that when the immunogenicity in one group is the same as in another group, the efficacy is thought to be comparable [9]. The bivalent vaccine has been reported to be protective against HPV6 and 11 infections [131, 132], but this was not confirmed in a Dutch cross-sectional study [133], or only partially [134].
These strong cross-protective effects are claimed to be caused by the use of the AS04 adjuvant in the bivalent vaccine, showing higher antibody levels than just aluminum hydroxide salt adjuvanted vaccines [135]. Cross-protection seems to be restricted to phylogenetically related HPV types of the vaccine types [118, 126-130], presumably due to the shared/similar neutralizing epitopes. Vaccine efficacies for non-vaccine virus types are however still lower compared to that of the vaccine types. This degree of cross-protection by VE appears to be consistent with

detection of cross-neutralizing antibodies [136]. It however remains difficult to interpret if these cross-reactive antibodies levels are related to protection or effectiveness as a correlate of protection is still lacking [137].

Since the implementation of HPV vaccines into immunization programs worldwide, several countries have reported on the impact and effectiveness of the HPV vaccines. Results of population-based vaccine effectiveness studies were first described in Australia. This was one of the first countries that implemented nationwide (quadrivalent) HPV vaccination and reported a VE of 86% (95% CI 71-93) against the prevalence of HPV types 6, 11, 16 and 18 combined, for three-dose vaccinated women compared with unvaccinated women [138]. More recent data just appeared from Scotland, were routine vaccination with the bivalent vaccine of 12 to 13 year old girls led to a dramatic reduction in all grades of cervical intraepithelial lesions, equating a VE of 80% or even higher [139]. In countries were the quadrivalent vaccine was implemented not only a reduction in cervical abnormalities but a strong reduction in anogenital warts was found as well [140-142].
Besides the beneficial effects of HPV vaccination among women, also beneficial effects in non-vaccinated women [139, 143, 144] and non-vaccinated men are observed due to herd immunity effects [145, 146]. Models predict that elimination of HPV16 and 18 could be reached when a vaccination coverage of 80% is achieved, and herd effects would already be noticeable with a vaccine coverage as low as 20% [147].

The impact of dosing schedules on antibody levels

In 2014, the recommendation of the HPV-vaccination changed from a three-dose to a two-dose schedule for girls 9 to 15 years of age, based on the recommendations of the WHO and European Medicine Agency (EMA) [148]. The bivalent and quadrivalent vaccine both induce high antibody levels in a two- and a three-dose schedule, if these vaccines are at least given 6 months apart (Figure 8). HPV18 antibody levels after a two-dose schedule of the quadrivalent vaccine, however, are inferior to a three-dose schedule within 2 years, suggesting that two doses may induce protection on the long run [149]. Head-to-head trials of the bivalent and quadrivalent vaccine according to a two-dose schedule show that the bivalent vaccine induces 1.7 fold higher antibody levels for HPV16, and 5 fold higher levels for HPV18 compared to the quadrivalent vaccine [150]. Two doses of the nonavalent vaccine induce antibody levels equivalent to that of the quadrivalent vaccine [151].
High levels of cross-reactive antibodies of non-vaccine HPV types have been found after both bivalent and quadrivalent vaccination in two- and three-dose vaccinated women, although being higher in bivalent vaccine recipients than in quadrivalent recipients, and remained significantly above those from unvaccinated individuals [144, 152, 153].

Multiple studies showed that a persistent HPV16/18 infection was rarely found, 0%-<1% for 12 months, among participants who received any HPV vaccination, regardless of the number of doses given [154-158]. Post Hoc analyses in the Costa Rica Vaccine trial [155] and IARC India HPV vaccine trial [157] showed cumulative incidental HPV16/18 infections of 1.5% and 1.6% in one-dose recipients respectively, compared to 5.3% and 0.9% in three-dose recipients respectively. In the IARC trial, the rates of persistent HPV16/18 infection was 0% in one-dose recipients compared to 0% and 0.2% in the two- and three-dose trial, respectively. Neither of the studies

has so far reported efficacy against other HPV-associated endpoints, such as precancerous lesions or anogenital warts. Thus, the outcomes of these studies suggested that one-dose of the HPV vaccine could already be effective against vaccine-type HPV infection while significantly reducing the costs in vaccine supply and simplifying delivery, especially in low-income countries. One-dose studies mostly report on seropositivity, antibody levels and antibody stability. Just a few studies also report on antibody avidity and neutralizing antibody levels [156-160]. HPV16 and HPV18-specific antibody levels were significantly higher in participants who received multiple vaccine doses compared to one-dose vaccinated participants. However, antibody levels in the one-dose group were significantly higher than in non-vaccinated controls with a natural infection. The low antibody levels following an one-dose vaccination might be of limited clinical relevance, as in absence of a correlate of protection for HPV vaccination, it is hard to specify a specific antibody level as an endpoint.

At this time, there are several randomized controlled trials designed and on-going to compare the efficacy and/or immunogenicity of a single dose of the HPV vaccine; in Costa-Rica (ESCUDDO; NCT03180034), Kenya (KEN-SHE; NCT03675256), the Gambia (HANDS; NCT03832049) and Tanzania (DoRIS; NCT02834637) [161], which will provide us answers about one-dose efficacy.

Table 3 Vaccine efficacies of the current licensed HPV vaccines against both vaccine and non-vaccine types. Adapted from Harper *et al*.[127]

	Cervarix	Gardasil	Gardasil9
Among women 15/16- 26 years			
4-6 months HPV16/18 infection	94% (92-96)	96% (83-100)	Na
6 month HPV31/33/45/52/58 infection	Na	18% (5-29)	96% (94-98)
6 month HPV31 infection	77% (69-830	46% (15-66)	96% (91-98)
6 month HPV33 infection	45% (25-60)	NS	99% (95-100)
6 month HPV45 infection	74% (58-84)	NS	97% (92-99)
6 month HPV51 infection	17% (4-28)	Na	Na
6 month HPV52 infection	Na	NS	97% (95-99)
6 month HPV58 infection	Na	NS	95% (91-97)
CIN2+ related to HPV16/18	98% (88-100)	98% (94-100)	Na
CIN2+ related to HPV31	88% (68-96)	70% (32-88)	100% (40-100)
CIN2+ related to HPV33	68% (40-84)	NS	100% (33-100)
CIN2+ related to HPV39	75% (22-94)	NS	Na
CIN2+ related to HPV45	82% (17-98)	NS	NS
CIN2+ related to HPV51	54% (22-74)	NS	Na
CIN2+ related to HPV52	Na	NS	100% (67-100)
CIN2+ related to HPV58	Na	NS	NS
CIN2+ caused by any HPV type	62% (47-73)	22% (3-38)	63% (35-79)
CIN3+ caused by any HPV type	93% (79-99(	43% (24-57)	Na
AIS caused by any HPV type	100% (31-100)	Na	Na
Among women older than 25 years of age			
6 month infection or diseases related to HPV16/18	91% (79-97)	85% (68-94)	Na
6 month HPV31 infection	66% (25-86)	Na	Na
6 month HPV45 infection	71% (34-88)	Na	Na

Vaccine efficacies are presented with 95% confidence intervals.
Na= not applicable/ available, NS= not significant, AIS= adenocarcinoma in situ

Cellular immunity to prophylactic vaccines

Cellular immunity to HPV vaccines have been less well studied as antibody levels, but show us that the bivalent and quadrivalent vaccines give an HPV-specific B and T cell responses [162-164]. Age at vaccination but not vaccine dose was found to impact memory B cell formation, whereas CD4 T cell memory formation was found to be influenced by dose and not related to age [165]. Bivalent vaccine recipients showed higher numbers of memory B cells after vaccination compared to the quadrivalent vaccine recipients [135, 162].

Also HPV31 and HPV45 specific CD4+ T-cells and memory B-cell responses were detected up to 36 months post vaccination with the bivalent vaccine. The findings for cross-reactivity against HPV31 and 45 are not surprising as HPV31 is closely related to HPV16 and HPV45 to HPV18. The level of cross-reactivity is, however, much higher with the bivalent vaccine compared with the quadrivalent [166]. Cellular immunity after a single vaccine dose has been reported in just one study, showing that HPV-specific T cells are detectable up to six years post vaccination but vary between HPV types and dosage groups [167]. It can be speculated that the higher efficacy of the bivalent vaccine than that of the quadrivalent vaccine can be attributed to a more potent induction of memory B-cell responses and more cross-reactive antibody responses, presumably due to their reactivity to a broader epitope array.

Vaccine implementation in the Netherlands and Caribbean Netherlands

At the time of introduction, the bivalent vaccine was implemented in the Dutch National Immunization Program (NIP) according to a three dose schedule (0, 1 and 6 months). In 2009, the Netherlands started with a catch-up campaign for girls born between 1993 and 1996. From 2010 onwards, girls were invited for vaccination in the year that they turn 13. In 2014, after the new recommendation of the WHO, FDA and EMA the Netherlands changed to a two-dose schedule (0 and 6 months). Already since the implementation in 2009 the introduction of HPV vaccination is monitored. Vaccination coverage increased during the first years of the program till 2014, however from 2015 this uptake declined, varying between 45 and 60% [168].

The Netherlands has three Dutch overseas municipalities; Bonaire, St. Eustatius and Saba, also known as the Caribbean Netherlands (CN). These islands have a diverse ethnic population of approximately 25,000 people. Here, HPV-vaccination has been included in the National Immunization Program since 2013. At first the quadrivalent vaccine was introduced on St. Eustatius and Saba in 2013, followed by the bivalent vaccine on all three islands in 2015 in a two-doses schedule for 9-10 year old girls. The vaccination coverage on these islands varied between 28-67% in 2018 [169]. A population-based cervical cancer screening program, however, has not been introduced in CN thus far.

In 2019, the Dutch Health council advised to implement a sex-neutral vaccination and a catch-up campaign for all individuals up to the age of 26 [170]. This advice is planned to be implemented from 2021 onwards, and will also apply to the BES islands.

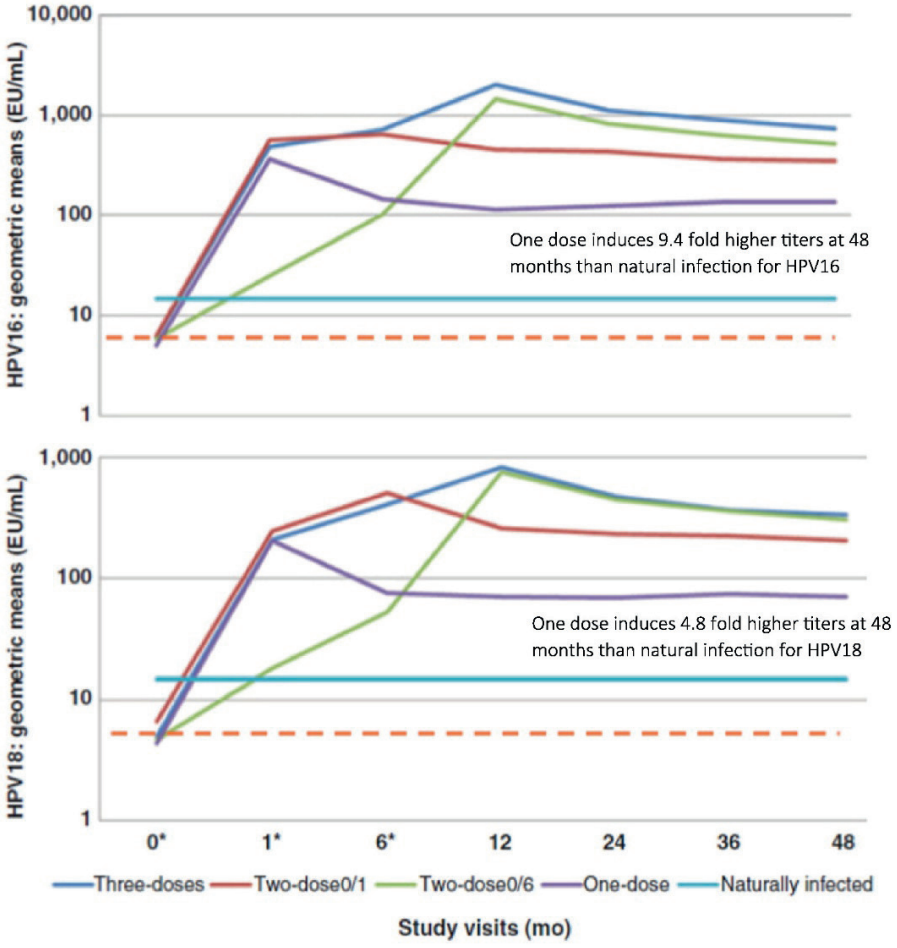

Figure 8 HPV16 and HPV18 antibody levels following different dosing schedules and natural infection. Adapted from Harper and DeMars[127]

Scope and outline

This thesis examined the humoral and cellular response to HPV in non-vaccinated and vaccinated individuals. The spontaneously-induced HPV-specific humoral response after infection was assessed in population-based studies.

In **chapter 2**, we examined the possible vaccine-induced changes in HPV-seroprevalence among the HPV unvaccinated Dutch population aged 0-89 years by comparing the HPV-seroprevalence before the introduction of the HPV vaccine with data of approximately six years post-implementation of the national HPV vaccination program. This revealed an increase in exposure of hr-HPV types in women and a rather stable exposure in men. No clear effects on herd immunity were observed within this rather short time frame after vaccine introduction combined with a suboptimal vaccine coverage.

In **chapter 3**, we conducted a cross-sectional in the Caribbean Netherlands, comprising of the islands Bonaire, St. Eustatius and Saba as the incidence of cervical cancer is high in the Caribbean while the sero-epidemiological data, key to the development of preventive programs, is scarce. Here we determined the immune status of the Dutch BES islands population just after introduction of HPV vaccination. High seroprevalence of multiple hr-HPV types were observed among women, indicating a relative high-risk for (precursors of) HPV-related cancers, and stressing the need for routine cervical cancer screening in CN.

We further assessed the humoral responses in vaccinated individuals. High antibody levels are thought to be important for protection against HPV infections, however a correlate of protection is still lacking. In **chapter 4**, we explored the longitudinal relation between hr-HPV antibody levels and HPV infections among vaccinated individuals. Antibody levels up to 9 years post vaccination with the bivalent vaccine in a three-dose schedule were high and persisted for both vaccine- and nonvaccine virus types. No consistent differences in type-specific antibody levels were observed between infected and non-infected women one year pre-infection.

In view of new reduced dosing schedules that are considered to be used, we aimed to gain more insight into humoral and cellular immune responses after just a single dose of the HPV vaccine in **chapter 5**. Therefore these responses were evaluated after one, two and three doses of the bivalent HPV vaccine. The one-dose of the bivalent vaccine indeed is immunogenic, but to a lesser extent as compared to two- or three doses. This indicates that girls receiving just one-dose might be at higher risk for waning immunity to HPV in the long-term.

Several studies show a higher immunogenicity for the bivalent vaccine than for the quadrivalent and nonavalent vaccine. In **chapter 6** we investigated the kinetics of innate and adaptive immune responses directly after vaccination with either the bivalent or nonavalent HPV vaccine. Insight in these responses would aid the interpretation of the different working mechanisms of the vaccines, and the induced adaptive responses observed. Moreover, for the first time in-dept immunological responses between the bivalent and the nonavalent HPV vaccine were studied. A strong monocyte response and plasma cell expansion was observed upon primary vaccination of both vaccines, which coincided with high antibody levels. HPV-specific antibody levels and memory B- and T cell responses were higher in the bivalent vaccinated women, which could be an explanation for the stronger cross-protection of the bivalent vaccine.

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CHAPTER 2

CHANGES IN HPV SEROPREVALENCE FROM AN UNVACCINATED TOWARDS A GIRLS-ONLY VACCINATED POPULATION IN THE NETHERLANDS

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Hella Pasmans[a], Joske Hoes[a,]Liza Tymchenko[a], Hester E. de Melker[a], Fiona R.M. van der Klis[a]

[a]Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3720 MA Bilthoven, the Netherlands.

ABSTRACT

Background In the Netherlands, bivalent HPV-vaccination was included in the National Immunization Program for 12-year old girls in 2010 (vaccination coverage 45-60%). We examined possible changes in HPV-seroprevalence in the HPV-unvaccinated Dutch population aged 0-89 years comparing pre-vaccination data with data of approximately six years post-implementation of national vaccination.

Methods Serum samples of men and women were used from two cross-sectional population-based serosurveillance studies performed before (2006-07, n=6384) and after (2016-17, n=5645) implementation of HPV-vaccination in the Netherlands. Seven high-risk HPV-specific antibodies (HPV16, 18, 31, 33, 45, 52, and 58) were tested in a virus-like-particle-based multiplex-immunoassay.

Results Type-specific HPV-seroprevalence has increased in women between 2006-07 and 2016-17. Also a higher seroprevalence for at least one type in women >15 years was found in 2016-17 (31.7%) compared with 2006-07 (25.2%). In men, overall HPV seroprevalence remained similar, however a lower seroprevalence was found for HPV16 in 2016-17 (7.5%) compared with 2006-07 (10.6%).

Conclusions Our results indicate an increase in high-risk HPV types in women and a rather stable exposure in men. No clear effects of the strategy of girls-only vaccination were observed in men, probably because of the short time after introduction combined with suboptimal coverage.

Impact No herd immunity observed yet in a population with a suboptimal HPV-vaccination coverage.

INTRODUCTION

Human papillomavirus (HPV), a virus capable of infecting the epithelial cells of the mucosa, is the cause of anogenital warts and cervical cancer [1]. Besides cervical cancer, HPV is also linked to various other cancers in the anogenital tract and oral cavity [2]. By routine HPV vaccination and effective cervical cancer screening programs, countries can reduce the burden of HPV-related disease.

All current globally available vaccines provide protection against HPV types 16 and 18, e.g. and are included in the current bivalent vaccine. HPV types 6 and 11 are added in the quadrivalent vaccine and the nonavalent vaccine included additional HPV types 31, 33, 45, 52, and 58. Vaccination against HPV has been implemented in many countries, with the primary aim to protect women against cervical cancer. In the Netherlands, the bivalent HPV vaccine was implemented in the Dutch National Immunization Program (NIP) as a girls-only vaccine for 12-year-olds in a three-dose schedule in 2010, and is currently still being used, protecting them against HPV types 16 and 18. Additionally, a catch-up campaign was initiated for girls from the birth cohorts 1993-1996 (i.e. 13-16 year-olds) in 2009. From 2014 onwards, the Netherlands shifted to a two-dose schedule (starting from birth cohort 2001 onwards). The HPV vaccination coverage in girls in the Netherlands varied from 2009-2017 from 45-62% [3].

To gain information about previous HPV exposure, HPV serology is established as an important tool for population-based studies [4]. This provides a view on type-specific cumulative lifetime exposure to HPV. Antibodies against HPV L1 virus-like-particles (VLPs) remain stable over time, and therefore reflect past infection and cumulative exposure. However, not everyone who contracted HPV will seroconvert, and the rate of seroconversion is known to be sex-dependent [5]. After HPV vaccination, HPV-specific antibodies are 10-100 times higher than (natural) infection-induced antibodies in serum [6], and therefore could be used to monitor vaccine uptake.

We assessed the (natural) infection-induced HPV seroprevalence for seven high-risk (hr) HPV types in the Dutch population in 2006/2007 (i.e. 4 years before the introduction of HPV) and 2016/2017 (i.e. 6 years post vaccination implementation). In addition, we investigated the effects of the introduction of the HPV-vaccination on the seroprevalence of HPV types in our population.

MATERIALS AND METHODS

Study Design

Serum samples from two cross-sectional population-based serosurveillance studies performed from February 2006 to June 2007 and from September 2016 to October 2017 in the Netherlands were used for this study. Participants were 0–79 years of age in the 2006-2007 survey (n=6384), and 0-89 years of age for the 2016-17 cohort (n= 5645). Study designs have been previously described in-detail [7, 8]. Briefly, the randomly invited participants were asked to fill in a questionnaire and to provide a blood sample. Questionnaires of both surveys included data on demographic characteristics, ethnicity (first and second generation migrants), vaccination history and sexual behavior. Vaccination history was determined via the individuals' registration booklet

and the Dutch vaccination registration Praeventis [9]. The questionnaire used in 2006-7 was extended in the 2016–17 survey with more questions regarding sexual behavior. Information related to sexual behavior was only available from participants older than 14 years of age in both the 2006-07 study and the 2016–17 study.

We obtained written informed consent from all participants and/or their guardians before participation. The studies were conducted in accordance with recognized ethical guidelines (the Declaration of Helsinki) and were approved by an institutional review board 'The Medical Ethics Committee Noord-Holland' in the Netherlands (METC number: ISRCTN 20164309 and M015–022).

Serological measurement

Serum samples of both surveys were stored at −80°C until analysis, samples were measured at random for age and sex. For the measurement of HPV-specific IgG serum antibodies against L1 virus-like-particles (VLP) of HPV16, 18, 31, 33, 45, 52, and 58, a VLP-based multiplex immunoassay (MIA) was used as previously described [10]. GSK (2006-07 survey) and MSD (Merck Sharp& Co, Kenilworth, NJ) (2016-17 survey) produced the HPV-VLPs used in these studies. Briefly, VLPs were conjugated to seven distinct fluorescent microspheres via amine coupling. Serum samples were 1/50, 1/100 or 1/10000 diluted and incubated with the VLP-coupled microspheres. HPV-specific IgG serum antibodies were detected using a secondary goat anti-human phycoerythrin-labeled antibody. Four in-house control sera and an in-house standard were used on each plate. The in-house standard (IVIG, lot LE12H227AF, Baxter) was calibrated against reference serum of GSK for all the seven HPV types. HPV-specific IgG antibodies were analyzed using the Bioplex system 200 with Bioplex software (Bio-Rad Laboratories, Hercules, CA). Samples were assumed to be seropositive above cut-offs according to the 99% Frey-method (with 99% one-sided t-values, based on concentrations measured in children of 1-10 years old (n=859)[11] and found to be 9, 13, 27, 11, 19, 14, and 31 Luminex Units/ml (LU/ml) [10] for HPV16, 18, 31, 33, 45, 52, and 58, respectively. As samples from 2016-17 were measured using a different batch of VLPs than those used in 2006-07, a correction formula was applied on the data of the 2016-17 survey. This correction formula was based on re-testing of a random subset of 160 samples of the 2006-07 samples with the new VLPs. The correction formula was applied to the 2016-17 antibody measurements in order to align them with the 2006-07 measurements.

Statistical analysis

Data analyses were conducted using SAS version 9.4 and GraphPad Prism version 8.0.2. Women who were vaccinated against HPV according to the vaccination registry (n=228) were excluded from analysis. Additionally, women under 31 years of age and with arbitrary antibody concentration cut-off of >100 LU/ml for HPV16 and >50 LU/ml for HPV18 were considered to be 'highly likely to have been vaccinated' and were excluded from the analyses (n=18). Characteristics of the study population were compared among the 2006-2007 cohort and the 2016-2017 cohort using Chi-squared tests. Seroprevalence for 'any' or 'all' hr-HPV-type(s) refer to the seven hr-serotypes that have been measured in this study. The study design (i.e. a two-stage cluster sampling method including specific regions and municipalities from which participants were invited) was taken into account in the analyses, as well as weights determined proportional to the reference population (Dutch population, 1st of January 2007 and 1st of January 2017, respectively) taking into account sex, age, ethnic origin, and urbanization degree. Seroprevalences were calculated

per age-cohort and as large differences already have been observed between men and women [10], analyses were stratified for men and women. Crude seroprevalences of the different cohorts, age groups and/or sexes were compared using Monte Carlo simulations. Parameters of the beta distribution for both seroprevalences were estimated and used in the simulations to obtain p-values. Geometric mean concentrations (GMCs) were calculated amongst HPV16 and HPV18 seropositive individuals from both cohorts, taking the study design into account. P values of <0·05 were considered statistically significant.

The associations between HPV seropositivity (positive for at least one out of the seven HPV types) in sexually active individuals older than 14 years of age who were not vaccinated and demographic characteristics (age, ethnic origin, degree of urbanization, education level, and socio-economic status), was examined for the 2016-2017 cohort for men and women separately. Additionally, associations with (sexual) behavior characteristics were taken into account, including: BMI, alcohol consumption, smoking, having a steady partner, age of sexual debut (being defined as the first time of vaginal/penile intercourse), condom use at last sex act, number of partners in the last six months, lifetime number of partners, and reported history of STI (note: participants with missing values for a specific variable were allocated to a unknown category). We used generalized estimation equation (GEE) logistic regression models with a log link function and robust error variance. The incorporation of a GEE with exchangeable correlation structure accounted for dependency of multiple HPV types within an individual. First, univariate logistic regression analyses were conducted to study characteristics associated with HPV-seropositivity. Variables that had $p<0.1$ in univariate analyses were included in the multivariate analysis and backward selection (dropping variables one-by-one) was then applied. Hence, a multivariate model only including independently associated risk factors ($p<0.05$) remained.

To study the differences in seroprevalence between the 2006-2007 and 2016-2017 cohort more closely, a pooled dataset was created including all HPV-unvaccinated participants from both cohorts. Again, the association between HPV seropositivity in sexually active individuals older than 14 years of age and demographics and sexual behavior characteristics was studied, in addition to the variable defining the cohort. Only characteristics available from both surveys were considered for inclusion in the model. Using a Poisson regression with robust error variance, we first calculated the crude prevalence ratio (PR). Next, we included the variables of interest to adjust for differences between the two surveys resulting in an adjusted prevalence ratio (aPR). The analyses were performed for seroprevalence of any HPV type as well as type-specific. In addition, we stratified the analyses for men and women; we assumed that if herd effects on seroprevalence were to be observed this short after HPV vaccine introduction, this would be amongst men (first-order effect), in particular younger males. Therefore, we looked also into the aPR for younger males (15-39 years of age).

RESULTS

Study and participant characteristics

We tested 5645 serum samples, with corresponding response rates of 13.2% for men and 18.4% for women from the 2016-17 survey, and 6384 serum samples, with corresponding response rates 28.9% for men and 34.7% for women from the 2006-07, which were tested previously

[10, 12]. Study characteristics were stratified for sex. In the 2016-17 survey, for both men and women, participants of 15 years and older were higher educated and had a higher net monthly income in comparison to both men and women in the 2006-07 survey. The mean age of sexual debut for people under 25 years of age was similar between the different surveys and sexes. However, age of sexual debut across all ages was lower in the 2016-17 survey compared with 2006-07 survey, for both men and women. Additionally, the percentage of participants reporting to have a current steady partner was lower in 2016-2017, while "the number of sex partners in the last six months" and "ever having been diagnosed with a sexually transmitted disease (STD)" were higher in the 2016-17 survey compared with 2006-07 (Table 1).

HPV seroprevalence

Age-specific seroprevalence and GMC in an unvaccinated population, by sex and survey

An increase in seroprevalence for any hr-HPV type was observed in women in the age cohort from 15-19 years old, which reflects the median age of sexual debut. In the 2016-17 survey, seroprevalence for any type increased from 3.0% (10-14 years old) to 30.5% (20-24 years old) and 33.7% (25-29 years old) and peaked at 37.0% in the 30-39 year old. The greatest rise was seen for HPV16 and HPV18. This increase in seroprevalence was much more gradual for men, and mainly in the 2006-07 survey was most pronounced for any hr-HPV type and HPV16 (Figure 1). Samples sizes of age cohorts can be found in Table 1 and Supplementary Table 1.

Low seroprevalences were observed in children 0-14 years of age in both sexes and surveys. In the 2016-17 survey the highest seroprevalences in children (0-14) were detected for HPV16 and HPV18 (Figure 1).

In the older female age groups, overall seroprevalence decreased from the age of 49 years onwards in the 2006-07 survey and from 60-69 years onwards in the 2016-17 survey. Age-specific higher seroprevalence for any hr-HPV type was observed in 2016-17 compared with 2006-07, being significant in age groups 30-39 (p=0.0108), 50-59 (p=0.0406) and 60-69 (p=0.0056) years of age. A lower seroprevalence for any hr-HPV type was observed in the age group 10-14 (p=0.0118) in 2016-17 compared with 2006-07. In men, a lower age-specific seroprevalence for any hr-HPV type was observed in 2016-17 compared with 2006-07, being only significant in the age groups 10-14 (p=0.0056). No significant difference was found for the age-specific seroprevalence for any hr-HPV type excluding 16 and 18 in the age groups 10-14 and 15-19 years of age between the two surveys, p=0.091and p=0.1206, respectively.

Overall HPV seroprevalence from unvaccinated individuals 15 years and onwards

Unvaccinated female participants older than 15 years of age showed significantly higher seroprevalence for any hr-HPV type in 2016-17 compared with 2006-07; 31.4% (95% CI 29.1-33.7) and 25.2% (95% CI 23.1-2.3) respectively. For men from 15 years of age and older, seroprevalence for any hr-HPV type was similar between the 2006-07 and 2016-17 surveys; 19.7% (95% CI 17.9-21.6) and 20.3% (95% CI 18.4-22.1) respectively. In women, also seropositivity for one up to all seven types was significantly higher in 2016-17 and hr-type specific. Type-specific HPV16, HPV18, HPV31 and HPV58 were higher in 2016-17 compared with 2006-07, which was also true for the combinations HPV16 and 18, HPV16 or 18 and HPV16 and/or 18 (Table 2).

Table 1 Sociodemographic and sexual behaviour characteristics of participants from 15 years of age without vaccination, with a blood sample for HPV IgG antibody determination in the Netherlands, by sex and survey

Sociodemographic characteristic	Men 2006-07 % (n) N=1937	Men 2016-17 % N=1911	*p value*	Women 2006-07 % N=2535	Women 2016-17 % N=2415	*p value*
Age groups, years						
15-19	6.87 (133)	5.67 (110)		6.51 (165)	1.98 (43)	
20-24	7.12 (138)	10.36 (198)		8.92 (226)	4.93 (107)	
25-29	6.50 (126)	9.00 (172)		8.76 (222)	9.36 (203)	
30-39	14.97 (290)	15.07 (288)		16.76 (425)	20.2 (438)	
40-49	14.51 (281)	13.55 (259)		14.20 (360)	18.44 (400)	
50-59	15.07 (292)	14.49 (277)		16.65 (422)	16.74 (363)	
60-69	18.79 (364)	16.27 (311)		17.16 (435)	16.04 (348)	
70-79	16.15 (313)	12.55 (240)		11.05 (280)	10.01 (217)	
80-89		2.93 (56)			2.31 (50)	
Educational level[a]			<0.0001			<0.0001
High	29.17 (565)	40.24 (769)		23.59 (598)	35.04 (760)	
Middle	29.94 (580)	28.78 (550)		31.52 (799)	29.74 (645)	
Low	39.34 (762	25.64 (490)		43.35 (1099)	28.82 (625)	
Unknown	1.55(30)	5.34 (102)		1.54 (39)	6.41 (139)	
Net monthly income			<0.0001			<0.0001
<850/<970	5.94 (115)	502 (96)		8.72 (221)	5.3 (115)	
851-1150/ 971-1.335	7.80 (151)	6.65 (127)		9.47 (240)	9.04 (196)	
1151-1750/ 1.356-1.969	19.00 (368)	11.93 (228)		17.87 (453)	14.66 (318)	
1751-3050/ 1.970-3.314	32.27 (625)	28.52 (545)		25.68 (651)	29.28 (635)	
3.051-3.500/ 3.315-3.500	7.02 (136)	8.16 (156)		6.11 (155)	6.69 (145)	
>3.501	11.31 (219)	26.95 (515)		8.36 (212)	20.89 (453)	
Unknown	16.68 (323)	12.77 (244)		23.79 (603)	14.15 (307)	
Ethnicity			0.0307			0.0018
Dutch	81.78 (1584)	79.96 (1528)		81.85 (2075)	78.98 (1713)	
First generation migrant	10.84 (210)	13.50 (258)		11.76 (298)	15.26 (331)	
Second generation migrant	7.38 (143)	6.54 (125)		6.39 (162)	5.76 (125)	
Smoking						
Yes		48.77 (932)			43.11 (935)	
No		42.96 (821)			47.76 (1036)	
Unknown		8.27 (158)			9.13 (198)	
Alcohol						
Yes		76.35 (1459)			64.04 (1389)	
No		14.70 (281)			26.09 (566)	
Unknown		8.95 (171)			9.87 (214)	
BMI						
<18.5		1.99 (38)			1.89 (41)	
18.5-25		43.22 (826)			43.52 (944)	
25-30		33.18 (634)			26.97 (585)	
≥30		10.52 (201)			14.98 (325)	
Unknown		11.09 (212)			12.63 (274)	
Current steady partner			0.0005			<0.0001
Yes	79.50 (1540)	77.60 (1483)		75.31 (1909)	77.04 (1671)	
No	19.05 (369)	19.05 (364)		22.72 (139)	18.86 (409)	
Unknown	1.45 (28)	3.35 (64)		1.97 (50)	4.1 (89)	
Ever had sexual intercourse			<0.0001			<0.0001
Yes	89.93 (1742)	91.63 (1751)		90.49 (2294)	95.67 (2075)	
No	6.87 (133)	8.37 (160)		5.48 (139)	4.33 (409)	
Unknown	3.20 (62)	0.00 (0)		4.02 (102)	0.00	
Median age at sexual debut (<26 years of age)	16.9	16.8		16.7	16.7	
Age at sexual debut			<0.0001			<0.0001
<17 years	12.80 (248)	18.32 (350)		17.32 (439)	24.57(533)	
17-19 years	24.37 (472)	31.08 (594)		30.73 (779)	34.76 (754)	
≥20 years	31.96 (619)	28.57 (546)		27.14 (688)	24.85 (539)	
Unknown	30.87 (598)	22.03 (421)		24.81 (629)	15.81 (343)	
Number of partners last 6 months			<0.0001			<0.0001
0	14.09 (273)	11.15 (213)		15.35 (389)	16.32 (354)	
1-2 partners	62.36 (1208)	68.13 (1302)		60.79 (1541)	67.31 (1460)	
>2 partners	0.98 (19)	2.30 (44)		0.79 (20)	0.83 (18)	
Unknown	22.56 (437)	18.42 (352)		23.08 (585)	15.54 (337)	
Lifetime sexual partners						
1-2 partners		38.72 (740)			47.63 (1033)	
3-5 partners		21.04 (402)			23.19 (503)	
6-9 partners		10.57 (202)			8.21 (178)	
≥10 partners		12.87 (246)			8.85 (192)	
Unknown		16.80 (321)			12.13 (263)	
Condom use last time sex			<0.0001			<0.0001
Yes	8.00 (155)	14.08 (269)		7.65 (991)	13.05 (283)	
No	56.01 (1085)	69.65 (1331)		53.25 (1350)	73.95 (1604)	
Unknown	35.98 (697)	16.27 (311)		39.09 (991)	13 (282)	
Ever had sexual transmitted disease			0.0016			<0.0001
Yes	3.82 (74)	6.17 (118)		5.44 (138)	8.16 (177)	
No	89.00 (1724)	82.42 (1575)		86.04 (2181)	79.07 (1715)	
Unknown	7.18 (139)	11.41 (218)		8.52 (216)	12.77 (277)	

[a] Educational level was used for participants 0-11y, active education was used for participants 12-25y, and highest accomplished educational level was used for participants >25y. Low=no education, primary school, pre-vocational education (VMBO), lower vocational education (LBO/MBO-1), lower general secondary education (MAVO/VMBO). Middle=intermediate/ secondary vocational education (MBO-2-4), higher/senior vocational education (HAVO), pre-university education (VWO/Gymnasium); High=higher professional education (HBO), University BSc., University MSc., Doctorate
Missing: ethnicity n=13

For men, this was true for the combination HPV16 or 18, positivity for more than two hr-HPV types and type-specific HPV18, 31, 33, 45, 52, and 58. For HPV16 a lower seroprevalence was seen in 2016-17 (7.5%, 95%CI 6.5-8.5) compared with 2006-07 (10.6%, 95%CI 9.2-12.0). Just as for the combination HPV16 and 18, HPV16 and/or HPV18 and positivity for more than one hr-HPV type (Table 2). HPV16 was also most prevalent in both surveys, followed by HPV18, HPV45, and the rest of the types (Table 2). Only a very small percentage of the males were seropositive for all seven hr-HPV types, 0.6% and 0.3% for 2006-07 and 2016-17, respectively.

HPV type-specific antibody concentrations among seropositive individuals

The age-specific HPV16 geometric mean concentrations (GMCs) of (natural) infection-induced seropositive women as well as of seropositive men were comparable in all age cohorts between both studies. No differences were found between the GMCs of the HPV16 and HPV18 seropositive individuals between 2006-07 and 2016-17 (Supplementary Figure 3).

Risk factors for hr-HPV seropositivity

For women, the univariate analysis showed an association for HPV seropositivity for any hr-HPV type with middle educational level, being a 1st or 2nd generation migrant, having a lower income, ever used alcohol, not having a steady partner, lower age of sexual debut, having more than two sexual partners last 6 months, history of reported STD, and having more than 2 sexual partners during lifetime. In the backward selection model low and middle educational level, 1st generation migrants, more than 2 sexual partners during lifetime, and history of self-reported STDs remained and were independently associated with seropositivity for any hr-HPV type (Table 3).

For men, the univariate analyses showed only an association of seropositivity for any hr-HPV type with history of self-reported STDs (Table 3).

Pooled risk factor analysis associated with HPV-seropositivity for the 2006-07 and 2016-17 surveys

HPV seropositivity for any hr-HPV type for women from 15 years onwards was 25.2% in 2006-07 and 31.4% in 2016-17. After pooling both surveys, and adjusting for demographic characteristics (age, sex, urbanization, education, income, ethnicity) and sexual risk factors (age of sexual debut, number of partners during the last 6 months, history of STDs) this resulted in a smaller, but still significant, difference between 2006-07 and 2016-17, (aPR 1.16, 95% CI 1.02-1.32). Before adjustment of any variables, all seven hr-HPV types were significantly higher in 2016-17 compared to 2006-07. However, after adjustment for the demographic characteristics and sexual risk factors the differences remained only significant for HPV16 (aPR 1.29, 95% CI 1.07-1.55), HPV18 (aPR 1.31, 95% CI 1.01-1.70), HPV31 (aPR 1.54, 95% CI 1.111-2.14) and HPV52 (aPR 1.27 95% CI 1.00-1.62)(Table 4).

For men from 15 years onwards, HPV seropositivity for any hr-HPV type was significantly lower in 2016-17(18.2%) compared with 2006-07(20.3%). After adjustment for the demographic characteristics and sexual risk factors this did not remain significant (aPR 0.99, 95% CI 0.83-1.17). Before adjustment, HPV16, HPV18 and HPV31 were significantly lower in 2016-17 compared with 2006-07 and HPV45 was significantly higher in 2016-17 compared with 2006-07. This difference only remained significant for HPV16 after adjustment (aPR 0.71, 95% CI 0.55-0.91)

(Table 4) and HPV45 (aPR 1.47, 95% CI 1.13-1.92). Zooming in on men in the age cohort of 15-39, a non-significant decrease for HPV16 between 2006-07 and 2016-17 was observed (aPR 0.84, 95% CI 0.52-1.37).

