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HUMAN PAPILLOMAVIRUS AND OTHER MOLECULAR MARKERS IN CANCER OF DIFFERENT OROPHARYNGEAL SUB-SITES

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Stockholm 2018

Human papillomavirus and other molecular markers in cancer of different oropharyngeal sub-sites

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended in CCK Lecture Hall
Cancer Center Karolinska, R8:00, Karolinska University Hospital, Solna

Friday December 7th 2018 9:30

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The art decorating my cover page was painted by my grandma, Kathleen Finck, the year before I was born. Unfortunately, I didn't get the chance to ask her about her thoughts behind this painting. However, to me it symbolizes that every life is beautiful, and that caring for one another will make you grow as a person.

ABSTRACT

Background: Human papillomavirus (HPV) is a known risk factor for oropharyngeal cancer (OPSCC), and over the past few decades OPSCC has increased drastically due to an HPV epidemic. The oropharynx contains different sub-sites, where sub-sites rich in lymphoid tissue, such as the tonsils and the tongue base, are suggested to be more prone to harbor an HPV infection, and cancer of these sub-sites is more often HPV-positive (HPV[+]). Interestingly, patients with an HPV[+] tonsillar or base of tongue cancer (TSCC or BOTSCC) generally have a better survival compared to patients with corresponding HPV-negative (HPV[-]) tumors. However, HPV does not have the same prognostic value in the other OPSCCs, and has been suggested to differ even depending on the histology of normal tissue surrounding TSCC. Moreover, we have previously shown that low levels of HLA class I (which presents antigens to the immune system) in HPV[+] TSCC and BOTSCC was associated with a good prognosis, whereas the opposite was shown in HPV[-] tumors. Since current treatment often leads to severe side effects, de-escalation trials for patients with a predicted excellent prognosis would be an attractive alternative. Therefore, there is a need to understand the differences between OPSCC sub-sites, and find biomarkers that together with HPV status would identify patients that could benefit of de-escalated or targeted therapy.

Aims: To identify new prognostic markers in OPSCC and to study the importance of sub-dividing OPSCC. To study expression of proteins involved in antigen processing and presentation, in HPV[+] and HPV[-] OPSCC, and how expression is affected by irradiation.

Results: In paper I and II, the expression of the antigen processing machinery (APM) components were evaluated both in the nucleus and in the cytoplasm. We showed that LMP10 and LMP7 had prognostic value in both HPV[+] and HPV[-] TSCC and BOTSCC. We also found that APM components TAP2, LMP2, LMP7, and LMP10 were commonly suppressed in both HPV[+] and HPV[-] TSCC and BOTSCC, and that LMP2 and LMP7 expression was correlated to HLA class I expression. In paper III, we found that radiotherapy had the ability to increase cell surface HLA class I expression in some HPV[+] head and neck cancer cell lines, without an observed change at the transcriptional level. Our results from paper IV, show that HPV was significantly more prevalent in TSCC and BOTSCC as compared to the other oropharyngeal sub-sites. As described in paper V, the histology adjacent to TSCC varies and can be divided into TSCC, where normal tonsil-like adjacent tissue is present (specified TSCC (STSCC)), and absent (non-specified TSCC (NSTSCC)). We show that HPV is significantly more common in STSCC compared to NSTSCC. HPV[+] STSCC patients have a better clinical outcome compared to HPV[+] NSTSCC patients. However, no differences in clinical outcome was observed in patients with HPV[-] STSCC and NSTSCC.

Conclusions: This thesis provides increased understanding of differences between HPV[+] and HPV[-] status in the context of OPSCC sub-sites. In addition, we have identified several prognostic biomarkers that together with HPV status and OPSCC sub-site, can contribute to improved personalized medicine for patients with OPSCC.

SCIENTIFIC PAPERS INCLUDED IN THESIS

I. Tertipis N, **Haeggblom L**, Nordfors C, Grün N, Näsman A, Vlastos A, Dalianis T, and Ramqvist T. Correlation of LMP10 Expression and Clinical Outcome in Human Papillomavirus (HPV) Positive and HPV-Negative Tonsillar and Base of Tongue Cancer. *PLoS One. 2014; 9(4): e95624.*

II. Tertipis N, **Haeggblom L**, Grün N, Nordfors C, Näsman A, Dalianis T, and Ramqvist T. Reduced Expression of the Antigen Processing Machinery Components TAP2, LMP2, and LMP7 in Tonsillar and Base of Tongue Cancer and Implications for Clinical Outcome. *Transl Oncol. 2015 Feb; 8(1): 10-17.*

III. **Haeggblom L**, Nordfors C, Tertipis N, Bersani C, Ramqvist T, Näsman A, Dalianis T. Effects of irradiation on human leukocyte antigen class I expression in human papillomavirus positive and negative base of tongue and mobile tongue squamous cell carcinoma cell lines. *Int J Oncol. 2017 Mar 16.*

IV. **Haeggblom L**, Ramqvist T, Tommasino M, Dalianis T, Näsman A. Time to change perspectives on HPV in oropharyngeal cancer. A systematic review of HPV prevalence per oropharyngeal sub-site the last 3 years. *Papillomavirus Res. 2017 Dec;4:1-11.*

V. **Haeggblom L**, Attoff T, Hammarstedt-Nordenvall L, Näsman A. Human papillomavirus (HPV) and survival of patients per histological sub-site of tonsillar squamous cell carcinoma (TSCC). *Cancer Medicine. 2018 Mar 23.*

SCIENTIFIC PAPERS NOT INCLUDED IN THESIS

I. Sivars L, Landin D, Rizzo M, **Haeggblom L**, Bersani C, Munck-Wikland E, Näsman A, Dalianis T, Marklund L. Human papillomavirus (HPV) is absent in branchial cleft cysts of the neck distinguishing them from HPV positive cystic metastasis. *Acta Otolaryngol. 2018 Sept; 138(9):855-858*

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X.   **Haeggblom L**, Franzén J, Näsman A. Human polyomavirus DNA detection in keratoacanthoma and Spitz naevus: no evidence for a causal role. *J Clin Pathol. 2017 May;70(5):451-453*

XI.   Sivars L, Landin D, **Haeggblom L**, Tertipis N, Grün N, Bersani C, Marklund L, Ghaderi M, Näsman A, Ramqvist T, Nordfors C, Munck-Wikland E, Tani E, Dalianis T. Human papillomavirus DNA detection in fine-needle aspirates as indicator of human papillomavirus-positive oropharyngeal squamous cell carcinoma: A prospective study. *Head Neck, 2017 Mar;39(3):419-426*

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XIII.   Nordfors C, Vlastos, A, Du J, Ährlund-Richter A, Tertipis N, Grün N, Romanitan M, **Haeggblom L**, Roosaar A, Dahllof G, Dona Mg, Benevolo M, Ramqvist T, Munck-Wikland E Dalianis T. Human papillomavirus prevalence is high in oral samples of patients with tonsillar and base of tongue cancer. *Oral Oncol, 2014 May:50(5)491-7*

XIV.   Nordfors C*, Grün N*, **Haeggblom L***, Tertipis N, Sivars L, Mattebo M, Larsson M, Häggström-Nordin E, Tydén T, Ramqvist T And Dalianis T. Oral human papillomavirus (HPV) prevalence in high school students in two municipalities in Sweden. *Scand J Infect Dis. Nov;45(11):878-812013*

XV.   Nordfors C*, Grün N*, Tertipis T*, Ährlund-Richter A, **Haeggblom L**, Sivars L, Du J, Nyberg T, Marklund L, Munck-Wikland E, Näsman A. Ramqvist T And Dalianis T. CD8+ and CD4+ Tumour Infiltrating Lymphocytes in Relation to Human Papillomavirus Status and Clinical Outcome in Tonsillar and Base of Tongue Squamous Cell Carcinoma. *Eur J Cancer. 2013 Jul;49(11):2522-30.*

CONTENTS

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APM	Antigen processing machinery
BOTSCC	Base of tongue squamous cell carcinoma
β_2M	B2-microglobulin
CDK	Cyclin-dependent kinase
CD3$^+$	Cluster of differentiation 3 positive
CD8$^+$	Cluster of differentiation 8 positive
CNA	Copy number alterations
DFS	Disease free survival
DSS	Disease specific survival
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
ER	Endoplasmic reticulum
E1-7	Early regions 1-7
E2F	E2 factor
FDA	Food and Drug Administration
FFPE	Formalin fixed and paraffin embedded
FISH	Fluorescent in situ hybridization
FOXP3	Forkhead box P3
HLA	Human leukocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HPV$^+$	Human papillomavirus positive
HPV$^-$	Human papillomavirus negative
HR	Hazard ratio
IARC	International Agency for Cancer Research
ICD	International classification of diseases
IFN-β	Interferon beta

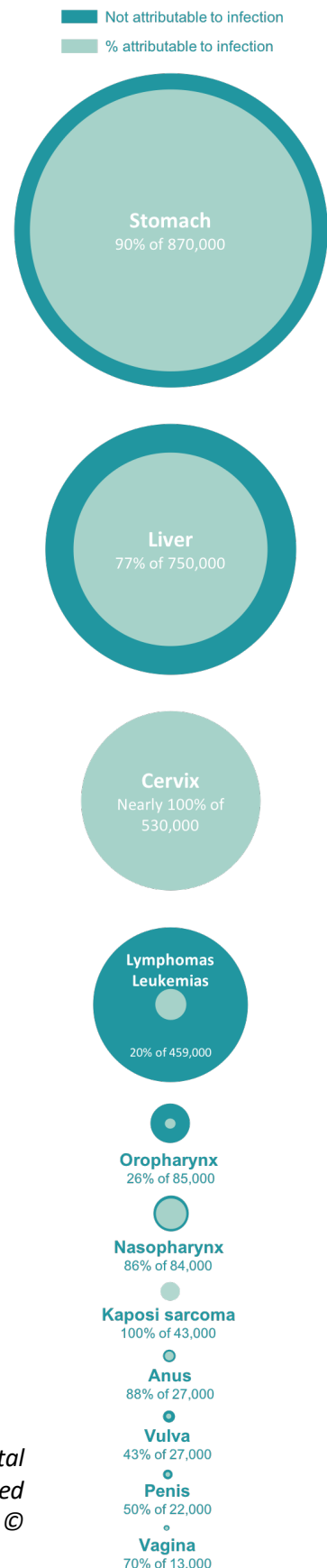|	
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IR	Ionizing radiation
LCR	Long control region
LMP	Large multifunctional protease
L1-2	Late regions 1-2
MHC	Major histocompatibility complex
NPC	Nasopharyngeal cancer
NSTSCC	Non-specified tonsillar squamous cell carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
ORF	Open reading frame
PCR	Polymerase chain reaction
PI	Propidium iodine
pRb	Retinoblastoma protein
p16	$p16^{INK4a}$
p16$^+$	$p16^{INK4a}$-positive
p16$^-$	$p16^{INK4a}$-negative
RRP	Recurrent respiratory papillomatosis
RT-PCR	Real time polymerase chain reaction
STSCC	Specified tonsillar squamous cell carcinoma
TAP	Transporter-associated protein
TIL	Tumor infiltrating lymphocyte
Treg	T regulatory cell
TSCC	Tonsillar squamous cell carcinoma
UICC	Union for International Cancer Control
VLP	Virus-like particle

1 INTRODUCTION

1.1 INFECTIONS IN CANCER

Cancer is one of the most common causes of death in humans, with about 14 million new cancer cases, and 8 million cancer-related deaths in year 2012 alone [1]. Overall, about 16% of all cancers are associated with infections by viruses, bacteria or parasites, where however this can vary 10-fold depending on geographical region and infectious agent [1–3]. In general, this fraction is higher in low-income countries and can often affect a younger population compared to in high-income countries [1–3].

The majority of all cancers caused by pathogens are due to viral infections. Human papillomaviruses (HPV) are a major culprit responsible for > 550 000 cases per year and known to cause cervical cancer and other anogenital cancers such as cancer of the penis, vulva, vagina, and anus, as well as cancer of the head and neck region. Other important human viruses related to cancer development are hepatitis B and C virus that can cause liver cancer, Epstein-Barr virus (EBV) that can cause e.g. lymphomas and nasopharyngeal carcinomas [4–7]. Human T-lymphotropic virus-1 (HTLV-1) associated to adult T-cell leukemia, Kaposi sarcoma herpesvirus (KSHV) causative of Kaposi's sarcoma, and Merkel cell polyomavirus (MCV) which is associated to Merkel cell carcinoma, are other cancer related infectious agents that are not as common as the afore mentioned ones [4–7].

Figure 1. *Proportions of common cancers caused by infections, in total numbers and percentages assumed to be caused by infections, based on global data from 2008. Adapted from the Cancer Atlas. Copyright © 2014 The American Cancer Society, Inc. All rights reserved [5, 8].*

Legend:
- Not attributable to infection
- % attributable to infection

Stomach
90% of 870,000

Liver
77% of 750,000

Cervix
Nearly 100% of 530,000

Lymphomas Leukemias
20% of 459,000

Oropharynx
26% of 85,000

Nasopharynx
86% of 84,000

Kaposi sarcoma
100% of 43,000

Anus
88% of 27,000

Vulva
43% of 27,000

Penis
50% of 22,000

Vagina
70% of 13,000

Infection driven cancers can also be due to bacterial infections, where e.g. *Helicobacter pylori,* is known to cause nearly 90% of all stomach cancer, or parasite infections, such as e.g. *Schistosoma haematobium* that is associated to bladder cancer [1, 5]. In figure 1 the most common cancers caused by infectious agents, according to data from De Martel et al., as adapted from the American Cancer Society and Cancer Atlas, are presented [5, 8].

Of notice is that viral carcinogenesis is a side effect of the induction of cell proliferation by many viruses. In order to increase viral production, viruses often have proteins that induce cells to go from a resting to a proliferative state [9]. This induction of cell proliferation can together with mutations in cellular genes lead to cancer. However, this only occurs in a small minority of infected cells. A frequent misperception is that viruses cause cancer due to increased transmission and viral burden, this is however not true. Only a minimal proportion of infected humans actually develop tumors, and those that do rarely serve as a source of transmission. Transmission of most of these, often very common viruses, is asymptomatic or associated with mild symptoms, although exceptions exist, and most infections do not lead to neoplasia [9, 10].

Very broadly, this thesis will focus on human papillomaviruses and their role in head and neck cancer.

1.2   THE EMERGENCE OF TUMOR VIROLOGY AND HPV DISCOVERY

As early as in 1842 an Italian physician named Rigoni-Stern published a paper where he had studied the death certificates of women from Verona during the period 1760-1839 and noticed that women that had been married, widowed or prostitutes had a much higher incidence of cervical cancer compared to women that had been virgins or nuns. Hence, he concluded that the cancer was related to sexual contact [11].

Moreover, in the early 20th century the scientist Payton Rous performed the famous groundbreaking experiment with hens leading to the discovery of a transmissible avian tumor virus. From a hen with a spindle-cell sarcoma, Rous retrieved the tumor, grinded and filtered it thoroughly through a Berkefeldt filter that would hinder both cells and bacteria to pass through. He thereafter injected the cell-free filtrate into a second hen, which later also developed a similar chest tumor, suggesting that something small enough

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to pass the filter (i.e. a virus) would be the cause of tumor growth. In 1966, Peyton Rous was awarded the Nobel Prize in physiology and medicine, for his discoveries. This was however not the first tumor virus detected, Ellerman and Bang from Denmark, just before Rous, discovered that a filterable extract could transmit leukemia between chickens. However, this discovery received little attention because leukemia was not recognized as a neoplastic disease until many years later [12].

