Occurrence of human papillomaviruses (HPV) types in HPV related cancer and in the genital and oral tracts of young adults

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OCCURRENCE OF HUMAN PAPILLOMAVIRUSES (HPV) TYPES IN HPV RELATED CANCER AND IN THE GENITAL AND ORAL TRACTS OF YOUNG ADULTS

Juan　Du

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Stockholm 2012

To my family

献给我最爱的家人

Abstract

Human papillomavirus (HPV) is associated to cancer of the uterine cervix, the third most common cancer among women, but also to head and neck squamous cell carcinoma (HNSCC), the sixth most common type of cancer in the world. HPV occurs in most cervical cancer (CC). In HNSCC, HPV is most frequently observed in oropharyngeal squamous cell carcinoma (OSCC) where tonsillar squamous cell carcinoma (TSCC) and the base of tongue account for 70-80% of the cases. It has also been shown that OSCC has increased in many Western Countries and we have shown HPV to be responsible for the increase of TSCC in Stockholm. In recent years, two vaccines were introduced against HPV, Gardasil (Merck) and Cevarix (GSK), both efficient against infection with HPV type 16 and 18 and Gardasil against HPV 6 and 11 as well, and these will change the prevalence of HPV types at different sites.

The aim of this thesis was to investigate the role of HPV in the increase of tongue base cancer in the Stockholm. In addition, we wanted to obtain different base lines for the prevalence of different HPV types for cervical cancer and in the genital and oral tracts in young adults in the Stockholm region. Finally we wanted to compare different HPV E6 variants in TSCC and CC as well as in cervical samples (CS) from healthy young women.

The first paper showed that the prevalence of HPV in base of tongue cancer in Stockholm increased from 58% in 1998-1999 to 84% 2006-2007 with HPV 16 dominating (86%). The parallel increase in incidence and proportion of HPV positive base of tongue cancer suggests HPV may contribute to this increase similar to that previously shown for TSCC.

The second paper showed a very high prevalence of HPV with 92.9% in all uterine cervix cancer cases, with 93.3% and 91.4 % in SCC and ADC, respectively. All HPV positive cases harbored HR types, either alone or as multiple infections. HPV 16 and 18 dominated, followed by HPV 33, 31, 45 and 56, in cervical cancer in the Stockholm region. Public HPV vaccination should inhibit a large proportion of HPV 16 and 18 positive tumors.

The third paper revealed a high HPV prevalence in 544 analyzed cervical samples from non-vaccinated young women aged 16-23 years of age and 70% were positive for HPV and 62% were positive for HR-HPV types. Over a third (34.7%) of the women was infected with HPV 16 followed by HR-HPV types 51, 18, 52 and 73. The prevalence of HPV, as well as HR-HPV infection appeared to increase with age in women aged $17 - 21$y, and then decrease. The data indicates that HPV vaccination in an early age can prevent HPV 16 and 18 infections and demonstrates the need for further monitoring of the prevalence of HR-HPV types.

The fourth paper showed that 9.3% (9.2% for women and 9.8% for men) of the 483 oral samples from young adults were HPV-positive, with 7.2% being positive for HR-HPV types. HPV 16 was the most common (31%) followed by HPV 59 and HPV 51. Among these 174 women that were tested both for genital and oral HPV infection, oral infection was more frequent in women with (17.1%) as compared to those without (4.4%) genital infection (p=0.043) and there was a high HPV type concordance between the oral and genital locations.

The fifth paper showed several patterns of HPV 16 E6 with the HPV E6 variant R10G was relatively common (19%) in TSCC, absent in CC and infrequent (4%) in CS, indicating significant differences of HPV 16 variants in TSCC compared to CC and CS which has not been observed before. Furthermore, the well-known L83V variant was very common in TSCC (40%) as it was in CC (31%) and CS (29%). The majority of HPV 16 (>90%) belonged to the European phylogenetic lineage and its derivatives. No significant relation between R10G variant and survival of TSCC patients was observed.

In conclusion, we have demonstrated that HPV infection may play a role for the increase in base of tongue cancer, and HPV 16 and 18 are highly prevalent in CC and CS. HPV 16 is frequently found in oral samples indicates that HPV vaccination will potentially be useful to combat some of these tumors. Finally, we have shown differences in HPV 16 E6 variants between the TSCC and CC sites.

List of Publications

I. Attner, P.*, Du, J.*, Näsman, A., Hammarstedt, L., Ramqvist, T., Lindholm, J., Marklund, L., Dalianis, T. and Munck-Wikland, E., The role of human papilloma virus (HPV) in the increased incidence of base of tongue cancer. Int J Cancer. 126(12):2879-84 (2010). (*contributed equally)

II. Du, J., Näsman, A., Carlsson, J., Ramqvist, T. and Dalianis, T.: Prevalence of Human Papillomavirus (HPV) types in cervical cancer 2003 – 2008 in Stockholm, Sweden, before public HPV vaccination Acta Oncologica, 50(8):1215-9 (2011).

III. Ramqvist, T.*, Du, J.*, Lunden, M., Ährlund-Richter, S., Ferreira, J., Marions, L., Sparén, P., Andersson, S. and Dalianis, T.: Pre-vaccination prevalence of human papillomavirus types in the genital tract of 15-23 year old women in a youth clinic in Stockholm, Sweden. Scandinavian Journal of Infectious Diseases. 43(2):115-21 (2011). (*contributed equally)

IV. Du, J., Nordfors, C., Ährlund-Richter, A., Sobkowiak, M., Romanitan, M., Andersson, S., Ramqvist, T. and Dalianis, T.: Oral human papillomavirus (HPV) prevalence in young men, and in women with and without cervical HPV infection attending a youth clinic in Sweden. Emerg Infect Dis., 18(9):1468-1471 (2012).

V. Du, J., Nordfors, C., Näsman, A., Sobkowiak, M., Romanitan, M., Dalianis, T. And Ramqvist, T.: Human papillomavirus (HPV) 16 E6 variants in tonsillar cancer in comparison to those in cervical cancer in Stockholm, Sweden. PLoS One. 2012;7(4):e36239.

Contents

Abbreviations

AA	Asian-American
ADC	Adenocarcinomas
Af-1	African-1
Af-2	African-2
As	Asian
ASCUS	Atypical squamous cells of undetermined significance
bp	Base pair
CC	Cervical cancer
CGH	Comparative genomic hybridization
CIN	Cervical intraepithelial neoplasm
COX-2	Cyclooxygenase-2
CR	Conserved region
CS	Cervical samples
CTL	Cytotoxic T-lymphocyte
DSF	Disease-free survival
E	European
E6AP	E6-associated protein
E6BP	E6-binding protein
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
HLA	Human leukocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human Papillomavirus
HR	High risk
HSIL	High-grade squamous intraepithelial lesions
HSPG	Heparan sulfate proteoglycans
hTERT	Human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
ICD	International Classification of Diseases system
IFN	Interferon
IRF	Interferon regulatory factor
LCR	Long-control region
LR	Low risk
LSIL	Low-grade squamous intraepithelial lesions
L83V	Leucine to valanine at amino acid 83
MAGUK	Membrane associated guanylate kinase
MHC	Major histocompatibility
NA1	North American 1
ORF	Open reading frame
OSCC	Oropharyngeal squamous cell carcinoma
Pap	Papanicolaou
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
R10G	Arginine to glycine in amino acid 10
RRP	Recurrent respiratory papillomatosis
SCC	Squamous cell carcinomas
SILs	Squamous intraepithelial lesions

SNOMED	Systematised nomenclature of medicine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSCC	Tonsillar squamous cell carcinoma
URR	Upstream regulatory region
VEGF	Vascular endothelial growth factor
VLP	Virus-like particles

1. Introduction

In 2007, the first vaccine against human papillomavirus (HPV) types 16, 18, 6 and 11 were introduced, thus protecting against cervical infection with these HPV types and also this way against some cervical cancers and condylomas. In this thesis, we have focused on analyzing for the presence of different HPV types and in some case their variants in tumors of the oropharynx, tumors of the uterine cervix and also in the cervical and oral tracts of young adults. However, before going into details of the work, a brief introduction to the field of HPV and oropharyngeal and cervical cancer will be presented.

1.1 Human Papillomavirus

1.1.1 History

It is always easier to find a virus in a place where one has a pathological change, such as a wart or a disease. This creates an interest in the disease with the aim to cure the individual of the disease by clearing the virus infection. In humans, the first reported transmission of warts from one individual to next one became evident around the turn of 20th century [1]. Much later a study that analyzed the potential oncogenic capacity of HPV in Epidermodysplasia verruciformis was published in 1972 by Stefania Jablonska [2]. Soon after, in the mid-1970s, that Harald zur Hausen proposed that HPV played an important role in causing cervical cancer, a hypothesis, which brought him the Nobel Prize in Physiology or Medicine [3-6].
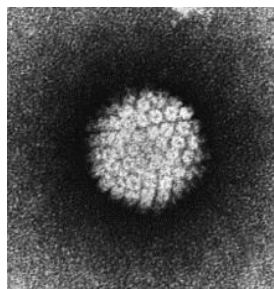
Figure 1. A scanning electron micrograph of an HPV virion
Source: Laboratory of Tumor Virus Biology

Thereafter numerous of investigations were done on HPV and the heterogeneity of HPV was realized in 1976 and 1977 [7-9]. One year later, Jablonska and Gerard Orth discovered HPV 5 or HPV 8 in skin cancers of epidermodysplasia patients [10]. In the 1980s, different HPV types from cervical warts and laryngeal papillomas (HPV 6 and 11) as well as from cervical cancer (HPV 16 and HPV 18) were isolated and identified by

Harald zur Hausen and his colleagues [11-15]. These reports were then followed by numerous studies identifying the molecular functions of HPV and its relation to cervical carcinoma (Fig. 1). Until now, over 150 different types of HPV have been identified and HPV is not as mysterious or elusive as it was originally in the eyes of molecular researchers [16, 17].

1.1.2 The viral particle

There are, as mentioned above, more than 150 different HPV types. Some types are mucosal, while others are found mainly in the skin. All HPV particles are non-enveloped and have a virion of approximately 55nm in diameter, that contains a circular double-stranded DNA genome of almost 8000 base pairs (bp) and with six to eight open reading frames (ORFs) (Fig. 2) [18]. The genome can be divided into three regions depending on their functions: the long-control region (LCR) or upstream regulatory region (URR), the early region and the late region. The LCR regulates transcription and the expression of early and late region genes and viral replication. The early region encodes for the E1, E2, E4-7 non-structural proteins, they and their functions will be described in more detail below. The late region encodes the L1 and L2 transcript, which make up the major capsid protein L1 and the minor capsid protein L2. It is known that L1 can self-assemble into virus-like particles (VLPs) either alone, or together with L2 [19-21], and assembly of L1 into VLPs is now used in the present HPV vaccines [22].
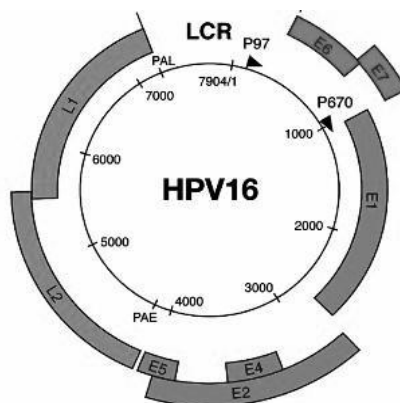
Figure 2. HPV 16 genome
Source: Clinical Science. 2006 (110): 525-541

1.1.3 HPV types and variants and taxonomy

The international committee on taxonomy of viruses has suggested that HPV forms one "family". This family consists of different phylogenetic branches or subgroups and in total there are 5 different subgroups. Each major phylogenetic branch or subgroup is referred as a "genus" with 40-50% nucleotide sequence diversity to the other subgroups (e.g. Alpha-papillomavirus, Beta-papillomavirus, Gamma-papillomavirus, Nu-

papillomavirus and Mu-papillomavirus) (Fig. 3). Minor branches or closely related groups are called "species" and have a 30-40% nucleotide sequence diversity compared to other species in the same subgroup (e.g. HPV species 2). A species is divided into different "types" when the L1 gene differs by over 10% or if there is around 10-25% diversity for overall nucleotide sequences (e.g. HPV 16). Very few HPVs are separated into "subtypes" which is when the nucleotide sequence differs between 2% and 10% (e.g. HPV 68A/B). The term "variant" refers to isolates of the same type that have less than 2% nucleotide sequence differences from the original HPV reference or prototype clone (e.g. E-T350G for HPV 16 E6)[23]. There are also intratype variants, and these have been described for HPV 16 E6 which has been classified into five major distinct lineages as European (E), Asian (As), Asian-American [24], African-1 (Af-1) and African-2 (Af-2) may have unique molecular and clinical properties [25, 26].
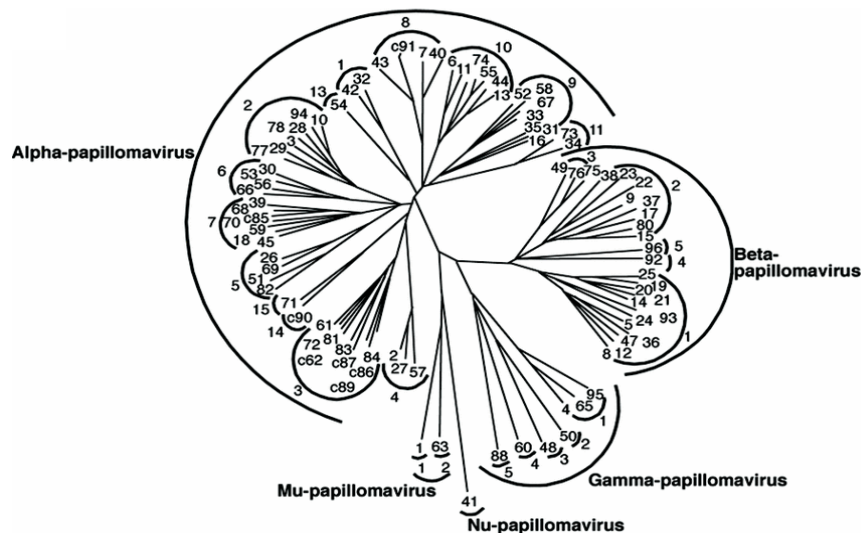
Figure 3. Phylogenetic tree of HPV
Source: Clinical science. 2006 (110): 525-541

1.1.4 Life cycle and transmission

HPV infects different epithelial tissues and this can result in microlesions. Some HPV types infect mainly cutaneous tissues and induce warts, while other HPV types mainly target mucosal tissues of the cervical and oral tracts. Depending on the oncogenic potential, various mucosal HPV types are divided into "High-risk HPV (HR-HPV) types" which can be potentially carcinogenic to humans and these include e.g.: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82; "Putative High risk HPV" which types are possibly carcinogenic to humans and these include e.g.: HPV 26, 53 and 66; and "Low-risk HPV (LR-HPV) types" that are currently not assumed to be carcinogenic and these include e.g.: HPV 6, 11, 42, 43, 44 and 70 [27]. During the years depending on the

accumulated information, some HPV types are placed in different groups. In this thesis, in order to compare the different subprojects, the distribution of the different HPV types into HR, putative HR and LR types will be described as reported by Munoz et al[27]. However, recently a new classification has been presented by International Agency for Research on Cancer (IARC) Vol 100B.
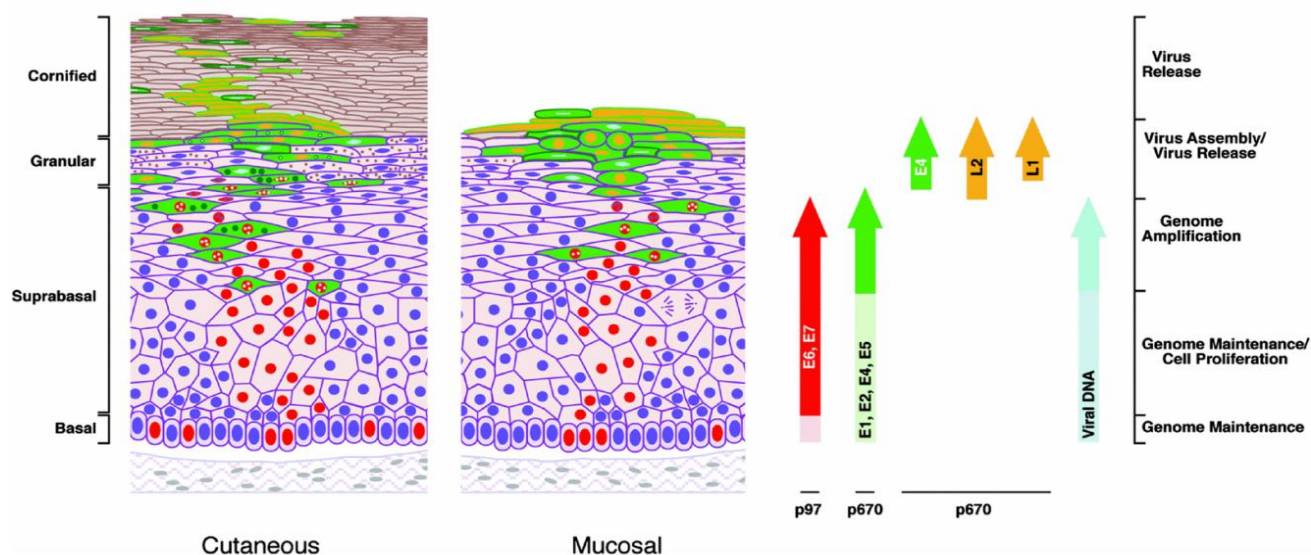
Figure 4. Organization of the HPV genome and the virus life cycle
Source: Clinical Science. 2006 (110): 525-541

The life cycle of HPV is highly regulated and tightly related to the differentiation program of human keratinocytes, and productive viral particles mainly target basal differentiated layers, which initially are exposed by microwounds (Fig. 4). Besides targeting stratified squamous epithelia, HPV can also infect the periphery of junctions between different types of epithelial cells such as e.g. the transformation zone of the cervix uteri. The whole process of replication occurs in suprabasal differentiated layers. The entry of HPV is not fully understood, however after the HPV particle enters, the infected basal cells maintain HPV DNA in a low copy number (approximately 20-100 copies) as nuclear plasmids, and this early process is defined as the establishment of the non-productive infectious state [28]. This state can be eventually cleared or persist for many years, and then it requires that the viral genome is maintained over cell divisions. This so-called maintenance state is also a prerequisite for HR-HPV types for developing cancer.