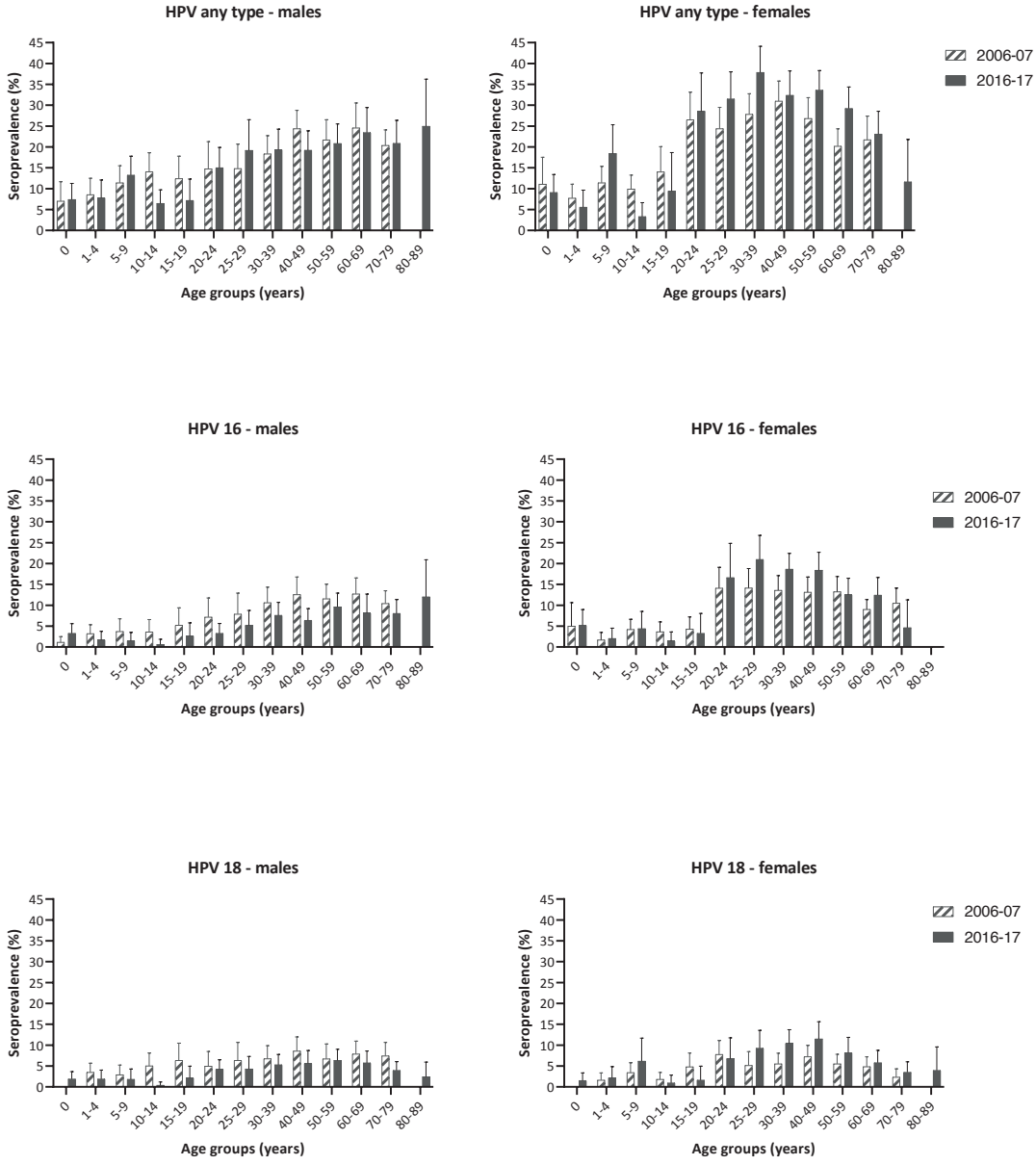

Figure 1 Age-specific seroprevalence (%) (with 95% confidence intervals) of any high-risk type human papillomavirus (HPV) IgG antibodies for men (a) and women (b), HPV 16 for men (c) and women (d) and HPV18 for men (e) and women (f) in the unvaccinated general population of the Netherlands.

Table 2 Weighted seroprevalence for seven high-risk HPV types and combinations in the total population of the Netherlands from 15 years of age without vaccination - stratified by sex and survey

Total population from 15 years of age, without vaccination		Men (2006-07) (n=1937)	Men (2016-17) (n=1916)	p-value	Women(2006-07) (n=2535)	Women(2016-17) (n=2177)	p-value
High-risk HPV types							
	HPV16	10.6 (9.2-12.0)	7.2 (6.2-8.2)	0.0000	11.9 (10.3-13.6)	15.8 (13.9-17.7)	0.0006
	HPV18	7.2 (6.1-8.2)	5.0 (3.8-6.2)	0.0078	5.6 (4.7-6.4)	7.9 (6.7-9.1)	0.0006
	HPV31	2.5 (1.7-3.4)	1.4 (0.9-1.9)	0.0158	3.3 (2.4-4.2)	5.2 (4.1-6.3)	0.009
	HPV33	6.0 (4.7-7.2)	5.4 (4.4-6.3)	0.4338	6.5 (5.5-7.6)	8.0 (6.3-9.7)	0.116
	HPV45	6.8 (5.4-8.1)	9.6 (8.2-10.9)	0.0022	7.5 (6.2-8.9)	9.6 (8.2-10.9)	0.0304
	HPV52	5.4 (4.4-6.3)	5.1 (4.1-6.0)	0.6576	6.9 (5.9-8.0)	8.7 (7.1-10.2)	0.0610
	HPV58	3.7 (2.8-4.5)	3.0 (2.1-3.9)	0.2914	4.5 (3.6-5.3)	6.4 (5.2-7.5)	0.0052
HPV combinations							
	HPV16 and 18	5.7 (4.9-6.6)	2.8 (2.0-3.6)	0.0000	3.6 (2.9-4.4)	4.8 (3.8-5.9)	0.0572
	HPV16 or 18	6.3 (5.1-7.5)	6.6 (5.5-7.7)	0.7376	10.2 (8.8-11.6)	14.1 (12.4-15.7)	0.00040
	HPV 16 and/or 18	12.0 (10.6-13.5)	9.4 (8.2-10.5)	0.0026	13.8 (12.1-15.5)	18.9 (17.0-20.7)	0.0000
	Positive for at least 1 hr HPV types	20.3 (18.4-22.1)	19.3 (17.7-21.0)	0.4378	25.2 (23.1-27.3)	30.1 (27.7-32.4)	0.0052
	Positive for at least 1 hr HPV types excluding HPV16 and 18	13.6 (11.7-15.5)	15.3 (13.7-16.8)	0.1826	17.9 (16.0-19.8)	21.0 (18.9-23.2)	0.0234
	Positive for more than 1 hr HPV types	9.8 (8.8-10.8)	7.3 (6.0-8.7)	0.0066	10.4 (9.1-11.7)	14.4 (12.3-16.4)	0.0004
	Positive for more than 2 hr HPV types	5.2 (4.0-6.3)	4.3 (3.4-5.2)	0.2078	4.9 (3.9-6.0)	7.8 (6.4-9.3)	0.001
	Positive for 7 hr HPV types	0.6 (0.2-0.9)	0.3 (0.0-0.5)	0.1686	0.3 (0.1-0.5)	0.7 (0.2-1.1)	0.1146

Table 3 Risk factor analysis for any high-risk type HPV IgG seropositivity among sexual active and unvaccinated participants from 15 years of age in the Netherlands, by sex .

Males (n=1751)		Univariate model		Multivariate model	
Risk factor		OR	95% CI limits	OR	95% CI limits
Age					
	15-19	Ref		Ref	
	20-24	0.60	0.18-2.01		
	25-29	0.68	0.21-2.26		
	30-39	0.98	0.31-3.08		
	40-49	0.94	0.30-2.99		
	50-59	1.04	0.33-3.29		
	60-69	1.01	0.32-3.16		
	70-79	0.94	0.30-3.00		
	80-89	0.93	0.25-3.46		
Education[a]					
	High	Ref		Ref	
	Middle	0.86	0.61-1.20		
	Low	1.11	0.81-1.54		
	Unknown	0.81	0.44-1.46		
Net monthly income[b]					
	<850/<970	Ref		Ref	
	851-1150/971-1335	0.68	0.31-1.48		
	1151-1750/1970-3314	1.59	0.77-3.30		
	1751-3050/1970-3314	1.18	0.59-2.35		
	3051-3500/3315-3500	1.82	0.84-3.95		
	>3501	1.17	0.59-2.32		
	Unknown	1.01	0.48-2.11		
Ethnicity[c]					
	Dutch	Ref		Ref	
	1st generation migrant	1.00	0.69-1.44		
	2nd generation migrant	0.78	0.42-1.45		
Smoking ever					
	No	Ref		Ref	
	Yes	0.95	0.72-1.25		
	Unknown	0.98	0.61-1.59		
Alcohol use					
	No	Ref		Ref	
	Yes	1.04	0.70-1.56		
	Unknown	0.92	0.53-1.61		
BMI					
	<18.5	Ref		Ref	
	18.5-25	1.06	0.34-3.34		
	25-30	1.23	0.39-3.87		
	>30	0.88	0.26-2.93		
	Unknown	1.03	0.32-3.41		
Current steady partner					
	No	Ref		Ref	
	Yes	1.12	0.74-1.71		
	Unknown	1.08	0.47-2.49		
Age of sexual debut					
	<17 years	Ref		Ref	
	17-19 years	1.21	0.84-1.73		
	≥ 20 years	0.86	0.58-1.27		
	Unknown	1.18	0.76-1.84		
History STD					
	No	Ref		Ref	
	Yes	**1.70**	**1.11-2.61**		
	Unknown	0.88	0.58-1.34		
Condom use					
	No	Ref		Ref	
	Yes	0.77	0.50-1.17		
	Unknown	0.76	0.46-1.26		
Partners last 6 months(sexual)					
	0	Ref		Ref	
	1-2	0.95	0.62-1.45		
	>2	0.84	0.37-1.91		
	Unknown	1.03	0.58-1.83		
Partners lifetime (sexual)					
	1-2	Ref		Ref	
	3-5	0.83	0.57- 1.21		
	6-9	0.97	0.62-1.50		
	>10	1.34	0.94-1.91		
	Unknown	1.45	0.92-2.28		

Females (n=2075)		Univariate model		Multivariate model	
Risk factor		OR	95% CI limits	OR	95% CI limits
Age					
	15-19	Ref		Ref	
	20-24	1.90	0.50-7.23		
	25-29	2.18	0.59-8.05		
	30-39	2.16	0.60-7.78		
	40-49	2.12	0.58-7.68		
	50-59	2.19	0.60-7.91		
	60-69	1.83	0.51-6.65		
	70-79	1.19	0.32-4.36		
	80-89	0.69	0.15-3.10		
Education[a]					
	High	Ref		Ref	
	Middle	**1.36**	**1.08-1.70**	**1.47**	**1.17-1.85**
	Low	1.10	0.88-1.38	**1.40**	**1.09-1.78**
	Unknown	1.17	0.78-1.75	1.22	0.83-1.80
Net monthly income[b]					
	<850/<970	Ref		Ref	
	851-1150/971-1335	1.14	0.75-1.73		
	1151-1750/1970-3314	0.93	0.62-1.39		
	1751-3050/1970-3314	**0.68**	**0.46-0.98**		
	3051-3500/3315-3500	**0.45**	**0.28-0.73**		
	>3501	**0.61**	**0.41-0.90**		
	Unknown	0.73	0.48-1.10		
Ethnicity[c]					
	Dutch	Ref		Ref	
	1st generation migrant	**2.27**	**1.83-2.81**	**2.47**	**1.97-3.10**
	2nd generation migrant	**1.57**	**1.04-2.36**	1.27	0.85-1.91
Smoking ever					
	No	Ref		Ref	
	Yes	1.20	0.99-1.45		
	Unknown	**1.49**	**1.07-2.06**		
Alcohol use					
	No	Ref		Ref	
	Yes	**1.29**	**1.03-1.62**		
	Unknown	**1.61**	**1.15-2.27**		
BMI					
	<18.5	Ref		Ref	
	18.5-25	1.07	0.52-2.20		
	25-30	1.16	0.56-2.40		
	>30	1.29	0.61-2.72		
	Unknown	1.38	0.65-2.91		
Current steady partner					
	No	Ref		Ref	
	Yes	**0.71**	**0.59-0.90**		
	Unknown	0.96	0.58-1.57		
Firsttime					
	<17 years	Ref		Ref	
	17-19 years	**0.77**	**0.61-0.95**		
	≥ 20 years	**0.52**	**0.0-0.67**		
	Unknown	0.79	0.58-1.07		
History STD					
	No	Ref		Ref	
	Yes	**2.92**	**2.27-3.77**	**1.54**	**1.18-2.01**
	Unknown	1.22	0.93-1.60	1.04	0.74-1.47
Condom use					
	No	Ref		Ref	
	Yes	1.07	0.82-1.40		
	Unknown	0.88	0.62-1.26		
Partners last 6 months(sexual)					
	0	Ref		Ref	
	1-2	0.92	0.73-1.17		
	>2	**3.03**	**1.58-5.78**		
	Unknown	0.91	0.64-1.29		
Partners lifetime (sexual)					
	1-2	Ref		Ref	
	3-5	**2.22**	**1.75-2.81**	**2.03**	**1.60-2.59**
	6-9	**3.71**	**2.80-4.90**	**3.47**	**2.58-4.67**
	>10	**6.93**	**5.30-9.06**	**6.23**	**4.68-8.29**
	Unknown	**2.72**	**1.93-3.83**	**2.55**	**1.66-3.93**

[a] According to definition of CBS in 2018.[B] Left 2006-07 survey, right 2016-17 survey. [C] Country of birth or country of birth of parents.

Table 4 Pooled analysis of the 2006-7 and 2016-17 survey after adjustments sociodemographic characteristics

	Men N= 3493		Women N= 4369	
	HPV seropositive n (%)	aPR (95% CI)	HPV seropositive n (%)	aPR (95% CI)
Any HPV type				
2006-2007	366 (21.0)	Ref	596 (26.0)	**Ref**
2016-2017	361 (20.6)	0.99 (0.83-1.17)	989 (33.1)	**1.16 (1.02-1.32)**
HPV16				
2006-2007	192 (11.0)	**Ref**	279 (12.2)	
2016-2017	137 (7.8)	**0.71 (0.55-0.91)**	363 (17.5)	**1.29 (1.07-1.55)**
HPV18				
2006-2007	130 (7.5)	Ref	128 (5.6)	**Ref**
2016-2017	98 (5.6)	0.77 (0.57-1.05)	187 (9.0)	**1.31 (1.01-1.70)**
HPV31				
2006-2007	49 (2.8)	**Ref**	80 (3.5)	**Ref**
2016-2017	29 (1.7)	0.66 (0.38-1.12)	128 (6.2)	**1.54 (1.11-2.14)**
HPV33				
2006-2007	111 (6.4)	Ref	151 (6.6)	**Ref**
2016-2017	96 (5.5)	0.89 (0.65-1.22)	186 (9.0)	1.08 (0.84-1.38)
HPV45				
2006-2007	123 (7.1)	**Ref**	184 (8.0)	**Ref**
2016-2017	180 (10.3)	**1.47 (1.13-1.92)**	222 (10.7)	1.16 (0.92-1.45)
HPV52				
2006-2007	103 (5.9)	Ref	167 (7.3)	**Ref**
2016-2017	92 (5.3)	0.95 (0.68-1.32)	212 (10.2)	**1.27 (1.00-1.62)**
HPV58				
2006-2007	70 (4.0)	Ref	113 (4.9)	**Ref**
2016-2017	54 (3.1)	0.71 (0.47-1.06)	152 (7.3)	1.28 (0.96-1.70)

DISCUSSION

In this study, we assessed the (natural) infection-induced seroprevalence of seven hr-HPV types in the Dutch population before and six to seven years after the introduction of a girls-only bivalent HPV vaccination program, with an uptake varying over the years between 42 to 61%. Surprisingly, HPV seroprevalence in female age cohorts of 15 years and older has increased in a ten year time period, mainly due to a significant increase in HPV16, 18, 31 and 58. In men, however, seroprevalence for any hr-HPV type remained similar with a decreasing trend found for HPV16 and increasing trend for HPV45.

We restricted the analyses to unvaccinated individuals thereby estimating naturally acquired and cumulative type-specific HPV exposure. The increase in seroprevalence with age for women was in line with the age of sexual debut. The peak in HPV-seropositivity was highest in women aged 30-39, which has been reported in other publications [13-15]. This peak in seroprevalence around ten to twenty years after sexual debut might reflect repeated exposures resulting in a subsequent increase of the seroconversion rate to induce a detectable antibody response [16].

In the 2006-07 survey, the seroprevalence in middle-aged and older women declined at an earlier age than in the 2016-17 survey, where levels started to decrease from 70 years and onwards. In males, this decline in HPV seropositivity is not seen. The slight decrease of seropositivity observed in older women could be explained by waning of antibodies which was suggested by Geijferstam *et al.* [4]. This would mean that seroprevalence is underestimating lifetime cumulative exposure. Alternatively, it could reflect a cohort effect, which is more likely as this effect is not seen in both sexes. Indeed, although age of sexual debut is similar, the sexual behavior pattern differs in the younger women having more lifetime sexual partners than the older women in this cohort [17].

Hr-HPV antibodies, albeit at very low concentrations, could be detected in children, which confirms other population studies [10, 16]. These antibodies might be derived from vertical or horizontal transmission [18].

In both surveys, a lower seroprevalence is observed in males compared to women, which is conform other population studies [10, 13, 15, 16, 19]. It is unlikely that the overall lower seroprevalence seen in males is due to lower infection rates, because males reported a significantly higher number of lifetime sexual partners compared to females in the 2016-17 cohort. HPV DNA prevalence studies showed similar results among both sexes [20, 21]. The fact that women display a higher seroprevalence then men is likely to be explained by the anatomic site of the HPV infection, influencing its immune response. Infections at the epithelium of the cervix and anal tract induce a higher immune response in comparison to infections that occur at the keratinized epithelia, such as genital skin. [22-25].

Seroprevalence for HPV16 was highest of all HPV types in both surveys, which is in accordance with other population studies [15, 16, 26-29]. In a ten-year time period HPV type-specific seroprevalence for HPV16, 18, 31, and 58 has increased in the female population of 15 years and older in the Netherlands. In addition, being seropositive for one up to all seven types increased

over the years. This is possibly explained by the observed change towards a higher number of sexual partners in the last 6 months and history of self-reported STDs in the 2016-17 survey compared to the 2006-07 survey.

The risk factor analysis was restricted to the HPV unvaccinated, sexually active population from 15 years onwards and stratified for sex. For women several behavioral factors, such as number of lifetime partners, history of STDs, and ethnicity, were independently associated with HPV seropositivity, which was also found in other studies [10, 15, 30, 31]. Alcohol use and smoking were only associated with HPV seropositivity in the univariate model. This is especially interesting with respect to the current increased risk in HPV-associated head and neck cancers [32, 33]. In these studies, alcohol use is often allied together with tobacco use, however, in our study we could not find an association with smoking or alcohol use in the multivariate model. Other studies find varying results, showing a negative association [29], a positive association [14] [34], or no association at all [35]. Thereby leaving the relation between smoking and HPV seropositivity unclear.

In the male part of our study, we only found history of self-reported STDs to be significantly associated with HPV seropositivity in the univariate analyses. In the backward selection model this factor did not remain independently associated with HPV seropositivity anymore. Comparison with other studies is challenging as most population studies combine men and women in their risk factor analysis. Studies including separate male analysis reported a variety of associated factors. Most consistent findings were associations related to age [13, 31, 36-42], number of male anal sexual partners [30, 40, 41, 43] and (self-reported) circumcision [44]. Number of male anal sexual partners and (self-reported) circumcision were unfortunately not included in our questionnaire. In addition, some of these studies were performed among men who have sex with men (MSM), which is considered a high-risk population with specific behavior, complicating direct comparison.

Also after pooling both surveys, and adjusting for demographic and sexual risk factors the increase in HPV seroprevalence in women in the years after the introduction of the HPV vaccine remained significant.

An interesting finding is the decreased HPV16 seroprevalence between the two surveys for the Dutch male population. Although this might be due to herd immunity of the girls-only vaccination program, among 15-39 year old men we observed a not statistically significant decline, while they seem likely to be the first age groups benefitting from the girls-only vaccination. In the even younger age-group of 10-14 year old boys we did find a difference between the two surveys for any hr-HPV type, but when excluding the vaccine types this difference was not significant anymore. This indicates that the, albeit minor, difference in seroprevalence in the 10 year period was mainly attributable to the vaccine types. Please note that in this age group the seroprevalences could not be adjusted as questionnaires including sexual behavior were only filled in by people above 15 years of age. In Australia, with high vaccine uptake percentages, herd protection impacts on seroprevalence in males (15-39 years of age) from a girls-only vaccination program were clearly shown five years after introduction of HPV vaccination [45]. Moreover, even a benefit for the non-vaccinated females was observed [46]. In contrast, no reduction was found

in HPV seropositivity in males followed by a girls-only vaccination program in a study in the US with comparable vaccination coverage in a girls-only program as in the Netherlands [47]. With a vaccination coverage of approximately 50% among vaccine-eligible girls in the Netherlands in 2016-17, herd effects on seroprevalence in the male part of the population might therefore be less pronounced. Nevertheless, in recent analysis among STI clinic visitors in the Netherlands both first order herd immunity effects among unvaccinated males as well as second order herd immunity effects in unvaccinated women were found [48]. However, this was measured through infection rates where effects can be detected earlier than by seroprevalence.

A strength of this study is that we compared two surveys with a broad age range, one before and one six to seven years after introduction of the HPV vaccination program, thereby enabling us to evaluate this program at a population level. An additional strength of this study is that an identical methodology and antibody assay is used between the two surveys. It must be kept in mind that the VLP sources have changed over time and could possibly cause some variance, for which we corrected by using QC and bridging. Furthermore, this study used two-stage cluster sampling strategy, including oversampling of minorities [7, 8], therefore being representative of the total Dutch population.

The use of different techniques and associated cut off levels hinders the comparison with other (population) studies. International standardization for all hr-HPV types, in addition to HPV16 and 18 which are applied in this study, could help to overcome this difficulty in future studies. Besides this, it could be argued that HPV seropositivity is not a conclusive marker for cumulative exposure. HPV has the capability to evade the host immune system and as a consequence, detectable HPV-specific antibodies in serum are only developed in approximately 50-70% of HPV-infected individuals [22]. Thus, seroprevalence will underestimate the actual lifetime HPV exposure and infection rate. Moreover, it should be noted that questions regarding sexual behavior were among the least well-completed. Self-reporting of sexual behavior could lead to bias due to social desirability and this was also illustrated by our risk factor analysis for some variables (e.g., a high unknown category).

HPV prophylactic vaccination programs are most effective when offered to non-sexually active pre-adolescents. In the Netherlands, the age of receiving the HPV vaccine is approximately twelve years old and recently the Health Council has advised to lower the age to nine years. Our data supports this change as HPV seropositivity begins to increase markedly after ten years of age. In addition, the Health Council also advised to implement a sex-neutral HPV program which will be effective from 2021. On top of that, a catch-up vaccination will be offered to all young adults up to 26 years of age [49].

To conclude, our data showed that HPV infection-specific seroprevalence in women has increased in the Netherlands in a ten-year period. In men, however, seroprevalence for any hr-HPV type remained similar, a decrease was found for HPV16 and an increase for HPV45. Whether the decline in HPV16 is a first sign of a herd effect remains uncertain since a less pronounced effect was observed in men aged 15-39 years of age, where we would have expected that a herd effect would be visible first. Future seroprevalence studies will be interesting to capture the effect of a longer follow up period after introduction of the girls-only program and possibly effects of the sex-neutral vaccination.

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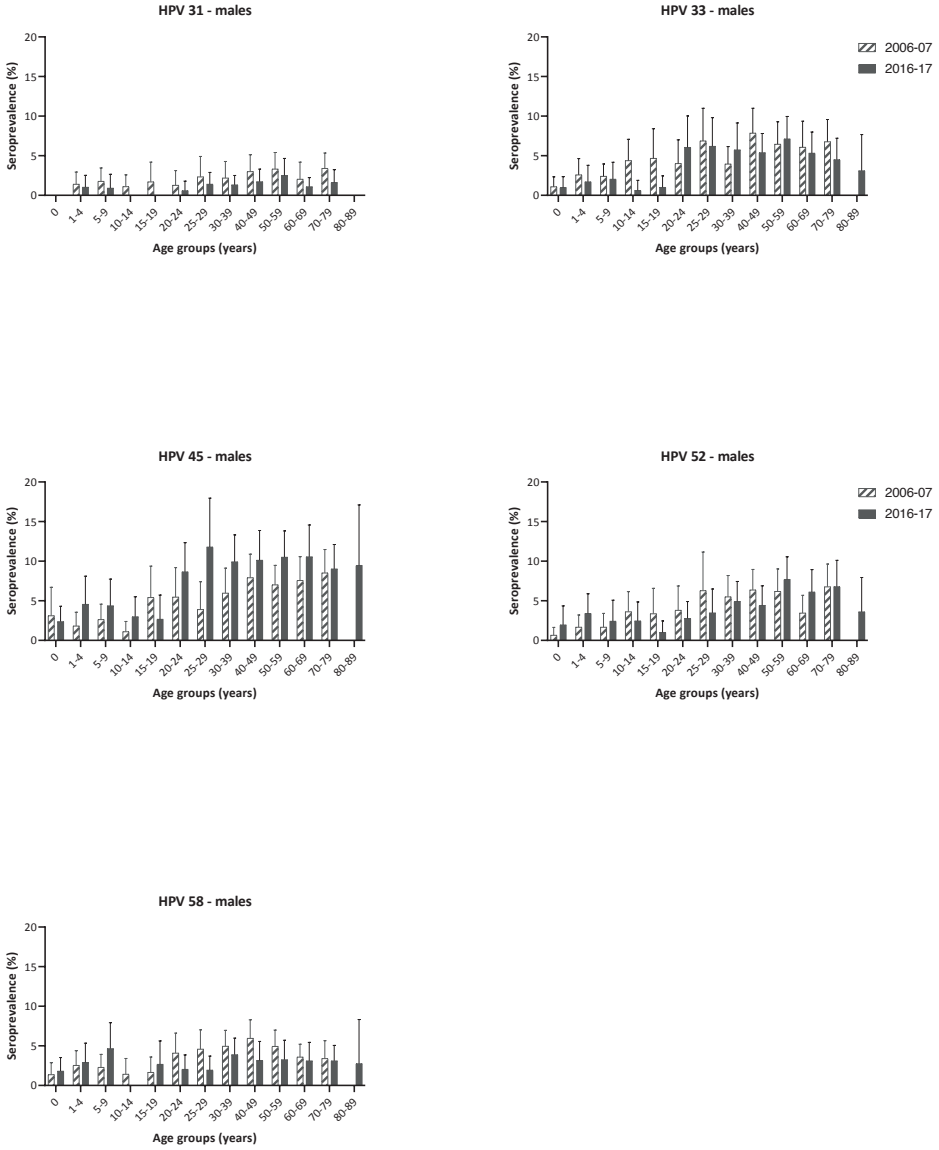
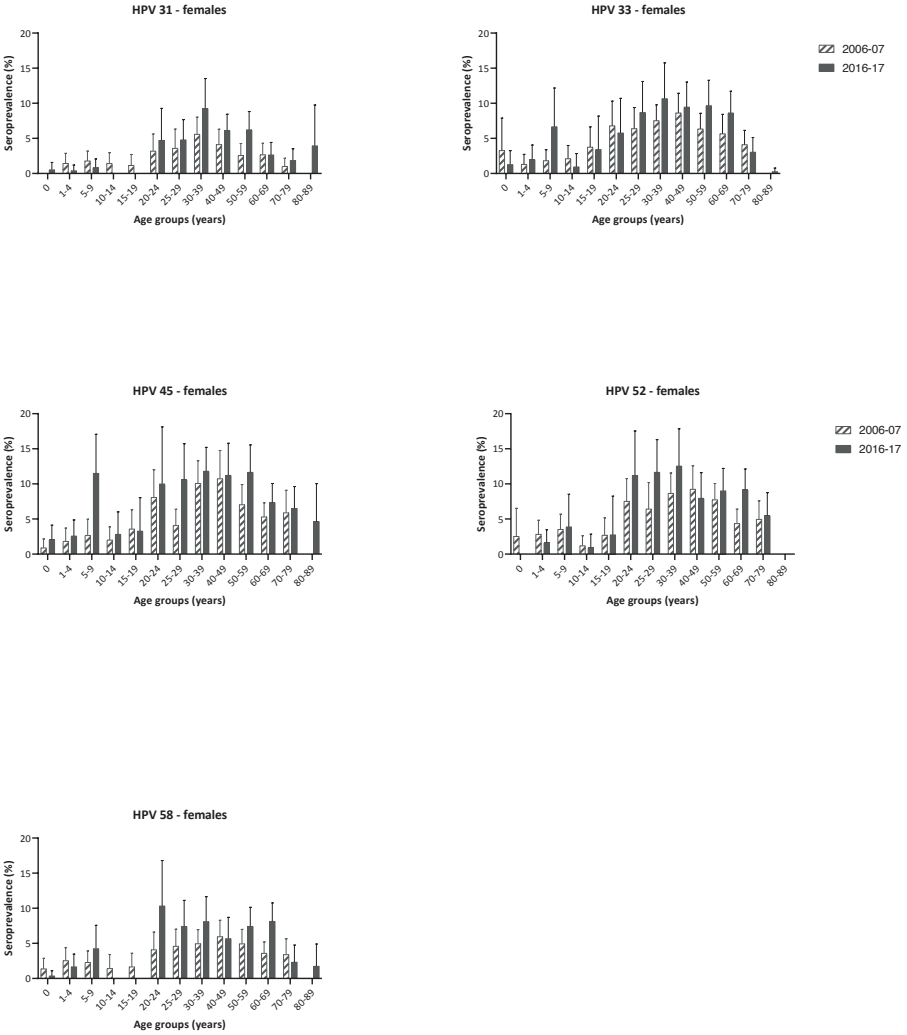
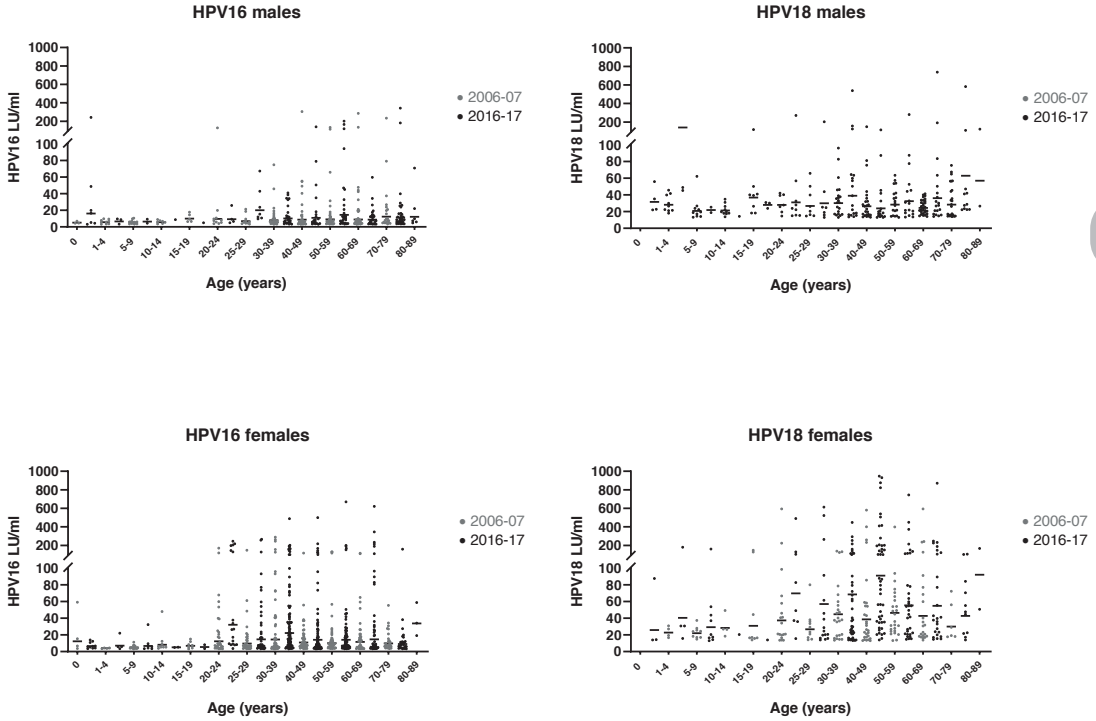
SUPPLEMENTARY MATERIALS



Supplementary Figure 1 Age-specific seroprevalence (%) (with 95% confidence intervals) of HPV31(a), 33(b), 45(c), 52(d) and 58(e) in the unvaccinated male population of the Netherlands.



Supplementary Figure 2 Age-specific seroprevalence (%) (with 95% confidence intervals) of HPV31(a), 33(b), 45(c), 52(d) and 58(e) in the unvaccinated female population of the Netherlands.



Supplementary Figure 3 Age-specific geometric mean concentration (GMC) (with 95% confidence intervals (CI)) of HPV16 antibodies in men (a) and women (b) and HPV18 in men (c) and women (d) in the Netherlands.

Supplementary Table 1 Sample sizes of the total population under 15 years of age without vaccination, by sex and survey

Total population under 15 years of age, without vaccination	Men (2006-07) N	Men (2016-17) N	Women (2006-07) N	Women (2016-17) N
0 year	187	202	159	195
1-4 years	267	171	247	167
5-9 years	314	170	306	157
10-14 years	206	167	226	118

CHAPTER 3

HIGH SEROPREVELANCE OF MULTIPLE HIGH-RISK HUMAN PAPILLOMAVIRUS TYPES AMONG THE GENERAL POPULATION OF BONAIRE, ST. EUSTATIUS AND SABA, CARIBBEAN NETHERLANDS

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Hella Pasmans [a]1*, Regnerus A. Vos [a],1,*, Liza Tymchenko [a], Alcira V.A. Janga-Jansen d, Sharda Baboe-Kalpoe [b],Koen Hulshof [c], Hester E. de Melker [a], Fiona R.M. van der Klis [a]

*Both authors contributed equally to this paper.

[a] Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
[b] Department of Public Health, Public Entity St. Eustatius, Cottageroad z/n, Oranjestad, St. Eustatius, Caribbean Netherlands, The Netherlands
[c] Department of Public Health, Public Entity Saba, The Bottom, Saba, Caribbean Netherlands, The Netherlands
[d] Department of Public Health, Public Entity Bonaire, Kaya Neerlandia 41, Kralendijk, Bonaire, Caribbean Netherlands, The Netherland

ABSTRACT

Background Incidence and mortality of human papillomavirus (HPV)-related cancers differs geographically, with high rates in Caribbean countries. Seroepidemiological data provide information on lifetime cumulative HPV exposure and contributing risk factors, but has not been available yet for Caribbean Netherlands (CN), comprising the islands Bonaire, St. Eustatius and Saba. Therefore, a cross-sectional population-based serosurveillance study was performed in this (recently girls-only HPV-vaccinated) population in 2017.

Methods Blood samples from participants (n=1,823, 0-90 years) were tested for seven high-risk (hr)-HPV-specific IgG-antibodies using a VLP-based multiplex-immunoassay. Risk factors for HPV-seropositivity were analysed among persons unvaccinated aged ≥15 years who ever had sex (n=1,080).

Results Among unvaccinated individuals aged ≥15 years, overall seropositivity was high (34.0%), with over half of them being seropositive for ≥2 hr-HPV types, and HPV16 and 52 being most prevalent (13%). Seroprevalence was substantial higher in unvaccinated women (51%) than men (18%), predominantly peaking in women aged 20-59 years, and was highest on St. Eustatius (38%). Besides age and sex, sexual risk factors were associated with HPV-seropositivity.

Conclusions In accordance with the Caribbean region, seroprevalence of multiple hr-HPV types was high in CN. These data corroborate the decision regarding introduction of a sex-neutral HPV-vaccination program and the relevance for considering a population-based cervical cancer screening program.

INTRODUCTION

Human papillomavirus (HPV) is the most common sexually transmitted pathogen in men and women worldwide, approximately infecting 80% of people at some time. Over 200 different HPV genotypes have been identified, of which 40 can infect the genital tract [1]. Persistent infection with high-risk (hr)-HPV types can lead to anogenital- and oropharyngeal cancers, of which cervical cancer is the most prevalent. Annually, 680,000 HPV-related cancers are estimated to occur worldwide, including 570,000 cervical cancer cases [2]. Hr-HPV types 16 and 18 are mostly detected in women and thereby responsible for 70% of all cervical cancer cases [3].

Incidence and mortality of HPV-related diseases differ geographically. For cervical cancer this can be largely explained by presence of organized prevention programs. Caribbean countries, that mostly lack vaccination and cervical cancer screening programs, have a higher than world average incidence and mortality rate with 15.2 and 8.5 per 100,000, respectively, whereas, e.g., in Western Europe this is below average with 6.8 and 2.1 per 100,000, respectively [2, 4, 5]. In Caribbean Netherlands (CN) – consisting of the three Dutch overseas municipalities Bonaire, St. Eustatius and Saba, comprising a diverse ethic population of ~25,000 people – HPV-vaccination has been included in the National Immunization Program since 2013. The quadrivalent vaccine was introduced on St. Eustatius and Saba in 2013, and bivalent vaccine on all three islands in 2015 (two doses for girls aged 9/10 years of age), with coverage in 2018 ranging between 28-67% across islands [6]. A population-based cervical cancer screening program, however, has not been introduced in CN thus far.

Insight into the population-based HPV serostatus provides information on age- and sex-specific lifetime cumulative HPV exposure and past infections of (vaccine-relevant) circulating genotypes, and can be linked to contributing risk factors. Moreover, these insights can serve as a guide for policymakers in their development of future HPV preventive programs, such as consideration of a population-based cervical cancer screening or as a baseline for future evaluation of the vaccination program. For instance, by estimating vaccine uptake (since vaccine-induced antibody levels are far higher than after natural infection), and monitoring changes in epidemiological dynamics of HPV infection after vaccination, including (indirect) herd effects in those ineligible for vaccination (by comparing age- and gender-specific serological profiles pre- and post-vaccination) as well as impact on other HPV types by the vaccine used (cross-protection/replacement) [7]. However, such data have not been available for CN yet; hence, by means of a representative serosurveillance study conducted in this (recently girls-only HPV-vaccinated) population in 2017 for the first time, we describe the seroprevalence of seven hr-HPV types (16, 18, 31, 33, 45, 52, 58) and associated risk factors for HPV-seropositivity.

MATERIALS AND METHODS

Study design and sample collection

A cross-sectional population-based serosurveillance study (Health Study Caribbean Netherlands) was conducted by the National Institute for Public Health and the Environment of the Netherlands (RIVM) in mid-2017. Details of the survey methods, data collection and inclusion have been described previously [8]. Briefly, on Bonaire, St. Eustatius and Saba, an age-stratified

sample, with age strata 0-11, 12-17, 18-34, 35-59 and 60-89 years, was randomly drawn from the population registry (PIVA-V, January 1, 2017). A total of 7,768 eligible individuals were invited. All procedures performed were in accordance with the 1964 Declaration of Helsinki and its later amendments. The Medical Ethics Committee Noord-Holland in the Netherlands approved the study (METC number: M015-022), and, prior to participation, signed informed consent was obtained from all participants aged ≥12 years and, if <18 years of age, also from their parents or legal guardians. In total, 1,900 participants were included in this study (response rate 24.5%).

Participants donated a blood sample – via a finger- or heel prick using the dried blood spot (DBS) method on air-dried filter paper (Whatman® 903 protein saver cards) – and completed a questionnaire on sociodemographics, sexual behaviour (from 15 years of age) and other factors possibly related to HPV infection. Information on HPV-vaccination was collected via vaccination certificates or, if unavailable, retrieved from local public health department if obtainable. Women up till 30 years of age without any documented vaccination record were considered vaccinated if their antibody concentration was within a range of vaccinated adolescent girls from a large cohort measured at the same laboratory (i.e., HPV16 ≥100 Luminex units (LU)/mL and HPV18 ≥50 LU/mL (Hoes et al.; submitted)).

Serological measurements

Blood samples were air-shipped to the laboratory of the RIVM and stored instantly at -80°C awaiting analyses. For the detection of HPV-specific IgG-antibodies levels against HPV L1 virus-like-particle (VLP) 16, 18, 31, 33, 45, 52, 58, a VLP-based multiplex-immunoassay was used, as previously described [9] (VLPs were kindly donated by MSD (Merck & Co, Inc, Kenilworth, NJ). In short, following standard protocol, a 3.2 mm (1/8 inch) punch was taken from the DBS and incubated in phosphate-buffered-saline containing 0.2% Tween-20 and 1% bovine serum albumin (i.e., assay buffer) at 4°C overnight on a shaker to release serum, resulting in a 1:200 dilution [10, 11]. If detection was out of range, samples were further diluted to 1:20,000 in assay buffer. HPV-specific antibodies were detected using R-phycoerythrin conjugated goat anti-human IgG after incubation with VLP-conjugated beads (Bio-Rad Laboratories, Hercules, CA). Blanks, four in-house controls and a standard were used consistently. HPV-specific IgG-antibodies were analyzed using the Bioplex200 system and software (Bio-Rad Laboratories, Hercules, CA), measured in arbitrary LU/mL (and for HPV16 and 18 converted to international units (IU)/mL by dividing LU/mL by 2.8 and 3.3, respectively). Samples were assumed to be seropositive above cutoffs determined via a method by Frey et al. [12] (with 99% one-sided t-values based on n=215 controls, aged 1-6 years from the present study), namely: HPV16: ≥9 LU/mL, HPV18: ≥15 LU/mL, HPV31: ≥9 LU/mL, HPV33: ≥11 LU/mL, HPV45: ≥27 LU/mL, HPV52: ≥19 LU/mL, HPV58: ≥17 LU/mL.

Statistical analyses

Data were analysed in SAS v.9.4 (SAS Institute Inc., Cary, NC) and R v.3.6. Overall seroprevalence and geometric mean concentrations (GMC) for IgG-antibodies against the seven hr-HPV types among the total population were estimated. These data were weighted, taking into account island, sex, age group, country of birth (and for Bonaire neighbourhood too), in order to match the population distribution of each island as of January 1, 2017. Differences in seroprevalence of

HPV-specific antibodies between islands, sex and age were determined by estimating the parameters of the beta distribution of these seroprevalence rates using the methods of moments [13]. Risk ratios, their corresponding 95% confidence intervals (CI) and *p* values were estimated by Monte Carlo simulations of both seroprevalence estimates. Differences in the GMC between islands, sex and age were identified by calculating the differences in logarithmic (ln)-concentrations and tested via a *t*-test. Age-specific seroprevalence, GMC and 95% CI were determined for CN, per islands and sex, and stratified for HPV-vaccination. Seroprevalence for 'any' or 'all' hr-HPV-type(s) refer to the seven hr-serotypes that have been measured in this study. Statistically significance was set at $p<0.05$.