When it comes to human tumor viruses, the first one to be associated with tumor development was Epstein-Barr virus (EBV) named after Michael Anthony Epstein and Yvonne Barr, the discoverers of the virus in 1964. The virus was isolated from Burkitt's lymphoma tumor cell lines [13]. Twenty years later, in 1984, finally the death certificate study by Rigoni-Stern was confirmed by Harald zur Hausen and colleagues, by identifying sexually transmitted high-risk HPVs in a high number of cervical cancer cases, indeed suggesting that HPV is associated to cervical cancer. This finding was rewarded with the Nobel Prize in medicine and physiology in 2008 [11, 14]. Today a total of seven human tumor viruses have been identified, together with a number of tumor viruses identified in other species, and some of these viruses have been shown to cause several different cancer types [7]. Most relevant for this thesis is that the International Agency for Research on Cancer (IARC) in 2007 acknowledged HPV type 16 (HPV16) as a risk factor for carcinogenesis of the cervix, vulva (basaloid and warty tumors), vagina, penis (basaloid and warty tumors), anus, oral cavity and oropharynx [4]. The latter was partly based on e.g. studies by the Dalianis group, which were first to show specific associations between tonsillar and base of tongue cancer and HPV [15, 16], as well as e.g. studies by Gillison et al., showing a correlation between HPV and oropharyngeal cancer [17].

1.3   HUMAN PAPILLOMAVIRUS

1.3.1   Introduction

Human papillomaviruses (HPVs) are small circular double stranded DNA viruses, encapsulated in a non-enveloped icosahedral capsid, that belong to the *Papillomaviridae* family [18]. There are many different HPV types, and so far, more than 200 types have been identified, and this number is still increasing. HPVs are divided into low-risk and high-risk types. The low-risk HPVs do not induce cancer, although some of them have the ability

to produce genital warts or skin warts. There are 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), which have the ability to cause cancer, and some that potentially are high-risk HPV-types, where however more evidence is needed to prove causation. HPV16 is the most common type causing the majority of all HPV related cancers [4]. HPVs are very common pathogens and most people are infected at some point in their life and clear their infections without any symptoms arising. However if a high-risk HPV infection persists over a longer time period there is a risk that the interference of the virus with the host cells may lead to cancer development [19, 20].

1.3.2 Classification

HPVs are divided into five major genera: alpha-papillomavirus, beta-papillomavirus, gamma-papillomavirus, mu-papillomavirus and nu-papillomavirus.
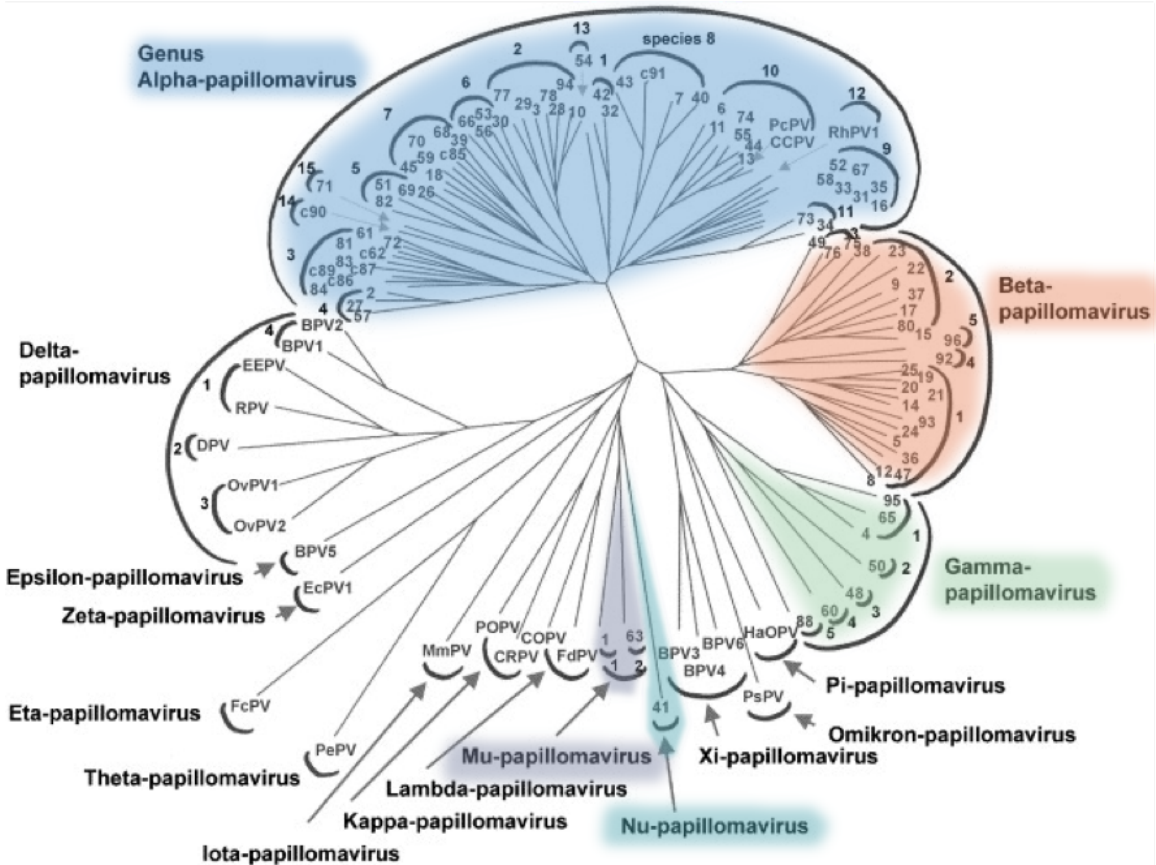

Figure 2. *Phylogenic tree containing the sequence of 118 different papillomavirus types. The colored clusters are the different HPV genera and the numbers at the ends of each branch identify each HPV type, and c-numbers refer to candidate HPV types. Remaining abbreviations refer to animal papillomavirus types. Adapted from Elsevier: Virology, de Villiers et al., 2004 [21].*

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HPV classification is based on the nucleotide sequence of the open reading frame (ORF) coding for the L1 capsid protein [20, 21]. Between different genera less than 60% of the L1 region sequence is the same. Within the same genus there are different species that share between 60%-70% nucleotide identity. Furthermore, within a species the HPVs are divided into types, where they share between 71-89% nucleotide identity, while if having above 89% nucleotide identity, two assumed different HPV types would instead be classified as belonging to the same type [20, 21]. New HPV types are given a unique number after the genome has been sequenced and studied, and they are then registered with the International HPV Reference Center, which, is since 2012 situated at the Karolinska Institute in Sweden, after previously having been established in Germany [20].

The different genera present with various behaviors and are associated with different diseases, affecting either mucosal or cutaneous epithelia. The alpha-papillomaviruses are known to cause both cutaneous and mucosal lesions in humans and primates and both high-risk and low-risk HPV types fall into this genus. Notably, HPV16, the most common cancer associated HPV type, is an alpha-papillomavirus. The beta-papillomaviruses can cause cutaneous lesions in humans, yet mainly exist in a latent form in the general population and can become symptomatic during immune suppression. Gamma-, mu-, and nu-papillomavirus genera also cause cutaneous lesions in humans [21]. Presented in figure 2, as adapted from de Villiers et al. [21], is a phylogenic tree of 118 different papillomavirus types, where also papillomavirus genera that infect other species are illustrated. All types that infect humans are presented with a number, whereas remaining types are known to infect other animals.

1.3.3 The genome and viral life cycle of HPV

1.3.3.1 The HPV genome

The HPV genome is built up by double-stranded circular DNA of roughly 8000 base-pairs and consists of an early and a late region as well as a long non-coding control region. The early region contains six proteins (E1, E2, E4, E5, E6 and E7), which are mainly involved in various functions of the viral life cycle, and the late region coding for two proteins (L1 and L2), which are structural proteins coding for the major capsid (L1) and the minor capsid (L2) [4]. An illustration of the viral genome of HPV16, and a model of the viral particle, are presented in figure 3A and 3B, respectively.
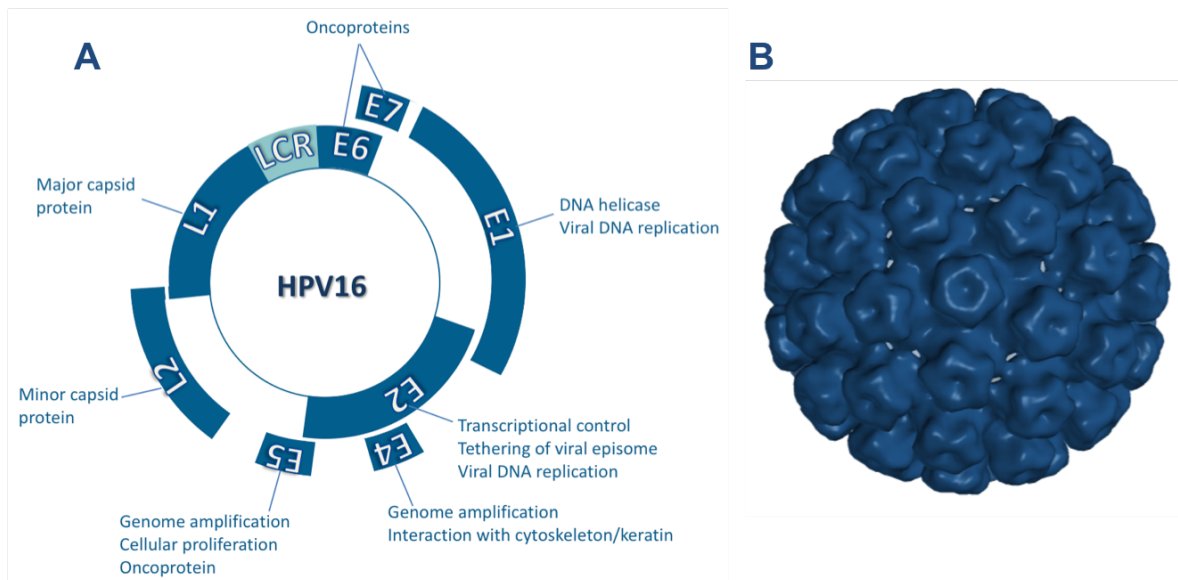
Figure 3. A) Viral genome of HPV16. B) Illustration of the HPV viral particle.

1.3.3.2 The viral life cycle

The viral life cycle is assumed to start when the virus, via a microlesion in the skin or mucosa gets access to and infects the basal layer of epithelial cells. Exactly how the virus enters the cell is not entirely known. Experimental models suggest however, that the two capsid proteins L1 and L2 access the basal lamina, and there interact with heparin sulphate proteoglycans, and possibly also laminin, resulting in a structural change in the viral capsid, facilitating binding to a secondary receptor on basal keratinocytes [22]. This is then followed by viral internalization and subsequent transport of the viral genome into the host nucleus. In the nucleus viral DNA can be found in an episomal form (non-integrated), which is most common during infections without cancer, or in an integrated form (integrated into the host genome), which is mainly associated to HPV induced cancer [23]. Studies also suggest that it is during wound healing, i.e. active cell division, that the virus genome actually is able to get incorporated into the cell nucleus. Once the virion is internalized it undergoes endosomal transport and uncoating. Thereafter the viral life cycle is tightly linked to the maturation of the infected keratinocytes [22, 24].

During the establishment of the infection, the initial viral DNA replication in the host cell starts and will increase the number of viral DNA copies to 10-100 copies per cell [25]. During the maintenance phase the viral copy number is controlled and remains stable around 10-100 copies per cell, both during cellular differentiation and proliferation.

Finally, in the differentiated epithelium, the viral genome will be amplified to around 1000 copies per cell, these are then encapsulated by L1 and L2 prior to shedding of the viral particles from the cell [25, 26]. The viral life cycle of HPV16 is displayed in figure 4, as adapted from Doorbar et al. [22].
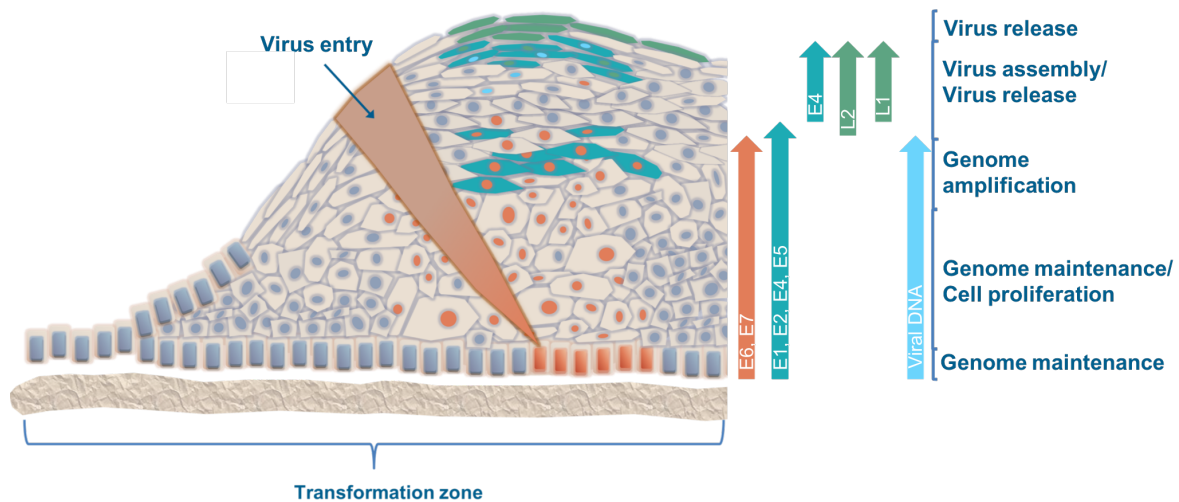

Figure 4. *The viral life cycle of high-risk HPV16 and the different phases of viral protein expression. Adapted from Doorbar et al. [22].*

1.3.3.3   E1 and E2 viral proteins

E1 and E2 are the two most important viral proteins during early infection and are involved in initial replication for establishing an infection, followed by controlling the infection during the maintenance phase [27]. E2 has one DNA-binding and one protein-binding domain connected by a flexible hinge region and can form a homodimer that can bind to four sites of the long control region (LCR). Three of these sites are located next to the origin of replication and are necessary for E1-activated viral replication. E2 binds to E1 and together they form a dimer that binds to the viral origin of replication, and thereby the host cell's DNA replication machinery is recruited [27, 28].

1.3.3.4   E6 and E7 viral proteins

The HPV genome has the ability to remain episomal in the cells, or become integrated into the host genome, the latter most often being a random event that sometimes can lead to the loss or disruption of viral genes needed for transcription [22]. Among these, E2 is the most important viral gene encoding a transcription factor, which is the key regulator and inhibitor of E6/E7 abundance [28]. E6 and E7 together are in high-risk HPV types regarded

as oncogenes. They are important for abrogating normal cell cycle control and pushing the cells to proliferate through the binding of E6 to p53 and the binding of E7 to retinoblastoma protein (pRb) [29]. The role of E6 and E7 in cell transformation and carcinogenesis is described in more detail below in section 1.3.5. In e.g. cervical cancer the majority of cancer cases show HPV integration into the host chromosome, where the viral integration site often lies within the E1 and E2 genes. Loss of functional E6/E7 regulation by E2 leads to a persistent expression at a high level of these genes and is also the reason for accumulation of genetic errors that eventually may lead to the development of cancer [22]. In head and neck cancer, integration of HPV and disruption of regulatory E1 and E2 genes has also been reported, however different studies show different numbers on how common this event is in the sub-sites of this cancer type [26, 30].

1.3.3.5 *E4 viral protein*

E4 is a late protein expressed from the early region of the genome and is expressed at high levels forming several E4-derived proteins. E4 has the ability to associate to and disrupt the cytoplasmic keratin network, however the biological importance of this function is yet to be determined. Nonetheless, several studies suggest that E4 is involved in viral release and that it could be involved in cell cycle arrest, and thereby virion amplification success [31, 32].

1.3.3.6 *E5 viral protein*

E5 is suggested to indirectly contribute to genome amplification by changing the cellular environment. E5 is a transmembrane protein containing a cytoplasmic C-terminus, and is assumed to be able to form pores, to interfere with apoptosis, as well as the intracellular trafficking of endocytic vesicles [22, 33]. E5 has also been suggested to be an oncoprotein, and its role in carcinogenesis is discussed in more detail in section 1.3.5.3 below.

