1

General introduction

Genital Human Papillomavirus infections

Human Papillomavirus (HPV) infection is one of the most common sexually transmitted infections worldwide. Up to 80% of sexually active individuals will be infected with HPV at some time during their life [Baseman 2005]. Data from large global meta-analyses indicate that at a given point in time 10.4% of women worldwide are positive for cervical HPV DNA. The prevalence of HPV is higher in less developed countries (15.5%) than in more developed regions (10.0%) [Clifford 2005; de Sanjose 2007; WHO/ICO information Centre 2011].

Overall HPV is responsible for little more than 5% of the global cancer burden, the majority of which can be ascribed to cervical carcinoma and less than 0.7% of which is accounted for by cancer of the penis, vulva, vagina and anus [Parkin 2006]. Remarkably, the number of HPV (non-smoking or tobacco) related oropharyngeal cancers has more than doubled in the past 20 years [Evans 2010; Nasman 2009]. Chronic and persistent infection with HPV substantially increases the risk of development of premalignant en malignant cervical lesions and is acknowledged by the World Health Organization as a necessary cause of development of cervical cancer [Bosch 2002; Walboomers 1999; zur Hausen1996].

Cervical cancer is the third most common cancer in women worldwide with 529.800 new cases in 2008, accounting for 8% of the cancer deaths among females [Ferlay 2010; Jemal 2011]. More than 85% of these cases and deaths occur in less developed countries, mainly due to the lack of routine cervical screening programs. The highest incident rates are reported in Eastern-, Southern- and Western Africa, South-Central Asia and South America. The development of cervical cancer will take 10 to 15 years after sexual debut and HPV infection [Schiffman 2005a; Snijders 2006]. In the majority of women an HPV infection is transient and will be cleared within 2 years [Moscicki 2001; Plummer 2007]. Only a small proportion of women (~10%) will stay persistently infected for several years and are at high risk to develop (pre)malignant lesions [Kjaer 2006; Khan 2005; Schiffman 2005b]. According to the Bethesda system, precursor lesions are referred to in cytopathology as either ASC-US (Atypical squamous cells of undetermined significance), low-grade squamous intraepithelial lesions (LSIL, mild dysplasia) or high-grade squamous intraepithelial lesions (HSIL, moderate or severe dysplasia) [Solomon 2002]. Cervical intraepithelial neoplasia (CIN) grade I, II or III is the histopathologic equivalent (Figure 1) [Snijders 2006]. About 85-90% of LSIL will spontaneously regress. When persisting and progressing to HSIL and left untreated, 30% will develop in to invasive cervical carcinoma [McCredie 2008; Wheeler 2008].

Classification and prevalence of HPV types

More than 100 different HPV types have been identified and catalogued so far, of which about 30 to 40 types are known to infect the mucosa and skin of the genital tract [Bernard 2005; Chan 1995; de Villiers 2004]. The genital HPV types have,