Risk factors were determined for hr-HPV-seropositivity among sexual active and HPV-unvaccinated participants from 15 years of age. Generalized estimating equations with an exchangeable correlation structure was used. Each hr-HPV type was treated as a separate endpoint accounting for multiple antibodies against hr-HPV types per person and ultimately estimating the exposure effect on hr-HPV-seropositivity as a whole. Risk factors included in the model were: island, sex, age group, ethnicity, residency in CN, educational level, smoking, alcohol consumption, body mass index (BMI), having a steady partner, age at sexual debut, sexual partners, sexual preference, condom use, oral contraceptive use, history of sexual transmittable disease(s) (STD) (note: participants with missing values for a specific variable were allocated to a missing category). In univariate analyses, all variables were adjusted for multiple hr-HPV types, and sex and age group thereby taking into account the survey design. Variables in univariate analyses with a $p<0.10$ were included in the multivariate analysis and backward selection (dropping variables one-by-one manually) was then used to identify risk factors in which a $p<0.05$ was considered statistically significant associated. Crude and adjusted odds ratios (ORs) and 95% CIs were estimated as well as unweighted seroprevalence and 95% CI for all studied factors.

RESULTS

Study characteristics

Sociodemographic study characteristics have been described in-depth elsewhere [8]. Shortly, 1,823 persons, aged 3 months to 90 years, donated a blood sample from which HPV-specific IgG-antibodies could be determined and filled-out the questionnaire (Table 1). There were 820 (45%) men and 1,003 (55%) women, and in accordance with the sampling, the largest part resided on Bonaire (*n*=1,124 (62%), followed by St. Eustatius (*n*=478 (26%)) and Saba (*n*=221 (12%)). Most people originated from the Dutch overseas territories (comprising CN, Aruba, Curaçao and St. Maarten) and Suriname (*n*=1,309, 72%), followed by Latin America and other non-Western countries (*n*=280, 16%), and indigenous Dutch & other Western countries (*n*=221, 12%). People from the Dutch overseas territories and Suriname were relatively often present in the study sample of St. Eustatius (82%), whereas this was the case for those from indigenous Dutch and other Western countries (22%) and Latin America and other non-Western countries (16%) on Saba – following their population composition [14]. In total, 102 women were vaccinated against HPV (*n*=73, 27 and 2 in age groups 9-14, 15-19 and 20-29 years, respectively), with relatively most on St. Eustatius (*n*=40 (8%)) and Saba (*n*=17 (8%)), as routine HPV-vaccination was introduced two years earlier than on Bonaire.

Table1 Sociodemographic and sexual behaviour characteristics of participants with a blood sample for HPV IgG antibody determination in the Health Study Caribbean Netherlands, by island

Sociodemographic characteristic	Bonaire *n* (%) *n*=1,124 (61.7)	St. Eustatius *n* (%) *n*=478 (26.2)	Saba *n* (%) *n*=221 (12.1)	Total *n* (%) *n*=1,823
Sex				
Men	503 (44.8)	221 (46.2)	96 (43.4)	820 (45.0)
Women	621 (55.2)	257 (53.8)	125 (56.6)	1,003 (55.0)
Age groups, years				
0-14	373 (33.2)	183 (38.3)	58 (26.2)	614 (33.7)
15-24	125 (11.1)	53 (11.1)	22 (10.0)	200 (11.0)
25-34	110 (9.8)	62 (13.0)	24 (10.9)	196 (10.7)
35-44	78 (7.0)	34 (7.1)	25 (11.3)	137 (7.5)
45-64	259 (23.0)	90 (18.8)	52 (23.5)	401 (22.0)
65-90	179 (15.9)	56 (11.7)	40 (18.1)	275 (15.1)
Ethnicity[*]				
Dutch overseas territories & Suriname	799 (71.2)	384 (82.1)	126 (57.5)	1,309 (72.3)
Indigenous Dutch & other Western countries	143 (12.7)	30 (6.4)	48 (21.9)	221 (12.2)
Latin America & other non-Western countries	181 (16.1)	54 (11.5)	45 (20.6)	280 (15.5)
(Maternal) educational level[b]				
High	170 (15.1)	68 (14.2)	85 (38.4)	323 (17.7)
Middle	297 (26.4)	126 (26.4)	45 (20.4)	468 (25.7)
Low	570 (50.7)	232 (48.5)	80 (36.2)	882 (48.4)
Unknown	87 (7.7)	52 (10.9)	11 (5.0)	150 (8.2)
HPV vaccination[c]				
Yes	45 (4.0)	40 (8.4)	17 (7.7)	102 (5.6)
No	1,079 (96.0)	438 (91.6)	204 (92.3)	1,721 (94.4)
Among participants from 15 years of age *(n_{total}=1,209)*				
Steady partner				
Yes	458 (61.0)	178 (60.3)	89 (54.6)	725 (60.0)
No	264 (35.1)	91 (30.9)	60 (36.8)	415 (34.3)
Unknown	29 (3.9)	26 (8.8)	14 (8.6)	69 (5.7)
Ever had sexual intercourse				
Yes	631 (84.0)	249 (84.4)	140 (85.9)	1,020 (84.3)
No	77 (10.3)	12 (4.1)	11 (6.7)	100 (8.3)
Unknown	43 (5.7)	34 (11.5)	12 (7.4)	89 (7.4)
Among participants from 15 years of age who had sexual intercourse *(i.e., excluding those without) (n_{total}=1,109)*				
Median age at sexual debut	18 (16-20)	17 (15-18)	18 (16-19)	17 (16-19)
Age at sexual debut				
<18	213 (31.6)	94 (33.2)	57 (37.5)	364 (32.8)
≥18	233 (34.6)	67 (23.7)	63 (41.5)	363 (32.7)
Does not want to answer	87 (12.9)	45 (15.9)	12 (7.9)	144 (13.0)
Unknown	141 (20.9)	77 (27.2)	20 (13.6)	238 (21.5)
Lifetime sexual partners				
1	110 (16.3)	22 (7.8)	26 (17.1)	158 (14.3)
2-4	150 (22.3)	53 (18.7)	27 (17.8)	230 (20.7)
≥5	102 (15.1)	34 (12.0)	40 (26.3)	176 (15.9)
Unknown	312 (46.3)	174 (61.5)	59 (38.8)	545 (49.1)
Ever had sexual transmitted disease				
Yes	30 (4.5)	12 (4.2)	16 (10.5)	58 (5.2)
No	644 (96.6)	271 (95.8)	136 (89.5)	1,051 (94.8)

[a]Dutch overseas territories include the islands: Bonaire, Saba and St. Eustatius (i.e., Caribbean Netherlands), and Aruba, Curaçao and St. Maarten. Within ethnic group indigenous Dutch and other Western countries, n=147 (66%) were indigenous Dutch. Within ethnic group Latin America and other non-Western countries, n=261 (93%) were born in Latin America

[b]Maternal educational level was used for participants 0-11y, active education was used for participants 12-25y, and highest accomplished educational level was used for participants >25y. Low=no education, primary school, pre-vocational education (VMBO), lower vocational education (LBO/MBO-1), lower general secondary education (MAVO/VMBO). Middle=intermediate/ secondary vocational education (MBO-2-4), higher/senior vocational education (HAVO), pre-university education (VWO/Gymnasium); High=higher professional education (HBO), University BSc., University MSc., Doctorate

[c]*n*=71 women were vaccinated against HPV according to the vaccination registry and *n*=31 without vaccination records were highly likely to be vaccinated based on IgG antibody concentration and age, see method section for detailed definition (in age groups 9-14, 15-19 and 20-29 *n*=73, 27 and 2 women were vaccinated, respectively).

Missing: ethnicity n=13

Questions related to sexual behavior were completed from age 15 years (*n*=1,209). 60% reported to have a steady partner and 84% ever had sexual intercourse. Among the latter, median age of sexual debut was 17 years (interquartile range (IQR) 16-19). Men had an earlier sexual debut (17 (IQR: 15-18)) than women (18 (16-20)), being lowest for men on St. Eustatius (16 (IQR: 14-18) and Saba (16 (IQR: 15-18). Overall, 16% reported to have had ≥5 lifetime sexual partners. For Saba this percentage (26%) was higher than Bonaire (15%) and St. Eustatius (12%), however, nearly 50% of participants did not complete this question (mostly on St. Eustatius (62%)). Five percent had a self-reported history of a STD (chlamydia was most reported (*n*=35), followed by gonorrhea (*n*=15)), being highest on Saba (11%).

Seroprevalence and GMC

Overall seroprevalence and GMC in CN

Seroprevalence for any of the seven hr-HPV types in CN (0-90 years, *n*=1,823) was 31.3% (95% CI 28.6-34.0) and amounted to 29.7% (95% CI 26.9-32.4) in those unvaccinated (*n*=1,721). GMCs for all hr-types in vaccinated individuals were significantly higher than in unvaccinated individuals, especially for vaccine types HPV16 (GMCs of 246.8 vs. 0.56 IU/mL, respectively) and HPV18 (74.7 vs. 0.74 IU/mL, respectively) (all $p<0.0001$) (Figure 1). Focusing on unvaccinated participants from age 15 years (*n*=1,180), overall seroprevalence was 34.0% (95% CI 30.8-37.3), and antibodies against HPV16 and 52 were detected mostly (both 13.1%), followed by HPV58, HPV18, HPV31, HPV45 and HPV33 (8.9-12.7%) (Table2a). Over half of those seropositive were positive for ≥2 hr-HPV types and a small proportion (2%) was positive for all hr-types.

Overall seroprevalence and GMC, by sex and island

Among those unvaccinated from age 15 years, seroprevalence for any hr-HPV type was significantly higher in women (51.4%) than in men (18.1%) (Table 2a). The same accounted for hr-type specific GMCs (all $p<0.0001$) and hr-type specific seroprevalence, and this sex difference was observed on all islands. HPV16 and 52 were most common in women (20%), and HPV58 (8%), 16 and 52 (both 7%) in men. Women were over 3-fold more often seropositive against ≥2 hr-HPV types than men (28.8 vs. 8.8%), yet seropositivity against all hr-types did not differ between sexes.

St. Eustatius displayed a higher seropositivity against any hr-HPV types (38.4%) as compared to Bonaire (33.4%) and Saba (33.1%) (Table2b), and this was due to a higher seropositivity in both men (23.0%) and (unvaccinated) women (55.7%) on this island. Overall GMCs for all hr-types were also highest on St. Eustatius, and significantly higher for HPV16, 18, 31, 33 and 58 as compared to Bonaire, and for HPV33 and 58 compared to Saba (all $p<0.05$). Also, with exception of HPV52 – which was highest on Bonaire – seropositivity against all other six hr-types was highest on St. Eustatius (with HPV16, 31 and 58 being highest), attributable to higher seroprevalence in men as compared to Bonaire and Saba. Interestingly, seropositivity against all hr-types on St. Eustatius was higher for men (6.8%) than women (1.6%), whereas this was not the case on the other islands.

Table 2 Weighted seroprevalence for seven high-risk HPV types and combinations in the total population of Caribbean Netherlands among those unvaccinated[a] and from 15 years of age, by sex (**a**) and island (**b**)

a)	**Seroprevalence (95% CI)**						
	Overall *n*=1,180		**Men** *n*=505 (42.8%)		**Women** *n*=675 (57.2%)		*P* value[b]
High-risk HPV types							
HPV16	13.1	(11.0-15.2)	6.8	(4.4-9.3)	19.9	(16.5-23.3)	**<0.0001**
HPV18	11.8	(9.7-13.8)	5.6	(3.3-7.9)	18.5	(15.1-21.9)	**<0.0001**
HPV31	10.9	(9.0-12.8)	5.6	(3.4-7.9)	16.3	(13.5-19.7)	**<0.0001**
HPV33	8.9	(7.1-10.8)	6.0	(3.5-8.5)	12.2	(9.4-14.9)	**0.001**
HPV45	9.4	(7.6-11.3)	5.3	(3.1-7.5)	13.9	(11.0-16.9)	**<0.0001**
HPV52	13.1	(10.8-15.4)	7.1	(4.3-9.9)	19.7	(16.2-23.2)	**<0.0001**
HPV58	12.7	(10.5-14.9)	7.5	(4.7-10.3)	18.4	(14.9-21.8)	**<0.0001**
HPV combinations							
HPV16 and 18	5.5	(4.1-7.0)	3.9	(2.1-5.7)	7.4	(5.2-9.5)	**0.02**
HPV16 or 18	19.3	(16.8-21.9)	8.5	(5.8-11.3)	31.1	(27.1-35.1)	**<0.0001**
Positive for 1 or more high-risk HPV types	34.0	(30.8-37.3)	18.1	(14.0-22.2)	51.4	(47.1-55.7)	**<0.0001**
Positive for 2 or more high-risk HPV types	18.1	(15.5-20.6)	8.8	(5.8-11.8)	28.1	(24.3-32.0)	**<0.0001**
Positive for 7 high-risk HPV types	2.0	(1.1-3.0)	2.3	(0.8-3.8)	1.8	(0.6-2.9)	0.61

[a]*n*=29 women were vaccinated (of which *n*=12 according to the registry and *n*=17 without vaccination records highly likely to be vaccinated (based on IgG antibody concentration and age, see method section for detailed definition))
[b]Statistically significant different ($p<0.05$) between men and women in bold type
CI=confidence interval

b)	**Seroprevalence (95% CI)**					
	Bonaire *n*=744 (63.0%)		**St. Eustatius** *n*=278 (23.6%)		**Saba** *n*=158 (13.4%)	
High-risk HPV types						
HPV16	**11.4**[st]	(9.0-13.8)	**20.7**[b]	(15.0-26.4)	18.3	(11.5-25.2)
HPV18	11.3	(8.9-13.7)	15.2	(10.3-20.2)	11.0	(5.7-16.3)
HPV31	**9.6**[st]	(7.4-11.8)	**16.4**[b]	(11.2-21.5)	14.9	(9.0-20.8)
HPV33	8.3	(6.2-10.5)	12.5	(7.8-17.2)	9.2	(4.5-14.0)
HPV45	8.9	(6.7-11.0)	12.6	(7.9-17.3)	9.9	(5.0-14.8)
HPV52	13.7	(11.0-16.4)	10.6	(6.3-15.0)	11.3	(6.1-16.4)
HPV58	12.3	(9.7-14.9)	16.6	(11.3-21.8)	10.0	(5.1-15.1)
HPV combinations						
HPV16 and 18	**4.9**[st]	(3.4-6.5)	**8.2**[b]	(4.2-12.2)	7.4	(3.0-11.9)
HPV16 or 18	17.7	(14.8-20.6)	27.7	(21.5-33.9)	21.9	(14.6-29.2)
Positive for 1 or more high-risk HPV types	33.4	(29.6-37.3)	38.4	(31.7-45.1)	33.1	(24.8-41.3)
Positive for 2 or more high-risk HPV types	17.7	(14.7-20.7)	20.8	(15.4-26.3)	17.0	(10.7-23.3)
Positive for 7 high-risk HPV types	1.5	(0.5-2.6)	4.4	(0.8-7.9)	3.4	(0.3-6.6)

[a]*n*=29 women were vaccinated (of which *n*=12 according to the registry and *n*=17 without vaccination records highly likely to be vaccinated (based on IgG antibody concentration and age, see method section for detailed definition))
[b]Statistically significant different from Bonaire ($p<0.05$) in bold type
[st]Statistically significant different from St. Eustatius ($p<0.05$) in bold type
CI=confidence interval

Age-specific seroprevalence and GMC in CN

In accordance with age of sexual debut, a sharp increase in seropositivity, i.e., a step-up, was observed from 6% in the 0-8 year-olds to 21.1% and 35.6% in the unvaccinated age groups 15-19 and 20-29 years respectively, with similar rise in GMC for all hr-types (Figure S1 and Figure1). Both GMC and seroprevalence peaked in age group 30-39 years (37.8%), and remained stable up till age group 50-59 years and declined in persons of 60 years and above to levels comparable to that of 15-19 year-olds.

Age-specific seroprevalence and GMC, by sex and island

Unvaccinated women had a substantially higher HPV seroprevalence for any hr-type as compared to men between 15-74 years of age (Figure 2). Likewise, a sex difference in seroprevalence and GMC for all seven hr-types was observed for age groups 20-74 years. Although the step-up among adolescents was noticeable among both men and women, it was most pronounced in women in whom seroprevalence increased considerably from 18.8% (in 9-14 years) to 39.8% in 15-19 years, and almost reached 60% in those aged 20-29 years – with greatest step-up seen

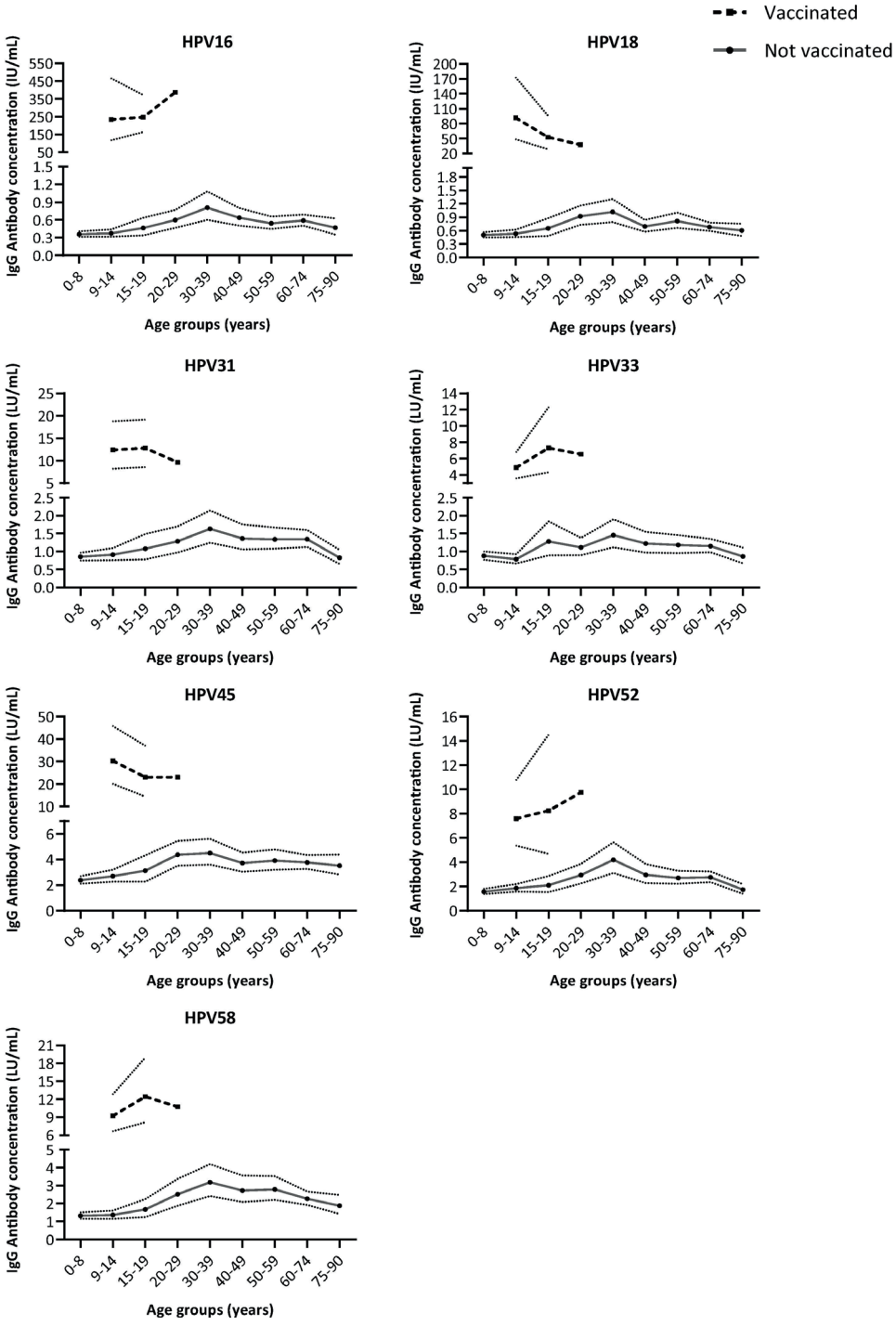

Figure 1 Age-specific geometric mean concentration (GMC) (with 95% confidence intervals (CI)) of seven high-risk types human papillomavirus (HPV) IgG antibodies in the general population of Caribbean Netherlands, 2017, by HPV vaccination. Note: 95% CI was not provided for vaccinated participants in age group 20–29 years due to the low number of participants in this group.

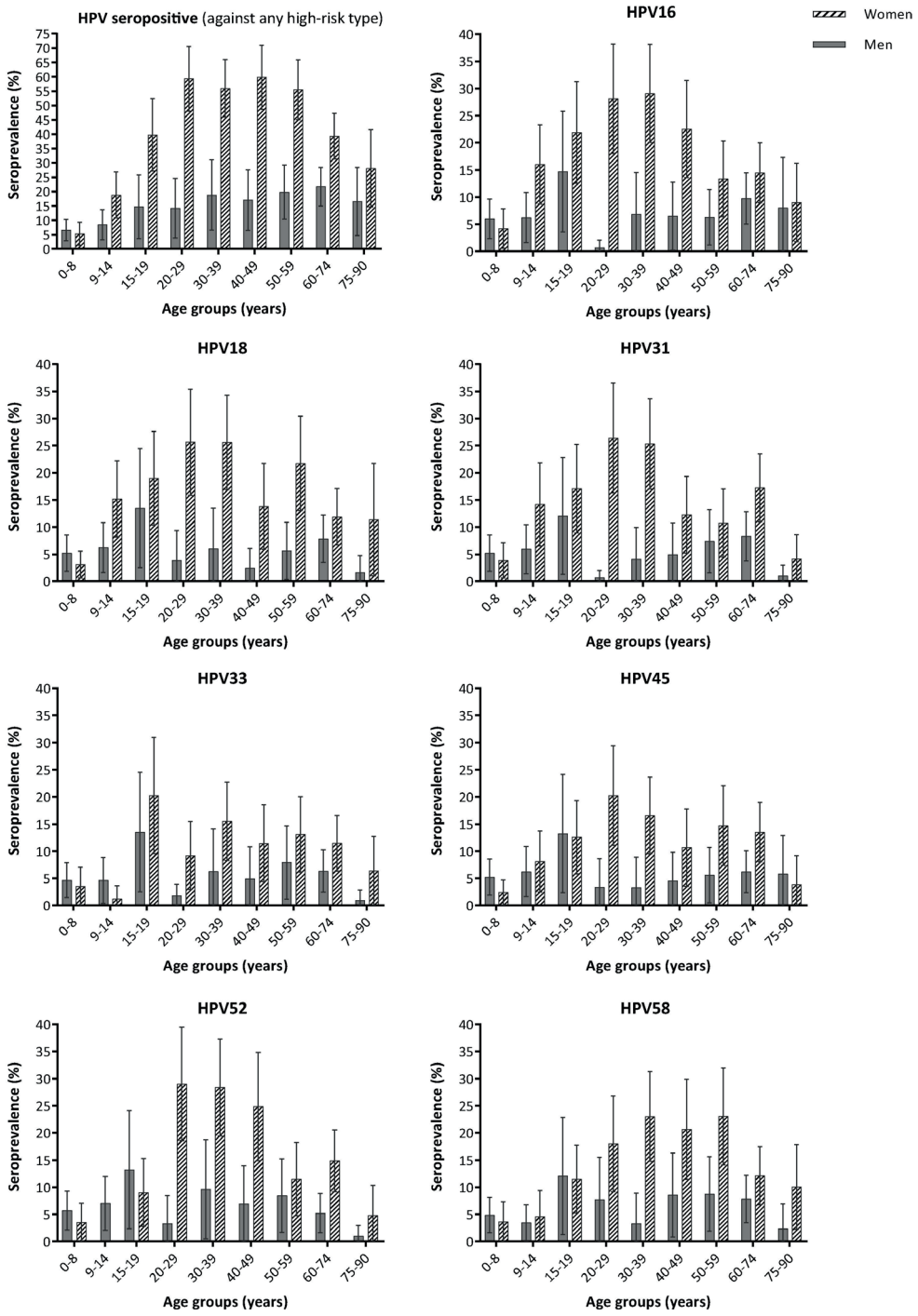

Figure 2 Age-specific seroprevalence (%) (with 95% confidence intervals) of any high-risk type and seven high-risk types human papillomavirus (HPV) IgG antibodies in the unvaccinated general population of Caribbean Netherlands, 2017, by sex.

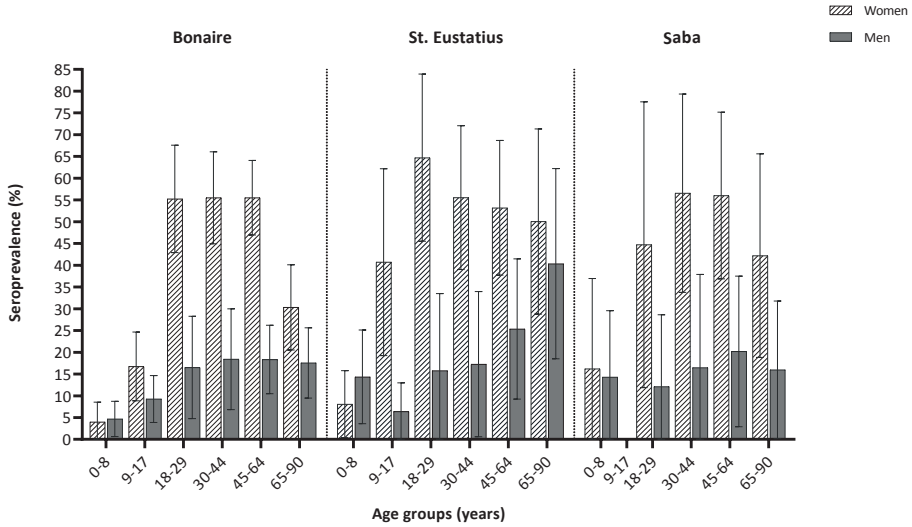

Figure 3 Age-specific seroprevalence (%) (with 95% confidence intervals) of any high-risk type human papillomavirus (HPV) IgG antibodies in the unvaccinated general population of Bonaire, St. Eustatius and Saba, 2017, by sex.

for HPV33 and HPV52. In women aged 20-39 years, seroprevalence was highest (all >25%) for HPV16, 18, 31 and 52. Remarkably, seropositivity for HPV58 in women rose gradually with age, peaking at 50-59 years (23%), and being highest among all seven hr-types in that age group. In men, highest seroprevalence was observed in 15-19 year-olds for all seven hr-types, with rates being similar to women in that age group for HPV45, HPV52 and HPV58.

Seroprevalence was remarkably high for any hr-type in 65-90 year-olds on St. Eustatius (45%) as compared to Bonaire (24%; p=0.008) and Saba (29%; p=0.13). This was primarily due to HPV16, 18 and 58 which were all >15% in this age group (Figure S2). Also, GMCs were significantly higher (data not shown). Specifically, besides women demonstrating a higher seroprevalence in this age group on St. Eustatius, men in particular had a higher seroprevalence as compared to those on the other islands (Figure 3) – with HPV16 and 58 being even higher in men than women on St. Eustatius (FigureS3). Interestingly, among the unvaccinated 9-17 year-olds on Saba no one was seropositive for any hr-type.

Risk factors for hr-HPV-seropositivity

Risk factors for hr-HPV-seropositivity was investigated in the HPV-unvaccinated sexually active participants from age 15 years (n=1,080) (Table 3). In univariate analyses the following variables were significantly associated with HPV-seropositivity: sex, age group, number of lifetime sexual partners and in the preceding year, and history of STD. In multivariate analysis, female sex was found to be the most pronounced determinant, followed by the number of lifetime sexual partners (2-4 and ≥5 vs. 1), being 25-34 years of age (vs. 15-24) and having a history of STD.

Table 3 Risk factor analysis for any high-risk type HPV IgG seropositivity among sexual active and unvaccinated participants from 15 years of age in the Health Study Caribbean Netherlands[a]

Potential risk factor for any high-risk type HPV seronegativity	*n* (%) *n*=1,080	% HPV seropositive (95% CI)	Univariate Crude OR[b] (95% CI)	*P* value[c]	Multivariate aOR[b] (95% CI)	*P* value[c]
Island				0.61		
Bonaire	672 (62.2)	37.5 (33.8-41.2)	Ref.			
St. Eustatius	261 (24.2)	39.5 (33.5-45.4)	1.12 (0.85-1.47)			
Saba	147 (13.6)	37.4 (29.6-45.2)	1.14 (0.81-1.61)			
Sex				**<0.0001**		**<0.0001**
Men	461 (42.7)	19.5 (15.9-23.1)	Ref.		Ref.	
Women	619 (57.3)	51.7 (47.8-55.6)	**2.94 (2.19-3.94)**		**3.34 (2.49-4.49)**	
Age group, years				**<0.0001**		**0.0007**
15-24	102 (9.4)	31.4 (22.4-40.4)	Ref.		Ref.	
25-34	189 (17.5)	54.0 (46.9-61.1)	**1.74 (1.09-2.80)**		**1.68 (1.04-2.73)**	
35-44	134 (12.4)	38.1 (29.8-46.3)	0.93 (0.55-1.57)		0.92 (0.54-1.59)	
45-64	383 (35.5)	37.6 (32.7-42.5)	0.99 (0.62-1.57)		1.00 (0.62-1.61)	
65-90	272 (25.2)	29.8 (24.3-35.2)	0.71 (0.43-1.17)		0.77 (0.46-1.28)	
Ethnicity				0.26		
Dutch overseas territories[d] and Suriname	675 (62.5)	37.9 (34.3-41.6)	Ref.			
Indigenous Dutch and other Western countries	178 (16.5)	32.0 (25.2-38.9)	0.85 (0.60-1.20)			
Latin America and other non-Western countries	227 (21.0)	42.7 (36.3-49.2)	1.18 (0.89-1.58)			
Resident of Caribbean Netherlands since, years of age				0.37		
0-10	529 (49.0)	38.4 (34.2-42.5)	Ref.			
11-17	25 (2.3)	44.0 (24.5-63.5)	1.23 (0.64-2.34)			
18-39	286 (26.5)	38.8 (33.2-44.5)	0.83 (0.62-1.11)			
≥40	189 (17.5)	34.4 (27.6-41.2)	0.92 (0.66-1.29)			
Missing	51 (4.7)	39.2 (25.8-52.6)	1.46 (0.79-2.69)			
Educational level[e]				0.90		
High	225 (20.8)	39.6 (33.2-46.0)	Ref.			
Middle	236 (21.9)	39.4 (33.2-45.7)	0.98 (0.68-1.40)			
Low	520 (48.1)	35.6 (31.5-39.7)	1.08 (0.81-1.45)			
Missing	99 (9.2)	43.4 (33.7-53.2)	1.05 (0.69-1.60)			
Current smoking				0.93		
Yes	163 (15.1)	33.1 (25.9-40.4)	Ref.			
No	888 (82.2)	39.0 (35.8-42.2)	0.93 (0.65-1.33)			
Missing	29 (2.7)	34.5 (17.2-51.8)	0.95 (0.40-2.26)			
Drunk alcohol in preceding year				0.33		
Yes	651 (60.3)	39.3 (35.6-43.1)	Ref.			
No	381 (35.3)	36.5 (31.6-41.3)	0.84 (0.66-1.07)			
Missing	48 (4.4)	31.3 (18.1-44.4)	0.85 (0.45-1.64)			
Body Mass Index				0.27		
Underweight	15 (1.4)	33.3 (9.4-57.2)	0.94 (0.41-2.13)			
Normal weight	280 (22.9)	32.1 (26.7-37.6)	Ref.			
Overweight	352 (32.6)	38.1 (33.0-43.1)	**1.39 (1.01-1.91)**			
Obesity	357 (33.1)	43.1 (38.0-48.3)	1.30 (0.96-1.77)			
Missing	76 (7.0)	35.5 (24.7-46.3)	1.12 (0.67-1.88)			
Steady partner				0.22		
Yes	689 (63.8)	38.9 (35.3-42.5)	Ref.			

No	327 (30.3)	35.8 (30.6-41.0)	1.03 (0.79-1.35)			
Missing	64 (5.9)	39.1 (27.1-51.0)	**1.70 (1.02-2.83)**			
Age at sexual debut, years				0.13		
<18	356 (33.0)	42.1 (37.0-47.3)	**1.39 (1.04-1.85)**			
≥18	359 (33.2)	37.3 (32.3-42.3)	Ref.			
Does not want to answer	140 (13.0)	37.1 (29.1-45.2)	1.34 (0.93-1.92)			
Missing	225 (20.8)	32.9 (26.7-39.0)	1.25 (0.88-1.76)			
Lifetime sexual partners				**<0.0001**		**<0.0001**
1	155 (14.4)	26.5 (19.5-33.4)	Ref.		Ref.	
2-4	225 (20.8)	38.7 (32.3-45.0)	**1.89 (1.25-2.85)**		**1.85 (1.22-2.79)**	
≥5	171 (15.8)	43.9 (36.4-51.3)	**2.42 (1.57-3.72)**		**2.24 (1.44-3.48)**	
Missing	529 (49.0)	39.1 (35.0-43.3)	**2.91 (1.99-4.24)**		**2.88 (1.97-4.19)**	
Sexual preference				0.81		
Heterosexual	812 (75.2)	37.3 (34.0-40.6)	Ref.			
Homosexual	14 (1.3)	35.7 (10.6-60.9)	1.03 (0.43-2.45)			
Bisexual	32 (3.0)	56.3 (39.0-73.5)	1.09 (0.65-1.84)			
Does not want to answer	74 (6.8)	37.8 (26.8-48.9)	1.35 (0.84-2.16)			
Missing	148 (13.7)	37.8 (30.0-45.7)	1.11 (0.77-1.61)			
Sexual partners preceding year				**0.043**		
0	244 (22.6)	32.0 (26.1-37.8)	Ref.			
1	557 (51.6)	41.7 (37.6-45.8)	**1.51 (1.09-2.10)**			
≥2	70 (6.5)	40.0 (28.5-51.5)	1.33 (0.79-2.26)			
Does not want to answer	48 (4.4)	29.2 (16.3-42.0)	1.51 (0.73-3.13)			
Missing	209 (14.9)	34.4 (28.0-40.9)	**1.81 (1.19-2.75)**			
Condom use last sexual intercourse				0.67		
Yes	166 (15.4)	38.0 (30.6-45.3)	Ref.			
No	613 (56.7)	37.7 (33.8-41.5)	0.81 (0.58-1.13)			
Does not want to answer	58 (5.4)	29.3 (17.6-41.0)	0.88 (0.46-1.69)			
Missing	243 (22.5)	40.7 (34.6-46.9)	0.87 (0.59-1.28)			
Oral contraceptive use last sexual intercourse				0.47		
Yes	117 (10.8)	49.6 (40.5-58.6)	1.32 (0.95-1.85)			
No	626 (58.0)	36.1 (32.3-39.9)	Ref.			
Does not want to answer	51 (4.7)	29.4 (16.9-41.9)	1.02 (0.55-1.90)			
Missing	286 (26.5)	38.8 (33.2-44.5)	1.09 (0.82-1.44)			
Ever had sexual transmitted disease				**0.01**		**0.02**
Yes	55 (5.1)	65.5 (52.9-78.0)	**1.68 (1.18-2.39)**		**1.64 (1.12-2.40)**	
No	1025 (94.9)	36.5 (33.5-39.4)	Ref.		Ref.	

[a]*n*=100 participants had not been sexual active, and *n*=29 women were vaccinated (of which *n*=12 according to the registry and *n*=17 without vaccination records highly likely to be vaccinated (based on IgG antibody concentration and age, see method section for detailed definition))

[b]Crude odds ratios were a priori adjusted for HPV high-risk type, sex and age group and significant (a)ORs are marked in bold type

[c]*P* values were determined by means of Chi-Square tests for GEE analysis, and significant *p* values (<0.1 in univariate and <0.05 in multivariate analysis) are marked in bold type

[d]Dutch overseas territories include the islands: Bonaire, Saba and St. Eustatius (i.e., Caribbean Netherlands), and Aruba, Curaçao and St. Maarten

[e]Active education was used for participants 15-25y, and highest accomplished educational level was used for participants >25y. Low=no education, primary school, pre-vocational education (VMBO), lower vocational education (LBO/MBO-1), lower general secondary education (MAVO/VMBO). Middle=intermediate/ secondary vocational education (MBO-2-4), higher/senior vocational education (HAVO), pre-university education (VWO/Gymnasium); High=higher professional education (HBO), University BSc., University MSc., Doctorate

Abbreviations: *aOR*, adjusted odds ratio; *CI*, confidence interval; *OR*, odds ratio; *Ref.*, reference category

DISCUSSION

For the first time we describe the seroepidemiology of IgG-antibodies against the hr-HPV types 16, 18, 31, 33, 45, 52 and 58 in the population of Caribbean Netherlands, situated in a region with a high incidence of HPV-related cancers [2, 4, 5]. Seropositivity for multiple hr-HPV types was high in the unvaccinated population, with antibody responses against HPV16 and 52 being detected mostly. In general, women had a nearly 3-fold higher seroprevalence compared to men, predominantly peaking in women aged 20-59 years. Seropositivity for six hr-types was highest on St. Eustatius, which was particularly attributable to older men. Besides age and sex, risk factors related to sexual behavior were found to be associated with HPV-seropositivity in unvaccinated participants from age 15 years.

The incidence of cervical cancer is high in Caribbean countries. Recent estimations on Suriname and neighbouring island Curaçao revealed that incidence is 22.4 and 13.4 per 100,000, respectively [15, 16], whereas this is lower in the Netherlands (7.5 per 100,000) [17]. Despite this high incidence, only few studies have been conducted on HPV seroepidemiology in the Caribbean region; data which is key in developing preventive programs. In CN, over one third of the unvaccinated population from age 15 years was seropositive against any hr-HPV type measured, and over half of them had detectable antibodies against multiple hr-types. HPV16 and 52 were the most common hr-types (both 13%), followed by 58, 18 and 31. These observations are within a broad range found in (the few) other studies conducted in the Caribbean region [18-20], with exception of Jamaica where an even higher seroprevalence (50%) was found for HPV16 [21]. Conversely, seropositivity in CN was higher as compared to Western countries [22-25], for instance in the Netherlands [9, 26]; a country in which girls-only vaccination has been introduced since 2009 and population-based cervical screening has been in place since 1996. Still, prior to vaccination, seroprevalence in the Netherlands was lower than the present estimates in CN, with higher rates among people from Latin America and Caribbean descent [9], similar to the present study.

HPV-specific antibodies could already be detected in young children who are not likely to be sexual active which is in accordance with other population studies [24, 27]. This implies that the route of HPV-transmission is not only by sexual contact, but also for instance via vertical or horizontal transmission and autoinoculation [28]. Further, participants who had been vaccinated displayed an significant antibody response against hr-HPV vaccine types 16 and 18 as well as against the non-vaccine types. This cross-reactivity has been observed by others [29-32]. Interestingly, no one was HPV-seropositive among the unvaccinated 9-17 year-olds on Saba. Although HPV-vaccination was introduced for 9 year-old girls on Saba in 2013 and vaccine coverage has been high since a herd effect due to vaccination might seem too early. As ~25% of this total age group on Saba responded in this study and the included numbers are low, future serosurveillance studies in CN should shed more light on this observation. Similar findings were not observed on the other islands; which might be explained by a lower vaccination coverage and very recent introduction of HPV-vaccination on Bonaire (two years prior to this study in 2015).

Female sex was the strongest predictor for being HPV-seropositive and women had a substantial higher overall seroprevalence than men in the total CN-population (51% vs. 18%). Seropreva-

lence (and GMC) rose quickly in adolescents and young adult women, corresponding to the age of sexual debut. This steep increase is in line with other studies [9, 19, 24, 33], and highlights the necessity to promote early education on HPV-vaccination and safe(r) sexual practices to prevent STDs in general. From age 60 years, seroprevalence decreased to rates comparable to 15-19 year-olds, possibly as a result of antibody waning or due to a cohort effect, i.e., decreasing sexual behavior over time, as earlier hypothesized [22]. Although the dissimilarity between sexes is in accordance with other studies, it was more pronounced than observed in other countries [9, 22]. After stratifying sexual risk behavior by sex, women were shown to have similar patterns as men (data not shown). It should be noted, however, that questions regarding sexual behavior were among the least well-completed, especially by men. Self-reporting of sexual behavior could lead to bias due to social desirability and this was also illustrated by our risk factor analysis for some variables (e.g., the missing category for lifetime sexual partners had the highest OR). It is known from literature, however, that Caribbean men more often report about multiple partnerships than women [34]. This could result in increased exposure to (multiple) HPV types in both sexes when compared to other populations. Subsequently, the fact that women display a substantial higher seroprevalence in this population might be explained by the different site of entry of the infection between sexes. As mucosal surfaces are infected in women predominantly, a detectable humoral immune response is more likely to be expected as compared to an infection at epithelial surfaces which mainly occur in men, as suggested by Desai and colleagues [22]. Hence, although increased sexual behavior in men will result in increased seropositivity, it will probably not be so pronounced as in women.

