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STUDIES ON THE INFLUENCE OF HUMAN PAPILLOMAVIRUSES (HPV) AND OTHER BIOMARKERS ON THE PREVALENCE OF OROPHARYNGEAL CANCER AND CLINICAL OUTCOME

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One must imagine Sisyphus happy.
A. Camus

ABSTRACT

Background. Oropharyngeal squamous cell carcinoma (OSCC), where tonsillar (TSCC) and base of tongue squamous cell carcinoma (BOTSCC) dominate, is increasing and is now the most common head and neck squamous cell carcinoma (HNSCC) in Sweden. Smoking and alcohol are risk factors for HNSCC, but in 2007, human papillomavirus (HPV) infection was also recognized as a risk factor for OSCC. Notably, HPV positive OSCC has a much better clinical outcome than and HPV negative OSCC. The last decade treatment of HNSCC, including OSCC, has been intensified with chemoradiotherapy, due to the generally poor prognosis of HNSCC. Such treatment, with more side effects, is likely needless for most HPV positive OSCC, but since around 20% of the patients do not do well, additional markers are needed to single out patients with an expected favourable outcome before possible de-escalation of treatment.

Aims. To follow incidence and HPV prevalence in OSCC over time in order to understand why OSCC has increased. To search for additional predictive biomarkers in HPV positive OSCC, to more safely de-escalate treatment.

Results: *In papers I-II* we showed that the prevalence of HPV in TSCC had increased significantly in Stockholm from 23% (1970-1979); to 28% 1980-1989; to 57% 1990-1999; to 79% 2000-2007. Notably, also during the 2000s there was a significant increase in HPV prevalence, from 68% 2000-2002; to 77% 2003-2005; up to 93% 2006-2007. Likewise, HPV prevalence also increased in BOTSCC, from 58% 1998-2001 to 84% 2006-2007. The increase in HPV prevalence was paralleled by an increase in incidence of TSCC and BOTSCC, and when estimating the HPV incidence in TSCC, the incidence had increased 7-fold from 1970 to 2006. *In paper III* we found that HPV positive TSCC had significantly more CD8+ and Foxp3+ tumour infiltrating lymphocytes (TILs), and that a high CD8+TIL count was correlated with a favourable clinical outcome in both HPV positive and negative TSCC. In addition, a high CD8+ count and a high CD8+/Foxp3+ ratio were significantly correlated with a favourable 3-year disease-free survival in HPV positive and negative TSCC respectively. *In papers IV-V*, we show that absence of "classical" HLA class I intensity staining was correlated with a favourable disease-free survival, disease-specific survival, overall survival and progression-free survival in patients with HPV positive OSCC, and to a worse clinical outcome in patients with HPV negative OSCC. Moreover *in paper V,* patients with HPV positive OSCC with absence of /or weak "classical" HLA class I intensity staining presented a very high survival that was independent of treatment regime. HLA-E, -G was also analysed in TSCC, but without any outcome correlation. In addition, HLA class II expression was analysed and found to be more common in HPV positive than HPV negative OSCC, but correlated to better clinical outcome only in the latter group.

Conclusion: A parallel increase in incidence as well as HPV prevalence in TSCC and BOTSCC was demonstrated suggesting HPV infection being responsible for the epidemic increase in OSCC. Finally, HPV positive TSCC or OSCC with high CD8+ TIL counts or with absent/weak HLA class I intensity staining presented a very good clinical outcome, suggesting that these markers could potentially be used to select patients for prospective randomised trials de-escalating oncological treatment.

LIST OF PUBLICATIONS INCLUDED IN THE THESIS

I. **Näsman A,** Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, Ahrlund-Richter S, Marklund L, Romanitan M, Lindquist D, Ramqvist T, Lindholm J, Sparén P, Ye W, Dahlstrand H, Munck-Wikland E, Dalianis T. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer. 2009;125(2):362-6*

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

III. **Näsman A,** Romanitan M, Nordfors C, Grün N, Johansson H, Hammarstedt L, Marklund L, Munck-Wikland E, Dalianis T, Ramqvist T. Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. *PLoS One. 2012;7(6):e38711*

IV. **Näsman A***, Andersson E*, Nordfors C, Grün N, Johansson H, Munck-Wikland E, Massucci G, Dalianis T, Ramqvist T. MHC class I expression in HPV positive and negative tonsillar squamous cell carcinoma in correlation to clinical outcome. *Int J Cancer. 2013;132(1):72-81*

V. **Anders Näsman***, Emilia Andersson*, Linda Marklund, Nikolaos Tertipis, Lalle Hammarstedt-Nordenvall, Per Attner, Tommy Nyberg, Giuseppe V. Masucci, Eva Munck Wikland, Torbjörn Ramqvist and Tina Dalianis. HLA class I and II expression in oropharyngeal squamous cell carcinoma in relation to tumor HPV status and clinical outcome. *Submitted*

* *Contributed equally*

RELATED PUBLICATIONS, NOT INCLUDED IN THE THESIS

I. Lindquist D, Romanitan M, Hammarstedt L, **Näsman A**, Dahlstrand H, Lindholm J, Onelöv L, Ramqvist T, Ye W, Munck-Wikland E, Dalianis T. Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7. *Mol Oncol. 2007;1(3):350-5*

II. Romanitan M*, **Näsman A***, Ramqvist T, Dahlstrand H, Polykretis L, Vogiatzis P, Vamvakas P, Tasopoulos G, Valavanis C, Arapantoni-Dadioti P, Banis K, Dalianis T. Human papillomavirus frequency in oral and oropharyngeal cancer in Greece. *Anticancer Res. 2008;28(4B):2077-80*

III. Lindell G, **Näsman A,** Jonsson C, Ehrsson RJ, Jacobsson H, Danielsson KG, Dalianis T, Källström BN, Larson B. Presence of human papillomavirus (HPV) in vulvar squamous cell carcinoma (VSCC) and sentinel node. *Gynecol Oncol. 2010;117(2):312-6*

IV. Attner P, Du J, **Näsman A**, Hammarstedt L, Ramqvist T, Lindholm J, Marklund L, Dalianis T, Munck-Wikland E. Human papillomavirus and survival in patients with base of tongue cancer. *Int J Cancer. 2011;128(12):2892-7*

V. Du J, **Näsman A**, Carlson JW, Ramqvist T, Dalianis T. Prevalence of human papillomavirus (HPV) types in cervical cancer 2003-2008 in Stockholm, Sweden, before public HPV vaccination. *Acta Oncol. 2011;50(8):1215-9*

VI. Attner P, **Näsman A**, Du J, Hammarstedt L, Ramqvist T, Lindholm J, Munck-Wikland E, Dalianis T, Marklund L. Survival in patients with human papillomavirus positive tonsillar cancer in relation to treatment. *Int J Cancer. 2012;131(5):1124-30*

VII. Du J, Nordfors C, **Näsman A**, Sobkowiak M, Romanitan M, Dalianis T, 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*

VIII. Du J, Nordfors C, Ahrlund-Richter A, Sobkowiak M, Romanitan M, **Näsman A**, Andersson S, Ramqvist T, Dalianis T. Prevalence of oral human papillomavirus infection among youth, Sweden. *Emerg Infect Dis. 2012;18(9):1468-71*

IX. Löfdahl HE, Du J, **Näsman A**, Andersson E, Rubio CA, Lu Y, Ramqvist T, Dalianis T, Lagergren J, Dahlstrand H. Prevalence of human papillomavirus (HPV) in oesophageal squamous cell carcinoma in relation to anatomical site of the tumour. *PLoS One. 2012;7(10):e46538*

X. Marklund L, **Näsman A**, Ramqvist T, Dalianis T, Munck-Wikland E, Hammarstedt L. Prevalence of human papillomavirus and survival in oropharyngeal cancer other than tonsil or base of tongue cancer. *Cancer Med. 2012;1(1):82-8*

* *Contributed equally*

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AIN	anal intraepithelial neoplasia
APC	antigen presenting cells
APM	antigen-presenting machinery
ART	accelerated radiotherapy
BOTSCC	base of tongue squamous cell carcinoma
CDK	cyklin-dependent kinases
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
COPV	canine oral papillomavirus
CRT	chemo-radiotherapy
CTL	cytotoxic T cells
DC	dendritic cell
DFS	disease-free survival
DNA	deoxyribonucleic acid
DSS	disease-specific survival
E	early region
E6AP	E6 associated protein
EGFR	epidermal growth factor receptor
ER	endoplasmatic reticulum
HART	hyper-fractionated accelerated radiotherapy
HC	heavy chain
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HRT	hyper-fractionated radiotherapy
IARC	International Agency of Research of Cancer
ICD	International Classification of Diseases
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
INF	interferon gamma
IRF	interferon regulatory factor
L	late region
LC	Langerhans cells
LMP	large multifunctional peptidase
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NCR	non-coding region
NK-cells	natural killer cells
ORF	open reading frames
OS	overall survival

OSCC	oropharyngeal squamous cell carcinoma
pA	poly-adenaylation
Pap test	Papanicolaou test
PCR	polymerase chain reactions
PFS	progression-free survival
PRR	pattern recognition receptors
Rb	retinoblastoma
RNA	ribonucleic acid
RT	radiotherapy
RT	reverse transcriptase
SCC	squamous cell carcinoma
siRNA	small interfering ribonucleic acid
TAP	antigen peptide transporter
TGF	tumour growth factor
Th	T-helper
TIL	tumour infiltrating lymphocyte
Treg	regulatory T-cell
TSCC	tonsillar squamous cell carcinoma
UICC	International Union Against Cancer
VIN	vulvar intraepithelial neoplasia
VLP	virus like particle
β2m	beta2 microglobulin

1 INTRODUCTION

Although there are reports from, as early as, 1876 describing transmission of tumours from one animal to another, the field of tumour virology is considered to start with Peyton Rous famous chicken experiments in 1909 (Figure 1). In these experiments, Rous was able to grind and filtrate a tumour, a sarcoma, from a chicken on a very fine pore filter, where no cells were able to pass, and then inject the filtrate into another healthy chicken. Some time later, Rous observed the injected healthy chicken developed a sarcoma. He then repeated the procedure and injected the filtrate from the newly "contaminated" chicken into another healthy chicken, and again this originally healthy chicken developed a tumour. The filtrate, that did not contain any cells, was later demonstrated to contain a virus, which sooner was named as Rous sarcoma virus (RSV), and the idea that cancer was just another example of an infectious disease was born.(Weinberg 2007; Moore *et al.* 2010)

The tumour virology field expanded in the beginning of the 20th-century, and in 1913 Fibiger reported that stomach cancer in rats was associated to bacterial agents, supporting the idea that cancer was an infectious disease. However, a few years later, Fibiger's work was dismissed – the rats did not get cancer, just metaplasia due to vitamin deficiencies – and the focus on the origin of cancer was shifted again, the researchers started to focus on chemicals as inducers of cancer instead.(Weinberg 2007; Moore *et al.* 2010)

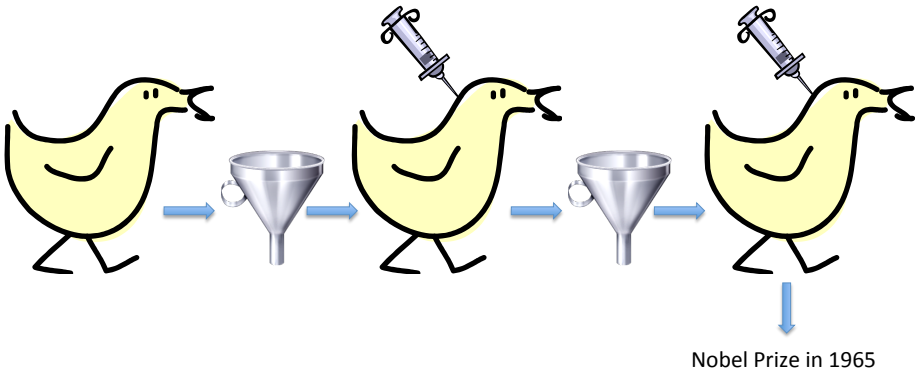


Figure 1. Experimental setup of Rous famous chicken experiment and the discovery of Rous sarcoma virus. The myth tells that a caring chicken farmer, living on Long Island, came up one morning to Dr. Rous with one of his chickens that newly had developed a tumour. However, instead of examining the chicken, Dr. Rous chopped the chicken's head off, extracted the tumour, filtrated it and injected it into another healthy chicken, which also developed a tumour. Rous then repeated the experiment serially and showed that the tumour-causing agent (later identified as Rous sarcoma virus) could be propagated. In 1965 he was awarded the Nobel Prize in Medicine for this discovery; whether the farmer was economically compensated for the chicken is still, though, shrouded in mystery.

After the initial detection of Rous sarcoma virus and all attention on tumour viruses, there came a time when the days of glory seemed to have passed for tumour virologists, but the research continued, and eventually more tumour viruses were discovered over the years. The first mammalian tumour virus to be detected was murine polyomavirus and this led to a revival in the interest of tumour viruses especially in mammals and of course especially in humans.(Moore *et al.* 2010)

Today, there are at least 7 discovered oncogenic viruses (belonging to 6 families) in man, and more viruses are to be expected as a consequence of new bio-molecular methods.(Moore *et al.* 2010) In this context, the detection of the association of human papillomavirus (HPV) and cervical cancer is a very famous example, where Professor Harald zur Hausen was awarded the Nobel Prize in Medicine in 2008 (Table 1).

Table 1. Tumour viruses in man and their association with malignancies presented in chronological order. Data obtained from (Moore *et al.* 2010).

Virus	Family	Associated malignancies	Year described
Epstein-Barr virus (EBV)	Herpesviridae	Burkitt's lymphoma, nasopharyngeal carcinoma, some Hodgekin's disease, some non-Hodgekins disease and some gastrointestinal lymphomas	1964
Hepatitis B virus (HBV)	Hepadnaviridae	Hepatocellular carcinoma	1965
Human T-lymphotropic virus I (HTLV-I)	Retroviridae	Adult T cell leukaemia	1980
High risk human papillomavirus 16 (HPV-16)	Papillomaviridae	Cervical, anal, penile, vulvar and head and neck cancer	1983
Hepatitis C virus (HCV)	Flaviviridae	Some hepatocellular carcinomas and some lymphomas	1989
Kaposi's sarcoma herpesvirus (KSHV or HHV8)	Herpesviridae	Kaposi's sarcoma	1994
Merkel cell polyomavirus (MCV)	Polyomaviridae	Merkel cell carcinoma	2008

The means of how human viruses cause tumours differs between viruses, but three main mechanisms can be distinguished and they are:

- The integration of the viral genome into the host genome
- The interaction of viral proteins with the host protein machinery
- Chronic inflammation

In the case of HPV, the virus of importance in this thesis, the published literature has focused on the second mechanism, and as we will see in the following chapters, this is the most important mechanism used by HPV to transform human cells.

Moreover, the focus of this thesis is on HPV and its role in oropharyngeal cancer. Therefore, we will now leave tumour virology as such, and a brief introduction to (1) HPV and (2) oropharyngeal cancer will be presented.

1.1 HUMAN PAPILLOMAVIRUS (HPV)

Viruses belonging to the family of *Papillomaviridae* are a group of small, non-enveloped viruses infecting mammals mainly asymptomatically, but also causing benign tumours/warts, and in some cases also neoplasias. In humans, so far over 150 different human papillomavirus (HPV) types have been identified and 12-18 of these are considered to be oncogenic.(IARC-Monographs 2009)

1.1.1 History

How the field of HPV in cervical, as well as head and neck cancer and other diseases emerged during the last century is beautifully described in a review by zur Hausen in Virology from 2009.(zur Hausen 2009) Here only a brief summary will be presented.

A high frequency of cervical cancer among prostitutes, but a very low in nuns and virgins, was noticed centuries ago, and was first reported in women followed during 1760–1839 in Verona Italy. As a consequence, during the 20th century, in parallel with the development of bacteriology/virology, many researchers tried to establish a link between cervical cancer and a sexually transmitted agent. Initially, attempts were made to link herpes simplex virus type 2, to the disease; but during the 1970´s HPV entered the scene. The first experiments that tried to establish a link between HPV and cervical cancer were initiated in 1972, but it was not until 1982 the first HPV type 16 sequences in cervical cancer were published. In 1983 HPV-16 DNA was isolated by southern blot from cervical cancer tissues, by the zur Hausen group.(zur Hausen 2009)

The rest is history, and today HPV is considered as a carcinogenic infectious agent, not only in cervical cancer, but also in some anal, penile, vulvar and head and neck cancers (discussed in 1.1.9).

1.1.2 Genome structure and viral particle

The Papillomaviridiae family consists of small (55nm in diameter), non-enveloped, double-stranded circular DNA viruses (Figure 2). The genome (~8000 base pair) can be divided into three major regions; the early region (E) that covers about 50% of the genome; the late region (L) that covers about 40% and the non-coding region (NCR) that covers approximately 10% of the genome (Figure 2). The early region contains the early open reading frames (ORF; ~genes) and encodes the early regulatory proteins E1, E2, E4, E5, E6 and E7 – and in HPV-31 also E8), while the late region encodes the late proteins L1 and L2, which together account for the viral capsid. Two poly-adenaylation (pA) sites separate the three regions, the early (pA_E) and the late (pA_L). The genome of HPV-16 is suggested to contain two major promoters, the early p97 and the late p670. The first is located upstream of E6 and is involved in the transcription of the early genes, while the latter is located within the E7 ORF and is responsible for the late transcripts.(Doorbar 2006; Zheng *et al.* 2006)

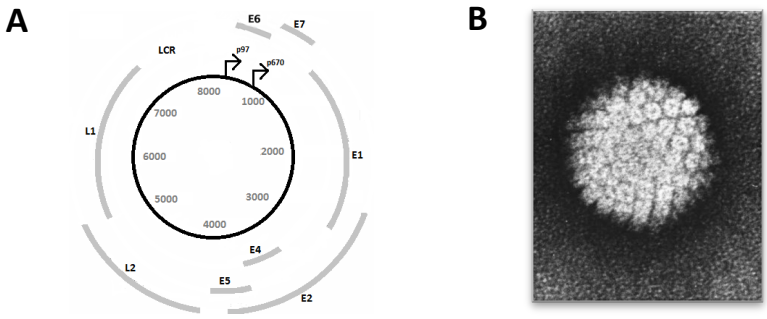


Figure 2. A proto-form of (A) the HPV genome (B) the icosahedral shaped viral capsid

1.1.3 Classification

As described above, all HPVs share a common genetic structure and some genetic parts are more conserved than other parts. One such well-conserved part, that is present in all papillomaviruses, is the L1 region, which is the framework in the papillomavirus classification.(de Villiers *et al.* 2004; Bernard *et al.* 2010)

The classification system is built up as a phylogenetic tree, in which the "family" is the root. Viruses belonging to the papillomavirus family, the *Papillomaviridae*, share the same structure and organisation, but the genetic sequence may diverge more than 55%. The family is divided into genera (alpha-pi), with 40-50% diversity to other genera; five of these genera (alpha, beta, gamma, Nu and Mu) are infectious in man – *i.e.* HPV. Branches within the genus are called "species". The species are divided into "types", and types within one species resemble each other more than 90% in their L1 region. Further separation may, in some cases, include sub-types (2-10% difference in L1) and variants (less than 2% diversity in L1). The most prevalent HPV type in head and neck and cervical cancer is HPV type 16 (HPV-16), which belongs to species 9 and the alpha-genus.(de Villiers *et al.* 2004; Bernard *et al.* 2010) Moreover, although there is scientific focus on the oncological potential of different HPV types, there are also data suggesting that intra-type variants (*e.g.* variants of E6 ORF) for HPV-16 may differ in oncogenic potential in cervical cancer.(Huertas-Salgado *et al.* 2011)

Another characteristic the HPV family is the division into cutaneous or mucosal HPVs, depending on which tissue the specific HPV type infects. Cutaneous HPVs have mainly been isolated from epithelial surfaces, *e.g.* from plantar warts (*e.g.* HPV-1) or patients with verruciform epidermodysplasia (*e.g.* HPV-8); while mucosal HPVs have been isolated from mucosal surfaces, *e.g.* laryngeal papillomas (*e.g.* HPV-6) and cervical cancer (*e.g.* HPV-16).(IARC-Monographs 2009) Another characterisation of HPV includes the separation into high-risk and low-risk types in cervical cancer. High-risk HPVs (*e.g.* HPV 16, 18, 31, 33, 35,) are more often associated with malignant tumours, while low-risk HPVs (*e.g.* HPV 6, 11, 34) are more seldom associated with neoplasias. There are also putative high-risk types, *e.g.* HPV 26, 53 and 66.(Munoz *et al.* 2003; IARC-Monographs 2009)

1.1.4 Transmission

HPV is mainly transmitted by skin-to-skin or mucosa-to-mucosa contact. In cervical infections, HPV is primarily sexually transmitted, and the risk of being HPV infected is correlated to the numbers of sexual partners, young age and early sexual debut.(Winer *et al.* 2003; Lenselink *et al.* 2008; Ramqvist *et al.* 2011b; Plummer *et al.* 2012) In oral/oropharyngeal infections, the route of transmission is still not fully elucidated. Although sexual contact (oral-to-genital) has been suggested as an important transmission route, other pathways, *e.g.* mother-to-child (at birth) and mouth-to-mouth (sometimes referred to as "kissing"), have been proposed.(D'Souza *et al.* 2009)

Nevertheless, how HPV infection is established, *i.e.* in which way the virus gains access to the basal layer is not fully understood. It has however been suggested that heparin sulphate proteoglycans and α-6 integrin may be of importance for binding of the virus to cells. Nonetheless, uptake of the virus after binding to human cells is slow, and upon endocytosis, facilitated by the viral E2 protein, viral DNA is transported to the nucleus.(Sapp *et al.* 2009; Schiller *et al.* 2010) In the nuclei, the viral genome is, usually, maintained as an episome.(Doorbar 2006)

Another issue that is not completely unravelled is whether the virus has a very specific tissue tropism. It has for a long time been accepted that some HPV types preferentially infect the epithelial cells within the transformation zone of the cervix, where the squamous epithelium turns into columnar epithelium. It has therefore been proposed that HPV has an affinity for "squamocolumnar junctions".(Moscicki *et al.* 2006) Likewise, in *e.g.* tonsillar cancer (discussed in 1.2.1), the corresponding site to the cervical transition site is suggested to be the crypts.(Kim *et al.* 2007) However, it is noteworthy to comment that there are no squamocolumnar junctions in the tonsils.