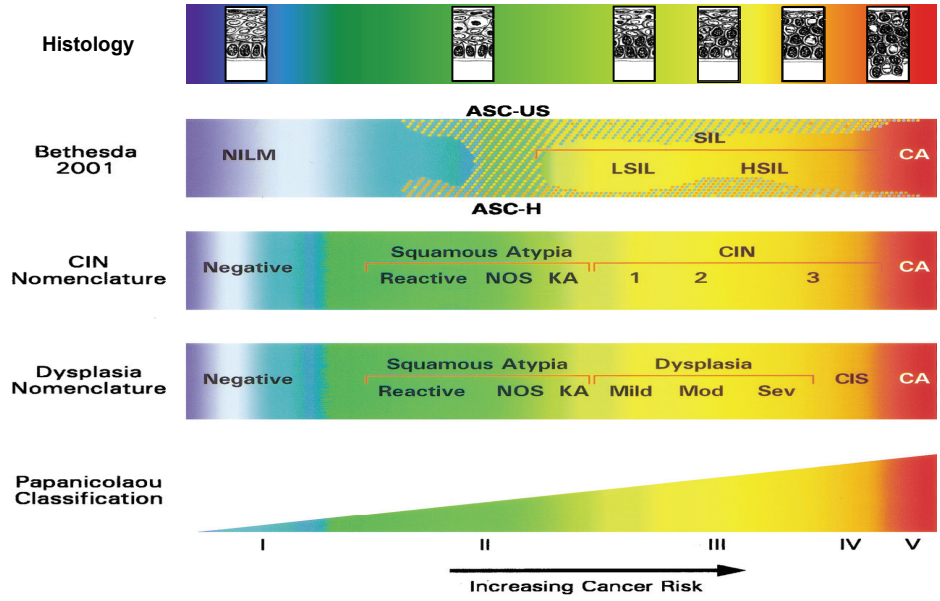

Figure 1. Schematic overview of the morphological alterations and comparative classifications of HPV-related microscopic abnormalities of normal cervical epithelial cells towards invasive cervical cancer. Modified from Sherman and Solomon et al. [Sherman 2003; Solomon 2002].

based on large epidemical studies, been subdivided into low-risk and high-risk or carcinogenic HPV types. Infections with low-risk HPV types (6, 11, 40, 42, 44, 54, 61, 70, 72, 81 and CP6108) have been associated with benign lesions of the anogenital region (e.g. condylomata accuminata, LSIL) and high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) are frequently associated with (pre) malignant cervical lesions [Munoz 2003]. Infection with one of the high-risk HPV types is prevalent in more than 96% of cervical carcinomas [Clifford 2003; Clifford 2006; de Sanjose 2010; Munoz 2004; Smith 2007b]. HPV16 and HPV 18 are estimated to account for 70% of cervical cancer cases worldwide (54.4% and 16.5% respectively), with only marginal differences between developed and less developed regions [WHO/ ICO information Centre 2011]. The other most common high-risk HPV types 31, 33, 35, 45, 52 and 58, account for another 20% of cases. The eight high-risk HPV types are not only detected most frequently in cervical cancer, but to a lesser extend also in pre-cancerous lesions and even in women with normal cytological findings (Figure 2). Respectively, 44.1% of high-grade lesions (HSIL), 18.5% of low-grade lesions (LSIL) and 2.7% of women with normal cytology are estimated to be HPV16 positive. The relative importance of HPV18, 31, 33, 35, 45, 52 and 58 vary a little by region and cytopathological result.

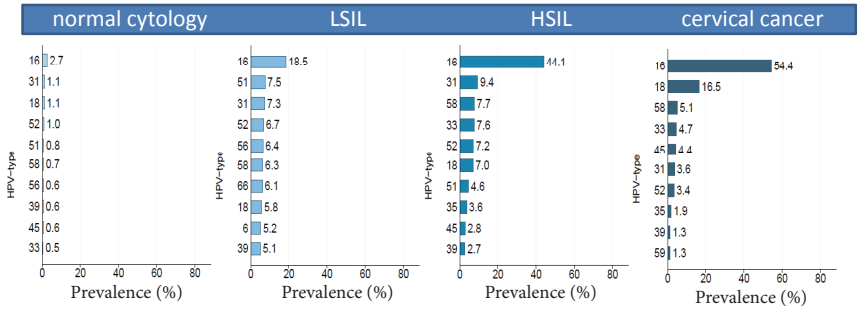

Figure 2. Ten most frequent HPV types among women with and without cervical lesions in the world. Modified from figure 22 of the summary report of the WHO/ICO information Centre on HPV and Cervical cancer [WHO/ICO information Centre 2011].