The productive stage starts when the daughter cells migrate away from the basal layers and differentiate. HPV infected cells delay the terminal differentiation program, and allow several cell cycles supporting viral replication with production of viral DNA at high

4

levels [28], for details see also figure 4. The E6 and E7 proteins are expressed at low expression levels, together with E1, E2, E4 and E5, which contribute to maintain the viral genome and cell proliferation. The late transcripts of capsid protein L1 and L2 as well as accumulated E4 and E5 allows HPV virions to assemble in the granular layer cells and later be shed from the epithelial surface to the environment [16, 29-31].

HPV infections are transmitted mainly by skin-to-skin or skin-to-mucosa contact. Cervical HPV infections are mainly transmitted by sexual contacts and the prevalence is correlated with the number of sexual partners [32, 33]. Each genotype has its own characteristic age specific peak transmission curve [34] and this will be discussed in more detail below. Less is known about oral HPV transmission. Although it is assumed to be sexually transmitted, it may also be transmitted from mother to child, most likely also by kissing [35, 36] .

1.1.5 Viral genes and proteins

The HPV genome consists, as mentioned above, of the early region encoding the non-structural proteins E1, E2, E4-7; the late region encoding the viral capsid proteins L1 and L2; and the regulatory region responsible for transcription and replication (URR/LCR). Below the genes and proteins of the HPV genome will be described.

1.1.5.1 E1

The E1 gene has the largest ORF, which is highly conserved, and has a weak binding affinity for a consensus motif repeated 6 times in the viral origin [16]. The E1 gene can be divided into three functional domains: the C-terminal enzymatic domain that includes ATPase/helicase activity; the variable-length central DNA-binding domain that recognizes specific sites in the origin of replication; and finally the N-terminal region that is essential for replicating DNA [37]. The N-terminal was shown to bind histone H1 and full length E1 can displace H1 from DNA [38]. The capacity of elongation is diminished when removing the E1 N-terminal, and this decreases production of new viral DNA [37, 39]. The C-terminal has been shown to be necessary and sufficient for interaction between E1 with the E2 DNA-binding domain [40, 41]. The E1 protein is generally expressed at a low level and is only efficiently active when E2 also is present.

1.1.5.2 E2

E2 plays several important roles in the viral cycle. The dimeric E2 protein is necessary for viral gene expression and viral DNA replication by binding to sites in the LCR as well as for regulation of transcription [42-44]. In addition, E2 together with E1 and cellular DNA forms an E1-E2-DNA complex, which enhances the ability to activate transcription and

replication [45]. High-level of E2 protein expression down regulates transcription of the oncogenes E6 and E7 [46]. In cervical carcinogenesis, disruption of E2 ORF due to integration of viral DNA is a late event, not occurring before CIN III lesions, and the loss of E2 repression can explain the increase of E6 and E7 transcription in late lesions [47-50].

1.1.5.3 E4

E4 originates from a viral transcript that consists of the first five amino acids of E1 fused to the E4 coding sequences, and is formed by a single splice between the E1 ORF and the E4 ORF [51, 52]. Moreover, the E4 protein is synthesized in the late phase of the viral life cycle and exclusively localized within the differentiating layer of human epithelium in a differentiation-dependent way [53, 54]. The E1 gene provides an initiation codon for E4 translation, which precedes transcription of capsid proteins L1 and L2 [55]. The E4 protein associates with multimeric structures in the cytoplasm and binds to the cytoskeleton [56-59]. In addition, E4 induces cell cycle arrest in G2 by a checkpoint pathway with the Cdc2-cyclin B complex [60-63]. In this case, cells infected with HPV continue to replicate during differentiation. A recent report suggested that E1^E4 proteins mediate keratin phosphorylation and ubiquitinilation, which may target the differentiation-dependent keratins [64]. Thus, E4 plays a significant role in the viral life cycle, and could be important to provide the best conditions for late viral functions in suprabasal keratinocytes.

1.1.5.4 E5

The E5 protein consists of 83 amino acids and is 10 kDa in size. It is a hydrophobic membrane bound protein, and in endoplasmic reticulum (ER), it disrupts the cell actin cytoskeleton and inhibits endocytic trafficking from early to late endocytic structures [65, 66]. Most of its biological effects are mediated through its N-terminal, which is also the most hydrophobic domain [67]. E5 is an oncoprotein that enhances immortalization of human keratinocytes and transforms fibroblasts as well as stimulates cell proliferation and increases viral gene expression together with E7 gene [68-70]. Moreover, the E5 protein is expressed in the lower part of the epithelium in low grade squamous intraepithelial lesions (SILs), while it is expressed throughout high grade SILs and in invasive squamous carcinoma [71]. In addition, E5 is mainly expressed in episomal viral DNA and deleted upon integration into the host genome [71].

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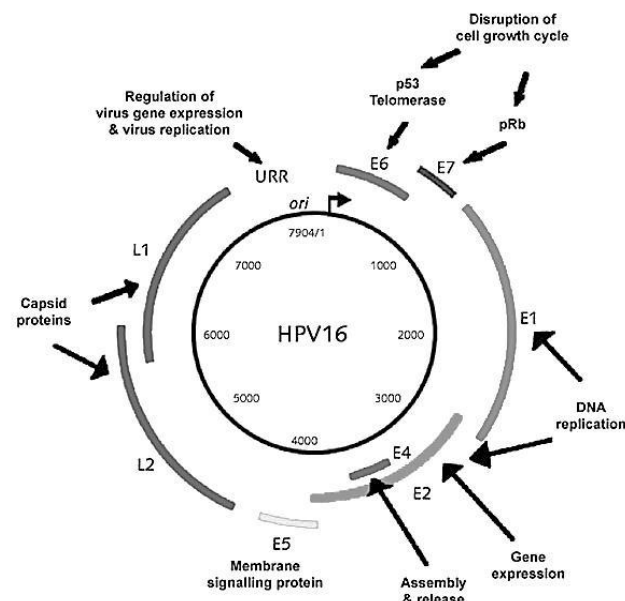
Figure 5. Functions of different viral gene and proteins

There are several pathways that E5 targets the cell, and one is the epidermal growth factor receptor (EGFR). The higher E5 expression, the lower internalization of EGFR and more activated EGFR receptors recycle back to the cell surface. This way, the E5 protein has the ability to up-regulate EGFR expression two to five-fold [70, 72]. E5 also prevents endosomal acidification and EGFR degradation by the association with the 16 kDa subunit of vacuolar proton-ATPase [73]. In an EGFR dependent way, E5 enhances the cell cycle into S phase and targets cyclooxygenase-2 (COX-2) [24, 74]. Other pathways are: inhibition of p21 expression at the transcriptional level [75], and overexpression of the vascular endothelial growth factor (VEGF) through MEK-ERK1/2 and PI3K/Akt pathways [76].

E5 also decreases expression of the major histocompatibility (MHCII) complex on the cell surface in response to interferon gamma (IFN-γ) treatment and reduces human leukocyte antigen-I (HLA-I) by binding to its heavy chain and preventing its expression [77, 78]. Together these findings demonstrate a role of E5 for avoiding immune surveillance and escaping elimination by the immune system.

1.1.5.5 E6

E6 gene is an important oncogene with many functions and consists of 151 amino acids, which are well conserved with two atypical zinc fingers containing two cysteines (Cys-X-X-Cys) [79]. Its best-known activity is to degrade the tumor suppressor protein p53, by binding to the conserved LXXLL motif from cellular E6-associated protein (E6AP) and this E6/E6AP complex targets the central region of p53, which then becomes

ubiquitinated and degraded by proteasomes [80-82]. Notably, p53 degradation is observed only in HR-HPV types and not in LR-HPV types [83]. Thus, HPV-mediated p53 degradation is an essential step for carcinogenesis [84]. E6 proteins are also involved in many other cell differentiation progressions [85]. Importantly, E6 protein binds transcriptional coactivators CBP/p300 in order to inhibit transcriptional activity and promotes HPV early transcription activities [86]. Moreover, HR-HPV E6 proteins have a PDZ-binding domain that interacts with membrane associated guanylate kinase (MAGUK) family members [87, 88]. This complex causes degradation of PDZ partners, promotes cell transformation and induces epithelial hyperplasia in vivo [89]. E6 can also associate with elimination of Bak to induce apoptotic in response to UV irradiation [90]. Furthermore, the E6 protein from HPV 16 has a high activity on the telomerase and human telomerase reverse transcriptase (hTERT) promoter, which may stimulate proliferation and prevent senescence of different types of cells [91, 92].

E6 can also affect interferon signaling. E6 inhibits IL-18 and interferon regulatory factor-3 (IRF-3), and it impairs Jak-STAT activation and decreases E-cadherin, which depletes Langerhans antigen-presenting cells in infected skin [93-96]. Last but not the least, E6 also has the ability to alter the differentiation of keratinocytes through the Notch signaling pathway and it also acts directly on G1/S transition [97, 98].

In summary, the biological functions of E6 have been studied extensively, but its association to the viral life cycle and cancer progression deserves further attention (Fig. 6).
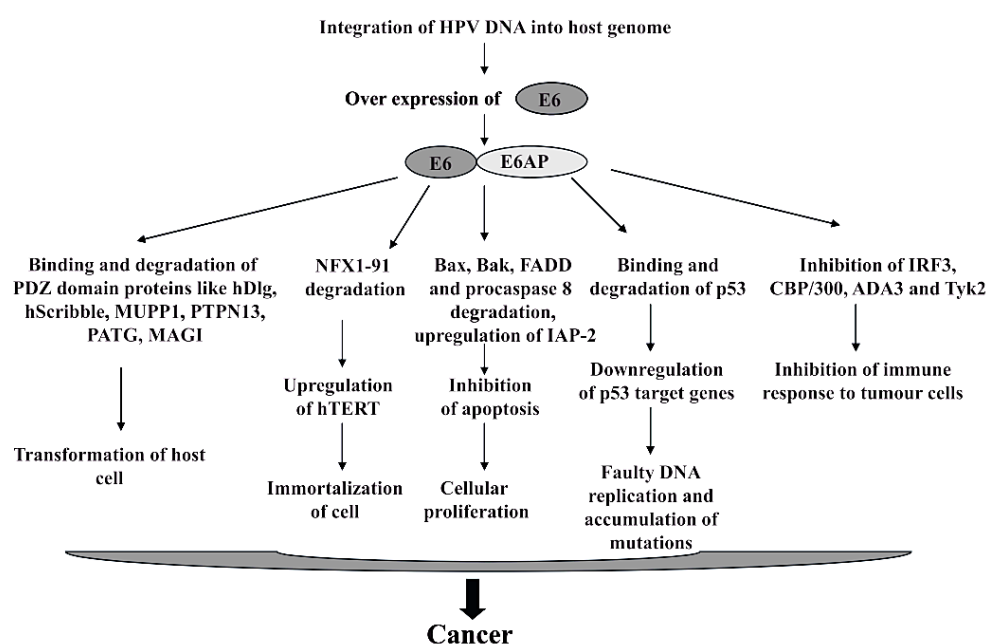
Figure 6. Multiple effects of the E6 protein
Source: J. Biosci. 2009 (34): 113-123

1.1.5.6 E7

E7 is one of the most important early genes and can target many cellular proteins as well as regulate the cell cycle and HPV replication efficiently. Many characteristics and biological activities of E7 have been discussed in reviews (Fig. 7) [85, 99, 100].

The E7 ORF encodes 98 amino acids and has homologies with the conserved regions 1 and 2 (CR1 and CR2) of adenovirus E1A protein and large T antigens of polyomaviruses [101]. The CR2 homology region contains LXCXE motif, which can bind to the tumor suppressor retinoblastoma (pRb1) and the related tumor suppressors p107 and p130 [102, 103]. These three termed pocket proteins modulate the transcriptional activities of the E2F family through multiple mechanisms [104]. The interaction between E7 with pRb can disrupt the formation of the pRb and E2F complex [105, 106]. This results in the release of active E2F, which in turn activates many other cell cycle genes like cyclin A and E and ends up in G1/S cell checkpoint dysregulation [107]. A difference in one amino acid was observed in pRb-binding domain of HR-HPV and LR-HPV, and this change contributes significantly with regard to the high affinity of HR-HPV binding to Rb [108]. Other studies demonstrate that HR-HPV E7 can induce pRb destabilization by proteasomal degradation, which is necessary for cellular transformation [109]. Of the other two pocket proteins, p103 and p130 have overlapping activates. P103 is more important for G1/S transition, while p130 controls more for the transition between G0 to G1. These two proteins together with pRB can regulate cell cycle progression [107, 110].
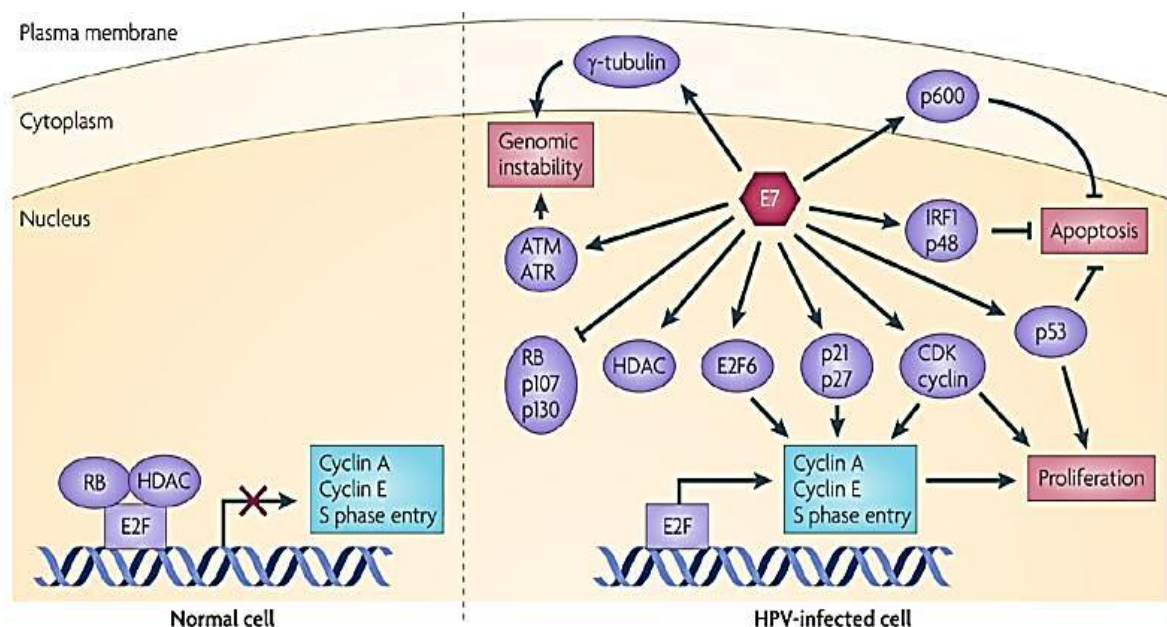
Figure 7. Multiple effects of E7 protein
Source: Nature Reviews Cancer. 2010 (10): 550-560

In addition to the above-mentioned main function for E7 on pRb, there are several other activities that are essential for HPV maintenance and proliferation. First of all, HPV E7 can interfere G1 growth arrest signaling mediated by p53 [111]. Expression of E7 proteins and their engagement of pRB increase the level of p53 in cells, and this can be counteracted by E6 [112]. Secondly, E7 can also associate $p21^{CIP1}$ and $p27^{KIP1}$ to abrogate the growth inhibitory activities of cdk2 in order to maintain a replication competent cellular and increase the level of cyclin A and E [113-116]. Notably, E7 expressing cells were also observed to have higher expression levels of $p16^{INK4A}$, which is an inhibitor of cdk4/cyclin D and cdk6/cyclin D and mediated G1 growth arrest [117]. This high expression level has in some reports been used as a diagnostic biomarker for HR-HPV infection [118].

E7 also abrogates transforming growth factor β (TGF-β) mediated growth inhibition [119] and promotes resistance to tumor necrosis factor α (TNF-α), which is an immune response mediator for viral infection [120]. It can also cause chromosomal instability [121, 122].

In summary, HPV E7 holds many abilities and activities with regard to cellular genome and signal pathways.

1.1.5.7 L1

The L1 ORF is highly conserved and major capsid protein L1 has an amazing property of in vitro self-assembly either alone or together with L2 and the ability of L1 to self-assemble has been utilized for vaccine production, which will be described in detail below [123]. The capsid of HPV consists of 360 L1 molecules with 72 subunits of pentameric capsomeres. L1 proteins from different types of HPV share similar motifs. HPV 16 L1 has been demonstrated to promote nuclear import [124]. Furthermore, it has function for viral entry by interacting with heparin sulfate proteoglycan (HSPG) and followed by internalization via clathrin-dependent endocytosis[16]. L1 positivity is more associated with Low-grade squamous intraepithelial lesions (LSIL) and LR-HPV types than with High-grade squamous intraepithelial lesions (HSIL) and HR-HPV [125, 126]. The loss of L1 expression in the more advanced lesions could be due to many reasons, and it is possible that the immune response selects for HPV infected cells lacking the L1 capsid protein.

1.1.5.8 L2

The L2 protein functions as an internal minor capsid protein and is associated with the outer shell L1 protein with up to 72 copies per capsid, although the exact number varies between different studies [127]. Nuclear localization signals on the L2 N- and C- terminal allows L2 to localize to the nucleus. The L2 protein plays essential functions in virus entry into the cells, localization to the nucleus, DNA binding, formation of capsid as well as viral stability [128-131]. In addition, the L2 protein can cause the re-localization and nuclear transport of the E2 protein [132]. Regarding vaccines, the L2 protein possibly contains regions that are more conserved across different HPV types and thus L2 could potentially be used for cross-protection in next generation vaccines against HPV [133, 134].

1.1.5.9 LCR

LCR is a non-coding region that includes several elements that control viral gene expression. HPV infection starts in the basal cells. Combinations of specific cellular factors interact with the LCR, and this initiates transcription of the viral E6 and E7 oncogenes. During malignant progression, the location of integration site, as well as the frequent loss of the E2 gene can have a dramatic effect on the viral LCR activity. When the repression of the LCR by E2 is lost, the activity of the E6 and E7 protein is promoted [135].