HPV-seropositivity for any hr-type was highest on St. Eustatius, followed by Saba and Bonaire, and seroprevalence (and GMC) for all measured hr-types, except HPV52, was highest on St. Eustatius too. Both women and men displayed higher seroprevalence rates on St. Eustatius as compared to the other islands, and particularly rates in men from 65 years of age were higher – predominantly due to HPV16 and 58. Increased sexual behavior on St. Eustatius most likely explains the difference between islands in general, and specifically among men. For instance, on this island, highest proportion for seropositivity against all seven hr-types was found in men as well as lowest age of sexual debut (16 years of age). The questionnaire data could not confirm this for other sexual risk factors, probably due to the high number of missing values for these variables on St. Eustatius.

Various potential risk factors for HPV-seropositivity were investigated in this study. Beside female sex, and being a young adult (25-34 years), increased number of lifetime sexual partners and a history of STD and are in line with other studies [19, 20, 35, 36]. Literature has been inconsistent on the influence of other factors, such as smoking, condom use, BMI and oral contraceptive use [18, 19, 36-40]. In this study, all these factors were not associated in our multivariate analysis, suggesting no relationship with HPV-seropositivity.

This study is subject to potential limitations. A direct comparison between HPV-serology studies is hindered by the use of different assays and methods [41]. Due to logistical reasons, we made use of the DBS-method in this study to collect our samples, and although we eluted these via a standardized and validated protocol, marginal difference with serum samples might not be inconceivable. Also, international standardization for all hr-HPV types, which has already been

done for HPV16 and 18 and applied in this study, could help to overcome this difficulty in future studies. Direct comparison of data was possible with the population-based study performed in the Netherlands [9, 26], which was conducted, measured and analyzed in a similar way. Additionally, our cutoffs were determined via a statistically valid and widely used method in the field of immunoassays. Moreover, in particular men aged 18-34 years were relatively hard to include in our study; a common phenomenon in population-based studies [8, 42]. Hence, especially on the smaller islands St. Eustatius and Saba, this limits stratifying for multiple variables, and due to possible loss of power one should not exclude potential related bias. To minimize this, we have weighted our sample on a set of sociodemographic characteristics corresponding to the island's population at the time of enrollment. Further, we cannot draw firm conclusions on the rate of current HPV infections in this study as not all infected persons will develop a quantifiable antibody response, seroconversion might be delayed or HPV DNA has been cleared [43]. Likewise, risk factors for HPV-seropositivity do not necessarily reflect determinants for current HPV-infections.

Our findings are of great importance for policy implications. Firstly, girls in CN are currently vaccinated twice at age 9/10 years and this age is justifiable by the observed step-up in seroprevalence of multiple (vaccine-relevant) hr-types in those 15-19 years, which is indicative for the age of HPV-exposure in this population. Secondly, in June 2019 the Dutch Health Council advised to expand the National Immunization Program by offering the HPV vaccine also to boys [44]. As the burden of HPV-related cancers among men is substantial in the Caribbean region [14] and HPV-seropositivity among men was shown to be significant too, a sex-neutral vaccination program in CN will lead to direct benefit of the male population. Thirdly, the high seroprevalence of multiple hr-types among adult women indicate towards a relative high-risk of (precursors of) HPV-related cancers and thereby underlines the need to consider routine cervical screening in CN and the potential value of a catch-up campaign.

Incidence of HPV-related cancers is high in the Caribbean region, and comprehensive and locally responsive cancer care is particularly challenging due to commonly under-resourced health-care systems, as highlighted by Spence and colleagues recently [45]. Besides the policy implications addressed, this study will be able to serve as a baseline for future investigations assessing the impact of a potential cervical cancer screening program and (catch-up) vaccination programs in CN, by estimating vaccine uptake and monitoring epidemiological dynamics of HPV infection in the population (i.e., direct and indirect effects as well as impact on circulating HPV types) [7]. Few seroprevalence studies have been conducted in this region and we hereby would like to emphasize the need for serosurveillance data since that would be the first step in developing evidence-based public health policy and could eventually prevent HPV-infections and associated diseases as a whole.

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SUPPLEMENTARY MATERIALS

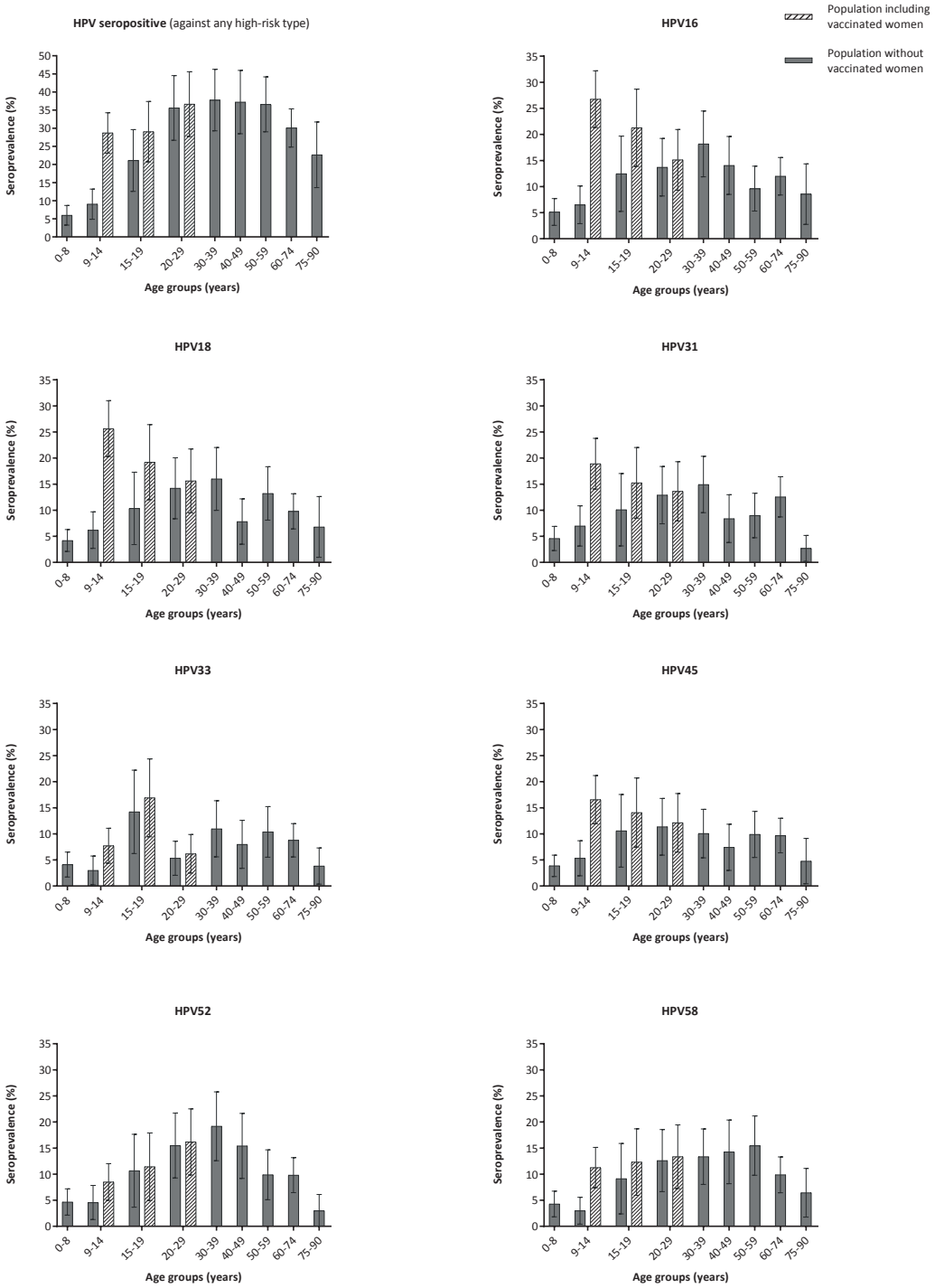


Figure S1 Age-specific seroprevalence (%) (with 95% confidence intervals) of any high-risk type and seven high-risk types human papillomavirus (HPV) IgG antibodies in the general population of Caribbean Netherlands, 2017, by HPV vaccination

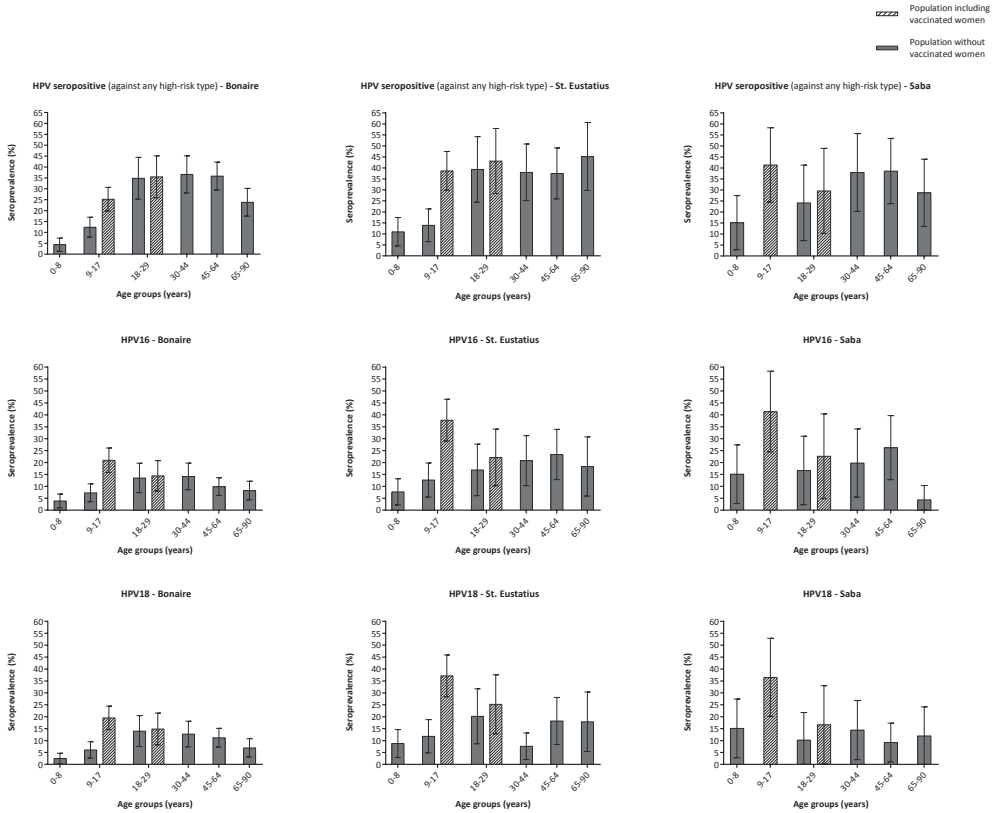
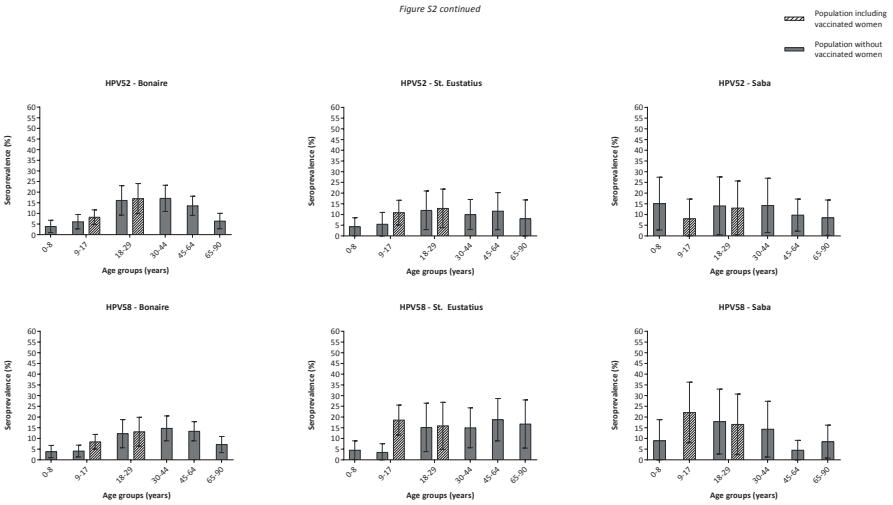

Population including vaccinated women
Population without vaccinated women
HPV seropositive (against any high-risk type) - Bonaire
HPV seropositive (against any high-risk type) - St. Eustatius
HPV seropositive (against any high-risk type) - Saba
HPV16 - Bonaire
HPV16 - St. Eustatius
HPV16 - Saba
HPV18 - Bonaire
HPV18 - St. Eustatius
HPV18 - Saba
Seroprevalence (%)
Age groups (years)
0-8
9-17
18-29
30-44
45-64
65-90

Figure S2 continued



Note: overview of number of participants in each age group in this Figure S2, stratified by island and vaccination

	Bonaire *n* (%) *n*=1,124 (61.7)		**St. Eustatius** *n* (%) *n*=478 (26.2)		**Saba** *n* (%) *n*=221 (12.1)	
Age groups, years	*Population including vaccinated women*	*Population without vaccinated women*	*Population including vaccinated women*	*Population without vaccinated women*	*Population including vaccinated women*	*Population without vaccinated women*
0-8	193	0	88	0	35	0
9-17	211	44	90	37	21	16
25-34	102	1	46	3	22	1
35-44	135	0	68	0	34	0
45-64	259	0	90	0	52	0
65-90	179	0	56	0	40	0

Figure S2 Age-specific seroprevalence (%) (with 95% confidence intervals) of any high-risk type and seven high-risk types human papillomavirus (HPV) IgG antibodies in the general population of Bonaire, St. Eustatius and Saba, 2017

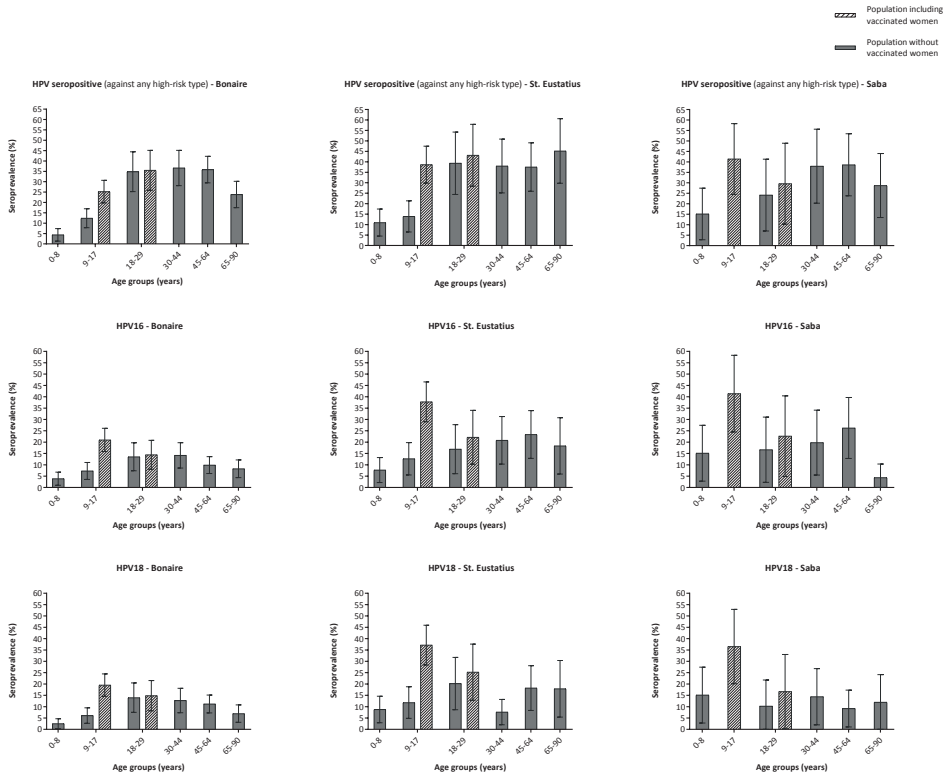


Figure S2 continued

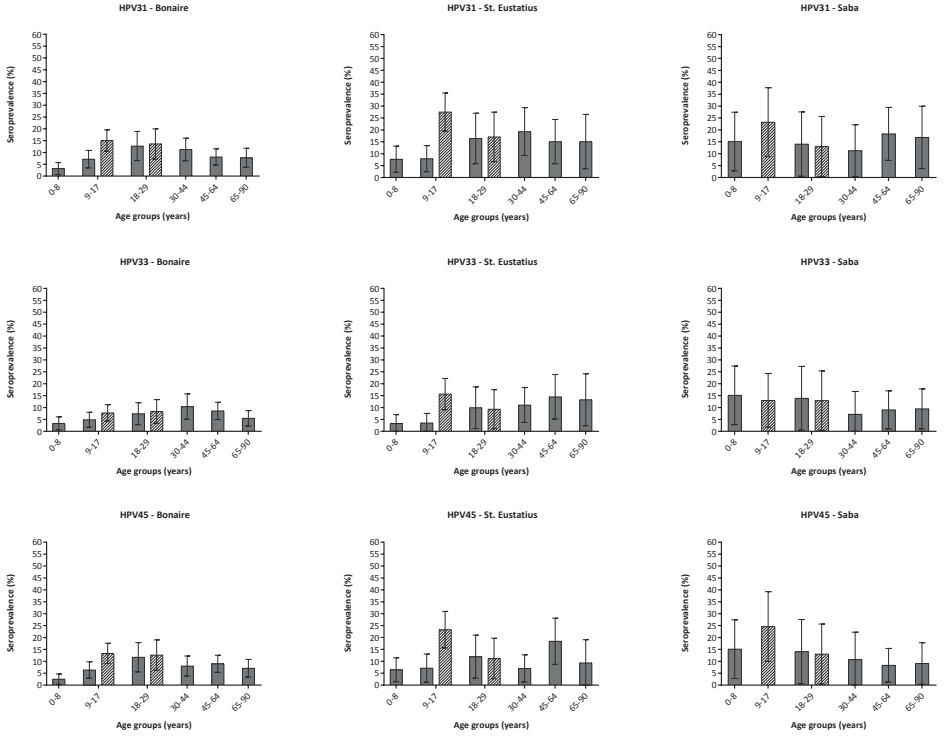
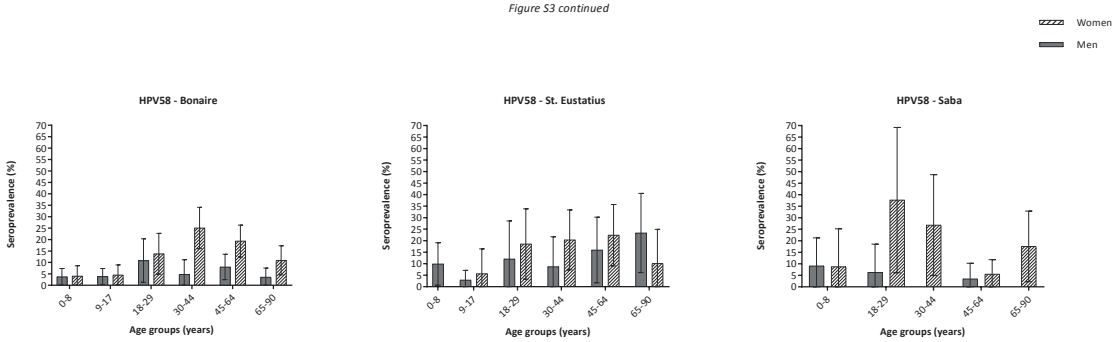


Figure S3 continued



Note: overview of number of unvaccinated participants in each age group in this Figure S3, stratified by island and sex

	Bonaire *n* (%) *n*=1,079 (62.7)		**St. Eustatius** *n* (%) *n*=438 (24.4)		**Saba** *n* (%) *n*=204 (11.9)	
Age groups, years	*Men*	*Women*	*Men*	*Women*	*Men*	*Women*
0-8	102	91	40	48	21	14
9-17	122	89	69	21	16	5
25-34	36	66	19	27	12	10
35-44	43	92	24	44	11	23
45-64	110	149	44	46	19	33
65-90	90	89	25	31	17	23

Figure S3 Age-specific seroprevalence (%) (with 95% confidence intervals) of seven high-risk types human papillomavirus (HPV) IgG antibodies in the unvaccinated general population of Bonaire, St. Eustatius and Saba, 2017, by sex

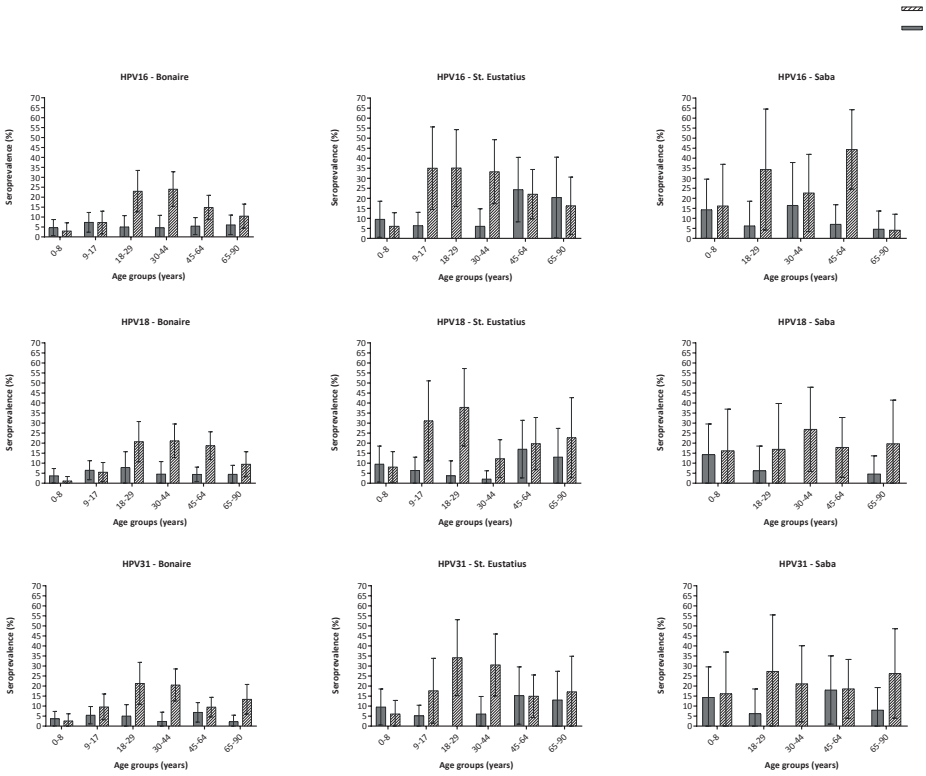
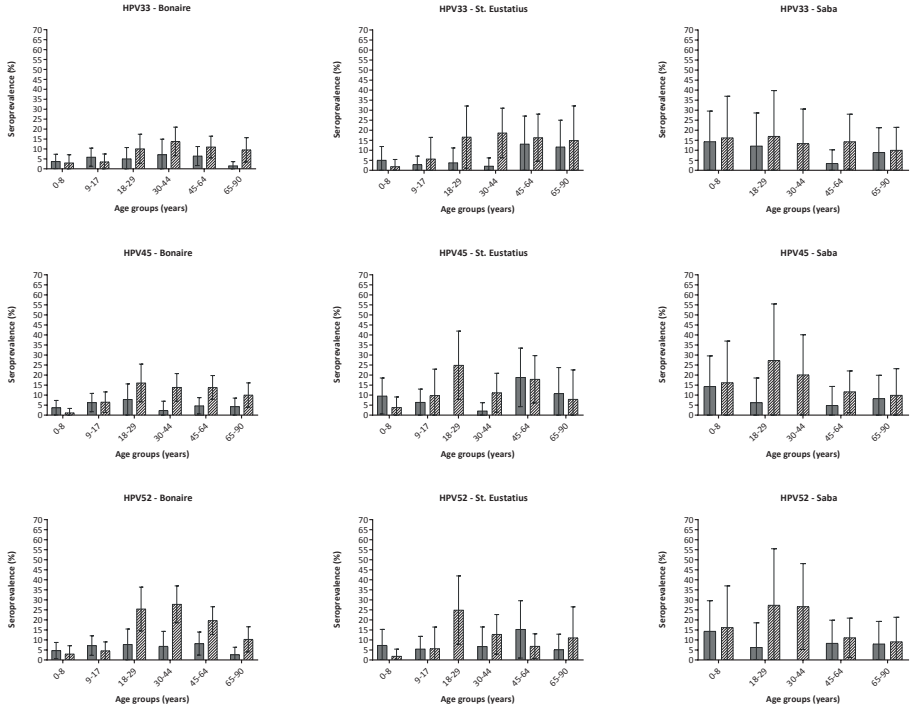


Figure S3 continued



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

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Joske Hoes[1,2], Hella Pasmans[1,3], Mirjam J. Knol[1], Robine Donken[4,5], Naomi van Marm-Wattimena[1], Rutger M. Schepp[1], Audrey J. King[1], Fiona R.M. van der Klis[1], Hester E. de Melker[1]

[1] Center for Infectious Disease Control, National Institute for Public Health and the Environment, 3721 MA Bilthoven, The Netherlands
[2] Department of Epidemiology and Biostatistics, VU University Medical Center, 1081 HV Amsterdam, The Netherlands
[3] Department of Medical Oncology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands
[4] Vaccine Evaluation Center, BC Children's Hospital Research Institute, Vancouver, BC V5Z 4H4, Canada
[5] BC Women's Health Research Institute, BC Women's Hospital + Health Center, Vancouver BC V6H 3N1, Canada

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.

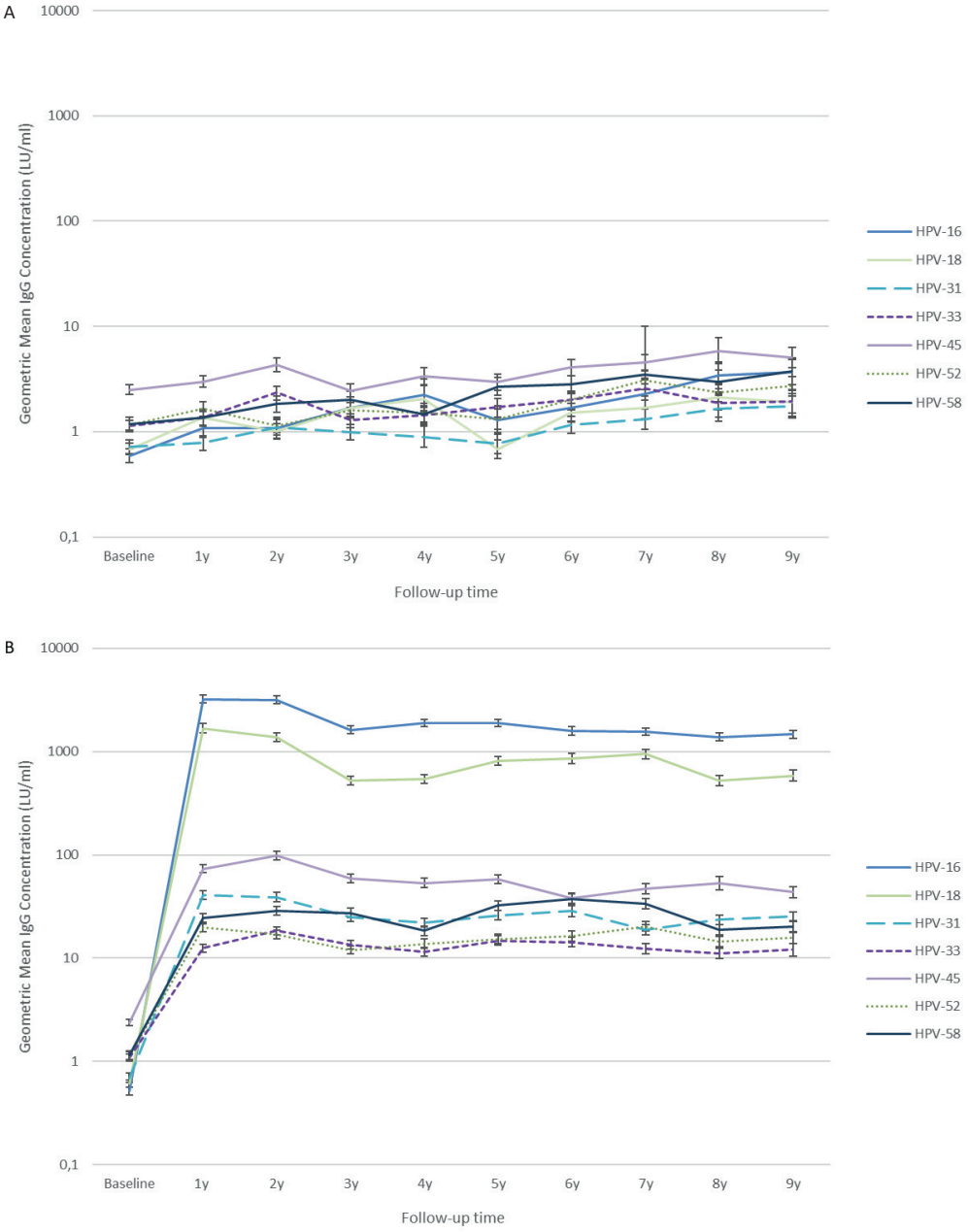

Figure 1 Geometric Mean antibody Concentrations of IgG among unvaccinated (A) and fully vaccinated (B) participants against seven different HPV types

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)

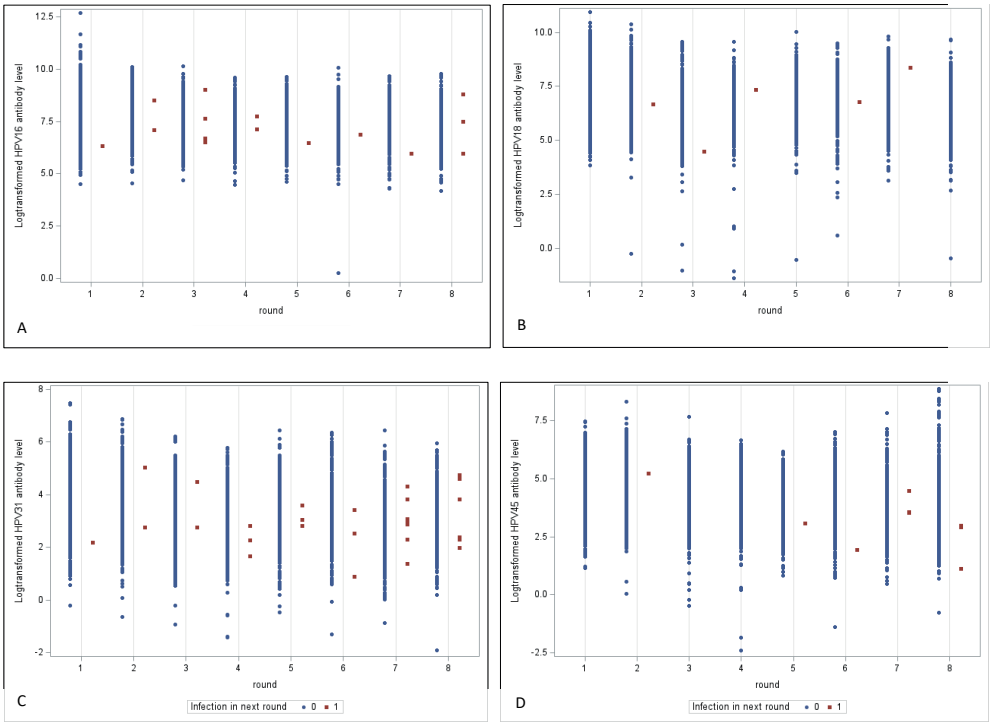

Figure 2 Antibody levels against HPV16 (A), HPV18 (B), HPV31 (C), and HPV45 (D) among vaccinated individuals with infections (red squares) and without infections (blue dots) in the subsequent year

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

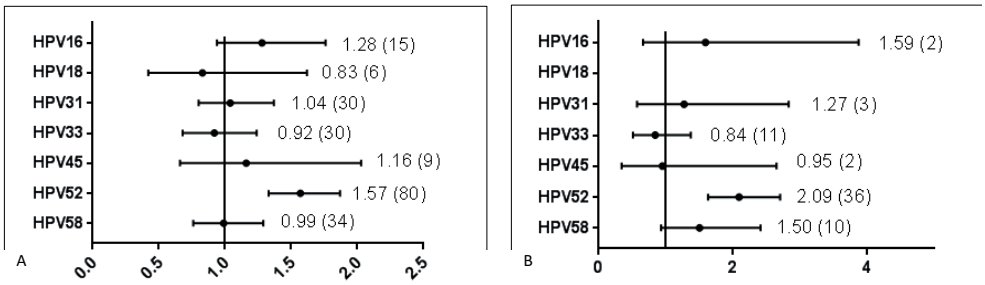

Figure 3 GMC ratio with 95% confidence interval one year pre-infection comparing non-infected vaccinated participants to infected vaccinated participants for incident infections (A) and persistent infections (B). The absolute number of type specific infections is in brackets.

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.

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CHAPTER 5

LONG-TERM HPV-SPECIFIC IMMUNE RESPONSE AFTER ONE VERSUS TWO AND THREE DOSES OF BIVALENT HPV VACCINATION IN DUTCH GIRLS

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Hella Pasmans[1,2], Tessa M. Schurink-van 't Klooster[1], Marjan J.M. Bogaard[1], Debbie M. van Rooijen[1], Hester E. de Melker[1], Marij J.P. Welters[2], Sjoerd H. van der Burg[2], Fiona R.M. van der Klis[1], Anne-Marie Buisman[1]

[1] Center for Infectious Disease Control, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3720 MA Bilthoven, the Netherlands
[2] Department of Medical Oncology, Leiden University Medical Center, Albinusdreef 2, 2333ZA Leiden, the Netherlands.

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 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.

Conclusion 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.

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.

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.

METHODS

Study Procedures

Samples were obtained from several studies. An overview is given of all groups in Figure 1. The Dutch national vaccination registry Praeventis was used to cross-sectionally select participants that had been vaccinated with the 2vHPV vaccine. The one-dose 2vHPV vaccinated girls were vaccinated between 2011 and 2016 (birth cohort 1998-2003), the two-dose vaccinated girls between 2010-2013 (birth cohorts 1997-200) and of the three-dose vaccinated girls vaccinated between 2009-2010 (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

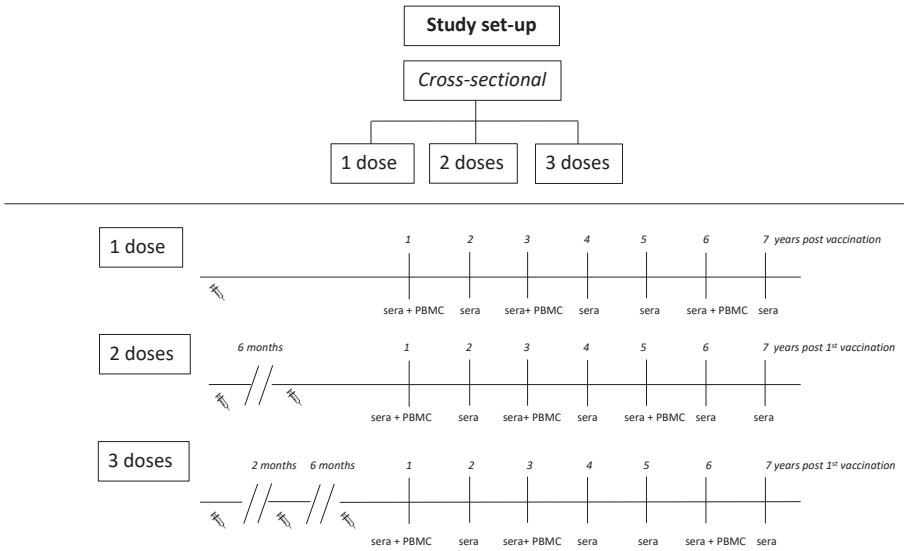

Figure 1 Study set-up and sampling scheme. Samples were cross-sectionally obtained one until seven years post the first vaccination from one-, two- and three dose 2vHPV vaccinated girls. The one-dose 2vHPV vaccinated girls were vaccinated between 2011 and 2016 (birth cohort 1998–2003), the two dose vaccinated 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).

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)(Figure 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.

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 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 minutes at RT. Antibodies that remain bound to the VLP-conjugated beads after treatment with NH_4SCN defines the avidity index.

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-specifc 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 $1x10^5$ 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.

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 $3x10^5$ 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 $3x10^5$ PBMCs. When no spots were detected the value <1 per $3x10^5$ was set at 0.5.

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 hours 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.

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.

RESULTS

Socio-demographics of study participants

A total of 890 girls, between 13-21 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.

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

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 (Figure 2A,B). Importantly, 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

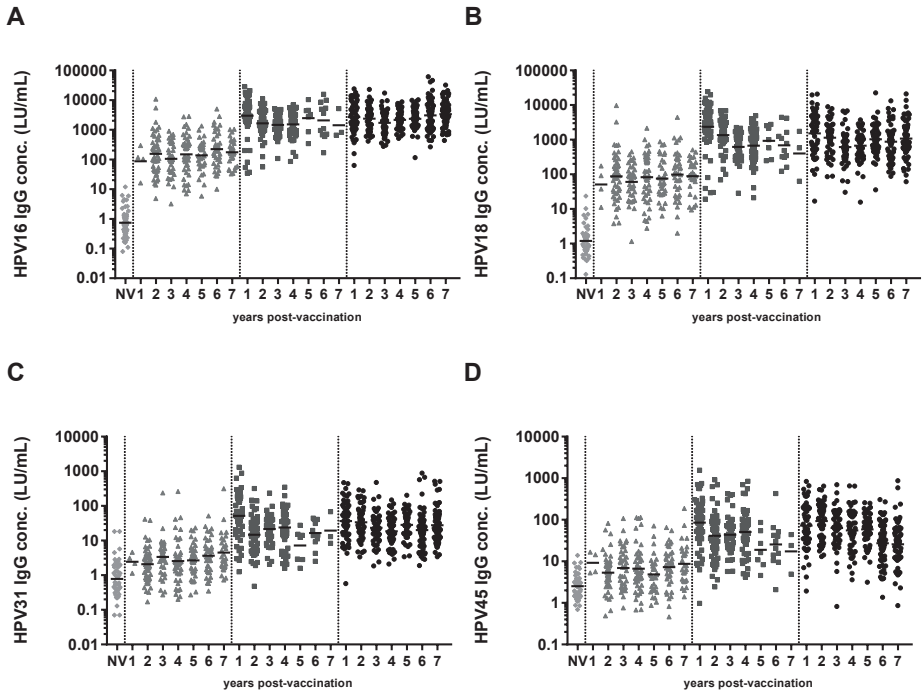

Figure 2 HPV16 (A), HPV18(B), HPV31 (C) and HPV45 (D) specific IgG antibody concentrations (LU/ml) of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls from one till seven years post-vaccination. The lines indicate the geometric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

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 (Figure 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 Figure 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 Figure 2 F,G).

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 Figure 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 sched-

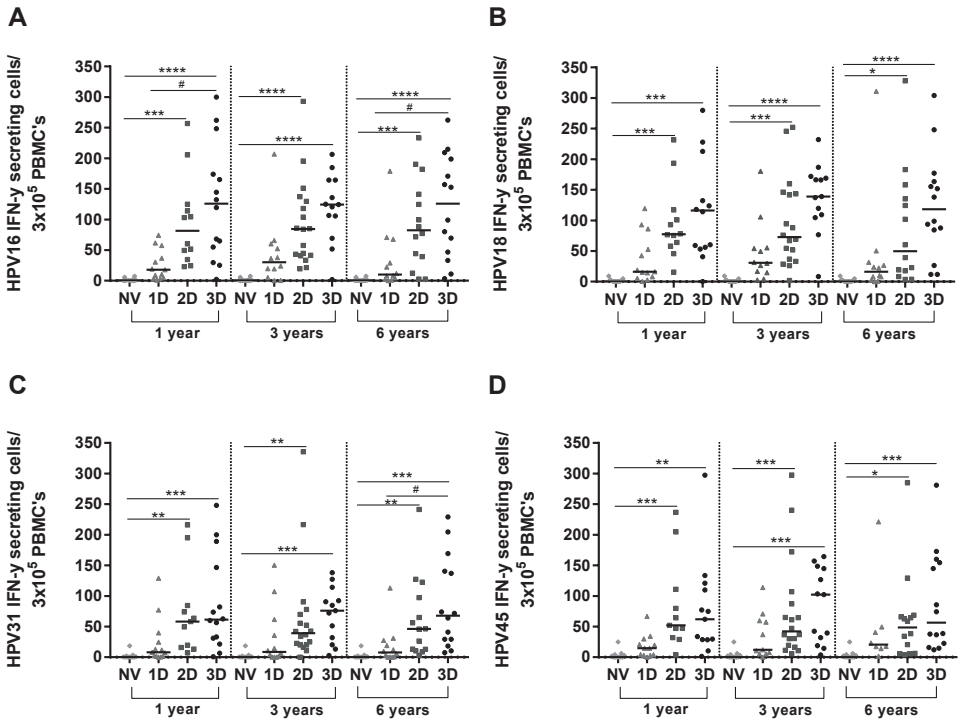

Figure 4 Numbers of IFN-γ producing cells for HPV16 (A), HPV18(B), HPV31 (C) and HPV45 (D) of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls at one, three and six years post-vaccination. The lines indicate the median (n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

ules at five years post-vaccination (data not shown).

