Chapter 1

Introduction
1 Human papillomaviruses

1.1 HPV genome and gene function

Human papillomaviruses (HPVs) belong to the family of Papillomaviridae. They consist of a double-stranded, circular DNA molecule of approximately 8.000 base pairs packaged in icosahedral particles of 45-55 nm in diameter.

The genome can be subdivided in three regions: a non-coding long control region (LCR), an early encoding region (E), containing 6 early genes and a late encoding region (L), containing 2 late genes (Figure 1). These eight genes are responsible for the translation of a series of functional proteins necessary for the viral life cycle.

The LCR, also known as the upper regulatory region, covers about 10% of the viral genome. Among individual HPV types the LCR shows substantial variation in nucleotide composition. By binding to this region transcriptional factors of the host cell and virus regulate viral gene expression. In addition, the viral origin of replication is located in the LCR.
Functions of HPV-encoded proteins are as follows (modified from IARC 2007):  
**E1**: ATPase and DNA helicase; recognizes and binds to the viral origin of DNA replication as a hexameric complex; necessary for viral DNA replication.  
**E2**: Main regulator of viral gene transcription; involved in viral replication; interacts with and recruits E1 to the origin.  
**E4**: Acts late in the viral life cycle; interacts with the keratin cytoskeleton and intermediate filaments; believed to facilitate virus assembly and release.  
**E5**: Induces altered regulation of cell proliferation; interacts with 16k subunit c of vacuolar ATPase; may activate growth factor receptors and other protein kinases; inhibits apoptosis; inhibits trafficking of MHC complexes to the cell surface.  
**E6**: Induces suprabasal DNA synthesis; induces telomerase; prevents cell differentiation; interacts with three classes of cellular proteins: transcriptional co-activators, proteins involved in cell polarity and motility; tumour suppressors and inducers of apoptosis, primarily p53.  
**E7**: Induces unscheduled cell proliferation; interacts with histone acetyl transferase; interacts with negative regulators of the cell cycle and tumour suppressors, primarily Rb (p105).  
**L1**: Major viral structural protein; self assembles into capsomeres and capsids; interacts with L2: interacts with cell receptors; encodes neutralizing epitopes.  
**L2**: Minor viral structural protein; interacts with DNA; interacts with ND10s; believed to facilitate virion assembly; may interact with cell receptors; encodes linear virus neutralising epitopes.

In the normal viral life cycle the proteins encoded by the viral E6 and E7 genes enable the virus to replicate by activating DNA synthesis and blocking an important apoptosis route in differentiated non-dividing host cells. However, deregulated expression of E6 and E7 in proliferating basal and para-basal cells, as seen in cervical cancer and its high-grade precursor lesions, results in uncontrolled proliferation and genetic instability. This phenomenon is associated with an abortion of the viral productive cycle, a condition often also referred to as ‘transforming infection’ to clarify the difference with a ‘productive infection.’

Deregulated E6 and E7 is considered the crucial step in the transformation process mediated by hr-HPVs and E6 and E7 are consequently referred to as viral oncoproteins that are indispensable for cervical carcinogenesis.

E7 has a number of cell cycle regulatory proteins as a target, among which the retinoblastoma susceptibility gene product, pRb, is most important. E7 binding to pRb causes inactivation of the latter, resulting in upregulation of genes involved in progression through the cell cycle at the G1/S boundary. In the normal viral life cycle E7 is mainly expressed in supra-basal, differentiated cells, where it induces S-phase re-entry of otherwise replication silent cells. This creates an optimal environment for vegetative viral replication in these non-dividing cells. The normal reaction of the host cell to this illegitimate induction of replication in differentiating
epithelial cells would be the triggering of apoptosis. This is, however, prevented by E6, which has the capability to target and inactivate p53 as well as other cellular proapoptotic proteins (like BAK). When their deregulated expression in proliferating cells becomes manifest, E6 and E7 together allow survival of cells displaying uncontrolled proliferation, which leads to genetic instability and accumulation of chromosomal alterations that can mediate further steps in the carcinogenic process.\textsuperscript{11,12,13,14}

Recently, p16 (INK4A), a cell cycle regulation protein, has been identified as a biomarker for hr-HPV-induced high-grade premalignant lesions characterized by deregulated E6 and E7 expression. Since p16 expression is regulated by an Rb-dependent negative feed-back loop continuous inactivation of Rb by hr-HPV E7 in proliferating cells results in increased p16 levels.\textsuperscript{15}

### 1.2 Classification of papillomaviruses

Within the last three decades more than 100 different genotypes of HPV have been recognised and completely described. An HPV type is classified as a new type in case of at maximum 90% nucleotide sequence identity determined over the open reading frames E6, E7, and L1 with other HPV types, whereas HPV subtypes are defined as having a sequence identity between 90% and 98% with existing types. HPV intratype variants are defined as having more than 98% nucleotide sequence identity with a given HPV type. These principles led to taxonomic groupings, which are widely accepted today (Figure 2, de Villiers\textsuperscript{16}). Taxonomic levels include family, genus, species, types, subtypes and variants.