1.1.6 Viral entry, replication, transcription, assembly and release

HPV binds to HSPG receptors of the basal epithelia cells and this changes the hidden L2 protein, which then is exposed to a secondary-binding site [131, 136]. After viral endocytosis, the L2-HPV genome complex is transported to the nucleus [137]. Viral DNA then remains present as extrachromosomal plasmids in very low numbers in the basal cells. This is the genome maintenance stage for viral infection. Only during squamous differentiation, with viral replication, will the infected cells express elevated levels of E6 and E7 followed by activation of the late promoter p670 in the upper epithelial layers. This allows differentiated suprabasal cells to stay in S phase and efficiently produce viral DNA (Fig. 8) [138-140]. The mRNAs expressing higher levels of E1, E2, E6 and E7 provides conditions for viral genome replication in different ways. The E1 and E2 proteins amplify the viral DNA, while the E6 protein down regulates p53 and the E7 protein binds to Rb and keeps the cells in S-phase, and all this supports viral DNA amplification. There are also many other transcriptional and post transcriptional regulations by the early and late promoters in LCR region or by mRNA itself [141-143].
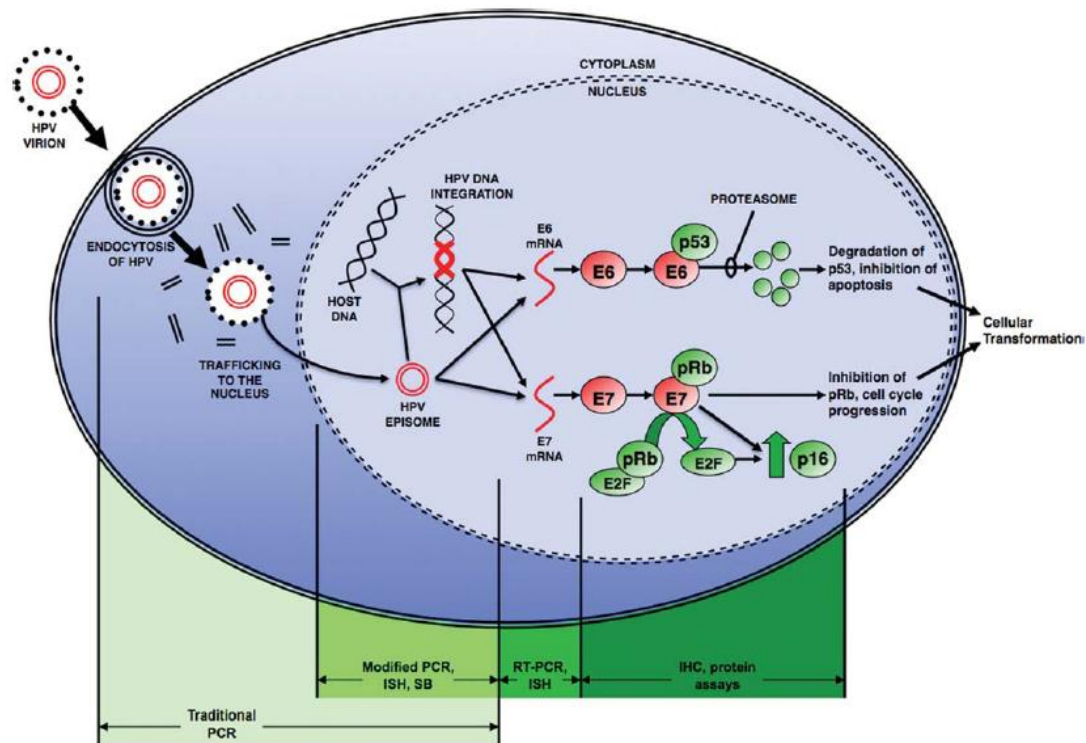
Figure 8. HPV infection in cell and the detection methods
Source: Laryngoscope. 2010 (120): 1756-1772

Along with the differentiation of the epithelial cells, the L1 and L2 genes are expressed in the upper epithelial layers. The virions become assembled in the cell nucleus and for this the capsid proteins are imported from cytoplasm to nucleus. Matured HPV are assembled in the highly differentiated cells, which require expression of the E2 protein and the L1 and L2 protein [144]. In the nucleus the genome is packed into the viral capsids, which then are transported to the a cell surface, where the E4 protein disrupts the keratin network in order to the release virus at the cell surface and thus allowing it to be shed from the epithelial surface [29, 59].

1.2 HPV-associated cancers

As mentioned above, HPV types can be divided into cutaneous and mucosal types and the latter are often divided into HR-HPV and LR-HPV. Cutaneous HPVs can not only cause skin warts on e.g. the hands and feet, but also cause epidermodysplasia verruciformis, and have previously been suggested to also cause non-melanoma skin cancer, however the latter is under debate recently [145, 146]. Mucosal LR-HPVs can cause condylomas in the genital tract [147, 148], and cause cervical dysplasia with low malignant potential but these are generally cleared within a few years [149]. Furthermore, LR-HPV can also cause warts in the upper airways e.g. recurrent respiratory papillomatosis (RRP) [150]. HR-HPVs can cause cervical cancer, many other anogenital carcinomas or pre-cancers, such as vulvar, vagina, anal and penile cancers [151-155], as well as oropharyngeal squamous cell cancer (OSCC) [156-158]. Below the association between HPV and some of these cancers is described.

1.2.1 HPV and cervical cancer

1.2.1.1 HPV and carcinogenesis in cervical cancer
Worldwide, cancer of the uterine cervix is the third most common cancer among women, with over 529,000 new cases and about 275,000 deaths in 2008, of which 88% from developing countries [157].
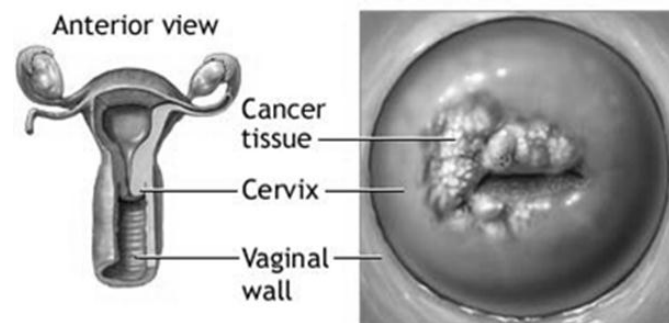
Figure 9. Cervical cancer site and tissue

In 1995, the IARC defined HPV 16 and HPV 18 as carcinogenic on the molecular level and by epidemiological studies [159]. Thereafter the carcinogenicity data was updated in IARC Volumes 90 and 100. It has now been established that HPV is strongly related with cervical cancer and its pre-cancerous conditions. All the carcinogenic HPV types belong to the alpha genus and there are lots of reports supporting the carcinogenicity of HR-HPV types in cervical cancer both in case-control studies, cohort studies and laboratory studies [160]. From statistic evaluation, persistent infections of HR-HPV is an extremely high absolute risk of CIN3 and cancer of the cervix [161]. Moreover, women tested negative

for HR-HPV are at low risk of cervical cancer comparing with HPV 16 and HPV 18 for more than 10 years [162].

1.2.1.2 Prevalence of HPV in cervical lesions and in cervical cancer

Cervical cancer is a cancer of relatively young people, with a mean age of patients around 45 to 50 years old. Normally micro-invasive cancer is not possible to detect and usually asymptomatic with limited symptoms such as e.g. vaginal discharge or irregular bleeding (Fig. 9). Most lesions are found as LISL in cervical tumor screening. When not discovered during screening, patients with advanced cancer usually contact the clinic because of pain or backache.

Overall around 83%-99.7% of all cervical cancers have been reported to be associated with HPV infection [161, 163-165]. Furthermore, a significant increase from 86% in earlier publications between 1990 and 1999 to 93% in later publications between 2006 and 2010 has been observed [163]. This may be due to the inclusion of more HPV types in the assay or to increased sensitivity of the assays. Among the HR-HPV types, HPV 16 is the most common type and it causes over 50% of all cervical cancer cases. HPV 18 is the second most common type in cancer of the cervix and is observed in about 15% of all the cases. Together, HPV 16 and 18 are responsible for approximately 70% of all cervical cancer cases, and the other HR-HPV types (e.g. HPV 45, 31, 33, 58 and 52) account for nearly all of the remaining cases [161, 163-166]. These latter types seem to have a weaker but still clear carcinogenic potential. Moreover, HPV 52 and HPV 58 are more common in Asia compared with other regions, while HPV 33 is more prevalent in Europe [163-165]. Less certain carcinogenic types like HPV 39, 51, 56 and 59 cause fewer cases of cervical cancer either by themselves or together with other types [163-165]. Some other HPV types like HPV 26 have been found to be active and carcinogenic, when in high viral loads in immune suppressed patients [167].

The vast majority of cervical cancer cases are squamous cell carcinomas (SCC), while adenocarcinomas (ADC) are somewhat less common. Several additionally rare cervical cancer types are seldom diagnosed. HPV 16 is more often observed in SCC than in ADC, while HPV 18 is more prevalent in ADC as compared to in SCC [163, 165, 168, 169]. Interestingly, countries with good pap smear screening programs report an increased proportion of ADC (~25% in most Western countries) compared to unscreened populations regardless of if there is a decreased prevalence of total cervical cancer cases [170, 171]. Besides, HPV 16 related types 31, 33, 35, 52 and 58 are also observed more frequently in SCC compared with ADC, while HPV 18 related type HPV 45 is more common in ADC than in SCC [165].

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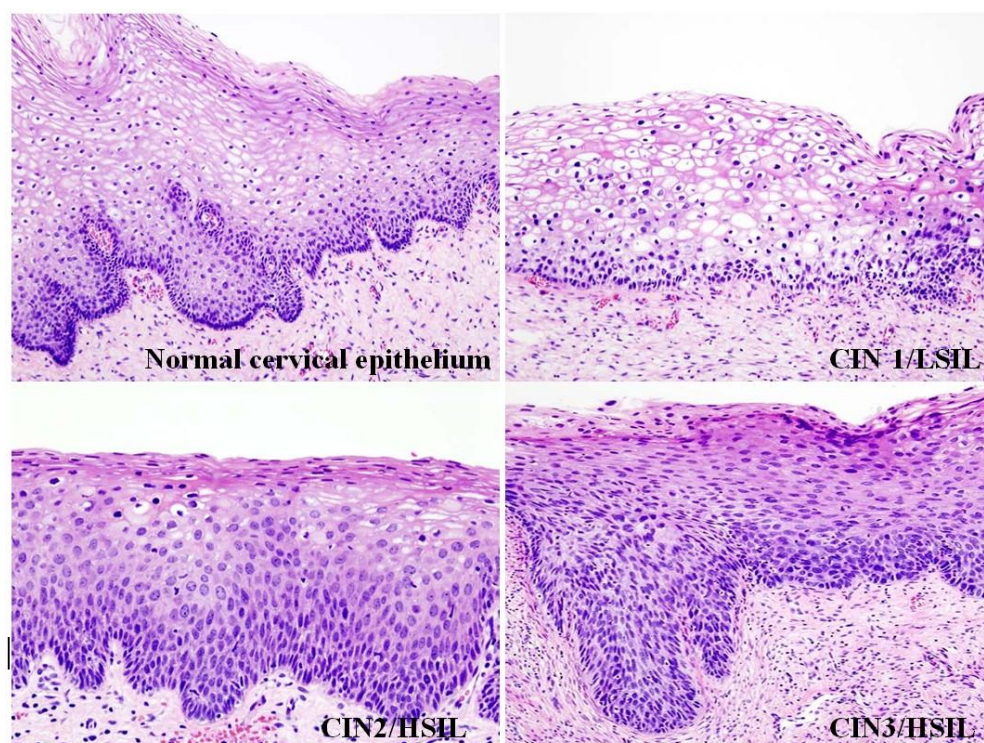
Figure 10. Histology grade and corresponding cytology of Cervical intraepithelial neoplasia (CIN)

In addition, HPV infection has been shown to be very common in young sexually active women in many reports both by others and by us in paper III and IV in this thesis [156, 172, 173]. Most of HPV infections are not visible. However, by colposcopy it is often possible to detect low-grade cervical intraepithelial neoplasm (CIN) (Fig. 10). Most HPV infections and low-grade CIN can be cleared by cell-mediated immunity without antibody detected. Only 10-15% of women do not clear their infections and thus have persistent HPV infections. This period can vary between three weeks to three years [174, 175]. As shown in Fig. 11, HPV is mainly episomal in CIN1 lesions with productive viral infection. Even at this stage, 80-90% of the infected women clear their HPV infections and their lesions, while the remaining 10-20% proceed to CIN2/3 [175]. In general, self-clearance of HR-HPV takes around 12-18 months [175]. From detection of HR-HPV until CIN3 development it usually takes three to five years and 30-40% of women with CIN3 proceed to cervical cancer which takes another ten to twenty years [176, 177]. However, the risk of cervical cancer for women with CIN3 women who receive an adequate initial treatment was reported to be 0.7% in 30 years compared with 50% in women who had persistent CIN3 in the past two years [176]. Thus only a minority of persistently HR-HPV infected women are at risk for cancer development. However, since many women are infected with HR-HPV, the women at risk for developing cervical cancer are numerous.
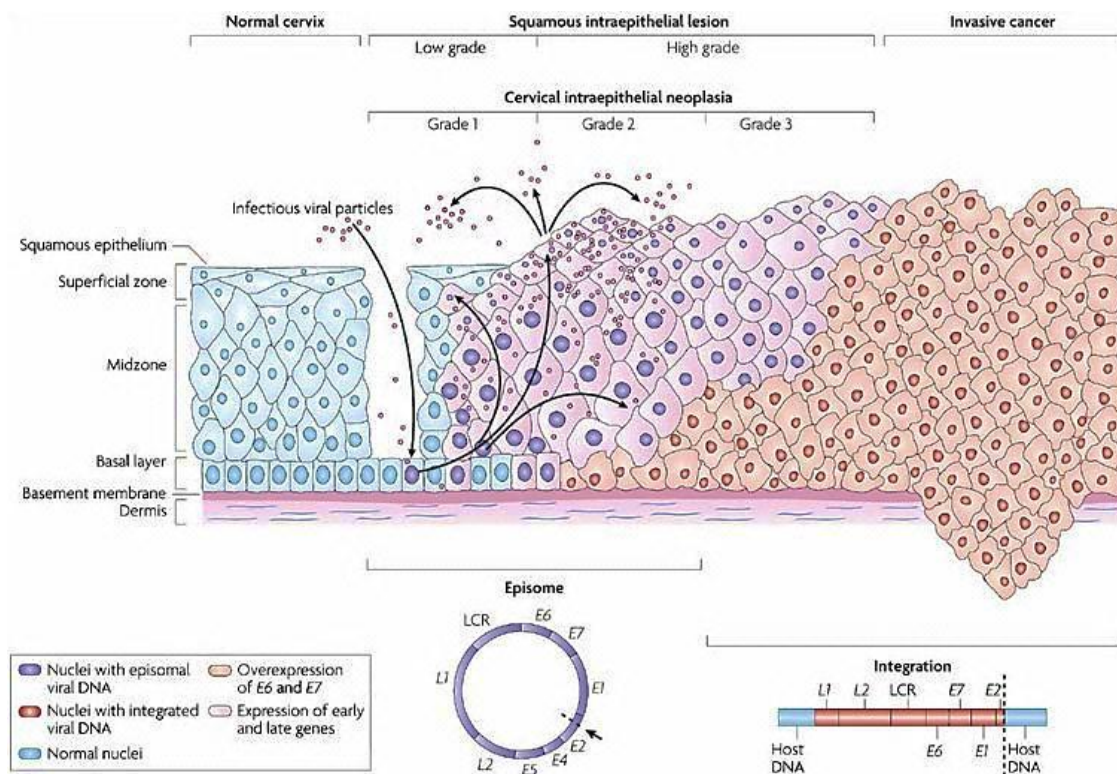
Figure 11. Pathogenesis steps of HPV infection
Source: Nature Reviews Cancer 2007 (7): 11-22

Furthermore, HPV infection as well as the most frequently and carcinogenic genotypes HPV 16 and HPV 18 are more commonly observed in invasive cervical cancer as compared to that in women with normal cytology or LSIL. HPV infection was observed in 71.1% of LSIL and much higher (up to 84.2%) in HSIL and most common (up to 87.6%) in SCC cases. The dominant type HPV 16 was also elevated from 18.7% in LSIL to 45.0% in HSIL and to 54.3% in SCC. HPV 18 was, similar to HPV 16, more prevalent in SCC (12.6%) than in HSIL (7.0%) and in LSIL (6.1%) [165, 178]. The data regarding HPV prevalence in cervical cancer in Stockholm has been included as Paper II and will be discussed more below [179].

1.2.1.3 Prevalence of HPV in normal cytology women and age distribution
HPV infection is very common and it was shown that approximately 10% of women in the world with normal cervical cytology harbored HPV and of these 23% were with HPV 16 and 8.5% HPV 18 (Fig. 12) [180, 181]. Consequently, HPV is one of the most common sexually transmitted infections, especially among the sexually active adults. The highest prevalence is observed in eastern Africa where nearly one third of women with normal cytology have HPV infection while the lowest prevalence is in South-Eastern Asia with 6.2% [181].
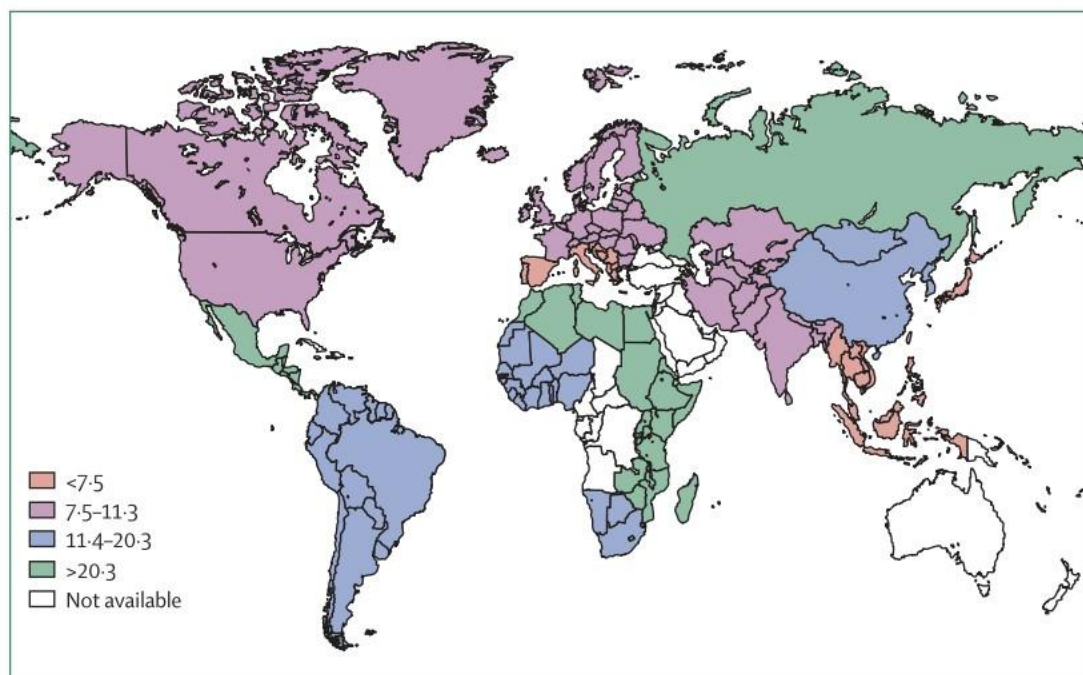
Figure 12. Distribution of HPV in worldwide
Source: Lancet Infect Dis. 2007 (207): 453-459

There is a similar pattern of HPV infection in different age groups in all regions. The prevalence is high in women younger than 34 years with the most frequently infected being the young and sexually active group below 25 years of age and then a decrease is observed between 35-54 years of age and this is thereafter followed with increase of HPV prevalence. An exception is in Asia, where the prevalence declines with increasing also at higher age. This prevalence of overall HPV is higher in less developed countries in all age groups than in more developed countries [181]. HPV prevalence and types in various age groups in Stockholm will be presented in paper III and IV and will be discussed more below [173].