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- (Figure 3) and IFN-γ producing cells were amplified by increasing doses of vaccines (Figure 4). After depletion of CD56+ NK-cells the IFN-γ producing cell numbers were similar (data not shown).

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 Figure 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 Figure 4AB) as well as of the Th1 cytokines IFN-γ and TNF-α (Supplementary Figure 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 Figure 5B). For IL-17A, IL-2 and IL-10 no differences were observed between the different dosing schedules (Supplementary Figure 5A,C,D). HPV18, - 31

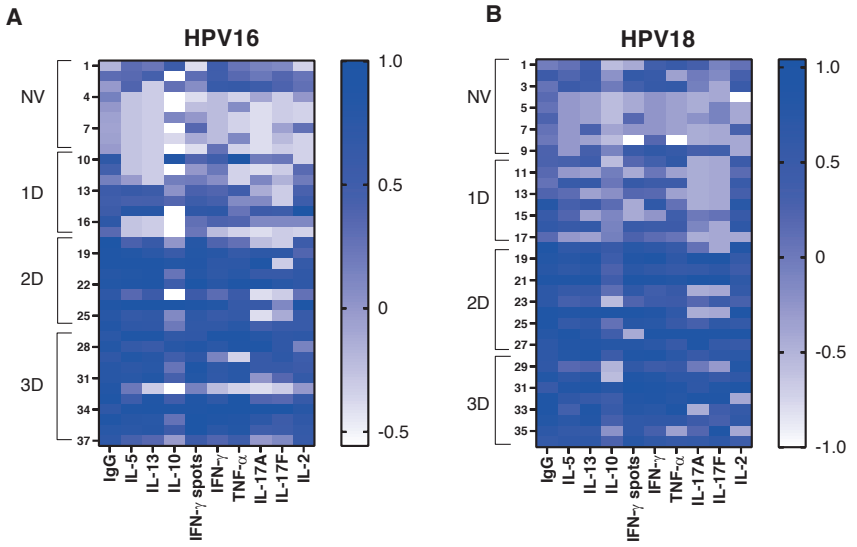

Figure 5 Heat map comparing IFN-γ producing cell numbers and cytokines in supernatants of PBMCs stimulated with HPV16 (A) or HPV18(B), as well as HPV16- or HPV18-specific IgG levels between non-vaccinated, one-, two- and three-dose 2vHPV vaccinated girls at one-year post vaccination. The normalized z-scores 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).

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 Figure 3). There were no differences between the Th1/Th2 ratios of the one-, two- and three-dose vaccinated girls (data not shown).

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.

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 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 100LU/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.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1 Geometric mean concentrations and seroprevalences, corrected for age, and corresponding confidence intervals for IgG antibody levels against seven HPV-types among girls who received an one-, two- or three-dose schedule of the 2vHPV vaccination from one up to seven years post-vaccination.

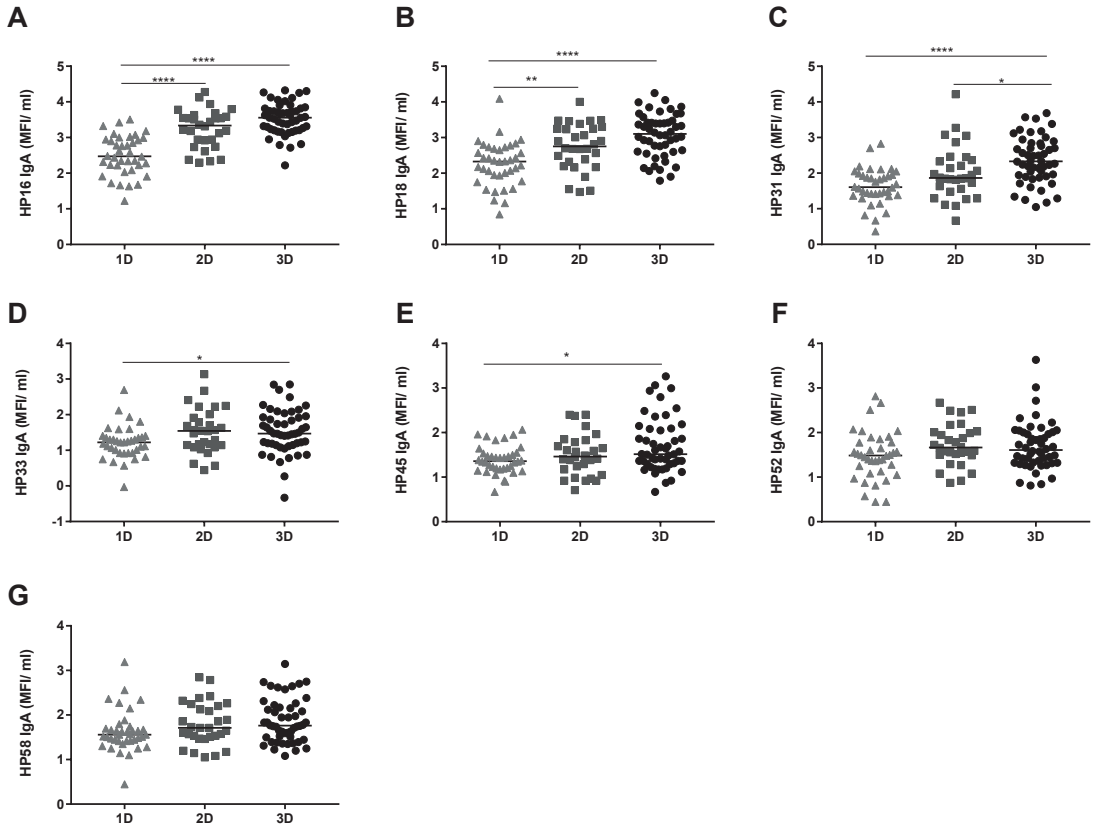
	NV	0-1 years					1-2 years					2-3 years				
Doses		1D	2D		3D		1D	2D		3D		1D	2D		3D	
IgG concentrations GMC (range)*				p-value		p-value			p-value		p-value			p-value		p-value
HPV16	0.7(0.5-1.0)	87.6 (12.7-606.4)	2983 (2009-4430)	0.013	2693 (1906-3805)	0.0114	156.2 (99.0-246.4)	1653 (1253-2182)	<0,001	2370 (1841-3051)	0.0011	105.4 (70.5-157.4)	1464 (1150-1864)	<0,001	1718 (1318-2241)	<0,0001
HPV18	1.2 (0.9-1.6)	50.9 (7.7-337.6)	2356 (1538-3609)	0.015	1522 (1023-2264)	0.0242	87.23 (55.1-138.2)	1360 (1022-1811)	<0,001	1139 (839.6-1545)	0.0015	60.44 (40.9-89.3)	619.8 (469.2-818.6)	<0,001	613.7 (449.1-838.6)	<0,0001
HPV31	0.8(0.56-1.1)	2.4 (1.0-6.2)	51.8(35.2-76.2)	0.001	36.43 (25.3-52.4)	0. 031	2.086 (1.5-3.0)	14.6 (10.3-20.9)	<0,001	33.66 (24.8-45.7)	0.0004	3.4 (2.3-5.0)	21.4 (15.5-29.6)	<0,001	23.3 (17.3-31.4)	0.4448
HPV33	1.2(0.9-1.5)	1.4(0.2-9.8)	15.3 (11.4-20.4)	<0,001	10.47 (7.7-14.2)	0.0936	1.34 (1.0-1.8)	8.0(5.8-11.0)	<0,001	18.04 (14.2-23.0)	<0,0001	1.6 (1.1-2.2)	8.2 (6.0-11.3)	<0,001	11.6(8.8-15.3)	0.0288
HPV45	2.5 (2.1-3.1)	9.2 (3.5-24.3)	85.7 (56.7-123.1)	0.006	72.44(50.9-103.1)	0.1276	5.3 (4.0-7.2)	41.05 (28.2-59.7)	<0,001	93.12(69.4-124.9)	<0,0001	6.9 (4.8-9.9)	43.8 (31.1-61.6)	<0,001	63.3 (46.1-87)	0.0242
HPV52	1.4 (1.0-1.9)	2.3 (0.3-17.2)	26.4 (19.3-36.0)	0.002	17.8 (13.1-24.3)	0.1513	1.9 (1.4-2.6)	13.25 (9.4-18.7)	<0,001	14.0 (10.5-18.6)	0.0063	2.9(2.0-4.2)	12.7(9.0-17.8)	<0,001	9.9 (7.6-12.9)	0.1049
HPV58	1.1 (0.8-1.5)	2.5 (0.4-14.6)	42.0 (29.5-59.7)	0.004	21.2 (16.1-27.9)	0.0384	2.3(1.7-3.2)	20.98 (14.1-31.3)	<0,001	27.5(20.8-36.3)	<0,0001	2.5 (1.8-3.3)	13.8 (10.1-18.9)	<0,001	24.4(17.7-33.6)	0.0019
Seroprevalence % (range)																
HPV16	2 (0.1-12.1)	100 (46.3-100)	100 (92.1-100)		100 (91.9-100)		97.9 (87.5-99.9)	100 (91.2-100)		100 (91.2-100)		97.5 (85.3-99.9)	100 (89.1-100)		100 (91.3-100)	
HPV18	2 (0.1-12.1)	75 (21.9-98.7)	100 (92.1-100)		100 (91.7-100)		89.6 (76.6-96.1)	100 (91.2-100)		100 (91.2-100)		92.5 (78.5-98.0)	100 (89.1-100)		100 (91.3-100)	
HPV31	0 (0-8.9)	0 (0-60.42)	73.7 (60.1-84.1)		60 (45.9-72.7)		4.2 (0.7-15.4)	29.4 (17.9-44.0)		58.8 (44.2-72.1)		2.5 (0.1-14.7)	52.5 (36.3-68.2)		43.1 (29.6-57.7)	
HPV33	4 (0.7-14.9)	0 (0-60.4)	68.4 (54.6-79.7)		47.3(33.9-61.7)		2.1(0.1-12.5)	37.3 (24.5-51.96)		78.4 (64.3-88.3)		5 (0.9-18.2)	42.5 (27.4-59.0)		51.0 (37.8-65.1)	
HPV45	0 (0-8.9)	0 (0-60.42)	84.21 (71.6-92.1)		87.3 (74.9-94.3)		10.4 (3.9-23.4)	72.6 (58.0-83.7)		94.1 (82.8-98.5)		20 (9.6-36.1)	82.5 (66.6-92.1)		92.2 (80.3-97.5)	
HPV52	4 (0.7-14.9)	0 (0-60.42)	66.7 (52.8-78.3)		65.5 (51.3-77.4)		4.2(0.7-15.4)	47.1 (33.2-61.4)		54.9 (40.5-68.6)		10 (3.3-24.6)	50 (34.1-65.9)		33.3 (21.2-48.0)	
HPV58	0 (0-8.9)	0 (0-60.42)	70.2 (56.4-81.2)		70.9 (56.9-82.0)		2.1 (0.1-12.5)	43.1 (29.6-57.7)		47.1 (33.2-61.4)		0 (0-10.9)	20 (9.6-36.1)		70.6 (56.0-82.1)	
Levels above 100LU/mL % (range)																
HPV16	0(0-8.9)	75(21.9-98.7)	93.0 (82.2-97.7)		98.2 (89.0-99.9)		58.3 (43.3-72.1)	98.0 (88.2-99.9)		100 (91.3-100)		52.5 (36.3-68.2)	100 (89.1-100)		100 (91.3-100)	
HPV18	0 (0-8.9)	25(1.3-78.1)	91.2 (80.0-96.7)		98.2 (88.8-99.9)		45.8 (31.6-60.7)	98.0 (88.2-99.9)		96.1 (85.4-99.3)		32.5 (19.1-49.2)	97.5 (85.3-99.9)		94.1 (82.8-98.5)	

	3-4 years					4-5 years					5-6 years				
	1D	2D		3D		1D	2D		3D		1D	2D		3D	
IgG concentrations GMC (range)*			p-value		p-value			p-value		p-value			p-value		p-value
HPV16	148.7 (93.9-235.2)	1523 (1177-1971)	<0,001	2155 (1764-2631)	0.0036	137.8 (92.3-205.9)	2461 (853.7-7097)	<0,001	2347 (1853-2972)	0.0004	224.3 (142.9-351.9)	2083 (850.4-5102)	<0,001	3051 (2248-4141)	<0,0001
HPV18	83.0 (51.5-133.8)	676 (495.6-922)	<0,001	668.5 (498.6-896.4)	0.0375	75.3(48.1-1)	923.9 (353.3-2416)	<0,001	1033 (760.9-1401)	0.0214	98.3 (58.8-164.2)	687 (327.7-1441)	<0,001	873.5 (635.8-1200)	<0,0001
HPV31	2.6(1.651-3.947)	23.9 (16.8-34)	<0,001	18.56 (13.72-25.11)	0.0166	2.7 (1.8-3.9)	7.2 (2.6-19.6)	0.071	28.34 (22.0-36.5)	0.1238	3.6 (2.5-5.3)	16.46 (9.1-29.9)	<0,001	20.02 (14.4-27.8)	<0,0001
HPV33	1.6(1.0-2.5)	9.9 (7.06-13.9)	<0,001	10.12 (7.617-13.44)	0.0063	1.6 (1.0-2.6)	1.9 (0.6-5.9)	0.86	15.5 (11.5-20.7)	0.6349	2.1 (1.3-3.5)	7.1 (2.9-17.2)	0.01	6.966 (5.0-9.8)	<0,0001
HPV45	6.6 (4.5-9.8)	501.0(35.7 6-72.6)	<0,001	55.54 (39.7-77.7)	0.0088	4.8 (3.5-6.8)	18.9 (7.2-49.7)	0.005	58.6 (45.1-76.1)	0.399	7.3 (5.0-10.5)	25.4 (11.2-57.8)	0.003	27.2 (20.0-36.9)	<0,0001
HPV52	3.2(1.9-5.5)	14.7(10.6-20.4)	<0,001	10.25 (7.4-14.1)	0.1282	2.4 (1.6-3.7)	5.2 (1.7-15.8)	0.275	12.9 (9.4-17.7)	0.5167	3.0(1.8-5.0)	11.8 (6.0-23.4)	0.002	8.9 (6.7-11.8)	<0,0001
HPV58	3.2(2.1-4.9)	18.7 (13.4-26.3)	<0,001	16.7 (12.1-23.2)	0.0369	2.9(1.8-4.7)	4.3 (1.8-10.0)	0.836	30.1 (22.5-40.1)	0.7804	3.8(2.3-6.0)	12.6 (5.8-27.4)	0.002	13.5 (9.9-18.5)	<0,0001
Seroprevalence %(range)															
HPV16	95.6 (83.6-99.2)	100 (91.4-100)		100 (91.1-100)		97.1 (83.4-99.9)	100 (56.1-100)		100 (90.77-100)		100 (88.8-100)	100 (69.9-100)		100 (91.3-100)	
HPV18	86.7 (72.5-94.5)	100 (91.4-100)		100 (91.1-100)		91.4 (75.8-97.8)	100 (56.1-100)		100 (90.77-100)		92.3 (78.0-98.0)	100 (69.9-100)		100 (92.5-100)	
HPV31	6.7 (1.7-19.3)	53.9 (39.6-67.5)		39.6 (26.1-54.7)		0 (0-7.8)	14.29 (0.75-57.99)		47.92 (33.52-62.64)		2.6 (0.1-15.1)	41.7 (16.5-71.4)		33.3 (22.0-46.8)	
HPV33	13.3 (5.5-27.5)	46.2 (32.5-60.4)		50 (35.4-64.6)		11.4 (3.7-27.7)	0 (0-43.91)		58.33 (43.28-72.07)		12.8 (4.8-28.2)	41.7 (16.5-71.4)		35 (23.5-48.5)	
HPV45	15.6 (7.0-30.1)	76.9 (62.8-87.0)		78 (63.7-88.0)		8.6 (2.2-24.2)	42.86 (11.81-79.76)		87.5 (74.06-94.81)		20.5 (9.9-36.9)	58.3 (28.6-83.5)		63.3 (49.9-75.1)	
HPV52	13.3 (5.5-27.5)	61.5 (47.0-74.4)		36.2 (23.1-51.5)		8.6 (2.2-24.2)	14.29 (0.75-57.99)		43.75 (29.77-58.72)		12.8 (4.8-28.2)	50 (22.3-77.7)		30 (19.2-43.4)	
HPV58	8.9 (2.9-22.1)	38.5 (25.6-53.0)		23.4 (12.8-38.4)		8.6(2.2-24.2)	0 (0-43.91)		43.75 (29.77-58.72)		2.6 (0.1-15.1)	33.3 (11.3-64.6)		18.3 (9.9-30.9)	
Levels above 100LU/mL % (range)															
HPV16	64.4 (48.7-77.7)	98.1 (88.4-99.9)		100 (91.1-100)		65.7 (47.7-80.3)	100 (56.1-100)		100 (90.8-100)		71.8(54.9-84.5)	100 (69.9-100)		100 (92.5-100)	
HPV18	46.7 (31.9-62.0)	96.2 (85.7-99.3)		96 (85.1-99.3)		34.3 (19.7-52.3)	100 (56.1-100)		97.92 (87.5-99.9)		51.3 (35.0-67.3)	100 (69.9-100)		98.3 (89.9-99.9)	

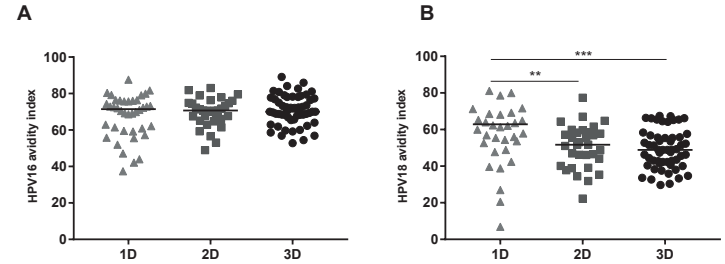
6-7 years					
	1D	2D		3D	
IgG concentrations GMC (range)*			p-value		p-value
HPV16	176.1 (117.5-263.8)	1418 (86.2-23305)	0.003	3365 (2621-4320)	<0,001
HPV18	88.7 (56.7-138.8)	401.2 (6.1-26466)	0.052	1076 (795.2-1456)	<0,001
HPV31	4.5 (2.7-7.4)	19.4 (1.25-302.3)	0.075	27.8 (20.6-37.5)	<0,001
HPV33	2.2 (1.3-3.9)	13.3 (1.7-107.3)	0.053	8.1 (6.0-11.0)	<0,001
HPV45	8.7 (5.6-13.6)	17.3 (1.0-287.7)	0.345	25.7 (18.68-35.4)	<0,001
HPV52	4.7 (2.9-7.8)	21.4 (4.4-104.3)	0.061	10.6 (7.8-14.3)	0.003
HPV58	3.8 (2.3-6.2)	16.5 (2.2-123.6)	0.070	14.3 (11.0-18.5)	<0,001
Seroprevalence %(range)					
HPV16	100 (85.0-100)	100 (31-100)		100 (92.8-100)	
HPV18	85.7 (66.4-95.3)	100 (31-100)		100 (92.8-100)	
HPV31	7.1 (1.3-25.0)	33.3 (1.8-87.5)		33.3 (22.7-45.8)	
HPV33	10.7 (2.8-29.4)	66.7 (12.5-98.2)		38.1 (26.4-51.2)	
HPV45	21.4 (9.0-41.5)	66.7 (12.5-98.2)		60.3 (47.2-72.2)	
HPV52	14.3 (4.7-33.6)	66.7 (12.5-98.2)		42.9 (30.68-55.9)	
HPV58	10.7 (2.8-29.4)	33.3 (1.8-87.5)		23.8 (14.4-36.5)	
Levels above 100LU/mL %(range)					
HPV16	71.4 (51.1-86.1)	100 (31-100)		100 (92.8-100)	
HPV18	50 (31.1-68.9)	66.67 (12.5-98.2)		96.83 (88.0-99.5)	

** P-values are indicated relative to the one-dose group at the same time-point.*

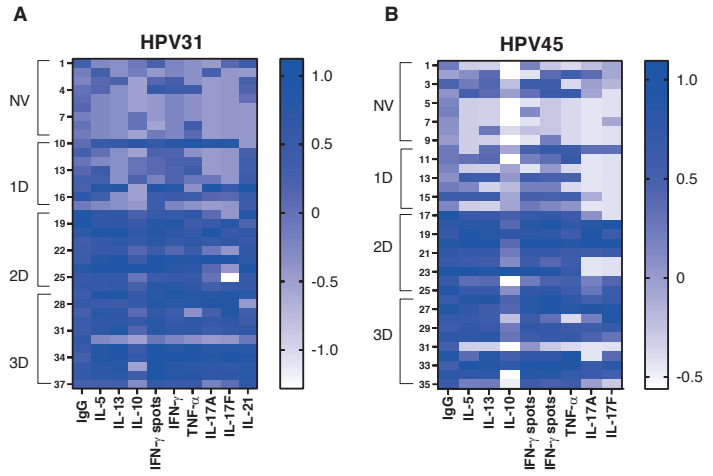
*** Years are time since vaccination.*



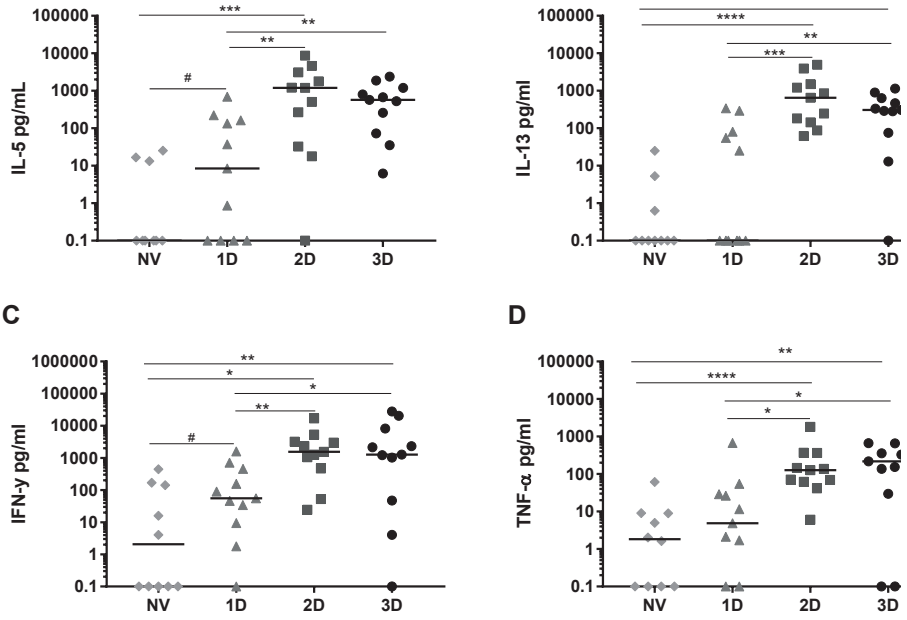
Supplementary Figure 1 HPV16 (A), HPV18(B), HPV31(C), HPV33(D), HPV45(E), HPV52(F) and HPV58 (G) IgA antibody concentrations (MFI/ml) of non-vaccinated (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) vaccinated girls at five years post-vaccination. The lines indicate the geometric mean concentration (n= 50 per group). *p<0.01, **p<0.001, ****p<0.00001



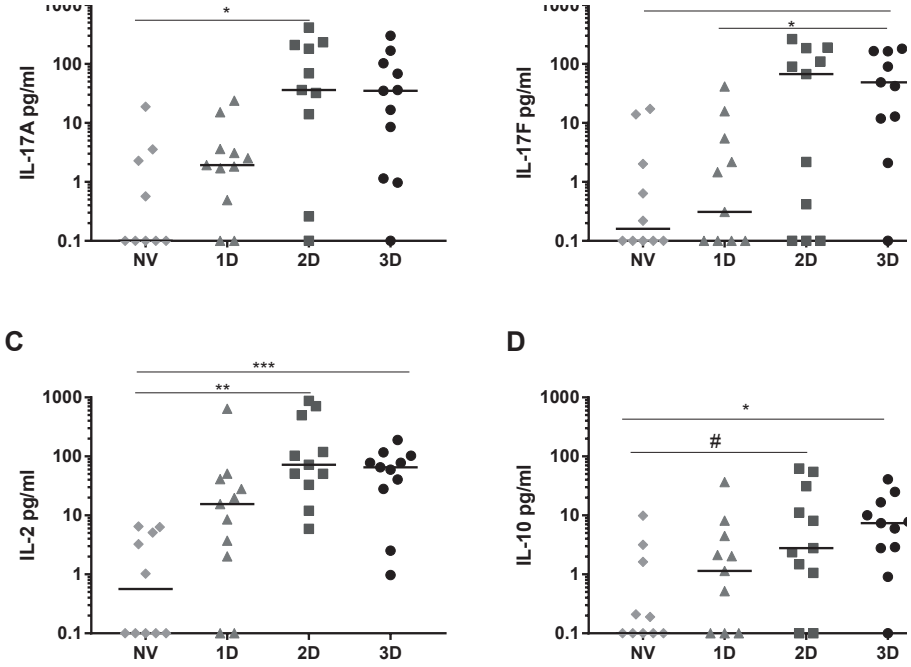
Supplementary Figure 2 Antibody avidity (%) for HPV16 (A) and HPV18 (B) of HPV-specific vaccine derived antibodies of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls. The lines indicate the median (n= 50 per group). **p<0.001, ***p<0.0001



Supplementary Figure 3 Heat map comparing IFN-γ producing cell numbers and cytokines in supernatants of PBMCs stimulated with HPV-31(A)andHPV-45(B) as well as type-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).



Supplementary Figure 4 Production of IL-5 (A), IL-13 (B), IFN-γ (C) and TNF-α (D) (pg/ml) in supernatants of PBMC s stimulated with HPV-16 of non (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls at one year post-vaccination. The lines indicate the median (n= 10-12 per group). #0.01>p<0.1,*p< 0.01,**p<0.001,***p<0.0001,****p<0.00001



Supplementary Figure 5 Production of IL-17α (A), IL-17F (B), IL-2 (C) and IL-10 (D)(pg/ml) in supernatants of PBMCs stimulated with HPV16 of non- (light grey diamonds), one- (grey triangles), two- (dark grey squares) and three-dose (black circles) 2vHPV vaccinated girls at one year post-vaccination. The lines indicate the median (n= 10-12 per group). *p<0.01, **p<0.001, ***p<0.0001.

CHAPTER 6

CHARACTERIZATION OF THE EARLY CELLULAR IMMUNE RESPONSE INDUCED BY HPV VACCINES AND ITS RELATION TO LONG-TERM HPV-SPECIFIC IMMUNITY

Submitted for publication

Hella Pasmans[a], Magdalena A. Berkowska[b], Annieck M. Diks[b], Bas de Mooij[b], Rick J. Groenland[b], Lia de Rond[a], M. Alina Nicolaie[a], Sjoerd H. van der Burg[c], Jacques J.M. van Dongen[b], Fiona R.M. van der Klis[a], Anne-Marie Buisman[a]

[a] Center for Infectious Disease Control, National Institute for Public Health and the Environment, 3721 MA, Bilthoven, The Netherlands
[b] Department of Immunology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, the Netherlands
[c] Department of Medical Oncology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, the Netherlands

ABSTRACT

Introduction Current human papillomavirus (HPV) vaccines consist of virus-like particles (VLPs) which are based on the L1 protein, but they are produced by different expression systems and use different adjuvants. We performed in-depth immunophenotyping of multiple innate and adaptive immune cells after bivalent versus nonavalent HPV vaccines.

Method Twenty pre-menopausal HPV-seronegative women were enrolled and randomized to receive three-doses of either the bivalent or the nonavalent HPV vaccine. Blood samples were collected at multiple time points from baseline up to 7 months after first vaccination. Four different Euroflow flow cytometry antibody panels were used to monitor >250 immune cell subsets. HPV-specific memory B- and T cells were determined by ELISPOT and HPV-specific antibody levels were measured by a VLP-based multiplex immunoassay.

Results The numbers of plasma cells expanded in the first week after both primary and tertiary vaccination in both cohorts. HPV16 and HPV18-specific antibody levels and memory B and T cell responses were higher in the bivalent cohort than in the nonavalent vaccines one month post third vaccination. For HPV31 and HPV45-specific antibody levels this pattern was reversed. Monocytes showed a clear expansion one day after vaccination in both cohorts but were significantly higher in the bivalent vaccine cohort. Large heterogeneity in responses of the other cell subsets was observed between donors.

Conclusion This pilot study showed a consistent response of monocytes and plasma cells after vaccination and a considerable variation in other circulating immune cells in both types of HPV vaccines between donors.

INTRODUCTION

A HPV infection is one of the most common sexually transmitted infections worldwide. Already over 200 different subtypes have been identified of which 15 are classified as oncogenic and are an important cause of anogenital and oropharyngeal cancers, but most importantly of cervical cancer. HPV16 and 18 are associated with about 70% of all cervical cancer cases, and 25% are associated with closely related HPV types within the groups A9 (HPV16-like: 31,33,35,52,58) and A7 (HPV18-like; 39,45,59,68) [1, 2]. Globally, around 800 women die of cervical cancer every day [3].

Currently, there are three licensed, highly efficacious prophylactic vaccines on the market providing protection against two (bivalent), four (quadrivalent), and nine (nonavalent) persistent HPV infections, and to some degree there is cross-protection against phylogenetically related non-vaccine types. All vaccines comprise virus-like particles (VLPs) which are based on the major HPV capsid protein L1. The antigens of the vaccines are produced in different expression systems; a baculovirus expression system is used for the bivalent vaccine and a yeast expression system for the quadrivalent and nonavalent vaccines. Moreover, the vaccines use different types of adjuvants. The bivalent vaccine is formulated with AS04, containing aluminum hydroxide salts and the TLR4 agonist MPL (3-O-deascyl-4'-monophosphoryl lipid A). The quadrivalent and nonavalent vaccines consist of aluminum salts as an adjuvant [4].

Several studies have investigated the differences in immunological responses to these different vaccines, and especially comparing the bivalent with the quadrivalent vaccine [5-9]. The majority of this work has focused on antibody responses, as they are thought to be the key mediators of protection against infection [10]. All three vaccine have been shown to induce robust antibody responses against the various vaccine types [9, 11, 12], but the bivalent vaccine has been shown to induce higher levels of HPV16/18-specific serum antibodies [6, 13] and more robust B cell responses [7]. The AS04 adjuvant is suggested to be of importance for this higher immunogenicity observed in bivalent vaccine recipients, as it is thought to induce a better Th1 response [14, 15].

To the best of our knowledge, innate cellular responses after HPV vaccination have not been studied so far. Insight in these responses would aid the interpretation of the different working mechanisms of the vaccines, and the induced adaptive responses observed.

The aim of this study was to increase our knowledge of early cellular immune reactions after vaccination with either the bivalent or the nonavalent HPV vaccine, and their possible involvement in/possible relation to HPV-specific long-term memory formation. Therefore, we investigated the kinetics of circulating innate and adaptive immune cell subsets by in-depth phenotyping after the first and last vaccination. Kinetics of circulating cells were related to the induction of long-term antibody and cellular memory responses upon vaccination.

METHODS

Study population and procedures

Participants were recruited among pre-menopausal female personnel of the Dutch National Institute for Public Health and the Environment (RIVM). Potential participants were invited to a

pre-study visit and asked to donate a finger prick blood sample to measure the presence of anti-HPV antibodies. During that visit they had the opportunity to ask questions about the study. HPV-seronegative (HPV16/18/31/45) women who were willing to participate were invited for further study consultation and signing the informed consent form according to the Declaration of Helsinki. The study was approved by the Medical Ethical Committees United, Nieuwegein, the Netherlands (study number: NL69015.100.19). Inclusion and exclusion criteria are listed in Supplementary Table 1.

Twenty women were enrolled and randomized in a 1:1 ratio to receive either the bivalent (GSK, Rixensart, Belgium) or the nonavalent (Merck, Sharp &Dohme, Kenilworth, NJ) HPV vaccine according to a three-dose schedule (0, 2 and 6 months). Whole blood and serum samples were collected at baseline (day 0) and at pre-defined time points following vaccination. Following primary vaccination, an initial cohort of five volunteers donated blood at eight visits (days 0 (baseline), 1, 2, 3, 6, 7, 10, and 14) to determine the most optimal sampling scheme (Figure 1A) for the remaining 15 donors (days 0, 1, 3, and 7). Then, all volunteers received the first and second booster vaccination and donated samples at day 80, 180 (booster baseline), 181, 183, 187 and 208 (Figure 1B).

Immunophenotyping by flow cytometry

PB-EDTA samples were processed within 2 hours from blood collection. Four different Euroflow flow cytometry antibody panels were used to monitor kinetics of over 250 immune cell subsets in peripheral blood; a dendritic cell (DC)-monocyte tube (MDC; Van der Pan ***et al.***,

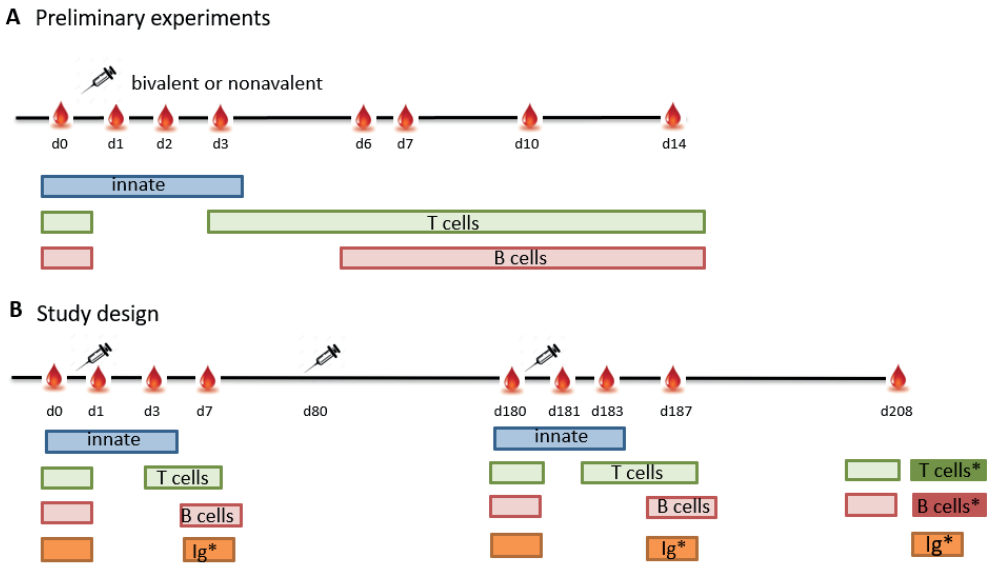


Figure 1 Study design; Twenty healthy HPV16/18/31/33/45/52/58 seronegative adult pre-menopausal women received either the bivalent or nonavalent HPV vaccine according to a three-dose schedule. (A) In the preliminary experiments a blood sample was taken just before vaccination (day 0), and after the first vaccination at days 1, 2, 3, 6, 7, 10 and 14 (B) In the study a blood sample was drawn just before (day 0), and after the first vaccination at days 1, 3, 7. At two months a second vaccination was given. Just before (day 180), an after the 3rd vaccination also blood samples were drawn at days 181, 183, 187 and 208. The colors indicate a which timepoints which immune components were studied, blue: innate cells, green: T cells, red= B cells, orange- IgG antibodies. *= HPV16/18/31/45-specific.

manuscript in preparation), a CD4 T cell tube (CD4T) [16], a CD8 cytotoxic T cell tube (CYTOX tube) and a B cell and plasma cell tube (BIGH)[17, 18]. Additionally, Perfect Count microspheres™ (Cytognos) were used according to the EuroFlow protocol (www.EuroFlow.org) for precise enumeration of cell numbers using the Perfect Count tube (PCT). The CD4 T-cell, CYTOX and the PCT were directly stained on whole blood, using either 100µl (T cells) or 50µl (PCT) of peripheral blood. For the CD4 T cell and CYTOX tube, 100µl PB was stained for 30min at RT in the dark with the corresponding antibody panels. After washing, 100 µl of Reagent A (Fix & Perm, Nordic MUbio, Susteren, the Netherlands) was added and incubated for 15 min at RT in the dark. Cells were washed and 100µl of Reagent B (Fix&Perm™ Nordic MUbio, Susteren, the Netherlands) and intracellular antibodies (Granzyme B, CD154) were added and incubated for 15 min at RT in the dark. After washing, cells were resuspended in 200µl PBS and acquired immediately or were stored at 4°C (max.1 hour) and measured on LSR-Fortessa or Fortessa X20 flow cytometers (BD Biosciences) (stain-lyse-wash protocol, followed by intracellular staining; protocols available at www.EuroFlow.org). The samples for BIGH and MDC tubes were processed according to the bulk lysis protocol (available at www.EuroFlow.org). Briefly, NH_4Cl was added to 1.5ml (WBC > $8x10^6$/ml) or 2.0 ml (WBC <$8x10^6$/ml) of PB to a final volume of 50mL, and was incubated for 15min on the roller bank at RT. Samples were centrifuged for 10 min at 800g, and washed twice in PBS/0.2%BSA/2mMEDTA/0.09%NaAz. Then, $10*10^6$ cells were stained for 30 min in the dark at RT with corresponding antibody panels (NB: the MDC tube was incubated rolling in the dark). The BIGH staining was followed with the Fix & Perm procedure for intracellular staining with Ig subclasses only. For the MDC tube, which did not require intracellular staining, 2mL of BD lyse (BD FACS™ Lysing solution, BD biosciences) was added, incubated for 10min in dark at RT, and washed. Then, cells were resuspended in 500uL of PBS and immediately measured on one of the BD Fortessa flow cytometers or after storage at 4°C for max 1h.

Flow cytometers were calibrated daily according to the EuroFlow guidelines. In short, the photomultiplier tube (PMT) voltages of the flow cytometer were set using BD™ Cytometer Setup and Tracking (CS&T) beads (BD Biosciences) and SPHERO™ Rainbow calibration particles (Cytognos), as previously described [19, 20]. For data analysis, Infinicyt software v 2.0 (Cytognos, Salamanca, Spain) was used.

The Euroflow antibody panels of the different tubes were designed for the PERISCOPE consortium and are elaborately described elsewhere, for the MDC tube (van der Pan ***et al.***, manuscript in preparation), for CD4 [16], the CYTOX and the B cells tube (patent file in preparation: N2023163, filing date 5 Nov 2019) [17]. The antibody panels and corresponding analysis strategies were designed for the PERISCOPE consortium [17]. Both absolute cell counts (cells/ µl) and ratios over baseline were used through the manuscript. Ratios at days 1, 3 and 7 were calculated over the pre-vaccination baseline (day 0) and ratios at days 181, 183, 187 and 208 were calculated over the pre-second booster baseline (day 180).

Detection of memory B cells by ELISPOT

From 18ml of blood collected one month post third vaccination, peripheral blood mononuclear cells (PBMCs) were immediately isolated and stored at -135◦C until analysis. After thawing, B cells were purified from PBMCs by a CD19+ selection kit (StemCell Technologies, Vancouver,

Canada) and stimulated polyclonally with CPG and cytokines for five days as described previously [21]. HPV16, HPV18, HPV31 and HPV45-specific ELISPOT-assays were performed by coating multiscreen-IP plates (Millipore, Burlington, MA) with PBS containing 20 ug/ml HPV16, 18, 31 or 45 VLPs. A suspension of $1x10^5$ B cells was added per antigen in triplicate per participant. Tetanus toxoid (7 flocculation units/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 analyzed 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 to value of 0.1.

Detection of IFN-Y producing cells by ELISPOT

Numbers of HPV-specific IFN-γ-producing cells were measured by ELISPOT. PBMCs were stimulated with VLPs; 4 μg/mL(HPV16, HPV31 and HPV45) and 2 μg/mL(HPV18), in triplicate, in $3x*10^5$ cells/well in AIMV medium (Gibco, Waltham, MA) containing 2% human AB-serum (Harlan, Indianapolis, IN), for 4 days at 37°C and 5% CO_2. Unstimulated and lectin-stimulated cells served as negative and positive controls, respectively. Subsequently, the number of IFN-γ-producing cells specific for HPV16, HPV18, HPV31 and HPV45 was measured using ELISPOT-assays as described previously [22, 23]. Spot numbers were counted using an Immunospot reader (version V3.0) and software (version V6.1) (CTL Immunospot S6 Ultra-V Analyzer, Bonn, 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 $1*x10^5$ PBMCs.