1.3.4 Transmission

The transmission route of HPV depends on the tropism of the specific HPV type. Transmission of cutaneous HPV infections, e.g. HPV1, mainly occurs through skin-to-skin contact, or through skin to surface contact. Cervical infections and other anogenital infections are mainly transmitted through sexual intercourse, or other mucosa-to-mucosa

contact. Oral infections have not been studied to such a great extent, but are also assumed to be transmitted through sexual contact, and possibly also through kissing [34, 35].

All transmissions have in common that the virus enters through epithelium microlesions and infects the underlying receptive basal cells [36]. The risk of acquiring an anogenital or oral infection is closely related to sexual activity and increases with e.g. high numbers of sexual partners and early sexual debut. Although use of barrier methods, such as condoms, will protect from infection to some extent, protection is not guaranteed since the virus can still be transmitted through contact with non-preserved infected areas.

A few studies also found vertical transmission from mother to child. This has been suggested to occur by prenatal transmission during pregnancy, or perinatal transmission during or immediately after birth through contact with the infected cells of the cervix and vagina [37, 38]. Pre-conceptual transmission during or immediately after fertilization of an oocyte, where HPV can be transmitted via sperms has also been suggested [39].

1.3.5 Carcinogenesis

The E6 and E7 proteins are the main proteins involved in cellular transformation and carcinogenesis caused by high-risk HPVs. These proteins have been extensively studied, mainly in cervical cancer, and many studies have shown that the severity of cervical lesions is correlated to the frequency of HPV integration, where a high number of integrations are suggested to lead to high-grade dysplastic lesions [29]. E6 and E7 are involved in deregulating a number of fundamental cellular events such as the cell cycle, apoptosis, senesces, DNA repair, and differentiation, leading to an accumulation of DNA damage and eventually cancer development. These processes will be described in more detail in following sections and in figure 5. If the immune system successfully eliminates the HPV-infected cells, despite the transforming properties of E6 and E7, there will not be enough time for accumulation of chromosomal abnormalities sufficient to cause a malignancy, and therefore most HPV-infections do not actually lead to tumor formation [29, 40]. Immunosuppressed individuals consequently have a higher risk of contracting HPV associated lesions (e.g. warts) and cancers [41].
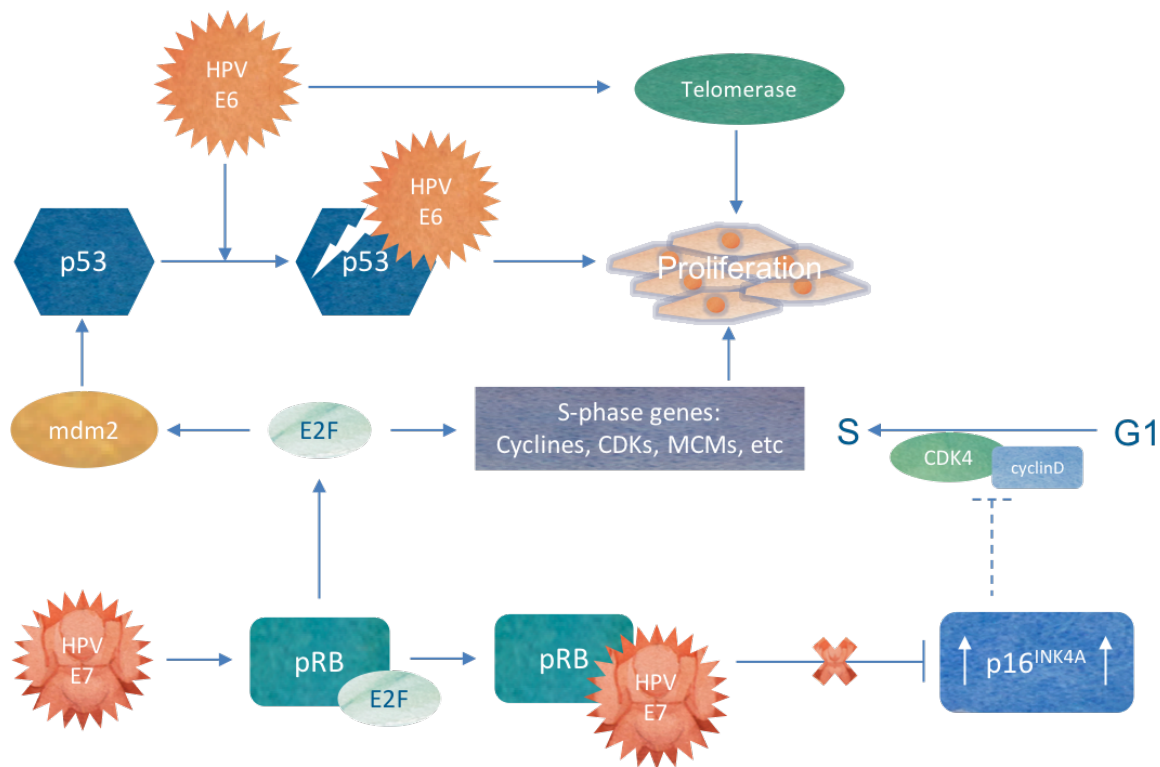
Figure 5. *High-risk HPV oncogenes E6 and E7 and their involvement in carcinogenesis.*

1.3.5.1 Viral protein E6 in carcinogenesis and its interaction with p53

E6 contributes to carcinogenesis by a very well-established mechanism, using the ability to cause degradation of the tumor suppressor protein p53 by the ubiquitin pathway [29, 42]. p53 is referred to as the guardian of the genome due to its important role in regulating the expression of genes encoding regulators of the DNA repair machinery, cell cycle, and apoptosis [43]. During cellular stress, p53 will trigger cell cycle arrest to allow time for DNA damage repair, or will trigger apoptosis in case the damage is too great, in order to ensure the integrity of the genome. Hence, E6 interference with p53 leads to uncontrolled proliferation of cells [29, 42]. In addition, in some high-risk, and to a lesser extent in some low-risk HPV types E6 is able to directly inhibit the expression of p53 regulated genes, such as the transcriptional co-activators CREB binding protein (CBP) and p300 [29]. Moreover, E6 and E6 associated protein (E6AP) interaction is involved in other HPV related events, such as transcriptional activation of the *hTERT* (human telomerase reverse transcriptase) gene, which encodes the catalytic subunit of the telomerase complex (figure 5) [29, 44].

1.3.5.2 *Viral protein E7 in carcinogenesis and its interaction with pRb and introduction of p16^{INK4a}*

The E7 protein has the ability to bind the tumor suppressor retinoblastoma protein (pRb) and its related proteins p130 and p107, which are proteins involved in cell cycle control [29, 45]. The function of pRb is to negatively regulate the proteins of the E2F family (E2F1-3), thereby keeping the cells in a quiescent state during the G0/G1 phase of the cell cycle. Binding of high-risk HPV E7 protein with pRb will lead to the degradation of pRb, which in turn will lead to an activation of E2F-regulated transcription. E2F1-3 regulate cyclin A and cyclin E, which are the positive regulatory subunits of cyclin-dependent kinase (CDK) complexes, and the accumulation of cyclins will lead to the activation of CDKs and cell cycle progression (figure 5). It has also been shown that E7 can directly bind some CDK inhibitors, leading to the disruption of their inhibitory function on the cell cycle [29, 45].

As an indirect effect of E7 interference with pRb, the tumor suppressor p16^{Ink4A} (p16) is upregulated. This is common in most HPV positive tumors, and therefore overexpression of p16 is often used as a surrogate marker for HPV infection. p16 normally functions as an inhibitor of the cdk4/6-cyclin D complex and is expressed in order to control cell replication and promote G1 growth arrest. When pRb is degraded due to E7 binding, p16 becomes upregulated and overexpressed, but will still not succeed in promoting growth arrest [46, 47]. It has also been suggested that p16 in HPV positive cells can be upregulated via another pathway independent of pRb interactions, since e.g. HPV16 E7 has also been shown to induce histone demethylase KDM6B (lysine demethylase 6B), which in turn leads to upregulation of p16 [48].

Of importance for this thesis, is that E7 has been proposed to have immune modulating functions. Expression of E7 has been associated to downregulation of major histocompatibility complex (MHC) class I expression, leading to a decrease in peptide presentation and cytotoxic killing. This is considered as a mechanism of immune escape [49, 50].

1.3.5.3 Viral protein E5 in carcinogenesis and its role in regulating molecules of the major histocompatibility complex

E5 in high-risk HPVs is suggested to be an oncoprotein due to its capabilities of interfering with classical MHC molecules. Thereby stopping them from occurring on the cell surface, and compromising the presentation of viral peptides of the infected cell [22]. It has been suggested to downregulate MHC class I (human leukocyte antigen (HLA) class I, in humans) by accumulation of MHC class I in the Golgi apparatus [51, 52]. The accumulation of MHC class I may be due to a lack of acidification in the Golgi apparatus and/or due to direct interactions between E5 and the heavy chain of HLA class I [51–53]. This would prevent MHC class I from translocating to the cell surface, leading to immune escape by avoiding clearance by e.g. T cells. Another study reported that E5 may have the ability to downregulate MHC class II in keratinocytes that have been treated with interferon gamma (IFN-γ) [54]. Finally, HPV E5 has also been shown to be capable of modulating epidermal growth factor receptor (EGFR) expression [55, 56].

1.3.6 HPV prevalence in the general population

Many HPV types are very common viruses, and as already mentioned, most infections do not actually lead to cancer. HPV has, depending on type, the ability to infect both mucosal and cutaneous cells of different parts of the body, however the following section will focus only on oral and cervical infections of the general population.

In a meta-analysis by Tam et al., the authors presented oral HPV infection data from sixty-six different studies, mainly from European and North American cohorts, yet remaining continents were also represented [57]. The overall oral HPV prevalence for all included HPV types was 7.7% and for high-risk HPV16 1.4%, and the incidence was 4.38 cases per 1000 person-months for all HPVs and 0.92 cases per 1000 person-months for HPV16. The authors also report that the oral HPV prevalence was higher in men (9.3%) compared to women (5.5%) and that the prevalence varied depending on geographical region. This meta-analysis excluded all studies, where only study subjects <18 year of age were included, however it did not report HPV prevalence differences per age group [57].

Similarly, in a study of oral HPV prevalence in the civilian US population with a majority of non-vaccinated individuals, Gillison et al. demonstrate an oral HPV prevalence of 6.9% in

men and women aged 14-69 years [58]. Interestingly, they also show a bi-modal pattern with two oral HPV prevalence peaks in mainly two different age groups. The first peak, was 7.3%, in individuals 30-34 years of age, and the second peak, was 11.4%, in individuals 60-64 years of age. Moreover, they show that oral HPV prevalence was lower in females compared to males, with 3.6% vs. 10.1% respectively [58].

Several studies performed by the Dalianis group report on oral HPV prevalence in youth (a population where HPV infections are very common) before and after the introduction of vaccination and catch-up vaccination against HPV in young girls and women [59–61]. Youth ranging between the ages of 15-23, visiting a youth clinic in Stockholm between 2009-2011, had an even higher oral HPV prevalence compared to the meta-analysis by Tam et al. just described, with a prevalence of 9.3%, with a similar prevalence in both males and females [59]. These study subjects had not yet been vaccinated against HPV and the risk of having an oral HPV infection was significantly higher if also presenting with a genital HPV infection [59]. In two follow up studies, including study subjects visiting the same youth clinic between 2013-2015, a great reduction in oral HPV prevalence was observed with now only 1.5% being HPV infected. Here 71% of the study subjects reported being catch-up vaccinated for HPV, yet not necessarily before sexual debut [60, 61].

The three last mentioned studies performed on subjects from a youth clinic in Stockholm, also presented cervical HPV infection data [59–61]. Cervical infection was much more common in this group of patients, compared to oral infection, and 74.1% of all girls/young women presented with a cervical HPV infection in the pre-vaccination study [59]. In the follow-up studies (2013-2015) cervical HPV infections were common both in the HPV catch-up vaccinated group (64.6%), and the non-vaccinated group (74.5%) [61]. However, the HPV catch-up vaccinated group showed significantly lower numbers of the two high-risk HPV types included in the vaccines compared to the non-vaccinated group [61]. HPV16 prevalence in the pre-vaccination study (2009-2011) was 35%, in the non-vaccinated group as compared to 5% in the HPV catch-up vaccinated group (2013-2015) and 18% among those that were not vaccinated during the same period [59–61]. The latter drop was likely due to herd immunity. A similar trend was observed for HPV18 prevalence, with 10% in the pre-vaccination study; as compared to 1% in the HPV catch-up vaccinated group and 4% in the non-vaccinated group during the later period. Whereas

prevalence of the HPV types not included in the vaccines have not changed much between the different time periods. This suggests that HPV catch-up vaccination has already had an effect on HPV prevalence among young people in Stockholm, both among the vaccinated population as well as, to a lesser extent, on the non-vaccinated population due to herd immunity [59–61].

In a meta-analysis studying worldwide HPV prevalence among women with normal cervical cytology, Bruni et al. in 2010, present that worldwide prevalence was 11.7% [62]. This study included studies published between 1995 and 2009, and hence the prevalence may have changed a bit since then. In this study Sub-Saharan Africa (24.0%), Eastern Europe (21.4%), and Latin America (16.1%) presented with the highest prevalence, whereas Western Asia (1.7%), Northern America (4.8%), and Southern Asia (7.1%) had the lowest prevalence. The prevalence for northern Europe was in this study 10%, which is quite a bit lower than that presented at the youth clinic from Stockholm. On the other hand, this meta-analysis showed a clear difference in regard to HPV prevalence in different age groups, where cervical HPV prevalence by far was highest in the youngest age group (<25 years) with a prevalence of 24%, and the least prevalent group was women 45-54 years of age (4.2%) [62].

As noted from the above, there is quite a large difference in HPV prevalence between the cervical and oral sites. The fact that HPV prevalence, and also the number of HPV copies is lower in the oral site as compared to the cervical site, could very much be due to that 0.5-1.5 liters of saliva are produced in the oral cavity per day [63].

It is important to follow the HPV prevalence of especially the oral and genital sites, in order to understand the possible future cancer burden and how the implementation of HPV vaccination will affect HPV prevalence in the population. In section 1.3.8.1 vaccination against HPV will be discussed in more detail.

1.3.7 HPV associated diseases

HPV has been associated with several different diseases, where cancer is the most severe outcome. In the following section the most common known HPV related diseases will be mentioned, but since head and neck cancer, or more specifically oropharyngeal cancer, is

a main part of this thesis this topic deserves its own chapter and will be mainly focused on in chapter 1.4.

1.3.7.1 Cervical cancer

Cervical cancer has been well established to be caused by HPV, and >99% of all cases have been associated to HPV infection, although other factors may have contributed to cancer formation [64, 65]. The most common HPV types associated to cervical cancer are HPV16, accounting for about half of all cervical cancer cases in Europe and the US, with HPV18, 31 and 45 accounting for another 25-30% of cervical cancer cases [65]. Cervical cancer is the fourth most common cancer in women and reports estimate 570,000 new cervical cancer cases in 2018, which is 6.6% of all female cancers. Moreover, 90% of all deaths due to cervical cancer occur in low and middle income countries, meaning that education, early diagnosis, prevention, effective screening methods and treatment need to be further improved or implemented in these countries in order to reduce the number of deaths [66]. Ultimately HPV vaccination will presumably prevent the vast majority of cervical cancer, but most likely it will take a long time before vaccination coverage in all areas will be high enough to protect women in the most vulnerable areas. This will also to a high degree depend on the attitudes towards HPV vaccination in the general population in different countries.

1.3.7.2 Other anogenital cancers

Not all genital cancers caused by HPV are limited to the cervix, other anogenital cancers include cancer of the vulva, vagina, anus and penis. In a report from 2017 by Martel et al. [6], the world-wide numbers of HPV related cancers from 2012 were presented, and 8,500 new vulvar, 12,000 vaginal, 35,000 anal (half occurring in men) and 13,000 penile cancer cases were assumed to be caused by HPV. The percentage of HPV related cancers is estimated to be 25% for vulvar, 78% for vaginal, 88% for anal, and 50% for penile cancer [6]. The most common HPV type in both vulvar, vaginal, anal and penile cancer is HPV16 [67–69]. Although there are screening methods for cervical cancer, there are no implemented screening methods for the other anogenital sites mentioned in this section [70, 71].