1.2.1.4 HPV and related biomarkers in relation to prognosis in cervical cancer and cancer development

Presence of HPV is useful biomarker for cervical cancer, because it can be easily followed. Moreover, the cyclin-dependent kinase inhibitor p16(INK4a) is over-expressed in nearly all HPV-transformed cells [182] and p16 overexpression was shown to correlate to better survival of patients after chemoradiation therapy with advanced-stage invasive cervical cancer [183]. In addition, E-cadherin, Ki67 together with p16 were shown to identify lesions with high risk of cancer progression and several other biomarkers have been explored[184-186]. Many microRNAs such as miR-21, miR-146a and miR-218, their relation to HPV and their roles as suppressors or oncogenes in cervical cancer and correlation to treatment response have been investigated sometimes with no concordant results, for details see review [187].

1.2.2 HPV and OSCC

Another HPV related cancer, which also will be presented in this thesis, is oropharyngeal squamous cell carcinoma (OSCC), a subgroup of the head and neck squamous cell carcinoma (HNSCC) group. HNSCC is the sixth most common type of cancer in the world and accounts for 100.000 cancer cases per year worldwide [157]. HNSCC includes cancers of the OSCC as well as e.g. the lip, the oral cavity, the nasal cavity, the paranasal sinuses, the hypopharynx, and the larynx (Fig. 13). The main risk factors for HNSCC are alcohol or tobacco abuse, other factors such as dietary factors and genetic factors may also count. In 2007, HPV was acknowledged by IARC for OSCC [188] based on reports by others and us [189, 190].

1.2.2.1 Carcinogenesis of HPV in OSCC

Similar to cervical cancer, it has been shown that individuals with HPV positive anogenital cancer have a higher risk to develop cancer in tonsils or oral cavity, which both OSCCs [191-193]. Furthermore, women with cervical cancer have an increased incidence of tonsillar cancer, and their husbands also have an increased risk of presenting tonsillar cancer and other upper aerodigestive tract cancers [194]. Taking into account the similarity in histology between oral mucosa and uterine cervix as well as that HPV infection in the cervix is transmitted by sexual contact, one can easily infer that oro-genital contact may be the dominant cause of HPV infection in the oral cavity [195, 196]. Furthermore, viral oncogene expression and viral integration in the cellular genome has been observed in tonsillar cancer [197, 198].
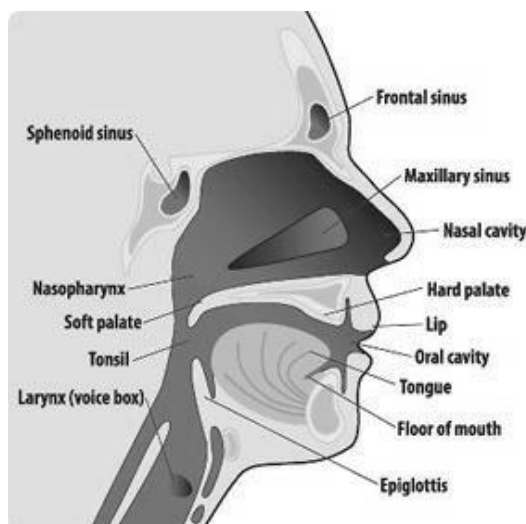
Figure 13. Head and neck cancer subsites

OSCC accounts for around 10% of all HNSCC and is dominated by cancer of the tonsil and the base of tongue which account for 70-80% of the cases, but also includes cancer in the oropharynx outside these regions and the soft palate [199].

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1.2.2.2 Prevalence of HPV in OSCC

The overall prevalence of HPV DNA in HNSCC is around 26%, while in OSCC it is around 36% or higher and varies at different subsites and geographical regions [200, 201]. The prevalence of HPV in OSCC has increased significantly from around 40% before 2000, to 64% 2000-2004, and 72% 2005-2009 [202]. Tonsillar cancer has the highest HPV prevalence with 45-90% of all cases positive for HPV [190, 201, 203-205]. In OSCC, similar to cervical cancer, HPV 16 is the most common type with up to 90% of the tonsillar cases being HPV 16 positive, while other HPV types such as HPV 18, 33, 35 or 45 account for the remaining HPV positive cases [158, 203, 205-207].

HPV has been suggested to be more frequently episomal in OSCC as compared to cervical cancer and this difference has been assumed to be due to possible differences in the mechanisms of action of HPV in cervical cancer and OSCC development [208]. However, then again by comparative genomic hybridization (CGH) show many similarities between tonsillar cancer and cervical cancer [209].

Recently, in many Western countries there has been an increase in the incidence of OSCC despite a decrease in smoking, and this increase has been suggested to be due to an epidemical increase of HPV positive OSCC [210]. Our group has reported an increase of the prevalence of HPV in tonsillar cancer from the 1970s to 2006, from 23% to 93% [203, 211]. In addition, in this thesis, we show a similar increase in the prevalence of HPV between 1998-2007 in base of tongue cancer (Paper I) and this will be presented in more detail below [203, 212].

1.2.2.3 HPV and related biomarkers in OSCC and correlation to prognosis

Patients with tonsillar or base of tongue cancer present no or few symptoms e.g. a sore throat, earache or discomfort when swallowing, irrespective of the HPV status of the tumors. However, patients with HPV positive OSCC are usually somewhat younger than those with HPV negative cancer and do not always have a history of smoking. HPV positive OSCC differs to some extent from HPV negative OSCC in that they are more often less differentiated and frequently show basaloid histopathology, but these differences are not sufficient to distinguish HPV positive and negative OSCC [213]. However, in HPV positive OSCC, similar to cervical cancer, p16 is often, but not always overexpressed [214, 215]. Furthermore, the presence of HPV, p16 overexpression and younger age in OSCC is correlated to a better clinical outcome [190, 210, 216-218]. These significant differences may be of importance for making decisions concerning treatment of patients with HPV positive and HPV negative OSCC. The better survival for patients with HPV positive OSCC could be due to that they are more sensitive to radiation therapy [217].

1.2.2.4 Tumor classification and staging

Classification of tumor stage in these papers was done according the International Union against Cancer. The Mandatory parameters are listed below.

T: size or direct extent of the primary tumor

    Tx: tumor cannot be evaluated

    Tis: carcinoma in situ

    T0: no signs of tumor

    T1, T2, T3, T4: size and/or extension of the primary tumor

N: degree of spread to regional lymph nodes

    Nx: lymph nodes cannot be evaluated

    N0: tumor cells absent from regional lymph nodes

    N1: regional lymph node metastasis present; (at some sites: tumor spread to closest or small number of regional lymph nodes)

    N2: tumor spread to an extent between N1 and N3 (N2 is not used at all sites)

    N3: tumor spread to more distant or numerous regional lymph nodes (N3 is not used at all sites)

M: presence of metastasis

    Mx: distant metastasis cannot be evaluated

    M0: no distant metastasis

    M1: metastasis to distant organs (beyond regional lymph nodes)

1.2.3 HPV variants and cancer development

As mentioned in the variants and taxonomy section, HPV can have branches or variants within the same HPV type, and this includes HPV 16 especially. In addition, most studies on HPV 16 variants were on E6 and E7. In cancer tissues, the HPV 16 E7 protein is often more conserved, while HPV 16 E6 shows more variations both with the major variant lineages and with single nucleotide alterations [219, 220]. The most frequent mutation in HPV 16 E6 is E-T350G (i.e. a T to G transition at nt 350), which causes an amino acid change, L83V (leucine to valanine at a.a. 83). The HPV 16 variant L83V was shown to have an association with viral persistence and cancer progression of cervical carcinoma in some studies, but this association is in need of further investigation [221-223]. Besides L83V, some other variants have also been investigated for their effects on cancer progression [224]. Although many studies have been performed on HPV 16 variants in cervical cancer, only few studies have analyzed the effects of these variants in OSCC [189, 225]. In paper V, we have studied different variants not only in cancers like cervical cancer and tonsillar cancer, but also in cervical samples from infected young adults.

1.3 Preventive measures, screening and vaccines

To prevent HPV induced cancer, it is important to clear HR-HPV infection. This can occur naturally, and if not by different interventions as presented below.

1.3.1 Host immune response to HPV

Most of HPV infections are cleared by the immune response and different immune cells are involved. Cellular immunity can be initiated by dendritic cells can present HPV antigens in the context of MHC class I and II in order to up-regulate the secretion of cytokine and priming of naive CD4+ and CD8+ T cells. Changes in the immune response towards HPV in the cervix can sometimes be associated with progression of cervical cancer [226]. CD4+ and CD4+/CD8+ ratio are often higher in CIN1 than CIN3 and invasive cancer, while CD8+ cells are more frequently observed in invasive cancer [227]. Moreover, in cervical cancer, HLA class I is frequently completely lost or down regulated, the NK cell population can be decreased and CD4+CD25+Foxp3+ regulatory T cells (Treg) activity can be increased, together demonstrating a poor immune response in cancer patients [228-230]. The immune response in head and cancer is most likely similar, but much less is known [231]. Also antibodies against specific HPV types can be detected after productive HPV and it is possible that these antibodies have an effect on diminishing/preventing viral spread, as has been demonstrated after HPV vaccination [232]. It has also been shown that anti-angiogenic therapy can in some cases promote the immune response by decreasing immunosuppressive regulatory T-cells, immunosuppressive cytokines and transcription factor (STAT3). Many other candidates for immune response and immunotherapy have been shown [231].

1.3.2 Screening for cervical cancer and potential other screening

The Papanicolaou (Pap) smear test is a screening test that was described already in 1940s and it is used for early detection of precancerous CIN stages in order to intervene and prevent the development of cervical cancer [233] (Fig. 14). Pap smear screening when used in different countries, normally starts at the ages between 20 and 25 and continues until the ages of 50 or 60. In Sweden, it is recommended that Pap smear screening should be done every third year for women aged 23-50 years, and every fifth year for women aged 51-60 years. However, it is in developing countries which lack organized Pap smear screening programs, that nearly 80% of all the cervical cancer cases are reported. On the other hand, in industrialized countries, Pap smear screening has effectively reduced the incidence and death cases of cervical cancer[234].
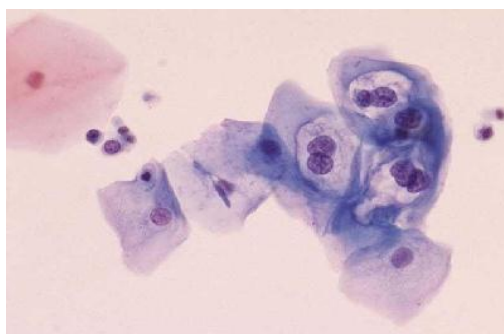
Figure 14. LSIL from Pap smear
Source: NCI Bethesda System web Atlas

The tight relation between HPV and cervical cancer, has now promoted HPV DNA testing on cervical swabs, which is used for identifying women with HPV infections or lesions. The sensitivity and specificity of HPV DNA testing is much better than cytology alone when used in women above 30 years of age, when the prevalence of HPV infection has decreased. However, in younger women below 30 years of age with a high prevalence of HPV infection it is not possible to use HPV testing as a screening method without cytology [160]. Incorporation of HPV DNA testing into existing screening programs is currently an important issue and different strategies that combine HPV DNA testing with screening need to be evaluated in order to avoid pitfalls and unnecessary costs [160].

It is also possible to detect HPV in the oral tract, by collecting mouthwash samples or taking tonsillar swabs [235]. However, if this is the most optimal way needs to be investigated further. In addition, whether there is a need for oral screening for HPV DNA should also be examined further.

1.3.3 Treatment for HPV-associated cancers

1.3.3.1 Treatment of cervical cancer and oropharyngeal cancer at the Karolinska University Hospital

According to a new International Federation of Gynecology and Obstetrics (FIGO) staging system [236], patients with cervical carcinoma lesions strictly confined to the cervix or with stromal invasion ≤ 4.0 cm in dimension (below stadium 1B2 in FIGO) are treated with surgery. Patients with cervical carcinoma invading beyond the uterus or have > 4.0 cm stomal invasion (above stadium 1B2 in FIGO) are treated with radiochemotherapy, including radiotherapy once per day and cisplation each week as well as brachytherapy (a form of radiotherapy, where the radiation source is placed inside or next to the area requiring treatment). Preoperative brachytherapy was more commonly used before.

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The main treatment for OSCC, including tonsillar and base of tongue cancer is external radiotherapy together with interstitial radiotherapy. Sometimes, chemotherapy is also used. Surgical treatment was more common over 20 years ago with radical excision and free flap coverage of the primary site. Now it is more often used for salvage surgery.

1.3.3.2 HPV-target treatment

HPV related cancers persistent to express HPV viral genes and oncogenes from HPV make them potential sites for targeted treatment. Inhibiting oncogene expression should be possible to prevent the growth of cancers. Medicines or therapeutic vaccines that interfere with the activities of viral genes, proteins or viral micro RNAs could potentially be used to combat HPV-positive cancers.

1.3.4 Vaccines against HPV

There are currently two HPV vaccines that been approved by the US Food and Drug Administration (FDA): Gardasil (Merck, USA) and Cervarix (Glaxo-SmithKline, UK). Both of them are based on VLPs, self-assembled from L1 proteins produced in eukaryotic expression systems, but they are combined with different adjuvants [237, 238]. In Gardasil the adjuvant used is amorphous aluminum hydroxyphosphate sulfate, while aluminium hydroxide and the immune modulator monophosphoryl lipid A (AS04) are used in Cervarix [239]. Gardasil is a quadrivalent vaccine against HPV 6, 11, 16 and 18 and Cervarix is a bivalent vaccine against HPV 16 and 18. Both of the vaccines are given by muscular injection with three doses for half year.

Lately, a large phase III trial study on Cervarix called PATRICIA was done which included 18,644 women from 14 countries and with a follow up of 34.9 months after the third dose. This study demonstrated that the vaccine efficacy associated with HPV 16/18 was 98.1% against CIN2+ if the women had normal cytology and were HPV DNA negative at the baseline. In fact, more specifically there was 100% protection against HPV 16 and 92.3% protection against HPV 18. Furthermore, in other studies Cervarix has shown a high cross-protection against HPV 31 and 45, with a 77.5% and 81.4% efficacy for protection against persistent HPV 31 and 45 infection, but notably with a 100% vaccine efficacy against CIN2+ or adenocarcinoma in situ caused by these two HPV types [240, 241]. Vaccine efficacy against CIN2+ associated with HPV 31, 33, 45, 52, 58 was over 68% and with all 14 oncogenic HPV types combined was more than 60% [240].

Gardasil trials have also been done, and in 2007, FUTURE I and FUTURE II were performed and involved 17,622 women. In these studies, a very high vaccine efficacy was

also observed. Gardasil prevented 98% of HPV 16/18 related high-grade cervical lesions if the women had not been previously exposed to either HPV 16 or HPV 18. Especially for HPV 18, this vaccine demonstrated a 100% vaccine efficacy against CIN2+, and over 95% efficacy for HPV 16 [242, 243]. However, if the women were HPV 16/18 DNA positive at the baseline, irrespective of serostatus, vaccine efficacy was only 5.8%. Nevertheless, it was higher and reached 35.2% if the women were HPV DNA positive and seronegative at study entry [242, 243]. Gardasil has been observed to decrease HPV 31 and HPV 45 infections by 43.6% for CIN1-3 and 58.7% for CIN2+ or adenocarcinoma in situ. The vaccine efficacy for CIN2+ associated with the 10 none vaccine HPV types (HPV 31, 33 ,35, 39, 45, 51, 52, 56, 58, 59) was 32.5% [244]. In addition, Gardasil was shown to be able prevent high-grade vulvar and vaginal lesions as well as cervical warts [243, 245].

All of these cross-protections may help to avoid cervical cancer for another 6-10% of the vaccinated women, but the next generation of vaccines would still need to protect against the remaining 20-25% cancer cases [246]. Both vaccines showed an antibody peak at month 7 with an initial decrease over the next year, but the levels then remained stable for seven to 10 years [247, 248]. High-level protection was observed for persistent infection with over 90% [240]. Modeling estimates indicate that the level will keep on for at least 12 years and perhaps life-long for most vaccinated women [249]. Notably, antibody levels were much higher with Cervarix than with Gardasil [250].

These vaccines are safe and it should be most cost-effectiveness to vaccinate young adults before they become sexually active. Recently one study indicated that HPV vaccination also reduced the risk of anal cancer and precancerous lesions in young men who have sex with men [251]. Probably the same holds true in head and neck cancer, but little has been done until now [252]. Considering the modest female vaccination rates and a broad range of disease outcomes, male vaccination was recommended to be given to boys ages 11-12 in the US. In Stockholm, general introduction of public HPV vaccination (Gardasil) of girls born 1993-1999 has just started now in 2012.

2. Aim

- To investigate possible changes of HPV prevalence of base of tongue cancer in Stockholm from 1998 to 2007.

- To evaluate the prevalence of different HPV types in cancer of the uterine cervix, including both squamous cell carcinoma (SCC) and adenocarcinoma ADC between 2003 and 2008 in Stockholm.

- To study the prevalence of different HPV types in the cervical tract of 15-23 year old women visiting a youth health clinic in Stockholm, before the public vaccination.

- To follow the prevalence of different HPV types in the oral tract among young women/men at a youth health clinic in Stockholm before public HPV vaccination.

- To explore the frequencies of different HPV 16 E6 variants in tonsillar squamous cell carcinoma (TSCC), in cervical cancer (CC), as well as in cervical samples (CS).

3. Study individuals, Materials and Methods

3.1 Study individuals

3.1.1 Patients

Patients with base of tongue squamous cell carcinoma diagnosed between 1998 and 2007 in Stockholm, were identified through the Swedish Cancer Registry using International Classification of Diseases system (ICD) 10, code C01.9. In total, 109 patients were identified, and 95 with available pretreatment samples were included in Paper I.