HPV-specific antibody levels

HPV16/18/31 and 45 specific IgG and IgA antibody levels in serum at day 7, 14, 180, 187 and in plasma at day 208 after the first vaccination were determined by using a VLP-based multiplex-immunoassay. All VLPs used in this study were provided at cost part by GSK. The VLP-based multiplex has been described elsewhere in detail [24]. In short, sera were incubated with HPV-specific VLP-conjugated beads (Bio-Rad Laboratories, Hercules, CA). HPV-specific IgG 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). For IgA, a 1/200 dilution of R-PE conjugated goat anti-human IgA (Jackson Immunoresearch, West Grove, PA) was used. HPV-specific antibodies were analyzed using the Bioplex-system 200 with Bioplex-software (Bio-Rad Laboratories, Hercules, CA). IgG antibody levels were expressed in Luminex units (LU) per mL. Semi-quantitative IgA antibody concentrations were expressed in mean fluorescence intensity (MFI).

The presence HPV16, 18, 31 and 45 specific IgG subclasses (IgG1, IgG2, IgG3, IgG4), was determined at day 187. Analysis was performed as described above, by using IgG-isotype-specific mouse anti-human R-PE conjugated secondary antibodies used in 1/500 dilution (IgG1), 1/100

(IgG2-4) (SouthernBiotech, Birmingham, AL). 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%.

Statistical analysis

Univariate summaries comprise geometric mean concentrations (GMC) with their 95% confidence intervals (CI). Bivariate summaries comprise Spearman correlations and the permuted version of the Spearman test for pairs of immune cells at each time point. Multivariate exploratory methods comprise principal component analyses (PCA) performed either per time point or longitudinally.

The permuted version of the Wilcoxon-Mann-Whitney non-parametric test was used to test the association between each immune cell and vaccination type, separately for each type point. The permuted version of the Wilcoxon sing-rank test was used to test the distribution of measurements over time. To compare the changes between two time points for each cell type, fold changes in numbers of cells were used. The calculations carried out comprised the fit of linear mixed models, where the outcome was the log-transformed measurement and determinants were time points and vaccination type. The random effects part was specified as a random intercept. When measurements comprised zero's, the linear mixed model was fit on the original scale of the measurement and differences were reported instead of fold changes. Additionally, the longitudinal measurements of each immune cell were used to calculate the area under the curve of each participant's trajectory; the resulting measures were used in an association study in relationship to the vaccination type. The results of the tests were corrected for multiple testing with the Benjamini-Hochberg (BH) method applied separately to each sub-study. Reported differences and associations are deemed "significant" if the adjusted p-value amounts to less than 0.1, according to a false discovery rate of 10% (FDR < 10%).

The PCA was performed stratified per time point and at any time point by means of packages FactoMineR and factoextra in R. The variables were a priori standardized to have mean 0 and variance 1.

Summary statistics were performed by means of Graphpad Prism V7 (GraphPad, San Diego, CA, USA), while the hypothesis tests were performed in RStudio by means of the coin package (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/, version 1.3).

RESULTS

Study cohort characteristics

A total of twenty pre-menopausal healthy women between 23 and 44 years of age were included in this study. One participant dropped out at day 1 after the first vaccination and was replaced with a new participant. Women received three doses of either the bivalent vaccine (n=10) or the nonavalent vaccine (n=10) at 0, 2 and 6 months (Figure 1). The age of the participants who received the bivalent vaccine (32.7y.o, 95%CI 28.0-37.4) did not significantly differ from those receiving the nonavalent vaccine (31.6y.o., 95%CI 26.4-36.8).

Baseline and determination of optimal sampling time points after vaccination

As the immune status at the moment of vaccination can possibly influence the immune response, the cellular composition at day 0 as well as day 180 was used as baseline for immediate vaccine effects (day 0 before primary vaccination and day 180 before the third vaccination). At baseline and booster baseline, there were no significant differences in the total absolute numbers of cells in all immune subsets between the two cohorts (Mann-Whitney test, p.adjusted.value <0.3). However, the comparison of immune cell subsets between day 0 and day 180 did show significant differences, all p.adjusted.values<0.05. This shows that the median measurement of the participants at day 0 is significantly different for the median measurement of the cellular subsets at day 180, both before and after stratification for vaccine cohorts (Supplementary Table 2).

Preliminary experiments in the first five participants were designed to determine the most optimal post-vaccination timepoints for blood sampling to measure innate and adaptive immune cell numbers. The numbers of innate cells were evaluated at baseline (day 0), day 1, 2 and 3 post vaccination, as innate cells are expected to react directly upon vaccination [25]. Numbers of monocytes peaked at day 1 post vaccination in donors vaccinated with the bivalent vaccine, as was reflected by 2 out of 3 donors showing 1.3-fold or higher increase at day 1, when compared to baseline. The numbers of DCs increased at day 1 (mostly bivalent vaccine) and day 2 (mostly nonavalent vaccine; Supplementary Figure 1A). Changes in the numbers of T cells were minor and not consistent between donors. Of those limited changes, the response peaked as early as day 3, while in 3/5 others it peaked at day 7. The same was true for the total numbers of B cells, which increased in 2/5 donors from day 6 onwards. Plasma cell numbers peaked at day 7 post vaccination in 4/5 donors (Supplementary Figure 1B), thereby showing a clear expansion (Supplementary Figure 2). Therefore, to limit the number of sampling to a maximum of 10 and include relevant time points for each major population, we decided to include the timepoints day 0, 1, 3 and 7 (also used by others [26]) after primary vaccination for studying the cellular kinetics in the remaining 15 participants (7 in the bivalent- and 8 in the nonavalent cohort).

Strong alterations in circulating monocytes after vaccination

To study how innate cells respond to vaccination, ratios over baseline numbers of cells were calculated for all of the innate cell subsets analyzed at day 1 and day 3. A significant increase in median numbers of monocytes was only observed at day 1 in the bivalent vaccinated women (1.17 95%CI 1.003-1.37(Figure 2A). After the third vaccination, a significant increase in the numbers of monocytes was seen at day 181 in both cohorts, 1.27 95%CI 1.05-1.55 and 1.28 95%CI 1.05-1.57, respectively. Depending on their maturation stage, monocytes can be divided into three phenotypically distinct subsets ranging from classical (cMo) to intermediate (iMo) to non-classical (ncMo)[27]. These different monocyte subsets followed a different pattern in time after vaccination per vaccine type (Figure 2B). The samples in the bivalent cohort showed a significant early rise in numbers of iMo (1.72, 95%CI 1.17-2.53) at day 1. This rise in iMo was not significant after the third vaccination with the bivalent vaccine. For cMo a significant increase was observed at day 181, 1.29 95%CI 1.08-1.55. In the donors of the nonavalent cohort, only after the third vaccination, a rise in numbers of cMo was observed (1.26 95%CI 1.04-1.52) (Figure 2B). Statistically significant differences between the two cohorts were observed at day 1 for the absolute numbers of total monocytes, p.adjusted.value<0.01, iMo p.adjusted.value<0.01, and

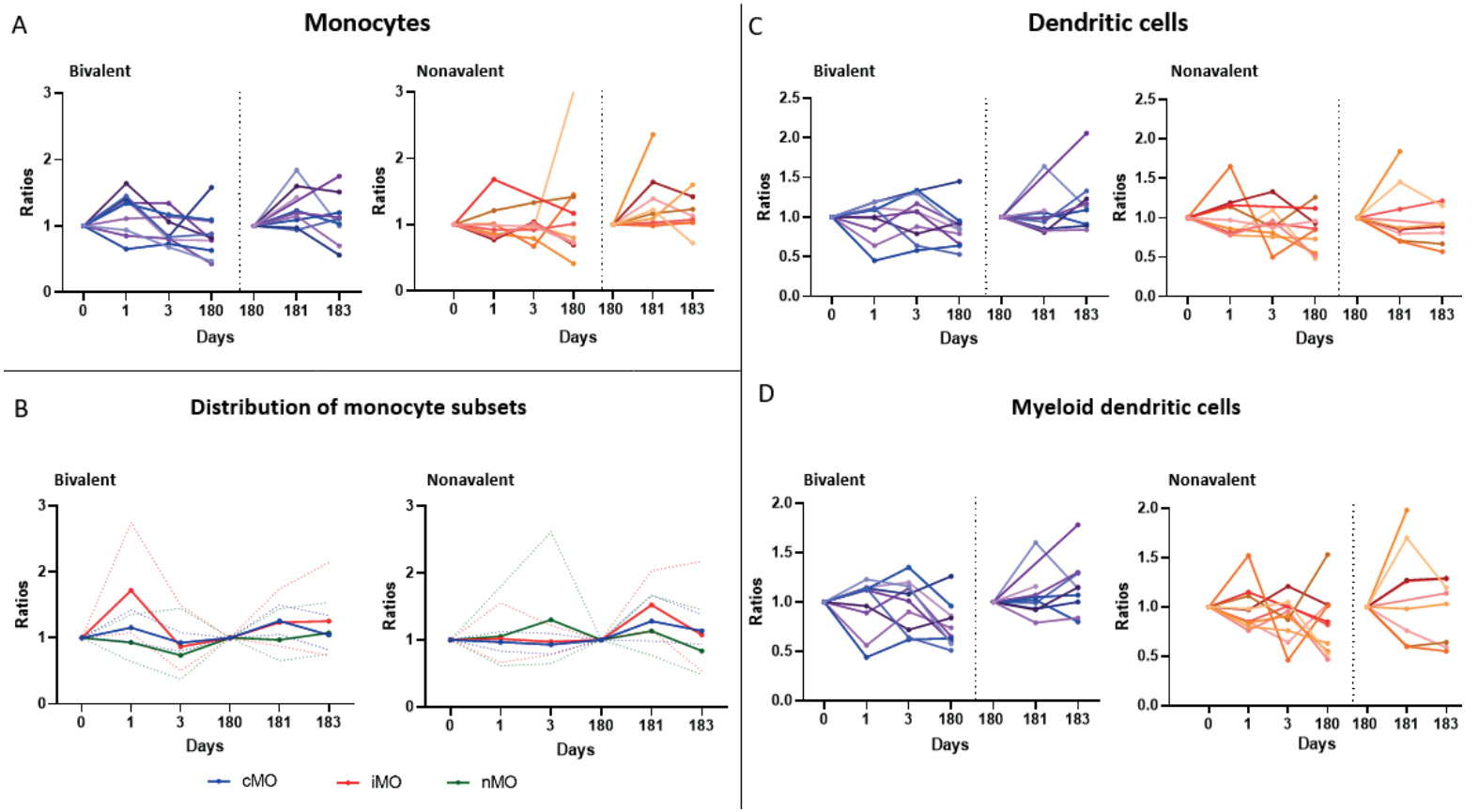


Figure 2 Monocytes (A), the distribution of monocytes (B), dendritic cells (C) and myeloid dendritic cells (D) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at days 0, 1, 3, 180, 181 and 183 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value. cMO= classical monocytes, iMO- intermediate monocytes and nMO are non-classical monocytes.

cMo p.adjusted.value<0.05. Overall, a higher number of circulating monocytes was observed upon bivalent vaccination than after nonavalent vaccination.

The total numbers of DCs (CD45+, CD33+, CD141+/-, HLA-DR+, FcERI+, CD14-, CD16-, CD303+/-) upon vaccination showed a heterogenous response between donors of both vaccine cohorts (Figure 2C). However, in some donors first an increase (day 1) and then a decrease (day 3) is observed, while in other donors this was reversed. In the nonavalent cohort the ratios of the DCs varied, but showed a non-significant trend at day 3 that suggest a decrease upon primary vaccination (0.86 95%CI 0.71-1.04). After the third vaccination this was similar (Figure 2C). The DCs can be subdivided into myeloid DCs (mDC) which mainly stimulate T cells, and plasmacytoid DCs which have a role in viral infections due to their interferon producing capacities [28]. We studied the ratios in numbers of mDCs (CD45+, CD33+, CD14- to dim+, HLA-DR+, FcERI+, CD16-CD141+/-) which showed a pattern similar to that of the total DCs (Figure 2D). Overall, no clear increase or decrease was observed over time in numbers of DCs in either cohort.

In the bivalent cohorts, median ratios of numbers of neutrophils increased at days 1 (1.46 95%CI 1.05-2.02) and 181 (1.42 95%CI 1.15-1.76) and returned to baseline or lower levels at days 3 and 183 (Supplementary Figure 3A). In the nonavalent cohort an increase was only observed after the third vaccination at day 181 (1.48 95%CI 1.18-1.85). Numbers of neutrophils differed significantly between the two cohorts at day 1 and 3 after primary vaccination, p.adjusted.value<0.01 and p.adjusted.value<0.05, respectively, being higher in the bivalent vaccinated cohorts.

Basophils are important in allergic response and eosinophils are involved in combating parasites [29], but are not likely to be involved in a response to vaccination. Basophils and eosinophils fluctuated independently of the primary vaccination with some differences between donors (Supplementary Figure 3BC). Surprisingly, after the third vaccination a significant increase for eosinophils was observed at day 183, with median ratios in numbers for bivalent and nonavalent donors of 1.44 95%CI 1.15-1.79 and 1.32 95%CI 1.04-1.68, respectively (Supplementary Figure 3C). Natural killer (NK) cells showed neither a significant increase or decrease at day 3 or day 7, depending on the donor, in both cohorts. However, after the third vaccination there was a clear peak at day 3 in 8 out of 10 donors of the bivalent cohort, this response occurred somewhat later in time (day 7) in the nonavalent cohort (Supplementary Figure 3D).

Heterogenous changes in T cell numbers after vaccination

CD4 T cell ratios of the bivalent cohort increased either at day 3 (2 donors) or day 7 (6 donors) post vaccination (up to 1.33 and 1.32 fold above baseline, respectively; Figure 3A), albeit no significant increase or decrease for median values was observed after primary and third vaccination. CD4 T cell ratios did not change after primary and third vaccination in the donors of the nonavalent cohort. The number of CD4 T follicular helper (TFH) cells, which help B cells in the formation of germinal centers, generally followed the kinetics of total CD4 T cells (Figure 3A). Overall changes for CD4 T cells and CD4 TFH cells were subtle, but homogenous between donors.

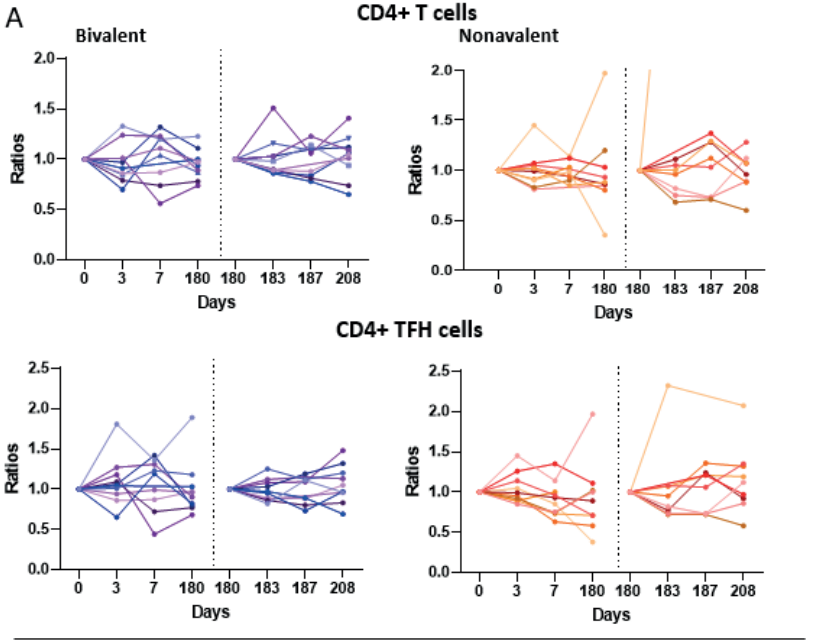
In general, the numbers of CD4 T-helper 1 (Th1) like cells (CD183+ CD194- CD196- CCR10-) decreased in the first week after primary vaccination in both the bivalent and the nonavalent cohort, with medians of 0.69 95%CI 0.56-0.86 and 0.79 95%CI 0.64-0.98 at day 3 and 7 respec-

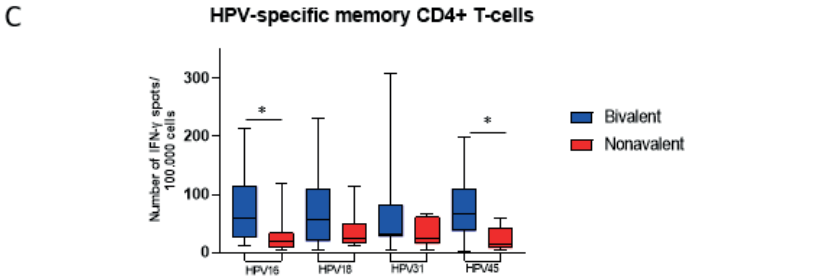
tively and for nonavalent donors 0.82 95%CI 0.66-1.02 and 0.90 95%CI 0.72-1.11 at day 3 and 7, respectively (Figure 3B). After the third vaccination, the median ratios in numbers of CD4 Th1-like cells increased to a similar extent in both cohorts at day 3 but the change was only significant in the nonavalent cohort 1.69 95%CI 1.06-2.71. The numbers of CD4 Th2-like cells (CD183- CD194+ CD196- CCR10-) in both the bivalent and nonvalent cohort had a heterogenous response. The numbers of CD4 Th17-like cells (CD183- CD194+ CD196+ CCR10-) showed a significant decrease at day 7 in the bivalent cohort of 0.44 95%CI 0.20-0.97. In the nonavalent cohort, an inconsistent pattern of the response in the first week after primary vaccination was observed. After the third vaccination, no significant changes were observed in both the bivalent and nonavalent cohorts. In the bivalent cohort, median ratios of numbers of CD4 Th22-like cells (CD183- CD194+ CD196+ CCR10+) showed a decrease at day 7, 0.38 95%CI 0.15-0.99. After the third vaccination an increase was observed, despite not being significant, 2.06 95%CI 0.92-4.60 and 2.05 95%CI 0.89-4.72 at day 183 and 187, respectively. In the nonavalent cohort a heterogenous response was observed upon primary vaccination between the participants. After the third vaccination this was the same. In Supplementary Figure 4, CD4 T regulatory (Treg) cells, CD8 T cells and gamma delta T ($TCR_{\gamma\delta}$) cells are shown, these cells fluctuated independently of vaccination and showed a high variation between donors (Supplementary Figure 4BCD).
Long-term HPV16/18/31 and 45-specific IFN-y producing cells were detectable in all donors at day 208. The bivalent cohort showed consistently higher numbers of specific IFN-y producing cells compared to the nonavalent cohort, being significant upon HPV16 and HPV45 stimulation, p=0.029 and p=0.026, respectively (Figure 3C).

Plasma cell expansion and maturation after 7 days post primary vaccination

In both cohorts the ratio of plasma cells (PC) above baseline showed a strong increase at day 7 after primary vaccination, up to 7.65 fold and 34.23 fold for the bivalent and nonavalent cohort, respectively (Figure 4A). This increase was significant in the nonavalent cohort (3.01 95%CI 1.347-6.60) and almost significant in the bivalent cohort (2.15 95%CI 0.98-4.71). After the third vaccination there was a non-significant trend towards an increase at day 187 in both cohorts (Figure 4A). In Figure 4B and Supplementary Figure 5, changes in ratios of numbers of different subclasses (IgG, IgA, IgD and IgM) of plasma cells are presented. In both the bivalent and nonavalent cohort, a significant increase is seen at day 7 for IgG1 subclasses, 3.03 95%CI 1.11-8.31 and 5.80 95%CI 2.12-15.90, respectively. In the nonavalent cohort a significant increase is observed for IgG2 and IgM, 2.27 95%CI 1.19-4.30 and 5.20 95%CI 1.53-17.72, respectively. A significant decrease was observed for IgG4 for both the bivalent and nonavalent cohort, 0.02 95%CI -0.02-0.08 and 0.03 95%CI -0.01-0.06, respectively. After the third vaccination, significant increases are only observed in the bivalent cohort at day 187, in IgG1 (3.11 95%CI 1.14-8.49) and in IgG3 (10.68 95%CI 3.81-29.94). In the nonavalent cohort no significance increase was observed, but for IgG3 an increasing trend was observed (2.97 95%CI 1.00-8.82). Furthermore, plasma cells can be divided into maturation stages based on the expression of CD20 and CD138. Most immature plasma cells are CD20+CD138-, then they become CD20-CD138- and most mature plasma cells are CD20-CD138+. All these maturation stages can be measured in blood [30]. Total plasma cells showed an increase in maturation phenotype (CD20-CD138+) at days 7 and 187 (data not shown).

An increase in numbers of memory B cells was not observed in the first week after primary and the third vaccination in both cohorts (Figure 4A). HPV-specific memory B cells for type 16, 18,





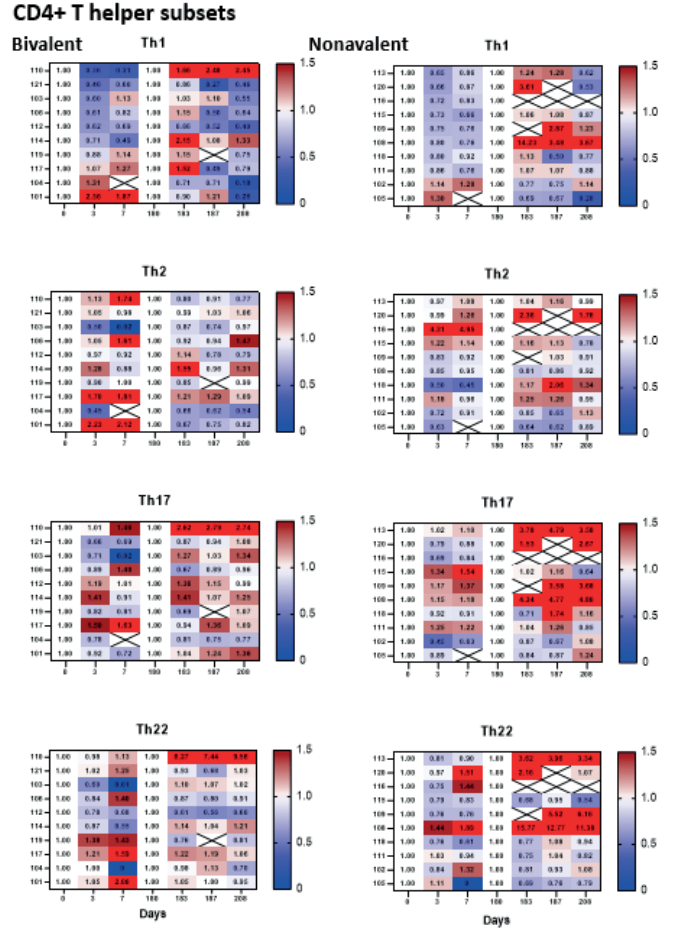


Figure 3 CD4+ and CD4+ TFH T cells (A) and CD4+ T helper subsets (B) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 3, 7, 180, 183, 187 and 208 post vaccination. Values >1.5 are depicted in bright red. HPV16/18/31/45 specific memory CD4+ T cells, X indicates non-reportable value at that timepoint (C) at day 208 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value. *p<0.05

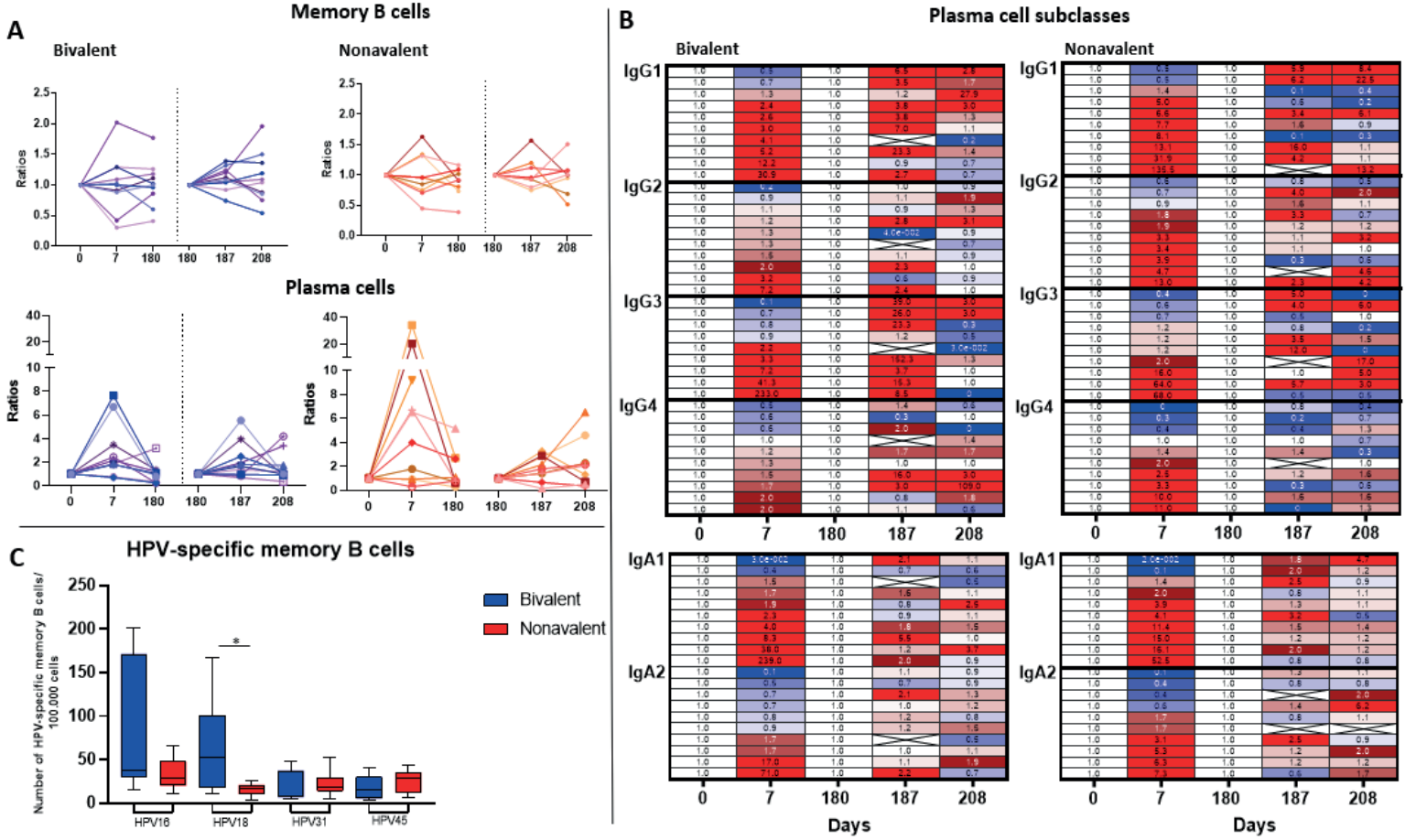


Figure 4 Memory B cells, plasma cells (A) and plasma cell distribution responses (B) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 7, 180, 187 and 208 post vaccination. Values >2.0 are depicted in bright red. HPV16/18/31/45 specific memory B cells, X indicates non-reportable value at that timepoint (C) at day 208 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value.*p<0.05.

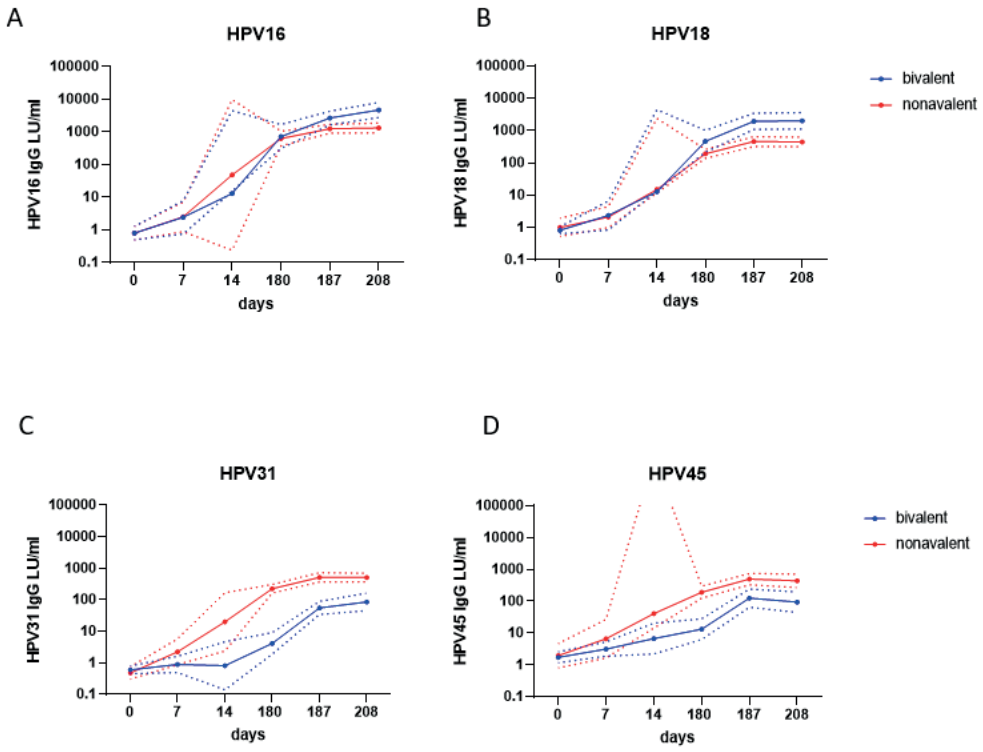


Figure 5 HPV 16/18/31/45 specific IgG antibodies IgG upon bivalent (blue) and nonavalent (red) vaccination at day 0, 7, 14, 180, 187 and 208 post vaccination. The line represents the GMC at each timepoint and the sashed lines represents the 95% confidence interval.

31 and 45 were all detectable at a month post third vaccination (day 208). The bivalent vaccinated donors showed significantly higher numbers of HPV18-specific memory B cells than those in the nonavalent cohort, p=0.035, and slightly higher but not significant for numbers of HPV16-memory B cells (Figure 4C).

High HPV-specific antibody responses in both vaccination cohorts

HPV-specific IgG antibodies were induced by both vaccines and their levels increased from day 7 up to day 187 after vaccination and seemed to plateau at day 208 (Figure 5). The GMC for HPV16 and 18-specific IgG levels was significantly higher in the bivalent cohort than the nonavalent cohort at day 208, p=0.0011 and p=0.0003, respectively (Figure 5AB). As HPV31 and HPV45 were not present in the bivalent vaccine, HPV31 and -45 IgG-specific antibody levels were significantly higher in the nonavalent cohort compared with the bivalent cohort, p<0.0001 and p=0.0015, respectively (Figure 5CD). HPV-specific IgA levels showed similar patterns as for IgG but did not show significant differences between the cohorts (Supplementary Figure 6). The most abundant HPV16,18,31,45 IgG subclass induced after both the bivalent and nonavalent vaccination was IgG1, followed by IgG3. Very small amounts of IgG2 and IgG4 were found. HPV16, 18 and 31 specific IgG1 distributions were significantly higher in nonavalent vaccinated women. HPV16, 18 and 31 specific IgG3 distributions were higher in nonavalent vaccinated women (Supplementary Table 3).

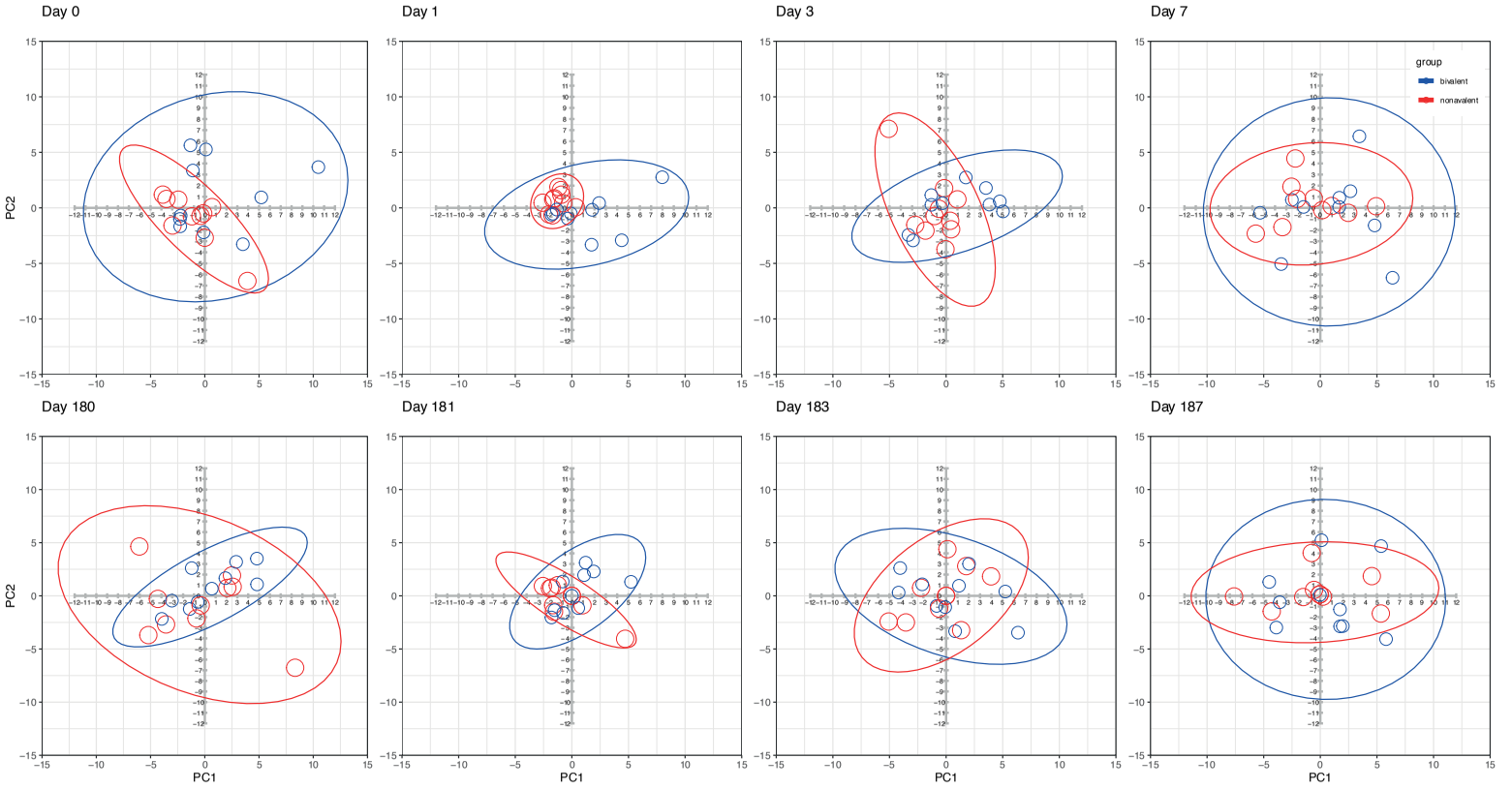


Figure 6 Visual representation of the principal component analysis where we describe the individuals by vaccination group, where group "bivalent vaccination" is denoted by 0 (blue) and group "nonavalent vaccination" is denoted by 1 (red) at days 0, 1, 3, 7, 180, `181, 183 and 187 post vaccination. The size of the eclipses visualizes the contribution of individuals to the first two principal components.

Individual responses post vaccination per donor in the major cell subsets in both vaccination cohorts

In Supplementary Figure 6 the absolute numbers of cells for the four major cell lineages (monocytes, B cells, plasma cells and T cells) over time are indicated per participant. The graphs are ordered based on the magnitude of plasma cell expansion. The donors showed a heterogenous response. In seven of the donors with a clear plasma cell expansion, no response in their numbers of monocytes was observed. In the remaining four donors with a less prominent plasma cells expansion an increase in the numbers of monocytes is observed. Six donors with a stable, or even slight decrease, in the numbers of plasma cells appear to have an increase in their numbers of monocytes. In the remaining two, no or a slight decrease in the numbers of monocytes is observed. Numbers of total B cells and CD4 T cells appear to remain similar to baseline levels overtime, and do not seem to be affected by vaccination.

Multidimensional comparisons of kinetics of different cell types

To study the long-term response, we looked for correlations between the IgG levels at day 208 with numbers of memory B- and T cells at day 208, CD4+ T cells and plasma cells at various timepoints, per HPV type, stratified per vaccine cohort. No significant correlations were found with HPV16-specific IgG levels at day 208 (Supplementary Table 4). We also studied the correlations between IgG subclasses and IgG subclass plasma cells at day 187, here also no significant correlations were found (data not shown).

By means of the PCA analysis, we determined at day 1 after primary and booster vaccination (days 181), the variation between all individuals was generally being explained by numbers of monocytes and mDCs, at days 3 and 183, this was equally explained by the numbers of innate cells and T cells. At day 7 the numbers of plasma cells explained most of the variance between individuals, while at day 187 this was explained by the numbers of CD4 T cells and B cells.

Also by means of PCA analysis, we determined potential differences between the two vaccine cohorts in the variation of induction of the various circulation numbers of cells during time after vaccination. We projected the input of all immune cell types at each time point onto 2 dimensions and analyzed the pairs of cells that contributed to differences in the phenotypes of cells between the vaccines. At day 1 the Wilcoxon-Mann-Whitney test of association showed evidence of differences in distributions of cells between the two vaccination groups, $p=0.04$. At this day only innate cellular subsets were measured, so this means that the vaccinations have a different effect on the expansion of innate cell subsets at day 1 after vaccination. At the other timepoints there was no evidence of differences in distributions of cellular subsets between the two groups (Figure 6).

DISCUSSION

In this study, we investigated the kinetics of various immune cell subsets in the circulation following primary and third vaccination with the bivalent and nonavalent HPV vaccines using in-depth immunophenotyping by means of state-of-the-art flow cytometry. These cellular kinetics were correlated to HPV-specific antibody levels and numbers of long-term memory B- and T cells and were compared between the bivalent and nonavalent HPV vaccines. The numbers of innate cells, especially that of monocytes, showed a clear expansion one day after vaccination in both cohorts and was significantly higher in the bivalent cohort than in the nonavalent cohort. In both cohorts, the numbers of plasma cells expanded in the first week after both primary and third vaccination, being significant only upon primary vaccination. In all other cellular subsets, a large heterogeneity in responses between the donors was observed. The HPV16 and 18-specific antibody levels and memory B and T cell responses were higher in the bivalent than the nonavalent cohort at one month after the third vaccination. This was opposite for HPV31 and -45 levels, which are not contain in the bivalent vaccine.

Innate cells, which form the first line of defense against infections, react quickly to vaccine antigens via pattern-recognition receptors like toll-like receptors (TLRs). The adjuvant AS04 is described to interact via monophosphoryl lipid A (MPL) with TLR4, which is frequently present on antigen presenting cells (APCs) [31, 32]. Innate cells, such as monocytes serve several functions within the immune system, most importantly phagocytosis, antigen presentation and cytokine secretion [33]. In this study, the numbers of innate cells have been determined at baseline and day 1 and day 3 after vaccination. Especially the numbers of monocytes, and their corresponding subsets, together with that of neutrophils, changed in the circulation at day 1 post vaccination. Monocytes and dendritic cells react upon vaccination as APCs and further migration to the secondary lymph nodes to be able to present the vaccine antigens, whereas the other innate cells studied have these capacities to a lesser extent [33]. Monocytes differentiate into cMo, iMo and nMo groups [34], in our study we found that absolute counts of iMo and cMo are higher in the bivalent cohort compared with the nonavalent cohort at the first day after vaccination. Which can be explained by the TLR4 agonist present in the adjuvants of the bivalent vaccine. Moreover, the PCA plots showed us that at day 1 the contribution of the monocytes of the bivalent vaccinated donors to the variance in the total observed response was greater than that of the nonavalent vaccinated donors. Although differences in magnitude of monocyte responses between cohorts are in line with the literature, we cannot exclude that part of these differences can be attributed to the differences in the kinetics of the immune response. Since changes in the innate compartment are very dynamic and the number of samples is limited, even a minor change in the response time may have a large effect on direct comparisons. Especially for iMo, that showed a clear peak in the bivalent cohort at day 1, this peak in numbers of iMo may reflect an effect of the vaccine in the activation of monocytes. We also observed higher numbers of neutrophils in the bivalent cohort than found in the nonavalent cohort within the first days after vaccination, suggesting that the nonavalent vaccine induces a more moderate innate immune response.

Dendritic cells (DC) are classical APCs and should become activated upon vaccination. However, DCs in blood are only found in an immature state and upon activation rapidly migrate to the lymph nodes, to infiltrate into the tissue at the site of administration [35]. We observed a

heterogenous response in numbers of circulating DCs directly after vaccination and in consequence we were unable to show clear kinetics in the DC response. Another study, making use of mass cytometry, did however show expansion of DCs upon vaccination [36], but an attenuated vaccine was used instead of a VLP-based viral vaccine and different timepoints (from day 5) were chosen. Mobilization of leukocytes into the circulation is highly likely to be specific for the type of vaccine, as also shown by the data presented here.

