Human papillomaviruses of skin and genital lesions

Johansson, Hanna K

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Human papillomaviruses of skin and genital lesions

Hanna Johansson

Department of Laboratory Medicine, Malmö

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FACULTY OPPONENT

Professor Ulf Gyllensten

Department of Immunology, Genetics and Pathology

Uppsala University

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Abstract

Around 5% of all cancer cases worldwide are caused by human papillomavirus (HPV) which has been established as the cause of cervical cancer and genital warts (condylomas). Cutaneous HPV types have been weakly associated with non-melanoma skin lesions such as squamous cell carcinoma (SCC) and actinic keratosis (AK).

We have analyzed skin and genital lesions for presence of HPV-DNA in order to search for unknown and known HPV types. As an attempt to identify women at risk for development of high-grade cervical lesions, HPV E6/E7 mRNA expression was analyzed among women with minor cervical abnormalities.

Metagenomic sequencing identified 15 known HPV types, four previously known HPV types and two novel putative types in cutaneous lesions (n=369) and swabs from top of the lesions (n=142) (SCC, AK and keratoacanthoma). The prevalence and viral load of characterized HPV155 and SE46 HPV-isolate was investigated in patients with different cutaneous lesions. HPV155 and SE46 were both found in 2% of patients. HPV178 was characterized from a swab of normal skin next to an actinic keratosis.

A baseline of 36 different HPV types of the alphapapillomavirus genus in 93.9% of condyloma swab samples before HPV vaccination was established. Extended testing among subjects initially negative for HPV (n=50), found 21 patients with cutaneous types of HPV, including novel HPV153.
After metagenomic sequencing (n=40) of HPV-negative condylomas, we detected four different HPV types of the alphapapillomavirus genus but also six known HPV types, three previously described putative HPV types and 22 novel putative types of the beta and gammapapillomavirus genera. Novel HPV175 and HPV180 were isolated from the condyloma. HPV153, 175 and 180 are all gamma types.

In the study of high-risk HPV mRNA expression of HPV-DNA positive minor cytological abnormalities (ASCUS; atypical squamous cells of undetermined significance, n=211, and CIN1; cervical intraepithelial neoplasia grade 1, n=131) we observed a high sensitivity (97.2%) but low specificity (10.2%) for detecting future high grade cervical lesions (CIN2+). Presence of HPV E6/E7 mRNA was associated with future development of CIN2+ among high-risk HPV DNA positive women with ASCUS and CIN1. The absence of HPV mRNA demonstrated a tendency for protection against future development of CIN3.

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Hanna Johansson

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Lund University, Malmö, Sweden

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FOR FUTURE ADVENTURES

Contents

Summary

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We have analyzed skin and genital lesions for presence of HPV-DNA in order to search for unknown and known HPV types. As an attempt to identify women at risk for development of high-grade cervical lesions, HPV E6/E7 mRNA expression was analyzed among women with minor cervical abnormalities.

Metagenomic sequencing identified 15 known HPV types, four previously known HPV types and two novel putative types in cutaneous lesions (n=369) and swabs from top of the lesions (n=142) (SCC, AK and keratoacanthoma). The prevalence and viral load of characterized HPV155 and SE46 HPV-isolate was investigated in patients with different cutaneous lesions. HPV155 and SE46 were both found in 2% of patients. HPV178 was characterized from a swab of normal skin next to an actinic keratosis.

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Populärvetenskaplig sammanfattning

Omkring 16% av alla cancerfall i världen beräknas vara orsakade av infektioner och omkring 5% av dessa orsakas av humant papillomvirus (HPV). HPV har etablerats som orsak till livmoderhalscancer och kondylom (könsvårtor). HPV-typer som förekommer på hud har förknippats med icke-melanom hudcancer såsom skivepitelcancer (SCC) men också ofarliga hudhudförändringar som aktinisk keratos (AK). HPV-typer brukar delas upp i sådana som hittas på slemhinnor och sådana som hittas på hud.

Vi har analyserat både hudskador och könsvårtor för förekomst av HPV-DNA, samt uttryck av HPV-mRNA bland celler från livmoderhalsen med lätta cellförändringar.

När vi använde en generell sekvenseringsteknik för att upptäcka DNA-virus bland hudförändringar (SCC, AK och keratoakantom) fann vi en större mångfald av olika HPV-typer från huden. Totalt identifierade vi DNA-sekvenser från 15 kända HPV-typer, fyra tidigare kända förmodade HPV-typer och två förmodade nya typer. Vi undersökte hur vanliga två av HPV-sekvenserna som hittades var (SE42 och SE46) i olika prover från hud. Det visade sig att dessa var ovanliga (påvisades hos 2% av patienterna) och hittades främst i pinnprover från hudytan. SE42 klonades och är nu erkänd som HPV155.

Vi har identifierat 36 olika slemhinne-HPV-typer i borstprov från ytan av kondylom. Dessa HPV-typer representerar en baslinje av vilka typer som är vanligast före införandet av HPV-vaccinationsprogrammet i Sverige. De tre vanligaste typerna var HPV6 (62%), 16 (13%) och 11 (10%). Efter utökad testning av kondylom som var HPV-negativa (50 st) påvisade vi 21 patienter som var positiva för HPV-typer som normalt hittas på hud, däribland nya HPV153.

Efter generell sekvensering (n = 40) av HPV-negativa kondylom upptäckte vi fyra olika HPV-typer utan också sex kända slemmhinne-typer, men också tre tidigare beskrivna förmodade HPV-typer och 22 nya förmodade hud-typer. Nya HPV175 och HPV180 isolerades.

I mRNA-studien tittade vi på uttrycket av HPV-mRNA i lätta cellförändringar bland celler från livmoderhalsen. mRNA visar på en aktiv infektion. Alla inkluderade kvinnor var HPV-DNA positiva vid starten av studien. Över 90% av

proverna var positiva för HPV-mRNA fastän endast ca 30% av kvinnorna utvecklade svårare cellförändringar, förstadier till livmoderhalscancer, under uppföljningsperioden på 4,5 år. Ett HPV-mRNA negativt prov från livmoderhalsen tycks skydda från utveckling av svåra cellförändringar.

List of papers

This thesis is based on following papers;

I. Unbiased approach for virus detection in skin lesions.

Bzhalava, D., Johansson, H., Ekström, J., Faust, H., Möller, B., Eklund, C., Nordin, P., Stenquist, B., Paoli, J., Persson, B., Forslund, O., Dillner, J., 2013. PLoS ONE 8, e65953.

II. Human papillomavirus typing in reporting of condyloma.

Sturegård, E., Johansson, H., Ekström, J., Hansson, B.G., Johnsson, A., Gustafsson, E., Dillner, J., Forslund, O., 2013. Sex. Transm. Dis. 40, 123-129.

III. Metagenomic sequencing of "HPV-negative" condylomas detects novel putative HPV types.

Johansson, H., Bzhalava, D., Ekström, J., Hultin, E., Dillner, J., Forslund, O., 2013. Virology 440, 1-7.

IV. Complete genome sequences of three novel Human Papillomaviruses; 175 178, and 180.

Johansson, H. and Forslund, O., 2014. Genome Announc. 2, e0043-14.

V. Presence of HR-HPV mRNA in relation to future high-grade lesions among women HR-HPV positive women with minor cytological abnormalities.

Johansson, H., Bjelkenkrantz, K., Darlin, L., Dillner, J., Forslund, O. *Manuscript.*

Abbreviations

aa	Amino acid
AK	Actinic Keratosis
ASCUS	Atypical squamous cells of undetermined significance
BCC	Basal cell carcinoma
bp	Base pair
CIN	Cervical intraepithelial neoplasia
EV	Epidermodysplasia Verruciformis
FFPE	Formalin-fixed paraffin-embedded
GS	Genome sequencer
HPV	Human papillomavirus
HSPG	Heparan sulfate proteoglycans
IARC	International Agency for Research on Cancer
KA	Keratoacanthoma
MDA	Multiple displacement amplification
NMSC	Non-melanoma skin cancer
nt	Nucleotide
ORI	Origin of replication
PCR	Polymerase chain reaction
PV	Papillomavirus
RCA	Rolling circle amplification
SCC	Squamous cell carcinoma
URR	Upstream regulatory region
WGA	Whole genome amplification

Introduction

Cancers caused by infectious agents

In 2008, it was estimated that 16% of new cancer cases worldwide was caused by infections. This estimate was based on the incidence of 27 different cancers from 184 countries (1). The three main chronic infections that cause cancer is Helicobacter pylori; a bacteria causing cancers of the stomach (2), hepatitis B and C virus that cause liver cancer (3, 4) and HPV which is the cause of cervical cancer (5, 6).

Oncogenic HPV types of the alphapapillomagenus are the cause of cervical cancer (5) and condylomas (7, 8). Oncogenic HPV types also cause other malign mucosal tumors such as anal, vulvar, and oral cancers (IARC, 2012). HPV is responsible for 30% of cancers caused by infections (1).

The established tumor viruses are, apart from HPV and Hepatitis B/C, the Epstein-Barr virus which is the cause of Burkitt's lymphoma (9), the Human herpesvirus type 8 causing Kaposi's sarcoma (10) and the Human T-cell lymphotropic virus (HTLV-1) causing adult T-cell leukemia (11). The Merkel cell polyomavirus has been linked to Merkel cell carcinoma of the skin, and is classified as a probable carcinogen (12, 13).

History

In 1933, the first papillomavirus was described; the Shope papillomavirus. It was later called cotton tail rabbit papillomavirus (CRPV) and was found to cause warts in rabbits (14). A few years later, it was discovered that warts induced by CRPV had potential for malignant transformation. In seven of ten domestic rabbits carrying papillomas for more than 200 days, cancer developed from the papillomas (15). They stated that "*the virus that gives rise to the rabbit papillomas must be looked upon as the primary cause of the cancers developing therefrom*".

In 1972, the link between human papillomavirus (HPV) and cutaneous wart like lesions of patients with a rare hereditary immunosuppressive disease;

Epidermodysplasia Verruciformis (EV) was discovered (16). A few years later, the first human papillomaviruses was isolated from skin warts (17, 18).

Viral particles resembling papillomaviruses were detected in genital warts (condylomas) in 1970 (19), but it was not until 1980 that HPV6 (the main cause of genital warts) was identified (20). Two virus groups had been suggested to play a role in the development of cervical cancer; herpes simplex virus (HSV) (21) and HPV due to its role in genital warts. The association between genital HPV and cervical cancer was made in the late 1970s by Harald zur Hausen and co-workers (22, 23) as they were unable to detect HSV in cervical specimens (6). The first oncogenic HPV (HPV16) was isolated in 1983 by hybridization of HPV11 to DNA from a cervical carcinoma (6). In 1999 it was established that HPV was the cause of virtually all cervical cancers worldwide (5). Harald zur Hausen was awarded the 2008 Nobel Prize in Physiology or Medicine for his discovery of human papillomaviruses causing cervical cancer.

Classification of papillomaviruses

Different papillomavirus (PV) isolates are describes as "types", and the classification are based on the sequence of the most conserved gene (open reading frame (ORF)) within the PV genome; the L1 gene (24). It is the similarity of the DNA sequence of the L1 gene that divides a new PV into genus, species, subtype or variant. PVs within the same genera share more than 60% similarity, and a novel PV shares less than 90% similarity to any other known PV type. The definition of a subtype is sharing 90-98% nucleotide similarity. Variants share more than 98% similarity (24).

A new HPV type will be recognized after cloning of the complete genome and requires that the DNA sequence of the L1 gene differs more than 10% from the closest related PV. The International HPV reference center (German Cancer Research Center, Heidelberg, Germany, 1985-2012. Karolinska Institutet, Stockholm, Sweden, from 2012) confirms novel HPV DNA sequences and assigns a number to a new HPV type.

HPVs are divided into five different genera; alpha, beta, gamma, nu and mu (Figure 1). The largest genera are the alpha, beta and gamma. Nu and mu are only comprised of three HPV types; 1, 41 and 63 (7, 25).

The HPV types can also be divided into mucosal HPV types that infect mucosa and cutaneous HPV types that infect skin. Generally, alphapapillomaviruses are detected on mucosal and beta- and gammapapillomaviruses are detected on skin.

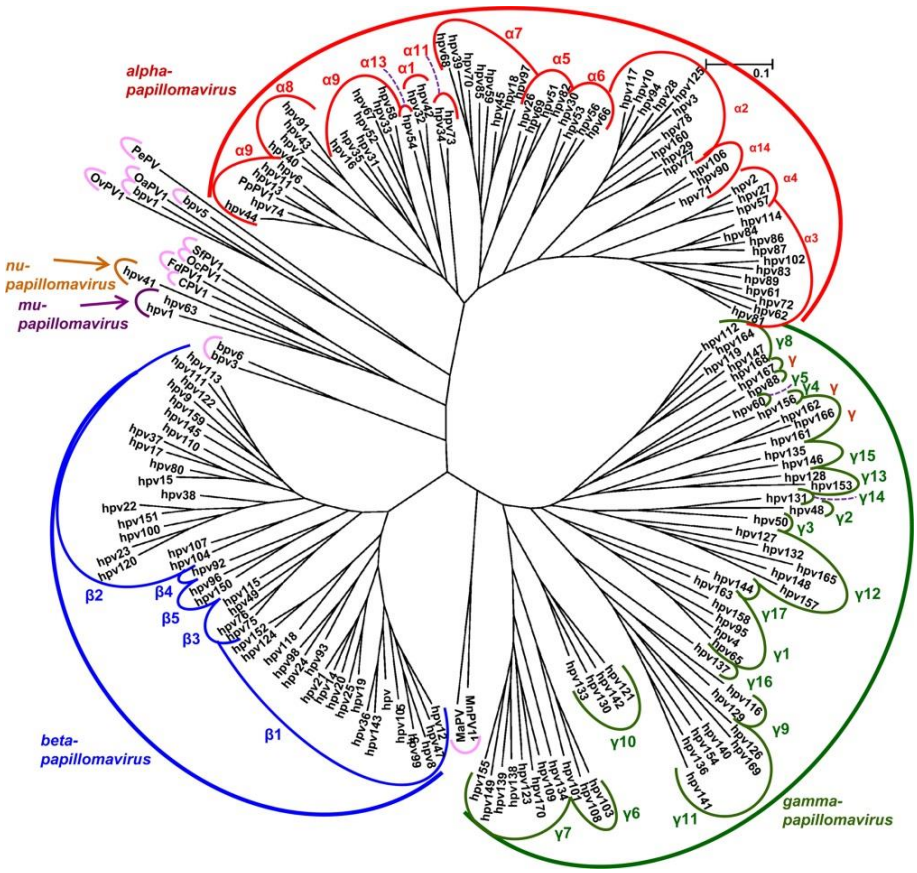

Figure 1. Phylogenetical tree based on the L1 ORF sequences of 170 HPV types, as well as single animal papillomaviruses Reprinted from de Villiers, Cross-roads in the classification of papillomaviruses, in Virology 2013; **445**:2-10, with permission from Elsevier.

Mucosal HPV

The alphapapillomavirus genus is composed of 65 different HPV types divided into 13 species commonly detected in mucosal. There are a few alpha types that are mainly detected on skin and belong to species 2, 4 and 8 (7, 24, 25). The alpha genus also includes some PV types infecting non-human primates (7). The alpha types are divided into high-risk (oncogenic/carcinogenic) and low-risk (non-oncogenic) types according to their ability to cause cancer (mainly cervical cancer) (26, 27). The International Agency for Research on Cancer (IARC) (2012) consider the following types as carcinogenic; HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. However, HPV types 68, 73 and 82 may be added to that list, with HPV66 as a probable high-risk type (26). HPV16 and 18 are the most commonly detected

HPV types in cervical cancer belong to species 9 and 7, respectively. The remaining HPV types of the alphapapilloma genus are considered as low-risk for cancer, such as HPV6 and 11 which are the main cause of condylomas (genital warts) (7, 8). Most low-risk types are found in species 1, 3, 4, 8 and 10.

There are two vaccines against HPV16 and 18 on the market; Gardasil® (Merck & Co) and Cervarix® (GlaxoSmithKline). Gardasil also protect against HPV6 and 11. They are both prophylactic vaccines based on virus like particles (without the viral genome) of the L1 protein.