Patients with cancer of the uterine cervix diagnosed between 2003 and 2008 at the Karolinska University Hospital were identified using Systematized Nomenclature of Medicine (SNOMED) coding system. With this system 215 patients were identified through Department of Pathology and Cytology and 160 with available pretreatment samples were included in paper II and 52 of these also included in paper V.

Patients with tonsillar squamous cell carcinoma diagnosed between 2000 and 2007 in Stockholm were identified through the Swedish Cancer Registry using ICD-7 code 145.0, since they were included in previous papers, where this ICD system had been used. Of the totally 228 identified patients during this period with available sufficient material, 108 were included in paper V. Clinical data on all patients and tumor characteristics were obtained from the medical records at the head and neck surgery, or the pathology departments at the Karolinska University Hospital. The studies were conducted according to ethical permissions 2005/431-31/4, 2005/1330-32 and 2009/1278-31/4 for head and neck cancer region, and 2008/813-31/2 for cervical cancer from the Regional Ethical Committee at Karolinska Institutet, Stockholm, Sweden.

3.1.2 Young adults aged 15-23 attending a youth health care center

Young women and men attending a youth clinic in the center of Stockholm participated in papers III and IV. Annually around 4000 young women and 800 young men from local area or attending the different universities of Stockholm, visit this clinic annually for birth control advice and for the treatment of sexually transmitted diseases. Participation in the study was voluntary and anonymous. Written consent was obtained and only data on the year and month of birth as well as HPV vaccination status were documented. The numbers of participants can seemingly be seen as low. However, the main reason was the lack of enrolment during periods of high workload and when asked most individuals participated. Paper III comprised the cervical cohort study, where 615 women were included, of which 65 young girls had been vaccinated with Gardasil or Cervarix and which was performed between December 2008 until February 2010. Paper IV comprised the oral cohort study which was initiated during the December 2009 to October 2011 and it included 408 women

and 82 men, all non-HPV vaccinated. The permissions for the studies of HPV prevalence in the cervical tract and for the studies of HPV prevalence in the oral tract, 2008/870-31/4 and 2009/1147-31/2 respectively, were approved by the Stockholm Regional Ethics Committee.

3.2 Materials

3.2.1 Cancer tissues

All base of tongue and tonsillar cancer pre-treatment samples were obtained as formalin fixed paraffin-embedded tissues. *All uterine cervical carcinoma tissue samples* were also obtained as formalin-fixed paraffin-embedded tissues. Furthermore, for verification of the original diagnosis a second pathologist reviewed all cancer specimens. In Paper I, 95 cases of base of tongue cancer biopsies could be obtained from the109 patients diagnosed between 1998 and 2007 in the Stockholm region. In paper II, 154 of the 215 cervical cancer cases, diagnosed 2003-2008, were verified to contain sufficient cancer material for further analysis. In paper V, 52 HPV 16 cervical cancer cases and 108 HPV 16 positive tonsillar cancer cases were included in the analysis for HPV 16 E6 variants. Disease-free survival (DFS) was defined as time from the date of diagnosis to the date of the last known occasion that the patient was disease-free, or the date of disease recurrence (local, regional or distant recurrence). Death without documented recurrence was censored at the date of death.

3.2.2 Cervical and oral tract samples from young adults

In paper III, 615 cervical samples were obtained by self-test or by a midwife using nylon flocked swabs and preserved in sterile tubes containing 5 ml SurePath preservation solution; these were stored at 4°C. In paper IV, 180 cervical samples were taken as described above and 483 oral samples with sufficient material from 401 women and 82 men were collected. Oral samples were obtained after 30 seconds mouthwash with 15ml 50% Listerine (Johnson and Johnson Consumer Nordic), stored at +4°C maximum 3 days, centrifuged at 6000g, 10 min, and the pellet stored at -20°C. In paper V, 51 HPV 16 positive of the 615 cervical samples above were included together with 52 and 108 respectively of the cervical and tonsillar cancer cases described above.

3.3 Methodology

3.3.1 DNA and RNA extraction (Paper I-V)

For paraffin-embedded cancer biopsies, DNA was extracted from $2\times$ 15 μm tissue slides with the Roche High Pure RNA Isolation kit (Roche, Roche Diagnostics Scandinavia AB, Sweden), but with the exclusion of DNase treatment, while RNA extraction was performed using $4\times$ 15 μm tissue slides and DNase digestion. In parallel, for every five slices a blank control sample without any DNA was taken and treated in the same way to exclude cross-contamination.

For cervical samples, DNA extraction was performed with the Roche High Pure PCR Template Preparation Kit. Oral sample DNA was extracted with Gentra Puregene Buccal Cell Kit (Qiagen, QIAGEN AB, Sweden), and dissolved in 100 μl DNA Hydration Solution.

3.3.2 HPV DNA and RNA detection by polymerase chain reaction (PCR) and real-time PCR (Paper I)

In paper I, in order to be able to compare with earlier HPV prevalence data for tonsillar cancer, presence of HPV DNA was analyzed by PCR using general primer pairs GP5+/6+ and CPI/IIG. GP5+/6+ primer pairs recognize L1 ORF at highly conserved sequences at 3' end and give a bind of around 140 bp fragment, while CPI/IIG primer pairs recognize E1 ORF and give a detection of around 188 bp fragment [253, 254]. In addition, all samples were tested using HPV 16 and HPV 33 type specific PCR primer which detect E6 and E7 ORF of HPV genome, respectively. Samples tested negative for general primers and HPV 16, were tested for the human housekeeping gene S14 to verify the presence of amplifiable DNA. All PCR products were visualized on agarose gels, and only products with the appropriate size were considered as positive. Samples positive for HPV with general primers and negative for HPV 16, were sequenced for determination of HPV type on the amplicon generated from these primers.

For analyses of HPV RNA, in paper I, cDNA was synthesized from 800 to 1,000 ng extracted RNA using SuperScript1 III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). A qualitative real-time PCR with a SYBR green protocol was used to detect HPV 16 E6 and E7 cDNA with E6 and E7 specific primers. E6 primers were 5′-GAGCGACCCAGAAAGTTACCA-3′ and 5′-AAATCCGCAAAAGCAAAGTCA-3′ (131 bp) and for E7 5′-ACCGGACAGAGCCCATTACAA-3′ and 5′-GTGCCCATTAACAGGTCTTCC-3′ (120 bp). cDNA was synthesised from extracted RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, USA) and 50 ng used analyzed in a quantitative real-time PCR with SYBR Green

Supermix (iQ SYBR Green Supermix, Bio-Rad, USA) and 10 pM of either E6 or E7 primers. Samples were run in triplicate with a standard dilution series for DNA quantification. Samples were only considered as positive or negative for HPV 16 E6 and E7 RNA expression. E6/E7 HPV RNA negative samples were further tested for amplifiable cDNA, using a SYBR green protocol with GUSB primers.

3.3.3 Luminex and Magpix (Papers II-IV)

For the studies in Paper II and III, the HPV genotyping kit from Multimetrix (Heidelberg, Germany), for use with a Luminex analyzer, was utilized for HPV typing [255]. With this kit 24 HPV types are analyzed in parallel: 15 HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), 3 putative HR types (26, 53 and 66) and 6 LR types (6, 11, 42, 43, 44 and 70) (Fig. 15). In this assay broad-spectrum GP5+/6 + primers (BGP5+ /BGP6 +) were utilized. The beta-globin gene was simultaneously assayed in order to verify the presence of amplifiable DNA.

Direct Hybridization Assay
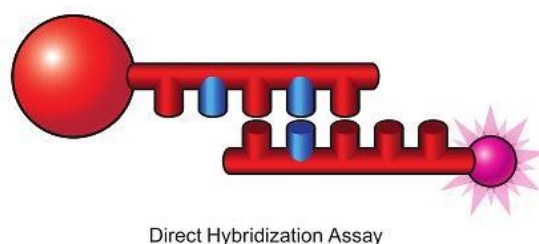
Figure 15. Typical bead and probe bind corresponding PCR products with fluoresces
Source: Luminex company webpage

For PCR amplification, 10 µl of sample DNA in a 50 µl reaction mixture containing biotinylated primers was included in the HPV genotyping kit and Qiagen Multiplex PCR Master Mix (Qiagen, Germany), according to the manufacturer's instructions. The amplification was performed in 40 cycles with an annealing temperature of 38°C for 90 s. Hybridization of PCR products to Luminex beads and subsequent steps were performed according to the manufacturer's instructions. The samples were evaluated using a Luminex 100 analyzer (BioRad Laboratories, USA). The cut-off limits proposed by the manufacturer were used with an important exception. To avoid false positivity for HPV 82 due to cross-reactions between HPV 51 PCR amplicons and the HPV 82 probe, for samples with a median fluoescence intensity (MFI) value for HPV 51>200, the cut-off value for HPV 82 was increased to background+60 MFI.
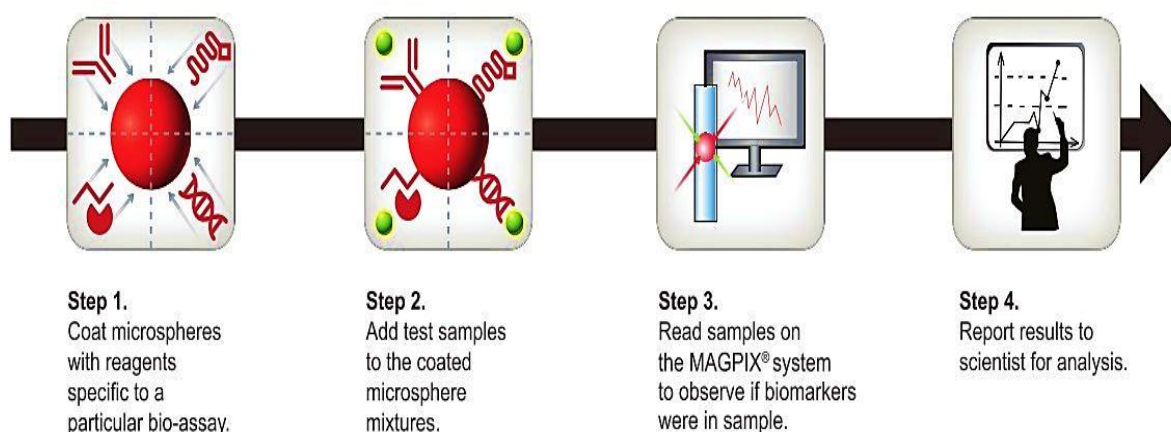
Figure 16. MAGPIX workflow
Source: Luminex company webpage

For the study in paper IV the primers and probes used in this assay were copied in our own lab. However, the design of the assay was originally from the group of Michael Pawlita [255]. Our assay was validated by retesting samples earlier analyzed with the commercial kit. The Luminex analysis earlier performed on a Luminex 100 instrument was from 2011 and onward analyzed using a MagPix instrument. This is a new instrument from Luminex and although there are differences in the functioning between the two instruments, all the steps are exactly same except that the beads are magnetic (Fig. 16). We have also compared the results obtained between these two instruments and confirmed that these are the same.

3.3.4 HPV E6 sequencing (Paper V)

For the analysis of HPV 16 E6 from cancer tissues two primer pairs covering the whole E6 region was utilized. 5'- CCGGTTAGTATAAAAGCAGACAT-3' together with 5'-TGCTGTTCTAATGTTGTTCC-3' amplifying bp 57-375 and 5'-GGAATCCATATGCTGTATGT-3' together with 5'- TGCAATGTAGGTGTATCTCC-3' amplifying bp 273-587. For cervical samples one primer pair covering the whole of E6 was utilized; 5'-CCGGTTAGTATAAAAGCAGACAT-3' and 5'-GTACCCTCTTCCCCATTGGT-3' amplifying bp 57-902. PCR was performed with annealing temperature at 49°C and products were purified using ExoSAP-IT (USB, USA) according to the protocol of the manufacturer. A PCR for sequencing was performed on the purified products with Big Dye^TM terminator (Applied biosystems, USA) and the amplicons were analyzed in an Applied Biosystems 3130 sequencer. Both DNA strands were sequenced and the DNA sequence and corresponding amino acid sequence was compared with the reference European HPV 16 sequence (reference code: NC_001526.1) using the Sci-Ed software (Science and Educational Software, USA).

3.3.5 Statistical analyses (Paper I, III-V)

Fisher's exact test (2-tailed) was used to compare proportions of HPV DNA-positive samples between different time periods, with regard to oral and cervical HPV prevalence, and with regard to differences in frequencies of different variants for tonsillar cancer (TSCC), cervical cancer (CC) or cervical samples (CS).

An independent, 2-sided t test was performed to compare the mean age between patients with HPV positive and HPV negative base of tongue cancers.

The associations of HPV status with TNM status, stage or differentiation were calculated using the Freeman-Halton extension of Fisher's exact test (2-tailed). All these analyses were done using VassarStats website for statistical calculations (http://faculty.vassar.edu/lowry/VassarStats.html) or GraphPad Software (http://www.graphpad.com/quickcalcs/index.cfm). Ninety-five percent confidence intervals (95% CI) were calculated using CIs for proportions without correction for continuity according to a method described by Robert Newcombe.

4. Results and Discussion

4.1 Paper I: The role of human papillomavirus (HPV) in the increased incidence of base of tongue cancer

4.1.1 Aim
The aim of Paper I was to investigate possible changes in the prevalence of HPV in base of tongue cancer between 1998 and 2007 in Stockholm.

4.1.2 Background
During the period of 1970-2007 an increase in the incidence of tonsillar cancer had been observed in Stockholm. Furthermore, it had been shown that in tonsillar cancer this increased incidence was most likely due to HPV infection, since there was a parallel increase in the proportion of HPV positive tonsillar cancer from 23% in the 1970s to 93% between 2006-2007 [203]. For this purpose we attempted to investigate the incidence of base of tongue cancer as well as the prevalence of HPV in base of tongue cancer between 1998 and 2007.

4.1.3 The incidence of base of tongue cancer has increased during the past ten years
Data from the Swedish Cancer Registry showed that the total age-standardized incidence of base of tongue cancer in Sweden had increased from 0.15/100,000 person-years during 1970-1974 to nearly 0.3/100,000 person-years between 1975-1979 and 1995-1999, and continued to increase to around 0.47/100,000 person-years in 2000-2007 (Fig. 17).
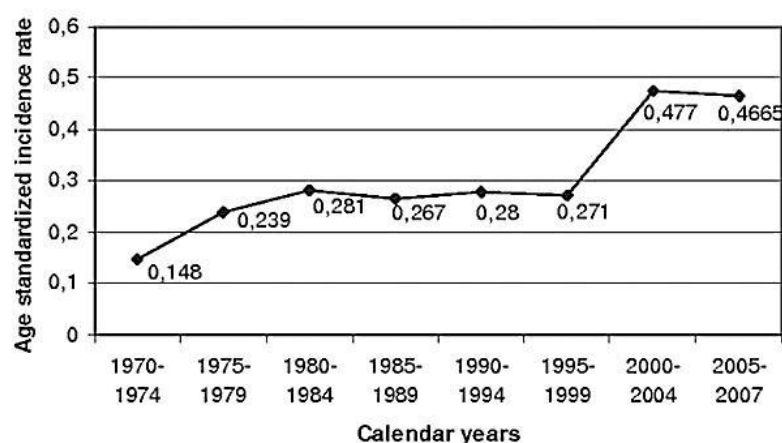
Figure 17. Age-standardized incidence rate of base of tongue SCC in Sweden between 1970 and 2007 (per 100,000 person-years)

4.1.4 The prevalence of HPV in base of tongue cancer has increased between 1998 and 2007

The incidence and prevalence of HPV were analyzed through the period of 1998 to 2007. HPV status was tested on 95 tumor samples with amplifiable DNA from base of tongue cancer patients diagnosed in Stockholm between 1998 and 2007. The prevalence of HPV was shown to have increased from 58% (15/26) during the period 1998-2001 to 75% (18/24) in 2002-2003 and to 85% (22/26) in 2004-2005 and 84% (16/19) in 2006-2007 (Tab. 1).

More specifically, HPV DNA was detected in 75% (71/95) of all the cases and of the positive cases, 86% (61/71) were HPV 16, 10% (7/71) were HPV 33, 2.8% (2/71) HPV 35 and 1.4% (1/71) HPV 58. There were no HPV double infections. In addition, by analyzing RNA expression from 20 randomly selected HPV 16 positive samples, the majority (17/20, 85%) were E6 and E7 mRNA positive, indicating that HPV was functional in the tumors.

Table 1. HPV prevalence in base of tongue cancer 1998-2007 in Stockholm

Years	Biopsies retrieved	% HPV DNA[1] detected from the retrieved biopsies (95% CI)
1998–2001	26	58%[2] (39–77)
2002–2003	24	75% (58–92)
2004–2005	26	85% (71–99)
2006–2007	19	84% (68–100)
Total	95	75%

[1]Presence of HPV DNA by PCR. [2]$p < 0.05$ compared with the frequency of HPV in 2004–2007.

Most patients (68%) with base of tongue cancer in this report were men. Patients with HPV positive and negative base of tongue cancer were similar in mean age, however patients with HPV positive cancer often presented with Stage IV tumors, lower T-stage, but higher N stage than those with HPV negative tumors.

4.1.5 Discussion

In this study, we were able to demonstrate that between 1998 and 2007 there was an increase in the incidence of base of tongue cancer as well as an increase in the prevalence of HPV positive base of tongue cancer.

The fact that incidence of base of tongue cancer had increased three fold between 1970 with 0.15/100 000 to 0.47/100 000 2007 was similar to that observed for tonsillar cancer which was 0.74 per 100,000 person-years between 1970-1979 to 1.65 per 100,000 person-years between 2000 and 2006 [203]. In parallel, we also observed an increase in the prevalence of HPV between 1998 and 2007 from 58% 1998- 2001 to 84% 2006-2007. Also here the percentage was similar to that observed in tonsillar cancer, since there the increase was from 57% in the 1990s to 93% between 2006-2007 and the incidence rate of HPV positive tumors almost doubled in each decade between 1970 and 2007 [203]. For tonsillar cancer we estimated that the number of HPV negative tumors had declined, and most likely the same holds true for base of tongue cancer, but this was not calculated.

