# Human papillomavirus testing and its application in cervical cancer prevention

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# HUMAN PAPILLOMAVIRUS TESTING AND ITS APPLICATION IN CERVICAL CANCER PREVENTION

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Abstract

Because of the strong causal relationship between persistent infections of human papillomavirus (HPV) and cervical intraepithelial neoplasia (CIN) and cancer, HPV-testing has been proposed for improvement of cervical screening programs, including triaging and follow-up after treatment for CIN. We developed two new methods for HPV-testing with genotyping: A high-throughput HPV genotyping method that uses mass spectrometry for detection of the products of type-specific mass extend reactions, and a method with particularly sensitive detection of a broad spectrum of HPV-types, also in the case of multiple infections, that uses type-specific probes coupled to fluorescent beads for detection on the Luminex platform.
The utility of HPV-testing was evaluated in 3 different studies:
A general primer PCR- based genotyping method and the commercial Hybrid Capture (HCII) assay were compared for sensitivity and specificity for detection of CIN in secondary screening and in follow-up after treatment for cervical dysplasia. The sensitivities were high for both methods, although somewhat higher for the PCR method, but the concordance between the methods was substantial.
The performance of HPV-genotyping for prediction of recurrence after treatment for CIN was compared to that of cytology. Only HPV-genotyping could predict all cases of CIN grade II or worse in histopathology, and all cases of CIN I or worse in cytology during follow-up had persistence of HPV.
The applicability of HPV-genotyping was also evaluated in a secondary screening setting. Different high risk HPV types had substantial differences in risk for presence of CIN III or worse among women with ASCUS and CIN I in cytology, suggesting that HPV typing could be useful for further optimization of ASCUS/CIN I triaging strategies.

In summary, 2 HPV-genotyping methods with different applicability have been developed and validated. We also conclude that HPV genotyping is useful both in secondary screening as well as in follow-up after treatment for CIN.

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# SUMMARY

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In summary, 2 HPV-genotyping methods with different applicability have been developed and validated. We also conclude that HPV genotyping is useful both in secondary screening as well as in follow-up after treatment for CIN.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Det är grundligt etablerat att livmoderhalscancer (cervixcancer) orsakas av infektion med humant papillomvirus (HPV). I Sverige såväl som i många andra länder har man infört nationell cytologisk cellprovskontroll för att förebygga utvecklingen av cervixcancer och dess förelöpare. Eftersom kopplingen mellan cervixcancer och HPV är så stark har man funnit att testning för HPV i) kan förbättra effektiviteten av cellprovskontrollen, ii) visa vilka patienter som, efter att ha haft oklar cytologi, ska remitteras för vidare utredning (sekundärscreening), och iii) vilka som löper störst risk för återfall efter kirurgisk behandling av cellförändringar, då ett negativt HPV-test indikerar en mycket liten risk för återfall.

I de samlade arbetena i avhandlingen har HPV-testning använts och utvärderats i sekundärscreening och i uppföljning efter behandling av cellförändringar. HPV-testning i sekundärscreening kunde mycket väl identifiera fall av höggradiga cellförändringar, och dessutom bekräftades att vissa HPV-typer medför högre risk för höggradiga cellförändringar än andra.

HPV-testning i uppföljning efter behandling kunde bättre förutsäga återfall än konventionell cytologi, och av de patienter som hade någon form av cytologiska cellförändringar under uppföljningen hade samtliga persistens av HPV. Även i detta testningssammanhang visades att vissa HPV-typer medför högre risk för höggradiga cellförändringar än andra. Sammantaget indikerar detta att sådan HPV-testning som dessutom kan skilja olika HPV-typer från varandra kan vara användbar för optimering av sekundärscreening, och också är av intresse för att få kunskap om persistens av HPV efter behandling då detta medför högre risk för återfall.

I avhandlingen beskrivs också två nya HPV-testningsmetoder, båda med kapacitet att urskilja ett antal HPV-typer. Den ena kan identifiera HPV-typer i stora provmaterial, och lämpar sig bäst när man behöver testa många prover under kort tid, t ex när man vill studera effekter som HPV-vaccineringen kommer att medföra på vilka HPV-typer som cirkulerar i befolkningen. Den andra metoden har högre känslighet för att skilja ut ett stort antal HPV-typer, men är inte fullt lika storskalig. Den lämpar sig för t ex rutintestning av kliniska HPV-prover.

# LIST OF PAPERS

**I**. **Söderlund-Strand A**, Dillner J, Carlson C.
High-throughput genotyping of oncogenic human papilloma viruses with MALDI-TOF mass spectrometry.
Clin Chem 2008;54:86-92*.

**II**. **Söderlund-Strand A**, Carlson J, Dillner J.
A modified general primer PCR system for sensitive detection of multiple types of oncogenic Human Papillomavirus.
Submitted.

**III.** **Söderlund-Strand A**, Rymark P, Andersson P, Dillner J, Dillner L
Comparison between the Hybrid Cature II test and a PCR-based Human Papillomavirus detection method for diagnosis and posttreatment follow-up of cervical intraepithelial neoplasia.
J Clin Microbiol 2005;43:3260-3266**.

**IV.** **Söderlund-Strand A**, Kjellberg L, Dillner J.
Human Papillomavirus persistence after treatment for cervical dysplasia with loop electrosurgical excision procedure and laser vaporisation.
Submitted.

**V.** **Söderlund-Strand A**, Eklund C, Kemetli L, Grillner L, Törnberg S, Dillner J, Dillner L.
Evaluation of the usefulness of typing in Human Papillomavirus-based triaging of borderline or low-grade cervical cytology.
Manuscript.

# ABBREVIATIONS

DNA: Deoxyribonucleic acid

ORF: Open reading frame.

bp: Base pair

VLP: Virus-like particle

LSIL: Low-grade squamous intraepithelial lesion

HSIL: High-grade squamous intraepithelial lesion

CIN: Cervical intraepithelial neoplasia

CIS: Carcinoma in situ

SCC: Squamous cell carcinoma

ADC: Adenocarcinoma

ASCUS: Atypical cells of undetermined significance

STD: Sexually transmitted diseases

ASCCP: American society for colposcopy and cervical pathology

IARC: International agency for research on cancer

PCR: Polymerase chain reaction

EIA: Enzyme immunoassay

RDBH: Reverse dot-blot hybridization

HCII: Hybrid Capture, second generation

MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight

MS: mass spectrometry

# INTRODUCTION

## HISTORY

The papillomaviruses (PV) belong to the family *Papillomaviridae* and are highly species-specific with a tropism for epithelial cells. The PVs are found among many species, and probably occur in most mammals and birds [1]. The first PV was identified in 1933, and was shown to cause warts in cottontail rabbits [2]. This particular virus, cottontail rabbit papillomavirus (CRPV), was later shown to induce malignant transformation [3]. Harald Zur Hausen proposed in the late seventies that human papillomavirus (HPV) can cause cervical cancer[4, 5], a discovery that in 2008 is awarded with the Nobel prize. A few years later the two most common HPV-types found in cervical cancer, HPV 16 and 18, were discovered [6, 7]. HPV was found so regularly in cervical cancer that it is considered essentially necessary for the development of cervical cancer [8, 9].

## CLASSIFICATION

There are more than 200 different HPV types, which are further divided into cutaneous types that infect the skin, and mucosal types that infect the mucosa. The HPVs are classified according to the DNA sequence of the L1 gene coding for the major capsid protein. If the DNA sequence differs more than 10% compared to the DNA sequence of the closest known HPV type it is considered a new type, if it differs 2-10% it is a subtype, and if the difference is less than 2% it is a variant[1]. The PVs are grouped according to genotype into genera where different genera have a similarity of the L1 gene of less than 60% (figure 1). The PV types in each genus are further divided into species that share 60-70% identity. The HPV types that infect the genital mucosa are grouped into the genus alpha-papillomavirus, and the HPV types that infect nongenital skin, also called cutaneous types, are found in the genera alpha, beta, gamma, mu, and nu. The genital-mucosa HPVs are further divided into “high-risk” and “low-risk” types according to their respective associated risk to induce cervical

cancer[10] . Since the genital-mucosa types are of particular interest for this thesis, the focus will henceforth be primarily on these types and most particularly on the high-risk types.

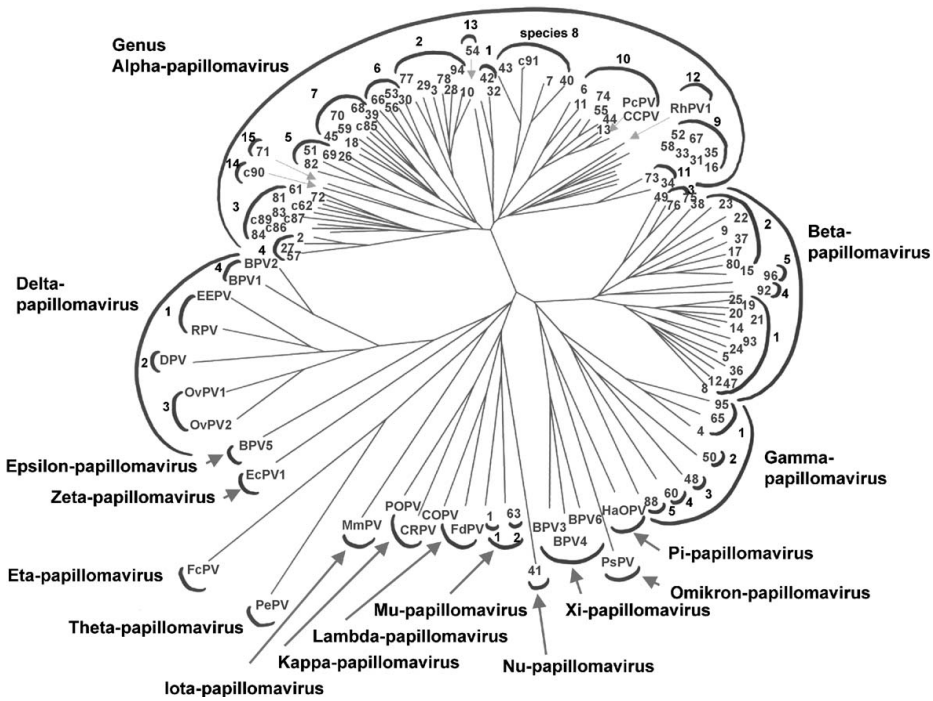


**Figure 1**. The phylogenetic organization of the papillomaviruses. Reprinted from de Villiers EM *et al,* Classification of papillomaviruses, in Virology 2004;324:17-27 with permission from Elsevier.

## MORPHOLOGY AND GENOMIC ORGANIZATION

The HPV virion is ~ 60 nm in diameter with a *T*=7 icosahedral capsid. The capsid is composed of 72 capsomers, and each capsomer is further composed of 5 monomers of the major capsid protein L1. There are 60 hexavalent capsomers which are surrounded by 6 other capsomers and 12 pentavalent which are surrounded by 5 other capsomers[11] . It has been suggested that there are 12 molecules of the minor capsid protein L2 in the capsid, and that these molecules are associated with the pentavalent capsomers[12] .

The genomes of PVs are well conserved, and nucleotide exchange events such as recombination or mutation very rarely occur[1] . The HPV genome is double-stranded DNA of approximately 8000 bp. Only one of the strands is actively transcribed, and transcription is tightly regulated by the differentiation state of the infected epithelial cell[13]. The genome is generally divided into 3 regions; the early region that encodes nonstructural viral regulatory proteins, the late region that encodes the 2 structural proteins, and the long control region (LCR) which is noncoding but contains the origin of replication and enhancer elements for regulation of gene expression[14] (figure 2). The high-risk HPVs contain 2 major promoters whose expression is regulated by differentiation. The early promoter controls expression in undifferentiated and differentiated cells and is active throughout stratified epithelium, whereas the late promoter is activated upon differentiation[15, 16]. In HPV 31, the early promoter is denoted p97 and is situated immediately upstream of the E6 gene, whereas the late promoter, p742, is situated within the E7 gene[15].

