Natural and vaccine derived immunity against the human papillomavirus

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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.

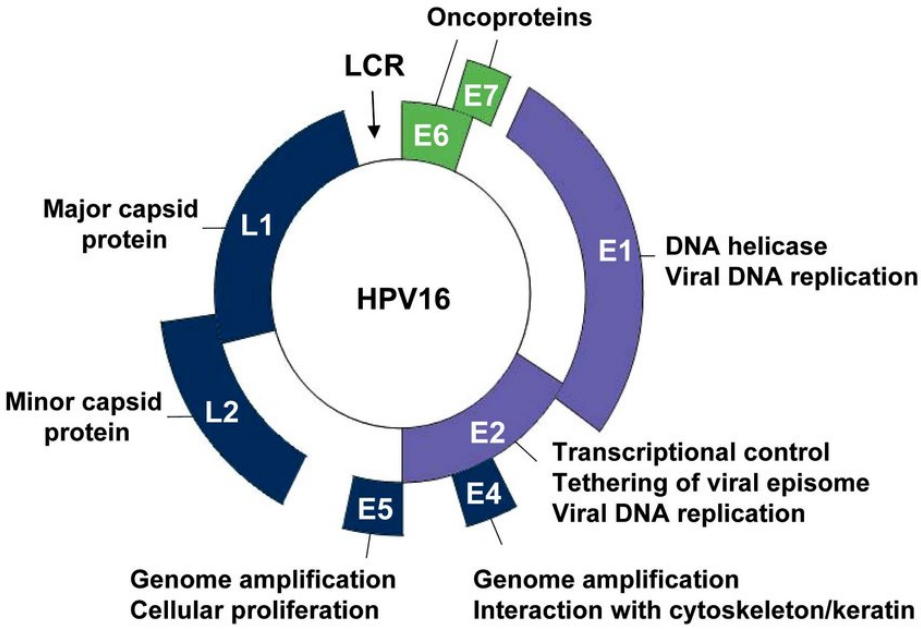

Figure 1 A schematic representation of the genomic organization of the HPV genome. Adapted from D'Abramo et al. [11]