Notably, only few research groups present data on the two OSCC subtypes tonsillar and base of tongue cancer separately. In our study, the HPV prevalence in base of tongue cancer was higher compared to reports from other groups, where an HPV prevalence of around 40-50% was observed [256-259]. However, in one of those studies only the presence of HPV 16 was analyzed, while in another report only 19 samples were analyzed, which is much more limited compared to our study, and in two other reports only samples obtained before 2002 were analyzed [256-259]. Nonetheless, our data with a rapid increase in HPV prevalence in base of tongue and tonsillar cancers during the past ten years was in line with that of others [260, 261]. Thus taken together our data and that of others are in line with that the increase of base of tongue cancer could be caused by HPV infection.

Among the HPV positive base of tongue cancer cases HPV 16 dominated and accounted for more than 80% of the cases and from randomly selected and RNA tested samples, there was a high E6 and E7 mRNA expression, indicating that the virus is functional and oncogenic in the tumors in this study. HPV 33 was the second most commonly observed HPV type in our study and accounted for 10% (7/71) of all the HPV cases. We do not know the reason for this, since this has not been shown for tonsillar cancer, where only one case of HPV 33 was detected in 83 samples. Furthermore, when analyzing the presence of HPV in the cervical and oral tract in young adults in the Stockholm area, HPV 33 infection was not among the most commonly observed types [173].

In the past there has been some controversy regarding that HPV as an etiological factor in OSCC. Some researchers claimed that HPV positive tumors were associated with sexual behavior and marijuana smoking, while HPV negative tumors were associated with tobacco smoking, alcohol use and poor oral hygiene and that similar to us that the increase of OSCC is due to HPV [210, 262]. In contrast, others found similar increased risks of developing

cancer associated with tobacco and alcohol use in patients with HPV positive and negative tumors [263, 264].

In this study, mean age of patients with HPV positive and HPV negative base of tongue cancer was similar, i.e. 63 years and 62 years respectively. This was different from a similar study by us conducted for tonsillar cancer, and from other reports with regard to OSCC, where patients with HPV positive cancer are usually somewhat younger than those with HPV negative cancer. We have no explanation for this difference.

There have been discussions whether HPV vaccination of boys as well as girls can be of benefit and be protective against head and neck cancer, and vaccination of both sexes was also suggested by Harold zur Hausen [265]. Some mathematical models demonstrate that vaccinating girls is cost-effective only if a high vaccination rate is acquired among girls [266, 267]. In HPV vaccination models it has been calculated that if the coverage of girls falls below 90%, which it most probably may do, a significant vaccination efficiency and cost effective improvement will only be seen when vaccination of boys is included [266, 267]. Furthermore, in Sweden, one study on rubella vaccination showed that rubella was only eliminated when both boys and girls were included in the vaccination program as compared to only vaccinating girls [268]. Furthermore, besides head and neck cancer, men who have sex with men may benefit, since the vaccine prevents both penile as well as anal cancer [269]. It should be noted that the cost of the HPV vaccines is an important part in the calculation of cost-effectiveness and since this cost has decreased as compared to some years ago earlier calculations may no longer be valid.

Although, not the subject of this paper, both previous to Paper I and later, others and we have shown that overall survival and disease-free survival for patients with HPV positive tonsillar and base of tongue cancers (and OSCC) was significantly better than that of those with HPV negative cancer [215, 270-275]. These data taken together demonstrate the importance of recalling that the numbers of HPV positive tumors may still be increasing and to consider HPV status when planning treatment for base of tongue cancer patients. It is possible that in the future patients with HPV positive tumors with additional biomarkers may be possible to treat with less aggressive treatment as compared to patients with HPV negative tumors.

In summary, the prevalence of HPV in base of tongue cancer has increased between 1998 and 2007. More studies following any changes in HPV prevalence in base of tongue cancer would be of interest as well as studies identifying new biomarkers that together with HPV status could be of use for individualizing patient treatment.

4.2   Paper II: Prevalence of human papillomavirus (HPV) in cervical cancer in Stockholm

4.2.1 Aim
The aim of Paper II was to gain information on the prevalence of HPV types in cervical cancer during between 2003 and 2008 in Stockholm, i.e. just prior to the introduction of public HPV vaccination in Sweden.

4.2.2 Background
In Europe, cervical cancer is the seventh most common cancer in women, with over 54 000 new cases and 25 000 deaths annually [157]. Up to 1300 new cases are diagnosed In the Nordic countries and about 450 new cases in Sweden are reported every year.

The major risk for developing cervical cancer is persistent infection with different oncogenic human papillomavirus (HPV) types. However, although there are global reports on the prevalence of different HPV types, there are few studies analyzing HPV types covering more recent periods in cervical cancer in Sweden. This study was initiated in 2009, the year public HPV vaccination was originally planned to start in Sweden.

4.2.3 HPV prevalence in both SCC and ADC
In total, 154 cervical cancer samples demonstrated amplifiable DNA and were analyzed for 24 different HPV types by the Luminex multiplex assay. The median age of the patients including both the SCC and ADC groups was 42 years. In total, 143/154 (92.9%) of the samples were HPV positive with all cases positive for at least one HR-HPV type. The majority 131/154 (85.1%) of the samples were positive for only one HPV type (Tables 2 and 3). Among those HR-HPV types, HPV 16 and 18 dominated and covered 90/154 (58.4%) and 29/154 (18.8%) cases and together accounted for 74.0% (114 cases) of all the samples. Other HR-HPV types either observed alone or together with other types are shown in Tables 2 and 3.

4.2.4 HPV prevalence in SCC
There were 111 (93.3%) cases among 119 cases of SCC that were positive for HPV in this study. HPV 16 and 18 were found in 76/119 (63.9%) and 12/119 (10.1%) of the SCC cases respectively and together they covered 83/119 (69.7%) of the cases (Tables 2 and 3). Other HPV types in SCC were listed in Table 2. Multiple infections without HPV 16 or 18 were observed in the four remaining cases as shown in Table 3.

Table 2. Distribution of different HPV types in CC, SCC and ADC

	Squamous carcinomas		Adenocarcinomas		Total	
	HPV positive cases (n)	HPV prevalence (%)	HPV positive cases (n)	HPV prevalence (%)	HPV positive cases (n)	HPV prevalence (%)
All	111/119	93.3	32/35	91.4	143/154	92.9
Age(year)						
20–39	45/49	91.8	14/14	100	59/63	93.7
40–59	30/31	96.8	13/15	86.7	43/46	93.5
Over 60	36/39	92.3	5/6	83.3	41/45	91.1
HPV type						
HR HPV						
16	76	63.9	14	40.0	90	58.4
18	12	10.1	17	48.6	29	18.8
31	5	4.2	0	0	5	3.2
33	7	5.9	0	0	7	4.5
35	1	0.8	0	0	1	0.6
39	2	1.7	1	2.9	3	1.9
45	3	2.5	2	5.7	5	3.2
51	2	1.7	0	0	2	1.3
52	3	2.5	0	0	3	1.9
56	4	3.4	0	0	4	2.6
59	2	1.7	0	0	2	1.3
68	1	0.8	0	0	1	0.6
73	2	1.7	0	0	2	1.3
82	1	0.8	0	0	1	0.6
Putative HR types						
53	1	0.8	0	0	1	0.6
66	1	0.8	0	0	1	0.6
LR HPV						
42	1	0.8	0	0	1	0.6
70	1	0.8	0	0	1	0.6
HPV16 and/or 18	83	69.7	31	88.6	114	74.0
HPV16 + 18	5	4.2	0	0	5	3.2
Multiple HPV types	10	8.4	2	5.7	12	7.8

4.2.5 HPV prevalence in ADC

Out of 35 ADC cases, 32 (91.4%) were HPV positive (Table 2). All included either HPV 16 or 18 (31/35, 88.6%) except one HPV 45 positive case (Table 3). Notably, HPV 18 and HPV 16 accounted for 17 (48.6%) and for 14 cases (40.0%) of the cases respectively. In addition, only 2/35 (5.7%) of the ADC samples were infected with more than one HPV type as demonstrated in Table 3.

4.2.6 Discussion

In this study, we reported a high prevalence of HPV (92.9%) in pretreatment uterine cervix cancer samples including both SCC and ADC of patients diagnosed between 2003 and 2008 at the Karolinska University Hospital in Stockholm. HPV 16 and 18 dominated and accounted for 74.0% of all the positive cases, and HPV 33, 31 and 45, followed them in prevalence. Data both in SCC and ADC implicated that the present HPV vaccines should be able to inhibit a majority of the cervical cancer cases in the area.

Table 3. Distribution of single and multiple HPV types in CC, SCC and ADC

HPV type	Squamous carcinomas (Number of cases)	Adenocarcinomas (Number of cases)	Total (Number of cases)
16	70	13	83
18	7	16	23
31	5	0	5
33	5	0	5
45	3	1	4
51	2	0	2
56	2	0	2
73	2	0	2
35	1	0	1
39	1	0	1
52	1	0	1
59	1	0	1
68	1	0	1
16+18	4	0	4
16+45	0	1	1
18+39	0	1	1
33+[53]	1	0	1
33+59	1	0	1
52+56	1	0	1
16+18+(70)	1	0	1
16+39+52	1	0	1
56+82+[66]+(42)	1	0	1

*Note: [] means Putative HR HPV types; () means LR HPV types.

The overall high HPV prevalence in cervical cancer in this study was comparable to the worldwide distribution, where 92.9% of all the cases are shown to be associated with HPV infection [163]. Our data were also similar to other studies in Europe with a presence of 88.9% (ranging from 70% to 97.8%) of HPV in cervical cancer [163, 164, 276-281]. Not unexpectedly when comparing with CIN data from Sweden, a slightly higher prevalence was observed in our invasive cancer study as compared to that found in pre-lesions where 36%, 63% and 80% of the CIN I, II and III cases contained HR-HPV [169, 282, 283]. Similar to what we observed, also a Swedish study on pre-lesions demonstrated that HPV 16 dominated (61%) in cervical cancer in situ, followed by HPV 33/52/58 with 24% in total and with HPV 18/45 infecting 12% of all the cases [284].

Our detection rate of both HPV 16 (66.5%) and HPV 18 (19.4%) was also within the ranges of HPV 16 and 18 prevalence both globally and within European countries. HPV 16 infection was the most dominate type both in total cervical cancer and in SCC cases among different studies [163, 164, 279]. Following HPV 16, the most common HPV types in our report were HPV 18, HPV 33, HPV 31 and HPV 45, and this was also in line with other studies with variations of the order depending on different geographic regions [163, 164,

279]. A slight higher prevalence of HPV 18 in our study than in overall European countries (10%) as well as a little higher HPV 18 prevalence than in ADC globally (32%-36.8%) was observed [163, 164]. Interestingly, only one case of HPV 35 and no HPV 58 infection were found in our study. However, they are the types, especially HPV 58, which vary a lot between different regions. As mentioned in the introduction section, HPV 58 is more frequent in Eastern Asia than anywhere else in Europe [163, 164].

Furthermore, in this study, HPV 18 accounted for proportionally more cases in ADC (17/35, 48.6%) than in SCC (12/119, 10.1%), while HPV 16 was less frequently observed in ADC (14/35, 40.0%) than in SCC (76/119, 63.9%) which is in concordance to the findings of other researches [163, 165, 168, 276]. In our study, many other high risk types e.g. HPV 33, HPV 31, HPV 51 and HPV 52 and the multi-infection cases were also more frequently distributed in SCC samples than in ADC. It has been suggested that HPV 18 could be more aggressive than HPV 16 and thus the difference in distribution of HPV 16 and 18 in ADC compared to SCC together with the presence of many more additional types in SCC could in part explain why ADC is usually more aggressive than SCC. This assumption would partly be in line with an earlier study from our group demonstrated that HPV 16 was much more common in early cancer stage of patients with surviving over five years than those of patients with a poor prognosis [282]. However, there could be completely other reasons for the differences in aggressiveness between SCC and ADC of which one could be that some SCC cases are detected earlier compared to ADC. Nonetheless, the fact that HPV 16 or/and HPV 18 infection was much higher in ADC (88.6%) samples than in SCC samples (69.7%) may affect the distribution and the occurrence of these cervical cancer types after the introduction of public HPV vaccination.

In Sweden, additional studies have explored the presence of HPV in ADC and similar to our study, HPV 18 was the most common type (52%) followed by HPV 16 (33%) in one of these studies [169, 285]. Furthermore, the presence of HPV was shown to be age-dependent, and was present in 89% of the patients below 40 years old, in comparison to in only 43% of the women above 60 years old [169, 285]. In our study, we did not see this trend with regard to age, but this could be explained by the fact that we only had a limited number of samples in patients over 60 years with ADC (n=6).

Recently, a study was done in Sweden which involved 2,772 cervical smears from 515 women with cervical cancer in situ and 315 women with SCC, with individually matched controls. The median follow-up years until diagnosis were around five to seven. They declared that persistence of HPV 16 infection given a relative risk of 18.5 for cancer in situ and 19.5 for SCC. In addition, in comparison to women that were negative for HPV 16/18

in their first smear the relative increase was 8.5-fold and 18.6-fold higher risk for developing cancer in situ and SCC, respectively. Infection with other HR-HPV types in the first smear was also associated with significantly increased risks for both cancer in situ and SCC [286].

Both vaccines (Gardasil and Cervarix) are highly efficient in preventing HPV infection as mentioned above. According to our data, HPV 16 and 18, both included in the vaccines dominated and accounted for 74.0% of all the cervical cancer cases. For these two types, both vaccines have a nearly 100% efficiency against cervical cancer with documented long-term protection in clinical trials [239, 240, 248]. Taking into account also the infections with the non-vaccine HR-HPV types (HPV 31, 33, 45 and 52) that potentially may be covered by cross protection, a large proportion of cervical cancer could be inhibited by the introduction of an HPV vaccination program in Sweden [240, 241, 244]. In this case, vaccination before exposure to HPV infection is extremely essential. However, it is possible that the distribution of HPV types in cervical cancer may change after the national immunization.

In a recent investigation in Sweden about attitudes towards HPV vaccination among parents with children aged 12-15 years old, the majority of the parents (76%) were willing to vaccinate their child if the vaccine was free of charge, in comparison to fewer parents (63%) if they had to pay for the vaccine. There was also higher willingness for vaccination if the parents had heard of HPV, as compared with those who never heard of HPV. Safety and efficacy of the vaccine were also strong correlates to the willingness to vaccinate [287]. All of this demonstrated the importance of educational information of HPV to the public before and in parallel with the vaccination.

To analyze HPV types in cervical cancer samples, we used Luminex, which is sensitive and covered 24 HPV types and several HPV types were found in the same tumor samples. This can result in difficulties to establish if only one of the HPV types triggers tumor development, but this can be a problem for nearly all the HPV detected methods. However, there are studies showing a correlation between HPV mRNA expression and the presence of HPV as well as the presence of HR-HPV and p16 expression [288, 289]. These correlations could thus provide additional factors that can be tested for in order to identify the real oncogenic HPV type in specific cervical cancer cases. In addition, treatments and situations e.g. smoking or drinking were not investigated in our assessment. Further studies on prognosis and survival data could be useful as well.

In summary, this Stockholm study described a high prevalence of HR-HPV and of HPV 16

and 18 in cervical cancer (including both SCC and ADC), which is comparable to many European studies. Therefore, potentially, a large proportion of cervical cancer should be possible to prevent by HPV vaccination if taking account of both the vaccine types and cross protection types. The acquired information thus highlighted that HPV vaccination in Stockholm and the introduction of HPV vaccine at early age should benefit women and prevent a considerable number of cervical cancer cases in the future.

4.3 Papers III and IV: Prevalence of HPV in the cervical and oral tract in young adults in Stockholm

4.3.1 Aim

The aims of Papers III and IV was to study the prevalence of different HPV types in the cervical and oral tract, by cervical tests or by mouth washing among young adults at a large youth health center in Stockholm before the introduction of public HPV vaccination in Sweden. In addition, oral and cervical HPV prevalence as well as possible HPV type concordance between the two sites were compared in women.

4.3.2 Background

Persistent HPV infection has been shown to associate and play an essential role in preceding cervical cancer as well as head and neck cancer. Besides, HPV infections can be present in the cervix for many decades. The presence of different cervical HPV types can be detected and followed in young healthy women. Probably the same holds true in oral tract. HPV vaccination with Gardasil and Cervarix prevents cervical infection with HPV 16 and 18 (HPV 6 and HPV 11 also in Gardasil). In Sweden, public HPV vaccination of girls 10-12 years of age has been initiated, which may change the prevalence of different HPV types in young adults as well as in HPV-related cancers. In this case, monitoring HPV prevalence among sexually active young adults form the health center is a good way to follow up and analyze possible changes in the prevalence of different HPV types. Prior to the introduction of public HPV vaccination, in order to acquire more information and obtain a baseline of cervical and oral HPV prevalence, two studies were performed.

4.3.3 HPV prevalence in cervical tract of young girls

In paper III, totally 615 samples were analyzed by an HPV specific Luminex multiplex assay. Six samples with limited material and 65 samples from women that had received one or more HPV vaccinations were excluded for further analysis. From the 544 non-vaccinated women, 70% of the genital samples were HPV positive and 62% for one or more HR-HPV genotypes. The 6 most common HR types were HPV 16 (34.7%), HPV 51 (10.7%), HPV 18 (10.1%), HPV 52 (9.9%), HPV 73 (9.4%) and HPV 39 (9.0%). Several other HR-HPV types (56, 59, 82, 31, and 33) were present at frequencies between 6.4% and 8.6%. Putative HR-HPV types 53 and 66 were found in 11.8% and 11.6% of the samples, respectively. The most common LR type was HPV 42 with 16.4%, while LR types HPV 6 and HPV 11 had a prevalence of 8.1% and 2.0%, respectively. The frequencies of the different genotypes in descending order are presented in Figure 18.