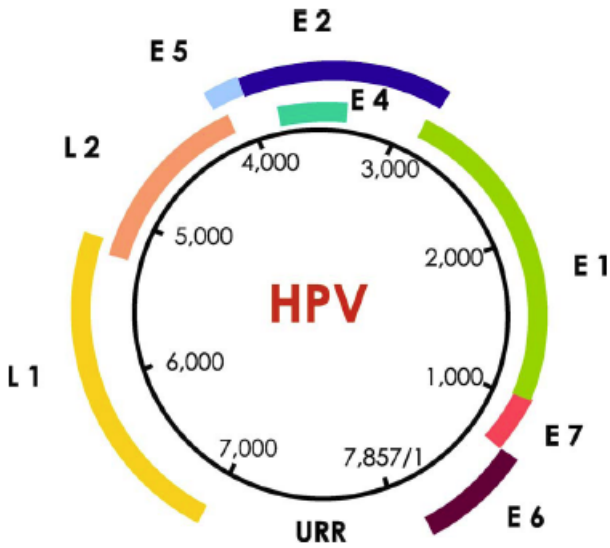


**Figure 2.** The genomic organization of HPV. URR is short for upper regulatory region, also commonly referred to as the long control region (LCR). Reprinted from Munoz N *et al*, Chapter 1: HPV in the etiology of human cancer, in Vaccine 2006;24:S1-S10 with permission from Elsevier.

## THE HPV REPLICATIVE CYCLE

The replicative cycle of HPV is closely linked to the differentiation of the HPV-infected epithelium. The PVs infect basal epithelial cells, which are the only cells in the epithelium that can divide. An uninfected basal cell would leave the basal layer, withdraw from the cell cycle and begin to differentiate, but in HPV-infected cells, the viral proteins can override the cell cycle arrest in order to allow for the production of new virions[17] (figure 3).

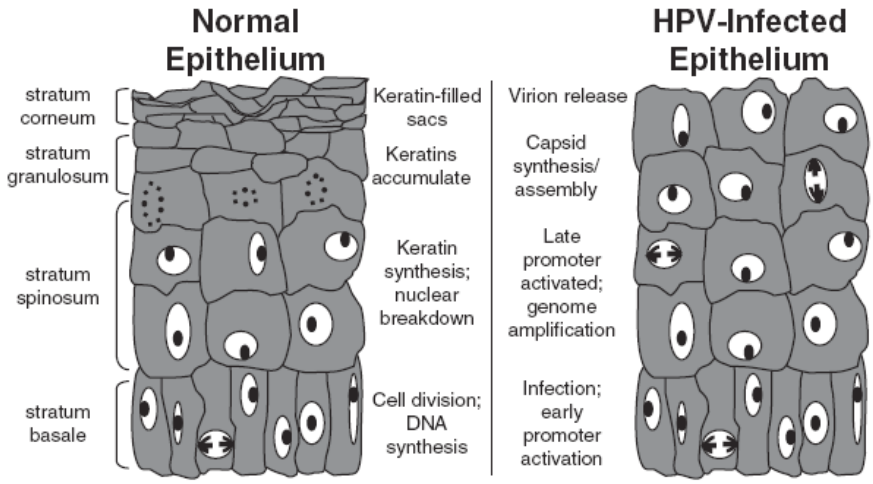


**Figure 3.** The differentiation process in normal epithelium (left) and the stages of HPV infection in HPV-infected epithelium (right). From Hebner CM and Laimins LA, Human papillomaviruses: Basic mechanisms of pathogenesis and oncogenicity, in Rev Med Virol 2006;16:83-97. Reprint permitted by Wiley Interscience.

It has not been clearly stated what the receptor(s) for virion attachment and uptake is, but several studies have proposed that it is mediated through heparin sulfate, possibly also involving a secondary receptor[18, 19]. Virion entry into cells is mediated via clathrin-dependent receptor-mediated endocytosis and has been shown to occur very slowly[20, 21]. Following infection, the virion is uncoated within the endosome, and then the HPV genome migrates into the nucleus where it is established as episomes, and the early promoter is activated[17]. Maintenance replication of the episome provides a low, steady copy number of 50-100 copies per cell that is kept stable during subsequent divisions of the host cell[22]. The HPV-infected basal cell migrates into the upper layers of the epithelium and starts to differentiate, although the cell cycle remains active mainly due to the action of the E7 protein[23]. During the differentiation process, the late promoter is activated which leads to late gene expression. In terminally differentiated cells, the viral genome is amplified to a high copy number and packaged into capsids whereafter progeny virions are released from the cell. HPV

is not believed to be cytolytic, and virions are shed together with desquamating cells from the uppermost layer of the epithelium[24].

## THE VIRAL PROTEINS

### E1

Both the E1 and E2 proteins are required for viral replication[25]. E1 binds to the origin of replication and is an oligomeric complex with viral DNA helicase and DNA-dependent ATPase as well as site-specific DNA-binding activities[26, 27]. For replication to occur, a co-operation between E1 and E2 is necessary since E1in itself has a low affinity for binding to the origin of replication. Only after E1 has formed a complex with E2 can there be an efficient binding to the origin of replication[28]. E1 is required for initiation and elongation of DNA synthesis[29] and is also presumed to recruit the cellular replication machinery to the viral replication origin by interaction with the cellular DNA polymerase[30, 31].

### E2

E2 is a DNA binding protein that modulates gene expression, but it also has other functions. It is constituted by 2 domains, one DNA-binding domain in the C-terminal region, and one transactivating domain in the N-terminal region[32, 33]. E2 functions as a repressor, for instance in viral transcription where it acts in repressing the activity of the early promoter in HPV 16 and 18[34, 35], but it also functions as an activator. It is crucial in viral replication by strengthening the binding of E1 to the origin of replication, and it is also required for plasmid maintenance in replicating cells. During genome segregation, E2 tethers viral genomes to mitotic chromosomes[36], which is mediated by the cellular protein Brd 4[37], but it has also been shown that the E2 protein of some types associate with the mitotic spindle rather than with chromosomes[38].

### E4

The E4 protein is found predominantly in differentiated cells even though its coding sequence is located in the early region of the genome. The E4 protein is translated

from the spliced transcript of E1^E4[39]. The expression of the E4 gene is upregulated in differentiated cells and correlates with the onset of vegetative viral DNA replication, but precedes the synthesis of the structural proteins[15, 40]. Several functions of E4 have been suggested; it could be important for facilitating the release of new virions by disturbing the cytokeratin matrix[41], and it has been suggested that synthesis of the E1^E4 protein could be important for activation of late viral functions and regulation of viral DNA amplification which was shown in HPV 31, but could not be demonstrated in HPV 11[42, 43]. Also for HPV 18 it has been shown that the E1^E4 protein is important for regulating late functions, and that there are type-specific differences between various E1^E4 proteins[44].

## E5

The genomes of the high-risk HPV types encode 3 proteins with transforming capacity; E5, E6, and E7, of which the E6 and E7 proteins have a significant role in malignant transformation. In contrast to the E5 gene of the bovine papillomavirus (BPV) 1 which encodes the primary transforming function, little is known about the biological mechanisms of the HPV E5 gene. The E5 protein is weakly oncogenic in tissue culture, and it has been shown to cooperate with the E7 gene to stimulate cell proliferation in primary rodent epithelial cells[45]. The E5 protein has been shown to increase cellular proliferation in the presence of epidermal growth factor (EGF) in rodent fibroblasts, and also to bind to the vacuolar proton-ATPase and thereby inhibit the acidification of endosomes, resulting in an increased recycling of EGF receptors to the cell surface[46, 47]. However, it has also been indicated that EGF receptors are not a target of E5, but rather that E5 modulates late viral functions through activation of proliferative capacity in differentiated cells[48]. Recently it was found when the HPV 16 E5 gene was expressed in human epithelial cells that the E5 protein affects several cellular pathways involved in cell adhesion, cell motility and mitogenic signaling[49].

## E6

The HPV E6 protein together with the HPV E7 protein are the main oncogenic proteins. The high-risk HPV E6 protein is a zink-binding protein, and it is mainly

produced early in infection[50]. E6 has several important functions. It interferes with the tumour suppressor protein p53 which in response to DNA damage activates expression of regulators that induce cell cycle arrest or apoptosis[51]. High-risk E6 forms a complex with the cellular ubiquitin ligase, E6AP, and thereby causes a rapid turnover of p53[52]. With reduced levels of p53, the restrictions on cellular DNA synthesis will be alleviated and there can be viral replication. Another function of high-risk E6 is the interaction with PDZ domain-containing proteins, which are cellular proteins that function in cell signaling and cell-cell adhesion, and are also involved in negatively regulating cellular proliferation. E6 can mediate the binding of PDZ proteins to the E6AP, resulting in their degradation, and it has been shown in a mouse model that interaction between E6 and PDZ domain-containing proteins is necessary for the induction of epithelial hyperplasia[53]. High-risk E6 can also activate hTERT, which is a catalytic subunit of telomerase[54]. This leads to increased telomeric length in epithelial cells which extends their life-span for production of new virions.

**E7**

The high-risk E7 protein binds zink and is phosphorylated by casein kinase II, primarily during the $G_1$ phase of the cell cycle[55]. High-risk E7 binds and degrades proteins of the Rb family, which are cell cycle regulators. The Rb proteins control the transition from $G_1$ to S-phase in the cell cycle through binding to the E2F transcription factors that can activate transcription of S phase components, leading to replication. Phosphorylation of Rb leads to release of the E2Fs and thereby transcription of S-phase genes. By binding and degradation of hypophosphorylated Rb, E7 hinders this cell cycle control, and E2Fs are released[56]. E7 binds class I histone deacetylases (HDACs), which are transcriptional corepressors[57]. Binding of HDACs leads to increased E2F transcription in differentiating cells, and subsequently to S-phase replication[58]. E7 can interact with cyclin-dependent kinase inhibitors involved in cell cycle arrest, for instance p21, and abrogate its actions, thereby hindering cell cycle inhibition[59]. It has also been shown that E7 can induce centrosome-related mitotic disturbances, leading to the exhibition of abnormal numbers of centrosomes in E7-producing cells[60].

### L1 and L2

The capsid proteins L1 and L2 are not expressed until late infection, in highly differentiated cells[61]. The capsid proteins are synthesized in the cytoplasm and are then transported into the nucleus for virion assembly. It has been suggested that L2 is important for viral capsid assembly since it can bind DNA and thereby introduce the viral genomes for encapsidation[62]. L2 has also been proposed to deliver the viral genome to the nucleus after uncoating[63].

## WHAT DIFFERENTIATES HIGH-RISK FROM LOW-RISK HPVs?

As already mentioned, not all genital-mucosa types can cause cancer, and this can be explained by the fact that the action of the E6 and E7 proteins shows some differences between high-risk and low-risk HPV-types. The implication of this has been shown in cell cultures, where the high-risk types can be distinguished from the low-risk types by the ability to transform or immortalize primary baby rat kidney epithelial cell cultures and keratinocyte cultures[64, 65], but also human keratinocytes[66]. Low-risk HPVs induce epithelial hyperplasia, produce viral progeny and contribute to the viral replicative cycle, but have a lower transforming activity and do not induce genomic instability[14]. Low-risk E7 binds to Rb but with a greatly reduced affinity compared to high-risk E7, and this is due to a single differing amino acid residue[67]. Low-risk E6 does not show any efficient interaction with p53, it lacks PDZ binding domains, and does not induce telomerase activity[51, 68, 69]. However, it has been suggested that even though low-risk E6 lacks the ability to immortalize cells, it can alter the cellular environment to allow for maintenance of HPV episomes in the cell[70].