Cutaneous HPV

HPV types of the beta and gammapapillomavirus genus are highly prevalent on skin (28-30). The betapapillomavirus genus is comprised of 51 different HPV types divided into five species. The gammapapillomavirus genus have expanded during recent years and are now the largest genus composed of 76 different HPV types and 26 species (hpvcenter.se, accessed 2014-08-04). Some species of the genus gamma only contain one HPV type. The HPV types of the Mu and Nu genera (HPV1 and HPV41, HPV63, respectively) are detected in cutaneous warts (24).

In addition, the identification of over 130 subgenomic sequences of putative gamma HPV types (FA-isolates) (31, 32), CUT-isolates (33) and isolates detected using the CP65 and CP70 primers (34) suggest a very large diversity of gammapapillomavirus types to be characterized in the future.

Expansion of the HPV family

The first HPV types; HPV1, 2, 3 and 4 was isolated from human warts in the 1970s using restriction enzyme digestion and gel electrophoresis (17, 18). The cloning of a full genome was performed in 1980 (35, 36). A few years later, HPV16 and 18 were isolated (6, 37). After the development of polymerase chain reaction (PCR), the conserved L1 gene and degenerate primers played a large role in the discovery of new HPV types such as the CP65/CP70 primer pair for detection of EV associated HPV types (34), GP5/6 primers for detection of mucosal HPV types (38), GP5+/6+ primers (39), the MY09/11 primers for detection of mucosal HPVs (40), the PGMY09/11 primers for broad spectrum detection (41) and the FA-primer pair for detection of mucosal and cutaneous HPV types (FA-isolates) (31) and the CUT primers for detection of both cutaneous and mucosal HPVs (33),. Recently, unbiased metagenomic sequencing has enabled further expansion of the papillomavirusfamily (42-45).

By 1989, 60 different HPV types were identified (46). In 2004, the number of identified HPV types were 93 (24). In 2010, an updated classification included 120 HPV types (7). An additional update recognized 165 HPV types (25). Now, about 40 years later since the first HPV types were identified, 195 different HPV types have been accepted by the HPV reference center with the highest number being 199 (www.hpvcenter.se, accessed 2014-08-04). Notably, HPV46, 55 and 64 have all been re-classified as subtypes of other HPVs and HPV79 was replaced by HPV91.

Genomic organization

Papillomaviruses are small non-enveloped with a diameter of about 60 nm. The circular genome (~7,200-8,000 bp) is double stranded DNA protected by an icosahedral capsid that is composed of two proteins; L1 and L2. The PV genome is divided into three regions; I) the early region starting with the gene E6 and ending with E2 or E5, II); the late region with genes L2 and L1 and III) the upstream regulatory region (URR) between the genes L1 and E6 (Figure 2). The URR is also called long control region (LCR) or non-coding region (NCR). Only one strand serves as template for transcription (47).

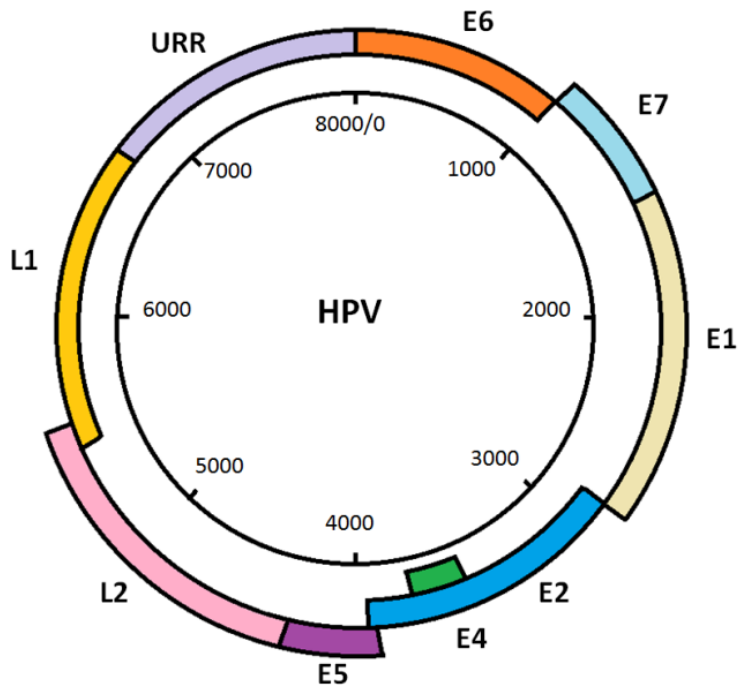

Figure 2. Schematic picture of the genomic organization of HPV.

HPV proteins

E6 and E7

E6 and E7 are the main oncogenic proteins of the HPV genome. Normally, induction of abnormal viral DNA synthesis in differentiated keratinocytes triggers a cellular defense mechanism that through apoptosis, differentiation and senescence eliminates such cells (48). The major role of the high-risk E6 is to eliminate the apoptotic response by mediating the ubiquitination, labeling for degradation, of p53 (49). p53 is a tumor suppressor protein that indirectly induce cell cycle arrest or apoptosis in response to DNA damage (50, 51). In addition, the HPV18 E6 has been demonstrated to degrade the pro-apopotic Bak protein (52).

Each cycle of DNA replication shortens the chromosomal telomeres to restrict the proliferative capacity of normal cells. High-risk E6 can induce the expression of hTERT (53, 54), a telomerase subunit that facilitate the life span of the cell and its immortalization.

HPV101, 103 and 108 lack the E6 open reading frame (55, 56). Interestingly, all three HPV types were isolated from cervicovaginal cells but phylogeneticaly cluster with HPV types of the gamma genus that normally infects skin.
The main function of the E7 protein is to bind and degrade proteins of the cell cycle regulating retinoblastoma (Rb) family. Degrading the tumor suppressor protein Rb maintains viral replication by promoting cell cycle entry in differentiated cells of the epithelia that otherwise would have seized division (57). The E7 of high-risk types bind and degrade pRb more efficiently than other HPV types (58).

Persistent infection seems to result in enhanced expression of HPV oncogenes E6 and E7 which are needed for malignant transformation (59).

E1 and E2

PVs rely on the host cell for replication; however, the PV genes E1 and E2 are required for efficient replication of HPV (60). The E1 protein binds to the origin of replication (ORI) and initiates replication by unwinding the DNA, recruiting DNA polymerases and other DNA binding proteins (61-63). To enhance the binding affinity to the ORI, E1 needs to form a complex with E2 (47, 64). Further, E1 is needed for elongation of the viral DNA synthesis (65).

E2 also has a role in viral DNA attachment to chromosomes to ensure that viral episomes are maintained in dividing cells (66), and function as transcriptional repressor of viral gene expression in keratinocytes (67, 68).

E4

The protein is generated from a fusion between the mRNA of the E1 and the E4 genes; the E1^E4 fusion protein. In some HPV types it is thought to facilitate virion release by destruction of the keratin network in the infected keratinocytes (69).

E5

In bovine PV, E5 is the main protein with capacity for malignant transformation (70, 71). However, little is known about the mechanism of E5 of HPVs. HPV16 E5 may play a role in immune evasion by down regulating antigen presentation on the surface of infected cells (72, 73). The E5 open reading frame is missing in most cutaneous HPV types (74).

L1 and L2

L1 and L2 compose the capsid surrounding the viral genome. L1, the major capsid protein, forms 72 pentameric capsomers with one L2, the minor capsid protein, under each pentamer (75).
The initial interaction between the papillomavirus capsid and the host is through L1 and heparan sulfate proteoglycans (HSPGs) (76-78). This interaction cause a small conformational change of the capsid that exposes L2, which indirectly allows the virus to bind another receptor on the keratinocyte cell surface (79).

Upstream regulatory region

The upstream regulatory region contains the origin of replication (ORI) and control signals for DNA replication and transcription (80).

The replicative life cycle of HPV

The skin consists of three layers; the epidermis on top followed by the dermis and hypodermis. The epidermis and the dermis are separated by a sheet of fiber called the basement membrane.

HPVs infect mucosal and cutaneous keratinocytes; squamous epithelial cells, and as PVs do not code for their own DNA-polymerase they have to rely on the host cell's replication machinery (60). As the replicative cycle of the skin's cells are so closely linked to the differentiation of the epithelial cells; the PVs must infect the cells close to the basement membrane; the only cells in the epithelium that can

divide. According to one model, PV reaches the cells just above the basement membrane through microabrasions, cracks, in the skin (77) (Figure 3). However, HPVs is also found in hair follicles which may allow the virus to reach dividing cells without microabrasions in the skin (81). During the normal skin generation process, the epithelial cells divide and migrate towards the surface of the skin. As they travel to the surface, the cells will differentiate and stop divide and eventually shed. However, the HPV infected cells will escape the cell cycle arrest and continue its cellular division to allow for production of new virions (82).

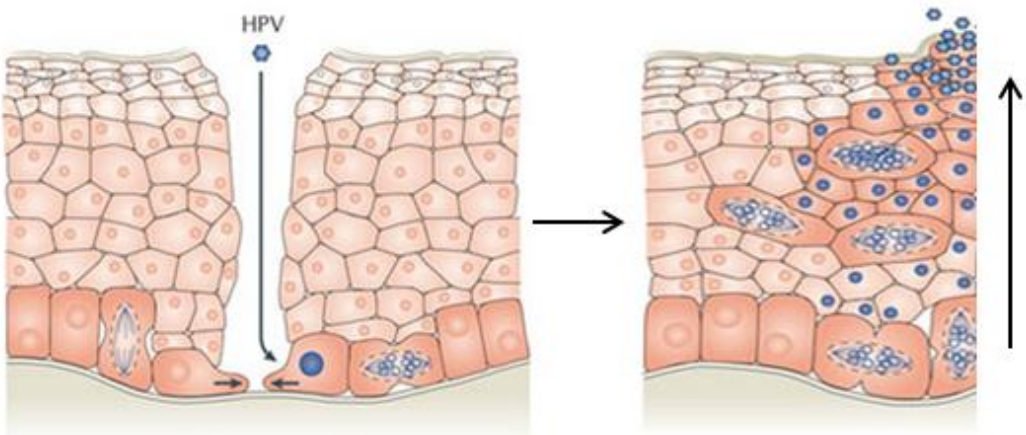

Figure 3. Overview of the papillomavirus life cycle. The basement membrane is seen as a thin grey line below the HPV infected cells. Newly produced HPVs are shed from the surface of the skin. Adapted from Banks et al., Human tumour viruses and the deregulation of cell polarity in cancer, in Nature Reviews, 2012; 12 877-886, with permission from the Nature Publishing Group.

HPV binds heparan sulfate proteoglycans (HSPGs) on the basement membrane via L1 (77), however, the binding mechanisms are not fully understood. After binding, the virus is transferred to a cell-surface receptor, enters the cell via endocytosis and is transported to the nucleus (83-85) where it is maintained in a low copy number, replicating with the division of the host cell (86).

When the infected cell leaves the differentiating compartment and exits the cell cycle, a large up-regulation of viral genes occurs, especially E6 and E7. These proteins will inhibit apoptosis, reactivate the cellular DNA synthesis and delay the differentiating program. As the cell cycle is reactivated the HPV genomes will be amplified to at least 1000 copies per cell. When reaching the top parts of the epithelium, the L1 and L2 proteins will be produced and infectious virions formed (87). HPVs are non-lytic and are shed together with dead skin cells from the surface of the skin (Figure 3).

HPV integration

One of the major challenges of the HPV is to establish long-term viral persistence in squamous epithelia as these cells constantly are renewed and shed (88). One key event in high-risk HPV-induced carcinogenesis is integration into the host genome. Integration usually occur near fragile sites of chromosomes (89) but may also occur elsewhere (90).

The expression of E6 and E7 is always maintained, but other parts of the HPV genome may be deleted (91), such as E1 and E2 (90, 92). Loss of E2 by integration may result in increased E6/E7 expression and stability of their mRNA, as E2 is a transcriptional repressor of E6 and E7, (93). Cells expressing E6/E7 from integrated HPV genomes have a selective growth advantage over cells with episomal HPV genomes (94).

HPV integration has been detected in low grade cervical lesions, but it is mostly mixed with the episomal form. Purely integrated HPV DNA is most common in high grade lesions and cancer (95).

HPV and genital infections

HPVs are one of the most common sexually transmitted pathogens in the world (IARC, 2007). At any time point, 11.7% of all women with normal cervical cytology will have a detectable HPV infection (96). Most sexually transmitted HPV infections are transient with a high rate of clearance the first months after infection (97, 98).

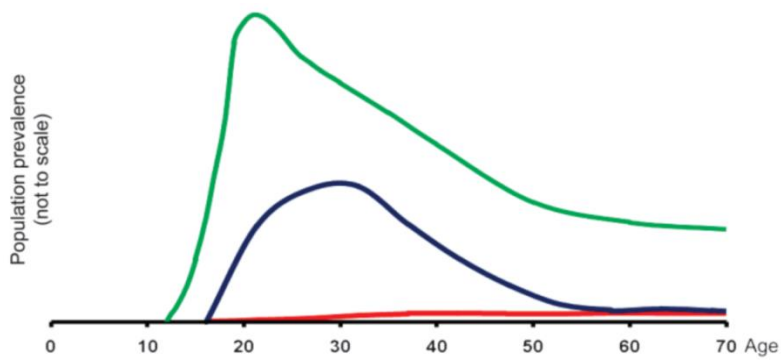

Figure 4. Incidence of female genital HPV infection (green), pre-cancer (blue) and cancer (red). Adapted from Schiffman et al., Human papillomaviruses testing in the prevention of cervical cancer, in J Nat Can Inst 2011; **5** 368-383, with permission from Elsevier.

The highest incidence of genital HPV infection in women is a few years after the average age at which women become sexually active and depends on geographical region. This peak is followed by a gradual decline (99) (100).

Condylomas

Condylomas, or genital warts, are a very common sexually transmitted disease caused by HPV, most commonly HPV6 and HPV11 (7, 8) of the genus alpha.

Condylomas generally grow in small groups of 5-15 papillomas (1-10 mm in diameter) on genital skin and surrounding mucosa and can occur anywhere on the genitalia, however, for men the condylomas mostly occur on the penile glans and shaft, for women they mostly appear on vulvovaginal and cervical areas. There are four different types of genital warts; condylomata acuminata and flat/macular lesions that grow on moist areas (non-keratinized epithelia) and papular warts and keratotic genital warts that grow on dry skin (keratinized epithelia) (101). A clinical diagnosis of condyloma is usually given by a physician experienced in sexually transmitted infection diagnostics and is based on clinical examination. Verification by histopathology is rarely used.

HPV6 and 11 are found in about 90% (range from 86-97%) of condyloma biopsies (8, 102-105). Large studies with condyloma swab samples detected HPV6 or 11 in 74-83% of samples, with 32-40% multiple infections (106, 107). A recent study showed that although 22 different HPV types of the genus alpha was found in and on top of condylomas, only HPV6 and 11 mRNA were detected in the lesions (105).
Vaccination against HPV16, 18, 6 and 11 in Australia has reduced new cases of genital warts among women aged 11-26 by 59% (108).

A study following young women for a mean 40 months showed that the incubation time from infection to visible warts was 1-6 months; with a median time to clearance with therapy of 5.8 months (109).

Precursors of cervical cancer

The earliest manifestations of cervical lesions are low grade cytological abnormalities, i.e. atypical squamous cells of undetermined significance (ASCUS) and cervical intraepithelial neoplasia grade 1 (CIN1). ASCUS is defined as poorly visualized cells which potentially can represent different grades of CIN or other infectious or non-infectious processes (110) such as fungal (e.g. *Candida vaginalis*), bacterial or parasitical (e.g. *Trichomonas vaginalis*) (111).

The Bethesda classification used in the US classifies the pre-malignant lesions as squamous intraepithelial lesions (SILs) or ASCUS. Low-grade SIL (LSIL) corresponds to CIN1, and high-grade SIL (HSIL) corresponds to CIN2 and CIN 3 (112).

CIN1 is the mildest form of pre-malignant cervical dysplasia according to European classification (113). The CIN lesions (between normal epithelium and invasive cancer) are classified histologically according to abnormal growth (dysplasia) of the squamous cells of the cervical epithelium. CIN1 represents mild dysplasia confined to 1/3 of the lower epithelium. CIN2 is moderated dysplasia confined to the basal 2/3 of the epithelium, and CIN3 corresponds to severe dysplasia which spans more than 2/3 of the epithelium (100). Cancer *in situ* spans the whole epithelium but is non-invasive.