1.1.5 Viral life cycle

During most infections with HPV, its gene products are highly regulated in means of levels, and time for expression, and HPV does not usually cause malignant tumours. A productive HPV infection can be separated into different stages, in which different viral proteins play specific roles and are more abundant than during other stages (Figure 3).

The viral life cycle starts at the basal membrane upon infection and with the viral genome being episomal. It has been suggested that the viral genome is replicated during the cellular S-phase and that the numbers of viral copies/cell are stable with around 10-200 copies/cell.(Doorbar 2006)

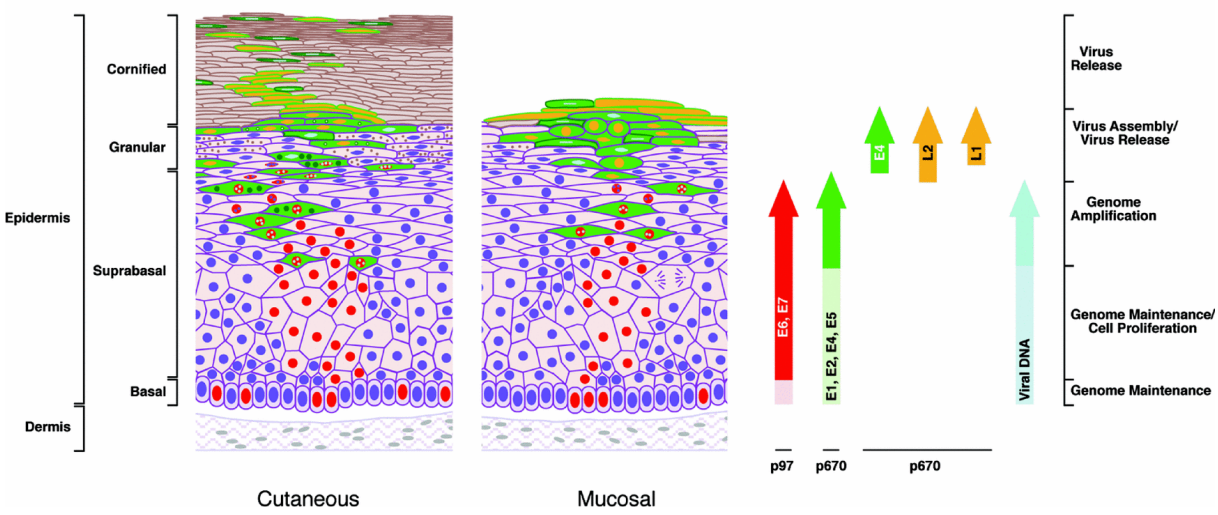


Figure 3. Viral life cycle. Viral protein and DNA expression in the different epithelial layers.(Doorbar 2006)

As mentioned above, transcription of viral proteins is initiated by the early promoter, p97 in HPV-16 (Figure 3 and 4). Transcription, both from the early and the late promoter, occurs from only one of the two viral strands and both generate long pre-mRNA strands, that later are spliced into viral mRNA, as illustrated in Figure 4.(Zheng *et al.* 2006)

Viral proteins E1 and E2 are the two most important viral proteins during early infection. E2 plays many crucial roles, *e.g.* it binds viral DNA in the NCR and recruits E1, which in turn recruits the cellular proteins necessary for DNA replication, *e.g.* α DNA polymerase.(Doorbar 2006) In addition, E1 also exhibits a helicase function, opening the viral DNA for cellular replication proteins, by binding to A/T rich regions in the NCR. E1 is therefore regarded as essential for initiation of viral replication. (Wilson *et al.* 2002) Upon cellular replication, following S-phase, E2 is suggested to play another important role, in that it anchors the viral episomes to mitotic chromosomes and allows the viral genomes to split equally.

E2 also plays an important role in regulating viral transcription. Low levels of E2 activate early transcription, while abundance of E2 inhibits early promoter transcription and it has been proposed that E2 can repress the TATA box binding protein (important for initiation of transcription).(Doorbar 2006; Zheng *et al.* 2006; Thierry 2009) As stated previously, the early promoter initiates transcription of E1 and E2 as well as E4-E7. It is also the only mediator for transcription of E6 and E7, which together are important for abrogating normal cell cycle control and pushing the cells to proliferate, and E6 and E7 will be discussed more extensively below with regard to transformation (section 1.1.7). Similar to E2, transcription of E4 and E5 by the early promoter is less efficient as compared to that initiated by the late promoter, as described below (Figure 3 and 4)(Moody *et al.* 2009).

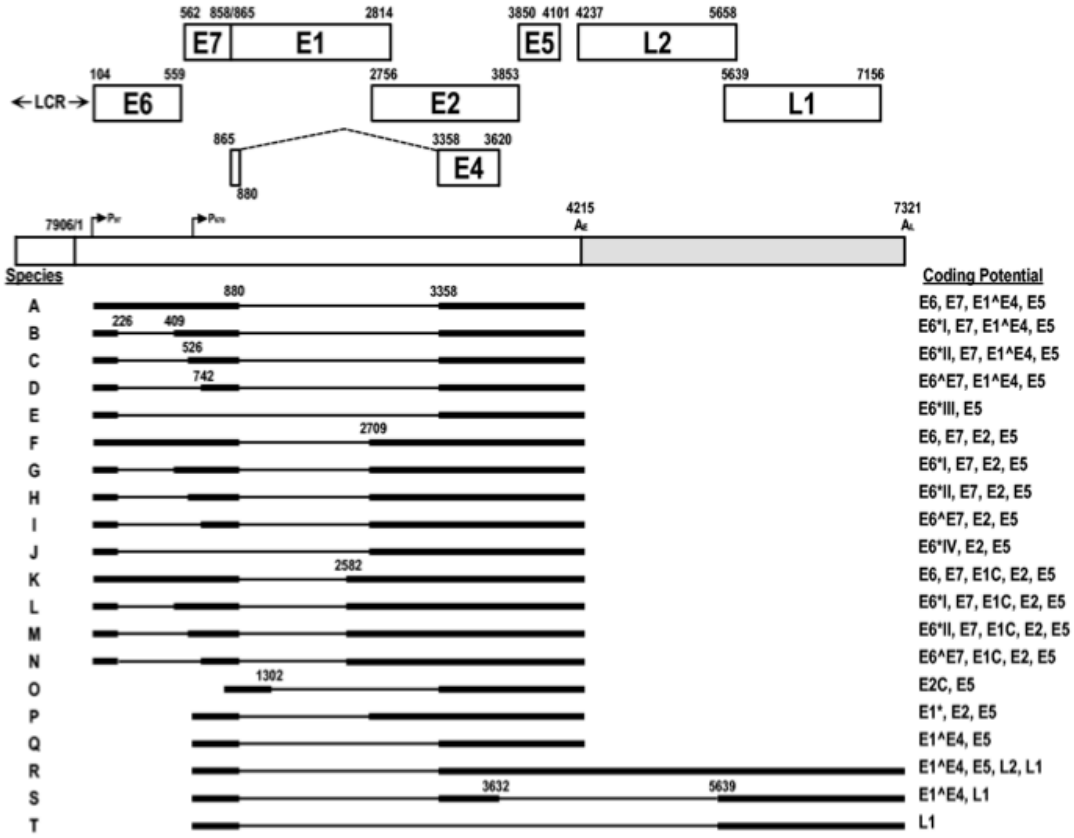


Figure 4. A schematic model of the HPV genome and its transcripts, presented here for HPV-16. The full genome (~8 kb) is presented with all the ORFs, the two promoters (p97 and p670), the two polyadenylation sites (for the early and late transcripts respectively) and the NCR (here presented as LCR). Moreover, the early and late transcripts are also presented below the viral genome. As indicated on the side, the possible splice-products are also presented.(Zheng *et al.* 2006)

Upon epithelial differentiation, transcription from the late promoter increases – which leads to high levels of E1, E1^E4, E5, L1 and L2 (Figure 3). The activity from the early promoter remains stable, giving low levels of E6 and E7 at all epithelial stages. Transcription of E1 and E2 changes also upon differentiation, and occurs not only from the early, but also from the late promoter. Moreover, in contrast to transcription from the early promoter, E2 levels do not down regulate transcription from the late promoter. As a consequence, the switch in promoter leads to higher transcription and replication, which gives a dramatic increase in viral copy numbers per cell.(Moody *et al.* 2009) However, which mechanisms that induce the promoter switch are still unrevealed.

During the viral life cycle, E4 is the most abundant protein. It is accumulated in the cytoplasm, and its expression is increased upon cellular differentiation.(Doorbar 2006) However, the role of E4 is still enigmatic, but some studies claim a role of E4 in viral replication, transcription of the viral capsid proteins, and the release in viral particles. (Wang *et al.* 2004; Nakahara *et al.* 2005; Wilson *et al.* 2007)

As mentioned above, another early protein that is transcribed more frequently from the late promoter is the E5 protein (Figure 3 and 4). This protein is small and hydrophobic

and is, therefore, preferably detected in the Golgi and the endoplasmatic reticulum.(Moody *et al.* 2010) Moreover, in vitro studies have demonstrated a link between E5 and *epidermal growth factor receptor* (EGFR), in that E5 may increase EGFR levels as well as modulate EGFR signalling pathways. It has therefore been suggested that E5 has the ability to exhibit a weak transforming activity.(Moody *et al.* 2009; Moody *et al.* 2010) Nevertheless, it should be noted that E5 is frequently deleted in cervical cancer (Tsai *et al.* 2003; Hafner *et al.* 2008) – suggesting that E5 is not necessary for cellular transformation. Another function of E5, and of interest for this thesis, is the ability of E5 to down-regulate human leukocyte antigen (HLA) class I expression (discussed in section 1.1.10.3.2). There are studies that suggest that E5 can accumulate HLA class I molecules in the Golgi apparatus; thereby reducing HLA class I expression and preventing the cells from cytotoxic T-cell clearance (for more details see section 1.1.10.3.2). In addition, a study by Zhang et al has also demonstrated that E5 is able to down-regulate HLA class II expression in interferon gamma (INF-γ) treated keratinocytes.(Zhang *et al.* 2003) Altogether, these studies suggest, that E5 may be able to decrease the immune recognition of HPV infected epithelial cells.

The last step in the viral life cycle is virus assembly and release. The newly synthesised viral genomes are packed into icosahedral capsids, formed by the viral L1 and L2 proteins. The capsid consists of 360 copies of the L1 protein and theoretically up to 72 copies of the L2 protein.(Sapp *et al.* 2009) As mentioned above, the L1 protein self-assembles (also discussed in section 1.1.11.1) and forms pentamers (capsomeres), and each viral capsid consists of 72 capsomeres. The L1 pentamers are formed in the cytoplasm and the virus particle in the nucleus. After packing, the virus particles are shed from the surface of the epithelium.(Doorbar 2006)

1.1.6 HPV induced carcinogenesis

As described above, E6 and E7 are expressed during the whole life cycle, but are more prominently expressed during the earlier stages of the viral cycle. E6 and E7 interact with the human cell cycle and drive it into S-phase, which enables the viral genome to replicate. A very simplified explanation of the abilities of E6 and E7 to induce S-phase is that E7 inhibits the Rb (retinoblastoma) protein, and E6 degrades the human p53 protein (Figure 5).(Leemans *et al.* 2011)

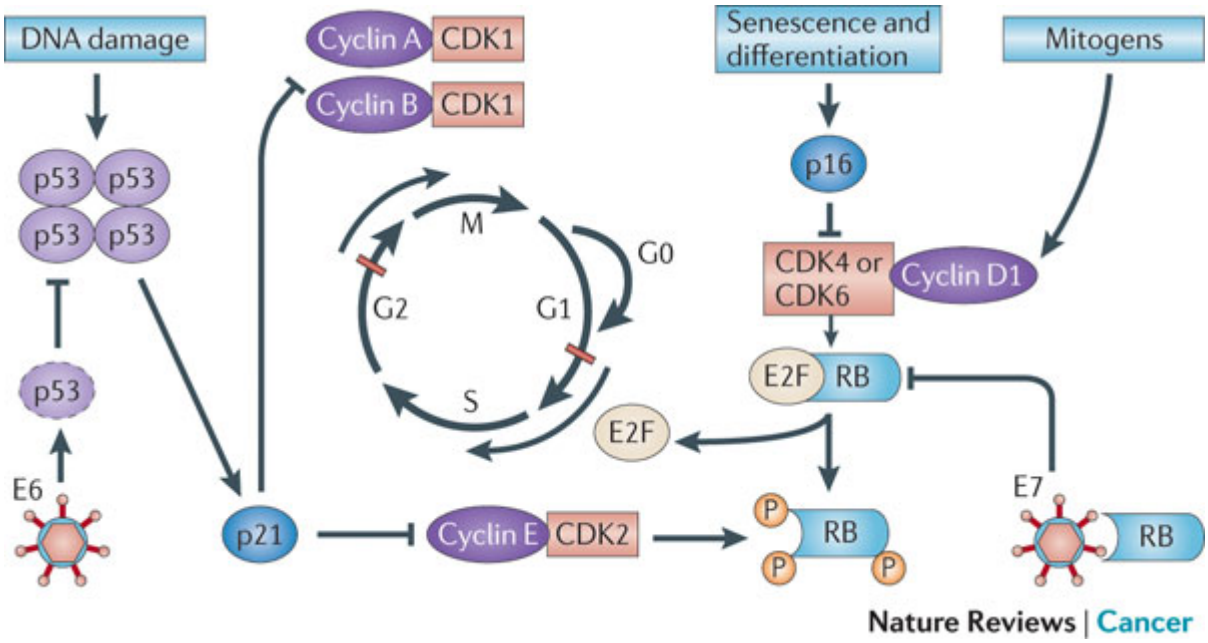


Figure 5. A schematic overview of cell cycle deregulation by HPV. For details, please see the text in section 1.1.6 (Leemans *et al.* 2011)

Normally, S-phase is induced by cyklins and cyklin-dependent kinases (CDK) upon mitotic signals. Upon such stimulation, the cyklin-CDK complexes phosphorylate the Rb protein (pRb), and inhibit its E2F binding. E2F transcription factors are then released and activated, and can *e.g.* induce S-phase genes. In an HPV infected cell however, pRb is inactivated by E7, and thereby S-phase is induced.(Doorbar 2006)

The natural inhibitor of the CDK4/CDK6-CyklinD complex, that promotes S-phase, is the $p16^{INK4a}$ protein (belonging to the INK4 family, one of two CDK inhibitor families in mammals).(Canepa *et al.* 2007) In an HPV infected cell, $p16^{INK4a}$ is often up-regulated (discussed in 1.2.7), which seems to some extent logic when studying figure 5. However, in a recent study by McLaughlin-Drubin and colleagues, the authors showed that up-regulation of $p16^{INK4a}$ in HPV positive cells was due to KDM6B, a histone demetylase induced by HPV-16 E7. For this reason, it was therefore suggested that E7 mediated $p16^{INK4a}$ expression is independent of pRb inactivation.(McLaughlin-Drubin *et al.* 2011)

Moreover, in a well functioning cell, inhibition of pRb and deregulation of E2F would cause p53 activation *e.g.* by another CDK inhibitor in the INK4a family, $CDKN2A^{ARF}$ ($p14^{ARF}$). Activated p53 should then normally lead to halted cell cycle and to apoptosis. (Polager *et al.* 2009) However, cell cycle halting and apoptosis are inhibited, by the fact that E6 binds to and degrades p53 (Figure 5).(Doorbar 2006)

During natural HPV infection, the levels of the oncogenic viral proteins E6 and E7 are low and are regulated by E2 levels (described in 1.1.5). However, in many tumours, the HPV genome may integrate into the human genome, causing a disruption in the E2 ORF. Such disruption can lead to loss of E2 and allowing higher E6 and E7 levels, due to loss of their regulation by E2.(zur Hausen 2000; Munger *et al.* 2004)

The above-sketched overview is, indeed, a simplification of the interactions between the viral oncogenic proteins and the cellular machinery. It is also important to bare in mind, especially in head and neck cancer, that additional transforming processes/factors may co-exist at the same time, *e.g.* HPV infection and smoking.(Gunnell *et al.* 2006) Below the E6 and E7 and their interactions with cellular proteins will be described in more detail as well as the role of viral integration.

1.1.6.1 E6

E6 is a well-conserved oncogene, and the protein consists of 151 amino acids. Although one of the most studied functions of high-risk HPV E6 is the binding - in complex with the E6 associated protein (E6AP) – to p53, which thereby becomes ubiquitinated and degraded, other functions of E6 have also been identified.(Munger *et al.* 2004) One other such function is the ability of high-risk HPV E6 to bind the PDZ-family protein. Such interaction may result in a degradation of the PDZ-domain resulting in deregulation of organization and differentiation and of the chromosomal integrity.(Feller *et al.* 2010) Other important functions include the ability of E6 to induce telomerase activity and mediate the degradation of pro-apoptotic proteins, *e.g.* Bak.(zur Hausen 2000)

As stated above, much research has been directed on understanding the link between high-risk HPV E6 and p53. Studies on low-risk HPV have also shown the ability of E6 to interact with p53, but without changing the turnover of p53.(Klingelhutz *et al.* 2012) Why this is the case has not been fully elucidated. However, it has been shown that high-risk HPV E6 can potentially bind to p53 at two locations, while low-risk HPV E6 binds only to one location, suggesting different interactions between E6 of high and low-risk HPV types and p53.(Ganguly *et al.* 2009)

The reason why high-risk HPVs are more oncogenic in some patients than others, have also gained a lot of interest the last years. Interestingly, a Nature paper some years ago (1998) investigating the role of polymorphism in p53, suggested that the E6 of HPV-16 and HPV-18 would be more prone to degrade p53 with Arg rather than Pro at codon 72. The authors also found that Arg p53 was more common among cervical cancer patients, compared to healthy controls.(Storey *et al.* 1998) However, other studies followed immediately and were not been able to support these findings.(Helland *et al.* 1998; Hildesheim *et al.* 1998; Josefsson *et al.* 1998)

E6 may also have an immune modulating capacity and this will be discussed below in section 1.1.10.

1.1.6.2 E7.

E7 is a 98 amino acid long protein with a plethora of functions. As already described above, one of its most important functions is to bind and destabilize pRb and the related tumour suppressors p107 and p130; which all three regulate the E2F family.

Furthermore, studies have shown that E7 of high-risk HPVs binds pRb with a 10 times higher affinity than E7 of low-risk HPVs, and this difference has been explained by a difference in one single amino acid.(McLaughlin-Drubin *et al.* 2009)

E7 also has the ability to inhibit CDK inhibitors *e.g.* p21 and p27 (Figure 5). In addition, it has been suggested that E7 has the ability to enhance integration of foreign DNA, resulting in an increased mutagenesis, although the mechanism for this is not fully understood. In addition, E7 of high-risk HPVs has the ability to induce chromosomal instability, as also shown for E6.(McLaughlin-Drubin *et al.* 2009) Finally, E7 has also an immune modulating capacity and this will be discussed in more detail below in section 1.1.10.

1.1.6.3 Viral integration.

In the normal viral life cycle, the virus is maintained and replicates as an episome. However, in high-grade lesions and in cervical neoplasias the viral genome frequently integrates into the human genome.(Moody *et al.* 2010) It has been concluded that integration is not regulated (*e.g.* by integrases, as in HIV) and occurs most likely randomly in the whole human genome, possibly with a preference for fragile sites and most likely a consequence of chromosomal instability.(Thorland *et al.* 2000; Thorland *et al.* 2003) Irrespective of the mechanism behind it, integration of the viral genome has been suggested as an important step for, but not a pre-requisite for the development of invasive cervical cancer.(Pett *et al.* 2007; IARC-Monographs 2009) Explanations for this statement have in the literature been two:

- Upon integration viral DNA sequences may be deleted. Although different sequences may be lost, E2 is often deleted – leading to deregulated E6 and E7.(Woodman *et al.* 2007; Collins *et al.* 2009)

- Expression of important regulatory cellular genes may be affected by insertion of viral DNA.(Ferber *et al.* 2003; Ragin *et al.* 2007a)

In cervical cancer, the published literature is consistent and today it is widely accepted that HPV genome integration occurs in ~90% of the cases.(Pett *et al.* 2007) Nevertheless, in head and neck cancer the data are still insufficient and although many studies have claimed that the genome is integrated, the methods used are often indirect and inadequate. Many studies have quantified E2 and E6 DNA and in cases where E6 dominates over E2, one has concluded the viral DNA to be integrated.(Venuti *et al.* 2000; Koskinen *et al.* 2003; Kim *et al.* 2007) However, since integration is a likely random event and any HPV sequence may be lost in the process, it should, be impossible to adequately assess the integration status of the viral genome just by gene quantification. Moreover, the ratio between E2 and E6 has often also been used to quantify the degree of integration in these studies described above. Reasoning this way, a ratio of *e.g.* 0.5 implies that 50% of the viral genomes are integrated and 50% are episomal. However, E2 expression from an episomal HPV genome should theoretically be able to repress both integrated and episomal E6 and E7 expression.