## ONCOGENESIS

The transforming capacity of high-risk HPVs is probably a consequence of a viral replication strategy that is driven by the necessity to perform replication in suprabasal, normally growth-arrested differentiated epithelial cells, and to establish long-term

maintenance in a tissue in which individual cells are rapidly turned over and shed[14]. Oncogenesis requires synthesis and action of the high-risk E6 and E7 proteins, as described in the section “the viral proteins”.

An important event in HPV-induced carcinogenesis is the integration of the viral genome into the host chromosome. Integration sites are randomly distributed over the genome with a predilection for genomic fragile sites[71]. As a consequence of integration, the E6 and E7 genes are consistently maintained, but other parts of the genome is lost or no longer functioning[72]. Since the E2 gene, which encodes a transcriptional repressor of the E6 and E7 genes, is disrupted during integration, the inhibition of E6 and E7 gene expression may be released. There is evidence for increased stability of the E6 and E7 mRNA after integration, and that integration brings about cellular growth advantages over cells with episomal HPV genomes[73, 74].

Not all cancers display integrated HPV genomes, and there is evidence that the integration occurs to different degrees depending on which high-risk type that is involved[75, 76]. The highest frequency of integration is seen in cervical cancer, a much lower frequency is found in high-grade cervical lesions, and in low-grade cervical lesions it is a rare event[71, 75], although integrated HPV 16 has been found in low-grade cervical lesions, especially among older women[77].

## HPV IMMUNITY

### Natural conditions

The replicative cycle of HPVs leads to a minimum of viral antigenic exposure to the host immune system. There are several reasons for this: i) HPV is a double stranded DNA virus with no RNA intermediate to stimulate an innate immune response, ii) the HPV proteins synthesized during early infection are primarily nuclear proteins not presented by the infected cell, iii) most HPV proteins are expressed at very low levels in the basal epithelium where they will have the largest exposure compared to the upper part of the epithelium, iv) HPV does not induce cell death which would lead to

activation of immune defenses, and v) the phases of HPV-infection does not include viremia[78]. Information about the cellular immune response has come from spontaneously regressing warts, where a large infiltrate is displayed in the epithelium of predominantly CD4+ cells but also of CD8+ T cells and macrophages[79]. Expression of the early genes in the cytoplasm of infected cells generates short HPV peptides that bind to human leucocyte antigen class I molecules and are then presented to cytotoxic T-lymphocytes (CTL). However, it is not clear if HPV-infected keratinocytes can activate CTLs directly[80].

The humoral response following HPV-infection is directed against conformationally dependent epitopes on the L1 capsid protein[81]. Antibody responses to HPV antigens other than L1 are minimal or absent in HPV-infected patients[78]. There is evidence that a genital HPV infection gives an antibody response in most but not all women, with type-specific differences in persistence of antibodies and also in timing of seroconversion related to detection of initial HPV DNA[82]. It has been shown that the antibodies can persist for many years[83].

### Therapeutic vaccination

Since the high-risk E6 and E7 proteins are the causing agents of oncogenesis, the therapeutic vaccine development has aimed at stimulating T cell responses against these proteins. Several types of vaccines have been evaluated such as peptide, protein, DNA or viral vector-based, which are all proven to be safe and immunogenic in patients, but there is often no correlation with clinical outcome[84].

### Prophylactic vaccination

Whereas the therapeutic vaccines target the E6 and E7 oncoproteins, the purpose of the prophylactic vaccines is to generate neutralizing antibodies against the L1 protein. The L1 protein can be synthesized in eukaryotic cells and self-assemble into virus-like particles (VLPs), and when used in vaccines, these VLPs elicit virus-neutralizing

antibodies in serum[85, 86]. VLPs induce type-specific antibody responses, with some exceptions[87-89].

Prophylactic vaccines have been used in several trials and have proven efficient for reducing the acquisition of HPV-infection[90, 91]. A vaccine containing L1 VLPs of the types 6, 11, 16, and 18 was approved by the US Food and Drug Administration in 2006. A bivalent vaccine that includes HPV 16 and 18 VLPs has also been approved in Europe[86]. Results from clinical trials of these vaccines show that both vaccines are highly immunogenic with seroconversion rates to all targeted HPV-types of over 98%[92]. The bivalent vaccine was 94% effective in preventing persistent HPV 16/18 infections, and showed 100% efficacy in preventing cervical intraepithelial neoplasia (CIN) development due to HPV 16 or 18, whereas the quadrivalent vaccine was 89% effective in preventing persistent HPV 6/11/16/18 infections, and showed 100% efficacy in preventing CIN development due to HPV 6/11/16/18 infections[92]. The durability of protection over time is yet to be seen.

## HPV-ASSOCIATED DISEASES

### Non-genital diseases

Skin warts, most commonly on the hands and feet, are benign papillomas caused by cutaneous HPV-types. Most warts regress spontaneously within 2 years, and are transmitted mainly by skin to skin contact. Patients with the rare disease epidermodysplasia verruciformis have an increased susceptibility to cutaneous HPV-infections, in which the warts do not tend to regress but may progress to squamous cell cancers[93]. The potential role of oncogenic HPVs in nonmelanoma skin cancer (NMSC), further subdivided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), has been the subject to some investigations. It has been shown that healthy skin may contain HPV[94, 95], and that removal of the superficial layers of NMSCs result in a drastic reduction of HPV-positivity[96], but also that HPV may act as a co-carcinogen with UV-light in NMSC development[93]. It has been proposed that

HPV induces oncogenesis but that it is not needed for tumour maintenance[97]. Taken together, the role of HPV in NMSCs is not yet proven.

There is evidence for HPV involvement in benign oral lesions such as oral warts[98, 99], but also in the development of cancer. Head and neck cancer commonly refers to SCC arising in the upper aerodigestive tract. It has been suggested that there is a causal association between HPV and a subset of head and neck cancers, for instance oral and oropharyngeal squamous cell carcinoma (OOSCC), and HPV 16 is the most frequently detected type[100-102]. HPV DNA has also been found in SCC of the esophagus and may play a role for the development of this cancer[103].

Recurrent respiratory papillomatosis (RRP) is a rare disease of lesions in the laryngo-pharyngeal system. Even though the lesions are benign, they may cause severe morbidity due to recurrences after surgical interference, and even mortality when the lesions are extended into the lower airways. In rare cases progress to cancer may occur. The incidence is 2 per 100 000 adults and 4 per 100 000 children, and most cases are caused by HPV 6 and 11, with HPV 11 reported as associated with clinical severity[104, 105]. The quadrivalent HPV-vaccine containing VLPs for HPV 6, 11, 16, and 18 is expected to have some impact on the incidence of RRP[105].

## Genital diseases

The primary route of genital HPV-infection is sexual intercourse, and infection with HPV is also one of the most common sexually transmitted diseases[103, 106].

Apart from warts on the skin, HPV also causes genital warts. Anogenital warts or condylomas occur anywhere on the external genitalia, and HPV is found in approximately 90% of condylomas; the most frequent type by far is HPV 6[107]. Condylomas are apparent in at least 1% of the sexually active population of the USA[103].

The development of vulvar cancer has been shown to have two etiologies of which one is related to HPV[108]. Most vulvar SSCs are preceded by an intraepithelial

precursor lesion called vulvar intraepithelial neoplasia (VIN)[103]. A role for HPV in the development of a large proportion of VIN and vulvar cancer has been established, and a prevalence as high as 80% has been reported in VIN[103, 109]. Also in vaginal cancer, HPV infection has been implicated as one of the risk factors[110].

Anal cancer is a rare disease with a reported annual incidence of 2.1 per 100 000 individuals in the USA[111]. It is preceded by anal intraepithelial neoplasia (AIN), and HPV is an important etiological factor in the development of anal cancer[112, 113]. HPV infection is also one of the risk factors for penile cancer, and the most commonly found HPV-type in penile cancer is HPV 16[114].

Cervical lesions and cancer will be dealt with in the following section.

## NATURAL HISTORY OF HPV IN CERVICAL LESIONS AND CANCER

### Epidemiology of HPV in women

The overall HPV-prevalence among women worldwide has been estimated to be 10%[115]. The rates of HPV are highest among adolescents; in one study 55% of the initially HPV-negative adolescents acquired HPV-infections within 3 years[116, 117]. The HPV prevalence is decreasing in women older than 35, but there is a second peak of prevalence in women aged 45 years or older[115]. According to the estimates by de Sanjose *et al*[115], approximately 291 million women are carriers of HPV DNA, and of these women 32% have HPV 16 or 18 or both. The HPV-types most commonly detected are similar to those detected in cervical lesions and cancer[115]. The incidence has been suggested to be higher for oncogenic HPV types than for non-oncogenic types[118]. In a study of HPV incidence, it was shown that 1 year after incident infection, 70% of the women were no longer infected, but the longer an infection persisted, the more difficult it was to clear it; if an infection had not resolved after 1 year, the probability to resolve it within 6 months was 11%[119].

Whether oncogenic and non-oncogenic HPV types have the same duration of detectability is inconclusive since some studies show similar duration, with the exception of HPV 16[118], while others show longer duration for oncogenic types[119, 120]. The role of multiple HPV infections in HPV persistence has been debated, but the results are contradictory since some find an association between infection with multiple types and persistence[119] whereas others do not[121].

## HPV type-distribution in cervical cancer: What types are "high-risk"?

During the years since detection of the causal relationship between HPV and cervical cancer, several efforts have been made to assess the risk associated with each HPV-type found in the genital tract and in cervical cancer. In 2003, Munoz *et al* used pooled data from 11 studies including almost 2000 women with cervical SCC and the same number of control women to perform an epidemiologic classification of HPV-types[10]. According to this investigation, HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 were categorized as carcinogenic, or high-risk types and HPV 26, 53, and 66 as probably carcinogenic, or probable high-risk types. These results were confirmed in 2005 by a study by Schiffman *et al* of HPV infection and incidence of CIN III and cervical cancer among 10 000 women in Guanacaste, Costa Rica[122]. There was a meeting at the international agency for research on cancer (IARC) in France in 2005 with the purpose to reassess the carcinogenicity of HPV, and the concluding classification was to categorize HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 as carcinogenic to human beings[123]. In 2006, Munoz *et al* did another reclassification were HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 were classified as high-risk types and HPV 26, 53, 66, 68, 73, and 82 were classified as probable high-risk types[124]. Besides these classification studies, several studies of the HPV prevalence and type distribution in cervical cancer have also been performed. In 2004, Munoz *et al* conducted a pooled prevalence analysis of HPV type-specific distribution in 3100 cervical cancer cases from 25 countries and found that the 15 most prevalent HPV-types in cervical cancer are, in descending order of frequency, HPV 16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 39, 51, 73, 68, and 66[125]. Three years later, in 2007, Smith *et al* performed a meta-analysis update on HPV type distribution in 14 600 cases of cervical cancer from all continents, and found the 8 most prevalent HPV-

types in cervical cancer to be 16, 18, 31, 33, 35, 45, 52, and 58, which confirms the findings from the prevalence study from 2004 by Munoz *et al*[125, 126].

Thus, even though many of the HPV-types found to be associated with high risk for cervical cancer are similar between the separate studies, there is some variation leading to a lack of an absolute consensus on exactly which HPV-types that should be considered high-risk.

## Epidemiology of HPV in men

Apart from contributing directly to the disease burden of men, HPV-infections in men also contribute to HPV-infections and subsequent cervical lesions in women[127, 128]. Several studies have focused on risk factors for male acquisition of genital HPV-infections. A high number of female sex partners is reported to be a risk factor[129-132], but also young age[130, 131] and not being circumcised[127, 130, 131]. Whether the use of condoms reduces the risk of HPV-infection is not clear since some studies find a lower risk associated with condom use[131-133] whereas other studies fail to do so[129, 134].

It has been shown that HPV-infections in men are multifocal and the incidence is higher than that in similar cohorts of women[135]. The type distribution is somewhat different compared to women[136]. However, it is difficult to compare studies due to differences in sample techniques and number of sample sites per patient[137].