1.3.7.3 Head and neck cancers

Head and neck cancers are a group of cancers that affect the following sites: the oropharynx, the oral cavity, the hypopharynx, the larynx, the epipharynx, the salivary glands, the lips and the nasal and sinus cavities, and therefore include a very diverse group of cancers [72].

It has been suggested that head and neck cancer can be divided into three genetic subgroups: tumors that contain transcriptionally active HPV, tumors that are HPV-negative (HPV$^-$) and have numerous copy number alterations (CNA)-high, and tumors that are HPV$^-$ but CNA-silent [73]. The HPV related tumors have been suggested to divide further into two distinct sub-groups: HPV-KRT and HPV-IMU. What characterizes these sub-groups is that, HPV-KRT has an increased expression of genes in keratinocyte differentiation, and in the oxidation-reduction process. HPV-IMU instead has a strong immune response, and mesenchymal differentiation [74]. Many cancer genes and pathways are likely involved in the progression of HPV$^-$ CNA-high tumors, where likely smoking is the main risk factor. Lastly, the CNA-silent group of tumors still have active p53 and pRb, as opposed to the previously described groups. However, the etiology of CNA-silent tumors remains unclear, yet aging is hypothesized to be a risk factor [73].

The incidence of head and neck cancer is more than 650 000 new cases each year, with a mortality rate of about 400 000 cases each year, making it a quite common cancer type with a poor prognosis [1, 75]. The main risk factors for head and neck cancers are smoking and excessive alcohol consumption, however in 2007, the International Agency for Cancer Research (IACR) announced that HPV also is a strong risk factor for some head and neck cancers. The by far most common HPV type associated to head and neck cancer, is HPV16, and some other observed HPV types are e.g. HPV31, 33, 35, 56, 58, 18 [76, 77]. HPV driven cancers within the head and neck region, are mainly found in the oropharynx, and more specifically in the tonsils and the base of tongue, where in Sweden about 70% are HPV positive (HPV$^+$) [78, 79]. Nasopharyngeal cancers (NPC) are known to be associated with Epstein-Barr virus infection [7]. However, NPC, has also been linked to high-risk HPV infection, where similarly to OPSCCs, HPV related NPCs most frequently present with nonkeratinizing histology [80, 81].

Worldwide HPV related head and neck cancers are estimated to 38,000 new cases per year, of which 29,000 (76%) are oropharyngeal cancers [6]. More details about oropharyngeal, tonsillar, and base of tongue cancer are found in chapter 1.4.

1.3.7.4 Warts

Warts are benign lesions that occur in the skin and mucosa and are caused by HPV infection. Although these lesions are benign, they can be uncomfortable and may need medical attention [82]. External genital warts (condyloma acuminata) are extremely common and are estimated to affect 500,000 to 1,000,000 individuals in the United States alone, each year. This means that approximately 1% of all sexually active individuals present with genital warts some time during their life time [82]. Non-genital warts (verrucas) are also extremely common, and affect the epithelial cells of mainly hands and feet, and are most frequently observed in children and teenagers [83]. The most common HPV types associated to genital warts are the low-risk types HPV6 and 11, causing about 90% of all genital warts. Warts are mainly treated by elimination of the growth, rather than treatment of the underlying infection, which unfortunately often results in recurrence within a few months [82].

1.3.7.5 Recurrent respiratory papillomatosis

Recurrent respiratory papillomatosis (RRP), is a rare disease, characterized by benign lesions that form along the upper aerodigestive tract [84]. As with genital warts, HPV6 and 11 are the most common types involved in this disease. RRP is a very unpredictable disease, where it at times presents with only mild symptoms or spontaneous remission, but also can present an aggressive disease with pulmonary spread, needing frequent surgical procedures in order to prevent airway obstruction [85]. Donne et al. show in a publication from 2016 that 1.42 per 100,000 individuals in the UK are affected by RRP, which is similar to the Armstrong et al. report (1.8 per 100,000 adults) back in 1995 [86, 87]. Armstrong et al. however also report a higher incidence among children (4.3 per 100,000), which often present with more severe RRP symptoms compared to adults [87].

1.3.8 Prevention of HPV related diseases

1.3.8.1 HPV vaccines

Since 2006, prophylactic vaccination against HPV has been available in Sweden, as well as in the US. Back then, the two available vaccines were Gardasil (Merck & Co.) [88], which was approved by the European Medicines Agency (EMA) and Food and Drug Administration (FDA) in 2006, and Cervarix (GlaxoSmithKline) [89], approved by EMA in 2007 and by the FDA in 2009. More recently, Gardasil9 (Merck & Co.) [90], was approved in 2014 by the FDA, and in 2015 by EMA. All three vaccines are made up of synthetically manufactured virus-like particles (VLPs) of the L1 epitope, yet differ in antigenic load, and adjuvant load and efficacy [88–90]. Cervarix is a bivalent vaccine offering protection against high-risk HPV types 16 and 18 [89], the most common HPV types found in cervical cancer, as does the quadrivalent vaccine Gardasil [88], which in addition protects against low-risk HPV types 6 and 11, the most common HPV types causing anogenital warts. Gardasil9, which is a nine-valent vaccine, protects against the following five HPV types: HPV31, 33, 45, 52, and 58, in addition to the four in Gardasil [90]. All three vaccines are suggested to be administered from 9 up to 25-26 years of age, and can be used in both girls and boys, although Cervarix is marketed for girls only [88–91].

In Sweden, HPV vaccination of 10-12 year old girls was introduced into the general vaccination program in 2010, and in 2012 HPV vaccination was administered through the school based vaccination program and as catch-up vaccination for women up to 20-26 years of age [92]. Today worldwide, over 80 countries have introduced HPV vaccination of girls in their general vaccination programs. Not surprisingly, these countries are mainly upper-middle income countries, and countries with the highest cervical cancer burdens unfortunately have not started with HPV vaccination programs [92, 93]. Several countries including Australia, Argentina, Austria, Canada, Denmark, and USA are examples that have also included/will include boys in their vaccination programs [92]. This will contribute to the prevention of HPV spreading, and decrease in cervical cancers, as well as other HPV-caused diseases including those that also affect men. The Public Health Agency of Sweden, has recommended the vaccination of boys, however so far unfortunately no decision has been made by the government to proceed accordingly [92].

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Gardasil and Cervarix provide protection against HPV HPV16 and 18 infections, and in countries with at least a 50% vaccination coverage of females, HPV16 and 18 have decreased by around 68% between the pre- and post-vaccination periods [93, 94]. In some countries, unfortunately the uptake has gone down because of reduced confidence in the safety of these vaccines [95]. In a recent review the authors have summarized the safety profile of 109 HPV safety studies, of which 15 were population-based studies, including in total over 2.5 million vaccinated individuals from six different countries [95]. All the studies presented an acceptable safety profile, where the injection site reactions were slightly more common with Gardasil9, as compared to Gardasil. They conclude that the benefits overweigh the risks from HPV vaccination [95]. Some of the more common side effects are injection-site swelling, injection-site pain, injection-site erythema and headache [88–90].

It takes many years from infection with HPV until actual cancer development occurs, therefore, so far it has not been possible to conduct efficacy studies with regard to the prevention of cancer. However, studies have shown reductions in precancerous lesions, providing reliable evidence for a likely reduction in cancer as well [96]. Whether HPV vaccines protect against oropharyngeal cancers is not clear, and since these cancers rarely present with precancerous lesions, there is so far no other way of measuring efficacy except for looking at oral infection rates. As mentioned in section 1.3.6, in two studies by Grün et al., oral HPV16 and 18 infections have been reduced after introduction of HPV vaccines [60, 61]. A larger study performed on 2,627 men and women 18-33 years of age, confirmed these results by showing an estimated 88.2% reduction in oral HPV prevalence of HPV16/18/6/11 in the vaccinated compared to the non-vaccinated groups [97]. These findings, suggest that the vaccine will also protect against HPV-related cancers of the oral cavity.

1.3.8.2 Screening

Screening programs for detection of pre-cancerous lesions in the cervix are well established and have reduced the cancer burden significantly [98]. Even though there now are vaccination programs implemented in many countries, it is still important to continue the screenings, because not all cancer-causing HPV types are covered by vaccination, and a large proportion of the population has not been vaccinated.

Commonly two screening methods have been used, conventional (pap-smears) and liquid-based cytology, where the latter is more common nowadays. Cells are obtained from the neck of the cervix and either spread out on a glass slide (conventional) or added into a small glass vial of preservative liquid (liquid-based) [98, 99]. This is an effective method, yet unfortunately has a high-risk of false positives. To compensate for this, HPV testing has been more and more implemented for detection of 13 high-risk HPV types. The sensitivity is much greater, however, there is a slight decrease in specificity. Moreover, HPV testing should only be used in women over 30 years of age, since the younger population have a high prevalence of transient HPV infections, yet a low prevalence of underlying high-grade lesions, which would lead to unnecessary treatments. Co-testing with both Pap-smear and HPV testing is most effective and has mainly been implemented in screening of women from 30 years of age. Younger females are in general offered Pap smears every 3rd year [98, 99].

There are no screening programs in place for HPV related cancers outside of the cervix uteri. However, there is ongoing research for early detection of head and neck cancers through the detection of early circulating antibodies towards HPV. A study by Kreimer et al. showed that 34.8% of patients were HPV16 E6 seropositive on average 6 years before diagnosis of an oropharyngeal cancer [100]. Moreover, screening of oral HPV infections may not be useful, since the detection signals for HPV in the oral cavity can be quite low, likely due to saliva production [101]. It has however, been shown that patients with HPV+ tonsillar and base of tongue cancer, often have much higher oral HPV signals [102].

1.3.9 HPV detection methods

There are several different ways of determining whether a sample is HPV positive (HPV+) or not. In this section, both direct methods (detection of viral DNA or RNA) and indirect methods (detection of serum antibodies against HPV, or proteins affected by the HPV infection) will be presented. Notably, the sample material to be analyzed plays an important role in what detection method is most optimal. Common types of sample materials for HPV testing are e.g. fresh, fresh frozen, or paraffin embedded tissues [103].

1.3.9.1 Detection of viral DNA or RNA

This is a direct method of HPV detection, where different polymerase chain reactions (PCRs) can be applied in order to detect viral HPV DNA or RNA. Most often this is applied in a way where the PCR product is visualized in order to determined HPV positivity. Most often so called general primers are used for the PCR reaction, which means that these primers ligate with highly conserved regions of the HPV genome, consequently being able to bind many different types of HPVs having this region in common [104]. Two common primer pairs are GP5+/GP6+ and MY09/MY11 that ligate with the L1 region [105, 106]. CPI/CDIIG is another primer pair that instead binds to the E1 region [107]. A drawback with these general primers is that they, due to mismatches in the primer sequence, amplify some HPV types better, whereas other types require a lot of viral copies to be detected. A way to overcome this is to use a combination of primers with some sequence variation, or a combination of different general primers (broad-spectrum GP5+/6+) as described by Schmitt et al. [108]. In the Luminex method used in some studies included in this thesis and described below, and in more detail in section 3.2.2, both a combination of broad-spectrum GP5+/GP6+ primers and several specific primers were used.

Following DNA amplification there are different ways of visualizing the possible HPV DNA product, and earlier this was often done by gel electrophoresis [46]. Fortunately today there are more efficient techniques and one technique, is a semi-quantitative probe-based method, utilizing Luminex technologies on a Magpix instrument, which will be described in more detail in section 3.2.2 [109, 110].

In order to ensure an active HPV infection, instead of DNA, often RNA from E6 and E7 is measured by e.g. hybrid capture or by cDNA synthesis followed by PCR amplification [110].

In situ hybridization is also a commonly used method, where a biotinylated probe hybridizes to the viral DNA, and the signal is then amplified for detection. The presence and location of the viral DNA can thereafter be evaluated by light microscopy [111].

1.3.9.2 Detection of p16^{INK4a} overexpression

As previously described above p16^{INK4a} (p16) overexpression is a result of the viral protein E7 interference and degradation of pRb, thus p16 expression often is used as a surrogate marker for HPV [46, 47]. By immunohistochemistry (IHC) using an antibody against p16, its overexpression can easily be determined. In general, it is very simple to determine p16-positivity, since the tumor in most cases is either 100% stained for p16, or not stained at all. In the literature, when using p16 overexpression as a surrogate marker, an arbitrary value of >70% stained tumor is considered as p16 positive, as well as HPV[+], in oropharyngeal cancer [112]. Since IHC and the evaluation of p16 is a very simple, cost-effective and a clinically friendly method, it is a popular way of determining HPV positivity. This method has however some downsides in regard to specificity for HPV driven tumors, when used without supporting methods [113, 114]. This will be discussed more in section 1.4.3.2. On the other hand, p16 overexpression combined with the presence of HPV DNA has been accepted as a robust way of determining HPV positivity [115].

1.3.9.3 Detection of serum antibodies against HPV

Serology is an indirect way of determining possible HPV infection. Here antibodies against the viral capsid proteins L1 or L2, or the oncoproteins E6 or E7 commonly are tested for. This method does however not provide tissue specificity and does not indicate whether the subject has an ongoing or previous infection [116]. However, as mentioned above, it has been shown that many patients with HPV[+] oropharyngeal cancer, present antibody responses against e.g. HPV16, more than a decade before the presentation of their oropharyngeal cancers [100]

1.4 OROPHARYNGEAL CANCER

Oropharyngeal squamous cell carcinomas (OPSCC) make up about 25% of head and neck cancers in Sweden [72]. Between 2008 and 2012, 1620 new OPSCC cases were reported, making up about 325 new cases per year [72]. A common first symptom for OPSCC is the discovery of a lump on the neck, often when diagnosed at a late stage. Other common symptoms are e.g. trouble swallowing, sore throat, and pain [117]. Risk factors for developing OPSCC are smoking or excessive alcohol consumption. Since 2007, HPV has also been acknowledged as a main risk factor for these tumors [4].

1.4.1 Anatomy and histology of the oropharynx

The oropharynx is located in the middle part of the pharynx behind the oral cavity and is covered by squamous cell epithelium. The anatomical sub-sites of the oropharynx are 1) the tonsils 2) the base of tongue, 3) the pharyngeal walls, and 4) the soft palate, including the uvula. Roughly the oropharynx can be divided into two categories: the "lymphoepithelial" sites including the tonsils and base of tongue, and the "non-lymphoepithelial" sites including the pharyngeal walls and soft palate. The location and anatomy of the oropharynx is illustrated in figure 6.