HPV biology and infection

HPV is a small non-enveloped circular double stranded DNA virus of almost 8000 base pairs belonging to the family of Papillomaviridae. The genome encodes for late structural genes (L1 and L2) and six early non-structural or regulatory genes (E1, E2, E4-E7). Briefly, L1 and L2 encode for viral capsid proteins, E1 and E2 enable viral transcription and the oncogenes E5, E6 and E7 modulate the transformation process. The L1 gene is the most conserved part of the genome between different HPV types and has been used to stratify the 118 HPV types identified so far in a phylogenetic tree (Figure 3) [de Villiers 2004; Doorbar 2006]. The genital HPV types belong to the

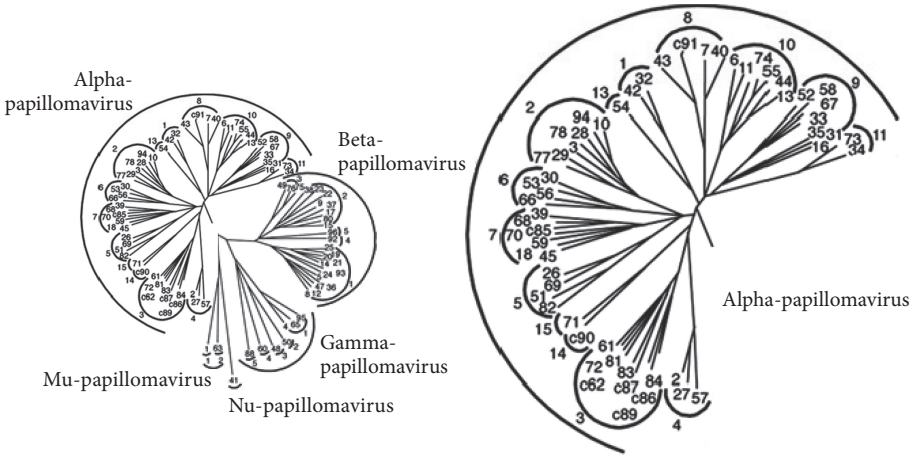

Figure 3. Philogenetic tree of HPV types. Alpha-papillomaviruses primary infect the genital mucosa and are subdivided in 15 different species or clades. HPV16, 31, 33, 35, 52 and 58 belong to the α9 species [de Villiers 2004; Doorbar 2006].

genus of alpha-papillomavirus, which is subdivided in 15 different species (subgroups). HPV16, 31, 33, 35, 52 and 58 belong to the α9 species or clade A9; HPV18 and HPV45 are a member of clade A7. Viruses within a species share between 71%-89% nucleotide homology within the L1 gene [de Villiers 2004].

Papilloma viruses probably infect the basal cells of the epithelium via micro-abrasions of the cervical transformation zone (i.e. squamous epithelium of the ectocervix to glandular epithelium of the endocervix). This leads to the expression of the early genes E1, E2, E5, E6 and E7 and replication of episomal viral DNA in the basal keratinocytes. Structural proteins L1 and L2 are assembled and mature virions are shredded and released from the superficial epithelium and may re-infect the host or patient (LSIL/ CIN I). Persistent infection with a high-risk HPV type might result in integration of the viral DNA into the host cellular DNA, and subsequently to overexpression of the E6 and E7 oncoproteins thereby promoting genetic abnormalities. These cells become dysplastic and if left untreated will progress to HSIL or (micro) invasive carcinoma [Doorbar 2005; Woodman 2007].

Immune responses to HPV

As described, infection with HPV is very common (lifetime incidence ~80%) and the majority of infected individuals will spontaneously clear the virus without even developing premalignant lesions of the cervix. Only a small proportion woman will become persistently infected and are at risk to develop HPV related malignancies. The immune system plays an important role in the balance between viral clearance (immunity) and viral persistence (immune tolerance).

Immunity is a result of the interaction between the innate or non-specific immune system and antigen-specific or adaptive immune system. The innate immune system provides immediate defense against infection by epithelial barriers, local inflammation and cytokines, complement system and phagocytes. Innate immunity has no memory, but is crucial for activating the adaptive immune response by antigen presentation. Adaptive immunity generates antigen-specific effector and memory cells (B and T lymphocytes). B cells play a role in humoral, or antibody related immune responses and T cells are involved in cell-mediated immunity.