A meta-analysis of 32 studies reported that high-risk HPV types is detectable in 43% (range 23-74%) of ASCUS and in 76% of CIN1 (range 55-89%) samples using Hybrid Capture II (Digene) (114). A systematic review of 423 studies showed that HPV-DNA of 48 different HPV types of the alphapapillomavirus genus was present in 52.1% of ASCUS and 74.2% of CIN1 lesions (28). It has been shown that for the vast majority (91%) of HPV-DNA positive women with ASCUS or CIN1, the HPV infection will clear within about 6-24 months, however, the remaining infections have high potential for persistence and progression to cancer (97).

It has been demonstrated that about 70 % of ASCUS and low grade lesions regress spontaneously (115). High-risk HPV testing is recommended as triage of ASCUS (116, 117), but not for CIN1 due to low clinical specificity.

Cervical cancer

Cervical cancer is the second most common cancer worldwide, with 83% occurring in the developing world. In developing countries, cervical cancer stands for about 15% of female cancers compared to 3.6% in developed countries (118). It is estimated that 4.8% of cancer cases globally could be caused by HPV every year (119). Cervical cancers are comprised of about 80% squamous cell carcinomas and 20% adenocarcinomas. Adenocarcinoma is neoplasia ("new formation") of epithelial tissue of glandular origin and is HPV DNA positive in about 70% of all cases (120). Cervical SCC usually occurs in the transformational zone, whilst adenocarcinoma arises from glandular tissue of the cervical canal (118).

The development to cervical cancer involves HPV transmission, viral persistence, progression to pre-cancer and invasive cancer. Reverse steps among pre-cancer stages can also occur (100, 121). Genital cancers require continued presence of

HPV for continued growth of cells and have at least one copy per cell of the causative HPV type (122, 123).

Women infected with HPV16 or 18 are considered to have increased risk for cervical cancer. HPV16 and 18 account for about 70% of invasive cervical cancers worldwide (124).

Established risk-factors for malignant conversion are high viral load of HPV16 (122, 123, 125) and persistence of infection (97, 126-130). Persistent infection seems to result in enhanced expression of HPV oncogenes E6 and E7 (59).
Other risk factors may be smoking (131), multi-parity (132), use of hormonal contraceptives (133), genetic factors (134), age at first intercourse and number of lifetime sexual partners (135), or other sexually transmitted diseases (136) such as herpes simplex virus type 2 (HSV-2) (137), *Chlamydia trachomatis* (138, 139) and HIV (140). A prospective serological study of women with cervical cancer showed that HSV had little or no association with cancer. However, previous exposure to *Chlamydia trachomatis* increased the risk for cervical cancer (141). Persistence of oncogenic HPV types is more likely among women with a previous *Chlamydia trachomatis* infection (142). HIV-1 has been classified as a cancer-causing agent by increasing the risk of cancer by immunosuppression (12, 13). HIV-infected women are about 5 times more likely to have cervical intraepithelial neoplasia, compared to HIV-negative women (140).

Invasive cervical cancers from 22 countries were analyzed for presence of HPV DNA using the MY09/11 PCR-primers (143). In this study, 93% of the invasive cervical cancers (formaline-fixed, paraffin-embedded) were positive for HPV DNA (866/932). Walboomers et al., speculated that the reasons that 7% of the samples were HPV DNA negative was that it is a false negative result, the carcinoma might not contain HPV DNA or that there is an absence of cancer cells in the sample analyzed. The HPV DNA negative samples was analyzed by HPV16 serology, betaglobin-PCR of the human genome to assess the DNA quality, HPV E7 specific PCR for 14 high-risk HPV types, consensus GP5+/6+-PCR (39) and CPI/II-PCR (144). Combining their results with the previous study of Bosch et al, the worldwide prevalence of HPV in cervical carcinomas was 99.7% (5).

There is a range of non-high-risk HPV types occasionally detected in high and low-grade cervical abnormalities. A systematic review found that HPV26, 67, 68, 69, 73 and 82 were slightly more prevalent in invasive cervical cancer than in normal cytology (0.27% in invasive cancer compared to 0.15% in normal cytology) (28). However, these HPV types have been classified as high-risk or probable high-risk by others (26).

Cervical cancer screening

Cervical cancer screening has reduced the incidence and mortality of cervical cancer, especially in high-income countries (145, 146). Organized screening programs use Papanicolau (Pap) staining of epithelial cells from the cervix where nuclear abnormalities can be detected. However, the accuracy of the Pap test (cytology) is only moderate with low sensitivity (30-87%) (147).

Infection with high-risk HPV is necessary for cervical carcinogenesis (5) and HPV-DNA testing has shown higher sensitivity then cytology testing (148). High-risk HPV testing is recommended as triage of ASCUS (116, 117).

HPV-DNA tests

HPV-DNA tests can be performed with commercial tests such as the Hybrid Capture II (Digene); detection of 13 high-risk HPV types without typing, the Linear Array Genotyping test (Roche); specific detection of 37 high- and low-risk HPV types, the Cobas test (Roche); 14 high-risk types with separate identification of HPV16 and 18 and the RealTime High-risk HPV test (Abbott); 14 high-risk types with specific detection of HPV16/18.

In-house tests are commonly PCR-based and use for example the GP5+/6+-PCR (39); with typing of 20+ HPV types, or the MGP-PCR with Luminex for typing of 39 high- and low-risk HPV types (149, 150).
An HPV-DNA test detects HPV-types in a sample without assessment of the activity of the HPV type.

HPV-mRNA tests

Expression of oncogenic E6/E7 proteins is necessary for malignant conversion of cervical cells (59, 151, 152). As 43% of ASCUS and 76% of CIN1 are HPV-DNA positive (114) and that up to 70% regress spontaneously (115), detection of E6/E7 mRNA of high-risk HPVs could potentially identify women at risk for development of cervical cancer (153).
Three examples of commercial HPV mRNA tests are the PreTect HPV-Proofer (NorChip) (154) and the NucliSENS EasyQ HPV test (Biomerieux) (155) that detects both HPV mRNA of HPV16, 16, 31, 33 and 45, and the APTIMA HPV E6/E7 mRNA assay (Hologic) that can detect mRNA of 14 high-risk HPV types. The APTIMA HPV E6/E7 mRNA assay has been approved by the American Food and Drug Administration (FDA).

HPV-negative genital infections

Some cervical cancers are HPV-negative as previously described (5, 143). The question "*do HPV-negative cervical carcinomas exist?*" was raised in an editorial

letter (156). This question could also be applied to condylomas, as swab samples was HPV-negative in one study (157). Other studies found no HPV-DNA in 1.2% (106) and 3.7% (158) of condyloma swab samples. Four explanations (cited below) were suggested (in addition of true negativity) (156) and these will be discussed with focus on condylomas.

> *1, "The specimens ultimately analyzed may not have been completely representative of the lesion".*

In the above mentioned three studies of condyloma swabs, all samples were analyzed for presence of human betaglobin to assess the quality of the samples as swab samples might contain too few cells. Also, the clinical diagnosis of condyloma might not be correct. One study reported simultaneous presence of condylomas and molluscs among 11% of patients with viral infection of the pubis (159). Molluscs are wart like nodules or raised skin caused by the sexually transmitted molluscum contagiosum virus (160).

> *2, "..integration of HPV DNA in the host cell genome in cervical carcinoma. This can result in the interruption, combined with deletions, of HPV DNA at integration sites".*

This is explanation is not likely applicable to condylomas with only episomal HPV genomes.

> *3, "Existence of still unidentified high-risk HPV types, undetectable with current general primer PCRs, cannot be excluded".*

Since 2010, only two alpha types have been characterized (HPV160 and HPV177), compared to 12 beta types and 42 gamma types. The use of metagenomic sequencing allows us to circumvent the PCR-amplifcation steps to unbiasely detect all DNA present in a sample, including unknown HPV types. Metagenomic sequencing of the vaginal microbiome of HIV positive women detected the complete genomes of two novel putatively HPV types which were most closely related to HPV101, 103 and 108; gamma types first identified in cervical samples, but not any novel HPV types of the alpha genus (45). Thus, it is not very likely that there are many unidentified oncogenic HPV types left to be discovered.

> *4, "Although general primer-based PCR methods detect a broad spectrum of HPV types, some genotypes show a slightly less efficient amplification".*

Such HPV types should theoretically be discovered by metagenomic sequencing. Some samples may also have a viral load under the detection level of the assay.

HPV and cutaneous infections

Non-melanoma skin cancer (NMSC) and other skin lesions

Two of the most common cancers among Caucasian populations are the non-melanoma skin cancers (NMSCs) basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC is approximately four times as common as SCC (161). Malign melanoma is a skin cancer that arise from melanocytes of the skin, it is an aggressive tumor that are likely to metastasize (162). In Sweden 2011, there were 3323 cases of malignant melanomas and 486 deaths reported. SCC and BCC were more common with 5775 and 39 835 cases, respectively, but with fewer deaths reported; 80 following SCC and no deaths following BCC. BCC was not reported in Sweden until 2004 (Skincancer, Swedish Cancer Foundation report, published in 2013).

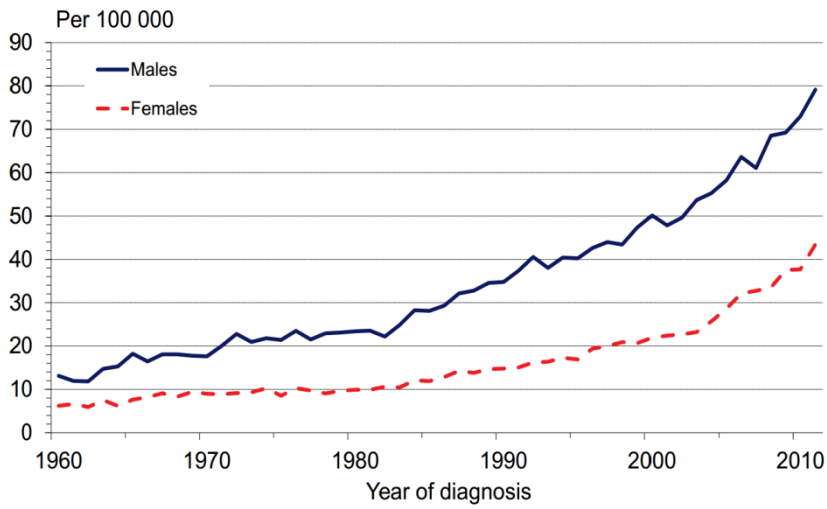

Figure 5. Incidence of non-melanoma skin cancer per 100 000 persons. Adapted from Cancer incidence in Sweden, 2011. Published by Socialstyrelsen.

SCC of the skin is one of the most rapidly increasing malignant tumors in Sweden over the last decade. An average increase of 4.7% per year for men and 6.9% per year for women has been seen the last 10 years (Cancer incidence in Sweden 2012, published by Socialstyrelsen).

BCC and SCC of the skin are both slow-growing tumors (163-165). BCC is locally invasive, but reports of metastatic BCCs are rare (about 1/4000 cases) (166). However, in the cases where BCC do metastasize, the morbidity and mortality rates are high (167). Metastatic SCC is more common and occurs in 3-10% of cases

(166). Tumors that metastasize are usually larger than non-metastasizing tumors (168).

The most well known risk factor for both BCC and SCC of the skin is ultraviolet light (163, 165). NMSC are most prevalent on sun-exposed parts of the body. For SCC, long term exposure to UV-radiation seems to be responsible for progression, for BCC it is short term burns (162, 169). Other risk factors for NMSC are older age, male sex, fair skin that tans poorly, red, blond or light-brown hair, blue eyes or light colored eyes and inherited skin conditions (166)).

Actinic keratosis (AK) is considered to be a precursor lesion for SCC of the skin (170, 171). AKs are confined to the epidermis and do not have metastatic potential (172).

Keratoachantoma (KA) is a benign skin tumor that is characterized by a rapid onset followed by spontaneous regression within a few months, usually within a year (173). KA strongly resembles SCC, it is debated whether KA is a benign variant of SCC or a separate unit. KAs are commonly treated as an SCC as a precaution (174, 175).

NMSC can cause disfigurement and the tumors can become painful, infected, inflamed and necrotic. As these lesions commonly are found in the face, they can cause functional impairment of the eye, eyelids and tear-ducts but also esthetical disfigurements and deformities ((166). The costs of treatment are high. Course of treatment of NMSCs are for example cryosurgery where the tumor is frozen by liquid nitrogen, lesion excision where the tumor is removed with different surgical techniques and radiation therapy which preserves the tissue (163, 164, 166).

HPV and healthy skin

HPV types of the beta-, and gammapapillomavirus genus are commonly detected on healthy skin (29, 30, 43, 176-178). Persistence, especially among HPV types of the genus beta has been seen in forehead swab samples (177) and in plucked eyebrow hair samples (179). HPV has also been detected in hair follicles (81) and in endocrinal ducts of the skin (180).

HPV and non-melanoma skin cancer

The link between HPV and NMSC was first observed among patients with the very rare hereditary immunosuppressive disease Epidermodysplasia Verruciformis (EV) (16). Patients with EV have a high risk of skin cancer and presents eruptions of

wart-like lesions typically caused by HPV types 5 and 8 (181). These wart-like lesions often progress to SCC at sun-exposed sites of the skin (182).

HPV5 and 8 belong to the betapapillomavirus genus originally called EV types. The E6 protein of HPV5, 8 and 38 has shown that it might contribute to UV-induced carcinogenesis by inhibiting DNA repair mechanisms (183-188). E6 of betapapillomavirus genus cannot degrade p53, however, HPV38 E6 can reduce the p53 signaling by inducing expression of a dominant negative inhibitor of p53; deltaNp73 (185). The p53 inhibition drives the cell through the cell cycle despite presence of thymidine dimers caused by UV-exposure. The continuation of the cell cycle may cause double-stranded DNA breaks and promote tumor formation. The degradation of pro-apoptotic Bak by beta-HPV E6 may help the HPV infected cells to avoid apoptosis in response to UV damage and high levels of double-stranded DNA breaks (183, 189).

Prevalence of cancers such as cervical cancer, NMSC, Hodgin's lymphoma and liver cancer are increased in patients receiving immunosuppressive therapy (190, 191). With the exception of NMSC, cervical cancer, Hodgkin's lymphoma and liver cancer are known to be caused by viruses. This suggests that the immunosuppression reduces the ability to control tumorigenic viruses. Among organ transplant recipients, NMSC is the most common post-transplant malignancy (161), about 65-250-fold increased risk for cutaneous SCC compared to the normal population (171, 191-195) and 10-fold increased risk of BCC of the skin (194).

HPV presence in NMSC of immunocompromised individuals can be up to 90%, particularly in SCC and benign AK lesions (196-199) (200). deVilliers et al., detected HPV DNA in 65% of AKs, 91% of SCC *in situ* and 91% of invasive SCC of renal transplant patients (196), whilst de Jong-Tieben et al., detected HPV-DNA in 80% of SCCs and in 93% of AKs (199). Studies that compared HPV prevalence in AK and SCC between immunosuppressed renal transplant patients and immunocompetent individuals showed HPV prevalence between 65-88% among immunocompromised, and between 27-54% among immunocompetent (200) (197, 198).

Cutaneous HPV types have been detected in several skin lesions among immunocompetent individuals, such as SCC (201-206), AK (204, 205) and KA (204, 206-209). However, the HPV types found have varied depending on which PCR-system that was used.

Skin biopsies of SCC, BCC, AK and seborrheic keratosis (SK) commonly contain multiple HPV types, but usually at very low viral loads (207, 210-214). HPV DNA of the betapapillomavirus genus has been associated with SCC (201, 202, 215, 216) and AK (217). However, the role of HPV in the development of NMSC among immunocompromised and immunocompetent individuals remains elusive.

A study by Forslund et. al,. showed that cleansing/stripping of the skin surface of the lesion with tape removed most of the HPV positivity without detectably altering the skin architecture (218), suggesting that many HPV DNA positive samples may reflect skin surface contamination and not an established infection. The HPV prevalence was 69% in swabs from top of the lesions of SCC, BCC and AK. After cleansing the lesions with tape, the HPV prevalence in the corresponding biopsies was only 12% (218).