Following the initial wave of the innate cells, the adaptive part of the immune system becomes activated. The numbers of CD4 T cells showed an increase from day 3 up to day 7 after primary vaccination in the bivalent cohort, which was less pronounced in the nonavalent cohort. This is in line with the observed HPV-specific IFN-γ responses. After the third vaccination no clear expansion was seen for both cohorts, similar to what was reported for antigen-specific T-cell upon booster vaccination with the hepatitis B vaccine [37]. Also, the individual plots show no clear effect of vaccination (bivalent or nonavalent) on numbers of CD4 T cells. CD4 TFH cells, which support the activation and differentiation of B cells into Ig-secreting plasma cells [38], showed a heterogenous response and were not found to be correlated to B cells in our study. The adjuvant present in the bivalent vaccine (AS04) has been shown to be capable of inducing a Th1 T-cell response in mice [14], whereas the adjuvant present in the quadrivalent and nonavalent vaccine mainly induced a Th2 T-cell response in animal models [39]. In contrast, human clinical data so far only show limited differences in vaccine-induced T cell (cytokine) profiles between the bivalent and quadrivalent vaccine, as assessed by expression of IL-2, IFN-γ, and TNF-a [40], which is confirmed by our data. Long-term HPV-specific IFN-γ producing cell levels, which we use as a measure for the HPV-specific T cell responses, were significantly higher for type 16 and 45 in the bivalent cohort when compared with the nonavalent cohort. This is in agreement with other studies [40, 41], that describe a trend towards higher IL-2 and TNF-α levels in bivalent vaccinated individuals than quadrivalent ones. So, although the aluminum adjuvanted vaccines (quadrivalent and nonavalent) also induces a Th1 response, this is higher in the AS04 adjuvanted vaccinated individuals. However, the precise underlying mechanism still needs to be unraveled and the relevance to protection .

Plasma cells, which are responsible for the production of antibodies, show a clear expansion at day 7 after primary vaccination with any of the two vaccines, which is also clear in the individual plots. Moreover, plasma cells also show to be the highest contributor to the variance in immune response observed at this timepoint in the PCA data. After the third vaccination this effect was diminished. It could be that we have missed the peak in plasma cells upon the third vaccination, since an expansion in plasma cells will be quicker due to already induced memory B cells. This was supported by a study showing a plasma cell expansion after the third dose of a rabies vaccination already at day 4 post booster vaccination [42], which was not included in our analysis.

Adjuvants, such as AS04, that activate TLRs are thought to induce antibody class switching [15]. In the bivalent cohort, an increase in specifically the subclasses IgG1, IgG2, IgG3 and IgA1 was observed. In the nonavalent cohort, IgG1, IgG2 and IgA1 showed the biggest increase. Remarkable was that IgG3 especially increased after the third vaccination compared with primary nonavalent vaccination. In contrast, our HPV-specific IgG subclass data show a significant higher contribution of IgG3 in bivalent vaccinated women than that in nonavalent vaccinated women.

Spearman correlation analysis did not show any correlations between IgG-subclass-specific plasma cells with HPV-specific IgG-subclass responses at day 187. This could either be explained by kinetics, as the antibodies induced by the plasma cells still need to be formed at day 187. Otherwise it could be that a part of the induced IgG-subclass specific plasma cells are not HPV-specific. In other studies, bivalent vaccinated women showed especially an IgG1 and IgG3 antibody profile [43], whereas quadrivalent, containing the same adjuvant as the nonavalent vaccine, showed high levels of IgG4 and IgA in addition to the IgG1 and IgG3 response [41, 44]. This, together with our results, suggests that the bivalent vaccine is better capable of inducing an IgG3 response. Since IgG3 is related to a potent pro-inflammatory response [45, 46], which could perhaps explain the higher immunogenicity of the bivalent vaccine compared to the nonavalent. HPV-specific memory B cells at one month after the third vaccination showed higher numbers for HPV16 and 18 in the bivalent cohort compared with the nonavalent cohort, which is in line with other studies comparing the bivalent and quadrivalent vaccine [5-7]. These studies also showed that the bivalent vaccine induce higher antibody levels for HPV16 and 18 than the quadrivalent vaccine [6, 8]. This is in line with our findings, where long-term HPV-specific IgG antibody levels for HPV16 and 18 were significantly higher in the bivalent cohort.

In individuals that received the bivalent vaccine, cross-reactivity and cross-protection against HPV types absent in the vaccine is observed and is mostly attributed to the AS04 adjuvant [7]. Although this is observed in many studies [6, 8, 9, 13], the mechanism explaining this is still lacking. Another additional explanation could lie in the difference in the structure of the L1 protein in the two different vaccines as besides the differences in adjuvants, also different L1 expression systems are being used for the synthesis of the HPV VLPs. The VLPs used in this study to measure HPV were made with the baculovirus expression system, resembling the VLPs of the bivalent vaccine. However, since others observed similar differences between bivalent and quadrivalent vaccinated individuals while making use of recombinant proteins [8], we do not expect that this would lead to any bias. The bivalent L1 proteins are produced using a baculovirus expression vector system and purified to be able to form VLPs. These VLPs consist of important conformation-dependent neutralizing epitopes, such as U4, V5 and J4, thereby closely resembling the native HPV virions [47, 48]. Also the shape of the VLPs produced in baculovirus was found to be more consistent [49], when compared to the VLPs produced in the yeast expression system [50]. So, presumably the adjuvant AS04 in the bivalent vaccine contributes to the high and a long-lasting immune response. Additionally, the conformation of the VLPs of the L1 produced protein in the yeast expression system might be less optimal for inducing cross-protective antibodies whereas those produced in baculovirus might have a better conformation that allows cross reactivity. Together with the potent innate response observed upon bivalent vaccination, this might cause the higher memory T cell responses that subsequently result in a plasma cell expansion and the corresponding antibody production.

The immune subsets, measured using the EuroFlow tubes, are not antigen-specific, providing us only with information about changes in blood cell numbers of multiple (>250) immune cell subsets upon vaccination. It allows us to monitor in-depth innate, B cell and T cell immune responses at different time points after vaccination in a highly standardized and reproducible manner. However, a limitation of this study was the high heterogeneity observed between do-

nors, together with the low sample size, therefore no clear cell responses, except for plasma cells, could be identified.

Both vaccines induced detectable B and T cell responses, although HPV-specific numbers were higher in bivalent vaccinated women, and lead to high levels of antibodies. Further research in this area could be performed by looking at HPV-specific B and T cells after vaccination. The B cell repertoire [51] could give us insight in the type of memory B cells that are formed upon either bivalent or nonavalent vaccination, possibly giving us an explanation of the observed cross-protection in bivalent vaccinated women.

The HPV field is just beginning to understand the potential implications of innate and/or adaptive immune signatures and adjuvant effects on the generation of effective adaptive immune responses.

To get an insight into the impact of the cellular response on the efficacy of the currently used vaccines may be of importance. This is needed for a more extensive insight in how the innate immune response is linked to long term immunity.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1 Inclusion and exclusion criteria of the EVI study

Inclusion criteria	Exclusion criteria
• Seronegative for high risk-HPV vaccine types (16,18,31,33,45,52,58); • Female; • Normal general health; • Pre-menopausal; • Willing to receive HPV vaccination; • Provision of written informed consent; • Willing to adhere to the protocol and be available during the study period.	• Present evidence of serious disease(s) within the last 3 months before inclusion requiring immunosuppressive or immune modulating medical treatment, such as systemic corticosteroids, that might interfere with the results of the study; • Chronic infection; • Known or suspected immune deficiency; • History of any neurologic disorder, including epilepsy; • Previous administration of serum products (including immunoglobulins) within 6 months before vaccination and blood sampling; • Known or suspected allergy to any of the vaccine components (by medical history); • Previous vaccination with any HPV vaccine; • Pregnancy; • Participation in another vaccination/ medicine study.

Supplementary Table 2 Corrected P values of the associations between the repeated measurements at day 0 and day 180, by the Wilcoxon signed-rank test

Cell subset	P.adjusted. value
Bcell	0
ImmatureBcell	0
NAIVECD5Bcell	0
NAIVEBcell	0
MemBcell	0
MemBcellIgMD	0
MemBcellIgG1	0
MemBcellIgG2	0
MemBcellIgG4	0
MemBcellIgA1	0
MemBcellIgD	0
PCIgM	0
PCIgG1	0
PCIgG2	0
PCIgG3	0
PCIgG4	0
PCIgA2	0
PCIgD	0
Tcells	0
CD4	0
CD4TFH	0
CD4TFHnaive	0
CD4TFHTh1	0
CD4TFHTh2	0
CD4TFHTh17	0
CD4Treg	0
CD4naive	0
CD4Th1	0
CD4Th2	0
CD4Th17	0
CD4Th22	0
CD4Th1.17	0
TCRgd	0
CD8Tcells	0
Nkcells	0
Leukocytes	0
Eosinophils	0
Neutrophils	0
Basophils	0
Monocytes	0
cMO	0
iMO	0
nMO	0
DC	0
mDC	0
HPV16IgG	5,54E-05
MemBcellIgA2	6,51E-05
PC	0,000138
PCIgA1	0,000916
CD4TFHTh1.17	0,020145
MemBcellIgG3	0,04081

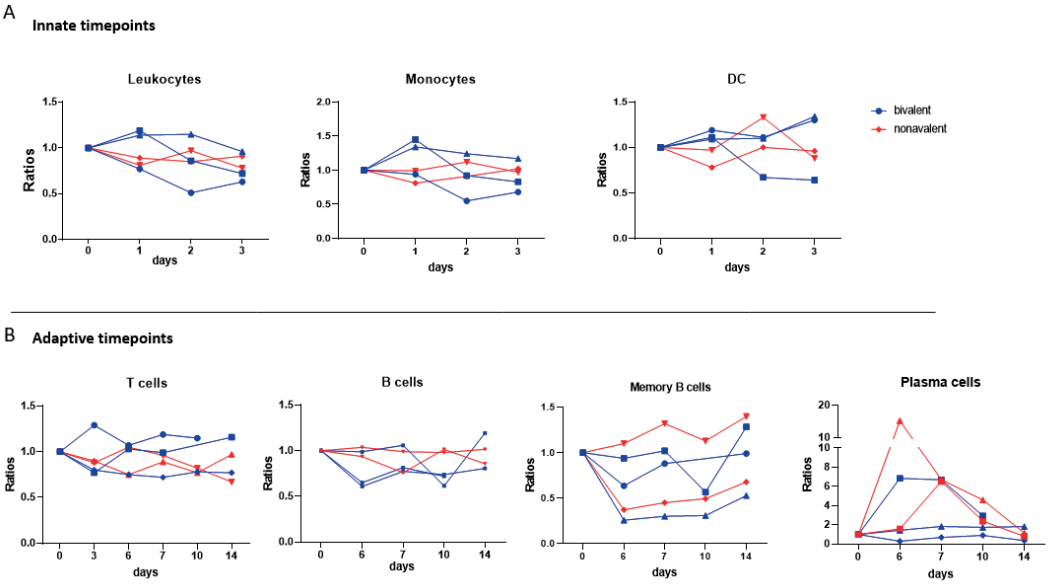
Supplementary Table 3 HPV16,18, 31 and 45-specicifc IgG-subclass percentages of the total HPV-specific IgG production

HPV16								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
Mean	53.92*	74.95*	0.11*	0.23*	45.9*	24.67*	0.07*	0.15*
95% CI	39.3-68.5	67.2-82.8	0.04-0.17	0.15-0.31	31.3-60.5	16.9-32.5	0.03-0.11	0.12-0.18
HPV18								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
Mean	49.7*	86.9*	0.15	0.17	50.0*	12.8*	0.13	0.16
95% CI	35.4-64.1	81.2-92.7	0.07-0.24	0.11-22	35.6-64.4	7.0-18.5	0.05-0.22	0.14-0.19
HPV31								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
mean	20.4*	79.9*	0.26	0.21	79.3*	19.7*	0.02*	0.17*
95% CI	13.6-27.2	72.7-87.1	0.0-0.62	0.13-0.29	72.4-86.2	12.5-26.6	0.0-0.06	0.15-0.19
HPV45								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
mean	68.6	76.7	0.16	0.28	31.2	22.6	0.07	0.46
95% CI	56.4-80.8	61.9-91.4	0.0-0.34	0.0-0.66	19.1-43.2	8.8-36.4	0.0-0.14	0.0-1.10

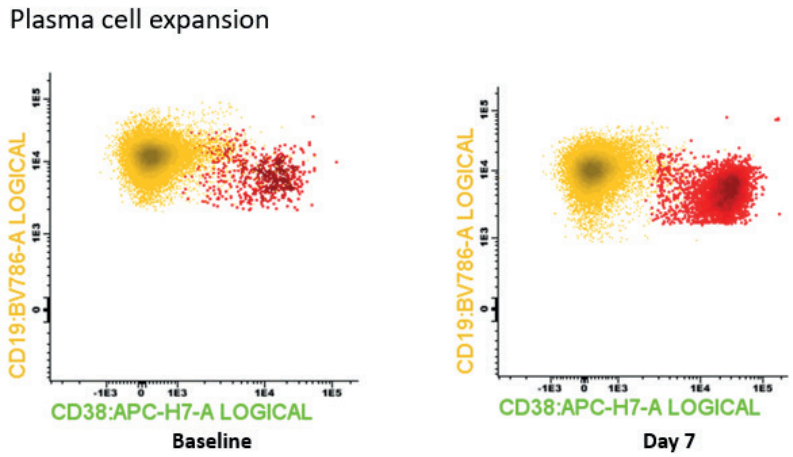
*$p<0.05$

Supplementary Table 4 Correlation between the long-term HPV16-antibody levels with different cellular subsets at various timepoints, stratified per vaccine cohort.

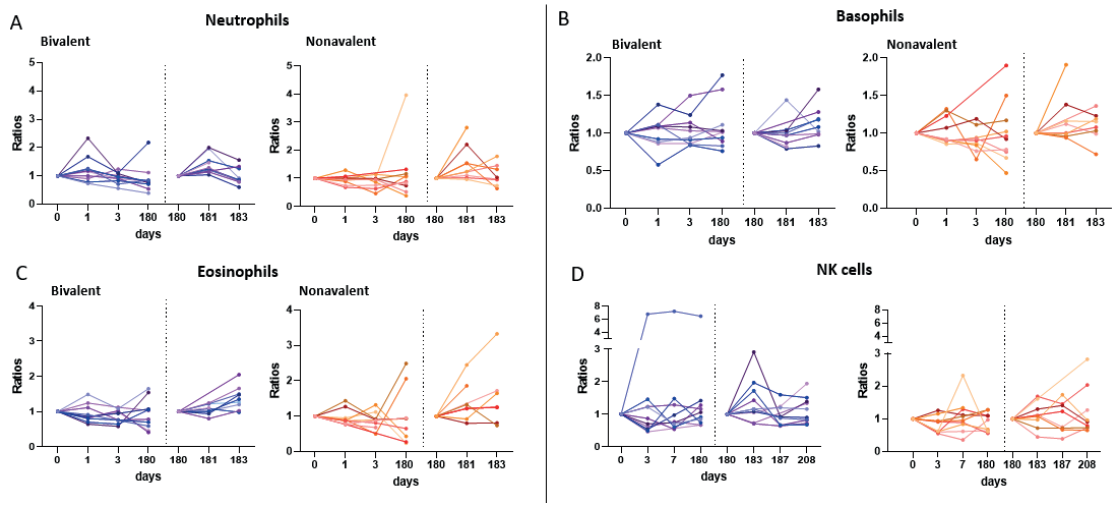
Cellular subset	Bivalent cohort		Nonavalent	
	R^2	P-value	R^2	P-value
Monocytes day 0	0.218	0.173	0.031	0.626
Monocytes day 1	0.065	0.476	0.009	0.789
Monocytes day 3	0.146	0.277	0.178	0.258
Monocytes day 180	0.149	0.270	0.025	0.662
Monocytes day 181	0.002	0.909	0.068	0.534
Monocytes day 183	0.017	0.742	0.046	0.611
DC day 0	0.162	0.249	0.095	0.388
DC day 1	0.068	0.467	0.010	0.787
DC day 3	0.041	0.575	0.054	0.549
DC day 180	0.003	0.889	0.052	0.526
DC day 181	0.036	0.626	0.001	0.931
DC day 183	0.007	0.827	0.013	0.786
CD4+ T cell day 0	0.041	0.573	0.002	0.894
CD4+ T cell day 3	0.002	0.911	0.002	0.902
CD4+ T cell day 7	0.002	0.911	0.027	0.650
CD4+ T cell day 180	0.008	0.811	0.001	0.947
CD4+ T cell day 183	0.022	0.680	0.073	0.518
CD4+ T cell day 187	0.001	0.999	0.001	0.941
CD4+ T cell day 208	0.054	0.520	0.004	0.866
HPV16 mem CD4+ T cell	0.002	0.906	0.056	0.540
B cell day 0	0.003	0.887	0.041	0.576
B cell day 7	0.050	0.535	0.096	0.383
B cell day 180	0.108	0.354	0.022	0.706
B cell day 187	2.62x10-5	0.990	0.050	0.598
B cell day 280	0.032	0.621	0.019	0.722
Plasma cell day 0	0.040	0.578	0.019	0.705
Plasma cell day 7	0.071	0.457	0.210	0.188
Plasma cell day 180	0.043	0.567	0.003	0.894
Plasma cell day 187	0.159	0.287	0.419	0.083
Plasma cell day 280	0.113	0.342	0.098	0.413
HPV16 mem B cell	0.045	0.555	0.304	0.124



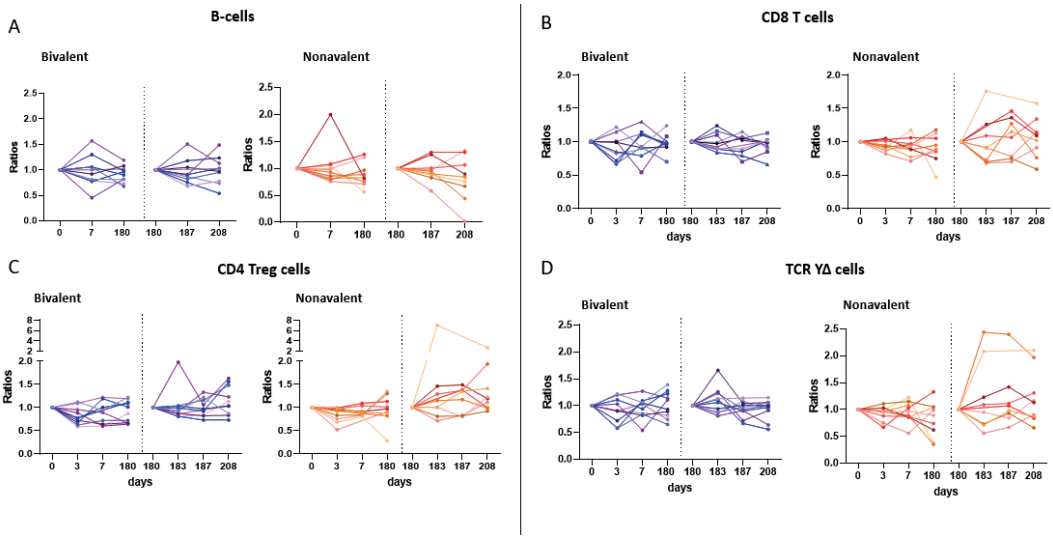
Supplementary Figure 1 Time finding results of the innate timepoints; leukocytes, monocytes and DCs at day 0, 1, 2 and 3 (A) and of the adaptive timepoints; T cells, B cells, memory B cells and Plasma cells at day 0, 3, 5, 6, 7, 10 and 14 after vaccination of either the bivalent (blue) or nonavalent (red) vaccine in the first five participants. Fluctuations of cells are presented as ratio compared to baseline value.



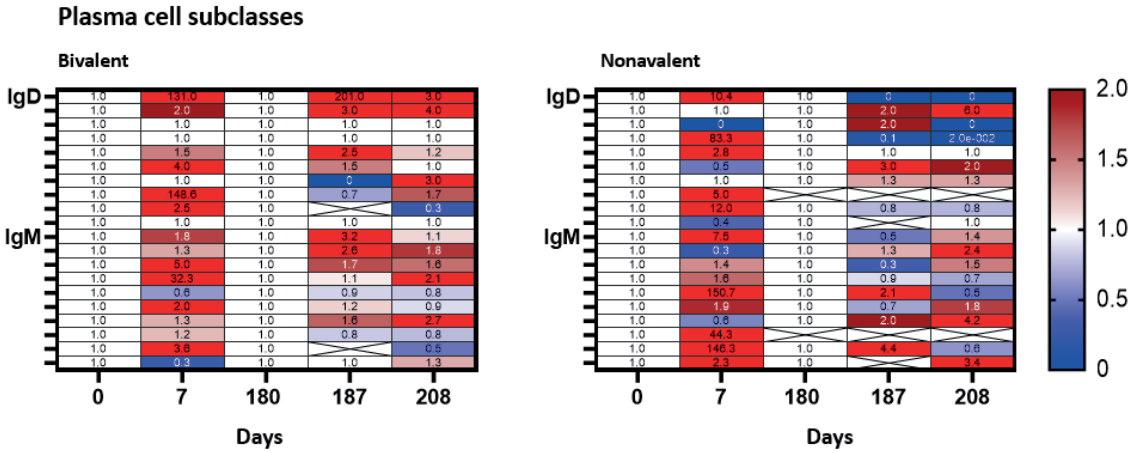
Supplementary Figure 2 Example of a plasma cell expansion from baseline to day 7 post vaccination of a bivalent donor. Plasma cells are depicted in red, memory B cells are depicted in yellow.



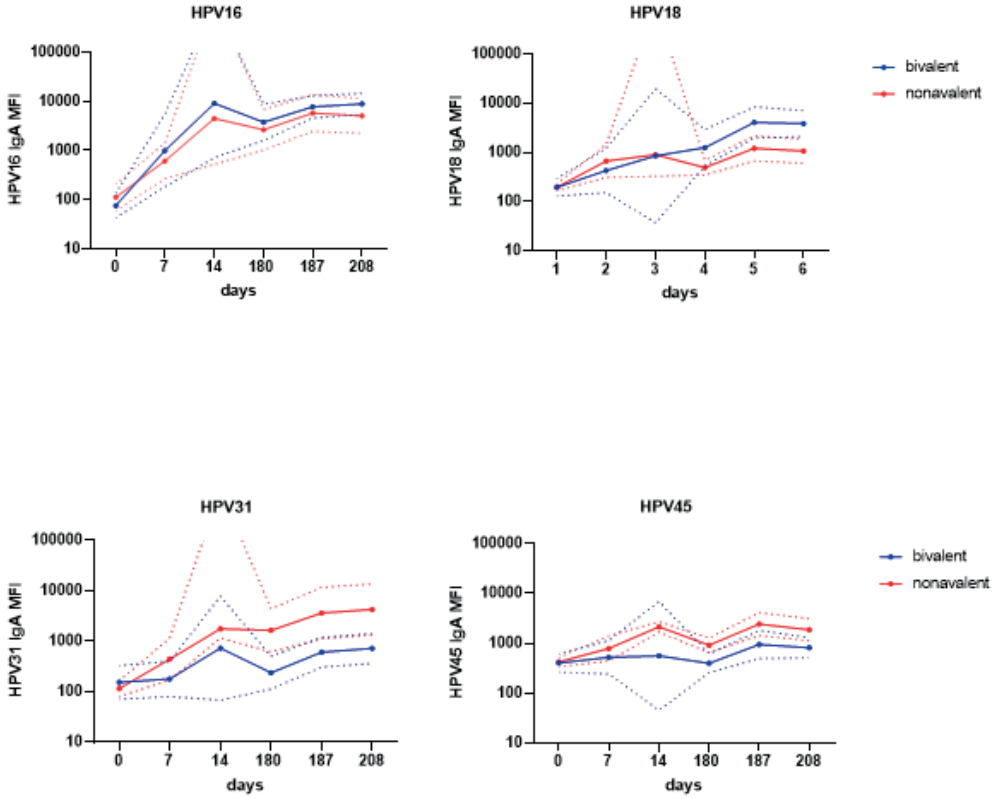
Supplementary Figure 3 Neutrophils (A), basophils (B), eosinophils (C) and NK cells (D) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 1, 3, 7 180, 181, 183 and 187 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value.



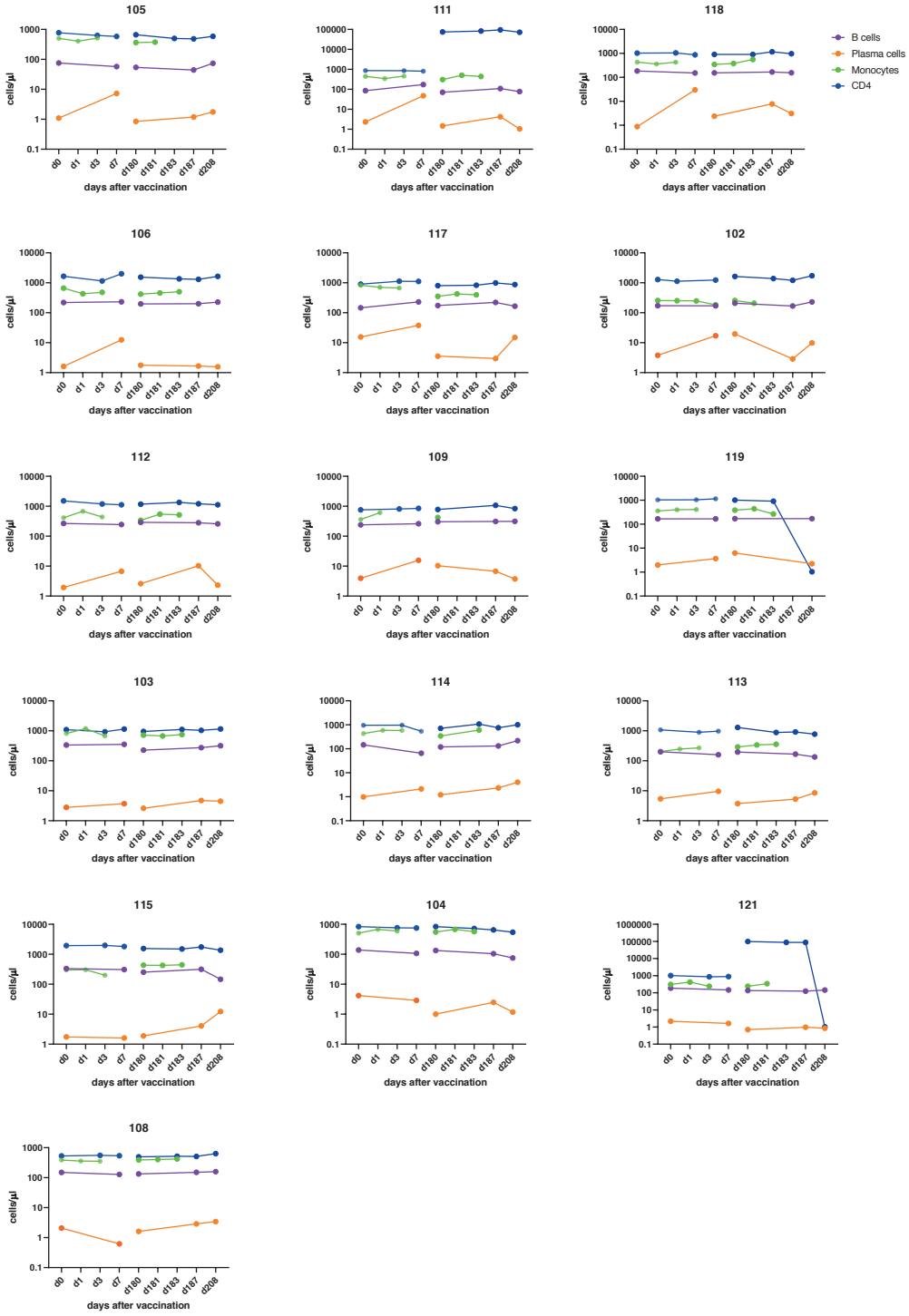
Supplementary Figure 4 B cells (A), CD8 T cells (B), CD4 Treg cells (C) and TCR YΔ cells (D) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 3, 7 180, 183, 187 and 208 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value.



Supplementary Figure 5 Plasma cell subclasses upon bivalent and nonavalent vaccination at day 0, 7, 180, 187 and 208 post vaccination. Values >2.0 are depicted in bright red.



Supplementary Figure 6 HPV 16/18/31/45 specific IgA antibodies IgG upon bivalent (blue) and nonavalent (red) vaccination at day 0, 7, 14, 180, 187 and 208 post vaccination.



Supplementary Figure 7 Absolute numbers of cells of the four most important cell lineages, monocytes (green), B-cells (purple), plasma cells (plasma cells) and CD4 T cells (blue), during time postvaccination per donor.

CHAPTER 7

GENERAL DISCUSSION

HPV is the most common sexually transmitted infection worldwide, and its presence is a necessary condition for the development of cervical cancer. Besides cervical cancer, HPV-infections are also known to cause other cancers situated at the anogenital or head and neck region, resulting in approximately 5% of all cancer cases worldwide. Most HPV infections are transient, and only 3-5% will eventually lead to (cervical) cancer in a long period of several decades, when no therapeutic intervention is performed. HPV vaccination was implemented in the National Immunization Program of the Netherlands in 2009/2010 and in that of the Caribbean Netherlands three years later. Both with the primary goal of reducing cervical cancer. This thesis described a series of studies on the immunogenicity of natural HPV infections and vaccine induced immune responses in the Dutch population. Here, I will elaborate on the most important findings from this thesis, describe their potential value and impact as well as speculate on future perspectives.

SUMMARY OF MAIN FINDINGS

In **chapter 2,** we assessed the possible changes in HPV-seroprevalence among the HPV unvaccinated Dutch population due to HPV vaccination by comparing the HPV seroprevalence in the pre- and post-vaccination era. This revealed an increase of high-risk (hr) HPV types exposure in unvaccinated women and a rather stable seroprevalence in men. For HPV16 a decrease in seroprevalence was found among men, that is unlikely to be due to herd immunity as we measured this at a rather short time frame after vaccine introduction in a period with a suboptimal vaccine coverage.Both the incidence and the mortality of HPV-related diseases differs geographically. Information on the HPV serostatus on the Caribbean Netherlands is not available yet. We performed a representative sero-surveillance study on these islands and described the results in **chapter 3**. We provided insight into population-based HPV serostatus, on lifetime cumulative HPV exposure and in past infections. A three-fold higher prevalence of multiple hr-HPV types was found among the female population when compared to men.

We further focused on studying the immune response in HPV vaccinated individuals. In **chapter 4** we described that HPV-specific antibody levels remain high and persistent for both vaccine and non-vaccine types. We observed this up to nine years postvaccination of a three dose vaccination schedule with the bivalent HPV vaccine in Dutch girls vaccinated at the age of 16. Although high antibody levels are thought to be important in the protection against an HPV infection, a correlate level of protection is lacking. Our study on the longitudinal relationship between antibody levels and HPV infections revealed no consistent differences between these two.

In 2014, the HPV vaccination program changed from a three-dose to a two-dose schedule. In view of even further reduced dosing schedules, we studied the humoral and cellular immune response after just a single dose of the bivalent HPV vaccine and compared this with a two- and three-dose schedule in **chapter 5**. One-dose of the bivalent vaccine resulted in elevated HPV-specific antibody levels up to seven years post vaccination. The levels of antibodies, however, were lower when compared to those induced by the two- or three-dose schedules. This lower antibody response coincided with lower numbers of memory B- and T-cells in one-dose recipients. This possibly indicates that that girls receiving one-dose might be at higher risk for waning protection to HPV in the long-term.

Currently there are three prophylactic HPV vaccines on the market, and several studies show that the immunogenicity of the bivalent vaccine is higher when compared with the quadrivalent and nonavalent vaccine. In **chapter 6**, we studied the in-depth kinetics of innate and adaptive cellular responses directly upon vaccination for the first time in a head-to-head comparison between the bivalent and nonavalent HPV vaccine. We observed strong monocyte responses the first day after primary vaccination, which were most pronounced in the bivalent vaccinated women. Also a clear expansion in plasma cells was observed in both cohorts in the first week after primary vaccination. HPV-specific antibody levels and memory B- and T cell responses were higher in the bivalent vaccinated women, with the exception of HPV31 and-45 specific antibody levels.

GENERAL DISCUSSION

Population based HPV seroprevalence

At a population level sero-surveillance studies, provide us information on clustering of groups of persons susceptible for HPV infection by risk factors and the impact of bivalent vaccination on the prevalence on other HPV types and herd immunity in men and unvaccinated girls.

HPV that enters the host by naturally occurring infection is capable of evading the immune system [1, 2], through the non-lytic life cycle of HPV and limited antigen presentation to lymph nodes. This results in limited but stable antibody production and seroconversion of only 40-60% of infected individuals [3-5]. HPV seroprevalence studies can be used to estimate the lifetime cumulative HPV exposure and experienced infections, whereas HPV DNA tests only detect clinical infections currently present. HPV seroprevalence studies are also easier to perform than HPV DNA studies by using vaginal DNA swabs. However, due to the limited immune response induced by HPV infection, HPV seroprevalence studies in naturally infected individuals do not represent an accurate measure of the number of infections, but actually underestimate them.
In **chapter 2**, we have observed that HPV seroprevalence among HPV unvaccinated women has increased in the Netherlands during the last decade. As we adjusted the results for demographic and/or sexual risk behavior, these factors cannot explain this increase. This may indicate that either the used questionnaire is not representative for the risk assessment for HPV seropositivity, or they were not answered in full honesty to intimate questions due to a social desirability bias, e.g. questions related to sexual behavior had the highest percentage of missing values. In men, HPV seroprevalence remained similar in this time period and for HPV16 even a decrease was found, which might be explained by herd immunity. However, in the specific age group (15-39 year old's) of men which most likely would benefit from girls-only HPV vaccination this decrease was not significant anymore, suggesting that herd immunity is unlikely. These findings were in agreement with observations in the United States, where just as in the Netherlands, there is a suboptimal vaccination coverage and no signs of herd immunity on the male population was observed after introduction of the HPV vaccine [6]. In contrast, the high vaccination coverage (>90%) in Australia resulted in herd immunity, impacting both (unvaccinated) males and females already at five years after the introduction of HPV vaccination [7, 8]. Thus the reason for a lack of herd immunity in the Dutch male population is probably due the suboptimal vaccination coverage of about ~ 50%, and in a lesser extent due to the short time period after introduction of HPV vaccination.

The incidence and mortality of HPV related diseases differ geographically [9], which can be largely explained by the presence of organized prevention programs, like screening and vaccination. In Caribbean countries, where vaccination and cervical cancer screening are mostly lacking, a higher incidence and mortality rate is observed when compared to the world average. The Caribbean Netherlands, comprised of the islands Bonaire, St. Eustatius and Saba (BES-islands) are public entities of the Netherlands. On the BES-islands, HPV-vaccination with the quadrivalent vaccine has been implemented in 2013 on St. Eustatius and Saba, and the bivalent vaccine on all three islands from 2015 onwards. So far, a population based screening program have not been introduced on these islands. As no data on protection against infectious diseases and associated risk factors were available for these islands, we have performed the Health Study Caribbean Neth-

erlands (HSCN) to monitor the National Immunization Program (NIP) (**chapter 3**). In **chapter 3**, we have described high HPV seroprevalence rates in the unvaccinated population, as described by other studies in the region [10], being highest in females in whom nearly three-times higher rates have been observed than found in men. These findings can be used as background information for future policy guidelines, since HPV vaccination is an outstanding method to be able to prevent cervical cancers on the long run. On the short term however, we speculate that cervical cancer screening has to be introduced on the BES islands, that certainly will reduce mortality due to cervical cancer.

To get a better understanding of the impact of the HPV-vaccination program, both in the Netherlands and on the BES-islands, it is of interest to continue the monitoring of the HPV seroprevalence and sexual behavior among the population every 10 year. In this way information can be generated about herd immunity, possible type replacement (HPV types not included in the vaccine) and changes in sexual behavior and which groups are at highest risk.

HPV seropositivity was higher in women than that in men in three consecutive population based studies (PIENTER) performed in 1995, 2006 and 2016 (**chapter 2 and 3**), that was in line with other population studies [11-15]. These differences between men and women are unlikely to be explained by different infection rates, since the similar infection rate between men and women was found in HPV DNA prevalence studies [16, 17]. The difference in HPV seropositivity between men and women is most likely caused by the different sites of virus infection. These are anatomically comprised of different epithelium types present at the penis and vulva/cervix, which are comprised out of either columnar or squamous and/or columnar epithelium respectively. The transformation zone in the cervix, where the squamous epithelium progressively undermines and replaces the columnar epithelium, displays metaplastic activity forming a risk for infection [18]. It is suggested that here HPV virions can easier reach the basement membrane and, therefore, are better capable of establishing a viral infection. The transformation zone also has the unique characteristic of facilitating pathogen recognition by the immune system, leading to a better HPV-specific immune response [19]. This could be an explanation to the observed differences in HPV seropositivity between men and women [3, 20-22]. This transformation has also been described in the anus, oropharynx and esophagus [23-25]. Therefore, if in the future questionnaires about sexual behavior would be extended with questions about vaginal, penile, anal and/or oral sex, we might get a better understanding of the role of the sites of entry of infection and corresponding seroconversion. It is important to note this information is accompanied with information about if this person is receptive and/or insertive during sexual intercourse.

We also observed HPV specific antibody levels in infants and young children, albeit being close to cut-off levels. In our and other population based studies [13, 15], these low levels were found up to the age of about nine years. There are various explanations for the presence of these antibodies in sexually naïve children. It is suggested that HPV-specific antibodies in infants are probably maternal IgG antibodies, however, since these antibodies wane in a few months after birth this explains it just partially [26, 27]. Infants could also be exposed to the virus via vertical transmission, for instance via the HPV infected genital tract of mothers during birth [28] or via an infected placenta or infected leukocytes cord blood [29]. Also horizontal transmission can occur, e.g. via breast-feeding [30] or oral/mucosal contacts [27, 31-33]. These data support the idea/

hypothesis that an HPV infection can be acquired early in life, possibly affecting an infection later in life. HPV-specific immunity in children is an unexplored area, as most focus is laid upon studying cervical infections in women. Just a few studies looked into the dynamics of HPV-specific immunity in children, showing that this was not related to the mothers HPV infection status. Especially oral infections were found to be most likely to occur in children [27, 34, 35]. This could lead to the induction of oral tolerance, or even a higher susceptibility for HPV exposure later in life but this still needs to be determined. However, in general the observed antibody levels in children are very low, and turned out to have no neutralizing capacities [36]. Presumably this will not interfere with vaccination later in life, as HPV vaccines are highly efficacious (~100%), meaning that this will also be the case in pre-exposed children.

HPV-seropositive associated risk factors were especially linked to sexual behavior, amplifying the need for prophylactic vaccination given before sexual debut. Our data supports the current advice of the Dutch Health council to vaccinate at an age of nine, as HPV seropositivity in the Dutch population begins to increase markedly after ten years of age.

In our studies we expressed antibody levels for HPV16 and 18 in international units (IU) per ml, according to an international reference serum for standardization of antibodies. This a prerequisite for comparisons and validation of various (population) serology studies, as currently different antibody detection techniques and associated cut off levels are being used. Adding of the current international reference serum for types 31, 33, 45, 52 and 58 is needed to make a broader comparison of differences in antibody levels to the several HPV-types in various laboratories worldwide and in follow up studies over time. The cut-off levels used in our studies (**chapter 2, 3 and 4**) to define seropositivity were determined by using a serum panel of children 1-10 years of age, assuming that they are predominantly HPV negative as they are sexually naïve. These cut-off levels are a fixed value per HPV-type, although the precise sensitivity and specificity is unknown by this method and could therefore be prone to misclassification bias. Other methodologies for instance by means of a mixture model could provide a more flexible approach, as it not defines one fixed cut-off value, until more is known of the serological response to HPV infection [37]. It is also argued to use sex-specific cut-off values [14, 38] as the infected epithelium differs. However, this only holds true if men would have only penile intercourse and not having anal intercourse. It has been described that the serological response upon anal infection is similar to that induced by a vaginal infection [20, 22]. Moreover, differences regarding HPV seropositivity that are present between men and women could be reduced and are perhaps not detectable anymore.

To get a better understanding in HPV-seropositivity, it is important to get a better insight in the immune response upon HPV infection. Most HPV infections are thought to be cleared or controlled to undetectable levels by the hosts immune response, whereas these infections persist in some individuals. A persistent (hr)-HPV infection is the major risk factor for the development of cervical cancer [39]. As described in **chapter 1**, HPV has several mechanisms to evade the host immune system, being an important step in persistence, but we do not know why this occurs just rarely. The first line of defense against infections is performed by innate immunity pathways and an efficient triggering of this innate response is a turning point between either viral clearance or virus persistence [40, 41]. Keratinocytes are a target for HPV infection and due to their expression of pattern-recognition receptors (PRRs) they can recognize microbial pathogens or damage

signals. PRRs include Toll-like-receptors (TLRs), which are capable of recognizing nucleic acids which are some of the microbial molecules that are accumulating during viral replication [42]. A high expression of TLR3, TLR7, TLR8 and TLR9 have been associated with HPV elimination, and are suggested to be predictors of HPV16 infection-clearance in women [40, 41]. Hr-HPV impairs important signaling pathways, like NF-kB and interferon-regulatory factor. This contributes to viral immune evasion and virus persistence [43-45]. In addition, polymorphisms in IL-1β, also affecting adaptive immunity [46], like IL-18 and inflammasome-related genes (NLR1 and NLR3) [47, 48] but not TLR9 polymorphisms, despite being a DNA sensor, were found to be associated with either viral clearance or persistence.