The pharyngeal walls and soft palate contain non-keratinized stratified squamous epithelium, with a supportive underlying lamina propria as well as muscular layer. The tonsils (palatine tonsils, lingual tonsils, adenoid tonsils, tubal tonsils and tonsillar pillars) and base of tongue together form a ring-like structure that often is referred to as the Waldeyer's ring, a region rich in lymphoid tissue [35, 118, 119]. Moreover, this region is also characterized by that its reticulated epithelium invaginates and merges with the underlying lymphoid tissue thereby forming crypts, which especially is observed in the tonsils, containing up to 30 crypts per tonsil, creating a large surface area [35]. The luminal surface of the crypts is fairly thin, allowing the lymphocytes to be very close to the lumen, and since the reticulated epithelium in some places lacks a basement membrane, this would allow increased lymphocyte infiltration and immune function.
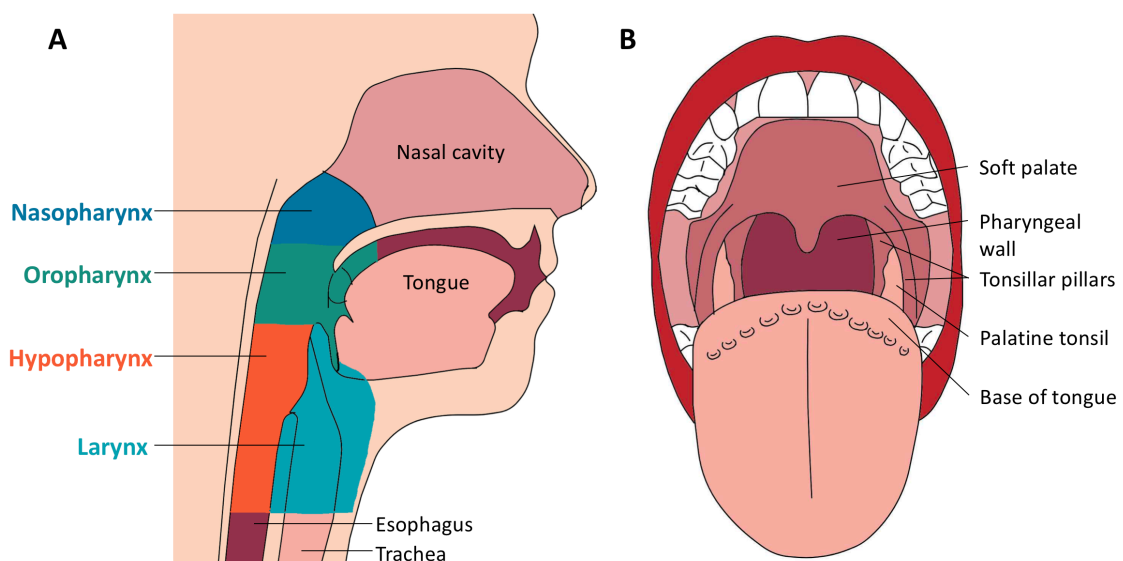

Figure 6. Anatomical schematics of A) the pharynx B) the oropharynx.

The tonsils and base of tongue are commonly referred to as containing "lymphoepithelium", because some areas are very densely infiltrated by lymphocytes and dendritic cells, making the epithelial layer barely distinguishable, as described above [35, 118, 119]. However, microscopically there is also some significant histological variability within these sites. Instead of "lymphoepithelium", some areas have a well-defined basal layer that separates the non-crypted epithelium from the connective tissue, which is similar to what is observed at the other OPSCC sites outside the tonsils and the tongue base. Moreover, the lymphoid nodules in the "lymphoepithelial" sites often present with germinal centers that consist of a lighter colored central area of proliferating B cells, and a denser area of resting B and T cells around the periphery [35, 118, 119]. Histological images of the "lymphoepithelial" and "non-lymphoepithelial" sites are shown in figure 7.
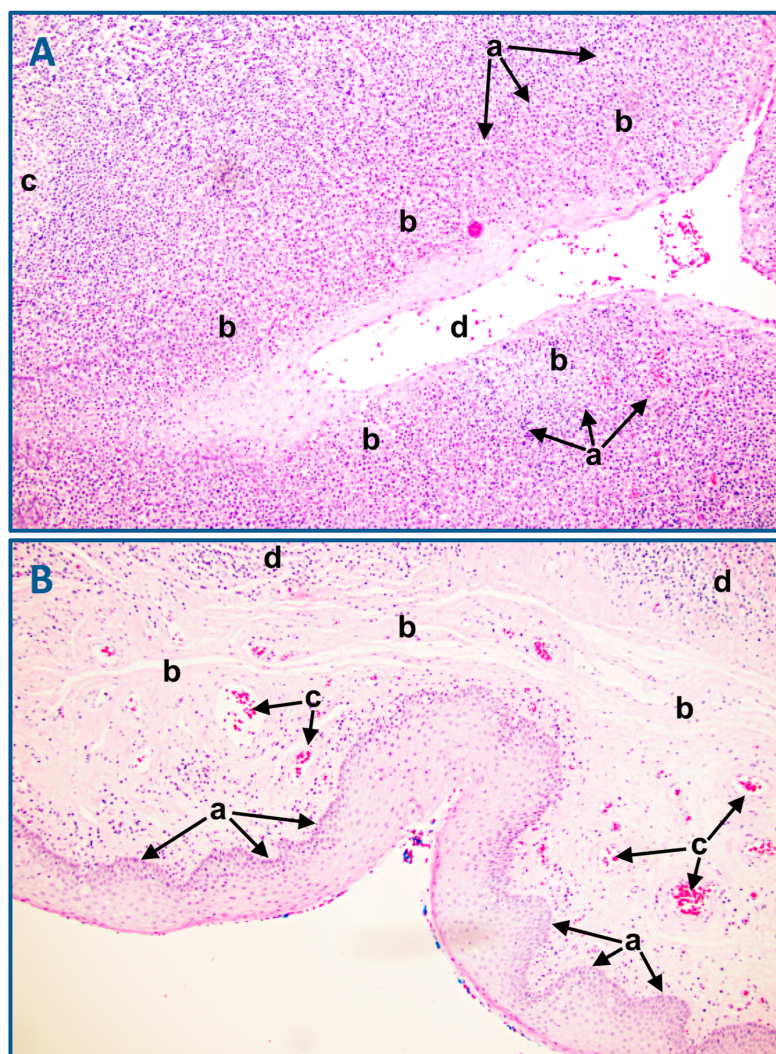

Figure 7. Histology of A) "lymphoepithelial" tissue with a) a barely visible basal cell layer, b) reticulated epithelium rich in infiltrating lymphocytes c) germinal centers, and d) invaginating crypts. B) "non-lymphoepithelial" tissue with a) a clearly visible basal cell layer, b) lamina propria with connective tissue containing c) vessels and d) lymphocyte aggregates.

1.4.2 Classification and tumor staging

As described in the previous section, there are different sub-sites of the oropharynx, and cancers arising from these sub-sites will be classified accordingly, following the International Classification of Diseases (ICD) system. Today the 10th revision of the ICD system is used (ICD-10), although the 11th edition was very recently released in June 2018 [120]. Table 1 describes the different cancers sites and corresponding ICD codes included in oropharyngeal cancer [121, 122]

Oropharyngeal cancers and most other cancers are graded according to the TNM Classification of Malignant tumors staging system, where T describes the anatomic extent of the tumor, N describes possible spread to regional lymph nodes, and M describes whether there is a distant metastasis [121]. Depending on what TNM classification a tumor has, its tumor stage can be determined. Tumor stages can range from 0 to IV, where stage 0 is when the cancer is still contained *in situ*, stages I and II when the cancer still is located in the organ of origin, stage III when the tumor has started to spread to regional nodes, and finally stage IV is in general when a distant metastasis has occurred [121]. However, most tumor types have their own TNM classification and staging guidelines.

Table 1. ICD-codes and sub-sites included in cancer of the oropharynx.

ICD-10 code	Definition
Anterior wall	
C01.9	Malignant neoplasm of base of tongue, unspecified
C10.0	Malignant neoplasm of vallecula
C02.4	Malignant neoplasm of lingual tonsil
Lateral wall	
C09.0	Malignant neoplasm of tonsillar fossa
C09.1	Malignant neoplasm of tonsillar pillar (anterior) (posterior)
C09.8	Malignant neoplasm of the overlapping tonsil
C09.9	Malignant neoplasm of tonsil, unspecified
C10.2	Malignant neoplasm of lateral wall of oropharynx
Posterior wall	
C10.3	Malignant neoplasm of posterior wall of oropharynx
Superior wall	
C05.1	Malignant neoplasm of soft palate, unspecified
C05.2	Malignant neoplasm of uvula
Unspecified	
C10.8	Malignant neoplasm of the overlapping oropharynx
C10.9	Malignant neoplasm of the oropharynx, unspecified
C11.1	Malignant neoplasm of the pharyngeal tonsils

Previously, following the 7th edition of the American Joint Committee on Cancer (AJCC) cancer staging manual, all OPSCCs were staged the same, but recently AJCC released the 8th edition, which for the first time took HPV status into account when determining the tumor stage [121, 123]. More specifically, the new staging system is based on p16 data, where p16-negative (p16[-]) OPSCCs are graded differently from p16-positive (p16[+]) OPSCCs. The main reason for this revision, was that HPV driven tumors in general have a very good prognosis, but very often present with regional lymph node metastasis, which following the previous staging system placed them in a high stage, although having a good prognosis. On the other hand, patients with non-HPV related tumors with a high stage often had a poor prognosis creating a large variability in prognosis within the same stages. Differences between the 7th and 8th edition of the Union of International Cancer Control (UICC) staging system (comparable to AJCCs cancer staging manual) for OPSCC are presented in table 2, as adapted from Taberna et al. [124]. The new TNM classification and staging system for p16[+] and p16[-] OPSCC, according to AJCCs 8th edition, are presented in table 3 [122].

Table 2. *Main differences between the 7th and 8th edition of UICCs TNM classification.*

Characteristics	7th edition TNM	8th edition TNM
Main N (lymph node) differences	N1: metastasis in a single ipsilateral lymph nodes, <3 cm	N1: ipsilateral metastasis in lymph node(s), <6 cm
	N2a: metastasis in a single ipsilateral lymph node >3 cm but <6 cm.	N2: bilateral or contralateral metastasis in lymph node(s), <6 cm*
	N2b: metastasis in multiple ipsilateral lymph nodes, <6 cm	
	N2c: metastasis in bilateral or contralateral lymph nodes, <6 cm	
Main T (tumor) differences	T4a: tumor invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate or mandible	T4: tumor invades any of the following: larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate, mandible, lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base or encases carotid artery§
	T4b: tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base or encases carotid artery	

*Because 5-years OS was similar among N1, N2a and N2b, they re-termed the N categories.
§Because 5-years OS was similar among T4a and T4b, they were no longer subdivided and it was re-termed as T4.

Table 3. *New TNM classification and pathological stage for p16+ and p16- OPSCC according to AJCCs 8th classification manual.*

p16-negative OPSCC stage

	T1	T2	T3	T4a	T4b
N0	I	II	III	IVA	IVB
N1	III	III	III	IVA	IVB
N2	IVA	IVA	IVA	IVA	IVB
N3	IVB	IVB	IVB	IVB	IVB

Patients with M1 are classified as stage IVC.

p16-positive OPSCC stage

	T1	T2	T3	T4
N0	I	I	II	III
N1	I	I	II	III
N2	II	II	III	III

Patients with M1 are classified as stage IV.

The new staging system has however been criticized due to basing HPV-positivity solely on p16 overexpression. In a study by Nauta et al., the new staging system was evaluated on a Dutch cohort, that indeed showed that the new staging system had a better predictive prognostic power compared to the old system in patients with p16+ OPSCC [125]. However, the same study also presented a subgroup of patients with p16+, yet HPV DNA-negative tumors, which had a worse overall survival. With the new staging system these patients would be given a false prognostic prediction, highlighting the importance of testing for both HPV DNA and p16 expression [125]. This issue will be discussed further in Paper IV. Others also show that the differentiation between stages in regard to survival, within the group of p16+ OPSCC patients, was not always significant [126, 127].

1.4.3 HPV in oropharyngeal cancer

Two of the most common OPSCCs are tonsillar and base of tongue squamous cell carcinomas (TSCC and BOTSCC) making up around 80% of all OPSCC. These are also the oropharyngeal sub-sites mainly associated to HPV infections, suggesting that HPV preferentially infects the "lymphoepithelial" parts of the oropharynx over the "non-lymphoepithelial" sites [72, 128]. OPSCC is also a male dominated disease, where around 70% or more of all OPSCC patients are male. Notably, HPV+ OPSCC, or mainly HPV+ TSCC and BOTSCC, differ both in clinical and biological aspects as compared to their

corresponding HPV[-] counterparts [129–132]. The median age of OPSCC patients is 62 years in Sweden, however patients with HPV[+] tumors are in general younger than those with HPV[-] tumors [72]. Moreover, patients with HPV[+] TSCC and BOTSCC have a considerably better prognosis compared to patients with corresponding HPV[-] cancer, with an >80% 3-year disease specific survival (DSS) compared to a 40-50% 3-year DSS for the corresponding HPV[-] groups [15, 101, 132].

There are also some differences in the genetic profiles between HPV[+] and HPV[-] head and neck squamous cell carcinomas (HNSCC) and OPSCC. Frequently affected pathways in HPV[+] cancer include RTK/RAS/PI(3)K signaling (e.g. *PIK3CA*, and *FGFR3*), differentiation (e.g. *NOTCH1* and *TP63*), and cell death (e.g. *TRAF3*) [133, 134]. In HPV[-] tumors instead loss of functioning TP53, and inactivation of *CDKN2A* (p16 encoding gene) are common mutations affecting the p53/pRb pathways. In a study by Smeets et al. genomes of HPV16^{+} and HPV[-] HNSCCs were compared in order to determine what different carcinogenic pathways are involved in the development of these tumors [135]. Here they present an integrated genetic progression model, including the proposed genetic events caused by HPV, and the genetic alterations driving tumor growth in HNSCC not caused by HPV. The model shows that, early common pre-cancer events leading to p53/MDM2 pathway inactivation are in HPV[+] tumors caused by E6, and in HPV[-] tumors due to TP53 mutation (17p loss). Secondary events lead to p16/CDK/pRb pathway inactivation, in HPV[+] tumors caused by E7 and in HPV[-] due to p16 inactivation (9p loss) or 3p loss. Late events, which is suggested to be when actual tumor formation starts, include the gain of 18q in HPV[+] tumors and the loss of 18q and 5q, and gain of 11q13 in HPV[-] tumors, as well as several common alterations. At this stage it is difficult to determine what pathways are involved [135]. In table 4, the main differences between HPV[+] and HPV[-] HNSCC, which are similar in OPSCC, are presented, as adapted from a review by Leemans et al. [136].

Table 4. Different clinical and biological characteristics of HPV⁻ and HPV⁺ HNSCC. Adapted from Springer Nature: Nature Reviews Cancer, Leemans et al., 2011 [136].

Feature	HPV-negative HNSCC	HPV-positive HNSCC	Refs
Incidence	Decreasing	Increasing	[78, 137]
Etiology	Smoking, alcohol abuse	Oral sex	[138]
Age	Above 60 years	Under 60 years	[137]
Field cancerization	Yes	Unknown	[139, 140]
TP53 mutations	Frequent	Infrequent	[135, 141, 142]
Predilection site	None	Oropharynx	[17, 143]
Prognosis	Poor	Favorable	[144, 145]

1.4.3.1 Epidemiology

Worldwide around 30% of oropharyngeal cancers are caused by HPV, which comprises 29,000 new HPV related oropharyngeal cancers per year [6].

The incidence TSCC and BOTSCC has risen drastically over the past few decades in Sweden, and this rise has been proposed to be due to an increased amount of HPV infections in the oropharynx [78]. At the same time there has been a drop in HPV⁻ OPSCCs, which most likely is due to a decrease in smoking among the Swedish population. More specifically there was a 7-fold increase in HPV⁺ TSCC from the 1970s to 2000s in Stockholm, Sweden [78]. However, data from 2000-2012 suggested that the incidence of HPV⁺ TSCC had reached a plateau in Stockholm. The incidence of HPV⁺ BOTSCC had also increased in Stockholm, with a 2-fold increase, but here the incidence did not seem to have reached a plateau [79, 132]. More recent unpublished data from 2013-2016, however suggests that the incidence of both TSCC and BOTSCC is still increasing, but at a slightly slower pace than before, and HPV prevalence has remained stable at around 70% [78]. From year 2000 to 2016 the incidence of TSCC and BOTSCC in Sweden has doubled from around 150 cases per year to around 350 cases per year. That the incidence of TSCC and BOTSCC is increasing is not unique for Sweden, but is observed also throughout North America, other parts of Europe, and Australia [146].

One theory as to why the incidence of HPV⁺ TSCC and BOTSCC reached a temporary plateau in Sweden was that sexual behavior during the HIV epidemic became more restricted. Using the incidence of sexually transmitted Chlamydia infection, as a marker for

sexual activity, a decrease of Chlamydia infections was noted in the Stockholm region between 1990 and 1995, during the intensification of the HIV epidemic [147]. This would be in accordance with a 20 to 30-year lag period between HPV infection and development of TSCC and BOTSCC. This lag period is however yet to be established.