## Risk factors for cervical cancer

Persistent infection with high-risk HPV types is the most recognized risk factor for the development of CIN and cervical cancer[10, 138-142]. But even though high-risk HPV infection is a necessary cause of cervical cancer[8, 9], it may not be the only cause since many women acquire cervical HPV infections but only a few of these progress to cervical cancer. A number of risk co-factors are therefore likely to be involved in the disease process.

**Parity**

It has been shown that a higher number of full-term pregnancies is associated with an increased risk for cervical cancer after adjustment for the number of sexual partners and age at first intercourse. Also early age at first full-term pregnancy was found to be associated with risk of cervical cancer[143]. When the analysis was restricted to high-risk HPV-positive women, the findings were similar[143].

**Tobacco**

Current tobacco smokers have been found to have a significantly increased risk of SCC compared to never smokers, and the risk increased with the number of cigarettes smoked per day but not with duration of smoking[144]. The same pattern was observed when the analysis was restricted to high-risk HPV-positive women. The proposed mechanisms for the effect of tobacco smoking in cervical carcinogenesis are a reduction of the cervical immune response, effects related to the metabolism of female hormones, and direct genetic damage caused by carcinogens in tobacco[144].

**Hormonal contraceptives**

The effect of hormonal contraceptives is not yet fully explored, but a meta-analysis of hormonal contraceptives and cervical cancer found that the risk of cervical cancer increases with increasing duration of contraceptive use, since 10 years use is associated with approximately twice the risk compared to that among never-users[145]. It was also suggested that this risk decreases after the use of oral contraceptives has ceased. A similar pattern of risk was observed among women positive for high-risk HPV[145].

**Other sexually transmitted agents**

An association between infection of *Chlamydia trachomatis* and an increased risk of SCC has been demonstrated[146-148], whereas the risk for adenocarcinoma (ADC) has not been shown to be associated with *Chlamydia trachomatis* [146, 148, 149].

Infection with herpes simplex virus type 2 (HSV 2) has also been suggested to be associated with increased risk of SCC[150], but the contrary has also been shown[151].

In the case of sexually transmitted diseases as co-factors, there is a possibility that they are indications of a higher risk-behaviour that increases the exposure to HPV rather than being true co-factors[152].

**Immunosupression**

Immunosupression caused by human immunodeficiency virus (HIV) or organ transplantation leads to an increased risk of cervical lesions and cancer when compared to healthy women, but the exact role of immunosupression in conferring increased risk is not known[153, 154]. HIV-infected women have been shown to be about 5 times more likely to have squamous intraepithelial lesions (SIL) than HIV-negative women[155].

**Genetic factors**

A familiary aggregation of cervical cancer has been observed with different heritability estimates. Couto *et al*[156] and Hemminki *et al*[157] found a higher risk of cervical cancer among women with an affected mother and/or sister. This risk was higher than among women with an affected grandmother or aunt[156], whereas Zelmanowicz *et al*[158] reported that a family history of cervical cancer was associated with an increased risk regardless of whether the affected relative was a mother, sister or daughter, but also that the familial aggregation due to shared environmental exposures could not be ruled out. Magnusson *et al*[159] found that cervical cancer development depends on genetic factors to a much higher extent than on shared familial environment.

## Cervical cancer development

Cancers of the cervix include squamous cell carcinoma (SCC), adenocarcinoma (ADC), adenosquamous carcinomas and the uncommon group neuro-endocrine tumours[160]. Of all cervical cancers, approximately 80% are SCC and approximately 20% are ADC, whereas the other categories are very uncommon[161]. High-risk HPV infection is involved in the development of SCC, but also the association between HPV and ADC is strong and suggests a causal relationship[162].

Most pre-malignant and malignant squamous cell neoplasias occur at the transformation zone, which is the part of the cervix where columnar epithelium transforms into squamous epithelium through a process called metaplasia[163].

Cervical cancer evolves from preexisting noninvasive premalignant lesions called cervical intraepithelial neoplasias (CINs) according to European classification, or squamous intraepithelial lesions (SILs) according to the Bethesda classification used in the USA[164]. These lesions are classified histologically on the basis of progressive atypia of epithelial cells, that is on the degree to which they have lost cytoplasmic maturation and exhibit cytologic atypia: CIN I corresponds to mild dysplasia, CIN II to moderate dysplasia and CIN III to severe dysplasia and carcinoma in situ (CIS). When relating the CIN-classification to the Bethesda system, CIN I corresponds to low-grade SIL (LSIL) and CIN II/CIN III to high-grade SIL (HSIL)[164, 165]. There is also the classification "atypical cells of undetermined significance" (ASCUS) that represents poorly visualized cells from an LSIL, HSIL or other infectious or non-infectious process[137]. In a review of natural history of CIN, it was reported that the approximate likelihood of regression of CIN I is 60%, of progression to CIN III 10% and of progression to invasion 1%, and corresponding approximations for CIN II are 40%, 20%, and 5%, respectively. The likelihood of CIN III regressing is 33% and of progressing to invasion greater than 12%[166].

It is debated whether cervical cancer generally develops from HPV-infected normal cervical epithelium via a sequence of CIN I- CIN II- CIN III lesions, or directly via a rapidly induced CIN III lesion[165], but it has been shown that LSIL and HSIL are distinct HPV infection processes[137]. LSIL appears to represent a transient manifestation of productive viral infection where the HPV-infected epithelium undergoes differentiation and exhibits only minor cellular abnormalities, whereas in the true cancer precursor HSIL, HPV infection of immature, replicating cells prevents differentiation leading to continued replication of immature cells and accumulation of genetic abnormalities that could lead to the development of cancer cells. LSIL may be established first, at the same time as or in the absence of HSIL[137]. The median time period from infection with HPV 16 to CIS among women with initially normal cytology has been estimated to be 7-12 years[167], but it has also been shown that some

CIN III lesions may develop within 2 years or less following normal cytology[168, 169]. The mean age of women with invasive cervical cancer is approximately 50 years while the mean age of women with HSIL is approximately 28 years, which suggests a long time period for cancer development to occur[137] (figure 4).

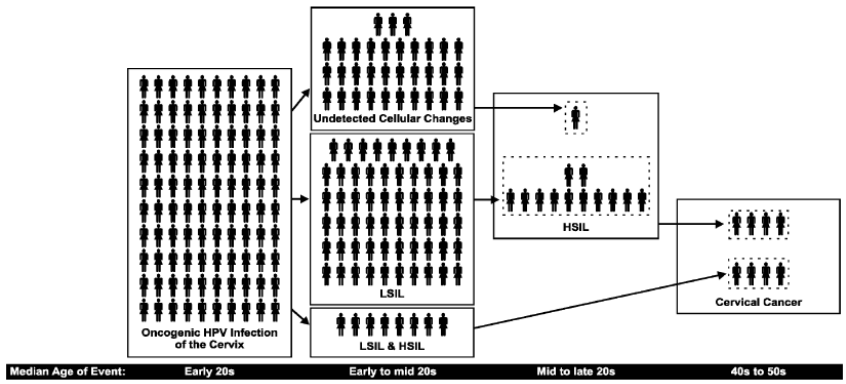


**Figure 4**. The natural history of cervical carcinogenesis. Reprinted from Baseman JG and Koutsky LA, The epidemiology of HPV infections, in J Clin Virol 2005;32:16-24 with permission from Elsevier.

Even though the HPV prevalence is high, the development of cervical cancer is a rare event occurring after a period of persistence with high-risk HPV. Approximately 80%-90% of all HPV infections resolve with time, leaving 10%-20% of individuals who do not clear the HPV infection which then becomes persistent[170]. Recent estimations of life time risk of cervical cancer in women with untreated CIN III are 31%-40%[171, 172]. Nevertheless, the majority of infections appear to be cleared by an effective immune response, and clearance of a high-risk HPV infection has been linked to cytological regression[173].

## Incidence of cervical cancer

Cervical cancer is the second most common cancer among women worldwide, and in the year 2000, the estimated global incidence of cervical cancer was 471 000 cases and 233 000 deaths[174]. Almost 80% of the cases occur in developing countries, where

the life time risk is about 2%, as compared to 1% in developed countries[174]. In Sweden, approximately 440 women were diagnosed with cervical cancer in the year 2006[175]. In 2007, Arbyn *et al* performed estimations of cervical cancer incidence and mortality in the European countries[176]. According to this, more than one in every 100 women in the 10 new states of the European Union dies from cervical cancer before the age of 75, which is twice as many as among women in the 15 old member states, probably due to lack of adequate screening in Eastern Europe[176] (figure 5).

The incidence of cervical SCC has been decreasing in recent years whereas the incidence of ADC and adenosquamous carcinoma has increased[162, 177, 178]. The increase of ADC may be an indication that current screening practices are not sufficient to detect some ADC precursor lesions[162]. These precursor lesions are often located high in the endocervical parts of the transformation zone which make them less accessible for sampling[179].

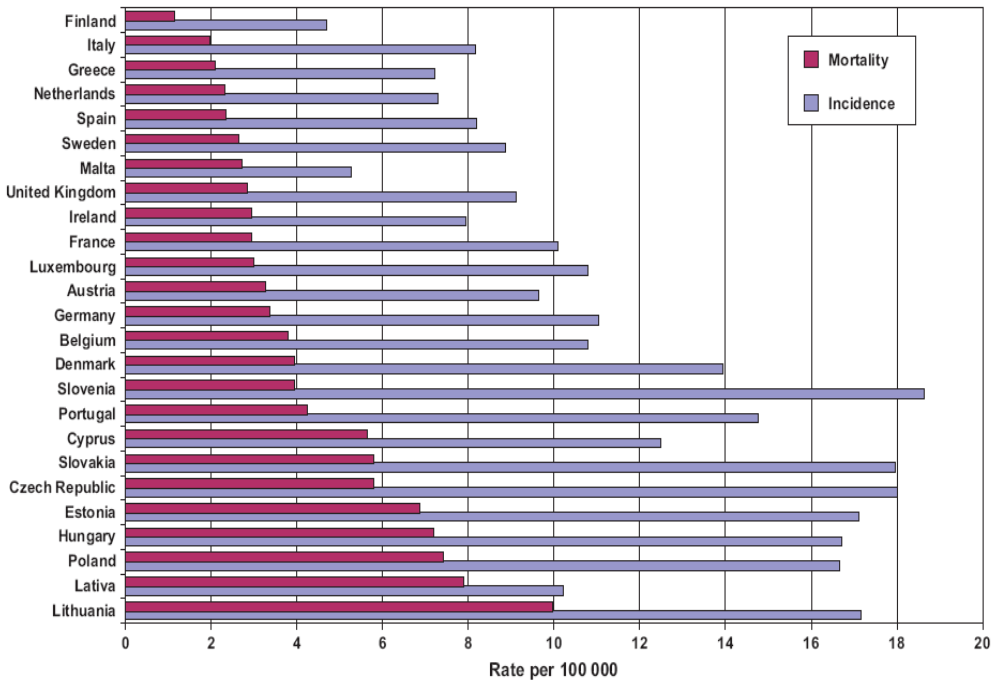


**Figure 5**. Age-standardized rates of incidence of and mortality from cervical cancer (/100 000 women years) in 25 EU member states, ranked by increasing mortality. The "new" member states that joined the EU in 2004 are Cyprus, Czech republic, Estonia, Hungary, Latvia, Lithuania, Malta, Poland, Slovakia, and Slovenia. Reprinted from Arbyn M *et al*, Burden of cervical cancer in Europe: estimates for 2004, in Ann Oncol 2007;18:1708-1715 by permission of Oxford University Press.