HPVs are strictly epitheliotropic. They can be divided in “mucosotropic” types, which are mainly found in the mucous epithelium of the oropharynx and anogenital tract and “cutaneous” types, according to their preference to infect the skin. Since 1993 the terms “low-risk” or “non-oncogenic” and “high-risk” or “oncogenic” were proposed in order to distinguish between HPV types mainly associated with benign versus malignant and premalignant conditions.\textsuperscript{17} In almost all cervical cancers and in cervical intraepithelial neoplasia grade 3 (CIN3) lesions hr-HPV is found, whereas in condylomata and a subset of CIN1 and CIN2 lesions low-risk types can be present.\textsuperscript{2,18} Lesions containing low-risk HPV types will never, or at maximum extremely rarely, progress into cervical cancer. Recently, 15 mucosal HPV types were classified as high-risk or carcinogenic on the basis of epidemiological criteria.\textsuperscript{2,18,19} These types are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. In addition three other HPV types (i.e. HPV 26, 53, and 66) are considered as probably carcinogenic. As low-risk were classified types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 82 and CP6108. The oncogenic (i.e. high-risk) genital HPV types belong to the genus alpha-papillomaviruses. Phylogenetically related oncogenic types HPV 18, 39, 45, 59 and 68 belong to species 7 and HPV types 16, 31, 33, 35, 52 and 58 to species 9.
1.3 HPV detection methods

Unlike many other microbiological agents, HPVs cannot be cultured. Therefore, several alternative methods evolved in the last 25 years for the detection, typing and quantitation of HPV, mainly in cervical specimens. Commercially available HPV antibodies against the L1 proteins have been employed for the detection of HPV using immunohistochemistry. By this way only intact HPV particles can be detected and this makes this method not useful for HPV detection in transforming infections because these infections are characterized by absence of virus production. Furthermore, it is difficult to detect the actual HPV oncoproteins because of the lack of sufficiently sensitive and specific monoclonal antibodies and by the very short half life of E6 and E7 gene products. As a consequence, the standard practical methods for the diagnosis of HPV infection are based on the detection of HPV nucleic acids, particularly HPV DNA. The former golden standard, Southern blotting, needs too much material and is not appropriate for routine detection of HPV in cervical smears.
Presently, the clinically most informative HPV DNA detection methods are based on the Hybrid Capture 2 (HC2; Digene®) principle and consensus polymerase chain reaction (PCR).20 HC2 uses a cocktail of HPV-specific RNA probes to generate hybrids which target HPV DNA sequences that are then captured with an antibody specific for RNA-DNA hybrids. Amplification of the signal is achieved by binding of hybrids to multiple conjugated antibodies that specifically recognise these DNA-RNA hybrids. The result is expressed on a scale of relative light units (RLU) read in an luminometer. In this assay two cocktails, comprising low-risk or high-risk types can be used. Because the RLU signal is proportional to the amount of HPV DNA present in the specimen, the HC2 assay cut-off has also been used to infer viral load on a semi-quantitative basis. Individual typing is not possible with HC2. HC2 is commercially available and FDA approved for use in primary cervical cancer screening in combination with classical cervical cytology or liquid based cytology in women over 30 years.21

A second hr-HPV test that is clinically validated in large randomized trials is based on PCR using consensus or general primers GP5+ and GP6+.22 These primers bind to highly conserved regions within the L1 gene of all genital HPV types. The amplified product shows enough variability in the region flanked by the primers for type-specific detection by hybridisation with oligonucleotides specific for the different HPV types. The most common read-out systems for this assay comprise enzyme immuno-assay (EIA) and reverse line blotting (RLB). The first assay is often used for the detection of hr-HPV types as a pool by using cocktails of oligoprobes specific for eg. hr-HPV types, whereas the latter assay can be used for individual genotyping. Other comparable consensus PCR tests in use include the PGMY, SPF10 LiPa and Amplicor (Roche) systems, which all target an area in the L1 gene. These assays display various analytical sensitivities for detecting hr-HPV types, but are, unlike GP5+/6+-PCR, not clinically validated in large trials for the detection of high-grade CIN lesions and cervical cancer in a highly specific manner. Nevertheless, the assays described have the potency to detect all relevant hr-HPV types, which is necessary for studies evaluating specific preventive or therapeutic vaccines. In general the sensitivity of the different HPV tests to detect viral DNA is variable and depending on many factors, such as size of amplimer and type of DNA polymerase enzyme used. It should, however, be noted that detection of very low levels of hr-HPV DNA generally does not reflect a clinically meaningful infection (i.e. infection giving rise to cervical cancer of high-grade precursor lesions), but rather a clinically irrelevant, i.e. transient or latent infection.23 Therefore, too sensitive methods have an intrinsic low positive predictive value for cervical (pre-)cancer because of the mere detection of transient/latent HPV infections. In order to be of clinical value for cervical screening, an HPV test should show an optimal balance between sensitivity and specificity for high-grade lesions, as is the case for hc2 and GP5+/6+-PCR.
2 Human papillomavirus and genital cancer