In paper IV, cervical samples were also collected, but this time they were collected in parallel, with oral samples obtained by mouth washes from totally 180 women in which

174 cases contained sufficient material from both sites. The prevalence of HPV type in the cervical and oral tracts was compared. Cervical HPV was detected in 129/174 (74.1%) women. The six most common HR-HPV types were HPV 16 (37.9%); HPV 52 (16.1%); HPV 51 (15.5%); HPV 18 (14.4%); HPV 56 (12.1%) and HPV 59 (12.1%); three detected putative HR-HPV types were HPV 53 (12.1%), HPV 66 (10.3%) and HPV 26 (0.6%); and the top three LR-HPV types were HPV 42(17.8%); HPV 6 (8.0%) and HPV 43 (5.7%).
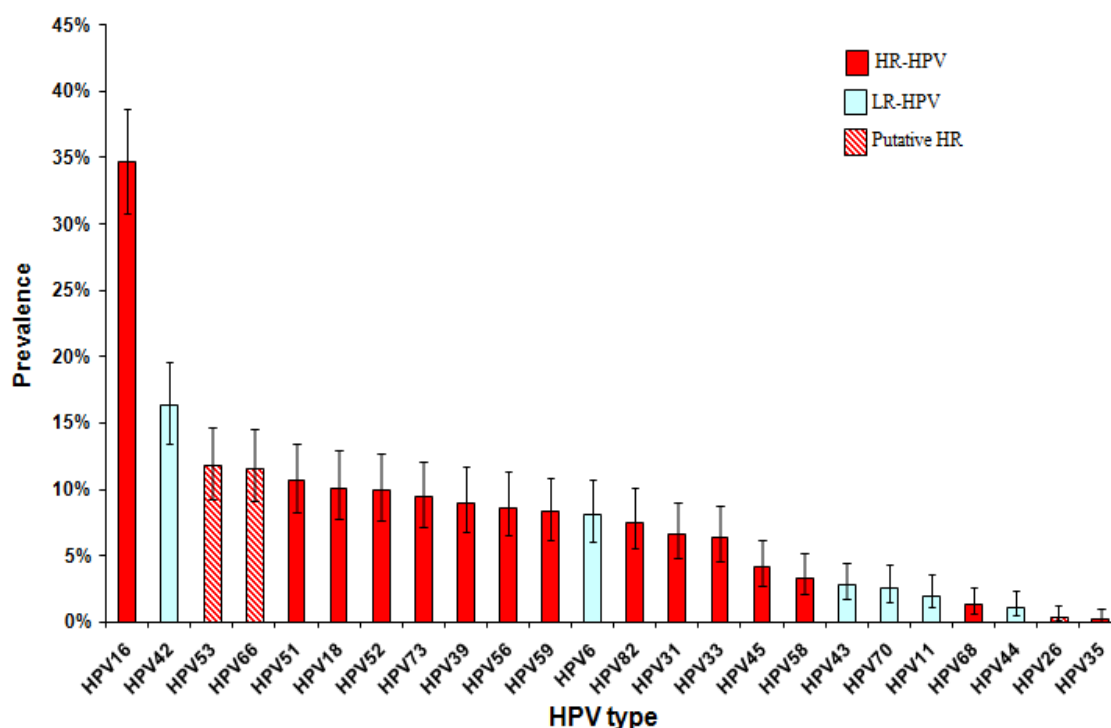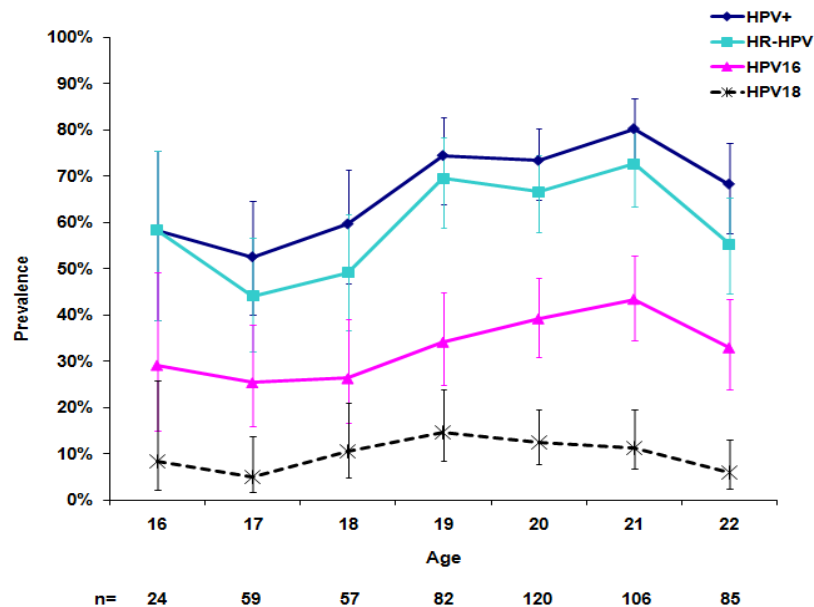

Figure 18. Prevalence of all HPV types in non-vaccinated women

4.3.4 HPV prevalence in cervical tract of young girls by age groups

The HPV prevalence for non-vaccinated women aged between 16 and 22 is demonstrated in Figure 19. The prevalence of HPV, as well as HR-HPV infection increased with age in women aged from 17 to 21 years old, and thereafter there was a decrease (Fig. 19). Our data indicates that the HR-HPV prevalence peaked in women aged 19 to 21 years old (67–73%) in our study group. The prevalence of HPV 16 also increased steadily to peak at age 21. However, it should be noted that the number of samples from each age group was relatively similar except for the 16-year old group, where there were fewer.

In addition, the majority (61%) of the samples were infected with more than one HR-HPV positive samples. The average number of HPV types among all the HPV positive samples was 2.1, and as many as eight different HR-HPV types were observed in one sample.

Figure 19. Prevalence of HPV types by age in non-vaccinated women, n denotes the total number of each age group

4.3.5 HPV prevalence in the oral tract of young women and men

In paper IV, out of the 490 oral samples with sufficient material for the analysis 9.3% (45/483) were HPV positive, with 9.2% (37/401) in women and 9.8% (8/82) in men. Furthermore, HR-HPV types were exhibited in 7.2% (29/401) and 7.3% (6/82) of women and men respectively
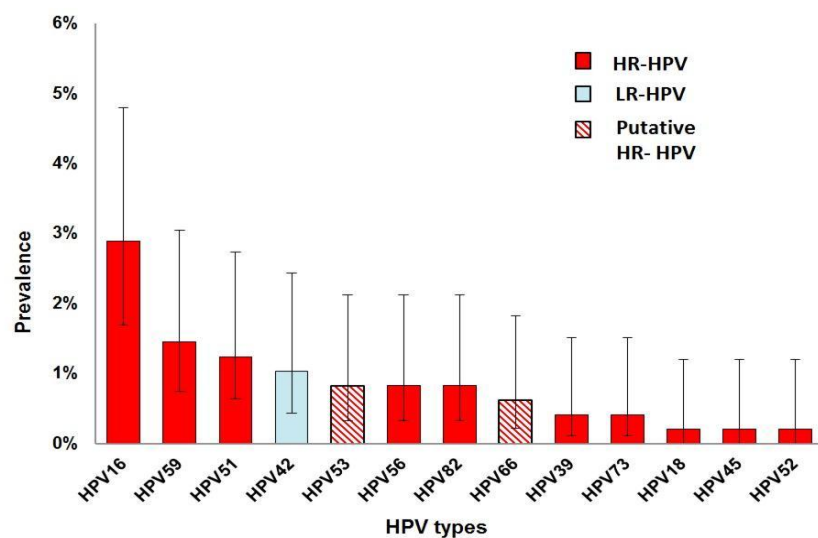
Figure 20. Prevalence of all HPV types in oral tract samples

Two or more HPV types were found in 15.6% (7/45) of all the oral samples. In total, 13 HPV types were detected as listed in Fig. 20 including ten HR-HPV types: HPV 16 (2.9%); HPV 59 (1.4%); HPV 51 (1.2%); HPV 56 (0.8%); HPV 82 (0.8%); HPV 39 (0.4%); HPV 73 (0.4%); HPV 18 (0.2%); HPV 45 (0.2%) and HPV 52 (0.2%); two putative HR-HPV types: HPV 53 (0.8%) and HPV 66 (0.6%); as well as one LR-HPV type: HPV 42 (1.0%).

4.3.6 HPV Prevalence of oral infection in relation to cervical infection

Notably, oral HPV infection was significant more common in women with (22/129, 17.1%) than those without (2/45, 4.4%) cervical HPV infection (p=0.043). In women with oral HPV infection, most of them (91.7%, 22/24) also had a cervical HPV infection. The only two oral HPV positive, with HPV negative genital samples were identified to have oral HPV 16 and HPV 42 infection.

For 20/22 (90.9%) samples with both oral and cervical HPV infection, there was high concordance in oral site with the HPV types in the cervical site (Table 4). Nonetheless, commonly detected types in the cervical tract were observed in the oral tract, but fewer HPV types were detected in the oral compared to the cervical tract. This is exemplified e.g. for women 10 and 13 both with several oral HPV types, where all types in the oral tract were detected in the cervical tract, but not vice versa (Table 4).

Table 4. HPV types detected in oral and cervical samples, in women being HPV positive in the oral tract

Women with oral and cervical HPV infection (n=22)	Oral samples			Cervical samples		
	High risk	Putative high risk	Low risk	High risk	Putative high risk	Low risk
1	**16***			**16***,18,51,82	53	
2	**16**			**16**,33, 45	66	43
3	**16**			**16**,18,39		6, 42
4	**16**			**16**,18,31,45,73		42,43
5	**16**			**16**,31,51,52,59	53	42
6	**16**			**16**,51		
7	**16**			**16**		42
8	**16**			**16**		
9	**18**			**18**		6
10	**39,51,56,59**			16,**39,51**,52,**56,59**,82	66	42
11	**51**			**51**		42, 43
12	**51**			**51**,82		
13	**56,59**			16,**56,59**		
14	**82**			18, 51,52,58,68,**82**	53	42
15	**82**			16,18,51,58,**82**	53	
16		**53**		33,45,51,82	**53**	
17		**53**			**53**	42, 43
18			**42**			**42**
19			**42**	45,56,59	66	**42**
20			**42**	16,33,52,56,59	53	6,**42**
21	82			16,33,45,73		11
22	73			16,18,52,56	53,66	
23	16					
24			42			

*Numbers in bold indicating type concordance between the sites.

4.3.7 Discussion

In papers III and IV, we could show that there was a high HPV prevalence (roughly 70%), with dominance of HR-HPV types, particularly with HPV 16, in the cervical tract of young women aged 15-23 years of age at a youth clinic in the center of Stockholm. In addition, we found an HPV prevalence of almost 10% in young men and women aged 15-23 years of age at the same clinic. Moreover, HPV infection in the oral tract was statistically more common in young women with a cervical HPV infection as compared to those without a cervical infection and there was a high type concordance between the two sites. Below, the discussion focuses on the presence of HPV at the two different locations.

4.3.7.1 HPV prevalence in cervical tract

In Paper III, 615 samples from the cervical tract of young women aged 15-23 from a youth health clinic were collected and analyzed for HPV types by Luminex assay. Among the 544 non-vaccinated women, as many as 70% were positive for any HPV, together with that 62% were positive for different HR-HPV types. When studying age distribution we observed that infection with HPV and HR-HPV increased from the age of 17 years reaching the highest prevalence (67-73%) among young women aged 19 to 21.

Our study shown a somewhat higher HPV prevalence compared to other studies performed on cervical samples from healthy women in Northern Europe, where the HPV prevalence varies between 23.5-50.2% below the age of 25 years [181, 290, 291]. The same holds true when comparing our data to those taking only into account women with normal cytology [181, 292]. Furthermore, our data showed a higher prevalence of HR-HPV types as compared to data from the Netherlands where HR-HPV was reported to be 21.2% to 44.7% among women 18-24 years of age [290, 292]. This could be explained by our samples here were collected from a rather selected study group that visit the youth health care center for various reasons e.g. contraceptives as well as cervical infections. The young adults there were considered to be a more sexually active group as compared to other young adults not attending the same center. However, our data was similar with a German study, reporting antibody HPV seroprevalence of 62% in a German women aged 15-34 years [34]. In addition, a study in Sweden of HPV genotypes in atypical squamous cells of undetermined significance (ASCUS) and LSIL reported an HPV prevalence around 70% in ASCUS in individuals aged 20-24 years [293]. Furthermore, the HPV 16 and HPV 18 prevalence in our study was higher than that reported by others, but more in line with a meta-analysis including a broader age group, where 23.3% were estimated to have HPV 16, and 8.5% HPV 18 [181]. This data is also comparable with the prevalence of HPV 16 and 18 in ASCUS, with 25% and 14% positive samples respectively in women 20-24 years of age [293]. However, there is very limited data on HPV infection with normal cytology to compare to, especially in Sweden. In this case, our paper provided a recent and large-scale

picture of HPV infection among young women in the center of a large city in Sweden. However, we could not include the cervical health status, or extended questionnaires besides age and HPV vaccination status in our study, due to the high work-load of the midwives at the youth clinic.

One other reason for the higher prevalence in our study could be methodological. We used a Luminex multiplex assay in combination with broad-spectrum BSGP5+/6+ primers, which have been shown to be 10-1000 more sensitive for several HPV types as compared to the often-used GP5+/6+ primers included in many other studies [294]. Since the high through put Luminex method is more sensitive for most HPV types, this can significantly reduce the potentially false negative cases obtained with some other methods, especially for HPV 73 and 82.

In comparison to our studies in cervical cancer (CC), there were many more types observed in the cervical samples, including both HR-HPV and LR-HPV. HPV 16 and HPV 18 were the most common types among the young healthy women, however not as dominate as in CC. This illustrates the oncogenic potential especially of HPV 16 and HPV 18 compared with other HR-HPV types. In fact, with regard to time for viral clearance, one follow up study on 252 women showed that around half of HPV 16 infection was cleared in five years, which was longer than that totally clearance of HPV 11, 43 and 51 infections, but shorter than for HPV 33 and HPV 44 which were not cleared [295].

Except HPV 16 and HPV 18, the other most common HR-HPV types in non-vaccinated women with prevalence over 9% were HPV 51, 52, 73 and 39. It is essential to be aware of that the difference among these none-vaccine types is minimal and it is not possible from this limited study to assess any differences important for future vaccine development. However, the next generation of vaccines should take consideration of these types, in order to reduce more lesions and cervical cancer as well as genital warts.

Furthermore, compared to other studies, a higher prevalence of HR-HPV types HPV 73 and 82 and LR-HPV type HPV 42 were also exhibited. This may partly be due to the more efficient detection of these types with Luminex Multiplex assay. Nevertheless, HPV 73 and 82 are not always tested for even in recent large studies on HPV prevalence in cervical samples. In Northern Europe, the prevalence of HPV 42 was below that of HPV 6 and roughly equal with that of HPV 11 [33, 290]. Still, there are some studies from other regions in Southern Europe and Africa finding a similar higher prevalence of HPV 42 than HPV 6 and HPV 11 in concordance with our findings in papers III and IV [181, 296-298].

The age distribution of HPV prevalence among women with normal cytology in various regions is shown in Fig. 21 where the first peak was below 25 years old. In paper III, over half of the women were HR-HPV positive already at 16 years of age. However, there were fewer individuals in this age group and they may have belonged to a more selected group than those from the older ages. The trend that both total HPV infection and HR-HPV infection peaked between 19-21 years old was in line with many other studies, although the HPV prevalence there was lower [290, 292, 293, 299, 300].
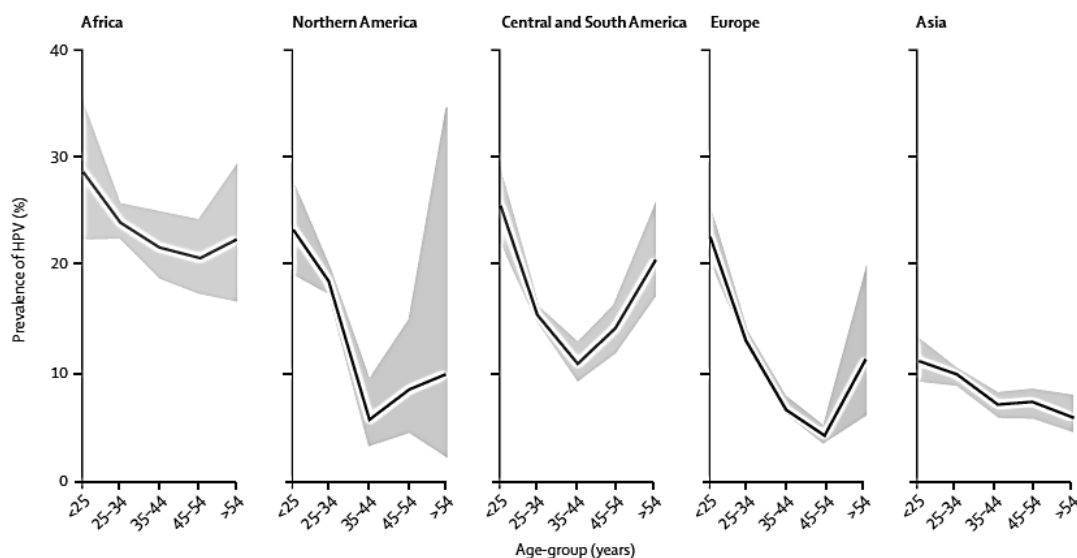
Figure 21. Age-specific HPV prevalence among women with normal cytology worldwide
Source: Lancet Infect Dis. 2007 (207): 453-459

HPV vaccines have been shown to be highly effective in preventing incident and persistent infection in fully vaccinated healthy young women as discussed above. However, this study, as well as other studies, data regarding the prevalence of common non-vaccine HPV types is essential for future HPV vaccine development. Moreover, in a group with a high HPV prevalence, it could be easier to rapidly detect changes in the prevalence of different HPV types after the introduction of public HPV vaccination. Studies similar to those in papers III and IV will be informative for which HPV types are present among young adults after public HPV vaccination of young girls, however they will not be able to provide which HPV types will occur in CC in the future. This will require other types of studies directly in CC. Nevertheless, the high prevalence of especially HPV 16 among women in papers III and IV demonstrates the need for vaccination at an early age, as is now being done in Sweden. Unfortunately, vaccination of women with pre-existing infection with HPV 16 or 18 is ineffective and has no therapeutic effect [301]. However vaccination may help for preventing further infections and genital warts or lesions, caused by other types that there could be cross-protection for in the present vaccines.

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After HPV vaccination in Sweden, the prevalence of HPV 16 and 18 will be lower and other changes may also be observed, e.g. due to the increased or decreased prevalence of non-vaccine HPV types, cross protection by the vaccines or due to global changes in the HPV type distribution. Nevertheless, how this will influence the presence of other common HR-HPV types remains to be seen. Further analysis on these ages group of young adults can be a good way to understand more about HPV infection and vaccination.

In summary, we found a high rate of HPV (70%) and HR-HPV (62%) infection in a cohort of 15-23 year old non-vaccinated women attending a youth health clinic. Moreover, 38% of HPV multiple cervical infections, as well as a high rate of HPV 16 and HPV 18 infections (35% and 10.1% respectively) was also demonstrated. Thus we conclude that HPV vaccination at the early age is urgently needed and that the vaccination program should prevent infection with HPV 16 and 18 in a high proportion of young girls. The high prevalence of other HR-HPV types besides HPV 16 and 18 demonstrates the need for further monitoring of the prevalence of HR-HPV types.