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

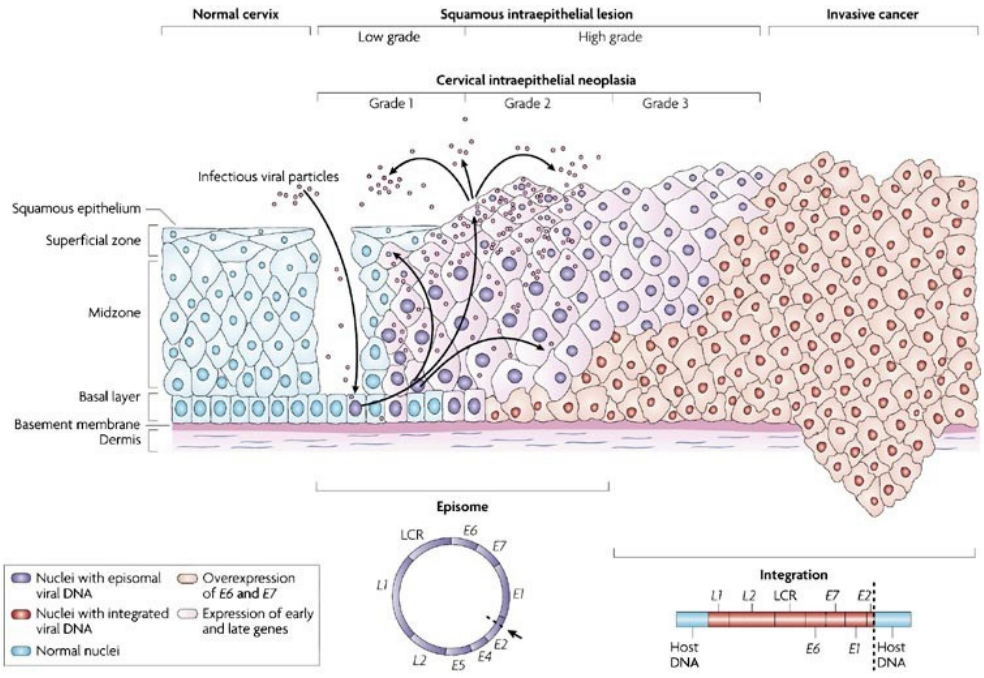

Figure 2 Schematic representation of HPV infection, progression and carcinogenesis. The different stages of a high-risk HPV infection are displayed, along with the transformations occurring in the cellular tissue. Adapted from Woodman *et al.* [18]

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].

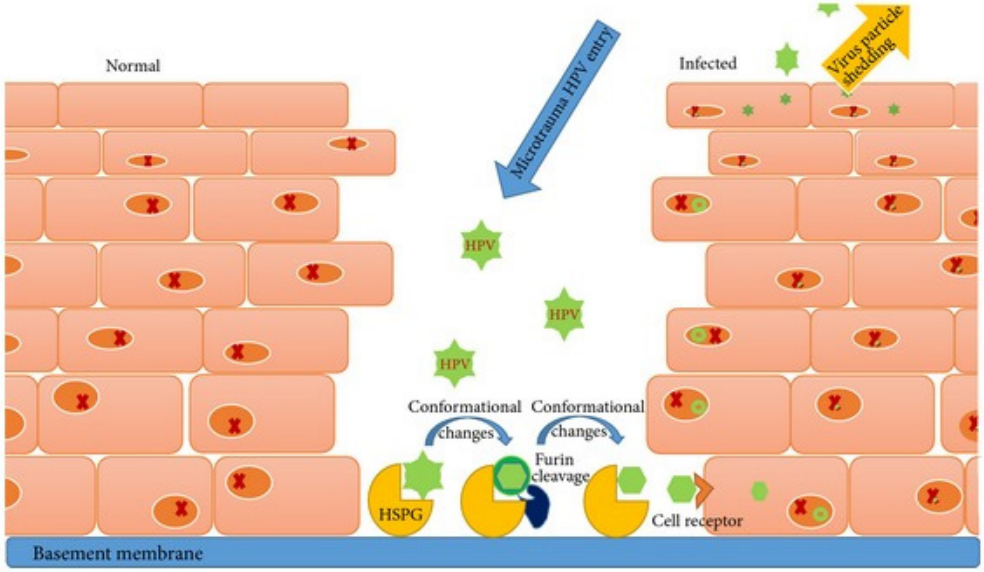

Figure 3 Schematic representation of the HPV infection into the basement membrane. Adapted from Deligeoroglou *et al.* [30].