2.1 Cervical cancer and precursor lesions

2.1.1 Epidemiology of cervical cancer

Cervical cancer is the second most common female cancer in the world. An estimated 437,000 new cases of invasive cancer of the cervix are diagnosed yearly, of whom 81% in developing countries. More than 233,000 women die each year from this disease, making it a major cause of death, accounting for 6-10% of all cancer related deaths in women. The age-standardized incidence of invasive cervical cancer is 18.7 per 100,000 women in developing countries and 11.3 in developed countries.

In the Netherlands 600 new cases of invasive cervical carcinoma were diagnosed in 2003, giving an age-standardized incidence rate of 6.9 per 100,000 women. In that year 258 deaths were reported, giving an estimated age-standardized mortality rate of 2.6 deaths per 100,000 women. Most likely as a result of population based cervical screening, the incidence of squamous cell carcinoma of the cervix in the Netherlands is decreased in the last decades by 2% yearly, demonstrating the preventability of the disease by screening.

In the 19th century Rigoni-Stern suggested that cervical carcinoma was a sexually transmitted disease. He based this suggestion on the fact that cervical carcinoma was non-existent in virgins and nuns, and highly frequent in prostitutes.

Epidemiological studies in the 1970s strongly supported the role of a sexually transmitted disease in the development of cervical cancer. In those years several agents were proposed including syphilis, gonorrhoea and type 2 herpes simplex virus (HSV-2).

Zur Hausen postulated in 1973 a link to HPV infections because the epidemiology of cervical cancer was similar to that of genital warts (known to be HPV-related). Some years later the role of HPV as the major infectious etiologic agent of cervical cancer of all histotypes (squamous cell carcinoma (SCC) as well as adenocarcinoma) was elucidated. Firstly, HPV 16 was isolated from an SCC and later it was shown that hr-HPV is necessary for the presence, maintenance and progression of CIN 3 to cervical cancer. By determining HPV-DNA in cervical samples in worldwide prevalence studies HPV was demonstrated in almost all cervical carcinomas.

These findings stimulated researchers to evaluate whether HPV testing could be used in the management of CIN lesions and as a primary screening test.

2.1.2 Histological and cytological classification of cervical cancer precursor lesions

More than 70% of cervical cancers are SCCs. About 10% to 25% of the cervical carcinomas comprise adenocarcinomas and adenosquamous carcinomas, being adenocarcinomas with focally
squamous differentiation. Adenocarcinomas originate from endocervical glands, located higher in the cervical canal. A small minority (<5%) of primary cervical cancers consists of carcinomas with neuroendocrine features, mostly small cell types.

The existence of precursor lesions of cervical carcinoma is known for a long time, with Broders being the first one to recognise carcinoma in situ as such in 1932.\textsuperscript{45} Later, in 1941, Papanicolaou and Traut demonstrated the usefulness of exfoliated cytology for the detection of these precursors.\textsuperscript{46} Richart was the first in the late 1960s to introduce the concept of cervical intraepithelial neoplasia.\textsuperscript{47} He described dysplasias as precursor lesions and assumed that cervical cancer would eventually develop from these lesions. Based on progressive atypia of epithelial cells and progressive involvement of the thickness of the epithelium he classified CIN as grade 1 when less than one third of the epithelial layer was involved, grade 2 when one to two thirds were involved and grade 3 when two thirds to full thickness involvement was present. In this system CIN 1 and CIN 2 are equivalent to mild and moderate dysplasia, respectively, whereas CIN 3 equals severe dysplasia and carcinoma in situ. In the late 1980s the Bethesda system was introduced in order to discriminate between lesions with a low or a high risk of progression to cervical cancer.\textsuperscript{48} Here, CIN 1 is classified as low-grade squamous intraepithelial lesion (LSIL) and CIN 2 and CIN 3 together as high grade SIL (HSIL). Modification of the Bethesda system was introduced in 2001 for a more efficient management of women with equivocal cervical cytology.\textsuperscript{49} The concept of CIN has also been extended to lesions from endocervical origin; atypical glandular cells (AGC) and adenocarcinoma in situ (AIS) are used in parallel in the updated Bethesda system.