The cellular immune system comprises many different subsets of T cells, including CD4+ T helper (Th) cells, CD4+ regulatory T cells (Treg) and CD8+ cytotoxic T lymphocytes (CTL). Th cells are key players in regulating immune responses and represent a much more heterogeneous population than previously suggested: Th1, Th2, Th9, Th17 and follicular T helper cells have been identified so far. Th1 cells regulate cell-mediated immune responses (i.e. activation of CD8+ cytotoxic T cells and effector cells of the innate immune system like macrophages and natural killer cells) and a.o. secrete IFNγ. Th2 cells produce interleukin (IL)-4, -5 and help the humoral effector immune response (i.e. help or stimulate B-lymphocytes to produce antibodies or

Immunoglobulin (Ig) to neutralize foreign antigen and prevent infection). Th17 cells play a key role in autoimmune diseases, mediate antitumor immunity indirectly by changing the tumor microenvironment (secretion of cytokines), and facilitate the recruitment of tumor-infiltrating CD8+ T cells and natural killer cells. Regulatory T cells play a role in immune tolerance by suppressing immune responses including CD8+ cytotoxic T lymphocytes and Th1 mediated responses. Both B- and T lymphocytes are capable of inducing immunological memory.

Innate immunity: immune evasion strategy of HPV

More and more aspects of immune mediated clearance of the HPV infection have become known, however the exact role of interactions of the (host) immune system and HPV remain undetermined. Time taken to spontaneous clearance of a high-risk HPV infection is a relatively long: generally 8 to 16 months [Trottier 2006]. HPVs are very successful in inducing chronic infections. The virus remains undetected by the host immune system for long periods of time because it is able to replicate in the stratified epithelia without inducing overt cell death, danger signals (cytokines) and local inflammation. Therefore triggering and activation of dendritic cells or Langerhans' cells (APC) and initiation of the adaptive immune response does not occur. There is no blood-borne phase or systemic viraemia since virus particles are shredded by the squamous epithelium through normal wear and tear, with no vascular or lymphatic channels nearby where immune responses are initiated. In addition, it has been reported that Langerhans' cells are reduced in women with CIN and do not get activated due to an altered phenotype [Fausch 2005; Hubert 2005]. Increased expression of Toll like receptors (TLRs) in cervical epithelium is associated with HPV clearance, while persistence was associated with reduced levels [Daud 2011]. High-risk HPV types down regulate the type 1 interferon (IFNα and β) secretion and thereby inhibit the activation of the adaptive immune response [Kanodia 2007]. A recent genome-wide expression profiling of HPV infected keratinocytes (KC) versus non-infected keratinocytes revealed that HPV is able to dampen the downstream signaling of the viral recognition receptors in infected cells. Many of the genes downregulated in HPV-positive KCs involve components of the antigen-presenting pathway, the inflammasome, the production of antivirals, pro-inflammatory and chemotactic cytokines, and components downstream of activated pathogen receptors. Notably, gene and/or protein interaction analysis revealed the down regulation of a network of genes that was strongly interconnected by IL-1β, a crucial cytokine to activate adaptive immunity [Karim 2011]. Together this shows that HPV can efficiently evade the innate immune system but many details and underlying mechanisms on how HPV mediates this remain unclear and require in depth study.

Adaptive immunity: role of humoral immune response in HPV infections

HPV type -specific antibodies produced by memory B cells may neutralize and opsonize the virus and prevent re- infection of the cervical epithelium. Serum-neutralizing antibody levels are low in natural infections and sero-conversion is detected within 8 to 18 months after infection [Carter 1996; Carter 2000; Dillner 1999; Ho 2004]. Not all

infected women sero-convert, HPV specific antibodies can be detected in 50 to 60% of women and are mainly directed against epitopes of the major capsid protein L1. The low or weak humoral immune response after natural infection is not surprising since the infection is intraepithelial and there is hardly systemic viraemia.