## Prevention of cervical cancer

The conventional mode of cervical cancer prevention is organized screening programs using Papanicolaou (Pap) staining of epithelial cells sampled from the cervix in the expectation that detectable nuclear abnormalities will be representative of histologically defined underlying lesions. Women with normal cytology continue with a fixed time-interval between sampling whereas women with abnormal cytology will be monitored through follow-up cytology or referral to further examination with

colposcopy, possibly followed by treatment[180]. Cervical screening using cytology has reduced cervical cancer incidence substantially. However, cytology has its limitations. A systematic review on accuracy of the Pap test indicated only moderate accuracy and the sensitivity was lower than generally believed[181]. The most critical limitation is its high false negative rate which implicates medical consequences[182]. Because of the strong causal relationship between persistent genital HPV-infections and cervical lesions and cancer, it has been suggested that HPV-testing should be included in screening. The accumulated experiences from epidemiological studies on HPV-testing point out that the negative predictive value of a negative HPV test is very high, and when combined with cytology it is >99%[183], that HPV-testing in comparison to cytology has a higher sensitivity for histologically confirmed HSIL[184], that high-risk HPV is present in a high proportion of normal cervical smears among women who are later to present with CIN II or III[185], and also that a single HPV test is more sensitive for underlying disease than one single cytology test[180]. It has been shown that the combined action of HPV-testing and cytology among women approximately 35 years of age reduces the incidence of CIN II/III[186].

So far, HPV-testing has been used for 3 screening purposes; i) as a complement to Pap smears in primary screening for detection of cervical lesions among asymptomatic women, ii) in triaging of women with abnormal Pap smears, either as a complement to cytology or as a substitute for the repeat smear, and iii) as follow-up after treatment of lesions for improved surveillance of recurrence, to permit more aggressive management of cases that are likely to recur because of persistent HPV-infections[182].

i) A comparison of HPV-testing and cytology in primary screening showed that the sensitivity was on average 27% higher with HPV-testing than with cytology alone, but the specificity was 8.4% lower for detecting high-grade lesions[182]. Also, the screening of women older than 30 tended to improve the performance of HPV-testing since HPV-infections in older women are less of a transient nature than among younger women. The use of HPV DNA testing in primary screening of women in this age-group, either as an adjunct to cytology or alone, will lead to a longer disease-free period of time after a negative result, suggesting the possibility of longer screening

intervals[180, 187]. Recently, a large cohort study of the long-term predictive value of HPV-testing and cytology showed that using HPV-testing, the screening interval could be 6 years among women with HPV-negative results[188]. Another important finding was that introducing HPV-testing into cervical screening will lead to an earlier detection of CIN III or worse, thus allowing for an extension of the screening interval[189]. HPV-testing in screening is already in use: In the guidelines for the management of women with abnormal cervical screening tests composed in 2006 by the American society for colposcopy and cervical pathology (ASCCP), it is recommended for women aged 30 years or more who have a normal cytology result but who are high-risk HPV-positive to do a repeat cytology and HPV-testing at 12 months[190]. If the HPV-test is still positive by then, a further examination with colposcopy is recommended.

Although more evidence on performance of HPV-testing in studies with HSIL and cervical cancer as outcomes is needed, HPV-testing is considered one of the most promising new technologies with the potential to improve cervical cancer screening[182].

ii) Recent reviews of HPV-testing in cervical screening have concluded that the utility of HPV DNA-testing for triage of atypical cytology could be useful[187, 191]. It has also been found that when using HPV DNA testing among women with LSIL in cytology one should be aware that high HPV positivity in a given population may compromise its effectiveness[180, 187]. However, for women over 35 years of age among whom the HPV prevalence is lower, HPV DNA testing could be useful for triaging of LSIL cytology[187].

iii) In follow-up after treatment for CIN, several studies show that HPV-positivity after treatment is associated with a higher risk for recurrence[192, 193], and that HPV-testing can be used to reduce the number of follow-up visits[194, 195]. According to the guidelines for management of patients with CIN by the ASCCP in 2006, the recommended follow-up after treatment for CIN II/III includes HPV-testing[196].

Another aspect of cervical cancer prevention is vaccination against the two most prevalent HPV-types found in cervical cancer, HPV 16 and 18, which cause approximately 70% of all cervical cancers[10].

## Treatment of cervical lesions

The purpose of the treatment of cervical lesions is to prevent progression to more severe disease.

To confirm cervical dysplasia detected by cytology, an examination referred to as colposcopy is performed using a lighted magnifying instrument for visualization after dilute acetic acid and iodine has been applied to the entire cervix to enhance any epithelial findings[197]. During the colposcopy, a punch biopsy of suspicious lesions can be performed to obtain a tissue sample for histopathologic examination.

As treatment options, there are ablative modalities that destroy the affected cervical tissue, or excisional modalities that remove the affected tissue and allow for histopathologic examination. Ablative modalities are for instance cryoptherapy and laser ablation, and excisional modalities are loop electrosurgical excision procedure (LEEP) and coldknife conization[196]. Consensus guidelines for the management of women with histology-confirmed CIN were provided by the ASCCP in 2006[196]. In women with a histological diagnosis of CIN I, follow-up with either repeat cytology at 6 or 12 months or HPV DNA testing every 12 months is the recommended approach. If the repeat cytology reveals ASCUS or worse or if the HPV-test is positive, colposcopy is recommended. For women with a histological diagnosis of CIN II/III, either excision or ablation are acceptable procedures, whereas for women with recurrent CIN II/III an excisional procedure is recommended[196].

Although surgery is a very effective treatment of cervical lesions, it does not necessarily eliminate HPV-infection from the cervical area, and it has been shown that among women treated for CIN, the risk of cervical cancer is 5 times greater than among the general population of women[198]. This suggests that, as adjunct to or during

follow-up after surgery, antiviral therapy could be useful[164]. The development of such anti-viral therapies targeting HPV protein functions or enhancing the ability of the host immune system to resolve infection or inducing apoptosis indirectly on HPV-infected cells is a subject for ongoing and future research[164].

## Why HPV-genotyping?

HPV-testing can be performed either as a test of the presence or absence of a certain group of HPV types, or as presence of specific genotypes, that is genotyping. Knowing what specific HPV-types that are present in an infection has been shown to be of great importance, both in cervical screening[140, 180, 189, 199] since it clearly appoints those at greater risk of developing CIN, and as a predictor for treatment failure during follow-up after treatment for CIN[199-201]. It has been shown that type-specific persistence identified women at increased risk of CIN more accurately than a single or repeated presence/absence test of an agglomerate of HPV types[180]. There is also evidence that the chance of clearance declined with longer duration of type-specific persistence and that the risk of CIN II or worse rose[202, 203].

Increasing evidence show that different HPV-types are associated with different risks for progression to high-grade CIN and cancer. The type associated with the highest risk is HPV 16[122, 140, 189, 202, 204]. Some studies have found that other types are also associated with a greater risk of CIN, such as HPV 18[199, 205], HPV 31 and 33[204, 206], although to a lesser degree than HPV 16. The differing risk associations with each type possibly reflect differences in oncogenic potential.

In line with the different risks for progression associated with each HPV-type, it has also been shown that the HPV-types most likely to persist are HPV 16[122, 189], but also HPV 18, 31, and 33[189].

Whereas these observations support the usefulness of HPV genotyping, the number of types to be targeted by the genotyping test must be determined: Genotyping in screening must detect the most common high-risk types, but it is important to realize that each addition of a new type increases the sensitivity for detection of CIN III or

worse, but at the expense of a decrease in specificity. This will lead to follow-up procedures for many more women but only to a slight increase of detected high-grade lesions[207]. It has been suggested that adding new high-risk types to the ones most commonly included in HPV-testing will probably have an irrelevant impact on screening[125]. The lack of HPV-genotyping tests on the market that have approval of the American Food and Drug Administration (FDA) is a second consideration. Thirdly, for the HPV-genotyping to be cost-effective, perceived risk must be matched to the appropriate medical management for safety in screening and follow-up[208]. Fourthly, optimal age for screening and duration of follow-up of type-specific infections must be determined. The age of 30 has been suggested as most women aged 30 or older have passed the peak of self-limited infections, i.e. they are more likely to have persistent HPV-infections that might progress into dysplasia than younger women[208]. Fifthly, can a gradient of risk for specific HPV-types be established? Current suggestions are to specifically genotype for HPV 16 and 18 and detect the other high-risk types as a group, since it has been shown that such HPV screening that distinguishes these two types from other oncogenic HPV-types may identify women at the greatest risk of CIN III or worse[205]. In the 2006 ASCCP consensus guidelines for the management of women with abnormal screening tests, it is found reasonable to refer cytology-negative, HPV 16/18 positive women over the age of 30 for colposcopy, while women with other high-risk types are invited in 12 months for renewed cytology and HPV testing, but the ASCCP awaits the FDA approval of a genotyping test before this management will be recommended[190]. At present it is clear that more data on the persistence of individual HPV-types and their risk of high-grade lesions and cancer is needed.

HPV-genotyping can be important not only in different screening settings, but also in the monitoring of the distribution of HPV-types that will be present after the introduction of HPV-vaccination. It is of great interest to investigate whether the prevalence of the vaccine types 6, 11, 16, and 18 will decrease, and if these types will possibly be replaced by other types.

## HPV-TESTING TECHNIQUES

An ideal HPV-test should be easy to perform, be highly reproducible, allow for high-throughput analysis and automation, have a sensitivity for high-risk HPV-types of at least 10 000 viral copies per sample, allow detection of multiple HPV-types, identify individual types, and provide information about the viral load of each type found[209]. Not many, if any, of the HPV-testing techniques currently in use can match this. There are, however, a great number of HPV-tests in use targeting the genital mucosa types, and these can be categorized into PCR-based tests and others, not PCR-based.

### HPV-testing not involving PCR

#### Nucleic acid hybridization

Some methods utilize DNA hybridization with no preceding amplification. In situ hybridization (ISH) uses labeled probes that specifically hybridize to intracellular HPV DNA. This method permits localization of the HPV infection in the sample, and has the advantage of preserving the morphological context for histopathological interpretation, but the sensitivity is low[210]. Also, discrimination between different HPV-types would require the use of type-specific probes in multiple ISH experiments[211].

The currently most utilized non-PCR based hybridization technique is the second generation of the Hybrid Capture system, HCII (Digene), which has FDA approval and is used in the USA as a triage test for women with ASCUS cytology to determine further management, and also as an adjunctive screening test with cytology for women over the age of 30. It tests for the high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 in aggregate using the high-risk probe cocktail, but it is also possible to test for the 5 low-risk types 6, 11, 42, 43, and 44 using the low-risk probe cocktail. The target HPV-DNA hybridizes to labeled RNA-probes in solution. These hybrids are captured on hybrid-specific antibodies bound to the wells of a 96-well microtiter plate and are detected by a specific monoclonal antibody and a chemiluminescent substrate, providing a semi-quantitative measurement of HPV-

DNA. The HCII assay has several advantages: It is easy to perform, it has a quite high throughput using the 96-well plate format and is suitable for automation. As it does not rely on target amplification it is relatively insensitive to cross-contamination. However, the HCII assay also has several limitations. Since it utilizes probe cocktails, it does not allow for genotyping. The detection limit is approximately 5000 genome equivalents, which makes it less sensitive than most PCR-based methods[211]. Cross-hybridization of the high-risk probe mix with HPV-types not represented in the probe mix has been reported for HPV-types 11, 53, 61, 66, 67, 70, 71, 73, 81, and 82[212-214]. The effects of cross-hybridization have been shown to decrease the accuracy of HPV-testing at least among women with equivocal or mildly abnormal cytology, where the specificity decreased substantially whereas the sensitivity did not increase[212].

A third generation of the Hybrid Capture test, HCIII, was recently developed. Also in this version, RNA-probes are used but together with biotinylated oligonucleotides directed to unique sequences within the target DNA for capturing into streptavidine-coated microtiter wells. Reduction of unspecific binding is achieved by the use of blocking oligonucleotides.

## PCR-based HPV-testing

PCR is a thermocycling process in which oligonucleotide primers flank the sequence region of interest to amplify DNA in the presence of a thermostable DNA polymerase, leading to an exponential increase of the target sequence. HPV-testing using PCR can be performed either as a one-step testing technique, or followed by a detection step. There are 2 common approaches of PCR-based HPV-testing using either type-specific primers or broad-spectrum primers.