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\textit{Table 1.} The CISOE-A (KOPAC-B) classification compared with the Bethesda 2001 and Pap classifications. Adapted from Bulk et al and Hanselaar et al.\textsuperscript{50,51} ASC-US: Atypical squamous cells of undetermined significance, ASC-H: Atypical squamous cells cannot exclude HSIL, AGC: Atypical glandular cells, LSIL: Low-grade squamous intra-epithelial lesion, encompassing CIN1, HSIL: High-grade squamous intraepithelial lesion, encompassing CIN2-3, and AIS: Endocervical adenocarcinoma in situ S stands for squamous epithelium, O for other abnormalities, E for endocervical epithelium.
Cervical cytology is used worldwide for screening and as a diagnostic tool in case of possible cervical disease. In the Netherlands the CISSE-A system (KOPAC-B) for the classification of cervical cytology is used since 1996.50,51,52 In this classification the C stands for composition, the I for inflammation, the S for squamous epithelium, the O for other abnormalities and endometrium, and the E for endocervical epithelium. Finally A stands for adequacy. The S, O and E define the Pap class. The relationship between cytological classification and histological classification of premalignant and malignant cervical lesions is presented in table 1.

2.1.3 **HPV-type prevalence in cervical cancer and precursor lesions**

Amongst the hr-HPV types HPV types 16, 18, 31, 33 and 45 are the most prevalent.2 There is a significant difference in the prevalence of different hr-HPV types in women with normal cytology, women with cervical cancer precursor lesions, especially the high-grade lesions CIN 2/3, and women with cervical cancer.53,54 In women of a screening population with normal cytology HPV 16 and HPV 18 account for about 30% of all hr-HPV infections, and HPV 16 alone for about 20%. In women with CIN 2/3 the prevalence increases to a larger proportion for these two types, ie. to about 50%, of which HPV 16 alone in 40%. In women with cervical cancer the proportion of HPV 16 and 18 is increased to 70%, and that of HPV 16 alone to about 60%. These findings are in line with the observations that particularly HPV 16 and to a lesser extent HPV 18, confer an increased risk of ≥CIN2/3.55,56,57,58,59

**Figure 3.** IARC results on prevalence of the four most common HPV types and of the risk estimates for squamous cell carcinomas worldwide (60).

2.1.4 **HPV and cervical carcinogenesis**

Cervical carcinogenesis is considered to be a multistep process, in which an hr-HPV infection represents the first step, but other important factors, like genetic host polymorphisms, viral characteristics such as genomic variation and the amount of viral DNA (viral load), and specific
genetic alterations are likely to contribute to the process as well. The fact that the prevalence of cervical HPV infections and HPV-induced lesions is much higher in immune compromised women suggests that the cellular immune system is normally an important factor in preventing cervical cancer. Only a subset of women (20%) infected with hr-HPV develop CIN lesions.\textsuperscript{61} It is thus important to realise that 80\% of the women with a hrHPV infection will have at maximum a transient infection and despite the fact that they may show cytological signs of their infection during follow-up they will show spontaneously clearance of the virus and normal cytology within two years.\textsuperscript{62,63} Similarly, the far majority of women with equivocal or mild Pap smear results who are HPV-negative, will become cytologically negative over the following 2 years and will not develop high-grade CIN lesions. It is believed that of the high-risk positive women only 1-2\% will get a transforming infection that ultimately develops into cervical cancer in at least 12-15 years.\textsuperscript{64,65}

Regarding the pre-malignant phase of cervical cancer there are two concepts. In the first and oldest concept cervical cancer develops through various stages of increasing severity of cervical dysplasia, thus starting as CIN 1 and slowly progressing via CIN 2 and CIN 3 to cervical cancer. In the second and newer concept high-grade CIN lesions, characterized by deregulated E6/E7 expression, develop much faster from normal cervical mucosa (2-3 years) and the preceding low-grade CIN stage is short lived, if existent at all during cervical cancer development. It then generally takes at least 8-10 years to progress from CIN 3 into invasive cervical cancer. In both concepts it is clear that once a CIN 3 lesion is established the risk of progression to cervical cancer is high.\textsuperscript{67-78}

\textbf{Figure 4.} HPV model in cervical cancer carcinogenesis (adapted from Snijders et al, 66).
2.1.5 HPV testing and viral load assessment for the detection of ≥CIN 2/3 lesions