Adaptive immunity: local and systemic cell mediated immune responses to HPV
80% to 90% of the genital HPV infections resolve with time, suggesting that the adaptive immune response eventually is activated and the infection is controlled by cellular mediated immune responses. Increased prevalence of persistent HPV infections and progression of disease in immunocompromised subjects illustrate the important role of cell mediated immune responses [Palefsky 2006]. A protective immune response to chronic viral infections comprises CD4+ helper T cells that activate and sustain the function of CD8+ cytotoxic T cells as well as innate effector cells [Cardin 1996; Harari 2006; Matloubian 1994; Walter 1995; Zajac 1998]. Immune responses in relation to progression and regression of HPV related diseases have intensively been studied and substantiate this notion. HPV-specific CD4+ and CD8+ T-cell responses have been detected in the peripheral blood of healthy, HPV-negative individuals (i.e. cleared infection in the past) and women with regression of their cervical lesions [Bontkes 2000; de Jong 2002a; de Jong 2004; Farhat 2009; Nakagawa 1997; Nakagawa 2000; Seresini 2007; Welters 2003; Welters 2006]. Immunohistological analysis of spontaneously regressing HPV-related genital warts demonstrated infiltration of large numbers of both CD4+ and CD8+ T cells and macrophages [Coleman 1994]. Woo et al. showed that infiltration of CD8+ cytotoxic T cells into LSIL lesions is related with subsequent regression of the lesion (i.e. protects) whereas the number of CTLs is substantially lower in persisting or progressing lesions [Woo 2008]. In *chapter3*, we will show that both HPV-specific CD4+ Th1/Th2 and CD8+ T cells are able to migrate from the circulation into the skin after intradermal peptide challenge in healthy individuals [van den Hende 2008]. In contrast, partial or complete failure of these types of HPV-specific immune responses is associated with viral persistence and progression of disease. Patients with cervical cancer and (recurrent) high-grade lesions (HSIL) either lacked or showed a dysfunctional and sometimes even suppressive systemic CD4+ T-cell response while CD8+ CTLs were rarely detected in the blood [Bontkes 2000; de Jong 2004; de Gruijl 1998; de Vos van Steenwijk PJ 2008; Nakagawa 2000; Steele 2005; Trimble 2010a; Youde 2000; Welters 2006]. The number of infiltrating CD4 and CD8 T cells is reduced in lesions progressing to HSIL or cervical cancer [Monnier-Benoit 2006; Woo 2008]. Trimble et al. recently showed that the lack of local or intra-lesional vascular expression of mucosal addressin cell adhesion molecule (MAdCAM) is correlated with the influx of CD8+ T-cells and regression of CIN2/3 lesions, whereas progressing lesions showed a dysregulated expression of MAdCAM [Trimble 2010b]. HPV-specific CD4 and CD8 T cells have been detected in tumor and cervical lesions as well as in tumor draining lymph nodes [de Vos van Steenwijk 2008; de Vos van Steenwijk 2010; Piersma 2008; van der Burg 2007]. Tumor infiltrating intraepithelial CD8+ T-cells is associated with a lack of lymph node metastases and a low CD8+/Treg ratio is an independent unfavorable prognostic factor in cervical cancer survival [Jordanova 2008; Piersma 2007].

HPV-specific regulatory CD4 T cells (Treg) are suggested to play a role preventing or suppressing immunological clearance of the HPV infection and associated malignancies [Adurthi 2008; Jordanova 2008; Molling 2007; Piersma 2007; van der Burg 2007].

Immunological approaches to combat HPV infections

Since infection with HPV is a necessary cause of development of cervical cancer, vaccination is assumed an effective mechanism to prevent infection and control HPV related disease. Two different treatment modalities have been developed: prophylactic vaccines that aim at prevention of HPV infection by antibodies or humoral immune responses and therapeutic vaccines that aim at regression of HPV induced (pre) malignant lesions by cell-mediated immune responses.