### Type-specific PCR

A type-specific primer pair only amplifies one HPV-type at a time. This means that several PCR-reactions would have to be performed separately for amplification of multiple HPV-types in one sample. To overcome this limitation, another PCR application can be used. One example is real-time PCR which can be used for a quantitative analysis of HPV-DNA using type-specific primers and probes[215], also in a

multiplex format, which allows for several HPV-types to be detected simultaneously[216-218].

**Broad-spectrum PCR**

For simultaneous amplification of multiple HPV-types, consensus or general primers that amplify a broad spectrum of types can be used. There are many broad-spectrum primer systems, but they all have in common that they target a conserved region in the HPV genome for amplification. Many broad-spectrum primers are designed for annealing to the L1 gene, which is the most conserved part of the HPV-genome (figure 6). Consensus primers exist also for other regions, exemplified by the CP primers targeting the E1 gene[219], but these have not been extensively used in clinical situations[211].

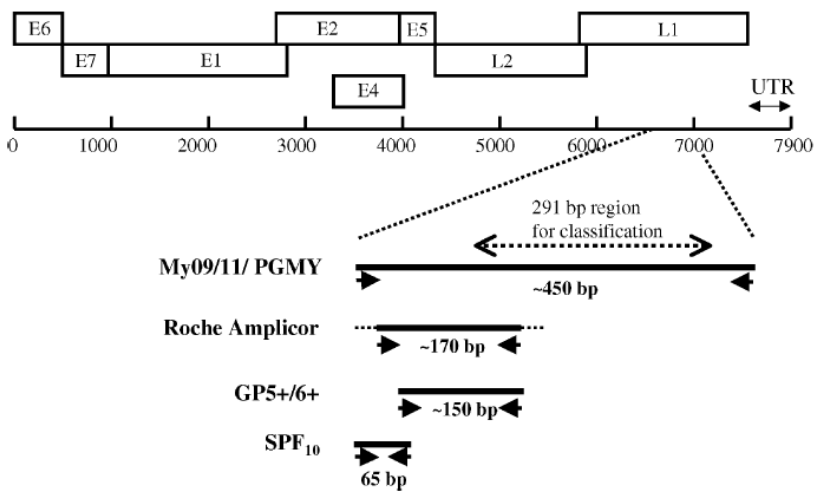


**Figure 6**. Target sites of commonly used broad-spectrum primers in the HPV-genome. The exact target site of the primers used in the Roche Amplicor test is not known. Reprinted from Molijn A *et al,* Molecular diagnosis of HPV infections, in J Clin Virol 2005;32:43-51 with permission from Elsevier.

The most commonly used consensus primers for HPV amplification can be categorized according to 3 different design approaches: i) a single consensus primer pair, ii) degenerate primers, and iii) sets of several consensus forward and reverse primers that either are not degenerated or may contain inosine.

i) Any single consensus primer pair fully complements few of the targeted type-specific HPV-sequences and contains mismatching nucleotides compared to the others. This is due to variability, even in very well conserved regions of the HPV-genome. To compensate for resulting mismatches, low and thus permissive annealing temperatures are used. Examples of the most extensively used single consensus primers are the GP 5/6 primers[220] and the elongated version, GP 5+/6+[221]. The amplicons are approximately 140 bp.

ii) Instead of lowering the annealing temperature to facilitate broad spectrum amplification, the approach of designing primers which contain degeneracies at some positions to compensate for the variability in the HPV genome can be used. A pair of degenerate primers comprise a quite complex mix of different combinations. Disadvantages with this approach are a low reproducibility in primer synthesis and a high batch-to-batch variation. A commonly used degenerate primer pair is the MY 09/11 primers[222] with an approximate amplicon size of 450 bp.

iii) The third approach is to design a set of multiple forward and reverse primers targeting the same region, but with fixed nucleotide variation at a few distinct positions to match as many HPV-types as possible. In some primer sets, nucleotides are exchanged for inosine that matches with any nucleotide although with low efficiency. The advantages over degenerate primers are that the reproducibility of primer synthesis is high, and the PCR can be performed at optimal temperatures. Widely used primer sets designed according to this approach are the PGMY 09/11 primers[223] that are modified from the MY 09/11 primers producing amplicons of 450 bp, and the inosin-containing SPF 10 primers[224] with amplicons of approximately 65 bp. Of all consensus primers mentioned in this section, these 2 sets have the highest analytical sensitivity[209].

A set of multiple primers is better for amplification of multiple HPV-types in a sample than a single consensus primer pair[209, 225]. If one type is present at a high viral load in the same sample as other types with a lower viral load, the kinetics of the PCR reaction will favour the dominating type at the expense of the less abundant types when a single primer pair is used.

### Amplicon detection

After the PCR reaction is completed, there are several methods for detection of HPV amplicons. A standard gel electrophoresis can be performed, but this will not provide any information on what type-specific HPV genomes that have been amplified. However, when gel electrophoresis is used in another format, typing data can be provided. An example of this is restriction fragment length polymorphism (RFLP), in which the PCR products are digested with restriction enzymes generating fragments of different sizes that can be separated by gel electrophoresis. A limitation with this method is that detection of multiple types that are present in different amounts in a sample is complex with a limited sensitivity.

Most detection techniques use hybridization between PCR products and probes. The original hybridization assay was Southern blotting used for HPV-typing in the early nineties[226]. According to this method, amplicons are electrophoresed and then transferred to a membrane. Labeled probes are added and hybridize to the amplicons. Since then, other hybridization methods have been developed that are less labour-intensive. In 1997, Jacobs *et al* described the application of an enzyme immunoassay (EIA) for detection of PCR-products[227]. In the HPV-EIA, one of the primers used in the PCR is biotinylated so amplicons can be captured onto streptavidin-coated microtiter plates. After denaturing, the unattached strand is removed by washing after which a cocktail of high- or low-risk type-specific labeled probes is added. Then conjugate is bound and hybrids can be detected after substrate addition[227]. In this assay, HPV-types are detected in aggregate, and the sensitivity is reported to range from 10 to 200 HPV copies, depending on the type.

There are also detection methods using reverse hybridization, i.e. immobilization of multiple oligonucleotide probes on a solid phase followed by addition of the amplicons in the liquid phase. An early example of this technique for detection of HPV DNA is the reverse dot blot hybridization (RDBH) assay[228]. In the RDBH, the biotinylated PCR-product is denatured and hybridized to type-specific probes that are immobilized on a membrane. Streptavidine-alkaline phosphatase conjugate is added and hybrids are visualized after addition of a substrate.

The most frequently used reverse hybridization methods are the line probe assay (LiPA)[229], reverse line blot analysis (RLB)[230, 231], and linear array (LA) (Roche molecular systems), all of which use the same general principle for detection: Multiple oligonucleotide probes are immobilized in parallel lines on a membrane strip. A biotinylated PCR-product is denatured and added to the strip for hybridization. After washing, the hybrids are detected by addition of a streptavidin-conjugate and a substrate generating colour at the probe line. Multiple HPV-types can be detected in this procedure, although the assays have a low throughput. The LiPA assay uses the SPF10 primers for detection of 25 HPV-types and is commercially available as INNO-LiPA (Innogenetics). The RLB assay using the MY 09/11 primers or PGMY 09/11 primers detects 27 HPV-types, but 37 HPV types when using the GP 5+/6+ primers. Both of these assays are similarly performed (see above) but the MY09/11 and PGMY 09/11 RLBs use individual, disposable hybridization strips for each PCR-product, whereas the GP 5+/6+ RLB uses a miniblotter system where membranes can be re-used. The RLB assay using PGMY 09/11 primers is also called line blot assay (LBA). The LA test is commercialized and uses the PGMY 09/11 primers for detection of 37 HPV-types. The LBA and LA tests were compared in a recent study showing that LA had higher sensitivity but lower specificity than LBA for detection of 2-year cumulative pre-cancer and cancer cases[232]. The LA has also been compared to the LiPA assay and it was found that the LA test detected more high-risk HPV types per sample than the LiPA assay, but that both assays are suitable for monitoring the impact of HPV 16/18 vaccines in clinical trials[233].

The commercial HPV-test Amplicor MWP (Roche molecular diagnostics) also uses the 96-well microtiter plate for detection of PCR-products of approximately 170 bp.

In the PCR-step a non-degenerate pool of primers is used targeting the L1 gene and also a primer-pair targeting the human HBB (haemoglobin, beta) gene. The microtiter plate is pre-coated with conjugated probes specific for 13 high-risk HPV types. The denatured amplicons are added to the wells, followed by addition of conjugate and a colorimetric substrate leading to visualization of hybrids. This assay simultaneously detects 13 high-risk HPV types in aggregate and the human HBB gene. A recent comparison between the Amplicor test and the HCII using cervical smears with diagnoses in the range of normal to high-grade SIL showed that the Amplicor test performance was similar to that of the HCII[234], whereas another study comparing the Amplicor test to PCR-based genotyping and HCII found that the performance of the Amplicor test was similar to the genotyping method but that both the Amplicor test and the PCR-based genotyping method had better performance than HCII[235]. The Amplicor test appears to show less cross-hybridization than the HCII and it also provides data on specimen eligibility, but it is more time consuming to perform.

A recently described HPV-genotyping method that utilizes hybridization is the bead-based multiplex genotyping method using the Bioplex 200 Luminex system (Biorad). Assays for presence of HPV proteins as well as for type-specific HPV DNA have been developed. For the latter, a PCR-based method was recently described in which biotinylated amplicons hybridize to oligonucleotide probes covalently linked to fluorescence-labeled polystyrene beads, which are internally dyed with various ratios of 2 spectrally distinct fluorophores creating an array of 100 different bead sets with specific absorption spectra[236] (figure 7). Individual oligonucleotide probes are coupled to different bead sets, allowing for up to 100 probes to be measured simultaneously. The biotinylated PCR-product is mixed with probe-coupled beads and after denaturation and hybridization, streptavidin-conjugated reporter molecules are added. Finally, beads are analyzed for both internal bead colour and reporter fluorescence using a Luminex analyzer. The signal for each type-specific probe is given as median fluorescence intensity (MFI).

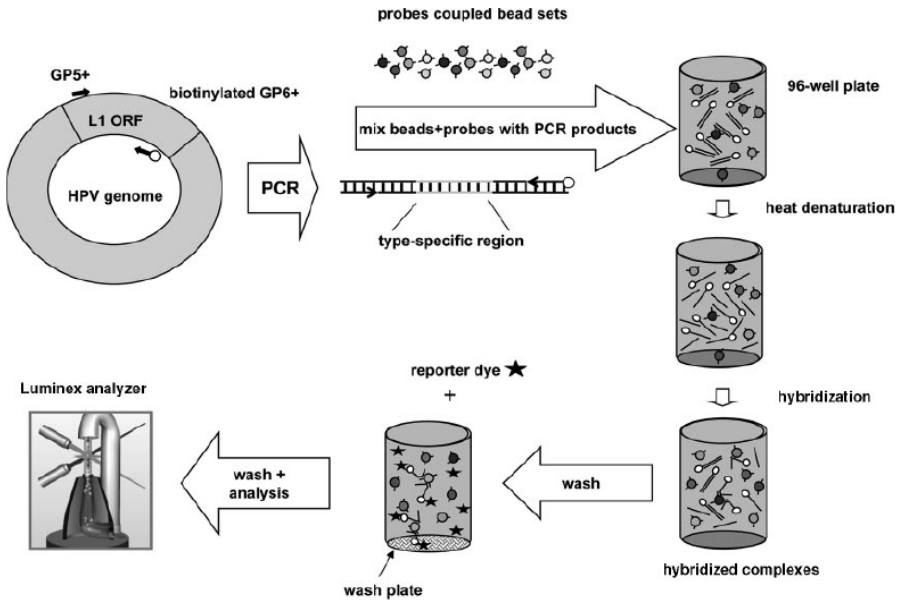


**Figure 7**. Schematic overview of the luminex-based HPV DNA genotyping method. The picture describes the use of the GP 5+/6+ primers, but the luminex system is not limited to the usage of this particular primer system. Reproduced from Schmitt M *et al*, Bead-based multiplex genotyping of human papillomaviruses, in J Clin Microbiol 2006;44:504-12 with permission from the American Society for Microbiology.