Nevertheless, a study by Mellin *et al*, using a more appropriate method (detecting human-viral junctions) showed HPV to be only episomal and more importantly some of the episomes had deletions. On the other hand, this study was small (11 tumours) and in some of these tumours there were a very high viral load.(Mellin *et al.* 2002) Obviously, the role of viral integration in the head and neck region needs more investigation.

1.1.7 HPV detection methods

Many different detection methods for HPV have been described, and which method that is used in a study or a clinical setting is often dependent on what kind of material/tissue that has to be analysed. However, there are also cases where different methods may be feasible, and in these cases there is often a scientific debate about the optimal method. In general, four main methodology categories can be distinguished 2 direct and two indirect): (1) direct detection of viral DNA/RNA in the tumour (2) direct detection of viral proteins in the tumour; (3) detection of serum antibodies (indirect detection method); and (4) detection of proteins that are up/down-regulated upon HPV infection (indirect detection method). Moreover, tumour material may also differ in that it can be fresh/fresh frozen or paraffin embedded. Below, a few examples of HPV detection methods are presented.

(1) Detection of viral DNA/RNA. Different types of polymerase chain reactions (PCRs) have been used to define a tumour as HPV positive.(Venuti *et al.* 2012) One of the most common methods used is a PCR with general (or consensus) primers and thereafter a system to visualize the products. General primers refer to that these primers can detect many different HPV types by binding to highly conserved regions within the viral genome.(Karlsen *et al.* 1996) Examples of such primer-pairs are the GP5/GP6 (bind to L1 region)(van den Brule *et al.* 1990); Gp5+/GP6+ (extended GP5/GP6 primers with 3nt – also binds the L1 region)(de Roda Husman *et al.* 1995); CPI/CDIIG (binds to the E1 region)(Tieben *et al.* 1993); MY09/MY11 (bind to the L1 region)(Karlsen *et al.* 1996) and SPF primer pairs (connect to the L1 region)(Kleter *et al.* 1998). Although these primer pairs bind conserved regions, there is still a considerable mismatch, which also varies between HPV types.(de Roda Husman *et al.* 1995) Therefore, some HPV types are more easily detectable than others.(Schmitt *et al.* 2008) To overcome this problem, the PCR program is, most often, designed with a lower annealing temperature – allowing the primers to bind with mismatches. However, a lower annealing temperature cannot totally solve the mismatch problem, and therefore a new consensus primer multiplex setup, the broad-spectrum GP5+/6+, has recently been proposed. In this multiplex PCR setup, the authors reported an increased sensitivity for at least 10 HPV types.(Schmitt *et al.* 2008) After DNA amplification, some researchers have used probe-based techniques to type the PCR product (*e.g.* commercially available Luminex or InnoLiPa), while others visualize the product on a gel and thereafter use HPV type specific PCRs or sequencing.(Mellin *et al.* 2002; Ramqvist *et al.* 2011b)

Another method, commonly used, is the *in situ* hybridization method. Here, a biotinylated probe is hybridised to the viral DNA and thereafter the signal,

and not the viral DNA as in the examples above, is amplified. The presence of, as well as the localisation of the viral DNA is then evaluated under a light microscope.(Venuti *et al.* 2012)

Other DNA/RNA methods described may include detection of viral E6 and E7 RNA, followed by cDNA synthesis that is followed by amplification and detection.(Venuti *et al.* 2012) There are also some very rare studies, where the presence of HPV DNA has been analysed for in serum from patients with *e.g.* head and neck cancer; but such detection methods should not be considered as sufficiently reliable.(Capone *et al.* 2000)

(2) *Detection of viral proteins in the tumour.* Many different antibodies against the viral oncoproteins E6 and E7 have been developed over the years, but due to low sensitivity and specificity these antibodies have never been of any value in clinical samples.

(3) *Detection of serum antibodies.* Methods detecting antibodies in sera against viral capsid proteins L1 and L2 as well as viral oncoproteins E6 and E7 have been used in *e.g.* epidemiological studies.(Mork *et al.* 2001; Poynten *et al.* 2012) However, these antibodies are never site-specific and could also be a result of an earlier infection.

(4) *Detection of the $p16^{INK4a}$ protein, which is up-regulated upon HPV infection.* Although there are many human proteins that are up/down-regulated upon HPV infection, overexpression of $p16^{INK4a}$ has by far most frequently been used to define HPV positive status in head and neck cancer. As described above, $p16^{INK4a}$ is up regulated as a result of degradation of pRb, caused by the viral protein E7.(Venuti *et al.* 2012) The advantages/disadvantages of using this marker in head and neck cancer will be discussed in section 1.2.7.

So far, the most common methods used in the head and neck cancer field are the use of PCR with GP5+/6+ primers, in situ hybridisation and the $p16^{INK4a}$ expression analyses. Although different algorithms for HPV detection have been proposed (discussed in section 1.2.7) the optimal HPV detection method/algorithm is still under debate.(Smeets *et al.* 2007)

1.1.8 HPV prevalence in healthy populations

HPV is one of the most common sexually transmitted diseases worldwide, and most sexually active individuals will be infected with HPV at some time point. Nevertheless, for the vast majority of infected individuals, HPV infection will pass asymptomatically and clear within 1-2 years.(Tota *et al.* 2011)

When analysing the prevalence of HPV infection in healthy individuals, the rates vary widely between infection site and study populations. An attempt to summarise the HPV prevalence in healthy women was recently made in a meta-analysis by Bruni *et al*. In this analysis the authors reviewed all published data from studies between 1995 and

2009 that included woman with normal cytology. The authors found a HPV prevalence of 7.2% worldwide (11.7% adjusted); but the prevalence differed immensely – from 1.6 to 41.9%. Geographically, the prevalence also varied, from around 30% in Eastern Africa, the Caribbean and Eastern Europe, while it was lower in Northern America, Western Europe and Asia.(Bruni *et al.* 2010) Another similar meta-analysis published in 2005, but during a different timespan, came to a similar conclusion.(de Sanjose *et al.* 2007) There also seems to be an age-specific distribution of HPV infection in females, with a first peak in women <25 years and a second in middle-aged women. (Franceschi *et al.* 2006; de Sanjose *et al.* 2007; Smith *et al.* 2008; Bruni *et al.* 2010)

Genital HPV infection in sexually active men is also very common, and varies also greatly between countries and populations, and can depend on the sampling and testing methods used as well as the anatomical site that is investigated (scrotum, shaft, glans and urethra). In addition, it has been suggested that male genital HPV prevalence, in contrast to that in females, remains close to constant after the peak-prevalence.(Smith *et al.* 2011; Tota *et al.* 2011)

Furthermore, anal HPV infection in healthy individuals has also gained scientific focus. Although most studies have studied HPV prevalence in HIV positive patients, a few studies have examined HPV in healthy individuals. In a longitudinal cohort study of healthy women in Hawaii, 70% of the studied women had >1 anal HPV infection during follow-up, and the majority cleared the infections swiftly. Furthermore, the investigators also stated that the anal and cervical HPV infections seemed to occur consecutively.(Goodman *et al.* 2008; Shvetsov *et al.* 2009; Goodman *et al.* 2010) In another Italian study, 28% of the investigated healthy sexually active women were infected with anal HPV; and similar figures were observed in the above-described Hawaii cohort at baseline.(Hernandez *et al.* 2005; Pierangeli *et al.* 2012) However, in heterosexual men, the prevalence of anal HPV infection has been reported to generally be low (<10%).(Vardas *et al.* 2011)

In addition, HPV prevalence in oral specimens has also been examined and so far the data suggest that <10% of healthy individuals have an oral HPV infection. However, also here there is a variation in HPV prevalence and different detection techniques are used (Kreimer *et al.* 2010; Rautava *et al.* 2011; Du *et al.* 2012; Gillison *et al.* 2012)

1.1.9 HPV associated malignant tumours and screening programs

The most commonly studied HPV associated malignant neoplasia is cervical cancer, where 83-99.7% of the cases have been suggested to be HPV positive.(Smith *et al.* 2007; de Sanjose *et al.* 2010; Li *et al.* 2011) On a regional level, similar numbers (92%) have been suggested in the county of Stockholm, Sweden.(Du *et al.* 2011) Nevertheless, other malignancies have also been associated with HPV over the years, including cancer of the anus (~80% HPV positive (De Vuyst *et al.* 2009; Hoots *et al.* 2009)), the vulva (~40% HPV positive (De Vuyst *et al.* 2009)), the vagina (~80% (De Vuyst *et al.* 2009)), the penis (~40% HPV positive (Tota *et al.* 2011)) and the head and neck region (discussed in 1.2.5). Many of these tumours are preceded by pre-stages

e.g.: cervical intraepithelial neoplasia (CIN) in cervical cancer, vulvar intraepithelial neoplasia (VIN) in vulvar cancer and anal intraepithelial neoplasia (AIN) in anal cancer. So far however, no pre-stages have been identified in head and neck cancer. In addition, other HPV associated tumour sites such as *e.g.* lung and oesophageal cancer has been suggested, but here the role of HPV is still inconclusive.(Lofdahl *et al.* 2012; Yanagawa *et al.* 2013)

Cervical cancer is today the only HPV associated disease that is included in a screening program, although there have been discussions to initiate screening-programs for some HPV associated tumours, *e.g.* for anal cancer.(Pierangeli *et al.* 2012) A systematic screening program for cervical cancer was initiated already in the 1960's in Sweden, and has proven to reduce the incidence of the cancer by 35-75%.(Bergstrom *et al.* 1999; Sigurdsson 1999; Hemminki *et al.* 2002) However, the previously most often-used method, the Pap smear test, has low sensitivity and specificity for pre-stages of cervical cancer. Therefore, alternative methods with potentially higher sensitivity have been proposed, *e.g.* liquid-based cytology – in which cells are suspended and spread in a thin uniform manner on a slide to facilitate microscopic interpretation.(Arbyn *et al.* 2008) Today it is questionable if Liquid-based cytology is indeed more sensitive than the classical Pap smear for cytology, but it does also offer the possibility to test for HPV status.(Strander *et al.* 2007; Arbyn *et al.* 2008; Froberg *et al.* 2013) Previously, HPV tests were controversial, and it was proposed that although detecting HPV was more sensitive, it was also less specific for detecting pre-stages of cervical cancer (CIN-II+).(Cuzick *et al.* 2006) Today, in many regions, HPV testing is gradually being introduced as a primary screening methods for women above 30 or 35 years of age, when genital HPV infection is less common in the general population as for those below 30.(Gyllensten *et al.* 2012)

1.1.10 Interactions with the immune system

HPV has similar to pathogens in general also developed mechanisms to avoid the immune system and, thus, avoid immune clearance. The expected human response to a viral infection is - indeed - described better elsewhere, and here only a brief and simplified recapitulation will be given.

1.1.10.1 General immune response

Keratinocytes, or other cells, may upon viral infection start to secrete pro-inflammatory cytokines, to attract antigen-presenting cells, such as dendritic cells (DCs) and macrophages. In addition, immature DCs - constantly sampling the surroundings for pathogens by pattern recognition receptors (PRR) - may engulf viral products. Upon DC activation, DCs mature and start to migrate to the regional lymph nodes and the foreign proteins within the DCs are degraded to peptides. These peptides are then presented on the major histocompatibility complex (MHC – often referred to human leukocyte antigen, HLA, in humans). Through presentation of peptides on MHC class I, or MHC class II antigens respectively, DCs bind to cytotoxic T cells (CTLs) or helper T-cells, in the presence of co-stimulatory molecules and bind to, interact with, and

activate their respective CD8+ or CD4+ receptors. Activated CD4+ T-cells start to proliferate and may mature into Th1 or Th2 cells depending on the cytokine environment. A milieu with IFN-γ drives Th1 cell production, while IL-10 and IL-4 inhibit Th1 cell production and promote the formation of Th2 cells. In a viral infection, a Th1 response is desired, which can, by *e.g.* the secretion of IFN-γ, induce maturation of CD8+ CTLs and activate macrophages and NK-cells.(Abbas *et al.* 2004) Nevertheless, HPV usually successfully constrains and inhibits the immune response by different mechanisms, which will further be described below.

1.1.10.2 Escaping the immune recognition by location of infection

As mentioned above, the HPV replication cycle is highly regulated and limited to differentiating keratinocytes, as discussed in section 1.1.5. Subsequently, HPV does not exhibit a blood borne phase and there is no obvious cytolysis, when the viral particles are released.(Tindle 2002) Additionally, due to sub-optimal codon usage by HPV, the viral proteins are kept low.(Zhao *et al.* 2005) Therefore, taken together, the immune system has a low chance to detect the infection.

Furthermore, data also suggest that the antigen presenting cells (APCs) in the epidermis, the Langerhans cells (LCs), are not activated by uptake of the HPV capsid, in contrast to the DCs in the dermis.(Fausch *et al.* 2002; Fausch *et al.* 2003; Da Silva *et al.* 2007) This way, the virus is able to hide from the immune system as long as the infection is localised to the epidermis. Moreover, it has also been proposed that the local cytokine milieu (*e.g.* TGF-β and IL-10), produced by the keratinocytes that are present when LCs mature, may promote LCs to polarise towards Th2 type immunity.(Brinkman *et al.* 2007) Such Th2 type immunity would result in an ineffective response against HPV infected cells.

Lastly, the antiviral and immune-stimulatory type -1 interferons (IFN-α and IFN-β) are inhibited by the HPV oncogenes. HPV E7 has been suggested to inhibit IFN-α signal transduction by binding of the interferon regulatory factor 9 (IRF-9) and preventing its translocation from the nucleus. Another function of E7 that has been proposed is the ability to inhibit IRF-1 activation of the IFN-β promoter. HPV18E7 has also been described to reduce IRF-1 genes, such as TAP-1 and monocyte chemotactic protein-1 (MCP-1). The HPV E6 protein has, on the other hand, been described to inhibit IFN-α signalling by preventing transcription through inhibition of IRF-3.(Tindle 2002; Stanley 2012) An interesting comment to the ability of HPV to down-regulate IFN-α and its importance in immune evasion is that IFN-α is used to treat genital warts. However, not all patients respond to this treatment, and there are data showing that responders have lower E7 levels than those that do not respond.(Arany *et al.* 1995)

1.1.10.3 HPV and antigen presenting machinery (APM)

1.1.10.3.1 MHC class I and class II

Recognition of viral peptides on MHC antigens is crucial for clearance of virally infected cells. In the majority of cases focus is directed towards the presentation of viral peptides on MHC class I molecules and recognition by CTLs. Nevertheless, prior to the presentation of viral peptides, a cascade of events takes place in the infected cell. Intracellular proteins, foreign as well as self-proteins, are degraded by the proteasome in the cytosol. (The activity of the ubiquitin-protease pathway may be modulated by IFN-γ, which induces proteasome sub-units (*e.g.* LMP-2, -7 and -10) as well as some proteasome activators.) After degradation, the peptides are transported and translocated to the ER by TAP-1 and TAP-2 molecules, with the purpose to be loaded to an MHC class I molecule. In parallel, the formation of MHC molecules is continuously on going, with the MHC class I heavy chain (HC) translocation to the ER lumen by Sec61. The HC binds to the chaperones Calnexin and BiP, and is thereby assisted in appropriate folding and the binding to the beta2 microglobulin (β2m) chain. Thereafter, Calnexin is dissociated and replaced by Calreticulin and Erp53, which stabilises the MHC molecule. Tapasin also binds to MHC class I molecules linking them with TAP1 and TAP2 proteins and, thereby allowing for building up of the peptide-loading complex. After the loading of MHC class I molecules with peptides, the whole complex is transported to the cell surface through the ER and Golgi. Sub-optimal loading of peptides to the complex, results in that MHC class I molecules exiting in the ER are degraded.(Seliger *et al.* 2000; Abbas *et al.* 2004; Seliger 2012)

As stated above, MHC class II antigens also bind peptides, and in this case mostly extracellular peptides, however since MHC class II antigens are almost exclusively expressed on APCs, *e.g.* monocytes, dendritic cells and B-cells this process is not clarified here in more detail.(Abbas *et al.* 2004) Nevertheless, as described later, HLA class II expression may be observed in some malignancies and has been shown to enhance tumour-specific immunity by bypassing the classical MHC class I pathway. (Glew *et al.* 1992; Glew *et al.* 1993; Armstrong *et al.* 1997; Armstrong *et al.* 1998; Zehbe *et al.* 2005)

1.1.10.3.2 HPV and MHC class I

Similar to many viruses, HPV has developed mechanisms to interact with and down-regulate the antigen presenting machinery (APM). However, in contrast to other viruses that may interfere at any of the above-described steps in the APM, HPV-16 has only been reported to interfere with MHC class I.(Hansen *et al.* 2009) Previous reports have shown the ability of the HPV E5 protein, which mainly is located in the Golgi, to bind the HC of MHC class I (independent of haplotype) and to retain it in the Golgi, however when the tumour cells were treated with interferon, the E5 mediated effect was lost.(Ashrafi *et al.* 2005; Ashrafi *et al.* 2006a; Ashrafi *et al.* 2006b; Campo *et al.* 2010) Other reports have investigated the role of HPV-16 E7, and found that the viral

protein may cause repression of the MHC class I promoter, leading to lower MHC class I levels on cell surface. Consequently, blocking of E7 with siRNA in SiHA and CaSki cell lines, increased MHC surface levels, while induction of E7 reduced the levels. MHC class I down-regulation increased NK-cell killing.(Georgopoulos *et al.* 2000; Li *et al.* 2006; Bottley *et al.* 2008)

1.1.10.3.3 MHC class I and II expression in HPV associated malignancies

Down-regulation of MHC class I expression is a well-known tumour evasion mechanism and may occur relatively frequently in human neoplasias, including *e.g.* breast, lung, ovarian, cervical and head and neck cancer.(Keating *et al.* 1995; Vitale *et al.* 1998; Atkins *et al.* 2004; Ogino *et al.* 2006; Ramnath *et al.* 2006; Han *et al.* 2008; Mehta *et al.* 2008) Moreover, loss of or down regulation of MHC class I expression has also been correlated with disease progression. Thus, in cervical cancer, MHC class I and II antigen expression is reported to decrease with disease progression as well as with metastatic disease.(Ryu *et al.* 2001) Similar results have been observed in other malignancies, *e.g.* ovarian cancer and melanoma.(Le *et al.* 2002; Carretero *et al.* 2008) These data are in line with older data, in which MHC class I was down regulated in metastasised neoplastic cells, as compared to primary tumour cells. Moreover, the metastatic down regulation was accompanied by loss of TAP-1 expression.(Cromme *et al.* 1993; Cromme *et al.* 1994b) Although no HPV proteins have been shown to interact with the APM proteins described above, studies have shown deregulated levels of *e.g.* LMP and TAP molecules in cervical cancer.(Cromme *et al.* 1994a; Evans *et al.* 2001; Mehta *et al.* 2008)

Nonetheless, down-regulation of HLA class I molecules has also frequently been linked with a poor clinical outcome in a variety of human tumours, *e.g* ovarian and head and neck cancer.(Meissner *et al.* 2005; Ogino *et al.* 2006; Han *et al.* 2008) However, in contrast, loss of HLA class I expression has also been linked to a favourable prognosis in some cancers, *e.g.* colon and breast cancer, as well as HPV associated cervical and head and neck neoplasias.(Madjd *et al.* 2005; Watson *et al.* 2006; Jordanova *et al.* 2008; Nasman *et al.* 2013) Frequently given explanations for the favourable prognosis in patients with HLA class I deregulated tumours often include alternative immune recognition possibilities, and often refer to NK-cell activity and the recognition of lack of MHC class I, *i.*e. none-self.(Jordanova *et al.* 2008) Similarly, explanations often given for the favourable prognosis in HLA class I expressing tumours often include an intact antigen presenting machinery that may induce an immune recognition.(Meissner *et al.* 2005)(Pages *et al.* 2011) However, it is important to note, that irrespective of possible mode for immune recognition, the prognosis is always poor if the patients are not treated.