The close association between hrHPV and cervical cancer has resulted in the idea to use hr-HPV testing on cervical scrapes for the detection of cervical cancer and its high-grade precursor stages. Several studies have clearly shown that application of hrHPV testing by HC2 or GP5+/6+-PCR in cervical screening improves the sensitivity and negative predictive value for high-grade cervical intraepithelial neoplasia and cervical cancer (≥CIN 2) compared to cytology alone, leading to a markedly earlier detection of these lesions.\(^{119-123}\) However, the positive predictive value for ≥CIN2 of even a clinically validated hrHPV test (i.e. HC2 and GP5+/6+-PCR) is somewhat lower than that of cytology because still a substantial number of test positive women do not have high-grade CIN lesions. Specifically, hr-HPV positive women with cytomorphologically normal smears fall in this category. As a consequence several groups have studied viral parameters that might predict which hr-HPV positive women will have or develop ≥CIN 2. Recent studies suggest that not the presence of viral DNA per sé, but the amount of viral DNA in a cervical scraping (i.e. viral load) might be a potentially relevant determinant for risk assessment of ≥CIN 2.\(^{23,25,26,27}\) Particularly for HPV 16 it has been demonstrated that increased viral loads are associated with an increased risk of ≥CIN 2, whereas reduced amounts of viral DNA reflect temporal infections, which are associated with absence of high-grade CIN lesions or regressive CIN lesions.\(^{28}\) Still, data for other types are limited and inconsistent\(^{29}\), obviously because of the relatively low number of women with non-HPV 16 types studied so far. In addition, a substantial overlap of viral load values exists amongst women with and without high-grade CIN. So far, this has precluded setting a viral load cut-off value for high-grade CIN on the basis of high viral loads that is informative at the individual level. A more likely possibility is setting a cut-off value for excluding high-grade CIN since low viral loads are likely to be associated with regression of CIN lesions when present or no development of CIN at all.

2.2 Penile cancer and its precursor lesions

2.2.1 Epidemiology of penile cancer

The incidence of penile cancer is much lower than that of cervical cancer. It is very low in developed countries, like the USA and Europe (age standardised incidence 0.3-1.0/100 000), but relatively high in developing countries, with the highest rates in sub-Saharan Africa, up to 1% by the age of 75 in Uganda.\(^{31,32,33,34}\) Almost all the cancers affecting the penis are SCCs.\(^{81,82}\) The incidence shows a continuous increase with advancing age, with an earlier onset in high incidence areas. The mean age at diagnosis is 60 years, with an age-standardized onset reaching its highest level after 70 years of age. Hence, the development of penile cancer appears to be a long-lasting process.
In the Netherlands 76 new cases of penile cancer were diagnosed in 2000, giving an age-standardized incidence of 1.0 per 100,000. This is comparable with incidence rates given by Parkin et al. for developed countries.\textsuperscript{31,79}

Penile cancer is particularly rare in populations practicing circumcision in early childhood, reducing the relative risk for penile cancer more than 10-fold.\textsuperscript{83} In Nigeria striking differences in the incidence of penile cancer have been found between two major populations in the country, one of them being Islamic, practicing childhood circumcision, and one of them being Christian, that do not. The same is true for India, where most penile cancers were diagnosed in the uncircumcised Hindu population, and only 0.02\% in the (circumcised) Muslim population. On the other hand, phimosis is associated with a high incidence of penile carcinoma. Dillner et al. demonstrated a 65-fold higher relative risk of penile cancer in men with phimosis in Sweden.\textsuperscript{84} Phimosis leads to chronic irritation and frequent bacterial inflammation of the preputium and the glans of the penis, probably caused by retention of desquamated epithelial cells and urinary products.

In addition to poor penile hygiene, as caused by phimosis, other risk factors are smoking, history of genital warts and more than 30 lifetime sexual partners.

\subsection*{2.2.2 Penile cancer and HPV}

In contrast to the consistent finding of HPV in cervical carcinomas, the reported prevalence of HPV in penile cancer is highly variable, with rates ranging from 15 to 71\%, depending on the sensitivity of the detection method and the selection of the tumor type. Penile carcinomas include several different histological subtypes. The majority of tumors are well-differentiated, keratinizing SCCs that resemble SCCs arising in non-genital skin. The second most common tumor subtype, verrucous carcinoma and the less prevalent variants, namely, basaloid carcinoma and warty carcinoma arise most frequently on the mucosal surfaces of the anogenital and oropharyngeal regions. On the penis, these tumors most often involve the glans. There is now strong evidence that differences in HPV prevalence are in part depending on the histological subtype of penile cancer. Although basaloid and warty carcinomas were found to be consistently associated with HPV presence, only a subset of keratinizing and verrucous penile cancers was positive for HPV DNA.