1.4.3.2 HPV positivity in oropharyngeal cancer

As already mentioned, there are several different ways of determining whether a sample is HPV[+] or not, and differences in the methods used result in various definitions of HPV-positivity [115]. Throughout the literature there are many different definitions for HPV-positivity and with a variety of HPV types included in the assays, this makes it unfortunately difficult to compare different studies resulting in that important details can be missed when using HPV as a prognostic marker [148].

Presently, HPV E6 and E7 mRNA detection in fresh frozen material, is assumed to be the most reliable method for determining an HPV driven cancer [115]. Unfortunately, fresh frozen material is not that commonly collected from tumor biopsies. Instead mostly formalin fixed and paraffin embedded (FFPE) tumor material is analyzed, since it is taken routinely for pathological evaluation and also tested for p16 expression by immunohistochemical (IHC) staining of an FFPE tumor section. Overexpression of p16 in OPSCC indicates in most, but not all, cases that the sample is HPV[+]. A study by Rietbergen et al. showed that patients with a p16[+]/HPV DNA[-] OPSCC had significantly poorer survival than patients with a p16[+]/HPV DNA[+] tumor [113]. Also, a second study by Rietbergen et al. showed that p16[+]/HPV DNA[-] OPSCC tumors presented with genetic patterns comparable to HPV DNA[-] OPSCC tumors [114]. These studies highlight the importance of additional HPV DNA testing when determining HPV positivity in OPSCC. Notably, also around 10-15% of non HPV-driven head and neck cancers present with p16 overexpression [149, 150]. Similarly, one also needs to be careful when only testing tumors for HPV DNA. Finding HPV DNA in the tumor does not prove that this cancer is HPV-driven. HPV can for example be present, but transcription may be absent or so low that it is below detection level. Moreover, carcinogenesis may have started before infection by HPV has taken place.

p16 overexpression in combination with presence of HPV DNA has been shown to be almost as sensitive as testing fresh frozen material for HPV E6/E7 mRNA expression [115].

This combination has therefore been accepted as an accurate measurement for an active HPV infection, making it more reliable to work with FFPE material and not needing to rely on fresh or fresh frozen samples in order to test for HPV E6/E7 mRNA overexpression.

It is of great importance to be sure that a tumor is driven by an active HPV infection, so that patients are not falsely categorized as having an HPV driven tumor [115, 125]. This is especially important when planning for de-intensifying therapy for patients that have a high probability for survival, or for targeting therapy for patients with a poor prognosis.

1.4.4 Treatment

In Sweden the main curative treatment modality for OPSCC is radiotherapy, but treatment varies in different geographical regions [72]. Depending on the location of the tumor, radiotherapy can in some cases be boosted by a high radiation dose limited to a small area by brachytherapy. Concomitant chemotherapy with cisplatin together with radiotherapy is considered in patients with tumor stage III-IV (according to AJCCs 7th edition) and radiotherapy together with targeted EGFR-inhibitor (cetuximab) has also been administered [72]. Since many OPSCC tumors are of a high stage, a harsher treatment with radiation together with chemotherapy or EGFR-inhibitors is very common [72].

One common side effects for patients treated for OPSCC with radiotherapy is mucositis that can lead to pain, difficulties to swallow, and can lead to nutritional issues [72]. Moreover, dysfunctional salivary glands leading to a dry mouth, is another common side effect, while osteonecrosis is a rare and later occurring side effect. In addition, surgery can lead to adverse effects such as nerve damage, pain, lymphedema, and cosmetic issues [72]. Therefore, due to the fact that patients with HPV+ TSCC and BOTSCC have a considerably better prognosis compared to patients with HPV- TSCC and BOTSCC, a possible de-escalation of treatment has been suggested for patients with HPV+ tumors in the future. In order to start treatment de-escalation, biomarkers are needed to identify which patients would benefit from a milder treatment, since not all patients with HPV+ tumors have a good prognosis [101, 151]. Potential biomarkers will be discussed in section 1.6.

Surgery is mainly performed on patients with small tumors of the uvula or soft palate. Sometime surgery also can be performed on small tumors of tonsils and base of tongue, this is however more uncommon since in most cases these tumors also present with regional lymph node metastasis. Moreover, the surgery of the base of tongue, location wise, is very difficult to perform with significant risks for morbidity, making surgery a fairly uncommon choice [72]. However, in some Scandinavian clinics, e.g. in Sweden, Finland, Denmark and Norway, Transoral robotic surgery (TORS) with its pros and cons has been introduced [152, 153].

There are also ongoing clinical trials, testing checkpoint inhibitors such as programmed cell death protein 1 (PD-1), PD-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitors, especially in patients with recurrent and/or metastatic disease [154]. The function of these inhibitors is to increase immune activation in the tumor in order to aid in tumor clearance.

1.5   SOME IMMUNOLOGICAL COMPONENTS

The immune system is very fascinating and complex, and can roughly be described as having two main lines of defense against foreign pathogens. Innate immunity is considered the first line of defense, which is very fast yet unspecific. Innate immunity means that this defense mechanism always is present in healthy individuals, and is comprised of e.g. epithelial barriers, dendritic cells, NK-cells, complement, and phagocytes [155]. The second line of defense is called adaptive immunity, which takes some time to get started, however is highly specific and effective [155]. Adaptive immunity is stimulated by foreign microbes, and adapts to the type of microbial invaders that are present. Common cell types belonging to the adaptive immunity are B-cells that have the ability to produce antibodies, and T cells that are effective in killing of infected cells [155]. The following sections will cover a few immunological aspects important for the work presented in this thesis. For more details on the immune system see [155].

1.5.1 Human leukocyte antigen class I and the antigen processing machinery

Human leukocyte antigen (HLA) class I and the antigen processing machinery (APM) are important components in making the intracellular pathogens, such as viruses, as well as normal host cell proteins, visible to immune cells, especially to those of the adaptive immune system, for further details see also [155, 156].

HLA class I molecules are present on all human nucleated cells, however are expressed in different amounts depending on what cell type they are present on [155, 156]. For example, on muscle cells the expression levels are very low, whereas on lymphocytes the expression level is very high [155, 156]. The main function of HLA class I molecules is to present foreign protein fragments from cytosolic and nuclear origin on the cell surface, to cytotoxic T-cells that have the ability to recognize and destroy the cell containing foreign antigens [155–157].

HLA class I molecules consist of a transmembrane α chain, that is noncovalently bound to a soluble protein β2-microglobulin (β_2M). At the amino-terminal end of the α chain, a peptide-binding cleft is found, which is where peptides roughly the size of 8-11 amino acids are bound and presented to cytotoxic T-lymphocytes [156–158]. The mechanisms that lead up to antigen presentation start by proteasome degradation of all ubiquitin-tagged proteins into small peptides from 3-22 amino acids long, and in turn these can be further degraded by peptidases in order to recycle the amino acids. However, a fraction of the peptides released by the proteasome will be taken to the endoplasmic reticulum (ER) by transporter-associated proteins (TAP) for further processing. Here the peptides will be trimmed into a length that fit the HLA class I machinery well, and thereafter the peptide-HLA complex is finally translocated to the cell surface for possible recognition by cytotoxic CD8$^+$ T-lymphocytes, as illustrated in figure 8 [156–158].

The function of the proteasome is to degrade foreign and self-proteins for presentation of peptides on HLA molecules as well as the recycling of amino acids. Immune cells or cells that have been stimulated by pro-inflammatory cytokines express larger amounts of immunoproteasomes, which is a specific type of proteasome [159, 160]. Upon cytokine stimulation three catalytic/proteolytic subunits of the ordinary proteasome are exchanged

by alternative subunits called large multifunctional protease 2 (LMP2), LMP7 and LMP10 and these thereby form the immunoproteasome instead of the constitutive proteasome (figure 8). Immunoproteasomes are responsible for degrading foreign proteins, such as viral and tumor antigens, rather than self-proteins [159, 160]. The immunoproteasome is mainly found in cells of the lymphoid organs, such as the lymph nodes and thymus. Here, these subunits present with peptidases more specific for processing proteins into peptides suitable for HLA class I presentation [159]. However, after IFN-γ stimulation due to e.g. a viral infection, the immunoproteasome also is found in other non-immune related cell types [160]. Although the known functions of the immunoproteasome are executed in the cytoplasm, studies (including Papers I and II) report nuclear localization as well [161–165].
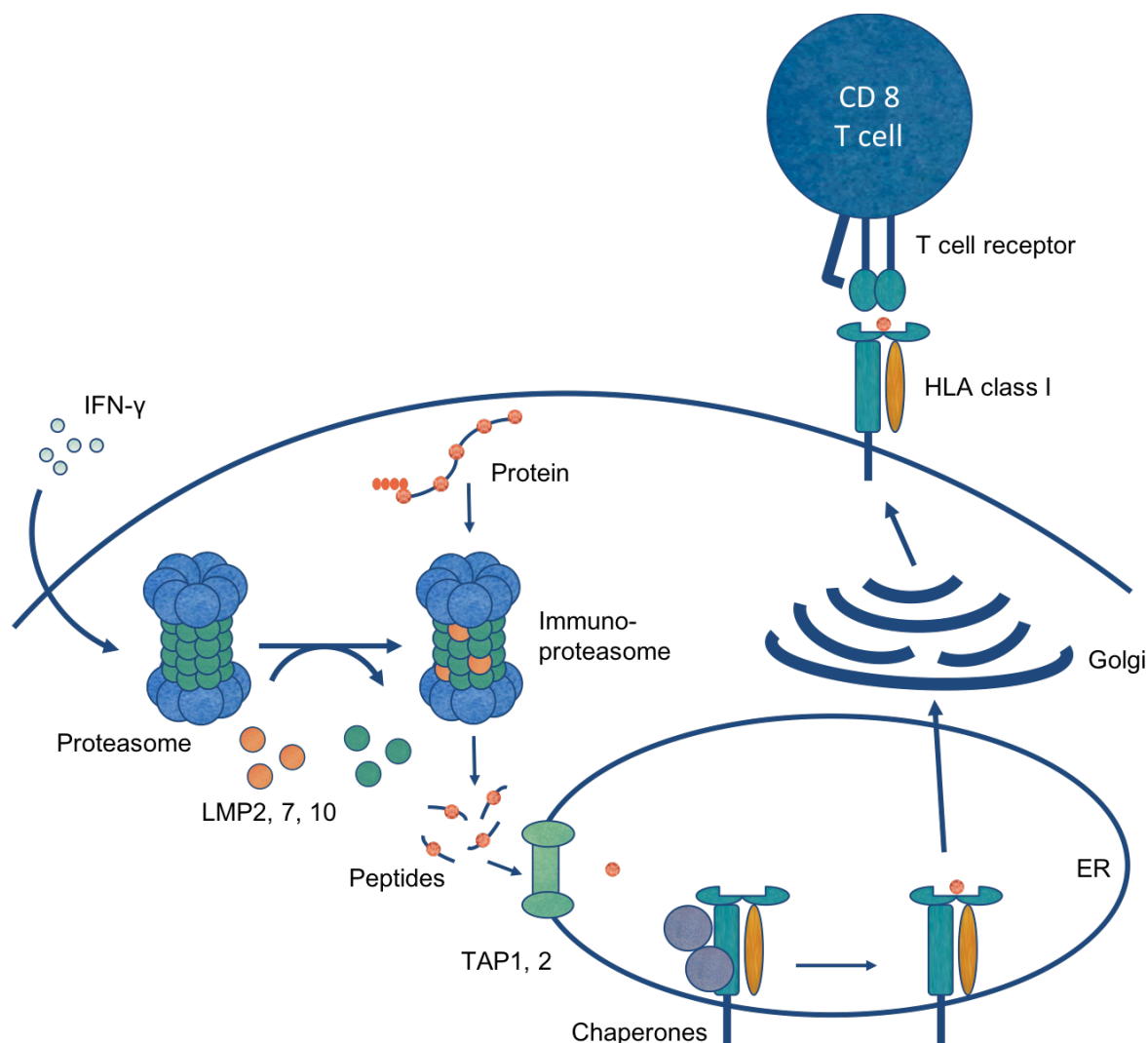
Figure 8. Interferon-gamma induced antigen processing via the immunoproteasome followed by HLA class I presentation to CD8⁺ T cells.

1.5.2 Tumor immune surveillance and tumor immune escape

The concept of tumor immune surveillance implies that the immune system can recognize and eliminate tumor cells [166]. Today there are many reports supporting this concept, e.g. some show that tumor T cell infiltration of different neoplasias often correlates with a favorable clinical outcome, suggesting that the tumor is kept in check by the immune system [167]. This has also been observed in OPSCC (described below in 1.6.2.1). Moreover, other reports have shown that individuals with high cytotoxic activity of their peripheral blood lymphocytes have a decreased risk of developing cancer [168]. Moreover, a lower cytotoxic activity has been linked to an increased risk of developing cancer [169]. Finally, it is also well recognized that immunosuppressed patients have an increased risk of developing malignant neoplasia – not only virus associated neoplasia [170].

As a tumor progresses there is a step-wise process leading to an equilibrium between immune-mediated tumor cell killing and novel mutations, and when this balance is disordered due to e.g. immune escape, this will allow for further tumor progression. Therefore, avoiding immune destruction of the tumor is now recognized as a hallmark of cancer development [171]. Roughly, ways to escape the immune system can be categorized as e.g. having the ability to induce immunosuppression, which is a process where the tumor itself, or the immune cells recruited to the tumor, creates a suppressive milieu [172]. The tumor may also lack susceptibility, which is a process where the tumor develops strategies to resist the cytotoxic mechanisms employed by T-cells and NK-cells. Moreover, lack of recognition, is also a known mechanism, which is when the tumor downregulates e.g. APM components and HLA molecules. Since this is a topic of this thesis, this will be discussed more in detail below [172].

1.5.3 Human leukocyte antigen class I and the antigen processing machinery in cancer

In HPV driven tumors, several papers have demonstrated that HPV has the ability to suppress HLA class I expression, and thereby assist HPV infected tumor cells to evade immune surveillance. HPV genes E5 and E7 are suggested to be involved in suppression of HLA class I expression [51–54, 173]. A study by Li Wei et al. showed that HaCaT cells transfected with HPV16 E7 reduced HLA class I expression by 50% compared to that in

non-transfected cells. Simultaneously they observed a 40% decrease in TAP1 expression, suggesting that E7 interacts with TAP1, and thereby inhibits its function [49]. Furthermore, a study by Myriam Gruener et al. showed that E5 had the ability to downregulate HLA class I expression, and they also proposed that E5 specifically targeted calnexin, a chaperone involved in HLA class I maturation and cell surface transportation, [174]. Moreover, in the same study they showed that in cells expressing calnexin, HLA class I expression was downregulated upon E5 cell transfection, whereas in calnexin deficient cells HLA class I surface expression did not change [174].

Suppression of HLA class I expression is however also a mechanism by which tumors non-related to viral infection, escape immune surveillance [175]. Some of the mechanisms by which HLA class I expression is suppressed include defects in β_2M synthesis, the loss of/mutations of genes encoding HLA class I heavy chains, abnormalities in components of the antigen processing machinery, or defective HLA class I regulatory mechanisms [176]. Furthermore, epigenetic changes have been found to affect changes in HLA antigen expression, components of the antigen processing machinery, and tumor antigen expression in tumor cells [176]. In several cancer types, including head and neck cancers, abnormalities in expression of immunoproteasome components LMP2, LMP7 and LMP10 have been described, which in turn leads to decreased number of presentable peptides, and reduction in HLA class I expression, i.e. immune evasion [177, 178]. Single nucleotide polymorphisms in LMP2 and LMP7 have been shown to be associated with a decreased overall survival in cervical cancer. Defective transcription factors, e.g. interferon response factor 1 (IRF1) and signal transducer and activator of transcription (STAT1), have been associated to loss of LMP2 upregulation [177, 178].