Prophylactic vaccination

Nowadays, two prophylactic HPV vaccines are licensed: Gardasil® and Cervarix®. Both use L1 VLPs as immunogens. Gardasil® is a quadrivalent vaccine consisting of HPV6, 11, 16 and 18 L1 VLPs and Cervarix® is a bivalent vaccine of HPV16 and HPV18 L1 VLPs. The vaccines make use of a different adjuvant. Both vaccines are administered by intramuscular injection on three occasions in six months and are well tolerated. Both vaccines are highly immunogenic and induce more than 98% sero-conversion and antibody titers 80-100 fold higher than detected after natural infection [Einstein 2009a; Villa 2006]. In fully vaccinated women, both vaccines are highly effective and provide protection of CIN lesions (associated with the HPV types in the vaccine) up to 6.4 and 8.5 years [Frazer 2011; Kjaer 2009; Paavonen 2009; Stanley 2010]. Vaccination will theoretically reduce 70% of the cervical cancer burden (e.g. HPV16 and HPV18 cervical carcinomas). These highly effective vaccines have been shown to reduce HPV-related disease. Nonetheless, long-term duration of protection after vaccination needs to be confirmed (ongoing long-term follow up studies). Mathematical models of the bivalent vaccine however suggest that antibodies can be detected up to 50 years after vaccination [Rowhani-Rahbar 2009].

In natural infections, the antibodies are type-specific and do not appear to be cross-protective [Palmroth 2010]. However, both VLP vaccines do not only induce type-specific antibodies but also cross-protection against related HPV types is observed (i.e. decrease of infections with related HPV types in vaccinated women compared to controls, but also cross-reactive and cross-neutralizing antibodies) [Bonanni 2009; Brown 2009; Jenkins 2008; Kemp 2011; Smith 2007a; Wheeler 2009]. Cross-reactivity has been observed between HPV16, 31 and 58 and HPV18 and 45. This is probably based on the high antibody concentrations as generated by the vaccines and the L1 epitope homology between the different HPV genotypes from the same species or clade.

Although prophylactic vaccination is currently licensed in most countries worldwide [WHO/ICO information Centre 2011], it will take many years before the prevalence of HPV infections among the population and HPV related (pre)malignant lesions will

decrease. Notably, current practice reveals that vaccination coverage among 13-17 year old females is lower as expected: little more than half of the young women (56.4%) in The Netherlands have been fully vaccinated and in the USA, only 26.7 to 32.0% of the young women have completed HPV vaccination [Centers for Disease and Prevention 2010 and 2011; de Hoogh 2011]. This suggests that it may take even longer before an effect of the vaccine at the population level will be noticeable. As mentioned, VLP vaccines were designed to prevent HPV infection. In women already infected with HPV, vaccination with the bivalent vaccine did not increase viral clearance, and therefore the vaccine has no therapeutic effect [Hildesheim 2007]. This underlines that there is still a need to develop therapeutic vaccines that aim at eradicating or reducing these lesions by cell mediated immunity.

Therapeutic vaccination

HPV-infected epithelial cells of HPV-associated (pre)malignant lesions overexpress E6 and E7 oncoproteins and therefore these proteins are attractive targets for the development of immunotherapeutic vaccines. Animal studies using transplantable tumors did show promising results (reviewed by [Frazer 2011]), however despite of all clinical trials (> than 40 publications) so far only Kenter et al. show a compelling therapeutic effect (e.g. 47% complete clinical response) after vaccination [Kenter 2009]. All literature reports presenting results of immunotherapeutic vaccines (i.e. adjuvant protein or peptide vaccines, recombinant viral vectors, and polynucleotide vaccines) in HPV-related clinical trials have been thoroughly reviewed by Frazer et al [Frazer 2011].