From recent comprehensive studies it can be concluded that transcriptionally active hrHPV is present in not more than about 40\% of penile carcinomas.\textsuperscript{90,119} The most common viral type identified in penile cancer was HPV 16, which is present in 60\% to 74\% of HPV positive penile cancers.\textsuperscript{82-86}
2.2.3 Precursor lesions of penile cancer

Precursor lesions of penile cancer are not as well identified as in cervical carcinoma. Similar to vulvar cancer there is an association between Lichen sclerosis et atrophicans (LSA) and penile cancer. Nasca et al. reported in a 10 year follow-up study of 86 men with LSA that nearly 6% of these men developed penile cancer. All these tumors were HPV negative. Hence, LSA is considered the precursor lesion of hr-HPV negative penile cancers.

Other precursor lesions with a high risk of progression to penile cancer are Bowen’s disease and Erythroplasia of Queyrat, both of which present histologically as penile intraepithelial neoplasia grade 3 (PIN3). Progression rates of these lesions to penile cancer of 5 to 10% have been reported. Most of these lesions are hr-HPV positive. Bowenoid papulosis is predominantly present in patients between 30 and 40 years of age. These lesions are normally multiple, small, well demarcated, red or pink papules on the penile shaft, glans or foreskin. Histologically, these lesions vary from PIN 1 to PIN 3. For Bowenoid papulosis the progression rate to penile cancer is less than 1%.

Based on the available data the same model for HPV-mediated carcinogenesis as widely accepted for cervical cancer is applicable to penile cancer, i.e. development of hr-HPV containing invasive cancer from progressive HPV-positive intraepithelial neoplasia lesions (CIN in women and PIN in men; Figure 5). This sequence of events is supported by data on age. The mean age of men with PIN1/2, PIN3 lesions and penile cancer is 35.8 years, 56.1 years and 65.3 years, respectively. These

![Figure 5. Penile carcinogenesis model (adapted from C.J.L.M. Meijer 2005).](image-url)
age differences are in line with the concept that it may take at least 20-30 years for the penile skin with a hr-HPV infection to develop into SCC via the various PIN stages. Moreover, the incidence of PIN and penile cancer shows the same age dependence as the prevalence of hrHPV.\textsuperscript{84,88} Still, the low prevalence of abovementioned clinical manifestations of PIN does not match with the burden of hr-HPV infections in the population. A more thorough investigation of penile lesions present in male sexual partners of women with CIN may unravel other lesions linked to hr-HPV that not only may serve as reservoir responsible for sexual transmission of HPV but also could be considered as precancerous penile lesions.

2.2.4 Penile lesions in male partners of women with CIN: clinical aspects and classification

Barasso et al. were the first to investigate male partners of women with CIN and to describe exophytic and flat lesions.\textsuperscript{97} Exophytic lesions were subdivided in papillomas, papular lesions, Pearly penile papules (PPPs) and condylomata, whereas flat lesions, often subclinical, were described as acetowhite lesions or maculae. Many other ways to describe male lesions were suggested, based on colposcopic appearance, margins of the lesions and colour. Some investigators tried to define PIN lesions as a separate class of lesions, appearing as pigmented, leucoplastic or erythroplastic papules. Indeed, HPV positivity in biopsies of these lesions was high.\textsuperscript{98} Some of the commonly found lesions in partners of women with CIN are specified below.

\textit{Condylomata} were first described by Buschke in the 19th century and are well known to clinicians. They may vary from small lesions to extremely large (see examples figures 6 to 8). These lesions typically contain a low-risk HPV type, predominantly HPV 6 or 11.

\textbf{Figure 6.} Example of small condylomata acuminatum.

\textbf{Figure 7.} Example of large condylomata acuminata in a immune-compromised patient.
Papular lesions are most of the times found near the frenulum as exophytic lesions and can be seen without acetic acid application (Figure 9).

Pearly penile papules are found around the corona, with a perimeter of 1 to 3 mm, presenting themselves in one or more rows (Figure 10).

Flat penile lesions are only seen after application of a 3 to 5% solution of acetic acid on a seemingly normal skin and show a whitish appearance. They are well demarcated, often slightly elevated and show often vascular patterns as in cervical lesions, like punctuation or mosaicism. Most of these lesions are to be found in the inner side of the preputium, the sulcus and the frenulum of the penis, but other penile locations like the shaft and the glans and other regions like perianal, scrotum and urethra have also been described. In biopsies of the penis these lesions exhibit a wide variety of histological diagnoses, reaching from squamous hyperplasia up to PIN3 (Figure 11-13).
Although the relation of condylomata, genital warts, and low risk HPV is well established the association between HPV and flat and papular penile lesions is not clear. Moreover, the relation of PPPs and HPV was only studied in a small number of men. PPPs are commonly seen in adolescence and are frequently a reason for uneasiness. Conclusively, with the exception of condylomata, comprehensive data on HPV presence in such lesions are lacking. In addition, absence of uniformity of classification of penile lesions hampers studies on their possible association with HPV. Coming to a practical classification system and performing a comprehensive HPV analysis are therefore major aims of this thesis.