Serological studies have shown an increased association for cutaneous SCC among subjects seropositive for antibodies to HPV types of the betapapillomavirus genus (219-222). Serological studies are thought to reflect past or present infection with HPV, how much of the viral capsid antigen that is presented to the immune system and the immune response of the host (223). Most serology studies have focused on HPV types of the genus beta; however, some studies have also included HPV types of the genus gamma. Significant risk of SCC was observed for HPV50 (gamma) (219), but increased risk for HPV types of the genus gamma have not been detected in other studies (222, 223).

Transcriptome sequencing of a series of HPV DNA-positive skin cancers did not find viral RNA expression, and state that HPV mRNA expression is not a factor in the maintenance of SCC of the skin (224).

Summary of papers

Aims

The purpose of the studies upon which this thesis is based on was to:

- Search for DNA virus among cutaneous squamous cell carcinoma, actinic keratosis and keratoacanthoma using metagenomic sequencing.

- Define the baseline distribution of HPV types in condylomas before introduction of vaccination program.

- Analyze "HPV-negative" condylomas using broad-spectrum PCR methods and metagenomic sequencing.

- Amplify, clone and characterize novel HPV types.

- Determine sensitivity and specificity of the APTIMA HPV mRNA assay for prediction of future development of high-grade cervical intraepithelial neoplasia among HR-HPV DNA positive women with ASCUS or CIN1 cytology.

Material and methods

Study material

Skin samples, paper I and IV

Four different patient series was used:

1. Formalin-fixed paraffin-embedded (FFPE) biopsies of SCC of the skin (n=28) and KA (n=72) from the department of Pathology at the Malmö hospital, Sweden. The biopsies were sectioned and de-paraffinized using xylene. The DNA was extracted by a phenol free-method (209).

2. Fresh frozen KA biopsies (n=92) from the department of Dermatology and Plastic surgery at the Norwegian National Hospital, Oslo, Norway. The DNA was extracted using the QIAamp DNA Minikit (Qiagen).

3. SCCs (n=85), AKs (n=92), BCCs (n=118) and SKs (n=46) collected for a hospital-based study in Sweden and Austria. All patients provided four different samples; a swab sample from top of the lesion, a swab sample from healthy skin, a biopsy from the lesion and a biopsy of healthy skin. The swabs were collected by a pre-wetted (0.9% NaCl) cotton-tipped swab that was rolled on the lesion or healthy skin. Biopsies were taken after cleaning the skin surface with tape to remove possible surface contamination. The DNA in the biopsies was extracted using a phenol free-method (31).

4. SCCs (n=35), AKs (n=22), BCCs (n=3), SK (n=1) and KAs (n=8) were collected for a Swedish hospital-based study. All patients donated swab samples and biopsies as described above.

Condyloma samples, paper II, III and IV

The condyloma swab samples were collected from 703 patients visiting the Centre for Sexual Health in Malmö, Sweden between 2006 and 2009. Four samples were also collected in Denmark. A sterile cervical cytobrush was dipped in sterile saline and brushed on the condyloma without prior cleaning. The brush was stirred in sterile saline and the cells pelleted. The cell-suspension was DNA extracted with the Magna Pure LC using the Total Nucleic Acid Kit (Roche).

Cervical samples, paper V

Liquid-based cytology (LBC) samples of minor abnormalities (ASCUS and CIN1) were identified in the Cytology and Pathology registries of the South Swedish Regional Cancer Centre. The LBC samples were collected in SurePath (BD) vials

between January of 2009 and December of 2010 within the national cervical screening program in Malmö, Sweden. The samples were frozen as pellets at -80°C and stored in the Malmö LBC biobank.

Methods

HPV DNA detection

Broad-spectrum PCR, paper II

HPV DNA have been amplified using broad spectrum PCR primer sets for amplification of mucosal HPV types; MGP-PCR followed by amplicon detection by Luminex (149, 150). The Luminex beads bind to the HPV-PCR products via type specific probes and recognize mucosal HPV types (high-risk for cancer types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68a and b, 73, and 82; probable high-risk for cancer type 66; low-risk for cancer types 6, 11, 42, 43, and 70). The Luminex probes of HPV26, 30, 40, 54, 67, 81, 89, 90, and 91 were added in February of 2008. The FAP59/64 primer pair was used for amplification of mucosal and cutaneous HPV types (31).

Real-time PCR, paper I

Specific HPV DNA sequences were amplified using real-time PCR (quantitative PCR; qPCR) using dual labeled probes (florescence and quencher).

Long range PCR, paper I, II and IV

Complete genomes of novel HPV types were amplified using either the High Fidelity kit (Roche) or the PrimeSTAR GXL DNA polymerase kit (TaKaRa).

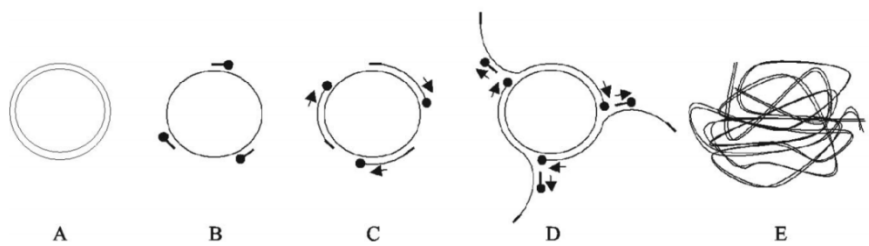

Figure 6. Overview of the whole genome amplification with focus on double stranded DNA (A). Random primers bind to the single stranded DNA and the amplification starts (B and C). When the polymerase reaches a downstream primer, strand displacement occurs and new primers can anneal to the displaced product (D). The end product is double stranded repeated copies of the DNA in the sample. Adapted from Rector el al., A sequence-independent strategy for detection and cloning of circular DNA virus genomes using multiply primed rolling-circle amplification, in Journal of Virology, 2004; 78:4993-4998, with permission from American Society for Microbiology.

Whole genome amplification, paper I, II, III and IV

Whole genome amplification (WGA) is also called multiple displacement amplification (Figure 6). It is based on rolling circle amplification (225) used to amplify circular DNA (226) but can also amplify linear DNA (227, 228). It was

developed to amplify all DNA in a sample, even for small amounts of starting materials, using random hexamer primers; however, the DNA has to be of good quality. The DNA polymerase used originates from bacteriophage phi29 (229, 230).

Metagenomic sequencing, paper I and III
Metagenomic sequencing, also called high-throughput sequencing or deep-sequecing, can be used to obtain an unbiased and comprehensive map of all DNA present in a sample without any prior amplification that requires information about the sequences that might be present.

The Genome Sequencer (GS) FLX and the GS Junior (Roche) are based on emulsion amplification and pyrosequencing. The genomic dsDNA is isolated and fragmented by nebulization where high-pressure nitrogen gas forces the sample into small droplets which shears the DNA into pieces (50-900 base pairs). The fragmented DNA is blunt-ended by removing overhanging 3' ends. The DNA is ligated between two adaptors. One of the adaptors is biotinylated which facilitates capture of these molecules to streptavidin-coated magnetic beads. Products without biotin will are washed away to isolate single stranded fragments (one for each bead). The beads are captured within droplets of emulsion oil that contains a PCR mix. The PCR reaction occurs within each droplet. After thermocycling, every bead will carry millions of copies of unique DNA. The emulsion is broken, and the beads are transferred onto a PicoTiterPlate (231). One bead will be deposited in each well of the plate. In each well, the DNA is sequenced using pyrosequencing (232). As the template is immobilized, solutions of A, T, G and C nucleotides can be added and removed from the reaction. The method is based on detection of the DNA polymerase using a chemiluminescent enzyme. Light is only produced when the added nucleotide complements the first unpaired base of the template.

The Ion Torrent sequencer (Life Technologies) does not use pyrosequencing. Instead, beads carrying single stranded DNA are sorted into wells and washed by A, T, G or C nucleotides. If the nucleotide is complementary, it will be incorporated and a hydrogen ion will be released. The change in pH are detected in each well and converted to base calls. No modified nucleotides or optics are used.

HPV mRNA detection

APTIMA HPV mRNA assay, paper V
The APTIMA HPV mRNA assay (Hologic) detects, but cannot distinguish between, E6/E7 mRNA of HPV16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68 in cervical cytology specimens.

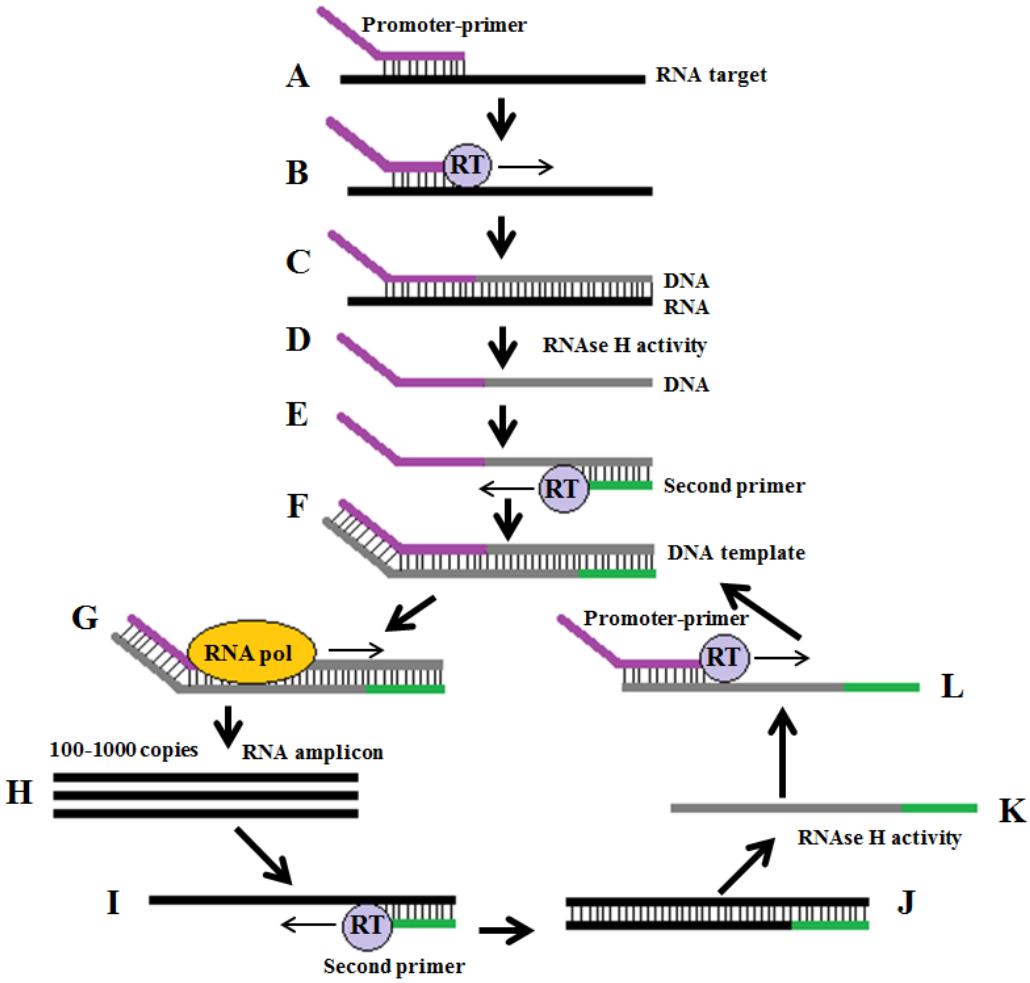

Figure 7. Schematic presentation of the transcription-mediated amplification (TMA) utilized in the APTIMA HPV mRNA assay. The reverse transcriptase (RT) creates a DNA copy of the RNA (A-C), and the RNA in the RNA-DNA hybrid is degraded by RNAse H (C-E). A second primer is added and amplification is performed by the RNA polymerase and the reverse transcriptase (E-L).

According to APTIMA instructions, SurePath specimens should be pre-treated with proteinase K included in the APTIMA Transfer Solution (ATS) kit. In the present study, 100 µL of the stored sample was used, instead of 1 mL SurePath specimen. The used 100 µL corresponds to about 1/4 of the original 1 mL SurePath sample. Briefly, 100 µL of the sample and 300 µL of the ATS were added to an APTIMA Specimen Transfer Tube (pre-filled with 2.9 mL buffered saline solution) and heated at 90°C for 15 min in a water bath.

During the automated assay, E6/E7 mRNA is captured on magnetic microparticles and amplified using transcription mediated amplification (TMA) (Figure 7). TMA is an amplification method that utilizes RNA polymerase, reverse transcriptase and

RNAse H activity to amplify RNA and DNA targets without thermal cycling. The amplicons are detected during the hybridization protection assay where light emissions are captured and measured. HPV amplicons coupled to labeled probes emits a glowing light that are detected.

Paper I

Metagenomic sequencing of cutaneous samples

Sequencing was performed on biopsies and swabs from top of the lesions of patients with SCC, AK or KA (as described on page 35). Samples were pooled (2-4 µL) in pools of 6-23 patients samples in each according to diagnosis and sample type and the pools were prepared using three different methods;

1. DNA in the size range from ~3-10 kb was extracted from an E-gel (Life Technologies) and the DNA was subjected to WGA (GenomiPhi High Yeild, GE Healthcare Life Science).

2. Before DNA extraction of the swab samples the viral capsids were separated from human DNA using ultracentrifugation. The DNA was extracted with the MagNA Pure LC using the Total Nucleic Acid kit (Roche) prior to WGA.

3. WGA only.

After WGA, the pre-treated smaller pools were pooled into seven larger pools according to diagnosis and sample type; I) 28 SCC (FFPE biopsies), II) 72 KA (FFPE biopsies), III) 92 KA (fresh frozen biopsies), IV) 82 SCC and 60 AK (swabs from top of the lesions), V) 41 SCC (fresh frozen biopsies), previously HPV-negative (216), VI) 44 SCC (fresh frozen biopsies), previously HPV-positive (216) and VII) 92 AK (fresh frozen biopsies).

The pools were sequenced using the 454 GS FLX Titanium (Roche). The pool of top of the lesion swabs of SCCs and AKs (number 4) that were only amplified by WGA were also sequenced using the Ion Torrent PGM sequencer (Life Technologies). Two different Ion Torrent PGM kits were used; one with 300 nucleotide (nt) read lengths and one with 400 nt.

The prevalence of HPV155 (SE42) and SE46 were investigated using real-time PCR on samples from 341 patients with SCC, AK, BCC, SK, and KA by the use of biopsies and swab samples from lesions and from healthy skin. HPV155 (SE42) were amplified and cloned from a swab from top of a lesion diagnosed as AK.

Paper II

HPV in condyloma

Condyloma surface swabs was tested for sample adequacy by assaying for the human betaglobin gene, 621 samples (376 males and 245 females) remained adequate. These samples were analyzed for HPV-DNA by amplification with the GP5+/6+ primers (39) (until April 15, 2008) and MGP primers (149) (from April 17, 2008). The PCR was followed by the bead based Luminex system (150) for identification of specific HPV genotypes.

A subset of samples was HPV negative but betaglobin positive. These samples were subjected to FAP-PCR (31). The FAP-PCR products were visualized on a gel and Sanger-sequenced. Samples still negative for HPV were amplified by Templiphi rolling circle amplification (GE Healthcare Life Science) (unbiased whole genome amplification, WGA) (233) followed by FAP-PCR, GP5+/6+ PCR or MGP-PCR and Luminex.

Paper III

"HPV-negative" condylomas analyzed by metagenomic sequencing

Forty samples of MGP-PCR "HPV-negative" condylomas (paper II) were pooled in 10 pools with four samples in each pool. The samples represent 21 woman and 19 men. The DNA in the pools was amplified using Genomiphi High Yield kit (GE Healthcare Life Science) (unbiased whole genome amplification, WGA). The pools were sequenced with a GS Junior. Three pools contained sequences of novel putative HPVs (denoted as SE-sequences). These samples were also sequenced individually by the GS Junior after whole genome amplification.

Paper IV

Characterization of three novel HPV types

In paper III we characterized the complete genomes of two novel putative HPV types from one single condyloma swab sample, SE87-complete and FA69-complete. The DNA in the sample was amplified using Genomiphi High Yeild kit (GE Healthcare Life Science) (unbiased whole genome amplification, WGA) prior to type-specific PCR. SE87 was amplified in three segments and FA69 as one entire fragment using the PrimeSTAR GXL DNA polymerase kit (TaKaRa, Japan). Using this method we also amplified the complete genome of novel a HPV type from a skin swab next to an AK.