Amplicons can also be detected using mass spectrometry (MS). The MS technique is used for sensitive analysis of a broad range of analytes, for instance peptides, lipids, and inorganic compounds. The mass spectrometers differ for instance in the source if ionization and in analyzers, but the main principle of analysis is shared by all instruments. MS using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument is often used to analyze biomolecules including peptides, proteins, oligosaccharides and oligonucleotides[237]. In a general MALDI-TOF MS analysis, the sample applied to a UV-absorbing matrix is exposed to laser irradiation, resulting in ions from the sample transferred into gas phase (figure 8). The ions are separated by virtue of their different flight times over a known distance. The lower the ion's mass, the greater the velocity and shorter its flight time, and the travel time can

then be transformed into the mass to charge (m/z) ratio. The ions are collected by a detector which converts the information into a mass spectrum[237, 238].

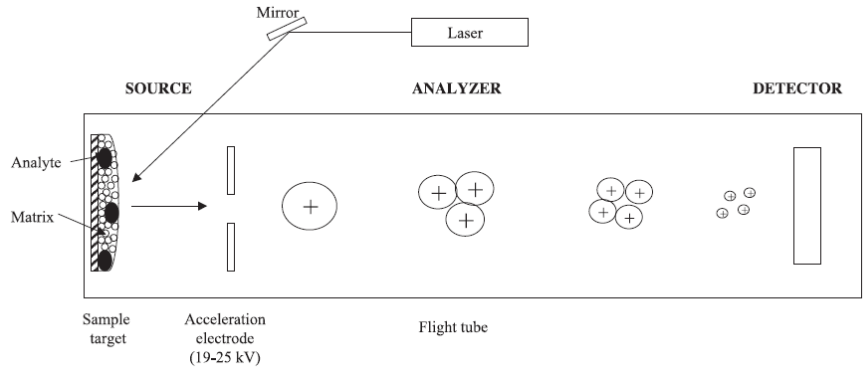


**Figure 8**. General principle of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Reprinted from Marvin LF *et al*, MALDI-TOF MS in clinical chemistry, in Clin Chim Acta 2003;337:11-21 with permission from Elsevier.

One of the applications for the MALDI-TOF instrument is genomic analysis, for instance of single nucleotide polymorphisms (SNP) in the human genome[239], but the use of MALDI-TOF MS for viral genotyping has not been extensively explored. So far, it has been used for typing of hepatitis B virus[240], hepatitis C virus[241], flaviviruses[242], human herpesviruses[243], and also for HPV which is described in paper I in this thesis. For genotyping of HPV, the commercialized Sequenom MassARRAY platform was used. It is normally used for SNP genotyping in which the nucleotide variability at a distinct locus is used to determine the presence or absence of a single base-pair mutation, but in the case of HPV genotyping, the technique was not used for discrimination between 2 alleles, but for the discrimination between 14 different HPV-types. According to our virus genotyping application of massARRAY, samples are submitted to a primary PCR using a consensus PCR-system, followed by dephosphorylation of the primary PCR reaction mix. Then, type-specific homogenous mass-extend (hME) primers with distinct molecular masses, one for each of the 14

targeted HPV-types, are added to the dephosphorylated reaction mix together with a nucleotide mix consisting of dATP, dCTP, ddTTP, and ddGTP, and other reagents, and a linear extension reaction is performed, using the amplicons produced in the primary PCR as template. In the presence of the specific target HPV type, the hME-primer will be extended by one or a few nucleotides producing extended hME-primers of distinct molecular masses, which will be detected in the MS analysis and presented as peaks in a mass spectrum. In the absence of a specific target HPV-type, only unextended hME primer can be detected.

The primary PCR targeting the HPV L1 gene generates amplicons of 160 bp. Within this short fragment, the sequences of all 14 hME primers and their extended products must be unique, both in the aspect of type-specificity to avoid cross-hybridization, but also in molecular mass so that each type-specific product can be distinguished from the others, and from non-extended primers.

## Reproducibility of HPV tests

Generally, the agreement between the most used HPV-tests is quite good, but several conditions, such as DNA extraction procedures, different sampling methods, differences in sample transport and storage, and the use of different DNA polymerases for the PCR reactions can affect test performance[209, 244]. To achieve improved reproducibility, validated protocols, reagents, and reference samples need to be further developed and more generally used[209]. Without worldwide standards for HPV-testing and reference samples, it is difficult to estimate and understand differences in HPV prevalence between natural studies of cervical cancer and to accurately assess the absolute risk associated with HPV infection[244]. A recent study using 4 different HPV-testing methods concluded that the individual limitations of each method need to be considered when genotyping is used for epidemiologic risk classification of individual HPV types, since the results may not be fully congruent between assays used for analysis of the same set of samples[245]. Another study where 4 HPV-genotyping methods were used for analysis of HPV-types with low prevalence found a moderate to low interassay agreement for the methods used, and concluded that the use of one

single assay in epidemiological as well as in clinical studies might lead to biased conclusions[246]. The use of a universally evaluated and accepted method or a combination of assays would lead to more accurate data[246].

A proficiency panel of HPV DNA for 16 HPV types as reference samples has been prepared and characterized by the WHO HPV Labnet Global Reference Laboratory. This panel provides the opportunity for different laboratories to assess the sensitivity and specificity of the locally utilized method in comparison to methods and applications used by other laboratories.

# AIMS OF THE PRESENT STUDIES

## PAPER I

To compare a newly developed detection method for high-throughput genotyping of high-risk HPV to the results of an established HPV-genotyping method using samples from women referred for colposcopy after atypical smears.

## PAPER II

To compare a newly developed PCR-system to an established PCR-system using a proficiency panel and clinical samples from secondary screening.

## PAPER III

To compare sensitivities and specificities of a PCR-based HPV-genotyping method and a commercialized HPV-testing method without genotyping capacity for their detection of CIN among women in secondary screening as well as for detection of CIN recurrence after treatment.

## PAPER IV

To evaluate a treatment method for cervical dysplasia using HPV persistence as outcome, and to investigate the efficacy of HPV genotyping to predict recurrence of high-grade CIN in follow-up after treatment for cervical dysplasia.

## PAPER V

To evaluate the usefulness of HPV genotyping in triaging of women with ASCUS or low grade cytology.

# MATERIALS AND METHODS

## PAPER I

A new PCR-based method for high-throughput analysis of 14 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) was developed using MALDI-TOF MS for detection. The comparison method was GP 5+/6+ PCR followed by RDBH (see the section "Amplicon detection"), for detection of the same 14 high-risk types as detected with the PCR-based MS-method. A total of 502 samples from women referred for a colposcopy-directed biopsy because of atypical smears (see paper III), and from follow-up after treatment of the same women were analyzed by both methods, and the results were compared to histopathologic and cytologic diagnoses.

In the present study, MALDI-TOF MS with the Sequenom MassARRAY system was performed as described in the section "Amplicon detection". The primary PCR-reaction included a set of 4 forward and 4 reverse primers, modified from the consensus primer pair GP 5+/6+ for improved annealing to 14 high-risk HPV-types, using the design approach of providing minimal repelling effects against mismatched templates. Also, a 5' 10-nucleotide extension was added to each primer for improved thermodynamic stability. In the PCR-program, 5 initial cycles with a permissive annealing temperature of 42$^{o}$C is followed by stringent brief annealing at 64$^{o}$C for the remaining 45 cycles. All new primers and the PCR-program were evaluated using gel electrophoresis at all steps.

Since the comparison method had a 5 times higher template input in the PCR, aliquots of the samples analyzed with the comparison method were concentrated 5 times before analysis with the PCR-based MS method.

A number of samples had discrepant results after the analyses with both methods. Aliquots of these samples were extraxted using proteinase K-digestion. The presence of human DNA in these re-extracted samples was demonstrated by real-time PCR

analysis with primers and probes for the human coagulation factor II gene, and the samples with positive results were re-analyzed with the MS-method and the comparison method. Ten samples with persistently discrepant results were sequenced using the modified forward primer mix. The sensitivity was also compared using 10-fold dilutions of 1-1000 copies per PCR reaction of plasmids with type-specific inserts for all 14 target HPV-types.

## PAPER II

The 8 primary PCR primers described in paper I were further improved by exchange of a forward primer and addition of a new forward and a new reverse primer, using the same design approach of providing minimal repelling effects against mismatched templates. The resulting 10 primers together with the optimized PCR-program (see paper I) form the modified general primer (MGP) PCR system.

For assessment of the performance of the MGP PCR system in comparison to the original primer GP5+/6+ PCR system, analysis of a proficiency HPV-panel and 592 clinical samples from women with ASCUS/CIN I in cytology (see paper V) was performed and also compared to histologic and cytologic diagnoses. Detection of amplicons was performed with bead-based multiplex genotyping using Luminex technology (see the section “Amplicon detection”). The probes used in the Luminex analysis provided detection of 14 high-risk and 7 low-risk HPV-types.

The proficiency panel contained plasmids with type-specific inserts for 14 high-risk types and 2 low-risk types in pools with 500 and 50 copies of each HPV- type per PCR-reaction as well as separate dilutions of 5 copies of HPV 16 and 18 per PCR-reaction. A further dilution down to 5 copies per PCR-reaction of all 14 high-risk types was also analyzed with both methods.

The results of the Luminex analysis were recorded as median fluorescence intensity (MFI), and signals were reported as the signal to cutoff ratio.

## PAPER III

A method comparison between the non-typing HCII assay and a GP 5+/6+ PCR-based genotyping method was performed using samples from 239 women referred for a colposcopy-directed biopsy because of atypical smears. Of these 239 women, 177 had treatment with conization. Samples for cytology and HPV-testing were obtained at the colposcopy visit and at a post-treatment follow-up visit. All samples were analyzed with i) HCII using the probe mix for 13 high-risk types (see the section "HPV-testing not involving PCR" and ii) PCR using the GP 5+/6+ primers followed by detection of biotinylated amplicons using EIA and RDBH of EIA-positive samples (see the section "Amplicon detection"). A separate PCR-EIA targeting the human HBB (haemoglobin, beta) gene was performed to determine quality of DNA in the sample. The HPV-test results were compared to the histopathologic diagnosis from the conization specimen and to cytological diagnoses at the follow-up visit.

## PAPER IV

A long-term follow-up study after treatment for cervical dysplasia was performed as an evaluation of i) the effectiveness of the treatment method using type-specific HPV persistence as outcome, and ii) the ability of HPV-testing, in comparison to cytology, to predict recurrence of high-grade CIN after treatment. The study included 178 women with abnormal smears who were referred for treatment. Follow-up visits were scheduled at 3, 6, 12, 24, and 36 months after treatment, with samples for cytology and HPV-testing obtained at all visits. All women were treated with loop electrosurgical excision procedure (LEEP) and laser vaporization. The HPV testing of 14 high-risk types was performed with GP 5+/6+ PCR followed by EIA and RDBH (see the section "Amplicon detection") with HBB-gene PCR-EIA as a control of sample quality. The ratio of CIN II+ over CIN I or less at treatment was calculated for each HPV-type.