There is increasing evidence that ionizing radiation (IR) has altering effects on the immune system, on the one hand dampening local immune responses due to clearance of lymphocytes from the affected area, on the other hand increasing T cell priming in draining lymphoid tissues [179, 180]. An increase in HLA class I expression has also been observed in e.g. multiple myeloma and renal cell carcinoma cell lines after irradiation [181]. Furthermore, it has been shown that cell surface HLA class I expression can be increased after transferring cell media from radiotherapy treated human breast cancer cells to non-treated cells, suggesting that the increase is due to secreted soluble factors,

such as immune stimulatory cytokines [182]. In the same study the authors also described interferon beta (IFN-β) as the mediating factor that had induced surface HLA class I expression. In a study by Reits et al. the authors showed, that radiotherapy had the ability to increase the intracellular peptide pool, which led to a subsequent upregulation of major histocompatibility complex (MHC) class I [183]. In paper III of this thesis, HLA class I expression in HPV⁺ and HPV⁻ head and neck cancer cell lines was evaluated after radiation therapy.

1.6 PROGNOSTIC BIOMARKERS

There are several precise definitions of biomarkers in the literature, however most definitions are very similar. The National Institutes of Health Biomarkers Definitions Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." [184]

In our group, we have focused on finding prognostic biomarkers for patients with HPV⁺ and HPV⁻ TSCC and BOTSCC that can be utilized before oncological treatment. The reason for separating HPV⁺ from HPV⁻ TSCC and BOTSCC is due to that HPV itself is a biomarker for a better survival in the patients with HPV⁺ TSCC and BOTSCC as compared to the corresponding HPV⁻ tumors, with 80% vs. 40-50% 5-year survival rate respectively [15, 101, 132]. Besides, the biological behavior of these tumor entities differs vastly, as described above. In addition, the expression of specific proteins may be related to the presence of HPV in the cell. Since HPV⁺ TSCC and BOTSCC have a better prognosis all proteins related to HPV will thus also be related to clinical outcome.

Furthermore, the impact of the presence of HPV depending on OPSCC subsite location and prognosis, has been studied in more detail in papers IV and V. Indicating the importance of the TSCC and BOTSCC sites, as well as the specific nature of TSCC.

1.6.1 Biomarkers in head and neck cancer

There are several biomarkers identified in head and neck cancer. In addition to HPV being a good prognostic marker in especially in TSCC and BOTSCC, high levels of plasma EBV DNA

in nasopharyngeal cancer patients has also been reported as a poor prognostic factor [185, 186]. Imaging, with 18F-fluorodeoxyglucose positron emission tomography (FDG-PET), can be used to study glucose metabolism, and a high uptake has been associated with cell viability and proliferation [187]. The quantitative parameter known as maximum standardized uptake value (SUV_{max}), is considered as a biomarker for head and neck cancers, and a high value is suggested to indicate an increased risk of progression, recurrence and poor survival [187]. Cancer-gene-targeted sequencing of head and neck cancers has shown that the loss of function in tumor-suppressor genes is more common than the gain of functions leading to oncogenes [188]. Furthermore, there are a handful of oncogenes important in head and neck cancer, that are immediately targetable with agents in clinical development, such as *FGFR, EGFR, MET, CCND1*, and *PIK3CA* [188].

1.6.2 Immune related biomarkers

1.6.2.1 *T cells in oropharyngeal cancer*

Common biomarkers that have been studied and established in several cancer types are the presence of CD4^{+} and CD8^{+} tumor infiltrating lymphocytes (TILs). Where a high number of TILs in general is favorable, due to increased tumor clearance by an active immune system [189–192]. In one of the studies not included in this thesis, we studied TILs as potential biomarkers in TSCC and BOTSCC and found that patients with a high CD8^{+} TIL count in HPV^{+} tumors, have a better 3-year overall and disease-free survival compared to patients with absent/low CD8^{+} TIL counts. A similar trend was shown for the HPV^{-} patient group, which however was not statistically significant. Furthermore, no prognostic value could be identified for CD4^{+} TIL counts in this study [192].

Regulatory T cells (Tregs) are immune cells with the ability to suppress the immune system. This is necessary to keep the immune system in balance and avoid autoimmunity [155]. A common mechanism for tumors to escape immune surveillance is the recruitment of Tregs. Forkhead box P3 (FoxP3) is a transcription factor expressed by Tregs, to which antibodies can bind in order to identify Tregs in for example tumors. The amount of tumor cells positive for FoxP3 has been used for predicting prognosis, invasiveness, and metastatic ability [193]. In a previous study by Näsman et al., the presence of FoxP3 and CD8 in TSCC was studied and found that a high ratio of CD8^{+} to FoxP3^{+} cells, meaning that FoxP3^{+} cells were in a great minority when compared to CD8^{+} cells, was correlated to a

better survival in both HPV^{+} and HPV^{-} tumors [194]. FoxP3 alone could however not predict prognosis, suggesting that CD8^{+} was the more important prognostic factor [194]. In a meta-analysis it was shown that in most included tumor sites, a high number of Tregs was associated to a poorer survival, whereas in some tumor sites, including head and neck cancers, a high Treg count was associated to better survival. These data suggest that the prognostic value of FoxP3 remains controversial [195].

1.6.2.2 HLA class I in oropharyngeal cancer

Another immunological biomarker that has been studied in several different cancers is HLA class I expression. Studies have, in many types of tumors, shown a downregulation of HLA class I, which is a way of immune evasion. In for example ovarian-, rectal-, and head neck cancer it has been shown that a low HLA class I expression relates to a poor prognosis [196–198]. Another study by Meissner et al., showed a tendency for head and neck cancer patients with low HLA class I expression in their tumors to have a decreased survival compared to those with tumors with normal HLA class I expression [199]. However, HPV status was not considered and the patient material was obtained from a mix of primary tumors and relapses [199]. Nevertheless, in a study performed earlier by Näsman et al. the absence of HLA class I expression was found to be a good prognostic marker for patients with HPV[+] TSCC [200]. This finding was interpreted to be due to high HPV activity, and more specifically possibly due to the downregulation of HLA class I expression by HPV E5 and E7. Not surprisingly, the opposite was true for patients with HPV[-] TSCC, where low HLA class I expression was correlated to a poor prognosis, which is in line with what has been shown in studies on other cancer types [201].

1.6.2.3 Antigen processing machinery components in oropharyngeal cancer

The study by Meissner et al. cited above, also showed that APM components LMP2, LMP7 and TAP1 often were downregulated or lost in cancer cell lines derived from head and neck cancer patients [199]. Moreover, LMP2, LMP7, and TAP2 had a significant prognostic value in that decreased expression in either of these markers presented with a poorer survival than in patients with normal expression levels [199]. In paper I the potential of LMP10 as a biomarker will be discussed, and in paper II potential biomarkers TAP1, TAP2, LMP2, and LMP7 were evaluated in HPV[+] and HPV[-] TSCC and BOTSCC.

2 AIMS

- To investigate antigen processing machinary components LMP2, LMP7, LMP10, TAP1, and TAP2 in correlation to HLA class I expression, and as potential prognostic biomarkers in HPV$^+$ and HPV$^-$ tonsillar and base of tongue cancer *(Papers I and II)*.

- To study the effects of radiation therapy on HLA class I expression, cell cycle, and apoptosis in HPV$^+$ and HPV$^-$ base of tongue and mobile tongue cancer cell lines, and the relation between HPV E5/E7 mRNA expression and HLA class I expression in the HPV$^+$ cell lines *(Paper III)*.

- To perform a systematic review and meta-analysis of the litterature in order to study the role of HPV per oropharyngeal cancer sub-site, and to investigate the importance of HPV detection method used *(Paper IV)*.

- To study the impact of histological context on HPV prevalence and survival in tonsillar cancer *(Paper V)*.

3 STUDY SUBJECTS, MATERIAL AND METHODS

3.1 STUDY SUBJECTS, MATERIALS AND STUDY DESIGN

The majority of studies performed in our group include FFPE pre-treatment tumor biopsies from TSCC and BOTSCC. From year 2000 and onward, the goal has been to collect samples, when available, from all of the patients with TSCC and BOTSCC diagnosed in the Stockholm region, enabling the performance of fairly large cohort studies. The samples have routinely been tested for presence of HPV DNA and p16 expression and additional tumor sections have been collected for various types of studies. Papers I and II, for example include samples from this cohort, used for IHC analysis, HPV DNA, and for Paper II also p16 data. Unfortunately, it is difficult to receive samples from all patients diagnosed with TSCC and BOTSCC in Stockholm. Some common reasons for exclusion have been e.g. the lack of FFPE samples due to cytology-based diagnosis only, lack of tumor material within the FFPE block, permission for use of tumor material in research was not granted by the patient, and that the FFPE block was missing at the time of collection. Before year 2000, a large amount of mainly TSCC samples have also been collected from the Stockholm region, here however the clinical data is less complete and p16 data is often missing. This cohort is however valuable in the way that these patients mainly have received radiation therapy as their only treatment, making it a more homogenous group of patients compared to patients diagnosed at a later time, who often have received a range of different treatment modalities. Samples included in Paper V are mainly retrieved from this earlier cohort.

Paper I & II. Both studies were retrospective cohort studies and included patients diagnosed with TSCC and BOTSCC between 2000-2007. When selecting tumor material for these studies, the aim was to include samples that in a previous study [200] had been tested for HLA class I expression. Initially a pilot study was performed on a small number of these patient FFPE TSCC and BOTSCC samples, staining by IHC for a number of APM components. Immunoproteasome component LMP10 showed most prognostic potential and was therefore further evaluated on a large patient cohort (Paper I). Several of the other APM components showed a correlation to HLA class I expression and were then further evaluated on a medium sized patient cohort (Paper II).

Paper I. TSCC (ICD-10: C09.0-9) and BOTSCC (ICD-10: C01.9), diagnosed at the Karolinska University Hospital during 2000-2007 were identified through the Swedish Cancer Registry. Of these, 278 patients with pre-treatment biopsies containing sufficient tumor material were available and included in this study. Of these, 258 patients were treated with intension to cure and could therefore be included in the survival analysis.

Paper II. Again, when selecting tumor material for this study, the aim was to include samples that in a previous study [200] had been tested for HLA class I expression. This study included two study cohorts, comprising a total of 151 patients with TSCC (ICD-10: C09.0-9) and BOTSCC (ICD-10: C01.9) treated at the Karolinska University hospital. The first set of samples consisted of 78 available TSCC samples from patients that were treated with the intention to cure, diagnosed between 2000-2006, all of which had been tested for HLA class I expression in a previous study [200]. The second set consisted of all 73 BOTSCC samples from patients diagnosed from 2000 to 2007, with available pretreatment biopsies, and 66 of these were treated with curative intent, while the rest received palliative treatment.

Paper III. This is an in vitro study examining the effects of radiotherapy on HLA class I expression. Ideally, three HPV$^+$ and three HPV$^-$ cell lines derived from TSCC or BOTSCC with and suppressed HLA class I expression would have been selected for this study. However, cell lines with these naturally occurring, specific properties do not exist. Moreover, it is also very difficult to establish OPSCC cell lines that retain their HPV infection. Therefore, in this study three HPV16$^+$ cancer cell lines, UM-SCC-47, UPCI-SCC-154, and UPCI-SCC-090, and one HPV$^-$ cancer cell line, UT-SCC-14, were studied. UPCI-SCC-154 and UPCI-SCC-090 were established from base of tongue squamous cell carcinomas, and UM-SCC-47 and UT-SCC-14 were established from lateral/mobile tongue squamous cell carcinoma. Moreover, UPCI-SCC-090 was established from a recurrence [202–204].

Paper IV. Since this study was designed as a systematic review and meta-analysis of the literature, only data from sample material already published was included. In total, 58 unique cohorts, meeting the inclusion criterion, and published between 2013-01-01 and 2016-10-31, from 64 scientific publications, were included in the analysis. Following inclusion criterion had to be met: separation by "lymphoepithelial" and "non-

lymphoepithelial" oropharyngeal sub-sites, reporting of HPV data by molecular tissue specific method (PCR, ISH or p16 IHC), in an un-selected cohort.

Paper V. This is a retrospective cohort study. For this paper, 203 patients diagnosed with TSCC (ICD-7: 145.0) between 1970 and 2002, with available hematoxylin and eosin stained tumor sections were evaluated. From these, 139 tumor sections contained sufficient normal tissue surrounding the tumor area for inclusion. This early cohort was mainly selected because hematoxylin and eosin stained tumor sections were readily available in our lab, from a previously published cohort [205, 206].

3.2 METHODS

3.2.1 HPV DNA and RNA extraction

All FFPE samples included in Paper I, II and V, have been tested for presence of HPV DNA, for details see each paper. DNA was extracted using the Roche High Pure RNA Paraffin Kit (Roche Diagnostics, Mannheim, Germany), according to manufacturer's instructions, with the exception of excluding the DNase treatment step. In Paper III, RNA was extracted from cell lines using the RNeasy® Mini kit (Qiagen, Venlo, The Netherlands), according to manufacturer's instructions. Samples were DNase treated using the RNase-free DNase set (Qiagen) to ensure DNA free samples. In order to avoid cross-contamination between samples, blanks were added and treated the same way as the samples, during both DNA and RNA extraction.

Methodological considerations: The Roche High Pure RNA Paraffin Kit used for DNA extraction is optimized for RNA retrieval, however DNA retrieval is also achieved by omitting the DNase treatment step. The reason for using an RNA kit instead of a DNA kit is to facilitate collection of both DNA and RNA from one sample.

3.2.2 HPV DNA detection

The HPV DNA detection method used in this thesis is mainly based on PCR methods using the general primers GP5+/6+ or broad-spectrum GP5+/6+ (bs-GP5+/6+) primers, targeting the conserved L1 region of the HPV genome. The GP5+/6+ primers were initially developed by de Roda Husman et al. [105] in order to increase detection sensitivity, and

was an extension by 3 nucleotides to the previously used GP5/6 primers [207]. However, these primers still had low sensitivity for some HPV types. Therefore, a multiplex PCR followed by a Luminex bead-based assay for 27 different HPV types was later developed by Schmidt et al. in the Michael Pawlita group, and is presently used in our group, although later with some added targets [108, 109].

Starting with the HPV DNA PCR method presently used (paper I and II), bs-GP5+/6+ together with specific primers for HPV16 E6 were used. As positive controls for HPV, DNA from SiHa cells, corresponding to 1, 10 and 100 HPV16 genomes per 5μl were utilized. In addition, specific primers for the house keeping gene β-globin were added to ensure presence of amplifiable cellular DNA. Moreover, as a negative control an RNase free water sample was included. For PCR amplification, 10 ng DNA for each reaction was used.

Amplified DNA was thereafter analyzed utilizing a bead-based assay on a Magpix instrument. This assay detects L1 DNA of 27 different HPV types, including all currently recognized high-risk and putative high-risk HPV types, as well as some low-risk types (HPV6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42-45, 51-53, 56, 58, 59, 66-70, 73 and 82). Detection of HPV16 E6 and β-globin was also included in the assay.