2.2.5 HPV detection in men

Compared to the studies performed in women, studies on HPV infections in men are scarce. Initially only the relatively insensitive DNA in situ hybridisation (DISH) technique has been performed on histological specimens, and its application was limited to visible lesions such as
condylomata, PPPs and lesions suspicious of penile intraepithelial neoplasia (PIN). With the use of PCR techniques sensitivity has increased considerably and high-volume testing of penile swap samples was possible.30,127 Subsequent studies on penile HPV infections showed highly variable results, and reported HPV prevalence rates range from 3% in Spain to 39% in Brazil.118 However, it should be realized that apart from demographic variations prevalence figures may depend on the sampling procedure and HPV detection method used. Most studies used samples of the penile foreskin, glans and shaft, but others reported also results using additionally scrapes from scrotum and perineum, urine samples and genital hairs.30 In addition, studies performed previously did not take into account the presence of penile lesions.

3 HPV as a sexually transmitted agent

3.1 HPV as STD

HPV infections are among the most common sexually transmitted infections in most populations, with calculations of exposure ranging from 70% in the US, to 95% in African populations displaying a high risk of malignant anogenital disease. In the last 20 years several epidemiological studies indicated sexual behaviour as the main cause for transmission of HPV to women. Characteristics were number of sexual partners, history of STDs, age at first sexual intercourse, barrier contraceptive use, co-infections and male sexual behaviour. However, the next step, blocking transmission of HPV by consistent condom use was never evaluated in a prospective study. In a retrospective study Wen et al. could correlate condom use to a reduced risk of acquiring condylomata. Some protection against CIN 2/3 lesions was suggested in a meta-analysis of Manhart and Koutsky regarding the use of condoms. In 2006 Winer et al demonstrated in young women a protective effect of condom use on hrHPV infections in a prospective randomised study.104,105,106 The prevalence of HPV DNA in the normal population and that of cervical cancer are both closely related to age, although the age profiles differ. On average the peak incidence of cervical cancer is 10-15 years later than that of the peak HPV prevalence. (Figure 13) HPV infections show the highest prevalence in sexually active women of about 20 years (>20%). In Europe prevalence of high-risk HPV peaks at 18-25 years and then declines to 2-3% later on. However, the main part of these young women will clear the infection within 8-14 months.107,108,109 Most and longest persistence is seen for HPV 16 infection.
Intact viral particles are necessary for an infection with HPV. Although the entering mechanism into the cell is not yet fully understood heparin sulphate proteoglycans are probably contributing to viral entry. The idea is that a minimal amount of viral particles is needed to induce an HPV infection ultimately resulting in clinically relevant lesions.\textsuperscript{108,109,110,111}

For HPV infections the productive viral life cycle starts in the basal layers of the genital epithelium. This basal layer can be reached either through chronic inflammation with erosion of the upper layers or microtraumata caused by sexual intercourse. Also in men an association between sexual behaviour and penile HPV infection has been found, and relevant determinants in this regard include sexual activity, number of lifetime sex partners, history of genital warts or other STDs, and not using condoms\textsuperscript{125} Castellsagué et al. demonstrated furthermore a protective effect of circumcision in men by showing that the risk of a penile HPV infection was three times lower in circumcised versus uncircumcised men.\textsuperscript{103} However, unlike the situation in women, no strong association between age and HPV infections in men is evident.\textsuperscript{116}

### 3.2 The male factor in cervical cancer

As genital HPV infections are considered the most common sexually transmitted infections it is imperative that men play an important part in the chain of infection. It is also plausible that men are a not only vector but also a reservoir of hr-HPV In studies performed between 1985

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{HPV infections peak at age 25–30 years, while cervical cancer peaks after age 40 years.}
\end{figure}
and 1993 by the IARC in countries with low, intermediate and high incidence rates of cervical cancer the prevalence of HPV was analyzed. A substantial part of the male partners of women with cancer was included in these studies. A striking difference was demonstrated between the presence of penile HPV in a high incidence country like Colombia and in a low incidence country like Spain. In these two countries the prevalence rate of penile HPV correlated even better with the cervical cancer rate than cervical HPV. The prevalence of penile HPV was five-fold higher in Colombia than in Spain, which corresponds with the eight-fold higher incidence of cervical cancer in Colombia. These data are consistent with the idea that a high prevalence of penile HPV infections is associated with a high cervical cancer incidence. On the other hand, circumcision reduced the risk of genital HPV and cervical cancer. Nevertheless, direct studies involving HPV transmission between sexual couples are lacking. In addition, although penile HPV infections have been studied by several groups a clinical substrate was so far missing. In this thesis we studied a cohort of sexual couples where the female partner, a woman with CIN, was the index patient and the male sex partner was the other investigated person. Attention was focused particularly on the clinical substrate of the males, which led to the discovery of hr-HPV associated flat penile lesions providing the major male source of HPV infections.