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

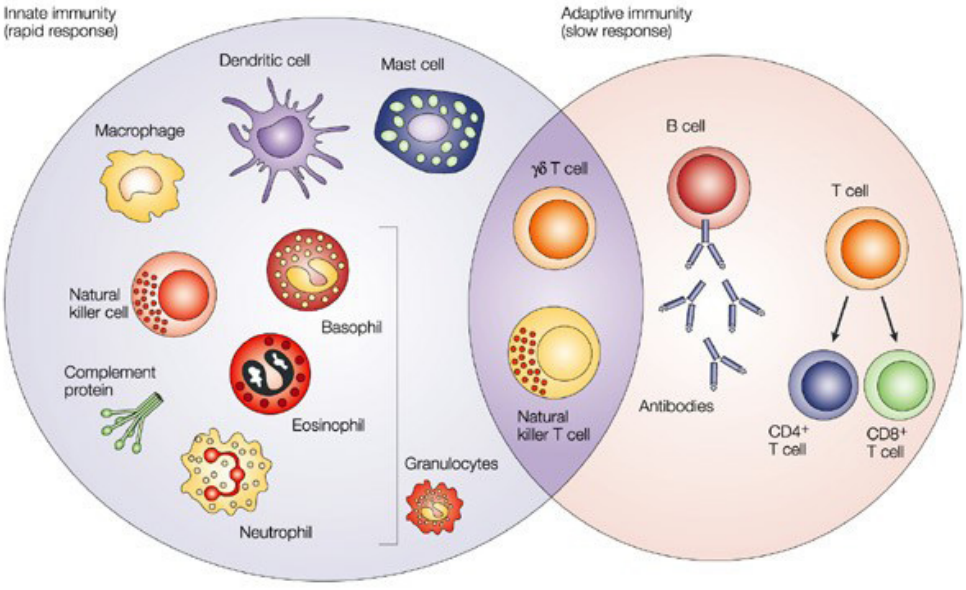

Figure 4 The innate and adaptive immune response. Adapted from Dranoff [47].

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

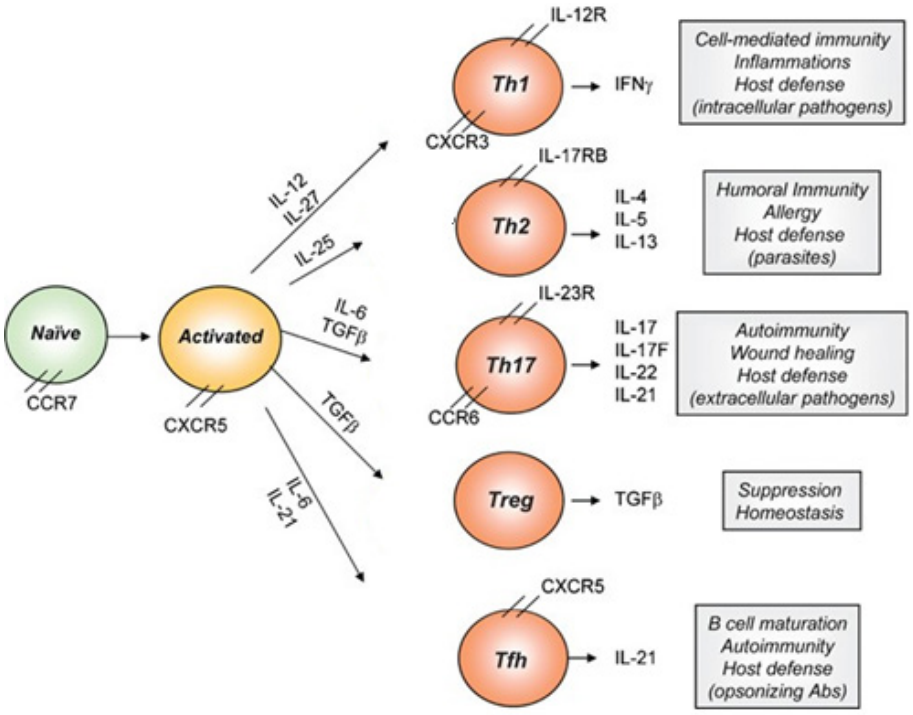

Figure 5 Differentiation of T helper cells, upon encountering antigen presented by professional APCs, naïve CD4+ T cells differentiate into different subsets which corresponding cytokine production, expression of transcription factors and chemokine receptors and immune regulatory functions. Adapted and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

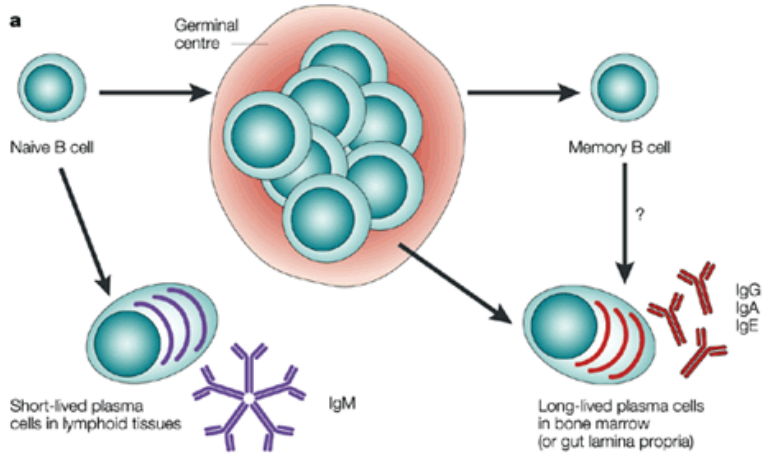

Figure 6 Development of memory B cells and plasma cells. Adapted from Gray. [71]

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.