1.1.10.3.4 Non-classical MHC molecules in HPV associated malignancies

It has been suggested that overexpression of non-classical HLA class I molecules -E and -G, sometimes observed in tumours, may modulate the immunological response. Hence, such overexpression has been correlated to tumour progression, although the

opposite has also been observed.(Wischhusen *et al.* 2007; Yie *et al.* 2007a; Yie *et al.* 2007b; Yie *et al.* 2007c; Amiot *et al.* 2011) HLA-G, that is normally expressed during embryonic development, may be up-regulated in *e.g.* renal cancer, and has *in vitro* been reported to inhibit the cytolytic functions of NK and T-cells.(Ibrahim *et al.* 2001; Carosella *et al.* 2011) Moreover, HLA-E, frequently overexpressed on tumour cells, has *e.g.* in cervical cancer been linked to tumour progression.(Marin *et al.* 2003; Goncalves *et al.* 2008) However, in HPV associated tonsillar carcinoma, neither the expression of HLA-E nor HLA-G influenced clinical outcome.(Nasman *et al.* 2013)

1.1.10.3.5 HPV and T-cell response

It has been suggested, and also to some extent stated above, that an effective T-cell response comprising both virus-specific CD8+ CTL and CD4+ IL-2 and IFNγ producing Th1 cells are required to eradicate HPV infected cells.(van der Burg 2012) This statement is consistent with that HPV induced progressive diseases are associated with a lack of such strong T-cell responses against the early viral proteins; and the tumours are often infiltrated with immune suppressive cells. More specifically, it has been shown that T-cells in cervical cancer do not produce granzyme B, that is crucial for the induction of target cell apoptosis, but do express PD-1, as a sign of exhaustion.(Bontkes *et al.* 1997; Karim *et al.* 2009; van der Burg *et al.* 2011) Moreover, the expression of CXCL12 has also been described to increase by disease severity in cervical cancer – resulting in even higher levels of Tregs.(Jaafar *et al.* 2009)

Consequently, in patients with cervical cancer, when observed, the presence of an HPV-specific Th1 response has been associated with a better clinical outcome and a better treatment response. Likewise, a high infiltration of granzyme B producing CD8+ cells in CIN has been linked to spontaneous regression in some patients.(van der Burg 2012) Moreover, the degree of CD8+ tumour infiltrating lymphocytes (TILs), or only TILs by itself, have been shown to correlate favourably to clinical outcome in a variety of tumours, including HPV associated malignancies.(Aaltomaa *et al.* 1992; Bethwaite *et al.* 1996; Sato *et al.* 2005; Galon *et al.* 2006; Nedergaard *et al.* 2007; Piersma *et al.* 2007; Nasman *et al.* 2012) Similarly, a high infiltration of Foxp3+ TIL has been linked to a worse prognosis in many different tumours, but also to a favourable outcome in others.(Ichihara *et al.* 2003; Badoual *et al.* 2006; Bates *et al.* 2006; Carreras *et al.* 2006; Salama *et al.* 2009; Shah *et al.* 2011).

1.1.10.3.6 HLA class I and II alleles and risk of HPV associated cancer

In a variety of diseases, studies have linked HLA alleles and their variants to the risk of developing a disease or to the prognosis of the disease. One example is that HLA-B27 is linked to an increased risk of developing Mb Bechterew or reactive arthropathy.(Sims *et al.* 2004) Correspondingly, similar attempts have been made to link HPV related malignancies to certain HLA types.

In this context, some words about the nomenclature of HLA may be of interest. The nomenclature of HLA class I also includes a letter that indicates the gene (*e.g.* -A) and a digit that indicates the allele. HLA-A, -B and -C are often referred to as "classical" genes, while the others, *e.g.* HLA-E and -G, are often referred to as "non-classical" genes. The loci of MHC class II genes are named with 3 letters; 1st: indicates the class (D), 2nd: indicates the family (M, O, P, Q and R) and 3rd: indicates the chain (A=α or B=β). Moreover, the first digit that follows the letter indicates the gene, which is followed by an asterisk and a number – that indicates allelic variants.(Marsh *et al.* 2010)

Most studies with regard to association between HLA and HPV related diseases have been made in cervical cancer, but the data are not completely consistent. Here only a few of many reports linking HLA alleles to an increased or a decreased risk for cervical cancer are presented. In some reports the presence of *e.g.* DRB1* is associated to an increased risk of developing cervical cancer, however the data is hard to overview. (Beskow *et al.* 2001; Hildesheim *et al.* 2002; Beskow *et al.* 2005; Chan *et al.* 2006; Castro *et al.* 2007; Ades *et al.* 2008; Madeleine *et al.* 2008; Castro *et al.* 2009; Hernandez-Hernandez *et al.* 2009; Matsumoto *et al.* 2012) Protective associations have also been shown. In a meta-analysis published some years ago, a protective effect of DRB1*1301 and DQB1*0603 against the development of cervical cancer was demonstrated.(Hildesheim *et al.* 2002)

1.1.11 Vaccines

Today, there are two commercially available prophylactic vaccines (Cervarix®, Glaxo-SmithKline, UK and Gardasil®, Merck, USA), both covering the two most common HPV types (16 and 18) worldwide in cervical cancer (~70%). However, up to date, no therapeutic vaccine has been introduced on the market, although extensive efforts have been made to develop such vaccines. Below, a brief summarization of the knowledge of the vaccines will be presented.

1.1.11.1 Prophylactic vaccines

The two today approved prophylactic HPV vaccines are both based on virus like particles (VLP), which are formed by self-assembled L1 proteins (discussed in 1.1.5). Gardasil®, which was FDA-approved in 2007, is a quadrivalent vaccine against two high-risk HPV types (HPV 16 and 18) and two low-risk HPV types (HPV 6 and 11). Cervarix, which was introduced some months later, is a bivalent vaccine against the high-risk HPV types 16 and 18. Although both vaccines consist of VLPs, the adjuvant that is administered with the VLPs and the production setting differs. In Gardasil®, the adjuvant consists of amorphous aluminium hydroxyphosphate sulfate and the vaccine is produced in yeast; in Cervarix® the adjuvant consists of aluminium hydroxide and the vaccine is produced in insects cells. Cervarix also contains monophosphoryl lipid A (MPA), leading to an activated innate immune response.(Schiller *et al.* 2008) Studies

have shown a very high efficacy in both vaccines (~100% prevention of HPV-16 and HPV-18 associated CIN III after 4-years) and that there is also some cross-protection against some other non-covered high-risk HPV types.(Munoz *et al.* 2010; Lehtinen *et al.* 2012; Malagon *et al.* 2012) Strong *in vitro* and *in vivo* data, as well as data from clinical trials suggests that the effector mechanism of both vaccines is mainly attributed to antibodies. Since Cervarix mainly induces a T helper 1 response, while Gardasil mainly induces a T helper 2 response, but the vaccines efficacies are basically the same, it is has been postulated unlikely that CD4 T cell effector functions play a major role in the protection. Likewise, although a strong CD8 T cell response against L1 is generated, it is regarded unlikely that cytotoxic T-cells are important protective mediators, especially, since L1 is expressed late in the viral life cycle (discussed in section 1.1.5).(Schiller *et al.* 2012) However, others have disagreed on this statement. (Dalianis 2013, personal communication)

As stated above, antibody mediated protection is suggested as the most important, and titres after HPV VLP vaccination are 80-100 fold higher that those observed after natural infection.(Frazer 2010) These high titres appear to remain high over time in both vaccines, but recent data imply that titres generated by Cervarix are more stable than titres generated by Gardasil. However, it is important to note that different assay methods have been used in the evaluations of the vaccines and therefore the results from the two vaccines cannot be directly compared.(Einstein *et al.* 2009; Schiller *et al.* 2009; Romanowski 2011) Moreover, there is an on-going debate on whether high antibody levels actually result in longer duration of protection. Based on the acquisition of higher antibody levels some have therefore argued that Cervarix is superior to Gardasil, while others have claimed the role of memory B-cells – which are induced in both vaccines (Villa 2011) – and stated that the vaccines should be considered as equal.

Nevertheless, up to date, no studies have investigated the preventive efficacy of the vaccines in head and neck cancer, and oropharyngeal cancer more specifically. However, some authors, *e.g.* Gillison 2008, have suggested the possibility of a protective vaccine effect in oropharyngeal cancer.(Gillison 2008) In her argumentation, also used by others, she refers to studies in which researchers have observed a protective effect against canine oral papillomavirus (COPV) infection in dogs after vaccination with VLPs.(Suzich *et al.* 1995) Other arguments used are the fact that oral HPV IgG specific antibodies have been detected in HPV seropositive HIV infected patients, in women with cervical cancer and in individuals attending a dental clinic.(Cameron *et al.* 2003; Buchinsky *et al.* 2006; Marais *et al.* 2006)

Conversely, others have been more guarded in their opinions. Since an intramuscular injection of the vaccine primarily induces an IgG response, while an IgA response should be more appropriate in a mucosal infection, the mode of action of the vaccines have been under discussion.(Mestecky *et al.* 2010) Two possible mechanisms have been proposed: (1) transduction of IgG antibodies (only described in cervix) and (2) exudation of antibodies to surfaces, where HPV binds to the basal membrane. It has however been suggested that exudation may play a great role, because the quadrivalent vaccine gives an excellent protection against genital warts (also in males) on cornified skin – a site where transfusion is very unlikely to occur.(Schiller *et al.* 2012) However, in oropharyngeal cancer, these above-described mechanisms are poorly studied.

1.1.11.2 Therapeutic vaccines

Up to date, there are no commercially registered therapeutic vaccines against HPV induced diseases in the clinic. However, many attempts have been made based on the hypothesis that tumours driven by foreign HPV antigens should easily be cleared by the immune system upon an induction of a strong Th1/cytotoxic T-cell response. Consequently, there should be a considerable possibility to clear HPV positive tumours by a therapeutic cancer vaccine. However, as discussed above (1.1.10), HPV could create an immunosuppressive milieu that may be hard to overcome. Furthermore, since HPV indirectly has a mutagenic potential, it could induce changes, resulting in that the tumour becomes more resistant to the immune system for other reasons.

Nonetheless, the choice of target is crucial and the early proteins E6 and E7 are often used. Therapeutic vaccines can be divided into 5 main categories; (1) peptide vaccines (2) protein vaccines (3) viral vector based vaccines (4) DNA vaccines and (5) dendritic cell (DC) based vaccines. Peptide and protein vaccines are easy to administer, but they often exhibit a low immunogenicity and require adjuvants, *e.g.* toll-like receptor ligands. In addition, peptide vaccines are mainly restricted to retained expression of specific HLA class I antigens, while protein-based vaccines, which can be potentially be processed into several peptides are not equally restricted to one specific HLA class I antigen. Viral vector based vaccines are in general more immunogenic, but pre-existing immunity and safety concerns are considered as drawbacks. Another vaccine approach is the use of DNA vaccines, which also are generally considered as safe and easy to deliver, but with low immunogenicity. Finally, the DC vaccine approach is also considered as safe, but is costly and time consuming. Below, some of the promising therapeutic vaccine approaches will be described in more detail.

Although an HPV specific CTL response has been detected in some patients included in therapeutic *peptide* vaccine trials against high-grade CIN/VIN or vulvar/cervical cancer, no correlation to clinical outcome has been observed.(Steller *et al.* 1998; van Driel *et al.* 1999; Muderspach *et al.* 2000; Ressing *et al.* 2000) Taking the data together, the use of short peptides has so far been discouraging. It is possible, that this poor outcome is partly due to the very specific HLA class I restriction of short peptides. More recently therefore, long peptides have gained more and more interest. Data from the groups of van der Burg and Melief suggest that the use of long synthetic peptides that span the whole HPV 16 E6 and E7 sequences may induce HPV specific CD4+ and CD8+ T-cell responses and also clinical responses in VIN. More specifically, after vaccination of women with VIN III with long HPV E6/E7 peptides, at the 12-months follow-up, complete responses and clinical responses were obtained in 9/19 and 15/19 respectively of the women, and were associated with a CD4+ and CD8+ T-cell type 1 response.(Kenter *et al.* 2008; Welters *et al.* 2008; Kenter *et al.* 2009; Welters *et al.* 2010)

Fused viral proteins (so-called fusion proteins) have also shown promising results in some but not all studies. As examples, Frazer el al used a fusion protein between E6 and E7 and observed decreased viral load in the vaccinated group, while Kaufmann *et*

al. used HPV 16 E7L1 chimeric VLPs and found no significant clinical effect.(Frazer *et al.* 2004; Kaufmann *et al.* 2007) Another fusion-protein vaccine approach is to combine a viral protein with a foreign, more immunogenic, protein, *e.g.* Hsp 65 from *Mycobacterium Bovis*. However, such studies have so-far shown diverse results.(Goldstone *et al.* 2002; Corona Gutierrez *et al.* 2004; Hallez *et al.* 2004)

1.1.12 Additional treatment modalities

Although the above-mentioned therapeutic vaccination may sound promising as a treatment modality, present clinically available treatment regimes in HPV associated malignancies are today only oncological treatment and surgery (discussed in section 1.2.8). However, novel and more specific therapies are emerging and possibly soon entering the everyday clinical scene. Such treatment modalities may include adoptive T-cell transfer of HPV specific T-cells, small molecules interfering with *e.g.* the viral protein E6 and drugs that modulate the immune response.(Stern *et al.* 2012) Although the mechanisms used in these treatments differ, they all have HPV as a direct/indirect target in common. Below some examples will be discussed.

1.1.12.1 Cidofovir

In Sweden Cidofovir is marketed as Visitide® and has CMV induced retinitis in AIDS patients as the only indication. The drug is an acyclic nucleoside phosphate and inhibits CMV replication selectively by inhibiting the viral DNA polymerase at concentrations 8-600 times lower than what is required to inhibit human DNA polymerases.(FASS.se 2013) However, Cidofovir has also been shown to reduce HPV E6 and E7 expression *in vitro* in 2 HPV-16 positive cell lines and to reduce the metastatic potential *in vivo*.(Amine *et al.* 2009) Furthermore, small studies using topical treatment with Cidofovir in pre-stages to vulvar or cervical cancer (VIN and CIN) have shown some promising results.(Snoeck *et al.* 2000; Tristram *et al.* 2005)

1.1.12.2 Imiquimod

Imiquimod, marketed as Aldara® in Sweden, is suggested to act through the Toll-like 7 receptor and thereby *e.g.* induce IFN-α and tumour necrosis factor beta (TNF-β). Studies have also suggested that topically applied Imiquimod may activate LCs in the epidermis, and upon such stimulation they may migrate to the lymph nodes and activate the adaptive immune system. The approved indications in Sweden for Imiquimod are actinic keratosis, basal cell carcinoma and condylomata accuminata.(FASS.se 2013)

Nevertheless, topical application of Imiquimod alone (discussed below) or as a combination therapy together with photodynamic therapy or together with a therapeutic HPV vaccine has been used outside its indications in clinical trials in VIN

patients.(Winters *et al.* 2008; Daayana *et al.* 2010) Two randomized controlled studies have recently been published, showing a favourable response in VIN patients treated with only Imiquimod compared to placebo.(Mathiesen *et al.* 2007; van Seters *et al.* 2008; Terlou *et al.* 2011) However, very few studies have examined Imiquimod treatment compared to surgery in VIN. In a retrospective study with a heterogenic study population by Bruchim *et al*, patients treated with surgery presented a better treatment response, but tended to relapse more frequently than those treated with Imiquimod.(Bruchim *et al.* 2007) Therefore, the role of Imiquimod in VIN and other HPV related pre-malignant diseases is still unknown and additional studies are needed.

1.2 OROPHARYNGEAL CANCER

Head and neck cancer includes neoplasias of the sub-sites of the mobile tongue, the lip, the oral cavity, the oropharynx, the salivary glands, the larynx, the hypopharynx, the epipharynx, the sinuses and the ear.(Munck-Wikland 2012) Oral and oropharyngeal malignancies represent 3-5% of all diagnosed cancers in the Western world, and are more common in some parts of Asia, where they represent up to 40% of all diagnosed cancer cases.(Parkin *et al.* 2002; O'Sullivan 2008) A common first symptom of oropharyngeal cancer is a lump on the patient's neck; consequently, these patients are often diagnosed at late stages. Other symptoms may include a sore throat, swallowing difficulties and pain.(Licitra *et al.* 2002; Hammarstedt 2008)

Classical risk factors for oropharyngeal cancer are smoking, alcohol abuse and betel nut chewing, but during the last decades an association of HPV infection and oropharyngeal cancer was detected.(Gillison *et al.* 2000; Mellin *et al.* 2000; Klussmann *et al.* 2001; Mellin *et al.* 2002; Genden *et al.* 2003; Ritchie *et al.* 2003; Hammarstedt *et al.* 2006) Based on some of those studies and additional evidence, in 2007, HPV was acknowledged by the IARC, as an additional risk factor for oropharyngeal cancer. (IARC-Monographs 2007)

1.2.1 Anatomy and histology of normal oropharynx

The oropharynx is located in the middle part of pharynx, and contains four distinct sub-sites: (1) the palatine tonsillar fossa and pillars, (2) the tongue base (3) the soft palate and (4) the pharyngeal walls. The oropharynx is covered by squamous epithelium. The lingual tonsil, the palatine tonsils and the inferior proportion of the nasopharyngeal tonsils (the adenoid pad) are often referred to as the Waldeyer's ring, since these structures form a circumferential ring in the oropharynx (Figure 6). The histology of the epithelium in the Waldeyer's ring is often referred to as "lymphoepithelium", in which the squamous epithelium invaginates and merges the underlying lymphoid tissue and forms crypts. These crypts are highly prevalent in the tonsils (10-30 crypts per tonsil), but are more rarely observed in the tongue base.(Syrjanen 2004; Duvvuri *et al.* 2009)

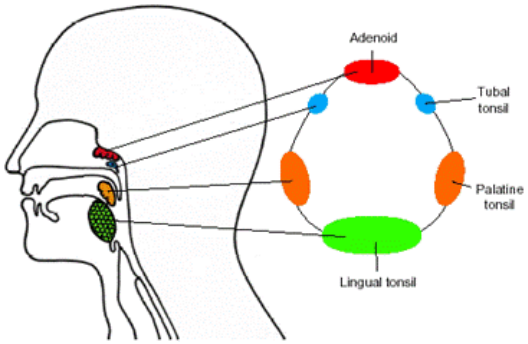


Figure 6. The Waldeyer´s ring, location and structures.

1.2.2 Oropharyngeal cancer – classification

Although oropharyngeal cancer may appear as a well-defined sub-type of head and neck cancer, different classification systems and different classification codes have been used in the literature, and in some scientific papers, oropharyngeal cancer is not defined at all.(Ritchie *et al.* 2003; D'Souza *et al.* 2007; Hammarstedt *et al.* 2007; Chaturvedi *et al.* 2008; Nasman *et al.* 2009) The standard classification of diseases is the International Classification of Diseases (ICD) system, which has developed and refined over the years and since 1994 ICD-10 is used. When the Swedish Cancer Registry was initiated in 1958, the ICD-7 was used and it is still used today. (Hammarstedt *et al.* 2007) Therefore, in order to compare *e.g.* incidence trends of oropharyngeal cancer over decades when using the Swedish Cancer Registry, all ICD-10 codes must be translated into ICD-7, thus making it somewhat complicated (Table 2).