Paper V

High-risk HPV mRNA among HR-HPV DNA positive minor cytological abnormalities

The study population was composed of high-risk HPV positive women diagnosed with ASCUS (n= 211) or CIN 1 (n= 131) with documented histological follow-up diagnosis.

We utilized the cytology and pathology registries of the South Swedish Regional Cancer Centre to identify index LBC samples of women aged 35 years or more, with cytological diagnoses of ASCUS or CIN1 and positive for a high-risk HPV type. The presence of high-risk HPV-DNA in the samples had previously been performed using MGP-PCR and Luminex at the Microbiology department of the Skane University Hospital in Malmö, Sweden.

Cervical LBC samples were included if they were DNA-positive for any HPV type covered by the APTIMA HPV assay. The APTIMA HPV assay detects, but cannot distinguish between, E6/E7 mRNA of 14 high-risk HPV types. The APTIMA HPV assay is known to cross-react with HPV types 26, 67, 70 and 82. Samples with these HPV types, except HPV70 were included in this study. Women were excluded if they did not have any subsequent histology registered within 4.5 years after the index cytology test. The most severe diagnosis at follow-up was recorded.
Except for some minor modifications (described on page 35) the APTIMA HPV assay was performed according to the manufacturer's instructions using the PANTHER platform.

Results and discussion

Paper I

Metagenomic sequencing of cutaneous samples

Samples prepared by E-gel or ultracentrifugation, only produced 124 viral sequence-reads, whilst most viral reads, 645, were detected in samples subjected to WGA only. The extra handling by E-gel and ultracentrifugation may have resulted in loss of material, compared to WGA only. Sequencing using the GS FLX (Roche) was performed on seven pools. Combination of the reads from the biopsies and the swabs, the GS FLX identified 662 reads, classified as related to HPV. Most reads were found in the pool of FFPE-KA biopsies with three different HPV related sequences, and in the pool of top-of-the-lesion swabs of SCCs and AKs with seven different HPV related sequences (250 and 387 reads, respectively). The Ion Torrent PGM was used to sequence the pools of swab samples from 82 SCCs and 60 AKs only pre-treated with WGA and identified 2744 and 762 HPV related reads using the 300 and 400 nt sequencing kits, respectively. We did not detect any HPV-related reads in the pool of HPV-positive fresh frozen SCC biopsies. These samples had previously been HPV-positive using FAP-PCR (216) which is very sensitive and can detect low levels of viral copies. Compared to our present study, other studies using FAP-PCR for HPV-DNA enrichment prior to metagenomic sequencing showed that FAP-PCR can detect low-levels of viral copies in large pools (204, 206, 234).

In total, sequences from 15 known HPV types and four previously described putative HPV types were detected. The latter include SE42, an already described subgenomic sequence originally detected using pre-amplification by FAP-PCR followed by metagenomic sequencing (204). In our present study, the GS FLX assembled two contigs of the SE42 genome, 763 and 6552 bp. The Ion Torrent PGM sequencing detected the complete genome of SE42. The complete genome of SE42 were amplified and cloned from a top of the lesion swab sample, from a patient with AK, and designated as HPV155 (7352 bp, gammapapillomavirus) (GenBank JF906559).

Two additional sequences related to HPV were also detected; SE46 and SE47. For SE46, we detected 22 reads using the GS FLX and 176 reads using the Ion Torrent. These sequences assembled into two contigs of 950 bp and 3774 bp. Only two sequence reads were detected of SE47 by the GS FLX, and none by the Ion Torrent. We were unable to amplify the complete genomes of SE46 or SE47 by type-specific primers.

Additional non-HPV-related sequences detected were human herpesvirus 8 (1 read), Epstein-Barr virus (5 reads), human endogenous retrovirus (10 reads), torque

teno virus (68 reads), human polyomavirus 6 (6 reads) and merkel cell polyomavirus (26 reads). Identification of these viruses demonstrates the broad detection capability of metagenomic sequencing.

We also performed a screening for HPV155 and SE46 in samples from 341 patients (biopsies of lesions, biopsies of healthy skin, swab samples of lesions, and swab samples of healthy skin) with cutaneous lesions (SCC, AK, BCC, SK, and KA) from patient-groups 3 and 4 (described on page 35) using real-time PCR.

Table 1. Detection and viral loads of HPV155 and SE46 in individual samples. Errata of table 3 in paper I.

		Viral copies/cell		**Viral copies/2.5µL sample**	
Diagnosis	**No. of HPV155 positive patients**	**Biopsy of the lesion**	**Biopsy of health skin**	**Swab from top of the lesion**	**Swab from healthy skin**
SCC (n=89)	3	- - -	$2.7x10^{-5}$ 3.8x10-4 -	26.5 10251 -	160.5 7235.5 0.5
AK (n=77)	2	1.8x-4 -	- -	6555a -	7 23
BCC (n=119)	2	- 4.8x01-5	- -	40 -	112 2.5
SK (n=48) & KA (n=8)	0	-	-	-	-
	No. of SE46 positive patients	**Biopsy of the lesion**	**Biopsy of health skin**	**Swab from top of the lesion**	**Swab from healthy skin**
SCC (n=89)	3	- - -	3.5x01-4 - -	11 17 -	16.5 30 2.5
AK (n=76)	3	- - -	- - -	- 13.5 4.5	11.5 110 23.5
BCC (n=121) & SK (n=47)	0	-	-	-	-
KA (n=8)	1	-	-	23.5	62

HPV155 was detected in 2% (7 of 341) patients with; SCC (n=3 of 89), AK (n=2 of 77) or BCC (n=2 of 119). HPV155 were detected in a biopsy of AK and BCC with a viral copy per cell of $1.8x10^{-4}$ and $4.8x10^{-5}$, respectively. HPV155 was also

detected in the swab of healthy skin of all seven patients with 0.5 to 7235 copies/2.5 µl template. SE46 was detected in 2% (7 of 341) patients with; SCC (n=3 of 89), AK (n=3 of 76) or KA (n=1 of 8). SE46 was not detected in any biopsies of healthy skin and only in one biopsy of healthy skin ($3.5x10^{-4}$ per cell). Viral copies per cell were determined by analyzing the human DNA with real-time PCR using primers and probe for the betaglobin gene (described in paper II). SE46 was detected in the swabs from healthy skin in all seven patents with 2.5 to 110 copies/2.5 µl template (Table 1). The viral load (viral copy per cell) could not be determined for the swab samples as some were negative for the betaglobin gene.

Conclusively, most of the viral DNA found represents known HPV types or known sub-genomic sequences of HPV. Metagenomic sequencing of skin lesions was useful for an unbiased assessment of viral DNA in these lesions.

Paper II

HPV in condyloma

Of the 621 condyloma swab samples, 96.3% were positive for HPV DNA and 93.9% positive for genital HPV types. The top five most common HPV types detected were HPV6 (61.7%), HPV16 (12.9%), HPV11 (10.3%), HPV42 (7.2%), and HPV66 (6.4%). Multiple infections were detected in 31.6% of all samples. HPV6 and 11 were detected in 71% of the condylomas, similar rates have been reported by others (106, 107). Simultaneous detection of both HPV6 and HPV11 were only found in six cases (0.01%). Low-risk types were detected in 76.3% of all condylomas but were slightly less common in women (71.8%) than in men (79.3%). High-risk types (with HPV16 being the most common with a prevalence of 12.9%) were detected in 34.9% of the samples and were more common among women (45.3%) than men (28.2%). Overall, 36 different HPV types of the alphapapillomavirus genus were detected.

After the initial routine investigation by MGP-PCR, 50 samples were HPV-negative. After extended analysis FAP-PCR and MGP-PCR we could conclude that 12 samples were indeed positive for HPV types that were not detected by the routine analysis. The most probable reason for this might be low viral loads in the samples. The most common genital type was HPV6 which were detected in seven of twelve samples. These additional HPV-positive samples were included in the baseline above. By the use of FAP-PCR we found that 21 samples were positive for HPV types or putative HPV types from the beta- and gammapapillomavirus genera. The putative HPV types represent 14 FA-isolates (generated by the FAP-primers). For one of these FA-isolates, the complete genome was amplified, cloned, and were assigned as HPV153 (GenBank JN171845) which belongs to the gammapapillomavirus genus,) and has a genomic size of 7240 bp.

This condyloma monitoring system has provided the largest, so far, reported condyloma series with comprehensive HPV typing before HPV vaccination introduction. A condyloma monitoring system will provide rapid feedback of the effectiveness of the vaccination-programs as it is a readily identifiable condition with short incubation time. A large spectrum of 36 genital HPV types were identified, which implies that condyloma monitoring systems will require methods capable to detect a broad range of HPV types in follow-up studies.

The HPV types of the beta- and gammapapillomaviruses, including HPV153, most probably represent HPV types residing in superficial layers of the skin or from healthy adjacent skin. This are possibly also true for all the HPV types from the genus alpha, apart from HPV6 and 11 who are known to cause condylomas (7, 8).

Paper III

"HPV-negative" condylomas analyzed by metagenomic sequencing

Combining the sequencing of the pools and the individual samples, 2207 reads of a total of 104,740 (2.1%) was related to viruses known to infect humans. Whole genome amplification can introduce errors in the sequence such as chimeras or genome rearrangements. We did indeed detect chimeric sequences (inverted segments), both automatically and manually. They were removed with the result that only 63% (1385/2207) of the sequences related to human viruses remained (with a read length of about 89-700 nt).

Of the ten pools, five pools remained HPV negative. As the samples did contain DNA, this could be due to misclassification of the clinical diagnosis, the lesion could truly be HPV negative, or the viral copy number might be below the detection limit of the assay. In one of the HPV negative pools we only detected reads of the Molluscum contagiosum virus subtype 1. We also detected Molluscum Contagiosum virus (only) in two of the individually sequenced samples originating from the same pool. Simultaneous condylomas and Mollusca have been observed (159). Condylomas are diagnosed using visual inspection and in a mix of condylomas and mollusca, all wart-like lesions might be classified as genital warts.

Five pools were HPV positive, three of which contained sequences of novel putative HPVs. These samples were sequenced individually. In total, 1337 reads were detected; 273 reads from the pools and 1064 reads from the individual samples. HPV6 were detected in two pools and two single samples. HPV6, together with reads of HPV58 which were detected in one individual sample and one read of HPV66, indicates that HPV can be missed by MGP-PCR and Luminex. Possible reasons why the known HPV types in those samples had not previously been detected by the initial general primer PCR include presence of viral variants with genomic alterations in the sequences targeted by primers and Luminex-probes, or

the viral loads were below the detection limits of the MGP-PCR Luminex assay. Indeed, HPV58 and HPV66 can be found in condylomas (106). In paper II, HPV58 in and HPV66 was detected in 2.1% and 6.4% of the samples, respectively.

We also detected reads of other HPV types and known HPV isolates belonging to the beta- and gammapapillomavirus genus, among them FA69, a FAP-PCR sub-fragment originally detected in a swab sample from a healthy forehead (235). We detected the complete genome of FA69 (GenBank KC108722, later named HPV180) that were assembled by 858 reads from one single sample (30 year old male). The size was 7356 bp and it belongs to the gammapapillomavirus genus. Eleven percent of the HPV related reads (145/1337) belonged to sub-genomic fragments of novel putative HPV types (SE-sequences). We detected 23 SE-sequences/contigs that belong to the genus gamma. Some may represent the same HPV types as the sequence fragments were scattered through the genomes. The entire genome of SE87 (gammapapillomavirus genus) was detected (GenBank KC108721, later named HPV175), with a genome of 7226 bp and was assembled by 80 reads detected in a single sample.

Overall, the analysis revealed that seemingly "HPV-negative" condylomas may contain known and previously unknown HPV types. The novel HPV related sequences detected are probably not causing the condyloma.

Characterization of three novel HPV types

HPV175, HPV178, and HPV180 all demonstrate a typical genome organization of cutaneous HPVs. The complete genomic sequences of HPV175 and HPV180 were identified using metagenomic sequencing (paper III) in a condyloma swab sample from a 30-year old male. HPV178 was isolated from a swab of healthy skin, next to an actinic keratosis of an 86-year old male, and discovered after an attempt to amplify the closely related HPV197. The complete genomic sequences are available in GenBank under these accession numbers: HPV175 [KC108721], HPV178 [KJ130020], and HPV180 [KC108722].

Paper V

High-risk HPV mRNA among HR-HPV DNA positive minor cytological abnormalities

The overall HPV mRNA prevalence among the ASCUS and CIN1 index samples was 92.1% (315/342), with 90% (190/211) and 95.4% (125/131) among those with ASCUS and CIN1, respectively. Twenty-nine percent (61/211) of women in the ASCUS group, and 34.3% (45/131) in the CIN1 group developed CIN2+ within 4.5 years of follow-up. The HPV mRNA test showed similar sensitivity for future CIN2+ and CIN3 in the ASCUS (96.7% and 100%) and CIN1 (97.8% and 100%) groups. However, the corresponding specificity was very low (5.4-12.7%).

Presence of HPV E6/E7 mRNA was associated with future development of CIN2+ among women with ASCUS and CIN1 (p=0.03). The absence of HPV mRNA demonstrated a tendency for protection against future development of CIN3 among women with ASCUS or CIN1. None of the 27 mRNA-negative but HPV-DNA positive women developed CIN3+ as compared to 43/315 of mRNA-positive women (13.6%) (p=0.06).

The specificity was low (5.4-12.7%), due to the large group of HPV mRNA positive women without progressive disease (over 60% of both groups). Almost half of the women in the ASCUS group and the CIN1 group were treated by conization or hysterectomy within the follow up time. Therefore, we cannot be sure that the treated women would develop a more severe diagnosis if left un-treated.

Other recent similar studies have shown lower sensitivity (mean 89.5%, range 77.8-100) but higher specificity (mean 47.8%, range 25-78) for development of future CIN2+ compared to our study (236-238).

Clear markers for progression are still needed to detect women at risk for development of high-grade lesions.

Novel HPV types characterized

Table 2. Novel HPV types within the genus gamma characterized within this thesis.

Genes	**E6**	**E7**	**E1**	**E2**	**E4**	**L2**	**L1**	**URR**
HPV153 (7420 bp) 71% similarity to HPV128 (L1)								
No. of nt**	429	288	1815	1200	432	1533	1533	504
No. of aa	143	96	605	400	144	511	511	
HPV155 (7352 bp) 77% similarity to HPV139 (L1)								
No. of nt	435	282	1818	1215	492	1554	1542	573
No. of aa	145	94	606	405	164	518	514	
HPV175 (7226 bp) 69% similarity to HPV60 (L1)								
No. of nt	441	291	1821	1209	498	1539	1512	485
No. of aa	147	97	607	403	166	513	504	
HPV178 (7314 bp) 68% similarity to HPV65 (L1)								
No. of nt	414	285	1824	1203	498	1593	1533	546
No. of aa	138	95	608	401	166	531	511	
HPV180 (7356 bp) 82& similarity to HPV121 (L1)								
No. of nt	429	294	1827	1170	375	1572	1557	545
No. of aa	143	98	609	390	125*	524	519	

*without start codon ATG. **including stop codon.

Table 3. The origin of isolation of the characterized types. Species information from hpvcenter.se.

HPV type	**GenBank accession**	**Previous name (when applicable)**	**Species**	**Index patient**			
				Lesion	*Type of sample*	*Age*	*Sex*
HPV153	JN171845	-	γ-13	Condyloma	Swab	27	Male
HPV155	JF906559	SE42	γ-7	Actinic keratosis	Swab	72	Female
HPV175	KC108721	SE87	γ-23	Condyloma	Swab*	30	Male
HPV178	KJ130020	-	γ-24	Skin next to SCC	Swab	86	Male
HPV180	KC108722	FA69	γ-10	Condyloma	Swab*	30	Male

* from the same sample.

Concluding remarks and future perspectives

We searched for unknown and known DNA virus among cutaneous squamous cell carcinoma, actinic keratosis and keratoacanthoma by the use of metagenomic sequencing. The majority of the detected viral DNA sequences represent known HPV types or known sub-genomic sequences of HPV of the beta- and gammapapillomaviruses.

We defined a baseline distribution of HPV types in condylomas before introduction of the HPV vaccination program. The diversity among the HPV types of the alphapapillomavirus genus was large, with 36 different types identified. The metagenomic sequencing of previously "HPV-negative" condylomas found that a substantial proportion of these did contain viral DNA and that a wide variety of different HPV types and novel putative HPV types of the beta- and gammapapillomavirus genus are present in swab samples of genital warts. The novel HPV types detected are probably not causative of the condylomas.