In the aforementioned phase II clinical trial, we have recently reported that patients with HPV-16 induced grade 3 vulvar intraepithelial neoplasia (VIN), treated with a highly immunogenic vaccine comprise HPV16E6 and E7 synthetic long overlapping peptides (HPV16 SLP) show clinical response [Kenter 2009]. At 24 months of follow up, complete regression of the HPV-induced lesions was observed in 47% of the patients. Success was paralleled by the induction of a strong and broad HPV-specific CD4+ and CD8+ T-cell response that peaked after the first vaccination [Welters 2010]. The size of the lesion at study entry was associated with clinical response, as non-responders displayed larger lesions and mount a weaker effector T-cell response, which coincided with a stronger HPV-specific regulatory T-cell response [Welters 2010]. Interestingly, this data sustained the notion that protection against chronic viral infections is mediated by both CD4+ and CD8+ T-cells as formulated in earlier reports based on studies of the spontaneous HPV-specific immune response in healthy individuals and patients with HPV-induced lesions [de Jong 2002a; de Jong 2004; Welters 2003; Welters 2006]. The induction of a local innate immune response might enhance the effect of the peptide vaccine in these larger lesions. Local pro-inflammatory stimuli (like Imiquimod) enhance the influx of CD8+ effector cells and suppress regulatory T cells, which might favor clinical outcome [Wagstaff 2007]. Recently, Daayana et al. reported that the effect of vaccine-induced responses was boosted by local treatment with Imiquimod of the VIN lesion [Daayana 2010].

Outline of the thesis

Infections with HPV are very common (~ 80% lifetime incidence) and the immune system plays an important role in the balance between viral clearance and viral persistence. The aim of this thesis was to gain further insight into HPV-specific cellular immunity in relation to the natural course of HPV related disease. HPV clade A9-specific T-cell responses were studied in relation to virological status (i.e. clearance or persistence) and clinical outcome (i.e. regression or progression of premalignant lesions). Furthermore, cross-protective immunity to related HPV types was so far only described for humoral immune responses. No data was available on cross-reactivity in HPV-specific cellular immune responses. Insight in this matter is important, not only for correct interpretation of analysis of HPV specific T-cell responses in relation to health and disease, but also to inform us whether the impact of therapeutic HPV-specific vaccines would be limited to the HPV types comprised (type specific vaccination) or could simultaneously target multiple high-risk HPV types.

To address these aims we conducted a detailed study of cellular immune responses against the highly immunogenic E6 antigen of the closely related HPV types of clade A9 (HPV16, 31, 33, 35, 52 and 58) and found that HPV-specific cross reactive CD4+ T cells are rare and are not likely to affect the interpretation of immune assays. The response pattern in HPV16 vaccinated VIN patients resembles that of healthy controls suggesting that vaccine induced CD4+ T-cell responses are unlikely to mediate cross protection and indicate that therapeutic efficacy of vaccines is type specific (*chapter 2*). *Chapter 3* reports on a pilot study of the use of a delayed type hypersensitivity (DTH) skin test to detect HPV-specific immune responses *in vivo*. This test might be useful to screen spontaneous and vaccine-induced responses to HPV in prospective cohort studies in low resource areas: where the prevalence of HPV infections is high and the access to specialized laboratories to perform *in vitro* assays is limited. Intradermal injection of HPV16 synthetic long peptides is safe and results in migration of HPV specific T cells into the skin as well as an increase of circulating HPV16 specific T cells. In both, *chapter 4 and 5* we report on HPV-specific T-cell responses in relation to virological and clinical outcome in three prospective cohort studies. *Chapter 4* describes the outcome in two developing countries (Haiti and South Africa) where the burden of HPV related disease is high. A prospective study in the United Kingdom on the natural course of HPV16 related low-grade cervical lesions is described in *chapter 5*. The outcome of these studies revealed that the failure of a HPV-specific T-cell response is associated with persistent infection of that specific HPV type and development of progressive disease. Interestingly, while no correlation could be detected between HPV type-specific immune responses and viral clearance, both studies revealed a trend between the regression of a low-grade lesion and the presence of type-specific immunity. Finally, the results of this thesis and directions for further research are discussed in *chapter 6*, and a summary in Dutch is provided in *chapter 7*.