Table 2. International classification of diseases translator

ICD-7 code		ICD-8 code		ICD-9 code		ICD-10 code	
code	*definition*	*code*	*definition*	*code*	*definition*	*code*	*definition*
				141.6	*Malignant neoplasm of lingual tonsil*	C02.4	*Malignant neoplasm of lingual tonsil*
145.0	*Malignant neoplasm of oral mesopharynx, Tonsil (excluding pillars)*	146.0	*Malignant neoplasm of oropharynx, Tonsils*	146.0	*Malignant neoplasm of the tonsils*	**C09.9**	*Malignant neoplasm of tonsil, unspecified*
				146.1	*Malignant neoplasm of tonsillar fossa*	**C09.0**	*Malignant neoplasm of tonsillar fossa*
				149.1	*Malignant neoplasm of Waldeyer's ring*	C14.2	*Malignant neoplasm of Waldeyer's ring*
145.7	*Malignant neoplasm of oral mesopharynx, Other specified parts of mesopharynx*	146.8	*Malignant neoplasm of oropharynx, Other specified parts*	146.2	*Malignant neoplasm of tonsillar pillars (anterior and posterior)*	**C09.1**	*Malignant neoplasm of tonsillar pillar (anterior and posterior)*
				146.3	*Malignant neoplasm of vallecula epiglottica*	C10.0	*Malignant neoplasm of vallecula*
				146.4	*Malignant neoplasm of anterior aspect of epiglottis*	C10.1	*Malignant neoplasm of anterior surface of epiglottis*
				146.5	*Malignant neoplasm of junctional region of oropharynx*	C10.8	*Malignant neoplasm of overlapping sites of oropharynx*
				146.6	*Malignant neoplasm of lateral wall of oropharynx*	C10.2	*Malignant neoplasm of lateral wall of oropharynx*
				146.7	*Malignant neoplasm of posterior wall of oropharynx*	C10.3	*Malignant neoplasm of posterior wall of oropharynx*
				146.8	*Malignant neoplasm of other specified sites of oropharynx*	**C09.8**	*Malignant neoplasm of overlapping sites of tonsil*
145.9	*Malignant neoplasm of oral mesopharynx, Part unspecified*	146.9	*Malignant neoplasm of oropharynx, Part unspecified*	146.9	*Malignant neoplasm of oropharynx, unspecified site*	C10.9	*Malignant neoplasm of oropharynx, unspecified*

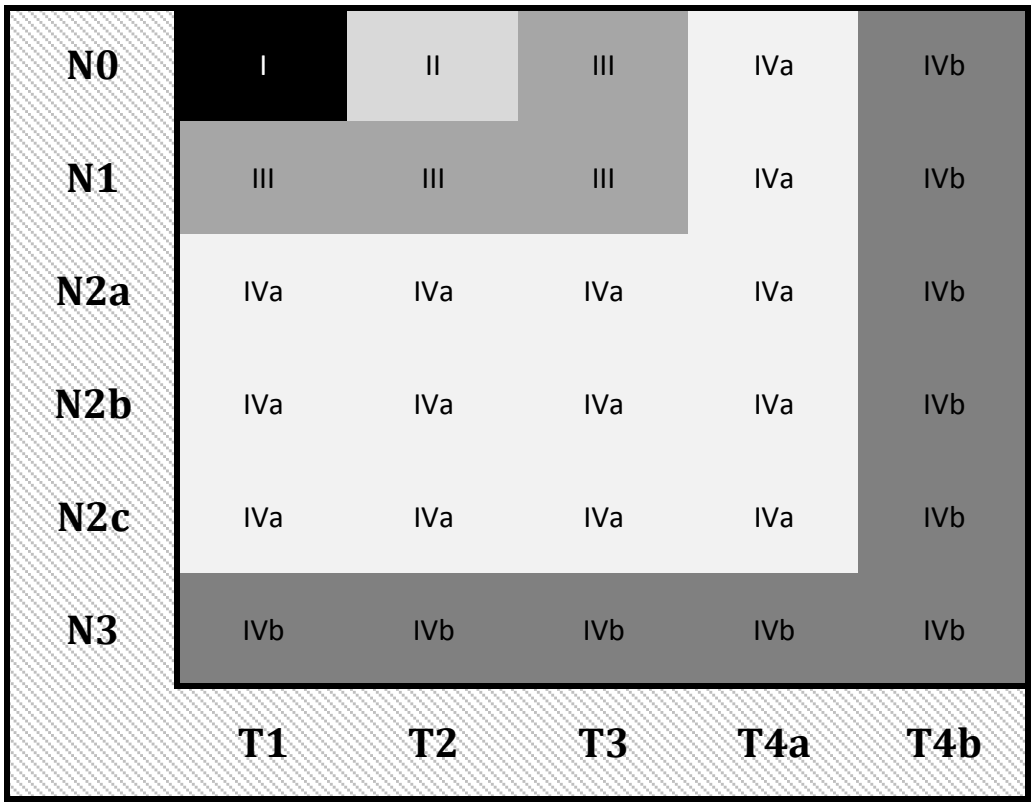
Codes that are underlined are used in paper I and III-V of this thesis

Clinical staging of oropharyngeal cancer is made according to the TNM-classification (International Union Against Cancer (UICC)). The latest classification system (7th Edition) is based on the size of the primary tumour (T-stage); presence, size, numbers of, and localisation of regional metastasis to lymph nodes (N-stage); and presence of distant metastasis (M-stage) (Table 3). The TNM-classification is then divided into stage I-IV (Figure 7).(Sobin *et al.* 2009)

Table 3. TNM classification of OSCC according to UICC

TNM classification		Definition	Comments
T	*TX*	Primary tumour can not be assessed	
	T0	No evidence of primary tumour	
	Tis	Carcinoma in situ	
	T1	Tumour <2cm in greatest dimension	
	T2	Tumour >2cm in greatest dimension	
	T3	Tumour >4cm in greatest dimension	
	T4a	Moderately advanced local disease	Tumour invades the larynx, deep/extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible
	T4b	Very advanced local disease	Tumour invades lateral pterygoid muscle, ptergoid palates, lateral nasopharynx, skull base, or encases the carotid artery
N	*NX*	Regional lymph nodes can not be assessed	
	N0	No regional node metastasis	
	N1	Metastasis in a single ipsilateral lymph node, <3cm in greatest dimension	
	N2a	Metastasis in a single ipsilateral lymph node, >3cm in greatest dimension, but <6cm in greatest dimension	
	N2b	Metastasis in multiple ipsilateral lymph nodes, <6cm in greatest dimension	
	N2c	Metastasis in bilateral or contralateral lymph nodes, <6cm in greatest dimension	
	N3	Metastasis in a lymph node, >6cm in greatest dimension	
M	*MX*	Distant metastasis cannot be assessed	
	M0	No distant metastasis	
	M1	Presence of distant metastasis	

Figure 7. TNM and stage (I-IVb) classification according to UICC



Patients with M1 (distant metastasis present) are classified as stage IVc

1.2.3 Histology

Squamous cell carcinomas cover >90% of all oropharyngeal malignancies, but other rare types, *e.g.* arising from the minor salivary glands, may also occur. Other infrequent tumours of oropharynx may include sarcomas (radiation-induced or spontaneous) and lymphomas (most common in palatine tonsils).(Duvvuri *et al.* 2009)

Histological studies suggest a unique entity of HPV related squamous cell carcinomas of the oropharynx, with non-keratizing carcinomas forming sheets, nests and trabeculae with pushing borders and a slight stromal response (Figure 8). In contrast, non-HPV associated oropharyngeal squamous cell carcinomas tend to form keratinizing tumours with polygonal (many sides) cells, rich of cytoplasm and distinct cell borders (Figure 9).(El-Mofty *et al.* 2003; El-Mofty *et al.* 2006; Chernock *et al.* 2009) Other histological classifications, very often used, are grading of tumours as well, moderate, poorly differentiated and undifferentiated; or grading of the tumours as "basaloid" or "non-basaloid". In well-differentiated SCC, there is a close resemblance to normal epithelial cells and a prominent keratinization, but in in poor differentiated SCC there is only a slight similarity to normal epithelial cells and keratinization is extremely scarce. Subsequently, the presence of HPV has been linked to low grade as well as to basaloid lesions.

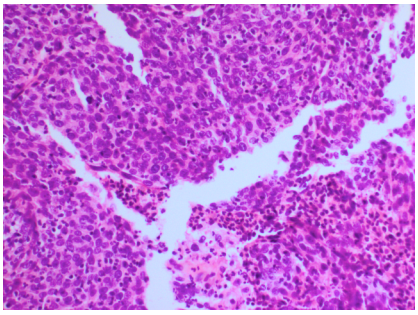


Figure 8. HPV related, non-keratizing squamous cell carcinoma.

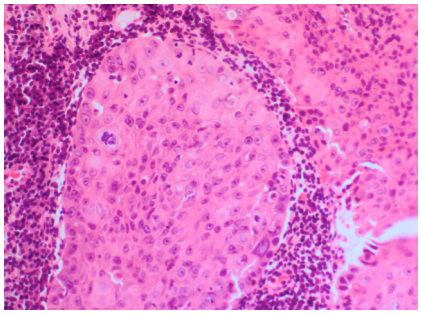


Figure 9. Non-HPV associated, keratizing squamous cell carcinoma with polygonal cells rich of cytoplasm and distinct cell borders.

1.2.4 Epidemiology

Annually, in Sweden, there are around 200-250 newly diagnosed cases of OSCC and the dominant sub-sites are tonsillar squamous cell carcinoma (TSCC) and base of tongue squamous cell carcinoma (BOTSCC), accounting for >90% of the cases.(Åberg 2012) While the incidence of head and neck cancer has generally decreased in Sweden, the incidence of OSCC has increased; and similar incidence trends have also been observed in many other Western countries. Moreover, this trend has been described to be most prominent in TSCC; in young (<60 years) and in male patients.(Frisch *et al.* 2000; Shiboski *et al.* 2005; Hammarstedt *et al.* 2007; Ryerson *et al.* 2008; Braakhuis *et al.* 2009; Reddy *et al.* 2010; Blomberg *et al.* 2011; Hocking *et al.* 2011; Olaleye *et al.* 2011; Saba *et al.* 2011; de Souza *et al.* 2012; Forte *et al.* 2012; McGorray *et al.* 2012; Åberg 2012)

1.2.5 Prevalence of and evidence for association to HPV infection

1.2.5.1 Initial studies

In 1983, Syrjänen and Syrjänen and colleagues published the first data indicating that sub-groups of HNSCC could be associated with HPV infection.(Syrjanen *et al.* 1982; Syrjanen *et al.* 1983) Although the Syrjänens focused mostly on oral and laryngeal squamous cell carcinomas the data highlighted the concept of HPV in HNSCC. Their initiative, initiated very many studies on the association of HPV with HNSCC in different locations and eventually there was extensive evidence supporting the presence of HPV in OSCC. Therefore, in 2007, the International Agency of Research of Cancer (IARC) declared that "there is strong epidemiological evidence for the casual role if HPV16 in the etiology of cancer of the oropharynx and tonsil".(IARC-Monographs 2009)

1.2.5.2 Prevalence of HPV in OSCC

However, the prevalence of HPV in OSCC varies, not only between study cohorts and countries; alcohol and smoking habits in the study population, but also over decades, age of study population and by how OSCC is defined (ICD codes used). Another factor that may contribute to the variation in HPV prevalence is that different methods are presently used to define HPV positivity (discussed in section 1.1.7)

Whereas a meta-analysis by Kreimer *et al* from 2005, including 27 studies and 969 patients from 19 countries, stated that 35.6% of all OSCC are HPV positive and HPV16 is the dominant type, there was a huge variation in the HPV prevalence figures reported. This is exemplified in that the HPV prevalence in TSCC was described to be as low as 12.6% in Taiwan, but as high as 79% of all TSCC cases in Stockholm, Sweden.(Kreimer *et al.* 2005; Tachezy *et al.* 2005; D'Souza *et al.* 2007; Kim *et al.* 2007; Charfi *et al.* 2008; Chien *et al.* 2008; Nasman *et al.* 2009; Hannisdal *et al.* 2010; Ribeiro *et al.* 2011; St Guily *et al.* 2011) Moreover, there is also evidence that the HPV prevalence has increased in OSCC during the last decades, suggesting that the above-mentioned increase in OSCC is due to HPV infection.(Hammarstedt *et al.* 2006; Ernster *et al.* 2007; Nasman *et al.* 2009; Attner *et al.* 2010) Accordingly, as discussed in paper I of this thesis, the HPV positive TSCC has increased 7-fold between 1970-2006 in Stockholm, while the incidence of HPV-negative TSCC has decreased from the 1980's.(Nasman *et al.* 2009) Another interesting study by Marklund and colleagues suggest that HPV prevalence in OSCC is highly dependent on which sub-site in OSCC that is studied. Thus, HPV prevalence has been reported to vary from only 16% in cancer of the oropharynx (ICD-10 C10) to up to 79% in TSCC (ICD-7 145.0) during a similar time period.(Nasman *et al.* 2009; Marklund *et al.* 2012)

There are very few studies examining the role of HPV in HIV infected OSCC patients. Some studies have proposed an elevated risk for developing OSCC in HIV infected patients, but this increase could potentially be explained by higher rates of smoking or by that HPV and HIV share sexual risk factors.(Grulich *et al.* 2007; Patel *et al.* 2008) Nonetheless, a recent published study investigates the risk of being infected with HPV in the oral cavity in HIV infected/non-infected individuals. The authors conclude that

HIV infected individuals have an increased odds of being infected with HPV positive and that HIV infected and non-infected individuals have different risk factors. Whilst presence of HPV in non-HIV infected individuals were associated to higher numbers of recent oral sex partners, HPV infection in HIV infected individuals was correlated to both the number of lifetime oral sex partners as well as the levels of CD4+ cells. It is therefore likely, that HPV positivity may primarily be due to persistent or reactivated HPV infection in HIV infected individuals, while more recent HPV infections may dominate in non-HIV infected individuals.(Beachler *et al.* 2012)

1.2.5.3 Biological data

There are several molecular/biological differences between HPV positive and HPV negative OSCC.(Gillison 2004; Dahlstrand *et al.* 2005) For example, HPV positive TSCCs tend to display fewer chromosomal aberrations as compared to HPV negative TSCC (Dahlgren *et al.* 2003). Moreover, $p16^{INK4a}$ (please see section 1.1.6 and 1.2.7) is more frequently overexpressed in HPV positive OSCC compared to HPV negative OSCC, while the opposite is documented for *e.g.* EGFR expression (see section 1.2.6). In addition, new high through-put methods have revealed other biological differences between HPV positive and negative OSCC, *e.g.* micro-RNA.(Lajer *et al.* 2011)

1.2.5.4 Epidemiological data

In addition to biological data (see above, sections 1.1.6 and section 1.2.3), there are also strong epidemiology data supporting the role of HPV in a subset of OSCC. Previous studies had shown that OSCC patients with HPV positive tumours were younger, smoke less, have a better clinical outcome (discussed below).(Lindquist *et al.* 2007; Fakhry *et al.* 2008; Gillison *et al.* 2008; Nasman *et al.* 2009; Attner *et al.* 2010; Dayyani *et al.* 2010) Although, in addition to the above, many studies have highlighted these risk factors, three studies of special interest may be worthwhile to mention. The first study from 1999 by Frisch *et al* showed a 4.3 fold higher risk for developing TSCC in patients with HPV associated anogenital cancer, while no corresponding increase in any other HNSCC was observed, suggesting the aetiological role HPV in TSCC and sexual behaviour as a risk factor.(Frisch *et al.* 1999) Another interesting study from 2000 showed that husbands to women with HPV induced cervical cancer had an increased risk of developing TSCC, implying the oncogenic role of and an important sexual transmission route for the virus.(Hemminki *et al.* 2000) A third, more recent study from 2008 by Gillison and her group, showed that increased numbers of sexual partners (vaginal/oral) was strongly associated with HPV-16 positive HNSCC, again supporting oral sex as a risk factor for TSCC.(Gillison *et al.* 2008)

1.2.6 HPV and impact of clinical outcome in OSCC

Today, the evidence for HPV having a favourable prognostic role in OSCC is to be considered as strong. There are large randomized control studies published, favouring HPV as a positive prognostic marker in OSCC. Examples of such studies are the DAHNCA 5 study, where $p16^{INK4a}$ positive (surrogate marker for HPV) OSCC patients

presented a better loco-regional control, disease-specific (DFS) and overall survival (OS) if treated with conventional radiotherapy.(Lassen *et al.* 2009) Another such large study by Ang et al showed that patients with HPV positive OSCC presented a significantly better overall and progression-free survival compared to that of patients with HPV negative OSCC, when treated with radio-chemotherapy.(Ang *et al.* 2010)

There are also numerous retrospective studies indicating a favourable prognostic role of HPV in OSCC and the correlation between survival and HPV in HNSCC was recently analysed in a meta-analysis by Ragin *et al.* In this analysis they concluded that patients with HPV positive HNSCC in general, and more specifically those with HPV positive OSCC, presented a better overall (OS) and disease-free (DFS) survival than those with HPV negative malignancies.(Ragin *et al.* 2007b) Interestingly, in most of the included studies in the meta-analysis, there was a discrepancy in prognosis and stage, *i.e.* tumour stage did not influence prognosis in patients with HPV positive OSCC. However, it is important to note that there was a huge variation in tumour types (i.e. paraffin embedded vs. fresh frozen), HPV detection methods, treatment regimes and outcome variables used in the different reports that were included in the meta-analysis. Furthermore, in many cases, other tumour sites than OSCC were included in the meta-analysis. Therefore, although the vast majority of retrospective studies suggest a better clinical outcome in patients with HPV positive OSCC, the data compiled in the meta-analysis should be interpreted with caution.(Ragin *et al.* 2007b) Nonetheless, one has to consider the evidence for a prognostic role of HPV infection in OSCC as strong.

The reason for the positive prognostic role of HPV in HNSCC and OSCC is, however, not fully elucidated. Although patient characteristics related to HPV positive OSCC, *e.g.* minimum exposure to alcohol and tobacco, good performance status, young age and no comorbidity, all have been liked to a favourable clinical outcome, the studies still suggest that HPV by itself is a prognostic factor.(Ang *et al.* 2010) Furthermore, there are retrospective studies that imply that patients with HPV positive OSCC are more sensitive to chemotherapy and/or radiotherapy, due to the presence of still functional and non-mutated p53.(Pryor *et al.* 2011) Another mechanism may include the, in general, absence of overexpression of EGFR in HPV positive OSCC, which has been correlated to radio-resistance in HNSCC in general.(Weinberger *et al.* 2006; Kong *et al.* 2009; Young *et al.* 2011)

1.2.7 HPV and $p16^{INK4a}$ overexpression in OSCC

As previously described in 1.1.6 and above, $p16^{INK4a}$ is frequently up regulated in HPV E7 expressing cells and, thus, $p16^{INK4a}$ has been proposed as a surrogate marker of HPV infection in OSCC. Consequently, many studies have linked $p16^{INK4a}$ overexpression to presence of HPV DNA in OSCC.(Weinberger *et al.* 2006; Reimers *et al.* 2007; Ang *et al.* 2010; Lewis *et al.* 2010; Thavaraj *et al.* 2011; Ukpo *et al.* 2011; Granata *et al.* 2012; Bussu *et al.* 2013) During the last decade, $p16^{INK4a}$ has gained more and more focus, and in 2007 Smeets *et al* first published an algorithm for HPV detection that included $p16^{INK4a}$.(Smeets *et al.* 2007; Hoffmann *et al.* 2010; Rietbergen *et al.* 2013) Unfortunately, this method has gained a lot of attention and has sometimes been

regarded as a standard detection method for transcriptional active HPV. In the Smeets 2007 study, the researchers tested out different HPV detections methods in paraffin embedded tumour samples and compared them to - what they defined as the gold standard - presence of E6 RNA by RT-PCR in fresh frozen tumours. The researchers ended up with presence of $p16^{INK4a}$ followed by GP5+/6+ PCR as the method with 100% sensitivity and specificity.(Smeets *et al.* 2007) However, the percentage of $p16^{INK4a}$ positive cells varied from 50-90% in the GP5+/6+ PCR positive group. Since the authors stated that presence of $p16^{INK4a}$ correlates to transcriptionally active HPV, these results raise the question whether HPV was active in only parts of the tumour. Another limitation of the study was the limited number of patients. It is also important to note, that data describing why HPV DNA may be present in OSCC, without being transcriptionally active (*e.g.* by methylation) are very scarce and were not discussed in the Smeets 2007 paper. Subsequently, recent reports have questioned this algorithm.(Hoffmann *et al.* 2012)

Furthermore, the definition of how one should define $p16^{INK4a}$ positivity has changed over the years. Initially, different cut-off levels were used, *e.g.* >10%; 50%; or "diffuse and strong intensity".(Lassen *et al.* 2011; Granata *et al.* 2012; Hong *et al.* 2012) However, in 2010, in a randomised controlled study, Ang *et al* used >70% $p16^{INK4a}$ positivity of the tumour cells as a cut-off. Later, the DAHNCA-group, that had previously used >10% as cut-off in their prospective studies, showed that only 15/711 (2%) of their $p16^{INK4a}$ positive OSCC changed p16 status if a cut off of 70% was applied instead. Consequently, "to optimise uniformity in prospective reporting of p16-IHC", the DAHANCA-group changed their cut-offs to >70% $p16^{INK4a}$ positivity.(Lassen *et al.* 2012) What is important to note however, is that there are no biological explanations for using the cut-off described, and one should also always be aware of the limitations when using IHC and setting such a precise cut-off (please see section 3.2.2.1). Another important point, also observed in our material, is that most HPV+ OSCC present with 100% $p16^{INK4a}$ positivity, and with only a minority presenting $p16^{INK4a}$ positivity between 10% - <100%.

Nonetheless, the expression of $p16^{INK4a}$ in correlation to clinical outcome, no matter the definition of overexpression or the biological mechanisms behind this expression, has been studied extensively in OSCC. Almost all published studies have shown a better survival in patients with OSCC with $p16^{INK4a}$ overexpression, as compared to those with OSCC without $p16^{INK4a}$ overexpression. This survival benefit seems to be consistent no matter which survival outcome and treatment modality that has been used.(Weinberger *et al.* 2006; Reimers *et al.* 2007; Kumar *et al.* 2008; Lassen *et al.* 2009; Ang *et al.* 2010; Lewis *et al.* 2010; Rischin *et al.* 2010; Ukpo *et al.* 2011; Granata *et al.* 2012) However, very few studies have investigated the prognostic impact of $p16^{INK4a}$ overexpression in HPV positive tumours.

1.2.8 Prognostic biomarkers in OSCC

Besides $p16^{INK4a}$ and HPV status, many different prognostic markers in OSCC have been proposed and the field is hard to overview. Many studies have attempted to

investigate standard prognostic markers, such as EGFR overexpression and p53 mutations. EGFR overexpression that often occurs in HNSCC in general - including OSCC - has in many studies been correlated to a worse prognosis, resistance to radiotherapy and a poor loco-regional control.(Ang *et al.* 2002; Burtness *et al.* 2005; Kumar *et al.* 2008) Likewise, several studies have suggested that HNSCC patients with p53 mutations have worse outcomes.(Erber *et al.* 1998; Licitra *et al.* 2006; Poeta *et al.* 2007) On the other hand, as mentioned above, it has been reported that HPV positive OSCC rarely overexpresses EGFR or presents with p53 mutations, as compared to HPV negative OSCC.(Westra *et al.* 2008; Hong *et al.* 2010)

Nonetheless, there are also plenty other markers *e.g.* CD8 tumour infiltrating lymphocytes and HLA class I expression (both topics of this thesis). Unfortunately, however, in the vast majority of biomarker studies in HNSCC, including OSCC, researchers have neither taken into account the HPV status of the tumour or specific location within HSCC. Recently, newer approaches such as screening for epigenetic changes are also emerging in parallel to global scanning methods. Therefore new markers are to be expected in HNSCC including OSCC.

Nevertheless, in the search of new prognostic biomarkers, irrespective of the marker, it is important to consider the site of the cancer as well as its HPV status.