The number of HPV types has expanded from 60 types in the year 1989 to 194 types today, where the latest major increase has been among the gammapapillomavirus genus. In accordance, from our studies above, five novel HPV types of gammapapillomavirus genus were characterized. HPV153 and HPV178 were discovered using PCR, whilst HPV155, HPV175 and HPV180 were discovered using metagenomic sequencing. Metagenomic sequencing appears to be a useful unbiased approach for expanding our knowledge of the diversity of HPVs. Future studies should preferentially sequence individual samples for a deeper assessment of viral DNA in each sample.

We determined sensitivity and specificity of the APTIMA HPV mRNA assay for prediction of future development of high-grade cervical intraepithelial neoplasia among HR-HPV DNA positive women with ASCUS or CIN1 cytology. We found that 92% of HPV-DNA positive samples were positive for HPV mRNA and demonstrated high sensitivity but low specificity of the HPV mRNA assay to detect future high-grade CIN. The specificity for future high grade CIN was low (5.4-12.7%), due to the high HPV mRNA positivity rates among the women without progressive disease. However, Presence of HPV E6/E7 mRNA was associated with future development of CIN2+ among high-risk HPV DNA positive women with ASCUS and CIN1. The absence of HPV mRNA demonstrated a tendency for

protection against future development of CIN3 among women with ASCUS or CIN1As most of the HPV-DNA samples also were HPV mRNA positive, markers for progression are still needed to detect women at risk for development of high-grade lesions.

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References

1. **de Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, Plummer M.** 2012. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. Lancet Oncol **13:**607-615.
2. **IARC.** 1994. Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum **61:**1-241.
3. **IARC.** 1994. Hepatitis viruses. IARC Monogr Eval Carcinog Risks Hum **59:**1-255.
4. **Blumberg BS, Larouze B, London WT, Werner B, Hesser JE, Millman I, Saimot G, Payet M.** 1975. The relation of infection with the hepatitis B agent to primary hepatic carcinoma. Am J Pathol **81:**669-682.
5. **Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol **189:**12-19.
6. **Durst M, Gissmann L, Ikenberg H, zur Hausen H.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A **80:**3812-3815.
7. **Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM.** 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology **401:**70-79.
8. **Gissmann L, deVilliers EM, zur Hausen H.** 1982. Analysis of human genital warts (condylomata acuminata) and other genital tumors for human papillomavirus type 6 DNA. Int J Cancer **29:**143-146.
9. **Epstein MA, Henle G, Achong BG, Barr YM.** 1965. Morphological and Biological Studies on a Virus in Cultured Lymphoblasts from Burkitt's Lymphoma. J Exp Med **121:**761-770.
10. **Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865-1869.
11. **Proietti FA, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL.** 2005. Global epidemiology of HTLV-I infection and associated diseases. Oncogene **24:**6058-6068.
12. **Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V, Group WHOIAfRoCMW.** 2009. A review of human carcinogens--Part B: biological agents. Lancet Oncol **10:**321-322.
13. **IARC.** 2012. Biological agents. Volume 100 B. A review of human carcinogens. IARC Monogr Eval Carcinog Risks Hum **100:**1-441.
14. **Shope RE, Hurst EW.** 1933. Infectious Papillomatosis of Rabbits : With a Note on the Histopathology. J Exp Med **58:**607-624.

15. **Rous P, Beard JW.** 1935. The Progression to Carcinoma of Virus-Induced Rabbit Papillomas (Shope). J Exp Med **62:**523-548.
16. **Jablonska S, Dabrowski J, Jakubowicz K.** 1972. Epidermodysplasia verruciformis as a model in studies on the role of papovaviruses in oncogenesis. Cancer Res **32:**583-589.
17. **Gissmann L, Pfister H, Zur Hausen H.** 1977. Human papilloma viruses (HPV): characterization of four different isolates. Virology **76:**569-580.
18. **Gissmann L, zur Hausen H.** 1976. Human papilloma virus DNA: physical mapping and genetic heterogeneity. Proc Natl Acad Sci U S A **73:**1310-1313.
19. **Oriel JD, Almeida JD.** 1970. Demonstration of virus particles in human genital warts. Br J Vener Dis **46:**37-42.
20. **Gissmann L, zur Hausen H.** 1980. Partial characterization of viral DNA from human genital warts (Condylomata acuminata). Int J Cancer **25:**605-609.
21. **Rawls WE, Tompkins WA, Figueroa ME, Melnick JL.** 1968. Herpesvirus type 2: association with carcinoma of the cervix. Science **161:**1255-1256.
22. **zur Hausen H.** 1976. Condylomata acuminata and human genital cancer. Cancer Res **36:**794.
23. **zur Hausen H.** 1982. Human genital cancer: synergism between two virus infections or synergism between a virus infection and initiating events? Lancet **2:**1370-1372.
24. **de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H.** 2004. Classification of papillomaviruses. Virology **324:**17-27.
25. **de Villiers EM.** 2013. Cross-roads in the classification of papillomaviruses. Virology **445:**2-10.
26. **Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ, International Agency for Research on Cancer Multicenter Cervical Cancer Study G.** 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med **348:**518-527.
27. **Lörincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ.** 1992. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet Gynecol **79:**328-337.
28. **Bzhalava D, Guan P, Franceschi S, Dillner J, Clifford G.** 2013. A systematic review of the prevalence of mucosal and cutaneous human papillomavirus types. Virology **445:**224-231.
29. **Antonsson A, Erfurt C, Hazard K, Holmgren V, Simon M, Kataoka A, Hossain S, Hakangard C, Hansson BG.** 2003. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. J Gen Virol **84:**1881-1886.
30. **Ma Y, Madupu R, Karaoz U, Nossa CW, Yang L, Yooseph S, Yachimski PS, Brodie EL, Nelson KE, Pei Z.** 2014. Human papillomavirus community in healthy persons, defined by metagenomics analysis of human microbiome project shotgun sequencing data sets. J Virol **88:**4786-4797.
31. **Forslund O, Antonsson A, Nordin P, Stenquist B, Hansson BG.** 1999. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol **80:**2437-2443.
32. **Forslund O.** 2007. Genetic diversity of cutaneous human papillomaviruses. J Gen Virol **88:**2662-2669.

33. **Chouhy D, Gorosito M, Sanchez A, Serra EC, Bergero A, Fernandez Bussy R, Giri AA.** 2010. New generic primer system targeting mucosal/genital and cutaneous human papillomaviruses leads to the characterization of HPV 115, a novel Beta-papillomavirus species 3. Virology **397:**205-216.
34. **Berkhout RJ, Tieben LM, Smits HL, Bavinck JN, Vermeer BJ, ter Schegget J.** 1995. Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. J Clin Microbiol **33:**690-695.
35. **Danos O, Katinka M, Yaniv M.** 1982. Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae. Embo J **1:**231-236.
36. **Heilman CA, Law MF, Israel MA, Howley PM.** 1980. Cloning of human papilloma virus genomic DNAs and analysis of homologous polynucleotide sequences. J Virol **36:**395-407.
37. **Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. Embo J **3:**1151-1157.
38. **Snijders PJ, van den Brule AJ, Schrijnemakers HF, Snow G, Meijer CJ, Walboomers JM.** 1990. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol **71 (Pt 1):**173-181.
39. **de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ.** 1995. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol **76:**1057-1062.
40. **Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM.** 1989. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells **7:**209-214.
41. **Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ.** 2000. Improved amplification of genital human papillomaviruses. J Clin Microbiol **38:**357-361.
42. **Mokili JL, Dutilh BE, Lim YW, Schneider BS, Taylor T, Haynes MR, Metzgar D, Myers CA, Blair PJ, Nosrat B, Wolfe ND, Rohwer F.** 2013. Identification of a novel human papillomavirus by metagenomic analysis of samples from patients with febrile respiratory illness. PLoS One **8:**e58404.
43. **Foulongne V, Sauvage V, Hebert C, Dereure O, Cheval J, Gouilh MA, Pariente K, Segondy M, Burguiere A, Manuguerra JC, Caro V, Eloit M.** 2012. Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. PLoS One **7:**e38499.
44. **Phan TG, Vo NP, Aronen M, Jartti L, Jartti T, Delwart E.** 2013. Novel human gammapapillomavirus species in a nasal swab. Genome Announc **1:**e0002213.
45. **Ameur A, Meiring TL, Bunikis I, Haggqvist S, Lindau C, Lindberg JH, Gustavsson I, Mbulawa ZZ, Williamson AL, Gyllensten U.** 2014. Comprehensive profiling of the vaginal microbiome in HIV positive women using massive parallel semiconductor sequencing. Sci Rep **4:**4398.
46. **de Villiers EM.** 1989. Heterogeneity of the human papillomavirus group. J Virol **63:**4898-4903.

47. **Mohr IJ, Clark R, Sun S, Androphy EJ, MacPherson P, Botchan MR.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694-1699.
48. **Evan GI, Vousden KH.** 2001. Proliferation, cell cycle and apoptosis in cancer. Nature **411:**342-348.
49. **Jones DL, Thompson DA, Munger K.** 1997. Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to HPV type 16 E7-induced apoptosis. Virology **239:**97-107.
50. **Werness BA, Levine AJ, Howley PM.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248:**76-79.
51. **Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129-1136.
52. **Thomas M, Banks L.** 1998. Inhibition of Bak-induced apoptosis by HPV-18 E6. Oncogene **17:**2943-2954.
53. **Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ.** 1998. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature **396:**84-88.
54. **Klingelhutz AJ, Foster SA, McDougall JK.** 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature **380:**79-82.
55. **Chen Z, Schiffman M, Herrero R, Desalle R, Burk RD.** 2007. Human papillomavirus (HPV) types 101 and 103 isolated from cervicovaginal cells lack an E6 open reading frame (ORF) and are related to gamma-papillomaviruses. Virology **360:**447-453.
56. **Nobre RJ, Herraez-Hernandez E, Fei JW, Langbein L, Kaden S, Grone HJ, de Villiers EM.** 2009. E7 oncoprotein of novel human papillomavirus type 108 lacking the E6 gene induces dysplasia in organotypic keratinocyte cultures. J Virol **83:**2907-2916.
57. **Gonzalez SL, Stremlau M, He X, Basile JR, Munger K.** 2001. Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. J Virol **75:**7583-7591.
58. **Gage JR, Meyers C, Wettstein FO.** 1990. The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. J Virol **64:**723-730.
59. **Doorbar J.** 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) **110:**525-541.
60. **Chow LT, Broker TR.** 1994. Papillomavirus DNA replication. Intervirology **37:**150-158.
61. **Sedman J, Stenlund A.** 1998. The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities. J Virol **72:**6893-6897.
62. **Lin BY, Makhov AM, Griffith JD, Broker TR, Chow LT.** 2002. Chaperone proteins abrogate inhibition of the human papillomavirus (HPV) E1 replicative helicase by the HPV E2 protein. Mol Cell Biol **22:**6592-6604.
63. **Conger KL, Liu JS, Kuo SR, Chow LT, Wang TS.** 1999. Human papillomavirus DNA replication. Interactions between the viral E1 protein and two subunits of human dna polymerase alpha/primase. J Biol Chem **274:**2696-2705.

64. **Sedman T, Sedman J, Stenlund A.** 1997. Binding of the E1 and E2 proteins to the origin of replication of bovine papillomavirus. J Virol **71:**2887-2896.
65. **Liu JS, Kuo SR, Broker TR, Chow LT.** 1995. The functions of human papillomavirus type 11 E1, E2, and E2C proteins in cell-free DNA replication. J Biol Chem **270:**27283-27291.
66. **Lehman CW, Botchan MR.** 1998. Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. Proc Natl Acad Sci U S A **95:**4338-4343.
67. **Bernard BA, Bailly C, Lenoir MC, Darmon M, Thierry F, Yaniv M.** 1989. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J Virol **63:**4317-4324.
68. **Demeret C, Desaintes C, Yaniv M, Thierry F.** 1997. Different mechanisms contribute to the E2-mediated transcriptional repression of human papillomavirus type 18 viral oncogenes. J Virol **71:**9343-9349.
69. **Doorbar J, Ely S, Sterling J, McLean C, Crawford L.** 1991. Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature **352:**824-827.
70. **Schiller JT, Vass WC, Vousden KH, Lowy DR.** 1986. E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J Virol **57:**1-6.
71. **DiMaio D, Guralski D, Schiller JT.** 1986. Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity. Proc Natl Acad Sci U S A **83:**1797-1801.
72. **Ashrafi GH, Haghshenas M, Marchetti B, Campo MS.** 2006. E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. Int J Cancer **119:**2105-2112.
73. **Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien PM, Campo MS.** 2005. E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. Int J Cancer **113:**276-283.
74. **Garcia-Vallve S, Alonso A, Bravo IG.** 2005. Papillomaviruses: different genes have different histories. Trends Microbiol **13:**514-521.
75. **Buck CB, Cheng N, Thompson CD, Lowy DR, Steven AC, Schiller JT, Trus BL.** 2008. Arrangement of L2 within the papillomavirus capsid. J Virol **82:**5190-5197.
76. **Giroglou T, Florin L, Schafer F, Streeck RE, Sapp M.** 2001. Human papillomavirus infection requires cell surface heparan sulfate. J Virol **75:**1565-1570.
77. **Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM.** 2009. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proc Natl Acad Sci U S A **106:**20458-20463.
78. **Joyce JG, Tung JS, Przysiecki CT, Cook JC, Lehman ED, Sands JA, Jansen KU, Keller PM.** 1999. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. J Biol Chem **274:**5810-5822.
79. **Day PM, Lowy DR, Schiller JT.** 2008. Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. J Virol **82:**12565-12568.
80. **Lace MJ, Isacson C, Anson JR, Lorincz AT, Wilczynski SP, Haugen TH, Turek LP.** 2009. Upstream regulatory region alterations found in human papillomavirus type 16 (HPV-16) isolates from cervical carcinomas increase

transcription, ori function, and HPV immortalization capacity in culture. J Virol **83:**7457-7466.
81. **Weissenborn SJ, Neale R, de Koning MN, Waterboer T, Abeni D, Bouwes Bavinck JN, Wieland U, Pfister HJ, Group E-H-U-C.** 2009. Prevalence and multiplicity of cutaneous beta papilloma viruses in plucked hairs depend on cellular DNA input. J Virol Methods **161:**280-283.
82. **Stubenrauch F, Laimins LA.** 1999. Human papillomavirus life cycle: active and latent phases. Semin Cancer Biol **9:**379-386.
83. **Horvath CA, Boulet GA, Renoux VM, Delvenne PO, Bogers JP.** 2010. Mechanisms of cell entry by human papillomaviruses: an overview. Virol J **7:**11.
84. **Raff AB, Woodham AW, Raff LM, Skeate JG, Yan L, Da Silva DM, Schelhaas M, Kast WM.** 2013. The evolving field of human papillomavirus receptor research: a review of binding and entry. J Virol **87:**6062-6072.
85. **Sapp M, Bienkowska-Haba M.** 2009. Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. FEBS J **276:**7206-7216.
86. **Hoffmann R, Hirt B, Bechtold V, Beard P, Raj K.** 2006. Different modes of human papillomavirus DNA replication during maintenance. J Virol **80:**4431-4439.
87. **Stanley M.** 2010. Pathology and epidemiology of HPV infection in females. Gynecol Oncol **117:**S5-10.
88. **Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K.** 2004. Mechanisms of human papillomavirus-induced oncogenesis. J Virol **78:**11451-11460.
89. **Thorland EC, Myers SL, Gostout BS, Smith DI.** 2003. Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene **22:**1225-1237.
90. **Ziegert C, Wentzensen N, Vinokurova S, Kisseljov F, Einenkel J, Hoeckel M, von Knebel Doeberitz M.** 2003. A comprehensive analysis of HPV integration loci in anogenital lesions combining transcript and genome-based amplification techniques. Oncogene **22:**3977-3984.
91. **Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol **61:**962-971.
92. **Romanczuk H, Howley PM.** 1992. Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. Proc Natl Acad Sci U S A **89:**3159-3163.
93. **Jeon S, Lambert PF.** 1995. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc Natl Acad Sci U S A **92:**1654-1658.
94. **Jeon S, Allen-Hoffmann BL, Lambert PF.** 1995. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J Virol **69:**2989-2997.
95. **Kulmala SM, Syrjanen SM, Gyllensten UB, Shabalova IP, Petrovichev N, Tosi P, Syrjanen KJ, Johansson BC.** 2006. Early integration of high copy HPV16 detectable in women with normal and low grade cervical cytology and histology. J Clin Pathol **59:**513-517.