Outlines of this thesis

During the period of January 1996 to June 2002 a randomised clinical trial was executed in the Albert Schweitzer hospital in Dordrecht, in which we studied the possible effect of condom use on the regression of cervical and penile lesions and the clearance of HPV in both sexual partners. This study was called the partner study. Women referred to the colposcopy clinic because of abnormal cytology were analyzed in the standard way with cytology, colposcopy and histology. When at least a mild dyskaryosis was demonstrated we asked these women to bring in their sexual partners and after explaining the goals of our study, both partners were included after mutual consent. In this way we were able to include 238 consecutive sexual couples. The male partners underwent at first visit a penoscopic examination of their penis, and eventually a biopsy when a clinically overt lesion was present. Scrapes from both partners, from cervix and penis, respectively, were tested by using the GP5+/GP6+ PCR EIA with the aid of cocktails of 14 high-risk and 6 low-risk HPV oligoprobes. In addition, DNA was tested for individual HPV types 16, 18, 31, 33, 6 and 11.

Randomisation for condom use was performed in blocks of 2 at a given time, independent of the CIN grade and the presence or absence of penile lesions. We gave condoms for free for at least three months, this period being proposed on the basis of the results of a pilot study done earlier. Both partners underwent the examinations as mentioned above at 3, 6 and 12 months.
and subsequently every 6 months if lesions and/or HPV were still present. Pictures of all lesions were made, allowing an independent interpretation at a later time point. Lifestyle and sexual behaviour were evaluated by questionnaires, starting from 1999. Furthermore, viral load of all baseline samples containing the most common hrHPV types was quantified in 2003, using type-specific real-time LightCycler PCR.

The partner study formed the basis for our aim to answer the following questions:

**What is the relationship between penile lesions and the presence of HPV?**

In chapter 2 we analyzed the presence of penile lesions in male sexual partners of women with CIN and their relationship with HPV. Flat penile lesions appeared the most prevalent lesions amongst HPV infected males, were strongly HPV-related and may represent productive HPV infections. Based on the results we propose a new classification for penile lesions that is simple and useful in clinical practice (*J Am Acad Dermatol* 2002; 47(3):351-7).

In Chapter 3 we studied the highly prevalent pearly penile papules (PPPs) in more detail. These PPPs were not related to HPV and apparently are a normal physiological skin formation of the penile corona and sulcus (*J Am Acad Dermatol* 2003; 49(1):50-4).

In chapter 4 we studied the prevalence of HPV and HPV-associated penile lesions in a male hospital population with non-STD complaints as well for comparison with male sex partners of women with cervical intraepithelial neoplasia (*Int J Cancer* 2005; 113(1):36-41).

**Is there a relationship between HPV viral load and the presence or absence of CIN lesions?**

In chapter 5 we assessed in a cross-sectional study the viral load of high-risk types 16, 18, 31 and 33 in women with normal cytology and women with abnormal cytology in whom a high-grade CIN lesion was diagnosed. We managed to define clinically relevant viral load thresholds for all five HPV types analysed below which high-grade CIN could be excluded (*Int J Cancer* 2006; 119(5):1102-7).

Given the occurrence of HPV transmission in sexual partners we subsequently evaluated the effect of condom use on the clinical behaviour of CIN and PIN in these couples.

**Does condom use influence the clinical behaviour of CIN and HPV status in women?**

In chapter 6 we evaluated in a prospective randomised clinical trial the influence of condom use on the clinical course of CIN and cervical HPV status in the females of sexually active couples. We could demonstrate a significantly faster regression of CIN 1 and CIN 2 lesions associated with clearance of HPV in the female partners in case of condom use (*Int J Cancer* 2003; 107(5):811-6).

**Does condom use influence the clinical behaviour of flat penile lesions and HPV status in men?**

In chapter 7, we evaluated in the same partner study the influence of condom use on the clinical course of flat penile lesions and penile HPV infections. We found a significantly faster regression
of flat penile lesions and clearance of HPV in male partners of couples that used condoms (Int J Cancer 2003; 107(5):804-10).

In the discussion (chapter 8) we put the data from the chapters into perspective and describe in more detail the differential diagnosis of penile lesions, the effect of condom use and possible drawbacks regarding viral load assessment in hr-HPV positive women without high-grade intraepithelial neoplasia.
References

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