1.2.9 Oncological treatment

The mainstay in HNSCC treatment has for decades been radiotherapy (RT) and/or surgery.(Vokes *et al.* 1993) However, over the last years, treatment has become more and more intensified with altered fractionated RT and/or systemic oncological therapies. Although the intensification may to some extent have improved survival in HNSCC patients in general, this has been to the cost of an increase in early and late side effects.(Pryor *et al.* 2011; Ramqvist *et al.* 2011a)

In order to improve loco-regional control, different altered fractionated RT regimens have been proposed in HNSCC (hyper-fractionated, accelerated and hyper-fractionated accelerated RT). While the generally accepted conventional RT fractionation is given as 1.8-2 Gy, 5 fractions/week up to a total dose of 66-70 Gy, reduced fractions (1.1-1.2 Gy) are given 2-3 times daily in hyper-fractionated RT (HRT). In accelerated RT (ART), the overall treatment time is reduced by an increase in the amount of doses per week and in hyper-fractionated accelerated RT (HART) the two above-mentioned strategies are combined. The rationale for ART is to reduce the repopulation of tumour cells and thereby improve the effectiveness of the therapy, while the rationale for HRT is to increase radiation dose without dramatically increasing side effects.(Zackrisson *et al.* 2003; Bourhis *et al.* 2006; Duvvuri *et al.* 2009)

In parallel, different systemic chemotherapy strategies have also been suggested in HNSCC, *i.e.* different cytostatic drugs, different drug combinations and different timing for drug administration. Traditionally, a commonly used standard chemotherapy regimen in HNSCC has been Cisplatin with/without 5-Flourouracil, although other

drugs have been described both in other combinations and as single drugs. Furthermore, chemotherapy may be given as "induction chemotherapy" (prior RT or surgery); as "concomitant chemotherapy" (synchronous to RT); or as adjuvant chemotherapy (after RT or surgery). In addition, EGFR-inhibitors may also be administered.(Duvvuri *et al.* 2009; Pignon *et al.* 2009)

Consequently, the treatment of HNSCC varies between different clinics and over time, which actually to some extent is reflected in paper V of this thesis. Nonetheless, over the past decades, researchers have tried to optimize treatment combinations in HNSCC patients.

Recently, a meta-analysis was published about altered RT fractionation in HNSCC, in which ART and HRT were compared to conventional RT (Bourhis *et al.* 2006). The authors concluded that patients treated with ART without total dose reduction and HRT presented a better loco regional control than those treated with conventional RT, but a statistical survival benefit was only observed between HNSCC patients with HRT and conventional RT. This may suggest that the total radiation dose may be of importance for treatment response in HNSCC.

The role of chemotherapy in HNSCC, and in oral and oropharyngeal cancer more specifically, has been evaluated in two meta-analyses by Pignon and colleagues from 2000 and 2009 (Pignon *et al.* 2000; Pignon *et al.* 2009); and in one meta-analysis from the Cochrane collaboration from 2011(Furness *et al.* 2011) respectively. No significant benefit in survival was observed in patients treated with induction therapy with platin-based chemotherapy compared to local radiotherapy in any of the studies. Similarly, no significant survival benefit was observed if adjuvant chemotherapy was applied. However, HNSCC patients treated with concomitant chemotherapy presented a significantly better survival than those treated with loco-regional treatment alone. (Pignon *et al.* 2000; Pignon *et al.* 2009; Furness *et al.* 2011)

Notably, similar survival benefits of the same order and magnitude were observed, no matter whether Cisplatin was used alone, or in combination with 5-Flourouracil, or other poly-chemotherapies including either a platin-based drug or 5-Flourouracil. However, as mono-chemotherapy, only Cisplatin presented a favourable clinical outcome, while other drugs led to inferior results.(Pignon *et al.* 2000; Pignon *et al.* 2009) Interestingly, Pignon and colleagues also compared direct and indirect concomitant chemotherapy to induction chemotherapy, and observed a benefit in survival for the concomitant treated HNSCC patients.(Pignon *et al.* 2009)

Although recent studies have focused on optimizing treatment in HNSCC in general, no prospective randomized control study has investigated the role of HPV in HNSCC, and OSCC more precisely; with regard to the different treatment approaches. As discussed previously, numerous studies have evaluated the prognostic role of HPV in OSCC and positive HPV status is consistently reported with a favourable DFS, DSS, OS and treatment response (section 1.2.6). Hence, the possibility to de-escalate the oncological treatment, *e.g.* RT only, in HPV positive OSCC has been discussed. Although there are a few studies up to date (2013-04-12) that are comparing different oncological treatments in HPV positive OSCC, there is no currently on-going study assessing

chemo-radiation compared to RT alone in HPV positive OSCC.(Chung *et al.* 2012; Mehanna *et al.* 2012)

1.3 UNANSWERED QUESTIONS AT THE TIME WHEN THIS THESIS PROJECT WAS INITIATED.

In 2005, when I first joined the research group as a medical student, the group had shown that HPV was associated with OSCC, that HPV positive and HPV negative OSCC were different entities, and that the former had better clinical outcome of the latter. However, IARC had not yet acknowledged HPV as a risk factor for OSCC.

Furthermore, much less was known about the epidemiology of HPV associated OSCC, despite that our clinical colleagues had clinically observed an increasing number of patients with OSCC.

During the same period in the US, treatment of all HNSCC - including OSCC - was being intensified with *e.g.* chemotherapy and altered fractionated RT regimes. This intensification of therapy was also reaching Sweden.

Obviously, with this background it was natural to look more into the epidemiology of OSCC and HPV positive OSCC, and this was the aim of the first two papers.

In addition, researchers started to speculate about the possibility to de-escalate the intensified treatment of patients with HPV positive OSCC. Nonetheless as described in the introduction, additional predictive markers were needed to better single out patients with HPV positive OSCC with a very low risk for relapse before de-escalating therapy. The aim of the last three papers was to identify useful biomarkers, in an immunological context, that could be used in combination with HPV status as a first step to better individualise future treatment.

2 AIMS

- **To over time (1970-2007), analyse the HPV prevalence and incidence of TSCC and BOTSCC, the two most common sub-sites within OSCC, in Stockholm, Sweden.**
  (Papers I and II)

- **To study the role of CD8+ and Foxp3+ tumour infiltrating cells in TSCC, in relation to HPV status and clinical outcome.**
  (Paper III)

- **To investigate the role of "classical" (A, B, C) and "non-classical" HLA class I (E and G) in both HPV positive and negative TSCC in correlation to clinical outcome.**
  (Paper IV)

- **To extend the previous HLA class I study, and investigate the role classical HLA class I as well as HLA class II antigen expression in a large cohort of OSCC, in correlation to HPV status and clinical outcome.**
  (Paper V)

3 MATERIAL AND METHODS

3.1 PATIENTS, MATERIALS AND STUDY DESIGNS

All studies included in this thesis were conducted according to the ethical permissions 2003/03-386; 2005/431-31/4; 2005/1330-32 and 2009/1278-31/4 from the Regional Ethical Committee at Karolinska Institutet, Stockholm, Sweden. Below, the patients included in this thesis, and their tumours will be described separately for each paper:

Paper I. All patients with TSCC (ICD-7 145.0) diagnosed between 2003-2007, in the Stockholm region, with available pre-treatment paraffin embedded biopsies, were included in the study. Patients were identified retrospectively through the local Stockholm Cancer Registry, from which the incidence data was obtained as well. The diagnoses were verified and patient data were obtained from the medical records. Moreover, data obtained (including HPV status) from a previous study by Hammarstedt et al (Hammarstedt *et al.* 2006), on patients with TSCC (also obtained through the Stockholm Cancer Registry) diagnosed between 1970-2002 in the Stockholm region, were also included here to compare HPV data prevalence/incidence over time.

Paper II. All patients with BOTSCC (ICD-10 C01.9) were identified between 1998 and 2007, through the Stockholm Cancer Registry, and in total 95 patients with available pre-treatment biopsies were included in the study.

Papers III and IV. Patients with TSCC (ICD-10 C09.0-9) diagnosed between 2000-2006 in the Stockholm region identified through the Swedish Cancer Registry, and with available pre-treatment biopsies were included in the study. However, here a "case-control" approach was applied. Patients with a poor clinical outcome were the cases and those with a favourable clinical outcome constituted controls. More specifically, from patients with TSCC biopsies (included in Paper I and Hammarstedt et al 2006), we included only those with biopsies that were: (1) HPV DNA and $p16^{INK4a}$ positive or (2) HPV DNA and $p16^{INK4a}$ negative. Patients with HPV DNA positive and $p16^{INK4a}$ negative of HPV DNA negative and $p16^{INK4a}$ positive tumours were excluded. In addition, patients that died of non-TSCC related causes (*e.g.* hanging) were also excluded. The two groups were then divided depending on their 3-year clinical outcome (progression-free survival), *i.e.* not dead of disease and recurrence-free for 3 years, thus resulting in four groups:

(A) Patients with HPV DNA and p16INK4a positive TSCC and favourable 3-year clinical outcome (n=98, in paper IV); a random sample of these (n=31 in paper III).

(B) Patients with HPV DNA and p16INK4a positive TSCC and a poor 3-year clinical outcome (n=21).

(C) Patients with HPV DNA and p16INK4a negative TSCC and a favourable 3-year clinical outcome (n=11).

(D) Patients with HPV DNA and p16INK4a negative TSCC and a poor 3-year clinical outcome (n=20).

In paper V. Here, all patients with OSCC defined by the ICD-10 codes C09.0-9; C01.9; C05.1-9; C10.0-9 and diagnosed during a period between 2000-2009 in the Stockholm region were included. However, due to technical reasons with the Regional Cancer Registry, the inclusion time spans for diagnosis were not completely overlapping. More specifically, patients with C09.0-9 and C01.9 were diagnosed between January 2000 - September 2009 and patients with C05.1-9 and C10.0-9 between January 2000 - January 2009. All patient records were also examined and patient characteristics and their clinical outcome were obtained.

3.1.1 Material considerations

3.1.1.1 The ICD-classification system

As described in the introduction, it is important to note that the classification system ICD has changed over time. Therefore, different codes are used in *e.g.* papers I and III, but both papers are analysing TSCC. In paper I the same ICD classification system (ICD-7) was used as in Hammarstedt et al (Hammarstedt *et al.* 2006), since it was a continuation of that study, and therefore some TSCC (ICD-10 C09.1 and C09.8) were not included in that study. This could have played a role with regard to the evaluation of HPV status (see Results and Discussion section 4.1). Nonetheless, all patients were identified through the Swedish Cancer Registry, which is regarded as almost 100% complete. Therefore, it is highly unlikely that OSCC patients were lost in our studies.

3.1.1.2 Study design

A population based retrospective cohort study design was applied in paper I, II and V and a case-control study design in paper III and IV. In paper III-V, survival measurements were analysed, but in different ways. In papers III-IV, patients with a favourable clinical outcome (progression-free survival) were compared to those with a poor clinical outcome, and this comparison was made both in patients with HPV positive and negative TSCC. In addition, disease-free survival (DFS) was also analysed with the Kaplan-Meier method. However, it is important to note that there was a selection of patients with regard to clinical outcome in papers III-IV. Besides, overall survival was not possible to assess, since patients dying of other causes were excluded. Nevertheless, in the following cohort study (paper V), the HLA results were verified and here other survival measurements were reported. Similarly, in another study from our group, we were able to verify the CD8+ results.(Nordfors *et al.* 2013)

3.1.1.3 Biopsies

All biopsies could not be retrieved from all patients diagnosed between the defined time spans in the above-mentioned papers and the major reasons for this were, to our experience, three: (1) The block was missing at the pathology department. (2) Too little tumour material in the biopsy. (3) The diagnosis was made by cytology only. The first two reasons were mainly at random and most likely do not affect the outcomes of the included papers (I-V). However, it is possible that the third reason may have affected the results of papers I and II. Patients diagnosed only by cytology are often treated with palliative intent and since there potentially could be more patients with HPV negative OSCC in the palliative group, there is a risk that we may have a bias in our sample. Nevertheless, it is also very important to note that very few patients were diagnosed based on cytology – and this is therefore most likely a theoretical rather than a real problem.

3.1.1.4 Treatment modalities.

Treatment modalities were collected from patient records and what gradually came apparent, especially in paper V, which is the most recent one, is that treatment modalities varied over time. For many years RT and surgery was the treatment of choice for OSCC, but (as described in 1.2.9) other treatment approaches have been introduced. The Karolinska University Hospital is no exception and the modalities have shifted. Thus, in the middle of the last decade, many patients were included in the ARTSCAN study, and randomised with regard to accelerated or conventional RT.(Zackrisson *et al.* 2011) Later, many patients were included in a phase II study with induction chemotherapy (Cisplatin, 5-Fu and Taxotere), accelerated RT and concomitant Erbitux that was followed/not followed by brachytherapy (ACCROBAT)(EUCTR2009-013438-26-SE). However, many patients were also treated outside these studies. Nevertheless, the reason why the treatment modalities differ between paper III-IV and V is mainly due to these studies. In addition, doses, schemes and drugs may differ between patients included in papers III-V and for this reason only a rough classification of treatment has been made in this thesis.

3.2 METHODOLOGY

The methods that are used in this thesis are considered as standard procedures in the field and are indeed better described in the included papers and elsewhere.(Nasman *et al.* 2009; Attner *et al.* 2010; Nasman *et al.* 2012; Nasman *et al.* 2013) Nevertheless, the debate is still on going with regard to which method is the most appropriate for detection of HPV. In papers I-V, HPV DNA PCR detection methods have been used. Below, this approach and potential limitations will be discussed in more detail. Some small comments regarding immunohistochemistry are also included.

3.2.1 HPV DNA detection

3.2.1.1 HPV PCR, with general and type specific HPV primers (Papers I-IV)

In papers I-IV, all samples have been tested with the commonly used consensus primer pairs GP5+/6+ and CPI/IIG as well as with the type-specific HPV 16 primer pairs (as previously described(Nasman *et al.* 2009)). As explained in the introduction, the different consensus primers target different sites of the HPV genome and the HPV 16 specific primers target the HPV 16 E6 ORF. By combining these primer pairs in an algorithm, we should, theoretically, be able to detect HPV genomes with deleted sequences in higher frequencies than if only one primer-pair was used.

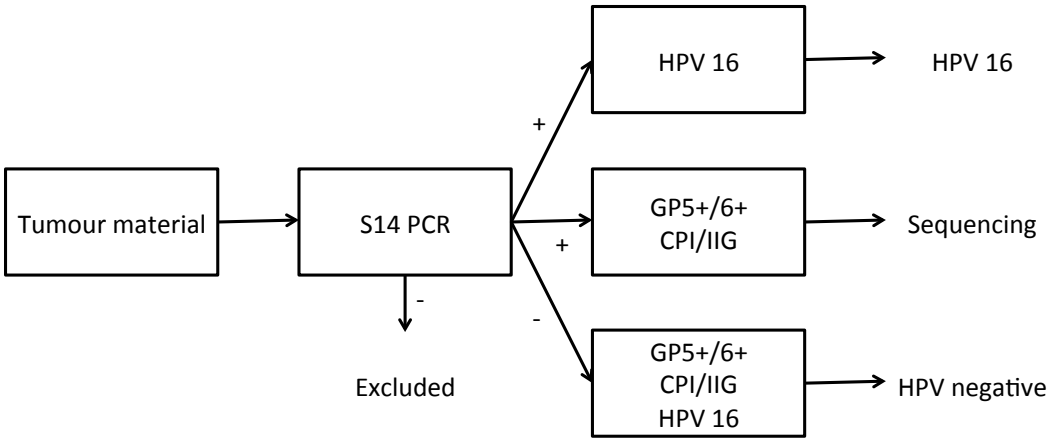


Figure 10. Algorithm for HPV detection in paper I-IV

Nevertheless, a drawback of our algorithm is the typing of HPV. Samples that were HPV 16 positive by the type specific primers were considered as only HPV 16, and samples that were GP5+/6+ and/or CPI/IIG positive, but HPV 16 negative were sequenced to determine the type. Therefore, in theory, in case of a double-infection including HPV-16, this would be recorded as an infection with HPV 16 (Figure 10). However, this was not the case, since as will be described below, when a Multiplex Luminex assay was used instead similar results with dominance of HPV 16 as a single infection were obtained.

3.2.1.2 S14 PCR for detection of amplifiable human DNA

In papers I-IV, to verify amplifiable human DNA, the S14 primer pairs were used (also described elsewhere) in all tumours. The S14 primers amplify a 127bp long sequence of the human ribosomal gene S14, which is in size close to that of the amplicons generated by the HPV detection primer pairs (GP5+/6+ ~130-150bp; CPI/IIG ~190bp; HPV 16 119bp). Consequently, these S14 primer pairs should be more appropriate than other housekeeping gene amplicons, which usually are double the size of the GP5+/6+ products. Nevertheless, the importance of using a housekeeping amplicon

approximately the same size as the viral amplicon is based on the assumption that the human and viral DNA degrades at the same speed - which we do not know.

3.2.1.3 HPV detection by Multiplex Luminex, Paper V

In paper V, all samples were tested with the Multiplex Luminex method, described elsewhere(Ramqvist *et al.* 2011b). This assay is more sensitive, since it uses the previously described broad-spectrum GP5+/6+ primers for amplification and it may detect up to 24 different types in the typing step. This was partly compensated for using a lower amount of DNA in the assay. Moreover, we also validated the method, by re-analysing samples included in paper I-IV with the Multiplex Luminex method. In view of the fact that that the typing of the different types are obtained simultaneously and that we detect very few cases of double-infection, suggests that the methods used in paper I-IV were adequate. In addition, the human beta-globin gene is used as a housekeeping gene.

3.2.1.4 General HPV detection considerations

One of the most important factors that may influence the result of the PCRs is the material and its condition. In our studies, we have used formalin fixed, paraffin embedded tumour tissues. The advantage using this kind of material is obvious; it is easy to get. The disadvantages vary, and include DNA quality and noise from tumour stroma and surrounding tissues. Formalin fixation results in DNA bridges, which can result in DNA breaks and shorter DNA fragments as compared to what is obtained in fresh frozen tumours. Another important factor is that infiltrating lymphocytes and surrounding stroma, as well as normal tissues can be present in the biopsies. Consequently, when loading the PCRs with the same amount of DNA (100ng in paper I-IV and 5-10ng in paper V), the ratio tumour/normal cell DNA may differ. To minimize these problems, the tissues were macro-dissected before DNA/RNA was extracted.

3.2.2 Immunohistochemistry

Immunohistochemistry (IHC) staining protocols are described in more detail in papers III-V. Nonetheless, although the staining procedures were similar, different evaluation criteria were used for the different antibodies.

3.2.2.1 P16 INK4a (papers III-V)

In the case of p16INK4a, which is discussed in the introduction section 1.2.7, two different cut-offs were used to define positivity. In paper III-IV, a cut-off of >75% was used and in paper V a cut-off of >70% was applied. The reason that we changed cut-off in the last paper is, as pointed out in the introduction, that two large prospective trials have used the latter cut-off and researchers in the field have started to consider this cut-

off as “validated”. However, here I would argue that such strict cut-off should be questioned for two reasons: (1) There is a lack of a biological reason for the cut-off and (2) There is a lack of such precision in the IHC evaluation. Another important general point, also applicable for p16INK4a staining, is that staining may vary within the tumour and IHC is just a “snap-shot”, looking at only one tiny slice of the tumour.

3.2.2.2 HLA staining (papers IV-V)

Different evaluation criteria were used for evaluation of HLA-A, B, C and HLA-E and HLA-G respectively, after staining with different specific antibodies for the different HLA alleles.

For HLA -A, -B and -C staining, the intensity was evaluated and scored as “absence of”, or as “weak” or as “normal” / “strong”. Thus, the intensity was graded similarly in paper IV and V, but the nomenclature was changed in the highest intensity staining. Fractions of HLA –A, -B and -C positive cells were also evaluated and scored on a 4-tier scale in paper IV (0%; 1-25%; 26-75% and 76-100%) and on 5-tier scale (0%; 1-25%; 26-50%; 51-75%; and 76-100%) in paper V. For HLA-E, intensity was scored as “absent, “weak”, “medium” and “strong” intensity and grading of fraction of positive cells was done according to the 5-tier scale used in paper V for HLA-A, -B and -C described above. For HLA-G, only absence of or presence of HLA-G was used. The reason for this discrepancy in evaluation was that the different antibodies gave different staining patterns and that others had used different scales previously.

Also, worthwhile to comment on, is that HLA intensity could vary within individual tumours from weak to strong/normal. Here, the most prominent intensity staining was used as intensity score. Consequently, one could argue that intensity staining is uncertain. To such statements, I would like to respond that the lowest intensity (absence of) is absolute and if intensity staining is correlated to *e.g.* prognosis, weak intensity always presents the intermediate group, flanked by normal/strong and absence of staining.

4 RESULTS AND DISCUSSION

4.1 INCIDENCE AND PREVALENCE OF HPV IN TSCC AND BOTSCC (PAPER I, II AND V)

In papers I and II, and to some extent in paper V, we wanted to over time, analyse the HPV prevalence and incidence of TSCC and BOTSCC, the two most common sub-sites within OSCC, in Stockholm, Sweden.

In papers I and II, the incidence numbers for the TSCC and BOTSCC were obtained from the Swedish Cancer Registry (Figure 11 A and C). The prevalence of HPV DNA positive TSCC and BOTSCC was also examined over time (Figure 11 B and D). In addition, the TSCC prevalence and incidence data were merged and standardised, in order to better present the development over time (Figure 12). In summary, the incidence and the HPV DNA prevalence figures has increased significantly over time. Below the numbers will be presented in more detail.