96. **Bruni L, Diaz M, Castellsague X, Ferrer E, Bosch FX, de Sanjose S.** 2010. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. J Infect Dis **202:**1789-1799.
97. **Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, Wheeler CM, Group A.** 2007. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. J Infect Dis **195:**1582-1589.
98. **Evander M, Edlund K, Gustafsson A, Jonsson M, Karlsson R, Rylander E, Wadell G.** 1995. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis **171:**1026-1030.
99. **Franceschi S, Herrero R, Clifford GM, Snijders PJ, Arslan A, Anh PT, Bosch FX, Ferreccio C, Hieu NT, Lazcano-Ponce E, Matos E, Molano M, Qiao YL, Rajkumar R, Ronco G, de Sanjose S, Shin HR, Sukvirach S, Thomas JO, Meijer CJ, Munoz N.** 2006. Variations in the age-specific curves of human papillomavirus prevalence in women worldwide. Int J Cancer **119:**2677-2684.
100. **Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE.** 2011. Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst **103:**368-383.
101. **Gross G, Pfister H.** 2004. Role of human papillomavirus in penile cancer, penile intraepithelial squamous cell neoplasias and in genital warts. Med Microbiol Immunol **193:**35-44.
102. **Ball SL, Winder DM, Vaughan K, Hanna N, Levy J, Sterling JC, Stanley MA, Goon PK.** 2011. Analyses of human papillomavirus genotypes and viral loads in anogenital warts. J Med Virol **83:**1345-1350.
103. **Brown DR, Schroeder JM, Bryan JT, Stoler MH, Fife KH.** 1999. Detection of multiple human papillomavirus types in Condylomata acuminata lesions from otherwise healthy and immunosuppressed patients. J Clin Microbiol **37:**3316-3322.
104. **Gissmann L, Wolnik L, Ikenberg H, Koldovsky U, Schnurch HG, zur Hausen H.** 1983. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Natl Acad Sci U S A **80:**560-563.
105. **Hawkins MG, Winder DM, Ball SL, Vaughan K, Sonnex C, Stanley MA, Sterling JC, Goon PK.** 2013. Detection of specific HPV subtypes responsible for the pathogenesis of condylomata acuminata. Virol J **10:**137.
106. **Aubin F, Pretet JL, Jacquard AC, Saunier M, Carcopino X, Jaroud F, Pradat P, Soubeyrand B, Leocmach Y, Mougin C, Riethmuller D, Group EDS.** 2008. Human papillomavirus genotype distribution in external acuminata condylomata: a Large French National Study (EDiTH IV). Clin Infect Dis **47:**610-615.
107. **Vandepapeliere P, Barrasso R, Meijer CJ, Walboomers JM, Wettendorff M, Stanberry LR, Lacey CJ.** 2005. Randomized controlled trial of an adjuvanted human papillomavirus (HPV) type 6 L2E7 vaccine: infection of external anogenital warts with multiple HPV types and failure of therapeutic vaccination. J Infect Dis **192:**2099-2107.
108. **Donovan B, Franklin N, Guy R, Grulich AE, Regan DG, Ali H, Wand H, Fairley CK.** 2011. Quadrivalent human papillomavirus vaccination and trends in

genital warts in Australia: analysis of national sentinel surveillance data. Lancet Infect Dis **11:**39-44.
109. **Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee SK, Kuypers JM, Koutsky LA.** 2005. Development and duration of human papillomavirus lesions, after initial infection. J Infect Dis **191:**731-738.
110. **Baseman JG, Koutsky LA.** 2005. The epidemiology of human papillomavirus infections. J Clin Virol **32 Suppl 1:**S16-24.
111. **Barcelos AC, Michelin MA, Adad SJ, Murta EF.** 2011. Atypical squamous cells of undetermined significance: Bethesda classification and association with Human Papillomavirus. Infect Dis Obstet Gynecol **2011:**904674.
112. **Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T, Jr., Young N, Forum Group M, Bethesda W.** 2002. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA **287:**2114-2119.
113. **Herbert A, Bergeron C, Wiener H, Schenck U, Klinkhamer P, Bulten J, Arbyn M.** 2007. European guidelines for quality assurance in cervical cancer screening: recommendations for cervical cytology terminology. Cytopathology **18:**213-219.
114. **Arbyn M, Martin-Hirsch P, Buntinx F, Van Ranst M, Paraskevaidis E, Dillner J.** 2009. Triage of women with equivocal or low-grade cervical cytology results: a meta-analysis of the HPV test positivity rate. J Cell Mol Med **13:**648-659.
115. **Alanen KW, Elit LM, Molinaro PA, McLachlin CM.** 1998. Assessment of cytologic follow-up as the recommended management for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions. Cancer **84:**5-10.
116. **Arbyn M, Ronco G, Anttila A, Meijer CJ, Poljak M, Ogilvie G, Koliopoulos G, Naucler P, Sankaranarayanan R, Peto J.** 2012. Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. Vaccine **30 Suppl 5:**F88-99.
117. **Cuzick J, Arbyn M, Sankaranarayanan R, Tsu V, Ronco G, Mayrand MH, Dillner J, Meijer CJ.** 2008. Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. Vaccine **26 Suppl 10:**K29-41.
118. **Parkin DM, Bray F.** 2006. Chapter 2: The burden of HPV-related cancers. Vaccine **24 Suppl 3:**S3/11-25.
119. **Forman D, de Martel C, Lacey CJ, Soerjomataram I, Lortet-Tieulent J, Bruni L, Vignat J, Ferlay J, Bray F, Plummer M, Franceschi S.** 2012. Global burden of human papillomavirus and related diseases. Vaccine **30 Suppl 5:**F12-23.
120. **Andersson S, Rylander E, Larsson B, Strand A, Silfversvard C, Wilander E.** 2001. The role of human papillomavirus in cervical adenocarcinoma carcinogenesis. Eur J Cancer **37:**246-250.
121. **Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S.** 2007. Human papillomavirus and cervical cancer. Lancet **370:**890-907.
122. **Moberg M, Gustavsson I, Wilander E, Gyllensten U.** 2005. High viral loads of human papillomavirus predict risk of invasive cervical carcinoma. Br J Cancer **92:**891-894.

123. **Schlecht NF, Trevisan A, Duarte-Franco E, Rohan TE, Ferenczy A, Villa LL, Franco EL.** 2003. Viral load as a predictor of the risk of cervical intraepithelial neoplasia. Int J Cancer **103:**519-524.
124. **de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, Tous S, Felix A, Bravo LE, Shin HR, Vallejos CS, de Ruiz PA, Lima MA, Guimera N, Clavero O, Alejo M, Llombart-Bosch A, Cheng-Yang C, Tatti SA, Kasamatsu E, Iljazovic E, Odida M, Prado R, Seoud M, Grce M, Usubutun A, Jain A, Suarez GA, Lombardi LE, Banjo A, Menendez C, Domingo EJ, Velasco J, Nessa A, Chichareon SC, Qiao YL, Lerma E, Garland SM, Sasagawa T, Ferrera A, Hammouda D, Mariani L, Pelayo A, Steiner I, Oliva E, Meijer CJ, Al-Jassar WF, Cruz E, Wright TC, Puras A, Llave CL, Tzardi M, Agorastos T, Garcia-Barriola V, Clavel C, Ordi J, Andujar M, Castellsague X, Sanchez GI, Nowakowski AM, Bornstein J, Munoz N, Bosch FX, Retrospective International S, Group HPVTTS.** 2010. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol **11:**1048-1056.
125. **Ylitalo N, Sorensen P, Josefsson AM, Magnusson PK, Andersen PK, Ponten J, Adami HO, Gyllensten UB, Melbye M.** 2000. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet **355:**2194-2198.
126. **Schlecht NF, Kulaga S, Robitaille J, Ferreira S, Santos M, Miyamura RA, Duarte-Franco E, Rohan TE, Ferenczy A, Villa LL, Franco EL.** 2001. Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. JAMA **286:**3106-3114.
127. **Ho GY, Burk RD, Klein S, Kadish AS, Chang CJ, Palan P, Basu J, Tachezy R, Lewis R, Romney S.** 1995. Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia. J Natl Cancer Inst **87:**1365-1371.
128. **Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV.** 2002. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol **55:**244-265.
129. **Kjaer SK, Frederiksen K, Munk C, Iftner T.** 2010. Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. J Natl Cancer Inst **102:**1478-1488.
130. **zur Hausen H.** 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer **2:**342-350.
131. **Appleby P, Beral V, Berrington de Gonzalez A, Colin D, Franceschi S, Goodill A, Green J, Peto J, Plummer M, Sweetland S.** 2006. Carcinoma of the cervix and tobacco smoking: collaborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the cervix from 23 epidemiological studies. Int J Cancer **118:**1481-1495.
132. **Munoz N, Franceschi S, Bosetti C, Moreno V, Herrero R, Smith JS, Shah KV, Meijer CJ, Bosch FX, International Agency for Research on Cancer. Multicentric Cervical Cancer Study G.** 2002. Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. Lancet **359:**1093-1101.
133. **Smith JS, Green J, Berrington de Gonzalez A, Appleby P, Peto J, Plummer M, Franceschi S, Beral V.** 2003. Cervical cancer and use of hormonal contraceptives: a systematic review. Lancet **361:**1159-1167.

134. **Magnusson PK, Lichtenstein P, Gyllensten UB.** 2000. Heritability of cervical tumours. Int J Cancer **88:**698-701.
135. **International Collaboration of Epidemiological Studies of Cervical C.** 2009. Cervical carcinoma and sexual behavior: collaborative reanalysis of individual data on 15,461 women with cervical carcinoma and 29,164 women without cervical carcinoma from 21 epidemiological studies. Cancer Epidemiol Biomarkers Prev **18:**1060-1069.
136. **Dupin N.** 2004. Genital warts. Clin Dermatol **22:**481-486.
137. **Smith JS, Herrero R, Bosetti C, Munoz N, Bosch FX, Eluf-Neto J, Castellsague X, Meijer CJ, Van den Brule AJ, Franceschi S, Ashley R, International Agency for Research on Cancer Multicentric Cervical Cancer Study G.** 2002. Herpes simplex virus-2 as a human papillomavirus cofactor in the etiology of invasive cervical cancer. J Natl Cancer Inst **94:**1604-1613.
138. **Anttila T, Saikku P, Koskela P, Bloigu A, Dillner J, Ikaheimo I, Jellum E, Lehtinen M, Lenner P, Hakulinen T, Narvanen A, Pukkala E, Thoresen S, Youngman L, Paavonen J.** 2001. Serotypes of Chlamydia trachomatis and risk for development of cervical squamous cell carcinoma. JAMA **285:**47-51.
139. **Smith JS, Bosetti C, Munoz N, Herrero R, Bosch FX, Eluf-Neto J, Meijer CJ, Van Den Brule AJ, Franceschi S, Peeling RW, study Imc-c.** 2004. Chlamydia trachomatis and invasive cervical cancer: a pooled analysis of the IARC multicentric case-control study. Int J Cancer **111:**431-439.
140. **Wright TC, Jr., Ellerbrock TV, Chiasson MA, Van Devanter N, Sun XW.** 1994. Cervical intraepithelial neoplasia in women infected with human immunodeficiency virus: prevalence, risk factors, and validity of Papanicolaou smears. New York Cervical Disease Study. Obstet Gynecol **84:**591-597.
141. **Arnheim Dahlstrom L, Andersson K, Luostarinen T, Thoresen S, Ogmundsdottir H, Tryggvadottir L, Wiklund F, Skare GB, Eklund C, Sjolin K, Jellum E, Koskela P, Wadell G, Lehtinen M, Dillner J.** 2011. Prospective seroepidemiologic study of human papillomavirus and other risk factors in cervical cancer. Cancer Epidemiol Biomarkers Prev **20:**2541-2550.
142. **Silins I, Ryd W, Strand A, Wadell G, Tornberg S, Hansson BG, Wang X, Arnheim L, Dahl V, Bremell D, Persson K, Dillner J, Rylander E.** 2005. Chlamydia trachomatis infection and persistence of human papillomavirus. Int J Cancer **116:**110-115.
143. **Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV.** 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst **87:**796-802.
144. **Tieben LM, ter Schegget J, Minnaar RP, Bouwes Bavinck JN, Berkhout RJ, Vermeer BJ, Jebbink MF, Smits HL.** 1993. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. J Virol Methods **42:**265-279.
145. **Hemminki K, Li X, Mutanen P.** 2001. Age-incidence relationships and time trends in cervical cancer in Sweden. Eur J Epidemiol **17:**323-328.
146. **Vaccarella S, Lortet-Tieulent J, Plummer M, Franceschi S, Bray F.** 2013. Worldwide trends in cervical cancer incidence: impact of screening against changes in disease risk factors. Eur J Cancer **49:**3262-3273.

147. **Nanda K, McCrory DC, Myers ER, Bastian LA, Hasselblad V, Hickey JD, Matchar DB.** 2000. Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: a systematic review. Ann Intern Med **132:**810-819.
148. **Cox T, Cuzick J.** 2006. HPV DNA testing in cervical cancer screening: from evidence to policies. Gynecol Oncol **103:**8-11.
149. **Soderlund-Strand A, Carlson J, Dillner J.** 2009. Modified general primer PCR system for sensitive detection of multiple types of oncogenic human papillomavirus. J Clin Microbiol **47:**541-546.
150. **Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T.** 2006. Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol **44:**504-512.
151. **Münger K, Phelps WC, Bubb V, Howley PM, Schlegel R.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol **63:**4417-4421.
152. **Hudson JB, Bedell MA, McCance DJ, Laiminis LA.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J Virol **64:**519-526.
153. **Cuschieri K, Wentzensen N.** 2008. Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. Cancer Epidemiol Biomarkers Prev **17:**2536-2545.
154. **Molden T, Kraus I, Skomedal H, Nordstrom T, Karlsen F.** 2007. PreTect HPV-Proofer: real-time detection and typing of E6/E7 mRNA from carcinogenic human papillomaviruses. J Virol Methods **142:**204-212.
155. **Jeantet D, Schwarzmann F, Tromp J, Melchers WJ, van der Wurff AA, Oosterlaken T, Jacobs M, Troesch A.** 2009. NucliSENS EasyQ HPV v1 test - Testing for oncogenic activity of human papillomaviruses. J Clin Virol **45 Suppl 1:**S29-37.
156. **Walboomers JM, Meijer CJ.** 1997. Do HPV-negative cervical carcinomas exist? J Pathol **181:**253-254.
157. **Sturegard E, Johnsson A, Gustafsson E, Dillner J.** 2008. [Condyloma typing important for follow up of HPV vaccination. A condyloma reporting project]. Lakartidningen **105:**3648-3650.
158. **Chan PK, Luk AC, Luk TN, Lee KF, Cheung JL, Ho KM, Lo KK.** 2009. Distribution of human papillomavirus types in anogenital warts of men. J Clin Virol **44:**111-114.
159. **Castronovo C, Lebas E, Nikkels-Tassoudji N, Nikkels AF.** 2012. Viral infections of the pubis. Int J STD AIDS **23:**48-50.
160. **Tyring SK.** 2003. Molluscum contagiosum: the importance of early diagnosis and treatment. Am J Obstet Gynecol **189:**S12-16.
161. **Oberyszyn TM.** 2008. Non-melanoma skin cancer: importance of gender, immunosuppressive status and vitamin D. Cancer Lett **261:**127-136.
162. **Boukamp P.** 2005. UV-induced skin cancer: similarities--variations. J Dtsch Dermatol Ges **3:**493-503.
163. **Alam M, Ratner D.** 2001. Cutaneous squamous-cell carcinoma. N Engl J Med **344:**975-983.
164. **Telfer NR, Colver GB, Morton CA, British Association of D.** 2008. Guidelines for the management of basal cell carcinoma. Br J Dermatol **159:**35-48.