4.3.7.2 HPV prevalence in oral tract

Considering the high prevalence of HPV the cervical tract reported in paper III, and the high HPV prevalence in oropharyngeal cancer, we were interested to examine the oral HPV prevalence in young men and women attending the same youth health clinic as in Paper III. A similar prevalence of HPV in women and men (9.3 and 9.8%) was observed, but a significantly higher HPV prevalence was found in women with (17.1%), as compared to those without (4.4%) cervical HPV infection. Moreover, most women with oral HPV infection had cervical HPV infection with type concordance.

There are much fewer studies on oral HPV infection and many of the studies then often include HIV infected individuals, with a much higher HPV prevalence (33%-45%), as compared to HIV negative individuals (15%-24%) [302-305]. Among the studies on HPV infection from oral mucosa of healthy individuals, a large variation can be observed depending on the populations included and the geographic regions that are studied. Some studies were found negative or reported a very low prevalence of HPV oral infection ranging from 2.9% to 4.5% [35, 306-311]. This may be due to differences between the techniques also. Similar with our data in paper IV, some other studies reported an oral HPV prevalence of 6.7%-14.3% [312-314]. However, in studies including only patients with HPV positive cervical samples or HPV positive lesions, a higher oral HPV prevalence (27%) was reported [315, 316]. HPV 16 was the most common oral HPV type in our data with 37.5% (9/24) among oral positive samples. This observation is supported by a systematic review data from 16 published papers [308].

Our findings with a similar oral HPV prevalence in men and women, and that women with cervical HPV infection had a higher oral HPV prevalence than those without cervical HPV infection were in concordance with other studies or reviews [308, 315, 317]. That we found the most common cervical HPV types also in oral samples, suggesting no major differences with regard to HPV types present in the cervical and oral tracts, was also in line with many other studies [313, 315, 316, 318]. Rintala M et al claimed that oral HPV infection of the spouse in a family increased the risk of persistent oral infection in the other partner 10 folds [315]. It has also been suggested that cervical HPV infection may increase the susceptibility to the oral infection, due to e.g. influences on the immune response, and this was supported by that the likelihood of detecting the same HPV types both in oral and cervical samples were three times higher in HIV positive women than in HIV negative women [313, 314, 319]. However, some other groups showed the opposite data and claimed HPV infection in genital region was independent of oral infection and immune suppression did not impact that much on HPV infection since treatment for HIV declined HIV-associated diseases dramatically, but not for HPV associate lesions [302, 306, 307, 314, 317].

Furthermore, most studies (similar to paper IV) comparing oral and cervical HPV infection found oral HPV infection was much less frequent than cervical HPV infection [304, 307, 314]. However, the difference may to some extent be because of differences in the material analyzed, and the continuous production of saliva causing viral DNA to be swallowed and lose the contact from the epithelial surface of the oral cavity may decrease the yield of virus in the oral samples. It is also possible that PCR inhibition in the oral samples may contribute to underestimate oral HPV infection [303].

Regarding the correlation between oral sexual behaviors, there are controversial reports. Some researchers claim oral sex is important while others find that open-mouth kissing could be associated with oral HPV infection[35, 306, 320]. Other researchers did not find similar association between sexual behaviors and oral HPV infection [304, 316, 317]. However, one should note that the age range of the individuals that are studied and the purification and detection methods varies also among the different publications, and there are no publications that study a limited age range of 15-23 years of age, which makes comparisons between our and the others studies difficult.

In conclusion, the prevalence of oral mucosal HPV infection is similar in men and women attending a youth clinic, and more common in women with, compared to those without cervical HPV infection. Furthermore, there is HPV type concordance in the oral tract when compared to the cervical tract, with dominance of HPV 16. The data suggest that both young girls and boys may potentially benefit from HPV vaccination.

4.4 Paper V: HPV 16 E6 variants in tonsillar cancer in comparison to those in cervical cancer and cervical infections

4.4.1 Aim
The aim of this paper was to examine if there were differences between HPV 16 E6 variants in tonsillar cancer (TSCC) and cervical cancer (CC) from patients diagnosed during a similar time period at the Karolinska University Hospital in Stockholm, Sweden. In addition, the data obtained from these tumors were compared to the frequencies of HPV 16 variants in cervical samples (CS) from healthy young women at a youth clinic in Stockholm.

4.4.2 Background
HPV has been classified into lineages as European (E), Asian (As), Asian-American (AA), African-1 and -2 (Af-1 and Af-2), and North American1 (NA1) depending on different geographical locations. In addition to the major variant lineages, variants with single nucleotide alterations are frequently found in tumors, which show different oncogenic ability in cancer progression. The most frequent variant in HPV 16 E6 is the T to G transition at nt 350 in E6, abbreviated as E-T350G, causing an amino acid change from leucine to valanine at a.a. 83 and often named L83V. This variant has been associated with viral persistence and cancer progression of cervical carcinoma in some studies [321-323]. Although many studies on HPV 16 variants have been performed in cervical cancer, very few of variants studies have been made in head neck cancer [189, 225].

4.4.3 Frequency of HPV 16 E6 variants
In total, 55 TSSC, 52 CC and 51 CS HPV 16 positive samples were analyzed with regard to HPV 16 E6 sequence. The L83V variant was the most common variant, and it was especially common in TSCC (25/55, 45%), thereafter followed by CC (16/52, 31%) and CS (15/51, 29%) respectively. However, for another variant E-A131G causing a change from arginine to glycine in a.a. 10 (often denoted R10G), a more prominent difference was found. This mutation was present in 22% (12/55) of TSCC, while completely lacking in the CC samples and only present in 4% (2/51) of cervical samples, and the differences between TSCC and CC (p=0.0003) and between TSCC and CS (p=0.0085) were significant.

To further validate the increased frequencies of R10G and L83V mutations in TSCC, 53 additional TSCC samples as listed in the Fig. 22 were evaluated for the R10G and L83V variants. When all the 108 samples were summarized, 19% (21/108) with R10G and 40% (43/108) with the L83V mutation were demonstrated. The majority (12/21, 57%) of the E6 R10G variants from TSCC also contained a L83V variant.
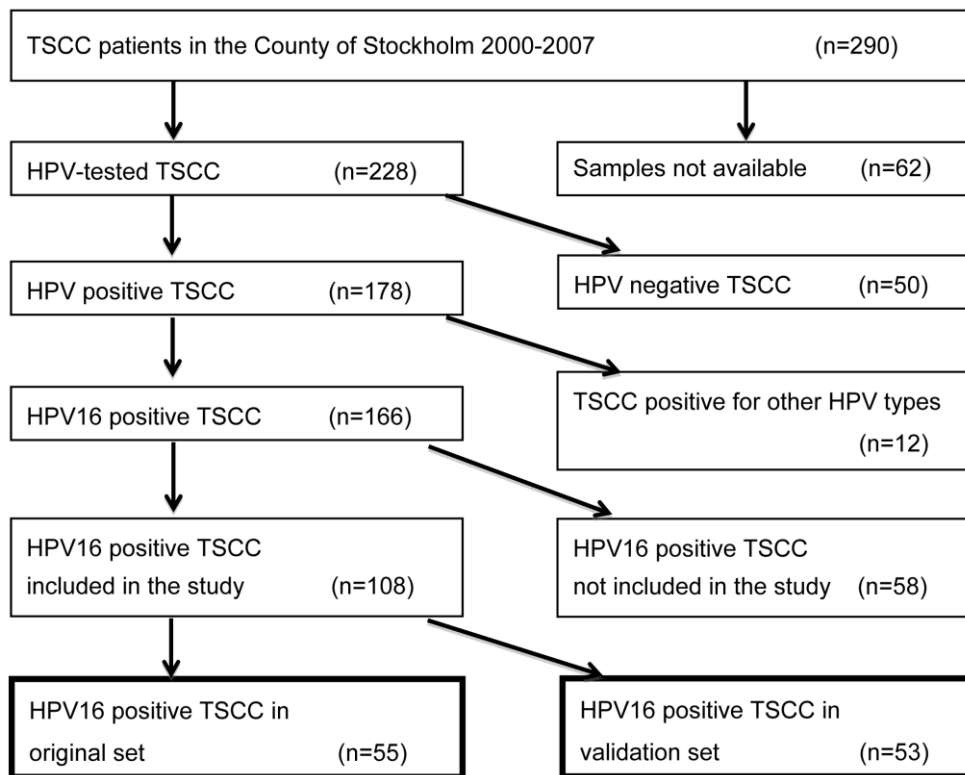
Figure 22. Flow chart of TSCC samples included

Mutations at other sites were less common for all types of samples, and only 68% (19/28) of the mutations affected the amino acid sequence. The most common variants were Q14H and H78Y, both present in 4% of CC and CS, while 7% in TSCC (details see support table in paper V).

4.4.4 Distribution of different phylogenetic lineages

The HPV 16 E6 European prototype was found in 38% of TSCC, 65% of CC and 59% of CS. However, when samples with the European type containing minor nucleotide differences (including R10G and L83V) were counted together, these figures increased to 93, 94 and 96% respectively. Other types e.g. Af-1 and 2, As. AA and NA were only found in few samples (details see support table in paper V).

4.4.5 Correlation of HPV 16 E6 variants in tonsqular cancer with clinical parameters

There were no significant differences when comparing patient and tumor characteristics based on the presence of R10G, L83V or absence of these variants in TSCC, although there was a tendency for tumors with R10G to have a lower T stage. In addition, there was no significant correlation in three years disease-free survival (DSF) between patients with presence or absence of the R10G variant or between TSCC patients with presence or absence of the L83V variant.

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4.4.6 Discussion

In this study, we have analyzed and compared the frequency of different HPV 16 E6 variants in TSCC, CC and CS and found HPV 16 R10G was relatively common in TSCC, absent in CC and rare in CS. In addition, the L83V variant was very common in all three categories of samples and HPV 16 European lineages dominated in all types of samples.

Studies on HPV variants in the head and neck region are very rare. One study on tumors of the upper aerodigestive tract found that the HPV 16 E6 L83V variant was present in five out of eight cases [225]. Another study on head and neck cancer found 23% of the HPV positive cases harbored the L83V variant and 11.5% the R10G variant [189]. However, to our knowledge no other study has compared the presence of HPV variants occurring in the head and neck and cervical tract regions. Our data in paper V showed that the rare HPV 16 E6 variant R10G was significantly more common in TSCC, while absent in CC and rare in CS in samples taken during similar time periods. To our knowledge, although observed in some studies, R10G variant was always rare in CC cases [220, 321, 324-326]. The reason for the high frequency of R10G variant in TSCC from Stockholm, as compared to CC is unknown. One explanation is that this HPV 16 variant is more common in the oral tract infection as compared to the genital area. Another possibility, that this variant of HPV 16 has a higher propensity to cause TSCC.

As mentioned in the introduction section, HPV 16 E6 together with E6AP forms a complex that specifically targets p53 and mediate p53 degradation, which is essential for tumorigenesis. The R10G mutation is in the N-terminal end. The N-terminal site from E6 a.a. 8 to 11 has also been reported to be nuclear localization signal, which is essential for inhibition of apoptosis and immortalization transformation [327]. One study performed on different variants in cervical cancers demonstrated that the R10G variant affected p53 binding and degradation as well as modified a B/T cell epitope [220]. More specifically, in one study it was shown that the E6 R10G variant dramatically alters the orientation of residues for interacting with the HLA-B7 peptide binding epitope, in this case influence cytotoxic T-lymphocyte (CTL) recognition as shown in Fig. 23. The author concluded the HPV 16 with E6 R10G variant could escape immune surveillance in patients with HLA-B7 [328]. Furthermore, cell transfection studies on oncogenic potential of variants demonstrated that both E6 with R10G and L83V retain the ability to abrogate growth arrest, and strongly reduce the steady state levels of p53 [329]. Nevertheless, while the E6 R10G variant has a decreased binding ability to E6-binding protein (E6BP), the E6 L83V variant has an increased binding affinity [224]. In addition, the E6 R10G variant has a reduced ability to induce Bax degradation, while L83V variant has an increased ability to induce Bax degradation compared to prototype HPV 16 E6 [224]. E6 with both the R10G and

L83V alteration, which was present in 9/12 TSCC this study, behaved similar to the prototype [224]. However, in another study, where cells were transfected by plasmids containing different HPV 16 variants the authors claimed that cells containing the L83V variant were more resistant to differentiation compared with the HPV 16 prototype, while the one with both L83V and R10G variant was less resistant [330]. In the present study no significant difference was observed regarding three years disease-free survival among the patients with TSCC carrying or without HPV 16 E6 R10G variant. In our study, the two TSCC patients that died in the third or fourth year with tumors containing the R10G variants also carried the L83V variant. All the TSCC patients with only the R10G variant are still alive after three years. However, the survival data are very limited in this study since the low number of cases (n=9) harboring only the R10G variant.
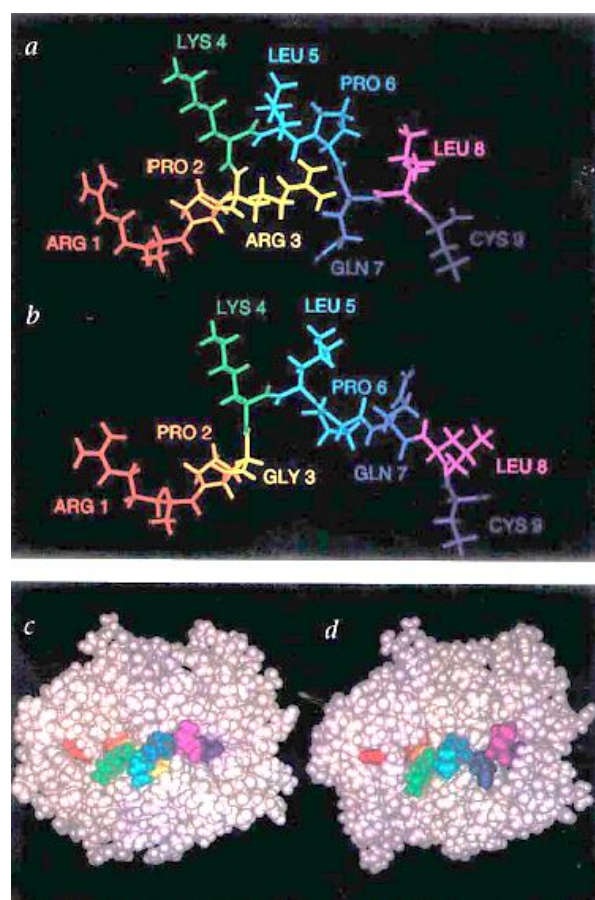
Figure 23. Computer models of peptides bound in the HLA-B7 class I molecule
Source: Nat Med. 1995 (1): p.464-470

Other variants of HPV 16 E6, especially L83V, differ geographically. Our data with L83V variant being the most common variant in CC was in line with many other observations, and our prevalence of the L83V variant were roughly in agreement with previous reports (44%-88%) in Europe [219, 321, 323, 324, 326]. In addition, the prevalence of the L83V variant was also comparable to data from head and neck region [189, 225]. The HPV 16 E6

variants were seldom analyzed in healthy infected young adults so that it is difficult for us make comparisons with our data. In some studies, an association between L83V and increased risk for cancer progression was demonstrated [321-323]. Furthermore, Zehbe et al demonstrated that genetic factors, e.g. the presence of specific HLA alleles may determine the oncogenic potential of L83V variant and increase the risk for developing cancer [322, 331]. However, in paper V, with a more limited number of patients, we did not find a correlation between the L83V variant and the three years disease free survival of the patients. Moreover, the prevalence of L83V was very similar in CC and CS in our study. Consistent with our data, the relation between L83V variant and cancer progression failed in some other studies [219, 325, 326, 332].

The European HPV 16 prototype, including the variants dominated in our study, and this is similar to other reports from Europe [219, 321, 323, 324]. Studies in American and Mexico have observed that more non-European variants e.g. AA and Af-2, and demonstrated that individuals with those variants had a greater risk for progressing to CIN3 or CC [222, 223, 333]. In addition, it has been shown that different HPV 16 lineages persist longer in race-related populations, i.e. E lineages persist longer in white women and Af lineages persist longer in African American individuals [334]. We could analyze for this option since the E lineage dominated in our study. However, European HPV 16 lineages from CC cases were about the same with the variants in CS from young women in paper V.

In conclusion, we found the rare R10G variant in HPV 16 was significantly more common in TSCC than in CC cases from patients admitted to the same hospital during the specific time period. In addition, in TSCC the R10G variant was frequently in combination with the L83V variant. There was no statistically difference in the presence of L83V the most common variant between CC cases and CS samples from healthy infected young adults regarding lineages. Finally, European lineages dominated in all categories of samples.

5. Summary and conclusions

- HPV infection, with dominance of HPV 16 is most likely the reason for the increase in base of tongue cancer in the Stockholm region.

- HPV 16 and 18 infections are the most common HPV types found in cervical cancer diagnosed between 2003 and 2008 in the Stockholm region cover approximately 70% of all cases.

- HPV infection is very common in the cervical and oral tracts (roughly 70% and 10% respectively) of young adults and HPV 16 is the most commonly found HPV type.

- The presence of HPV 16 E6 variants differs to some extent between the tonsillar and cervical cancer.

- The present HPV vaccines should be very useful in preventing most of the cervical cancer cases and if efficient to combat oral HPV infection they will prevent most HPV positive OSCC as well.

- The introduction of HPV vaccine at 10-12 years of age seems reasonable since already at the age of 16 many young women are infected with HPV.

- The prevalence of different HPV types should monitored since many changes in the prevalence of different HPV types can be expected.

6. Future perspectives

- The HPV vaccination program has recently been initiated in Sweden. This may drastically change the prevalence and distribution of HPV types in HPV-related cancers. Furthermore, due to increased travelling, additional changes in HPV types may occur. The most significant changes should be a lower prevalence of HPV 16 and 18, and a possible decrease of the cross protection types. In this case, more studies following any changes of HPV 16 and 18 together with other common HR-HPV types in HPV prevalence in head and neck cancer and cervical cancer would be of interest.

- Further studies on young adults should be performed with regard to the presence of oral and genital infection. This may help us understand the consequences of HPV vaccination and possible shifts in the presence of different HPV types.

- For patients with TSCC, there is an urgent need for pursuing the identification of new biomarkers e.g. non-smoking, HLA or other markers, which together with HPV status can potentially be used for predicting response to therapy, in order to better individualize patient treatment.

- Furthermore, studies are needed to investigate if the R10G variant may be more common in oral as compared to cervical samples. Functional studies could be done on the R10G variant in head and neck cells. In addition, other differences in the non-coding control region, E7 or E5 regions between the head and neck region and cervical region should be analyzed and compared.

7. Acknowledgments

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