## PAPER V

All women resident in the Stockholm County, Sweden, who on their organized, invitational smear had the cytological diagnoses ASCUS or CIN I between March 2003 and January 2006 were included in a randomized health care strategy. The two procedures compared were i) referral of all women with ASCUS or CIN I for colposcopy and biopsy (previous strategy) and ii) HPV-based triaging referring all women with ASCUS or CINI for a new visit with HPV-testing using the non-genotyping HCII assay with high-risk probes only (see the section "HPV-testing not involving PCR") . All HPV-positive women were referred for a colposcopy-directed biopsy, whereas HPV-negative women were scheduled for a new cervical smear 12 months later. All 15 ObGyn clinics in Stockholm County were randomised to colposcopy of all women (1567 women with ASCUS/CIN1) or to HPV triaging (1752 women with ASCUS/CIN1). Of the samples obtained in the HPV-triaging arm, 1595 were also analyzed using the GP 5+/6+ PCR followed by a bead-based multiplex genotyping method using the Luminex technology (see the section **"**Amplicon detection"). In the present study, this method was used for detection of the HPV-types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73, and 82. Real-time PCR amplification targeting the HBB gene was used as a quality control of the samples.

The results of the HPV genotyping analysis were compared to the HCII results as well as to the histopathologic diagnosis at a colposcopy-directed biopsy in the case this had been obtained, otherwise to cytology.

# RESULTS AND DISCUSSION

## PAPER I

The concordance between the MS method and the comparison method (GP 5+/6+ PCR followed by RDBH) was high, $\kappa$=0.945. The efficiency of type-specific HPV-detection with each method was compared: Among patients with a histopathologic or cytologic diagnosis of CIN I or worse, the results missed by RDBH were from 10 patients and the results missed by the MS method were from 9 patients. The MS method alone detected all cases of cancer, and also all HPV 68-positive results, and showed a slightly improved clinical sensitivity for detection of HPV in CIN II or worse over the comparison method. The analysis of the plasmid dilutions of 14 high-risk HPV-types revealed detection limits in the range of 1-100 copies per PCR-reaction for the MS method, and in the range of 1-1000 copies per PCR-reaction for the comparison method. The type-specific analytic sensitivity was higher or equal for the MS method over the comparison method for all 14 types except for HPV 31 and 39.

The MS method is automated using robotic pipetting and has a high throughput of 10x384 samples in 2 working days at a comparably low cost (about 14 SEK per sample). This, together with the clinical and analytical sensitivity, suggest that the MS method is useful for HPV-genotyping of large sample quantities, for instance in monitoring of the circulation of HPV-types in vaccinated populations.

## PAPER II

The MGP PCR system detected all 14 high-risk HPV types at the lowest level tested, 5 copies per PCR reaction. The comparison method, GP 5+/6+, detected HPV 16, 18, 56, 59, and 66 at 5 copies per PCR reaction, HPV 33, 35, 45 at 50 copies, and HPV 31 and 58 at 500 copies, whereas HPV 6, 39, 51, 52, 68, and a variant of HPV 35 were not detectable at these input levels.

According to the clinical sample analysis, there were 13 discrepant results that were GP+/MGP- and 240 results that were GP-/MGP+. All discrepant results for 12 types were only positive with MGP. Significantly more samples were positive for 14 HPV-types out of 21 with MGP, whereas GP5+/6+ did not detect any HPV-type significantly better than MGP. One sample was positive for HPV 56 with GP 5+/6+ only, and the signal to cutoff ratio was very high. This was probably due to the fact that with the MGP primers, one more mismatch between primers and template was introduced than with the GP 5+/6+ primers, and also because the MGP PCR program provides much more stringent annealing conditions than the GP 5+/6+ PCR program, thus being less permissive for non-specific amplification.

One woman was positive only after GP 5+/6+ PCR and had CIN I in histopathology, whereas 30 women were positive only after MGP PCR; of these women 1 had CIN I in histopathology and 8 had CIN II or worse.

MGP PCR detected 102 samples positive for >2 HPV-types, compared to 42 detected using the GP 5+/6+ PCR. This is in line with other findings that multiple primer systems are better for detection of multiple concordant HPV-types than single primer systems[225]. The full range of types that can be detected with the MGP PCR system is not fully explored.

Taken together, the MGP PCR system provides an improved amplification compared to the GP 5+/6+ PCR system for at least 14 types, among those the 2 most important carcinogenic types, HPV 16 and 18. The MGP primers also detected more multiple infections than the GP 5+/6+ primers. The MGP PCR system could be useful for primary HPV screening, for HPV triaging, for follow-up after treatment of cervical dysplasia, for epidemiological research and for monitoring the circulation of HPV-types in the vaccinated population.

## PAPER III

The concordance between the HCII assay and the GP 5+/6+ PCR-EIA genotyping method was substantial, both before (kappa=0.70) and after treatment (kappa=0.72).

The PCR-EIA method had higher sensitivity for CIN III in histopathology than HCII, 100% compared to 95.6%, respectively, probably due to the fact that the PCR-EIA method had a higher sensitivity. The specificity for CIN III in histopathology was 23.5% with PCR and 18.9% with HCII, i.e. very low. This can probably be explained by the fact that the samples were obtained from secondary screening, where many of the women have abnormal cytology/histopathology.

Both methods had the same post-treatment negative predictive values for CIN II or worse in cytology (99%) and for CIN III (100%). This confirms the value of HPV-testing in follow-up after treatment, since a HPV-negative test after treatment is associated with a very low risk of recurrence[195].

We found that both the HCII assay and the GP 5+/6+ genotyping test appear to be adequate for routine use in secondary screening as well as for follow-up after treatment.

## PAPER IV

The proportion of women with HPV clearance after treatment varies according to the treatment method used and the length of follow-up, as shown by several studies in which the rate of HPV persistence varied between 0%-35.1% [194, 200, 247-250]. At treatment, 129/178 women were HPV-positive. One year later, 10.8% were still HPV-positive with the same type as at treatment and 3 years later, 4.5% were persistently HPV-positive. During the follow-up period, 9 women had recurrence of histopathology-confirmed CIN II or worse. The HPV-clearance rate together with the number of recurrences indicate that the treatment method was not optimal.

Only HPV-testing identified all 9 women with recurrent histopathology-confirmed CIN II or worse: All of the 9 women had HPV type-specific persistence (100% sensitivity) whereas cytology detected 7 women out of 9 with recurrence (78% sensitivity).

Among the 129 women who were HPV-positive at treatment, CIN I or worse in cytology during follow-up was only found among women with HPV-persistence, either with the type found at treatment, or with a new type acquired during follow-up and detected at consecutive visits, whereas women who were HPV-negative or only transiently positive had normal cytology. These findings confirm the higher risk of cervical lesions in case of type-specific persistence, as found by others[141, 193, 251, 252].

HPV16/31/33 positivity was significantly associated with CIN II or worse rather than lesions of lower severity (OR, 3.05; 95% CI 1.23-7.76; P<0.05). These 3 HPV-types appear to be associated with an even higher risk of CIN than other high-risk types, which has also been shown by others[206].

## PAPER V

HCII testing using the high-risk probe mix found 1154/1595 women to be HPV-positive, and these women were referred to a colposcopy-directed biopsy. The PCR-based genotyping method found 1148 women positive for any high-risk type and 140 positive for any low-risk type.

The HPV-type with the highest sensitivity for CIN II or worse (CIN II+) and CIN III or worse (CIN III+) was HPV 16, detected in 42.2% of CIN II+ cases and 57.2% of CIN III+ cases but with only 23.8% of women testing HPV 16-positive. This type also had the highest odds ratio for CIN III+, 5.57 (95% confidence interval 4.03, 7.69). A combination of the 3 HPV types associated with increased risk for CIN III+, HPV 16, 31, and 33, resulted in an OR of 7.33 (95% CI 5.1, 10.53), a sensitivity of 76.7%, and a positive predictive value (PPV) of 23.8%, but were only detected in 36.3% of the women. In comparison, the HCII test had higher sensitivity, 97.8%, and OR, 19.73 (95% CI 7.28, 53.48), but lower PPV, 15.2%, and required referral of twice as many women, 72% as compared to 36%.

The sensitivity for detection of CIN III+ was 97.8% for HCII and 96.1% for any high-risk type with PCR. The difference in sensitivity is due to a number of women testing positive for only low-risk types according to the PCR-method, but testing positive

with HCII due to cross-reactivity between the HCII-probes and certain non-target low-risk types. The cross-reactivity of HCII is well established[214].

The addition of HPV 18 to HPV 16/31/33 did not increase the risk for CIN III+. Also for high-risk types other than HPV 16, 18, 31, and 33 no increased risk was observed. These 10 HPV-types were found among 29.6% of all women, but only among 16.7% of CIN III+ cases. If detection also of these HPV-types was required, it would result in almost twice as many women referred for colposcopy. A possible management of these women would include a repeat HPV test. For the use of HPV-testing for improvement of the ASCUS/CIN I triaging, it is necessary to ascertain the risks associated with each of the HPV-types considered "high-risk". Since at least some of them are not very common, it will take larger studies to investigate this, but based on the data provided in the present study, type-specific genotyping for HPV 16, 31, and 33 would be of interest and should be further explored.

In summary, we found that the different high-risk HPV-types have substantial differences in risk for presence of CIN II+, as shown among women with ASCUS or CIN I in cytology. The inclusion of genotyping, at least of some types, appears to be of particular interest for the further development of improved HPV-based triaging tests.

# CONCLUSIONS

Two newly developed and validated methods are described in this thesis, one that performs high-throughput genotyping for the main oncogenic HPV-types (PCR-based MALDI-TOF mass spectrometry detection), and one that has high sensitivity for a broad spectrum of HPV-types (the MGP PCR system followed by bead-based multiplex genotyping on the Luminex platform). The MALDI-TOF method is inexpensive with a cost for consumables of about 14 SEK per sample, and analyzes a large amount of samples, hundreds to thousands simultaneously, which makes monitoring of the effects of HPV-vaccination in terms of circulating HPV-types in the population feasible. For clinical HPV-genotyping, the MGP PCR method is a better option. The cost per sample is about 19 SEK, but the method can be applied also to low or moderate numbers of samples. The MGP PCR method has higher sensitivity than the MALDI-TOF method and it is easier to add new HPV-types to increase the range of HPV-types detected. However, it has a lower throughput and is not automated to the same extent. Thus, these methods fulfill different demands; either for clinical routine analysis of samples from screening or for large-scale epidemiological studies.

In the present work, the importance and applicability of HPV-testing, and especially HPV-genotyping, have been investigated in several ways:

i) HPV-testing was found to have a high sensitivity for detection of CIN II or worse, indicating that it is suitable for use in secondary screening and during follow-up.

ii) The results from the comparison between HPV-genotyping and cytology for prediction of recurrence during follow-up after treatment confirm the value of HPV-testing in this setting. HPV-genotyping was shown to be a better predictor of recurrence of histopathologic CIN II or worse than cytology, and only HPV-persistent women had CIN I or worse in cytology during follow-up. These findings also underline the higher risk of cervical lesions in case of type-specific persistence.

iii) When testing the relevance of HPV-genotyping in a secondary screening setting, we found that different HPV-types have large differences in risk for presence of high-grade lesions indicating that genotyping could be useful in secondary screening. Also in follow-up after treatment, HPV16/31/33 positivity was significantly associated with CIN II+ rather than lesions of lower severity. Thus, these 3 HPV-types appear to be associated with an even higher risk of CIN than other high-risk types, in post-treatment settings as well as in secondary screening.

Taken together, HPV-genotyping has a greater potential for optimization of screening and monitoring during follow-up than a non-genotyping test, even though more research is needed on which HPV-types that should be targeted in genotyping, and how to manage the genotyping information as a clinical routine.

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ALL my friends who have patiently gone through these years with me, and especially the past months when I transformed into a hermit. I will keep my promise to repay you all by presenting a really scary STD-lecture (with pictures) for your children when the time is right.

My parents for helping out.

The Strand children, the greatest gift: You have been ever so patient with me even when I was working the most and was unable to spend as much time with you as I would have preferred.

Ad **Oscar**:
Vir laudandus gaudeo te adesse.
Nec nisi tu nostris cantabitur ulla libellis,
ingenio causas tu dabis una meo.
*Ovidius*

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