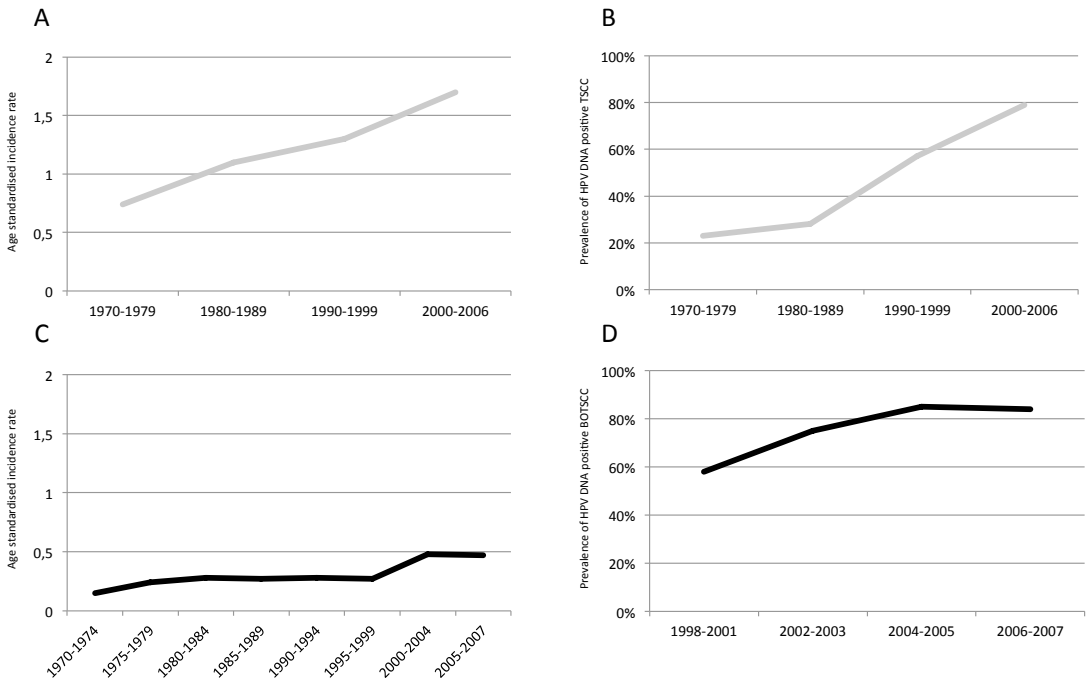


Figure 11. Incidence and HPV prevalence numbers for TSCC (grey) and BOTSCC (black) respectively. (A) Incidence of TSCC (ICD-7 145.0) and (B) prevalence of HPV DNA positive TSCC (ICD-7 145.0) over time as well as (C) incidence of BOTSCC (ICD-10 C09.1) and prevalence of HPV DNA positive BOTSCC (ICD-10 C09.1) over time.

In paper I, we show that the prevalence of HPV DNA positive TSCC has increased, from 23% in the 70´s to 29% in the 1980´s, (p=0.79) to 57% in the 1990´s (p=0.0025) and to 79% in 2000-2007 (p<0.0001). This increase was paralleled with an increase in TSCC incidence, which increased from 0.74/100,000 person years in the 1970´s to 1.65/100,000 person years in 2000-2006. In paper II, we showed a similar increase in prevalence of HPV DNA positive BOTSCC (from 58% 1998-2001 up to 84% 2006-

2007, p<0.05) as well as an increased incidence (from 0.15/100,000 person years in 1970-74 up to 0.47/100,000 person years in 2005-2007). In figure 11 A-D, these data are visualised.

Moreover, in paper I, the prevalence data was merged with the incidence data and the combination was subsequently standardised to the Swedish population of 1970. Here, we were able to show a 7-fold increase in HPV related TSCC, while HPV negative TSCC declined. The decrease in HPV negative TSCC is similar to that of lung cancer (and other smoking-related tumours in men) with a decrease from the 1980's. However, it is important to note that since we were not able to retrieve all biopsies from all TSCC patients, this is an estimate of the incidence of HPV positive and HPV negative TSCC.

Nonetheless, the data in papers I and II strongly suggest that the incidence of HPV induced OSCC has increased over time. Therefore, papers I and II together with a previous study from our group and a study from Colorado by Ernster et al, were the first studies to suggest such an increase.(Hammarstedt *et al.* 2006; Ernster *et al.* 2007; Nasman *et al.* 2009; Attner *et al.* 2010) As discussed in the introduction, other studies have followed and confirmed similar developments in other countries

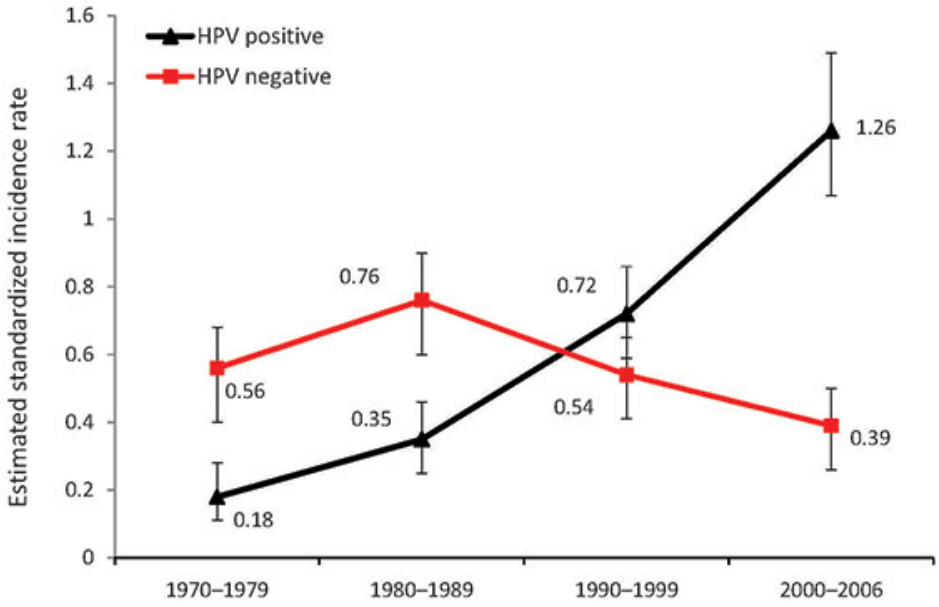


Figure 12. Estimated incidence of HPV positive and negative TSCC (ICD-7 145.0) over time.

Furthermore, as discussed previously in the introduction and in the material and methods section, the numbers obtained for TSCC and BOTSCC in papers I and II should be compared with caution, since different classification systems and time spans were used. Notably, in paper V (Table 1) all patients were classified by the same system and obtained at the same time. In this table, all patients diagnosed between 2000-2009 with available pre-treatment biopsies and treated with the intention to cure were included. Although it is not clearly stated in paper V, 77% of the TSCC (C09.0-9) and 73% of the BOTSCC (C01.9) biopsies were HPV positive between 2000-2009. The reason between the discrepancies between papers I and II on the one hand and paper V on the other hand is that a lower HPV prevalence was observed for the ICD-10

codes C05.1-9 and C10.0-9 used in paper V. The HPV prevalence development over time in the different ICD-10 diagnoses used in paper V is presented in figure 13.

In this figure, it becomes apparent that, although TSCC dominates in total numbers, the HPV prevalence is similar in TSCC and BOTSCC. Another observation is that the increased prevalence in TSCC is less obvious if the ICD-10.0-9 classification is used instead of ICD-7 145.0. However, the difference in HPV prevalence between 2000-2002 and 2006-2008 in ICD-10 09 OSCC is still significant ($p<0.05$) (Figure 13).

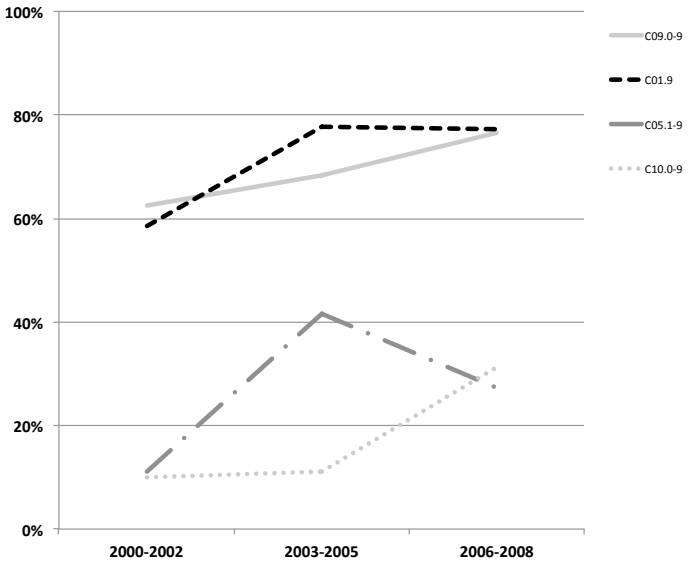


Figure 13. HPV prevalence in different sites of OSCC between 2000-2008 (data not published).

There is a remarkable difference in HPV prevalence within the different ICD-10 C09 codes. While the C09.0 (tonsils) has an HPV prevalence of 79% during 2000-2009, it is only 14% for C09.1 (tonsillar pillars). Moreover, during the same time span, C09.8 (large TSCC) and C09.9 (unspecified TSCC) had a prevalence of 63% and 57% respectively (data not published). Therefore, when stated in this thesis that soon almost all TSCC will be HPV positive, this should be interpreted in the context of ICD-7 145.0 and not ICD-10 C09.0-9.

Nevertheless, in both TSCC and BOTSCC >90% of the HPV positive cases, were accounted for by HPV16, which dominated completely, and in these cases E6 and/or E7 was present in the vast majority of cases (again > 90%). Furthermore, patients with HPV DNA positive TSCC and BOTSCC were often younger (papers I, II and V) and were non-smokers to a higher extent (paper V). They also had smaller tumours (papers I, II and V); but tended to have higher tumour stages (significant in paper II and V, but not in I) and tended to have less differentiated tumours (significant in paper I, but not in II and V).

Conclusion. *The incidence of TSCC and BOTSCC has increased and this increase has been paralleled by an increase in HPV prevalence, suggesting that HPV infection is responsible for this rise in TSCC and BOTSCC.*

4.2 TUMOUR INFILTRATING LYMPHOCYTES AND HPV STATUS IN CORRELATION TO CLINICAL OUTCOME.
(PAPER III)

In Paper III the aim was to study the role of CD8+ and Foxp3+ tumour infiltrating cells in TSCC, in relation to HPV status and clinical outcome.

In summary, a high CD8+ count as well as a high CD8+/Foxp3 ratio correlated to a favourable clinical outcome in both HPV positive and negative TSCC. In addition, HPV positive TSCC presented significant higher levels of TILs. Below the data is presented in more detail.

Patients with HPV positive TSCC with a favourable clinical outcome, defined here as progression free survival (see 3.1.2), had a higher mean CD8+ TIL count compared to those with a poor clinical outcome (61.4 vs. 19.4, $p<0.01$). Similarly had a higher mean CD8+ TIL count compared to those with a poor clinical outcome (61.4 vs. 19.4, $p<0.01$). Similarly, patients with HPV negative TSCC with a good clinical outcome had a higher mean CD8+ TIL count than those with a poor clinical outcome (19.4 *vs.* 4.7, p=0.001)(Figure 14A). However, when patients with HPV positive and negative TSCC with good and poor clinical outcome were compared with regard to numbers of Foxp3+ TILs, no differences were observed (HPV positive TSCC: 33.7 *vs.* 32.5, p=0.9; HPV negative TSCC: 11.7 *vs.* 16.4, p=0.6) (Figure 14B). When CD8/Foxp3 ratios were evaluated however, both patients with HPV positive and HPV negative TSCC with a good clinical outcome tended to have higher ratios than those with a poor clinical outcome (HPV positive TSCC: 3.0 *vs.* 1.5, p=0.09 and HPV negative TSCC: 8.2 *vs.* 1.1, p=0.10) (Figure 14 C).

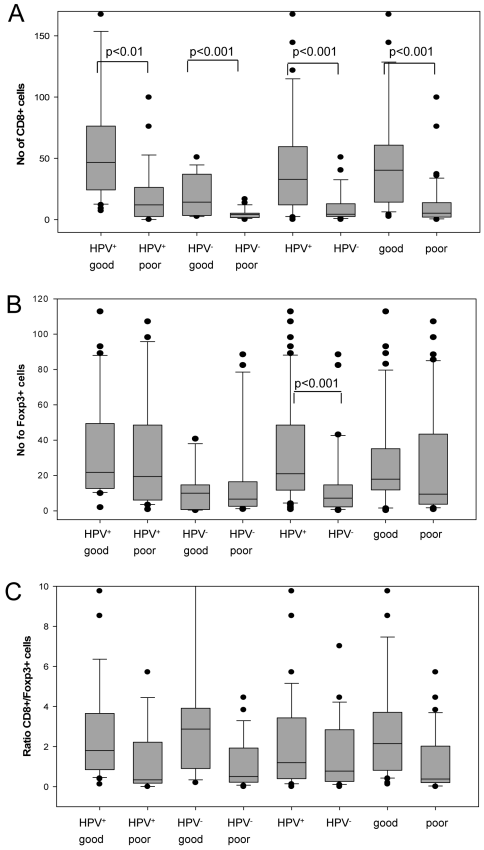


Figure 14. CD8+, Foxp3+ and CD8+/Foxp3+ ratio in correlation to HPV status and clinical outcome (good/poor) in TSCC.

(A) Mean CD8+ TILs levels in correlation to HPV status and clinical outcome; HPV status only; and clinical outcome only. A high CD8+ TIL mean count correlated to favourable outcome in patients with HPV positive and negative TSCC ($p<0.01$ and $p<0.001$ respectively); to HPV status in TSCC ($p<0.001$); and to outcome in general ($p<0.001$).

(B) Mean levels of Foxp3+ TILs in correlation to HPV status and clinical outcome; HPV status only; and clinical outcome only. A high Foxp3 TIL mean was associated with positive HPV status ($p<0.001$).

(C) Mean CD8+/Foxp3 ratio in correlation to HPV status and clinical outcome; HPV status only; and clinical outcome only. No significant differences were observed, but there was a trend a higher ratio to be correlated to a better outcome.

Additionally, HPV positive TSCC, as compared to HPV negative TSCC, had significantly higher mean levels of CD8+ TILs (45.6 *vs.* 9.9, p<0.001) and significantly higher mean levels of Foxp3 TILs (33.2 *vs.*14.7, p<0.001). However, no significant differences in CD8+/Foxp3+ ratios were observed (Figure 14 A-C).

A Kaplan-Meier analysis on disease-free survival (DFS) was also performed for patients with HPV positive and negative TSCC. Here, patients with HPV positive TSCC were divided into two groups based on median CD8+ TIL count. Patients with a high CD8+ TIL count had a significant better DFS than those with a count under the group median (log-rank test: 0.02). Similarly, patients with HPV negative TSCC with a high CD8+ TIL count had a better DFS than those with tumours with a low CD8+ TIL count, but here the differences did not reach significance (log-rank 0.17). If, however, the CD8+/Foxp3+ ratio was assessed, a high ratio (above 1) in HPV positive as well as in HPV negative TSCC was correlated to a better DFS (p=0.04 and p=0.03 respectively).

Moreover, in a Cox proportional univariable and multivariable analysis (adjusting for age, sex and stage) numbers of CD8+ TILs were used in patients with HPV positive TSCC, and the CD8+/Foxp3+ ratio was used in patients for HPV negative TSCC. In the HPV positive group, a high CD8+ TIL count was correlated to a favourable DFS both in the univariable and the multivariable analysis (HR 0.27, 95% CI 0.09-0.88 and HR 0.28, 95% CI 0.084-0.91 respectively). Likewise, in the HPV negative group, a CD8+/Foxp3+ ratio above 1.0 correlated with a favourable DFS, both in the univariable and the multivariable analysis (HR 0.27, 95% CI 0.073-0.99 and HR 0.21, 95% CI 0.057-0.81 respectively).

In summary, the results regarding TILs and clinical outcome are in concordance with previous published studies in HNSCC (Ogino *et al.* 2006) and other malignancies, *e.g.* cervical cancer.(Nedergaard *et al.* 2007; Piersma *et al.* 2007). In this study, we were also able to show the significance of HPV status and, as an indication of how important it is to separate HNSCC according to HPV when *e.g.* searching for new prognostic markers.

Finally, the expression of the cyclooxygenase-2 (Cox-2) enzyme was also examined in relation to infiltrating Foxp3+ cells. The rationale was that, Cox-2 has been suggested to indirectly induce up-regulation of Foxp3 expression, which is necessary for Treg function.(Baratelli *et al.* 2005; Sharma *et al.* 2005) However, no significant correlations between Foxp3 and Cox-2 were observed. Furthermore, in contrast to previously published papers in OSCC, Cox-2 expression did not by itself correlate to clinical outcome in either HPV positive or negative TSCC.(Chang *et al.* 2004) However, HPV positive tumours tended to have a stronger Cox-2 expression by intensity than HPV negative tumours (73% vs. 52%), and the difference was significant when adjusted for prognosis (p=0.049).

Conclusion. *Patients with HPV positive and negative TSCC with a high numbers of CD8+TILs have a better clinical outcome than those with tumours with low numbers of CD8+ TIL, indicating the number of CD8+ TILs could potentially be used a prognostic marker in TSCC– but this should first be validated in a prospective trial.*

4.3 EXPRESSION OF HLA CLASS I (-A, -B -C, -E AND -G) IN TSCC AND ITS CORRELATION TO CLINICAL OUTCOME.
(PAPER IV)

In paper IV, the aim was to investigate the role of "classical" (A, B, C) and "non-classical" HLA class I (E and G) in both HPV positive and negative TSCC in correlation to clinical outcome.

In summary, absence of/weak HLA –A, B and C expression was correlated with a favourable clinical outcome in patients with HPV positive TSCC, and to a worse clinical outcome in patients with HPV negative TSCC. No outcome correlations were observed for HLA-E and -G. The data are presented in more detail below.

Patients with HPV positive TSCC with absence of/weak HLA class I intensity presented a very favourable clinical outcome significantly more often than those with high HLA intensity (Table 4). Similar results were obtained if absence/presence of or localisation of HLA class I staining was evaluated (Table 4). However, no correlation between clinical outcome and HLA-E or HLA-G expression could be observed.

Table 4. HLA class I expression in HPV positive TSCC with good *vs.* poor clinical outcome.

Factor		Good clinical outcome[†] N (%)	Poor clinical outcome[†] N (%)	Odds ratio (95% CI)[††]	p-value
HCA-2[§] intensity	*Absent / Weak* *Normal*	77 (79) 21 (21)	8 (38) 13 (62)	1 5.9 (2.0 to 19)	p<0.001
HC-10[§] intensity	*Absent / Weak* *Normal*	68 (69) 30 (31)	8 (38) 13 (62)	1 3.6 (1.3 to 11)	p=0.011
HCA-2[§] presence	*Absent* *Present[‡]*	34 (35) 64 (65)	2 (10) 19 (90)	1 5.0 (1.1 to 47)	p=0.034
HC-10[§] presence	*Absent* *Present[‡]*	25 (26) 73 (74)	1 (5) 20 (95)	1 6.8 (1.0 to 295)	p=0.042
HCA-2[§] localization	*Absent* *Cytoplasmatic* *Membranous*	34 (35) 48 (49) 16 (16)	2 (10) 8 (38) 11 (52)	1 2.8 (0.6 to 14.1) 11.7 (2.3 to 59)	p=0.001
HC-10[§] localization	*Absent* *Cytoplasmatic* *Membranous*	25 (27) 46 (47) 27 (28)	1 (5) 11 (52) 9 (43)	1 6.0 (0.7 to 49) 8.3 (1.0 to 70.6)	p=0.09
HLA-E intensity	*Absent / Weak* *Moderate /Strong*	59 (60) 39 (40)	16 (76) 5 (24)	1 0.5 (0.1 to 1.6)	p=0.22
HLA-E presence	*Absent* *Present[‡]*	17 (17) 81 (83)	3 (15) 18 (85)	1 1.6 (0.3 to 16)	p=1.0
HLA-G presence	*Absent* *Present*	90 (92) 8 (8)	17 (81) 4 (19)	1 2.6 (0.5 to 11)	p=0.22

[*] HPV status obtained from previous studies

[†] Clinical outcome defined as recurrence free and alive 3 year after diagnosis (good) and as dead of disease or recurrence within 3 years after treatment (poor).

[††] Odds ratio of poor clinical outcome estimated using exact logisitc regression

[‡] Present referes to staining score 1-3

[§] Antibodies binding to classical HLA class I (A, B and C) heavy chain.

Conversely, in patients with HPV negative TSCC a higher intensity staining and presence of HLA class I expression as compared to weak intensity staining and absence of expression was correlated to a favourable clinical outcome. Expression of HLA-E and HLA-G did not correlate to outcome (Table 5).

Table 5. HLA class I expression in HPV positive TSCC with good *vs.* poor clinical outcome.