165. **Rubin AI, Chen EH, Ratner D.** 2005. Basal-cell carcinoma. N Engl J Med **353:**2262-2269.
166. **Preston DS, Stern RS.** 1992. Nonmelanoma cancers of the skin. N Engl J Med **327:**1649-1662.
167. **Ting PT, Kasper R, Arlette JP.** 2005. Metastatic basal cell carcinoma: report of two cases and literature review. J Cutan Med Surg **9:**10-15.
168. **Cherpelis BS, Marcusen C, Lang PG.** 2002. Prognostic factors for metastasis in squamous cell carcinoma of the skin. Dermatol Surg **28:**268-273.
169. **MacKie RM.** 2006. Long-term health risk to the skin of ultraviolet radiation. Prog Biophys Mol Biol **92:**92-96.
170. **Marks R, Rennie G, Selwood TS.** 1988. Malignant transformation of solar keratoses to squamous cell carcinoma. Lancet **1:**795-797.
171. **Boukamp P.** 2005. Non-melanoma skin cancer: what drives tumor development and progression? Carcinogenesis **26:**1657-1667.
172. **Cockerell CJ.** 2000. Histopathology of incipient intraepidermal squamous cell carcinoma ("actinic keratosis"). J Am Acad Dermatol **42:**11-17.
173. **Schwartz RA.** 2004. Keratoacanthoma: a clinico-pathologic enigma. Dermatol Surg **30:**326-333; discussion 333.
174. **Karaa A, Khachemoune A.** 2007. Keratoacanthoma: a tumor in search of a classification. Int J Dermatol **46:**671-678.
175. **Hurt MA.** 2004. Keratoacanthoma vs. squamous cell carcinoma in contrast with keratoacanthoma is squamous cell carcinoma. J Cutan Pathol **31:**291-292; author reply 292-293.
176. **Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG.** 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J Virol **74:**11636-11641.
177. **Hazard K, Karlsson A, Andersson K, Ekberg H, Dillner J, Forslund O.** 2007. Cutaneous human papillomaviruses persist on healthy skin. J Invest Dermatol **127:**116-119.
178. **Astori G, Lavergne D, Benton C, Hockmayr B, Egawa K, Garbe C, de Villiers EM.** 1998. Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. J Invest Dermatol **110:**752-755.
179. **de Koning MN, Struijk L, Bavinck JN, Kleter B, ter Schegget J, Quint WG, Feltkamp MC.** 2007. Betapapillomaviruses frequently persist in the skin of healthy individuals. J Gen Virol **88:**1489-1495.
180. **Egawa K, Egawa N, Honda Y.** 2005. Human papillomavirus-associated plantar epidermoid cyst related to epidermoid metaplasia of the eccrine duct epithelium: a combined histological, immunohistochemical, DNA-DNA in situ hybridization and three-dimensional reconstruction analysis. Br J Dermatol **152:**961-967.
181. **Orth G.** 1986. Epidermodysplasia verruciformis: a model for understanding the oncogenicity of human papillomaviruses. Ciba Found Symp **120:**157-174.
182. **Jablonska S, Majewski S.** 1994. Epidermodysplasia verruciformis: immunological and clinical aspects. Curr Top Microbiol Immunol **186:**157-175.
183. **Wallace NA, Robinson K, Howie HL, Galloway DA.** 2012. HPV 5 and 8 E6 abrogate ATR activity resulting in increased persistence of UVB induced DNA damage. PLoS Pathog **8:**e1002807.
184. **Viarisio D, Mueller-Decker K, Kloz U, Aengeneyndt B, Kopp-Schneider A, Grone HJ, Gheit T, Flechtenmacher C, Gissmann L, Tommasino M.** 2011. E6 and E7 from beta HPV38 cooperate with ultraviolet light in the development

of actinic keratosis-like lesions and squamous cell carcinoma in mice. PLoS Pathog **7:**e1002125.

185. **Accardi R, Dong W, Smet A, Cui R, Hautefeuille A, Gabet AS, Sylla BS, Gissmann L, Hainaut P, Tommasino M.** 2006. Skin human papillomavirus type 38 alters p53 functions by accumulation of deltaNp73. EMBO Rep **7:**334-340.
186. **Saidj D, Cros MP, Hernandez-Vargas H, Guarino F, Sylla BS, Tommasino M, Accardi R.** 2013. Oncoprotein E7 from beta human papillomavirus 38 induces formation of an inhibitory complex for a subset of p53-regulated promoters. J Virol **87:**12139-12150.
187. **Howie HL, Koop JI, Weese J, Robinson K, Wipf G, Kim L, Galloway DA.** 2011. Beta-HPV 5 and 8 E6 promote p300 degradation by blocking AKT/p300 association. PLoS Pathog **7:**e1002211.
188. **Muench P, Probst S, Schuetz J, Leiprecht N, Busch M, Wesselborg S, Stubenrauch F, Iftner T.** 2010. Cutaneous papillomavirus E6 proteins must interact with p300 and block p53-mediated apoptosis for cellular immortalization and tumorigenesis. Cancer Res **70:**6913-6924.
189. **Jackson S, Harwood C, Thomas M, Banks L, Storey A.** 2000. Role of bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. Genes Dev **14:**3065-3073.
190. **Vajdic CM, van Leeuwen MT.** 2009. Cancer incidence and risk factors after solid organ transplantation. Int J Cancer **125:**1747-1754.
191. **Lindelof B, Sigurgeirsson B, Gabel H, Stern RS.** 2000. Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol **143:**513-519.
192. **Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM.** 2007. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. Lancet **370:**59-67.
193. **Berg D, Otley CC.** 2002. Skin cancer in organ transplant recipients: Epidemiology, pathogenesis, and management. J Am Acad Dermatol **47:**1-17; quiz 18-20.
194. **Hartevelt MM, Bavinck JN, Kootte AM, Vermeer BJ, Vandenbroucke JP.** 1990. Incidence of skin cancer after renal transplantation in The Netherlands. Transplantation **49:**506-509.
195. **Moloney FJ, Comber H, O'Lorcain P, O'Kelly P, Conlon PJ, Murphy GM.** 2006. A population-based study of skin cancer incidence and prevalence in renal transplant recipients. Br J Dermatol **154:**498-504.
196. **de Villiers EM, Lavergne D, McLaren K, Benton EC.** 1997. Prevailing papillomavirus types in non-melanoma carcinomas of the skin in renal allograft recipients. Int J Cancer **73:**356-361.
197. **Harwood CA, Surentheran T, McGregor JM, Spink PJ, Leigh IM, Breuer J, Proby CM.** 2000. Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals. J Med Virol **61:**289-297.
198. **Shamanin V, zur Hausen H, Lavergne D, Proby CM, Leigh IM, Neumann C, Hamm H, Goos M, Haustein UF, Jung EG, Plewig G, Wolff H, de Villiers EM.** 1996. Human papillomavirus infections in nonmelanoma skin cancers from renal transplant recipients and nonimmunosuppressed patients. J Natl Cancer Inst **88:**802-811.

199. **de Jong-Tieben LM, Berkhout RJ, Smits HL, Bouwes Bavinck JN, Vermeer BJ, van der Woude FJ, ter Schegget J.** 1995. High frequency of detection of epidermodysplasia verruciformis-associated human papillomavirus DNA in biopsies from malignant and premalignant skin lesions from renal transplant recipients. J Invest Dermatol **105:**367-371.
200. **Harwood CA, Proby CM.** 2002. Human papillomaviruses and non-melanoma skin cancer. Curr Opin Infect Dis **15:**101-114.
201. **Asgari MM, Kiviat NB, Critchlow CW, Stern JE, Argenyi ZB, Raugi GJ, Berg D, Odland PB, Hawes SE, de Villiers EM.** 2008. Detection of human papillomavirus DNA in cutaneous squamous cell carcinoma among immunocompetent individuals. J Invest Dermatol **128:**1409-1417.
202. **Harwood CA, Surentheran T, Sasieni P, Proby CM, Bordea C, Leigh IM, Wojnarowska F, Breuer J, McGregor JM.** 2004. Increased risk of skin cancer associated with the presence of epidermodysplasia verruciformis human papillomavirus types in normal skin. Br J Dermatol **150:**949-957.
203. **Biliris KA, Koumantakis E, Dokianakis DN, Sourvinos G, Spandidos DA.** 2000. Human papillomavirus infection of non-melanoma skin cancers in immunocompetent hosts. Cancer Lett **161:**83-88.
204. **Ekstrom J, Bzhalava D, Svenback D, Forslund O, Dillner J.** 2011. High throughput sequencing reveals diversity of Human Papillomaviruses in cutaneous lesions. Int J Cancer **129:**2643-2650.
205. **Mackintosh LJ, de Koning MN, Quint WG, Ter Schegget J, Morgan IM, Herd RM, Campo MS.** 2009. Presence of beta human papillomaviruses in nonmelanoma skin cancer from organ transplant recipients and immunocompetent patients in the West of Scotland. Br J Dermatol **161:**56-62.
206. **Ekstrom J, Muhr LS, Bzhalava D, Soderlund-Strand A, Hultin E, Nordin P, Stenquist B, Paoli J, Forslund O, Dillner J.** 2013. Diversity of human papillomaviruses in skin lesions. Virology **447:**300-311.
207. **Forslund O, DeAngelis PM, Beigi M, Schjolberg AR, Clausen OP.** 2003. Identification of human papillomavirus in keratoacanthomas. J Cutan Pathol **30:**423-429.
208. **Lu S, Syrjanen SL, Havu VK, Syrjanen S.** 1996. Known HPV types have no association with keratoacanthomas. Arch Dermatol Res **288:**129-132.
209. **Vasiljevic N, Andersson K, Bjelkenkrantz K, Kjellstrom C, Mansson H, Nilsson E, Landberg G, Dillner J, Forslund O.** 2009. The Bcl-xL inhibitor of apoptosis is preferentially expressed in cutaneous squamous cell carcinoma compared with that in keratoacanthoma. Int J Cancer **124:**2361-2366.
210. **Vasiljevic N, Hazard K, Dillner J, Forslund O.** 2008. Four novel human betapapillomaviruses of species 2 preferentially found in actinic keratosis. J Gen Virol **89:**2467-2474.
211. **Vasiljevic N, Hazard K, Eliasson L, Ly H, Hunziker A, de Villiers EM, Norrild B, Dillner J, Forslund O.** 2007. Characterization of two novel cutaneous human papillomaviruses, HPV93 and HPV96. J Gen Virol **88:**1479-1483.
212. **Ekstrom J, Forslund O, Dillner J.** 2010. Three novel papillomaviruses (HPV109, HPV112 and HPV114) and their presence in cutaneous and mucosal samples. Virology **397:**331-336.

213. **Kullander J, Handisurya A, Forslund O, Geusau A, Kirnbauer R, Dillner J.** 2008. Cutaneous human papillomavirus 88: remarkable differences in viral load. Int J Cancer **122:**477-480.
214. **Forslund O, Ly H, Reid C, Higgins G.** 2003. A broad spectrum of human papillomavirus types is present in the skin of Australian patients with non-melanoma skin cancers and solar keratosis. Br J Dermatol **149:**64-73.
215. **Patel AS, Karagas MR, Perry AE, Nelson HH.** 2008. Exposure profiles and human papillomavirus infection in skin cancer: an analysis of 25 genus beta-types in a population-based study. J Invest Dermatol **128:**2888-2893.
216. **Forslund O, Iftner T, Andersson K, Lindelof B, Hradil E, Nordin P, Stenquist B, Kirnbauer R, Dillner J, de Villiers EM, Viraskin Study G.** 2007. Cutaneous human papillomaviruses found in sun-exposed skin: Beta-papillomavirus species 2 predominates in squamous cell carcinoma. J Infect Dis **196:**876-883.
217. **Plasmeijer EI, Neale RE, de Koning MN, Quint WG, McBride P, Feltkamp MC, Green AC.** 2009. Persistence of betapapillomavirus infections as a risk factor for actinic keratoses, precursor to cutaneous squamous cell carcinoma. Cancer Res **69:**8926-8931.
218. **Forslund O, Lindelof B, Hradil E, Nordin P, Stenquist B, Kirnbauer R, Slupetzky K, Dillner J.** 2004. High Prevalence of Cutaneous Human Papillomavirus DNA on the Top of Skin Tumors but not in "Stripped" Biopsies from the Same Tumors. J Invest Dermatol **123:**388-394.
219. **Waterboer T, Abeni D, Sampogna F, Rother A, Masini C, Sehr P, Michael KM, Pawlita M.** 2008. Serological association of beta and gamma human papillomaviruses with squamous cell carcinoma of the skin. Br J Dermatol **159:**457-459.
220. **Karagas MR, Nelson HH, Sehr P, Waterboer T, Stukel TA, Andrew A, Green AC, Bavinck JN, Perry A, Spencer S, Rees JR, Mott LA, Pawlita M.** 2006. Human papillomavirus infection and incidence of squamous cell and basal cell carcinomas of the skin. J Natl Cancer Inst **98:**389-395.
221. **Feltkamp MC, Broer R, di Summa FM, Struijk L, van der Meijden E, Verlaan BP, Westendorp RG, ter Schegget J, Spaan WJ, Bouwes Bavinck JN.** 2003. Seroreactivity to epidermodysplasia verruciformis-related human papillomavirus types is associated with nonmelanoma skin cancer. Cancer Res **63:**2695-2700.
222. **Andersson K, Michael KM, Luostarinen T, Waterboer T, Gislefoss R, Hakulinen T, Forslund O, Pawlita M, Dillner J.** 2012. Prospective study of human papillomavirus seropositivity and risk of nonmelanoma skin cancer. Am J Epidemiol **175:**685-695.
223. **Waterboer T, Neale R, Michael KM, Sehr P, de Koning MN, Weissenborn SJ, Sampogna F, Abeni D, Green AC, Bouwes Bavinck JN, Pawlita M, Group E-H-U-C.** 2009. Antibody responses to 26 skin human papillomavirus types in the Netherlands, Italy and Australia. J Gen Virol **90:**1986-1998.
224. **Arron ST, Ruby JG, Dybbro E, Ganem D, Derisi JL.** 2011. Transcriptome sequencing demonstrates that human papillomavirus is not active in cutaneous squamous cell carcinoma. J Invest Dermatol **131:**1745-1753.
225. **Fire A, Xu SQ.** 1995. Rolling replication of short DNA circles. Proc Natl Acad Sci U S A **92:**4641-4645.

226. **Dean FB, Nelson JR, Giesler TL, Lasken RS.** 2001. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res **11:**1095-1099.
227. **Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS.** 2002. Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci U S A **99:**5261-5266.
228. **Lage JM, Leamon JH, Pejovic T, Hamann S, Lacey M, Dillon D, Segraves R, Vossbrinck B, Gonzalez A, Pinkel D, Albertson DG, Costa J, Lizardi PM.** 2003. Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. Genome Res **13:**294-307.
229. **Garmendia C, Bernad A, Esteban JA, Blanco L, Salas M.** 1992. The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. J Biol Chem **267:**2594-2599.
230. **Blanco L, Bernad A, Lazaro JM, Martin G, Garmendia C, Salas M.** 1989. Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. J Biol Chem **264:**8935-8940.
231. **Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM.** 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature **437:**376-380.
232. **Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P.** 1996. Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem **242:**84-89.
233. **Rector A, Tachezy R, Van Ranst M.** 2004. A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J Virol **78:**4993-4998.
234. **Bzhalava D, Muhr LS, Lagheden C, Ekstrom J, Forslund O, Dillner J, Hultin E.** 2014. Deep sequencing extends the diversity of human papillomaviruses in human skin. Sci Rep **4:**5807.
235. **Antonsson A, Karanfilovska S, Lindqvist PG, Hansson BG.** 2003. General acquisition of human papillomavirus infections of skin occurs in early infancy. J Clin Microbiol **41:**2509-2514.
236. **Persson M, Elfstrom KM, Brismar Wendel S, Weiderpass E, Andersson S.** 2014. Triage of HR-HPV positive women with minor cytological abnormalities: a comparison of mRNA testing, HPV DNA testing, and repeat cytology using a 4-year follow-up of a population-based study. PLoS One **9:**e90023.
237. **Waldstrom M, Ornskov D.** 2012. Comparison of the clinical performance of an HPV mRNA test and an HPV DNA test in triage of atypical squamous cells of undetermined significance (ASC-US). Cytopathology **23:**389-395.
238. **Waldstrom M, Ornskov D.** 2011. Clinical performance of a human papillomavirus messenger RNA test (Aptima HPV Assay) on residual material

from archived 3-year-old PreservCyt samples with low-grade squamous intraepithelial lesion. Arch Pathol Lab Med **135:**1052-1056.