Thus, an inefficient innate immune response, thereby giving incorrect signals to the adaptive immune system, can lead to HPV viral persistence and eventually tumor progression. Why this innate immune response is different among individuals remains unknow so far. Explanations could lie in (epi)genetic changes, which have been observed for instance in transforming CIN lesions (reviewed in [49-52]), and deviations in host cell genes could accumulate over time being necessary for progression into cancer. Investment in studies to unravel this could help to elicit efficient therapies in HPV-related infections and tumors. On the other hand, the type of cells that are infected by HPV could be an important factor in progression towards lesions and cancer. Cells at the squamocolumnar junction (SJ) of the cervix have a unique gene-expression profile and biomarkers of these genes are highly present in high-grade CINs and cervical cancers[53]. Further research in exploiting the SJ phenotype can help us to better understand the risk in (early) cervical neoplasia.

Current Dutch vaccination program: bivalent vaccine in a two-dose schedule

The Netherlands has implemented HPV vaccination in their NIP in 2010 for 12 year old girls, together, with a catch up campaign which was offered to girls born between 1993 and 1996 in 2009. From 2014 onwards, the vaccination scheme changed from a three-dose schedule to a two-dose schedule. After implementation of a new vaccine into the NIP, its impact on the population is being monitored. Several factors can influence this impact, like 1) vaccination coverage, 2) the duration of the vaccine induced protection, 3) the hr-HPV types present in the vaccine, 4) rate of cross-protection and 5) the potential type replacement.

In the Netherlands, several studies are ongoing to monitor the effects on effectiveness and/or immunogenicity of the HPV vaccination program. In **chapter 4**, we describe persisting antibody responses against HPV16,18,31,33,45,52 and 58 up to nine years post vaccination. These findings were in line [54-56] with previous trials and other observational studies examining the immunogenicity of the bivalent vaccine. These high antibody responses also showed high vaccine effectiveness against incident persistent infections with HPV16,18,31,33 and 45 up to six years post vaccination [57]. No waning immunity to either vaccine type and cross-protective HPV types was observed. In several other countries observational studies also have shown a high vaccine effectiveness against infections and CIN lesions [58-61], as most of these countries start their cervical cancer screening at a younger age. In the Netherlands screening starts from 30 years onwards, consequently the first HPV vaccinated women will enter the cervical cancer screening program in the Netherlands in 2023. Cost-effectiveness models suggest that when HPV-screening is used as a primary tool, three life-time screens for vaccinated women are optimal [62]. All histological and cytological outcomes in the Netherlands are registered by the PALGA (Pathologisch- Anatomisch Landelijk Geautomatiseerd Archief) database and its linkage to the vaccine registration register (Praeventis) could be used to determine the vaccine effectiveness. This linkage is currently being done, and preliminary results showed that fully vaccinated women had a lower risk of developing atypical squamous cells of undetermined significance (ASC-US) and (H)SIL than unvaccinated women of the same age (Schurink-van 't Klooster; manuscript in preparation). The currently used HPV-based screening will still be the best method to detect cervical cancer cases, as it is not influenced by HPV prevalence in population [63].

Determining vaccine effectiveness by means of measuring persistent infections comes with several challenges. In the case of a natural infection our immune system is an important regulator in controlling HPV associated disease. The immune system either clears the infection or controls it by keeping it at a low copy number, thereby becoming latent [64]. Viral latency can be caused by an infection that did not reach a sufficient amount of viral load to be able to trigger the immune system. Latency can also be represented by an infection that is detected by the immune system, but subsequently not completely cleared. When the immune pressure subsides, the virus can reactivate. An infection could also become intermittently positive due to detection of a certain genotyping assay, while a more sensitive assay could detect a consistent persistent infection [19, 65]. Therefore it is difficult to differentiate between HPV infections that are latent or those that are simply below the level of detection of a certain test. Clinically validated genotyping assays, like the GP5+/6+ broad-spectrum PCR, consider the infections below their level of detection as not clinically relevant. This underlies the need that effectiveness measured with regard to CIN

and (cervical) cancer cases is of importance and must be awaited until 'real' effectiveness of the HPV vaccines to be established. Another challenge in determining effectiveness of HPV vaccination is with respect to other HPV related cancers, as mostly only cervical smears are sampled and not anal or oral samples. Efficacy of vaccination against anal intraepithelial neoplasia has been determined, and is as high as observed in CIN lesions [66, 67].

Measuring persistent infections requires longitudinal studies with standardized participation and follow-up, which is difficult to establish. For instance in the Netherlands, we achieved participation rates of about 10-20% for these type of studies. In the HAVANA study, written in **chapter 3**, a loss to follow-up was approximately 40%, being the biggest in the first two years of the study. Afterwards this follow-up became more stable with almost no loss of participation in the past years of follow-up. Although this possibly could lead to selection and/or habituation bias.

Male vaccination

Although still most of the HPV disease burden is caused by cervical cancer, HPV is also related to other morbidities affecting both men and women. These are less common than cervical cancer, but still result in various degrees of morbidity, mortality and costs. Even if all women were immunized, the transmission of HPV would still occur and be maintained among men who have sex with men (MSM).

Immunization of the male population will, besides to direct protection in males, result in a reduction of the risk of females being infected, through herd immunity. Sex-restricted vaccination demonstrated lower effectiveness, compared to universal vaccination [68]. Cost-effectiveness studies have shown that male vaccination is most cost-effective when female coverage is low. In contrast, a Dutch modeling study suggested that the most effective reduction of HPV infection is through increasing the uptake among girls, which is currently ~50% in the Netherlands, rather than including boys in existing programs [69]. Although this might be true, various approaches in communication strategies in the Netherlands have been conducted over the past years and numbers of vaccinated women are only slightly rising. Therefore, the introduction of male vaccination could benefit the Netherlands and significantly reduce disease burden because this might have a positive effect on the uptake by girls, as the focus would shift more towards HPV-related cancers than just cervical cancer. Target vaccination of only MSM, which will be at an older age, is likely to be less effective than vaccinating boys, as the prevalence of HPV among MSM is already high, especially at the anal site, at the time of vaccination [70] thereby being less effective. The MSM group will not benefit of female HPV vaccination. These data collectively argue for sex neutral vaccination. The Dutch Health Council advised to implement this from 2021 onwards [71].

In the Netherlands, potential side effects and sexual health aspects, believing that the girls were too young, of the vaccine were predominated in the HPV vaccine hesitance [72][73]. Other factors that could help to further increase the vaccination coverage is communicating honest about uncertainties and risks of vaccines and being transparent about how decisions are made within the NIP[74], enables parents to make an informed decision. Plain language must be used throughout communication to the public, as its wording, structure and design are so clear that the intended audience can easily find what they need, understand it and use it [75].

Monitoring of this introduction of sex-neutral vaccination is of high importance, as it is clear that vaccination can prevent cervical and anal cancer. If such a vaccination program would also prevent other HPV-associated cancers, like OPC, is still unknown as this has not been determined yet. Most OPCs are caused by HPV16. This type is present in all vaccines and effectively prevented anogenital diseases. It is suggested that this works just as efficient in OPCs, as in HPV vaccinated women a decrease in detection of HPV types is observed in the oral cavity [76-78]. However, clinical trials evaluating vaccine efficacy have been hampered by lack of data regarding incidence and clearance rates of oral HPV infections [79]. Persistent HPV infections result less often and slower to OPCs than cervical cancers. Therefore, more people and a longer follow-up time is needed to study the efficacy against OPC, making these trials highly expensive thereby hampering their initiation. The lack of an early clinical endpoint, since there are no well-defined oropharyngeal precancer lesions, also is a problem. However, initial studies suggest that vaccination might be effective in OPC [79, 80]. The presence of HPV16 E6 antibodies up to 10 years before cancer diagnosis is now suggested to be a biomarker, and could perhaps be used as an early marker to determine vaccine efficacy against OPC, as no precursor lesions are currently known [81].

One dose

Efficacy

Reduced dose HPV-vaccination schedules are of great interest in respect to the global health HPV burden, as this will reduce costs and simplify logistics. It will then become easier to reach women who are at the greatest lifetime risk of cervical cancer, and who are currently not being vaccinated [82]. Post-hoc analyses of original vaccine trials and population effectiveness studies among women who did not complete the full HPV vaccination scheme have suggested that one dose of the HPV vaccine is effective [83-86]. The protective effect against HPV16/18 infections was comparable, both for the bivalent and quadrivalent vaccine [59, 83, 85, 86]. However, cross-protection was observed after two and three doses with the bivalent vaccine, but not after one injection [59, 83], suggesting that an one-dose vaccination has a more limited efficacy to cervical cancer in general compared with two-or three doses.

Biological plausibility

The biological plausibility that HPV vaccines could be effective given in single dose is both supported by immunologic and virologic factors. The antibody levels detected against the vaccine targeted types are higher in most one dose recipients when compared to naturally infected individuals (**chapter 5**). This immunogenicity is largely attributed to the structure of the HPV vaccine antigen. HPV VLPs are composed of 360 ordered protein subunits forming a repetitive array of epitopes of 55nm on their surface. The interaction of these repetitive elements with B cell receptors on naïve B cells is exceptionally strong and leads to a consistent activation of memory B cells and long lived plasma cells (LLPCs) continuously producing antibodies for many years. Epitope spacing of 50 to 100Å appears critical for this, together with efficient trafficking to lymph nodes and efficient phagocytosis by antigen presenting cells. This leads to a potent and long lasting immune response, more closely resembling an acute virus infection rather than a simple subunit vaccine. In addition, as an infection is characterized by slow kinetics, the vaccine-induced anti-

bodies have more time to neutralize invading HPV virus [87]. In mice experiments, the transfer of HPV-specific antibodies at levels being even 100-fold lower than detectable in *in vitro* assays of vaccinated human beings, still was sufficient to protect against a HPV genital infection in this mice model [88]. It was therefore envisioned that antibody levels after a single dose with the HPV vaccine, although antibody concentrations are approximately 4-fold lower than two- and three dose vaccinated individuals, would not impair the efficacy of the HPV vaccines [87].

Immunogenicity

Immunogenicity studies show that antibody levels after a one dose schedule are lower when compared to two or three doses of either the bivalent or quadrivalent vaccine [84, 85], but still higher than after natural infection [84]. These findings are in line with what we find in **chapter 4**. Here, we also observed that these lower antibody responses coincided with lower production of T helper cytokines and lower memory T cell numbers, confirming results of Toh *et al.*[89]. Moreover, we showed for the first time that an one dose vaccination can induce HPV-specific memory B cells up to six years post vaccination, albeit that more doses resulted in a higher number of HPV-specific memory B cells. Follow-up data should clarify whether this lower immune response is also of clinical relevance. The lack of a correlate between the levels of HPV-specific antibodies and protection against HPV hampers further discussion on the minimum levels required to protect against infection. Randomized controlled trials, designed to determine the efficacy and/or immunogenicity of a one dose HPV vaccination are currently ongoing; in Costa-Rica (ESCUDDO; NCT03180034), Kenya (KEN-SHE; NCT03675256), the Gambia (HANDS; NCT03832049) and Tanzania (DoRIS; NCT02834637)[90]. In all these trials the nonavalent vaccine is also incorporated and more data concerning the efficacy against the five additional hr-types after just a single dose is expected soon. For the bivalent vaccine it was suggested that multiple doses are necessary to get an effective cross-protection against the non-16/18 HPV types, implying that a one dose strategy would not be an option. The nonavalent HPV vaccine on the other hand, generates antibody responses against all nine hr HPV types in one single dose. Thereby also closing the gap in costs, as at the moment the nonavalent is about twice as expensive as the bivalent vaccine.

Global cancer elimination

In 2018, the WHO Director General called a Draft Strategy to eliminate cervical cancer as a public health problem. This was approved by the World Health Assembly's in May 2020. The strategy outlines that cervical cancer is eliminated if there are less than 4 cases per 100,000 women and the timeline is that this should happen within the lifetime of today's young girls [91]. One of the prominent ways to achieve this is by increasing the vaccination uptake worldwide, ideally with a catch up in adults (sex neutral) to expand the proportion of immune individuals and thereby decreasing transmission.

There are several hurdles that hamper this. There is currently a HPV vaccine shortage, which is expected to be unsolved in the next five years. Nowadays, around 52% of all countries have implemented a HPV vaccination program, corresponding to the vaccination of 30% of 9-14 year old girls [91]. Eighteen percent of the global demand is currently used for males, and this number is only rising as more and more countries are implementing sex neutral vaccination. This percentage is for instance equal to no implementation in twelve LIC/LMIC. Therefore the Strategic Advisory Group of Experts (SAGE) of the WHO advised to postpone vaccination programs of

boys/men from 15 year old's onwards, thereby relieving the supply constraints in the short term and enable allocation in the countries with the highest HPV disease burden. Indeed, I agree with this that in the current situation of vaccine shortage, priority must be given to vaccinate women in LIC//LMICs with the highest burden of disease and most lives to be saved.

Another hurdle is the current global delivery infrastructure, especially in Sub-Saharan countries, making it not possible to vaccinate everyone and everywhere or at very high costs. HPV has for instance to compete with infectious diseases, like malaria and polio, for the global (research) funds.

Solutions to relief the current vaccine shortage, could be pausing the sex-neutral vaccination and catch up campaigns. This must however be done with extreme care, as it could help anti-vax audience to grab this as an opportunity to claim that there is something wrong with the vaccine. Therefore a delay in the introduction of a sex-neutral vaccination, rather than to pause a current program, might be better. HIC could argue that the HPV-related diseases in their countries, which is for instance the case in the United States is equal between men and women. This would lead to inequity on a country level, but not on a global level. As still 85% of all HPV-related diseases concerns cervical cancer. In addition to HPV vaccinations to prevent cervical cancer, cervical cancer screening programs also add to the prevention of cervical cancer by screening and treatment of pre-cancerous lesions. If we would only use the strategy of vaccinations, this would result in a 0.1% reduction in cervical cancer mortality in 2030 [91]. Combining both strategies, by scaling up of screening and treatment of pre-cancerous lesions would speed up this process. This would result in a reduction in mortality by cervical cancer of over 30%, in the same period of time [92]. In my view HIC are also better capable of affording and arranging a screening program, giving LIC/LLMC priority to vaccinations.

Another solution tackling both hurdles could be implementation of an one dose vaccination, as described in **chapter 5**, which will not contribute to further vaccine shortage. In my view this best can first be implemented in a HIC, with the argument stated above that here better monitoring and screening programs are in place, providing a 'safety net' if breakthrough infections would occur. If efficacy is then determined, one dose vaccination could simplify logistics and reduce financial costs.

We could also think of a way to save antigens and thereby relieving current supply constraints, by delivering the HPV vaccines in another way. All the HPV vaccines are currently administered by an intramuscular injection, but an intradermal injection might could save antigens, about to 1/5 up to 1/1000 of the current dose, which for instance has been done for the yellow fever and influenza vaccine [93-95].

So is this call for global cancer elimination rather a political statement instead of being realistic? My viewpoint is that as long as there is a vaccine shortage, there is not one perfect way to tackle all HPV related diseases. The global community therefore first has to decide with what aim we vaccinate; do we protect the individual or do we want to protect the population? Therefore to declare elimination seems a plan which is currently far from possible. Nevertheless, this does not mean there is no time for a combined action of vaccination and cervical cancer screening.

The perfect HPV vaccine

All currently licensed HPV vaccines, Cervarix, Gardasil and Gardasil 9, are very immunogenic and demonstrated to be highly effective [96, 97]. For all three VLP-based vaccines, formulation with adjuvants is essential to generate an effective immune response [98]. Adjuvants, like the classical aluminum salts are used in all three HPV vaccines and the AS04 adjuvant is also added in the bivalent vaccine. AS04 has the ability to stimulate TLR4, claiming to enhance APC maturation and a Th-1 mediated response [99]. For vaccine type-specific antibody levels, AS04 has proven the ability to induce higher responses than formulations only containing aluminum salt (**chapter 6**) [100, 101]. In **chapter 6**, we observed stronger HPV16 and HPV45-specifcic memory CD4 Th1 cell responses after vaccination with a AS04 adjuvanted vaccine. This may explain why the use of the bivalent vaccine results in higher antibody levels, as the interaction between HPV-specific CD4 Th cells HPV-specific B cells is required for B cell expansion and plasma cell formation. Plasma cells are responsible for the production of antibodies.

It is debated whether the AS04 adjuvant is responsible for the observed cross-protection in bivalent vaccinated individuals. Although this is observed in several studies [102, 103], the underlying mechanism remains unclear.
Another difference between the bivalent and the quadrivalent/nonavalant vaccine is the different L1 expression systems that are used to produce the L1 proteins for the HPV vaccines. A baculovirus expression vector system is used to produce the bivalent vaccine VLPs. These VLPs display important conformation-dependent neutralizing epitopes, such as U4, V5 and J4, thereby resembling the native virions in a close manner[104, 105]. Also the shape of the VLPs were found to be more consistent [106], when compared to the quadrivalent and nonavalent vaccine VLPs which are produced using a yeast expression system [107]. Potentially, this forms an alternative or additional explanation for the high antibody levels observed when the bivalent vaccine is used. It would therefore be interesting to study whether the VLPs formed in a yeast expression system together with the AS04 adjuvant would give the same immune response, as the VLPs formed in a baculovirus with AS04. At the same time, it is interesting to study which immune response is formed if the VLPs formed in a baculovirus are only adjuvanted with aluminum. In this manner, the impact of the adjuvants on the observed cross-protection of the vaccine may be revealed.

The limited amount of targeted HPV types in the bivalent and quadrivalent vaccine, is currently almost completely tackled by the nonavalent vaccine. Anogenital warts also have a large influence on the quality of life, impacting emotional well-being and sexual health [108] and its prevalence has been increasing in the Netherlands. Even after treatment, recurrences are high, leading to high treatment costs [109]. It is even stated that introducing a vaccine including type 6 and 11 in the NIP would lead to a more favorable cost-effectiveness of the vaccine [110]. Literature regarding the effect of the bivalent HPV vaccination against HPV6 and 11 infections and anogenital warts has been equivocal. There is no definitive answer as some studies find evidence for an effect [111-115], while others do not [116-120]. Although anogenital warts are not life threatening, considering to also include the protection of genital warts in the NIP could significantly decrease the economic burden and increase the quality of life.

Efforts are currently underway to design second-generation vaccines. Second-generation vaccines are aimed at generating a more broad, also mucosal, immune response in a more conve-

nient delivery mode. Currently, vaccines that currently have been tested in published clinical trials include purified VLPs delivered in the upper respiratory tract [121] with the aim to induce both serum IgG and secretory IgA in the female genital tract [122]. Commercial interest for this method, however, is limited as the delivery method still needs to improve to compete with subcutaneous injections. Preclinical studies with novel approaches are still at the preclinical phase. Other approaches have focused on L1 proteins expressed by existing live microbial vaccines. Cadila has generated HPV16 L1 recombinant of the Moraten Berna vaccine strain of measles virus, showing in mice induction of similar levels of measles virus antibodies as the parental vaccine strain and HPV16 antibody levels comparable to those induced after injection of purified VLPs [123]. This might lead to the addition of HPV to the current measles-mumps-rubella vaccination, which is already widely implemented, even in low-resource settings. Also an attempt with a recombinant *Salmonella* typhi vaccine is made, with hopeful results in mice [124]. These attempts hold promise for low-cost production and efficient delivery.

Vaccines based on the L2 protein, which is the minor capsid protein, are also under development. Antibodies to some L2 epitopes display a remarkable cross-neutralizing efficacy against a wide array of mucosal and cutaneous HPV types. Mouse and rabbit studies show that this broad neutralization is not only an *in vitro* artefact [125-127]. Sanofi Pasteur, together with its subsidiary Shanta Biotechnics, is initiating a clinical evaluation of a multimeric L2 peptide vaccine [128]. This holds promise for a broad protection against mucosal and cutaneous HPV infections by a relative inexpensive vaccine.

CONCLUSION

In this thesis, we showed that the use of HPV serology in big population studies is of importance for monitoring the HPV seroprevalence and the effects of HPV vaccination over time. The HPV antibody seroprevalence among women has increased in a 10-year time period in the Netherlands. Among males, seroprevalence remained similar and even a decrease for HPV16 was seen. Due to the short time after introduction combined with suboptimal coverage, this effect is unlikely to already be attributable to herd immunity. In the Caribbean Netherlands, there is a high seroprevalence of multiple hr-HPV types, especially among women. This indicates that there is a relative high risk of (precursors of) HPV-related cancers, thereby underlying the need to consider routine cervical cancer screening in Caribbean Netherlands. HPV antibody seroprevalence is increasing from ten years of age onwards in both the Dutch and the CN population, justifying the recent advice of the Health Council to lower the age at vaccination from 12 to 9 years of age. There is also a significant seroprevalence among the male populations albeit being lower than in women. This together with the lack of a clear induction of herd immunity by the girls-only vaccination justifies the advice of the Health Council to implement a sex-neutral vaccination program in the Netherlands.

We also showed that the bivalent HPV vaccine is highly effective and induces robust antibody responses up to nine years post-vaccination. Having a hr-HPV type infection was however not associated with HPV antibody levels before infection, thereby suggesting that likely also other (immunological) factors are of importance in determining the correlate of protection. A hr-HPV infection was associated with sexual risk behavior and smoking one year before infection.
In view of further reduction of HPV vaccination schedules, we studied both humoral and cellular antibody responses after different doses of the bivalent HPV vaccine. We found that the one-dose schedule induces detectable immunity up to seven years post-vaccination, but resulted in fewer B- and T-cell numbers and considerable lower antibody levels compared to two- or three doses. This might implicate that some of the girls receiving only one dose are at higher risk for unprotective immunity to HPV in the long term. However, a single dose vaccination is believed to significantly reduce the global cervical cancer disease burden, thereby also simplifying logistics and reducing costs which are of great importance for developing countries. A single dose would also not further constrain current vaccine shortages. To get a better understanding in the potential implications of the innate and adaptive immune response on the long-term responses, we studied the immune responses direct upon vaccination with the bivalent and nonavalent vaccine. Here we especially observed strong monocyte responses upon primary vaccination, being most potent in the bivalent vaccinated women. A clear expansion in plasma cells was observed in both vaccinated groups, and coincided with high long term antibody levels. HPV-specific antibody levels and memory B- and T cell responses were higher in the bivalent vaccinated women, with the exception of HPV31 and-45 specific antibody levels. This could be an explanation for the stronger cross-protection of the bivalent vaccine.

Finally, in the coming years important changes are expected regarding HPV screening and vaccination. The generation of vaccinated girls will enter the cervical cancer screening program and ultimate efficacy data will be available. The effectiveness of the one-dose schedule will become clear as clinical trials come to an end. In the Netherlands, a sex-neutral vaccination will have been

implemented in the near future. These changes will need to be monitored to provide scientific answers about the effectiveness and immunogenicity. For the current girls-only routine vaccination program, which is very effective, and efforts to try to increase its coverage are needed to generate higher health benefit for the total population. This thesis contains a variety of information about the natural and vaccine induced immunity against the human papillomavirus, follow up of the studies used in this thesis should be continued to get a better understanding of the 'real-world' evidence of HPV vaccination.

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APPENDICES

NEDERLANDSE SAMENVATTING

Het Humaan Papillomavirus (HPV) zorgt wereldwijd voor de meest voorkomende, seksueel overdraagbare infecties. Ongeveer 80% van de seksueel actieve populatie raakt ooit in zijn of haar leven besmet met HPV. De meeste HPV-infecties worden na zes tot twaalf maanden door het lichaam zelf opgeruimd. Sommige HPV-infecties blijven echter langdurig in het lichaam aanwezig. Dit noemen we 'persistente infecties'. Op dit moment zijn er al meer dan 200 verschillende typen HPV geïdentificeerd. Vijftien daarvan staan bekend als de zogeheten hoog-risico typen. Als je een persistente infectie hebt van een hoog-risico type dan kan dat leiden tot de ontwikkeling van baarmoederhalskanker. Ook andere kankers, zoals kanker aan anus, vulva, vagina, penis, mond en keel kunnen door HPV veroorzaakt worden. De typen HPV16 en HPV18 zijn de meest voorkomende hoog-risico typen. Zij zijn samen verantwoordelijk voor 70% van alle gevallen van baarmoederhalskanker wereldwijd.

HPV infecteert voornamelijk het gebied rondom de geslachtsdelen en de anus. Het slijmvlies hier is daarom de eerste verdedigingszone tegen een HPV-infectie. HPV heeft echter verschillende mechanismen ontwikkeld om het afweersysteem te omzeilen. Bij ongeveer 40 tot 60% van alle HPV-geïnfecteerde mensen worden antistoffen tegen HPV gevonden. In de rest van de gevallen zijn geen antistoffen tegen HPV vast te stellen.

De ontwikkeling van beginnende infectie tot baarmoederhalskanker duurt tien tot vijftien jaar. Als een HPV-infectie lang aanhoudt, kunnen er licht tot matig afwijkende cellen (cervicale intra-epitheliale neoplasie, CIN 2) zijn, die in de helft van de gevallen vanzelf weer door het lichaam opgeruimd worden binnen twee jaar. Matig tot ernstig afwijkende cellen (CIN3) hebben een kleinere kans om uit zichzelf te verdwijnen. Het bevolkingsonderzoek naar baarmoederhalskanker is gericht op het identificeren van vrouwen in de leeftijdscategorie van dertig tot zestig jaar die risico hebben op het ontwikkelen van baarmoederhalskanker. Door vroege opsporing van afwijkende cellen kan baarmoederhalskanker voorkomen worden. Naast een effectief screeningsprogramma kan middels vaccinatie een HPV-infectie en HPV-gerelateerde kanker voorkomen worden. Sinds 2007 zijn er zulke vaccins op de markt.

Momenteel zijn er drie effectieve vaccins beschikbaar die bedoeld zijn om een infectie met HPV te voorkomen, en daarmee ook bescherming te bieden tegen HPV-gerelateerde kankers. Ten eerste is er het bivalente vaccin dat beschermt tegen twee types HPV, namelijk HPV16 en HPV18. Het quadrivalente vaccin beschermt tegen vier types HPV, namelijk HPV6, HPV11, HPV16 en HPV18. HPV6 en HPV11 zijn zogeheten laag-risico HPV-typen, en veroorzaken nagenoeg alle gevallen van genitale wratten. Het meest recente vaccin is het nonavalante vaccin, bestaande uit negen types HPV. Naast bescherming tegen HPV6, 11, 16 en 18 biedt dit vaccin ook bescherming tegen de HPV typen 31, 33, 45, 52 en 58. Deze laatste vijf typen veroorzaken ongeveer 20% van de baarmoederhalskankergevallen wereldwijd. Alle drie de vaccins bieden goede bescherming tegen de typen HPV waarvoor ze bedoeld zijn. Daarnaast is er ook kruisbescherming tegen andere typen HPV aangetoond. Dit is het sterkst bij het bivalente vaccin.
In Nederland is in 2010 het bivalente HPV vaccin (Cervarix®, GlaxoSmithKline Biologicals) opgenomen in het Rijksvaccinatieprogramma met drie vaccinaties voor 12 jarige meisjes. Daarnaast is er in 2009 een eenmalige inhaalcampagne georganiseerd voor 13 tot 16 jarige meisjes om zich te

laten vaccineren. Vanaf 2014 krijgen meisjes voortaan nog maar twee vaccinaties, aangezien dit een even goede bescherming blijkt te bieden als drie vaccinaties. Vanaf 2021 zullen ook jongens gevaccineerd worden tegen HPV, en zal de leeftijd voor de start van vaccinatie omlaag gaan naar negen jaar.

Het afweersysteem is het verdedigingssysteem van ons lichaam tegen allerlei ziekteverwekkers zoals virussen, bacteriën en parasieten. Het afweersysteem kan onderverdeeld worden in een aangeboren- en een adaptief (verworven) deel. Het aangeboren systeem reageert snel, maar is niet zo specifiek voor een bepaalde ziekteverwekker. Het adaptieve deel, bestaande uit specifieke B-cellen en T-cellen komt wat langzamer op gang, maar kan zich daardoor beter aanpassen aan de ziekteverwekker en kan de reactie in de tijd ontwikkelen. Hierdoor wordt er een geheugenimmuniteit opgebouwd, waardoor het lichaam vervolgens langdurig beschermd is tegen die bepaalde ziekteverwekker. In dit proefschrift kijken we naar de reactie van ons afweersysteem in relatie tot HPV. We bekijken de hoeveelheid van HPV-specifieke antistoffen en in welke mate geheugencellen worden geïnduceerd na een HPV-infectie en na HPV-vaccinatie. Dit doen we op populatie- en individueel niveau.

HPV infecties in de populatie

Een infectie met HPV leidt in 50-70% van de gevallen tot een meetbare reactie van het lichaam; een detecteerbare antistofreactie. Deze reactie is gericht tegen het L1 eiwitkapsel van het virus. Antistoffen die opgewekt zijn na een HPV-infectie zijn relatief lang na infectie aanwezig in het bloed. Deze antistoffen kunnen gemeten worden en kunnen daarom gezien worden als een indicatie van HPV-infecties die in het verleden plaats hebben gevonden. Dit noemen we de serologische HPV reactie oftewel de HPV-serologie. In **hoofdstuk 2**, doen we verslag van onderzoek waarbij we gebruik hebben gemaakt van twee eerder uitgevoerde, grote populatiestudies, die een dwarsdoorsnede vormen van de Nederlandse bevolking, de PIENTER studies, en zijn uitgevoerd in de periode 2006-2007 en in de periode 2016-2017. De metingen van zeven verschillende HPV-antistoffen zijn onderzocht in deze studies. Daarmee hebben we de ontwikkeling van de HPV-seroprevalentie kunnen zien van een aantal belangrijke HPV-types in de Nederlandse populatie in de afgelopen tien jaar. Daarnaast kunnen we zien welk effect de introductie van het HPV-vaccin, in 2010, heeft gehad op de mate waarin HPV-infecties zich in de Nederlandse populatie voordoen.

In beide studies is te zien dat HPV aanwezig is in de leeftijdsfase van vijftien tot negentien jaar, de periode dat seksuele activiteiten over het algemeen starten zowel bij mannen als bij vrouwen. Ten opzichte van 10 jaar geleden hebben vrouwen vaker antistoffen tegen HPV en dit impliceert dat ze vaker geïnfecteerd zijn geweest. Bij mannen is de hoeveelheid antistoffen tegen HPV gelijk gebleven, met uitzondering van het type HPV16. Voor dit type is sprake van een daling. Het kan zijn dat dit de eerste signalen zijn van eventuele groepsimmuniteit naar aanleiding van de introductie van de vaccinatie van meisjes tegen HPV waar de mannelijke populatie indirect voordeel van heeft omdat HPV16 in het vaccin is opgenomen. Echter, de periode na introductie van HPV-vaccinatie is nog relatief kort en in combinatie met de lage vaccinatiegraad voor HPV van de afgelopen jaren, is groepsimmuniteit als verklaring voor deze bevinding niet heel waarschijnlijk.

De door HPV veroorzaakte kankers zijn wereldwijd niet evenredig verdeeld. In Afrika en het Caribische gebied komen HPV-gerelateerde kankers vaker voor dan in Westerse landen. Dit kan verklaard worden door het feit dat er in deze gebieden vaak geen bevolkingsonderzoek is gericht op baarmoederhalskankerscreening en vaak ook geen HPV-vaccinatieprogramma is. In 2010 hebben de eilanden Bonaire, Sint Eustatius en Saba een andere status gekregen. Daarmee valt de publieke volksgezondheid van deze eilanden onder de directe verantwoordelijkheid van de Nederlandse regering. Met behulp van de Health Study Caribisch Nederland (onderdeel van het PIENTER onderzoek), is een populatiestudie uitgevoerd in 2017 en is de prevalentie van infectieziekten voor Bonaire, Sint Eustatius en Saba voor het eerst in kaart gebracht. In **hoofdstuk 3**, hebben wij de HPV-seroprevalentie in Caribisch Nederland onderzocht. Er is hier een hogere HPV-seroprevalentie in vergelijking met Nederland met de hoogste cijfers op Sint Eustatius. Dit komt overeen met andere data uit deze regio. Meer dan de helft van de HPV-seropositieve personen blijkt geïnfecteerd voor twee of meerdere HPV-typen. HPV-infecties komen dus ook hier vaker voor bij vrouwen dan bij mannen. Deze informatie laat zien dat het voor deze drie eilanden van belang is over te gaan op de introductie van een sekse-neutrale HPV-vaccinatie en dat het belangrijk is bevolkingsonderzoek naar baarmoederhalskanker te gaan uitvoeren.

Afweerreactie na HPV vaccinatie

Gelijktijdig met de uitvoering van de inhaalcampagne van HPV vaccinatie in Nederland in 2009, is de studie 'HAVANA' (HPV Among Vaccinated And Non-vaccinated Adolescents) gestart met als doel het HPV-vaccinatieprogramma te monitoren. Binnen deze studie worden er jaarlijkse cervicale uitstrijkjes-, bloed- en cervicale secretiesamples afgenomen. In **hoofdstuk 4**, hebben we gekeken naar de longitudinale relatie tussen de hoeveelheid HPV-specifieke antistoffen en het aantal HPV-infecties onder gevaccineerde meisjes. We hebben gezien dat het bivalente vaccin hoge antistofconcentraties veroorzaakt en dat dit tot wel 9 jaar na vaccinatie hoog blijft. Dit geldt voor de twee HPV-types waartegen gevaccineerd wordt, maar ook voor enkele HPV-types die niet in het vaccin zitten. Er is echter geen verschil te zien tussen de hoeveelheid antistoffen per HPV-type van wel of niet geïnfecteerde meisjes.

Om inzicht te verkrijgen in de effecten van verdere reductie van het aantal prikken in het HPV-vaccinatieschema, hebben we in **hoofdstuk 5** de antilichaamreactie en cellulaire afweerreactie onderzocht na slechts een enkele prik van het HPV-vaccin in vergelijking met twee of drie prikken van het bivalente vaccin. De reductie van het vaccinatieschema naar een enkele dosis zou vooral een oplossing kunnen zijn voor (ontwikkelings)landen waar momenteel geen HPV-vaccinaties gegeven worden door de hoge kosten en de complexe logistiek. De schatting is dat 85 procent van alle gevallen van baarmoederhalskanker wereldwijd zich voordoet in landen waar geen vaccinatieprogramma is. Het geven van een enkele HPV-vaccinatie zou deze landen wel de mogelijkheid kunnen bieden om een HPV-vaccinatieprogramma te implementeren. In een studie waar ongeveer zeshonderd 12 tot 18-jarigen meisjes aan mee hebben gedaan, hebben we gekeken naar niveaus van HPV-specifieke antistoffen en bij een kleiner aantal deelnemers ook naar het geheugen van B- en T-cellen en van bijbehorende moleculen, cytokines genaamd. Bij meisjes die maar één keer gevaccineerd zijn, vinden we een langdurig aanhoudende HPV-specifieke antilichaamreactie. Deze is echter wel lager dan bij meisjes die twee of drie keer gevaccineerd zijn, en komt overeen met een verminderde HPV-specifieke B- en T-celreactie. Er zijn echter geen verschillen tussen de verschillende vaccindoseringen voor de kwaliteit van de

antilichaamreactie. Er is dus een aanhoudende afweerreactie tot jaren na een enkele vaccinatie, ook al is dit lager dan na twee of drie doseringen. Dit kan erop wijzen dat meisjes die maar een enkele vaccinatie hebben gehad, een grotere kans hebben dat hun HPV-specifieke afweer op de lange termijn sneller verdwijnt dan het geval zou zijn bij meerdere vaccinaties.

De drie verschillende HPV-vaccins die nu op de markt verkrijgbaar zijn, bestaan allemaal uit zogeheten virusachtige deeltjes (VLPs), die het L1 eiwitkapsel van het virus representeren. De drie vaccins maken gebruik van een verschillend 'adjuvans', een stof die aan het vaccin wordt toegevoegd om de afweerreactie van het vaccin te versterken. Het quadrivalente en nonavalente vaccin bevatten alleen aluminium als adjuvans, maar het bivalente vaccin gebruikt het adjuvans systeem 04 (AS04). Verschillende studies, die het bivalente met het quadrivalente vaccin vergeleken hebben, tonen een hogere antistofreactie aan na vaccinatie met het bivalente vaccin. In **hoofdstuk 6** hebben we een exploratieve studie uitgevoerd waar voor het eerst een vergelijking tussen het bivalente en het nonavalente vaccin wordt gemaakt in één studie. In deze studie kijken we naar de kinetiek van het aangeboren en het adaptieve afweersysteem direct na de vaccinatie. De bedoeling van dit onderzoek is de specifieke werking en de aansturing van het afweersysteem direct na vaccinatie beter te begrijpen. Gelijk op de eerste dag na vaccinatie zagen we vooral een sterke reactie van monocyten, witte bloedcellen die onderdeel zijn van het aangeboren afweersysteem, bij beide vaccins. Na zeven dagen is een sterke reactie in het aantal plasmacellen te zien. Plasmacellen zijn de meest uitgerijpte type B-cellen die de antistoffen produceren. Er zijn geen verschillen in de hoeveelheid van plasmacellen tussen beide vaccins, maar wel tussen de verschillende individuen. De hoeveelheid van HPV-specifieke antistoffen en HPV-specifieke B- en T-cellen zijn in beide gevaccineerde groepen aanwezig, maar het aantal ervan is hoger in de groep van de bivalent gevaccineerde vrouwen. Dit zou verklaard kunnen worden door het gebruikte AS04 adjuvans in het bivalente vaccin.

Samenvattend heeft dit onderzoek geleid tot meer kennis van HPV-immuniteit na een natuurlijke infectie en na een HPV-vaccinatie. HPV-serologie kan de afweerreactie op populatieniveau in kaart brengen, maar is ook een goede manier voor het bestuderen van verschillende karakteristieken van de HPV-afweerreactie na vaccinatie. Het bestuderen van de HPV-specifieke reacties van afweercellen zorgt voor een beter begrip van de werking van HPV-vaccins.

Toekomstig onderzoek richt zich vooral op de verschillende, belangrijke veranderingen op het gebied van HPV vaccinatie en het baarmoederhalskankeronderzoek. De eerste generatie van gevaccineerde meisjes gaan het screeningprogramma in, wat meer zicht kan geven op de uiteindelijke effectiviteit van het vaccin. Dit geldt ook voor de effectiviteit van een enkele HPV-vaccinatie als de klinische studies eindigen. De introductie van sekse-neutrale HPV-vaccinatie vindt in de nabije toekomst plaats, en zal gemonitord moeten worden om de effectiviteit en afweerreactie daarvan te kunnen bepalen. Wat betreft het huidige HPV-vaccinatieprogramma voor meisjes, dat erg effectief is, is vooral aandacht nodig voor het verhogen van de vaccinatiegraad om een grotere gezondheidswinst te behalen voor de gehele populatie. Dit proefschrift geeft inzicht in de natuurlijke en vaccin-geïnduceerde afweerreactie tegen HPV. Continuïteit van de studies in dit proefschrift helpt de werking van het HPV-vaccin beter te begrijpen.

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CURRICULUM VITAE

Hella Pasmans was born on April 4th 1992 in Valkenburg aan de Geul, the Netherlands. In 2010, she finished her secondary school at the Stella Maris College in Meerssen. Subsequently, she started her bachelor studies Nutrition and Health at Wageningen University and Research Center (WUR). After 3 years, she took a semester off to travel around New Zealand.

In 2014, she enrolled in the master program Molecular Nutrition and Toxicology at the WUR. She performed her master thesis on 'the effect of Desferrioxamine treatment on transferrin receptor, ferritin and hypoxia-inducible-factor-1-alpha protein level in C57BL/6JOlaHsd mice on a high fat diet' at the human and animal physiology group of the WUR, under the supervision of Dr. Jessica Hegeman and Prof. Dr. Jaap Keijer. For her first internship, she went to Instituto de Nutrición y Tecnología de los Alimentos (INTA) in Santiago, Chile. Here she developed a research in the lactation field called 'the effect of stress and cannabinoid receptor 1 antagonist during early lactation on insulin resistance in adult life of CD-1 mice', under the supervision of Prof. Dr. Miguel Llanos Silva. Hereafter she took some time off to travel around South America.

Her second internship she performed at the Danone Nutricia Research Institute in Utrecht, where she conducted a literature research on mycotoxins in relation to human health under the supervision of Dr. Prescilla Jeurink and Prof. Dr. Johan Garssen.

In October 2016, she started as a PhD candidate at the Centre of Infectious diseases of the National Institute for Public Health and the Environment (RIVM) in collaboration with the department of Medical Oncology at the Leiden Medical University Center (LUMC). Her supervisors were Dr. Anne-Marie Buisman, Dr. Fiona van der Klis and Prof. Dr. Sjoerd van der Burg. The research of her PhD focused on the natural and vaccine derived immunity against the human papillomavirus. The results obtained during her PhD are described in this thesis.

In November 2020, she started as a Scientist at the Clinical Immunology department at The Janssen Pharmaceutical Companies of Johnson& Johnson.

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