Factor		Good clinical outcome[†] N (%)	Poor clinical outcome[†] N (%)	Odds ratio (95% CI)[††]	p-value
HCA-2[§] intensity	*Absent / Weak* *Normal*	1 (9) 10 (91)	14 (70) 6 (30)	1 0.05 (0.001 to 0.46)	p=0.002
HC-10[§] intensity	*Absent / Weak* *Normal*	1 (9) 10 (91)	12 (60) 8 (40)	1 0.1 (0.001 to 0.7)	p=0.008
HCA-2[§] presence	*Absent* *Present*[‡]	0 (0) 11 (100)	8 (40) 12 (60)	-	p=0.028
HC-10[§] presence	*Absent* *Present*[‡]	0 (0) 11 (100)	5 (25) 15 (75)	-	p=0.13
HCA-2[§] localization	*Absent* *Cytoplasmatic* *Membranous*	0 (0) 0 (0) 11 (100)	8 (40) 7 (35) 5 (25)	-	p<0.001
HC-10[§] localization	*Absent* *Cytoplasmatic* *Membranous*	0 (0) 0 (0) 11 (100)	8 40) 7 (35) 5 (25)	-	p<0.001
HLA-E intensity	*Absent / Weak* *Moderate /Strong*	6 (55) 5 (45)	10 (50) 10 (50)	1 1.2 (0.2 to 6.8)	p=1.0
HLA-E presence	*Absent* *Present*[‡]	0 (0) 11 (100)	1 (5) 19 (95)	-	p=1.0
HLA-G presence	*Absent* *Present*	10 (91) 1 (9)	20 (100) 0 (0)	-	p=0.36

[*] HPV status obtained from previous studies

[†] Clinical outcome defined as recurrence free and alive 3 year after diagnosis (good) and as dead of disease or recurrence within 3 years after treatment (poor).

[††] Odds ratio of poor clinical outcome estimated using exact logisitc regression

[‡] Present referes to staining score 1-3

[§] Antibodies binding to classical HLA class I (A, B and C) heavy chain.

A Kaplan-Meier analysis on disease-free survival (DFS) was performed, both for patients with HPV positive and negative TSCC. In the HPV positive group, 100% of the patients with absence of HLA class I (HCA-2) remained disease-free 3 years after diagnosis, while this was the case for 90% of those with a weak, and for only 69% of the patients with normal intensity staining (log-rank test $p<0.001$). Similar results were obtained with the HC-10 mAb, with 100% of the patients with absence of HLA class I remaining disease-free 3 years after diagnosis, while this was the case for 90% of those with a weak, and for only 76% of the patients with normal intensity staining (log-rank test $p=0.01$).

In patients with HPV negative TSCC, a normal HCA-2 intensity was associated with a better 3-year DFS and 67% of the patients remained disease-free, while 40% of the weak intensity and none of the those with absence of HLA class I intensity staining remained disease free (log-rank test $p<0.022$). If HC-10 mAb was used instead, normal

intensity staining presented the best DFS (63% disease-free) followed by weak (33%) and absence of HLA class I intensity staining (0%)(log-rank test p=0.05).

Furthermore, when HCA-2 staining intensity (absence of/weak *vs.* strong) was used in a univariable Cox-regression model, we found that patients with HPV positive TSCC with normal intensity were 6.1 times more likely to have a recurrence in disease than those with absence of/weak HCA-2 staining intensity (HR 6.1, 95% CI 2.1-18.0, p=0.001). Conversely, patients with HPV negative TSCC with a normal HCA-2 intensity staining had a 5-fold decreased risk of relapse (HR 0.23, 95% CI 0.07-0.73, p=0.013). When HCA-2 intensity staining was adjusted for stage, age and sex in a multivariable analysis, absence of/weak staining was still correlated to a favourable DFS in the HPV positive group (HR 6.6, 95% CI 2.2-19.7, p=0.001), and to a worse DFS in the HPV negative group (HR 0.14, 95% CI 0.02-0.92, p=0.04). Similar results were obtained if HC-10 staining intensity was used instead, both in the univariable analysis (HPV positive TSCC: HR 3.9, 95% CI 1.3-11.5, p=0.012 *and* HPV negative TSCC: HR 0.27, 0.09-0.83, p=0.022) and the multivariable analysis (HPV positive TSCC: HR 4.1, 95% CI 1.4-12.2, p=0.011 *and* HPV negative TSCC: HR 0.31, 95% CI 0.08-1.2, p=0.09).

The fact that absent/weak MHC expression correlated with a very good prognosis in HPV positive OSCC was an unexpected finding. However, in *e.g.* breast cancer, a complete loss of MHC class I together with a favourable prognosis has been suggested to be due to NK-cell activity and an association between loss of MHC and resistance to apoptosis. Therefore, eradication of HLA lacking tumours by NK-cells could be an explanation of the favourable prognosis in these TSCC tumours. We therefore also assessed tumour infiltrative NK-cells (CD56 positive cells by IHC). The numbers of NK cells were low and no correlations to lack of HLA class I, HLA-G or to clinical outcome were observed (data not published).

Conclusion. *In summary, absence of and weak HLA-A, -B and -C intensity was associated with a favourable clinical outcome in patients with HPV positive TSCC, but with a worse in patients with HPV negative TSCC. These finings will be further discussed below in Paper V.*

4.4 EXPRESSION OF CLASSICAL HLA CLASS I AND HLA CLASS II IN OSCC AND ITS RELATION TO SURVIVAL
(PAPER V)

In paper V, the aim was to extend the previous HLA class I study in paper IV, and investigate the role classical HLA class I as well as HLA class II antigen expression in a large cohort of OSCC, in correlation to HPV status and clinical outcome.

In summary, absence of HLA –A, B and C expression in patients with HPV positive OSCC correlated to a very high disease-free survival (DFS), disease-specific survival (DSS) and overall survival (OS), independent of treatment regime. The opposite was observed in patients with HPV negative OSCC. HLA class II expression was correlated to a better prognosis in patients with HPV negative, but not in patients with HPV positive OSCC. The data is presented in more detail below:

In total, 551 OSCC patients were obtained from the Swedish Cancer Registry and 484 out of these were oncologically treated with the intention to cure. In total, 45 of the treated patients had no pre-treatment biopsies available. However, there were no differences between the 439 patients with biopsies and the 45 without biopsies with regard to patient characteristics, except for treatment – where patients with missing blocks were more likely to receive only RT as compared to patients with available blocks (p=0.007).

In total, 303/439 (69%) of the tumours were HPV positive and patients with HPV positive OSCC had a better DFS, DSS and OS as compared to those with HPV negative tumours, both in the univariable and multivariable analysis (adjusted for age, sex stage and tumour localisation) (Table 6).

Table 6. HPV status and survival

		Univariable analysis	Multivaraible analysis*
		HR (95% CI)	HR (95% CI)
DFS	*HPV negative (N=136)*	1 *(ref)*	1 *(ref)*
	HPV positive (N=303)	0.30 (95% CI 0.19-0.48)	0.30 (95% CI 0.18-0.50)
DSS	*HPV negative (N=136)*	1 *(ref)*	1 *(ref)*
	HPV positive (N=303)	0.23 (95% CI 0.15-0.36)	0.23 (95% CI 0.15-0.36)
OS	*HPV negative (N=136)*	1 *(ref)*	1 *(ref)*
	HPV positive (N=303)	0.26 (95% CI 0.18-0.37)	0.27 (95% CI 0.18-0.39)

* adjuseted for age, sex, stage and tumour localisation

Moreover, when classical HLA class I and HLA class II expression was analysed in HPV positive and negative OSCC there were significant differences. HLA class I expression was more often down-regulated and HLA class II expression was more often up-regulated in HPV positive OSCC, as compared to HPV negative OSCC (Table 7).

Table 7. Classical HLA class I and HLA class II staining in relation to HPV status

		HPV status				
		Positive		Negative		
		N	%	N	%	p-value[§]
Intensity of HCA-2 positive cells	*absent*	101	33%	24	18%	0.001
	weak	60	20%	45	33%	
	strong	142	47%	67	49%	
Fraction of HCA-2 positive cells	*absent*	101	33%	24	18%	0.009
	1-25%	33	11%	14	10%	
	26-50%	24	8%	16	12%	
	51-75%	33	11%	14	10%	
	76-100%	112	37%	68	50%	
Intensity of HC-10 positive cells	*absent*	60	20%	9	7%	0.001
	weak	73	24%	33	24%	
	strong	170	56%	94	69%	
Fraction of HC-10 positive cells	*absent*	60	20%	9	7%	0.001
	1-25%	24	8%	7	5%	
	26-50%	16	5%	4	3%	
	51-75%	39	13%	15	11%	
	76-100%	164	54%	101	74%	
Intensity of LGII-612.14 positive cells	*absent*	100	55%	82	45%	<0.001
	weak	29	73%	11	28%	
	strong	174	80%	43	20%	
Fraction of LGII-612.14 positive cells	*absent*	100	55%	82	45%	<0.001
	1-25%	26	72%	10	28%	
	26-50%	23	77%	7	23%	
	51-75%	34	74%	12	26%	
	76-100%	120	83%	25	17%	

[§] Chi-square test

Subsequently, the cohort was divided into a HPV positive and a HPV negative OSCC cohort and HLA expression was analysed in correlation to survival. In patients with HPV positive OSCC, absence of classical HLA class I expression was associated with a very high survival, while a strong intensity of the same was associated with a worse (Figure 15).

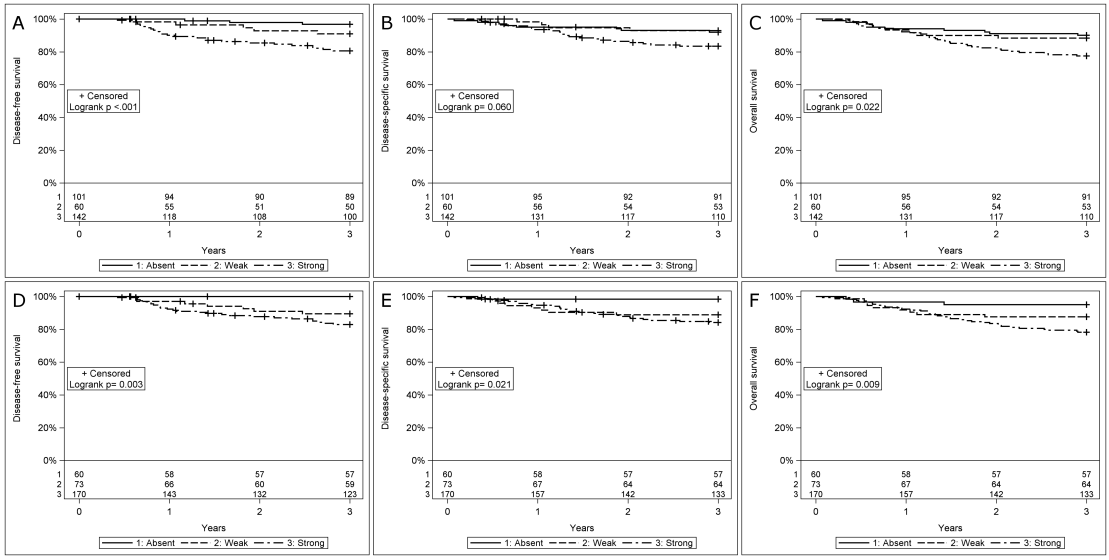


Figure 15. Kaplan-Meier curves for DFS, DSS and OS in patients with HPV positive OSCC. (A) DFS stratified for HCA-2 intensity, (B) DSS stratified for HCA-2 intensity, (C) OS stratified for HCA-2 intensity, (D) DFS stratified for HC-10 intensity, (E) DSS stratified for HC-10 intensity, and (F) OS stratified for HC-10 intensity.

Patients with HPV positive OSCC and absence of HLA class I intensity had a significantly better survival than those with a strong HLA class I intensity staining, while weak intensity staining presented an

intermediate survival (HCA-2: DFS p<0.001; DSS p=0.060; OS p=0.022; HC-10: DFS p=0.003, DSS p=0.021 and OS p=0.009).
The survival data was also analysed in a univariable and a multivariable model, adjusted for age, sex, stage and tumour site. Patients with HPV positive OSCC and absence of classical HLA class I staining still presented a better DFS, DSS and OS in the univariable (HCA-2: p=0.0019, p=0.062 and p=0.016 respectively and HC-10: all disease-free, p=0.025 and p=0.011 respectively) and in the multivariable (HCA-2: p=0.003, p=0.11 and p=0.033 respectively and HC-10: all disease-free, p=0.040 and p=0.024 respectively). Although the differences did not reach significant levels in the DSS analysis with the HCA-2 mAb, the trend was still similar to what was observed in the other survival measurements. Additionally, HLA class II was also analysed in correlation to survival, but here no survival associations were observed.

Consequently, the results in paper V showing that absent/weak HLA class I intensity staining in HPV positive OSCC was a prognostic favourable factor, was in line with and confirmed our previous findings in in paper IV for HPV positive TSCC. Nevertheless, the reason for the association between lack of HLA class I and a favourable survival is still unravelled. The down-regulation of HLA class I could, as described in the introduction, indeed be due to the repression of HLA by E7 or E5 and that the favourable prognosis is due to viral activity. Another explanation may be that the viral repression of HLA is overpowered by a possible increase in HLA class I expression after RT. Interestingly, previous studies have actually shown an up-regulation of HLA class I expression after RT in cervical cancer and also in myeloma cell lines.(Mikyskova *et al.* 2005; Chiriva-Internati *et al.* 2006) Similar results were also obtained in breast cancer, and here the authors concluded that the up-regulation was due to an increased peptide pool.(Reits *et al.* 2006) On the other hand, a study some years ago suggested that down-regulated HLA class I expression can be due to viral integration within the HLA foci.(Sheu *et al.* 2005) Such suggestions contradict the previous line of thoughts. Other explanations may include differences in the immune defence against HPV, and thus an immune selection, or due to NK-cell activity, which however is less likely here as discussed in paper IV.

Nonetheless, in the HPV negative OSCC cohort, the opposite results were obtained. In general, patients with HPV negative OSCC with a strong classical HLA class I expression presented a better survival than those with absence of or weak intensity staining. In addition, a strong HLA class II expression was in general associated with a better DFS, DSS and OS, both in the univariable and the multivariable analysis. Notably, similar results obtained in patients with HPV negative OSCC have previously been reported in other malignancies and highlight the atypical and very different nature of HPV positive OSCC compared to HPV negative OSCC.

Furthermore, oncological treatment (RT vs. CRT) was also examined in relation to HLA class I expression in patients with HPV positive OSCC. Interestingly, no differences in DFS, DSS and OS were observed between RT and CRT in patients with HPV positive OSCC with absence of or with weak HLA class I expression. More specifically, no differences were observed in the patients with absence of HLA class I expression with regard to DFS, DSS and OS (HCA-2: p=0.91, p=0.94 and p= 0.68 respectively; and HC-10: p=1.00, p=0.46 and p=0.20 respectively). Analogously, no

differences were observed in the patients with HPV positive OSCC with weak HLA class I intensity with regard to DFS, DSS and OS (HCA-2: p=0.15, p=0.88 and p=1.0 respectively; and HC-10: p=0.27, p=0.82 and p=0.99 respectively). In contrast to the other two groups, patients with HPV positive OSCC with a strong HLA class I staining intensity had a significantly better DFS, DSS and OS if treated with CRT than with RT (presented for DSS in Figure 16).

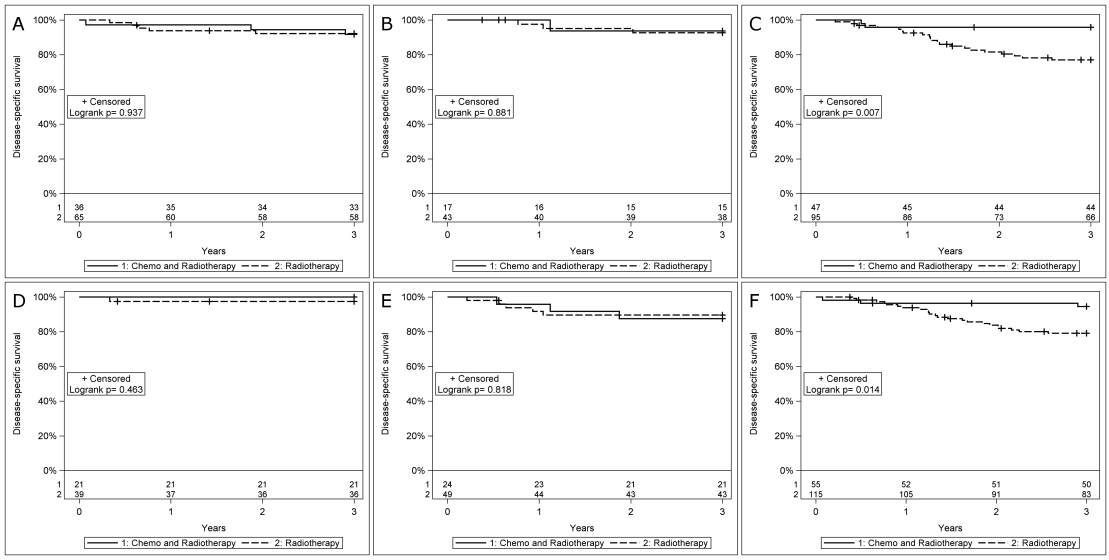


Figure 16. Kaplan-Meier curves for DSS in patients with HPV positive OSCC and different treatment regimes. (A) DSS in HPV positive OSCC with absent HCA-2 intensity stratified RT and CRT, (B) DSS in HPV positive OSCC with weak HCA-2 intensity stratified RT and CRT, (C) DSS in HPV positive OSCC with strong HCA-2 intensity stratified for RT and CRT, (D) DSS in HPV positive OSCC with absent HC-10 intensity stratified for RT and CRT, (E) DSS in HPV positive OSCC with weak HC-10 intensity stratified for RT and CRT, (F) DSS in HPV positive OSCC with strong HC-10 intensity stratified for RT and CRT.

It is important to note however, that there was most probably a selection bias for more patients with a poor clinical status receiving only RT than CRT, which probably explains the differences in the strong HLA class I intensity staining group. Nevertheless, irrespective of treatment with CRT or RT and a possible bias in selection of treatment, patients with HPV positive OSCC with an absence of, or weak HLA class I expression presented very high DFS, DSS and OS. In other words, even if patients with poorer health conditions were over-represented in the RT groups, still there were no differences in any survival measurements in the absence of and the weak intensity staining groups. Therefore, since patients with HPV positive OSCC with absence of HLA class I present with a very high survival, independent of treatment regime, these patients could potentially be the first to be randomized into a prospective experimental study for de-escalation of oncological treatment.

Conclusion. *Patients with HPV positive OSCC with absence of/weak HLA class I intensity staining have a better DFS, DSS and OS, independent of treatment regime, than those with HPV positive OSCC and strong HLA class I intensity staining. Furthermore, patients with HPV negative OSCC and a strong HLA class I and/or HLA class II expression have a better survival than those with HPV negative OSCC and absence of HLA class I and/or class II expression. Finally, HPV positive OSCC*

generally expresses lower levels of HLA class I and higher levels of HLA class II antigens than HPV negative OSCC.

5 CONCLUSIONS

- The incidence of TSCC and BOTSCC has increased and this increase has been paralleled by an increase in HPV prevalence, suggesting that HPV infection is responsible for this rise in TSCC and BOTSCC. (Papers I and II)

- Patients with HPV positive and negative TSCC with high numbers of CD8+ TILs have a better clinical outcome than those with low numbers of CD8+ TILs. (Paper III)

- HPV positive TSCC had in general higher numbers of CD8+ and Foxp3+ TILs as compared to HPV negative TSCC. (Paper III)

- Patients with HPV positive OSCC with absence of/low HLA class I intensity staining had a very high DFS, DSS and OS independent of treatment regime, and compared to those with HPV positive OSCC and a strong HLA class I intensity staining. (Papers IV and V).

- HPV positive OSCC generally expresses lower levels of HLA class I and higher levels of HLA class II antigens than HPV negative OSCC. (Papers IV and V).

- Patients with HPV negative OSCC with a strong HLA class I and/or HLA class II expression have a better survival than those with HPV negative OSCC with absence of HLA class I and/or class II expression. (Papers IV and V).

- Finally, we could confirm that patients with HPV positive OSCC have a better disease-free (DFS), disease-specific (DSS) and overall survival (OS) than patients with HPV negative OSCC. (Paper V)

6 FUTURE PERSPECTIVES

In papers I and II we demonstrated an increase in HPV positive TSCC and BOTSCC. Whether or not this trend will continue to increase in OSCC is not easy to predict, since smoking and alcohol (the other risk factors for OSCC) are still present in the Stockholm population. From the slope of the incidence and prevalence curves we have demonstrated in this thesis we may probably have reached the top of HPV prevalence in OSCC in Stockholm. However, this is still not certain. Furthermore, with a new era with public HPV vaccination of young women, and a possible heard immunity in males, it would, still be interesting to follow possible changes in the prevalence of HPV in general and in the prevalence of different HPV types in OSCC in the future.

In papers III-V we identified two potentially interesting immunological predictive markers (CD8+ TIL counts and HLA class I intensity staining) for clinical outcome. Our results were however obtained from observational retrospective studies where treatment was not randomised and this presents a limitation of our studies, although there are good reasons to assume that the results are valid. Besides, as discussed in the introduction, it is still uncertain how much the different treatment modalities influence survival. Nevertheless, a prospective randomised study should be conducted in patients with HPV positive OSCC with absence of HLA class I expression, or with high CD8 levels, or using a combination of both these markers, in which patients were randomised into controls (receiving CRT) and into cases (receiving RT only).

Lastly, the mechanisms behind the down-regulated HLA class I and the correlation to a favourable clinical outcome should be further studied. Is it due to a viral repression of intact HLA class machinery that is up-regulated upon treatment and overrides the viral repression? Or is it due to something completely else? Answers here may be useful in conducting future therapies.

As always, answers lead to new questions and challenges.

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