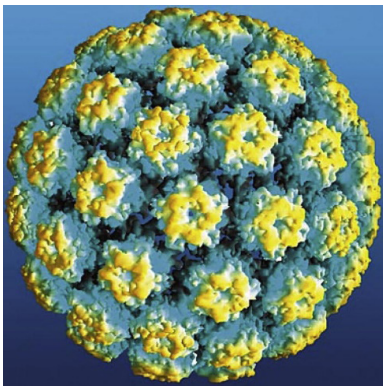

Figure 7 Atomic model of HPV16 L1 VLP, adapted from Schiller and Dowy, 2018 [78].

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.

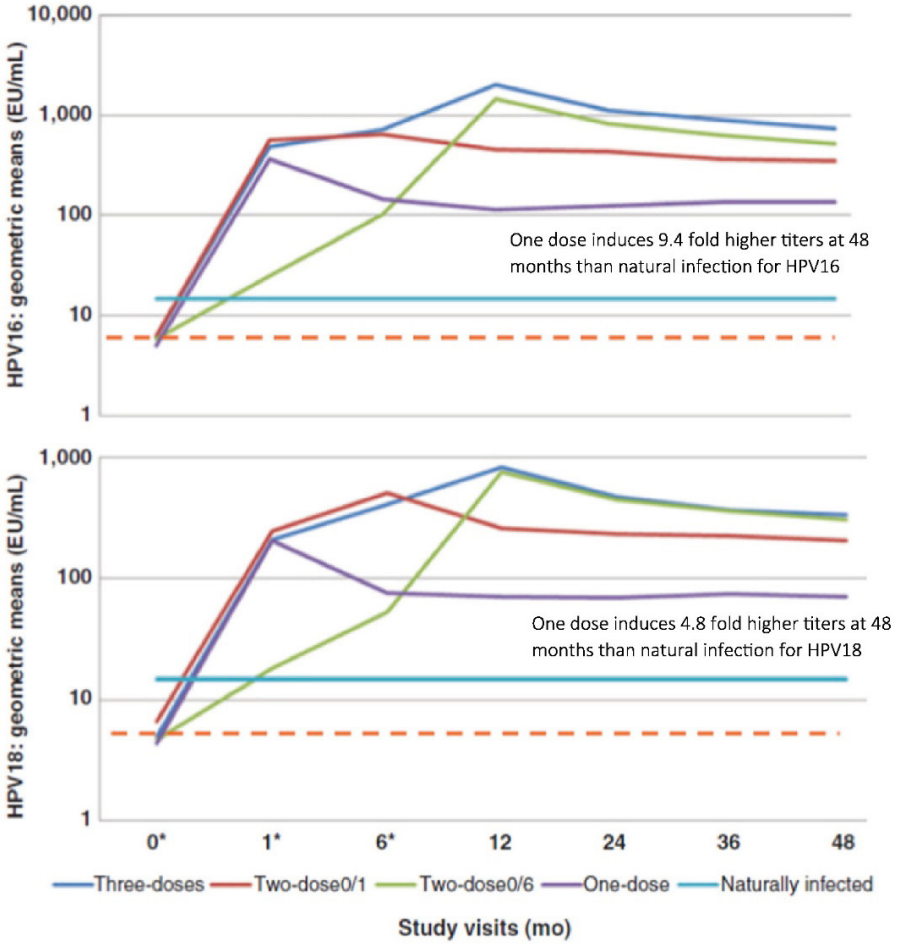

Figure 8 HPV16 and HPV18 antibody levels following different dosing schedules and natural infection. Adapted from Harper and DeMars[127]

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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