The amplified PCR product was mixed with HPV type specific probes (27 different) coupled to magnetic beads with a unique fluorescent color for each HPV type in the assay. Heating of the sample enables separation between DNA strands, followed by ligation of bead-coupled probes with corresponding HPV DNA strands, if present. The PCR product hybridizes to the bead-coupled probes and is detected by labeling one primer in each primer pair with biotin, to which a fluorescent conjugate can bind to, and thereby this fluorescent conjugate will be detected and analyzed. The Magpix instrument uses two lasers to evaluate the samples, the red laser evaluates the color of the beads determining which HPV-types are present, and the green laser detects the fluorescent conjugate and semi-quantifies the amount of HPV DNA. The results are reported back as median fluorescence intensity (MFI) values. As cut-off for HPV positivity 1.5 x background + 15 was used (figure 9) [109, 110].
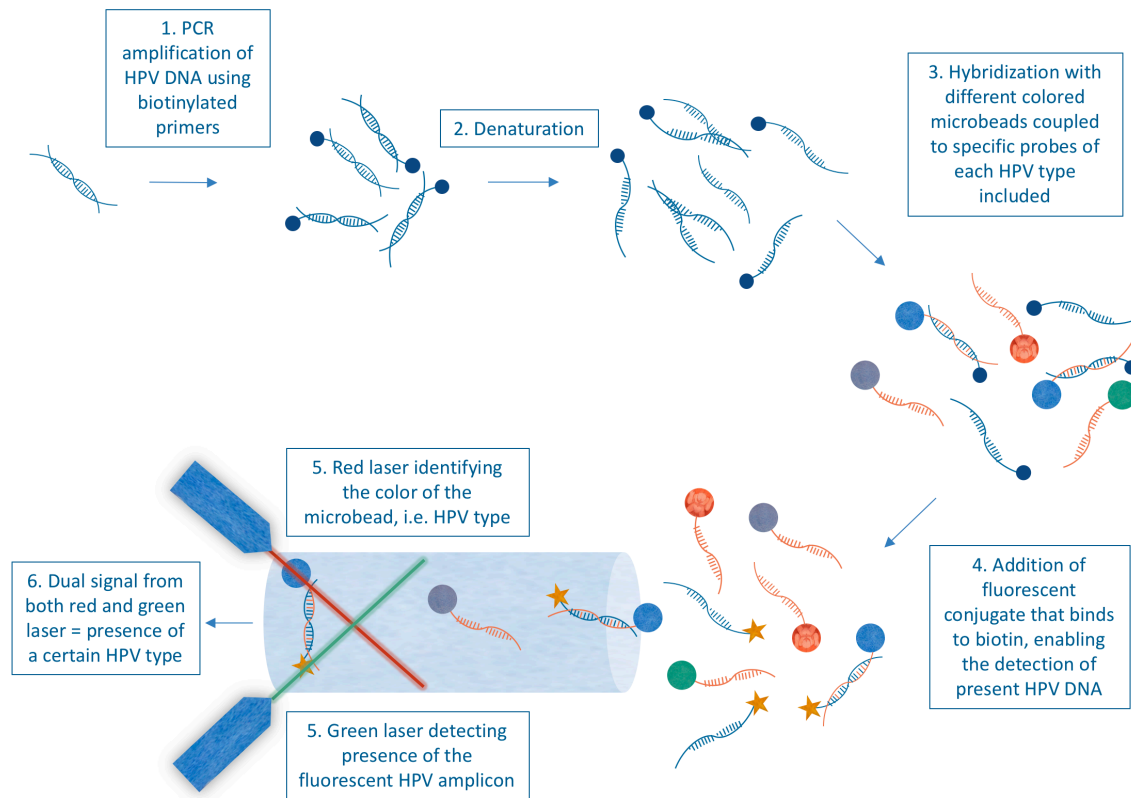
Figure 9. *HPV genotyping by a PCR based assay utilizing Luminex technologies.*

In paper V, in this earlier cohort for detection of HPV DNA, the older GP5+/6+ general primers had been used, together with another consensus primer pair, the CPI/CPIIG primers (targeting the E1 region), as well as HPV16 type specific primers targeting E6. Here, specific primers or the house keeping gene S14 were used to ensure presence of amplifiable cellular DNA. Gel electrophoresis was used for detection of amplified DNA at the correct amplicon length.

Methodological considerations: In the Papers I and II broad-spectrum GP5+/6+ primers were used. A drawback with general primers is that they in general, due to mismatches in the primer sequence, amplify some HPV types better, whereas other types require many more viral copies to be detected. A way to overcome this is to use a combination of primers with some sequence variation, or a combination of different general primers (bs-GP5+/6+) making the sensitivity more equal among different HPV types. However, at the time HPV testing was performed for Paper V, the Luminex assay together with bs-GP5+/6+ primers was not available. Instead HPV was tested using GP5+/6+ PCR and CPI/CPIIG PCR, followed by HPV type specific PCR for detecting. On the other hand, the sensitivity for

HPV16 was very high with this algorithm, and since the dominating HPV type in TSCC and BOTSCC is HPV16, this did likely not pose a problem for the studies included in this thesis. In addition, in paper V, two consensus primer pairs were used to exclude the possibility of false negative results due to loss of L1 region upon to viral integration. However, no additional cases were identified with the CPI/CPIIG primers (data not shown) as compared to using GP5+/6+ and HPV16 type specific primers only. Similarly, in the newer assay using bs-GP5+/6+ primers, HPV16 E6 specific primers were also used to exclude the possibility of false negative samples due to loss of L1 region upon integration.

Lastly, HPV genotyping using the bead-based Magpix assay is a good choice for evaluating large amounts of samples, since it allows for the detection of many different HPV types simultaneously, from the same sample, and is a very effective and a fairly cheap method.

3.2.3 Immunohistochemistry

Immunohistochemistry (IHC) comprised a large part of the methods for Paper I and II. For these studies, 4μm thin FFPE tumor sections were de-paraffinized in xylene and thereafter re-hydrated in decreasing concentrations of ethanol. Antigen-retrieval was achieved by boiling the sections in citrate buffer, followed by quenching of endogenous peroxidase activity using hydrogen peroxidase. Unspecific binding sites were blocked by treatment with horse serum. This was followed by an over-night incubation with the primary antibody, see next section for antibody details. In addition, for information regarding where the antibodies were purchased, please see the methodology part of the different papers. In order to detect specific binding of the antigen of interest, the sections were incubated first with a biotinylated secondary antibody, followed by incubation with an avidin-biotin enzyme complex (Vectastain Elite ABC kit (HRP), Vector Laboratories, Burlingame, USA). Antigen visualization was achieved by adding the substrate chromogen-39-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, USA) necessary for the enzyme of the avidin-biotin complex to produce a brown color. The slides were counterstained with hematoxylin, followed by de-hydration in increasing concentrations of ethanol and xylene. Mounted sections were evaluated by light microscopy by researchers blinded for clinical outcome.

For Paper I, the primary antibody MECL-1 (C-2) (anti-LMP10 antibody from Santa Cruz Biotechnology) was used. For Paper II, following primary antibodies were used: TAP1, rabbit polyclonal H-300; TAP2, rabbit polyclonal 44 H210, both from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); LMP2, rabbit polyclonal antibody ab3328; LMP7, ab3329, both from Abcam (Cambridge, United Kingdom). As secondary antibodies, BA-1000 anti-rabbit (1:200) and BA-2000 anti-mouse (1:200) both from Vector Laboratories (Burlingame, CA, USA), were used. By light microscopy, IHC staining of all five APM components were evaluated both in the nuclear and cytoplasmic compartment for intensity and percentage stained tumor cells. The intensity was scored as absent, weak, medium or strong/normal (similar to infiltrating immune cells). The percentage was scored as: 0%, 1-25%, 26-50%, 51-75% and 76-100% of stained tumor cells. In Paper II, the primary antibody p16^{INK4A} (clone: JC8, dilution 1:100, Santa Cruz Biotech, Dallas, USA) was used to determine p16 status. Samples were considered p16$^+$ if >70% of tumor were stained [145].

Methodological considerations Evaluation of the APM components was performed on both the nuclear and cytoplasmic compartment of tumor cells, as these APM components have been described to be localized to both the cytoplasm and nucleus. However, since the staining in the nucleus and cytoplasm was not clearly correlated, we decided to score these separately. There may also be some uncertainty concerning the consistency of evaluation relying on human visual estimations only. This uncertainty was minimized by letting two trained scientists evaluate the sections separately. When discrepancies were observed a consensus was reached, or a third researcher was consulted. Moreover, all studied APM components showed strong expression in both stromal tissue and tumor infiltrating lymphocytes, where the latter then served as positive internal controls. Notably, staining intensity may in some cases be hard to interpret, and interpretation may vary between samples. However, the presence of a strong internal control as described above, that can be related to tumor staining intensity, should in this study elicit a more stringent evaluation with increased reproducibility. The evaluation of p16 expression was very clear cut, where in the vast majority of cases, either 100% or 0% of tumor cells were strongly stained. p16 overexpression is therefore a very practical marker. However, as already mentioned earlier, p16 overexpression does still not always correlate with HPV DNA positivity. In Paper I and V, p16 expression was unfortunately not evaluated since we

earlier only assayed for presence of HPV DNA. This may have led to that a few tumors were wrongfully classified as being HPV-driven. However, in cohorts partly overlapping with the ones in Paper I and V, the combination of p16 overexpression and HPV DNA, gave few discrepancies from HPV DNA alone. This suggests that the impact of not evaluating both for the combined presence of HPV DNA and p16 overexpression, for the conclusion regarding the effects of the evaluated markers, or the differences of specified TSCC (STSCC) and non-specified TSCC (NTSCC), should be marginal.

3.2.4 Histological evaluation

In Paper V, TSCC hematoxylin-eosin stained sections were evaluated with regard to the histological context surrounding the tumor. Firstly, only sections with a representative amount of normal surrounding tissues were included. Secondly, these sections were separated into specified TSCC (STSCC) and non-specified TSCC (NSTSCC), as described first by Garnaes et al. [208]. STSCC was defined as having tonsillar crypts and tonsillar lymphoid tissue with germinal centers outside of the tumor area. NSTSCC was defined as lacking these characteristics in the normal surrounding tissue. However, while Garnaes et al. included tumors with no stroma in the NSTSCC category, we excluded these tumors from the analyses. None of the tumors assessed had arisen from the base of tongue.

Methodological considerations: Working with FFPE tumor sections, one always has to bear in mind that only a very thin section per fraction of the actual tumor is evaluated, and this section may not always be representative for the entire tumor. Especially in Paper V, when evaluating the histology surrounding the tumor, some samples had to be excluded just because no representative normal tissue was present, which in the patient of course would be there.

3.2.5 *In vitro* cell culture

In vitro studies on cancer cell line cultures were only used for the experiments in Paper III, and for further purchasing details and cell line characteristics, please see this paper. In short, the cell lines were handled as follows. Cell lines were cultured in either Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin streptomycin solution, or in minimum essential medium (MEM) with 1% non-essential amino acids and 0.1% gentamicin, both

containing 10% fetal bovine serum, and 1% L-glutamine. Stocks of all cell lines were grown in 75 cm^2 cell culture flasks with filter lids. From these the cells were then plated into 6-well cell culture plates for experiments and kept at 37°C with 0.5% carbon dioxide at 100% humidity. Medium was changed regularly (usually every 2-3 days) and the cell cultures were split using trypsin EDTA 1x when around 70-100% confluent. All cell lines were free from mycoplasma, tested using the Takara mycoplasma detection set.

Methodological considerations: Cell cultures are a very convenient way of studying biological mechanisms *in vitro*. However, the conditions for cells growing in a plastic flask are extremely different from the human body, and also the cells growing in cell cultures have been selected to grow well in cell culture settings. Therefore, *in vitro* studies only can give an indication of what may be happening *in vivo* and must be confirmed by *in vivo* studies before possibly being applied in humans.

3.2.6 Radiation

Paper III focused on the effects of radiation in different cancer cell lines, with the specific aim to study the influence of irradiation on HLA class I expression. The cells were irradiated using a Caesium-137 source, for different amounts of time depending on what dose was desired. Initially, different radiation doses, as well as fractionated doses, were tested out, and as a result of this calibration, one dose of 10 (Gray) Gy was used for the consecutive experiments. Standardized experiments were performed on cell lines plated in 6-well plates, with the goal of reaching 75% confluence upon radiation. To obtain this, different amounts of cells depending on each specific cell line were plated. Non-radiated controls were also prepared in the same way, with the exception of not being irradiated.

Methodological considerations: It is difficult to determine what a clinically relevant dose of radiotherapy translates into when treating cell lines, and perhaps a 10 Gy dose is fairly high. However, the dose was calibrated for these experiments, and allowed the majority of cells to still be viable after treatment, since a major goal of these experiments was to study the effect of irradiation on HLA class I expression in HPV$^+$ and HPV$^-$ cell lines, and to examine whether the two types of tumors responded differently. We assume that most cells receive the same radiation dose, however, small variations in radiation dose between the wells could occur depending on location of the well in relation to the radiation source.

3.2.7 Real-time PCR

In Paper III, real-time (RT) PCR was performed in order to determine presence of HPV16 E5 and E7, as well as HLA class I mRNA levels pre- and post-irradiation. For experimental details and sources of purchase, please see paper III. In total 0.1 µg of RNA was utilized for first strand cDNA synthesis using a First Strand cDNA Synthesis kit (ThermoFisher Scientific) using random hexamers as primers. The cDNA was thereafter prepared for RT PCR by preparation with SYBR-Green together with primers for HPV16 E5 and E7 as described by Ramqvist et al. [209], and primers for HLA-A on exon 3 as described by Villabona et al. [210]. Triplicates were included from each sample (treated and non-treated), for each gene of interest, and for GUS B (endogenous control). Triplicates containing primers from each gene of interest, mixed with water, were used as negative controls. The RT PCR program started at 50°C for 3 min followed by 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 1 min. Starting at 40°C and increasing by 0.5°C every 10 s until 120°C was reached, produced the melting curve needed for analysis. Radiated samples were compared to non-radiated samples by calculation of ΔΔCT values (delta delta cycle threshold values).

Methodological considerations: Measuring changes in mRNA for E5 and E7 expression is an indication, and an indirect way of following the potential of changes on the protein level after irradiation. It does not prove changes on the protein levels of E5 or E7 proteins, or that the interactions of these proteins with HLA class I expression are affected. However, we also assayed for HLA class I protein expression. Secondly, one has to bear in mind that SYBR green is not gene specific and binds to any double stranded DNA, also e.g. non-specific amplicons or contaminating DNA. Therefore, it is important to evaluate the melting curve, created at the end of RT PCR amplification, and compare to a positive control. A probe specific PCR, as e.g. TaqMan PCR, would not have this problem with possible non-specific amplification.

3.2.8 Flow cytometry

Flow cytometry was utilized to detect surface HLA class I expression, cell cycle analysis, and measurements of apoptosis, presented in Paper III. For further details and regarding

sources of purchase see paper III. In short, treated and non-treated cells were washed and collected in tubes after trypsinization, and stained and with the marker of interest together with appropriate controls. Cells were then analyzed for the markers of interest on the flow cytometer within an hour.

HLA class I expression was stained for, 48 hours after irradiation. More specifically, first the cells were stained with a live/dead cell marker (LIVE/DEAD® Fixable Near-IR Dead Cell Stain kit) in order to separate out live cells only, in the analysis. Thereafter the cells were stained with an HLA class I (A, B, C) specific antibody, clone W6/32, coupled to an Alexa Fluor 488 (anti-human) fluorescent dye. Unstained controls and isotype controls were included.

The DNA content of the cells was analyzed 24 hours after irradiation, in order to determine cell cycle changes depending on if the cells were irradiated or not. Here the cells were fixed by dropwise adding 100% ethanol, while vortexing them mildly and letting them incubate in the fridge overnight. This was then followed by staining for DNA content using propidium iodine (PI) together with RNase A, the latter in order to reduce RNA interference.

To distinguish apoptotic from normal cells, the cells were stained with a FITC Annexin V apoptosis detection kit I, which binds to phospholipid phosphatidylserines, a protein that in apoptotic cells becomes more exposed. This was done 96 h after irradiation. Both floating and adherent cells were collected, in order not to miss apoptotic cells that may have detached. Cells were stained for Annexin V coupled to a FITC fluorophore together with PI.

Methodological considerations: Although FACS can be used to measure a broad range of markers in one sample, for this thesis, fairy simple methods including very few parameters, have been used. One issue with FACS is that a single cell suspension is needed for the analysis of cells, which mostly works fine when analyzing cell lines, however some samples can be more prone to clump and then a lot of valuable cells may be lost for the analysis. Moreover, FACS is a method that in most cases only measures cell surface proteins, which means that information about intracellular expression is lost.

3.2.9 Systematic review and meta-analysis

Systematic reviews with meta-analyses are a great way of getting an overview of a certain research topic, and are also a common starting point for developing clinical practice guidelines [211].

The systematic review in Paper IV was performed by searching the database PubMed for publications published between 2013-01-01 and 2016-10-31 with the following search terms: (HPV OR Papillomaviridae[MeSH]) AND (oropharyngeal OR oropharynx OR tonsil OR tonsillar OR "base of tongue" OR "soft palate") AND (cancer OR carcinoma) AND (2016[DP] OR 2015[DP] OR 2014[DP] OR 2013[DP]). By consulting the PRISMA statement, which is a guide for performing good and valid systematic reviews and meta-analyses, the reviewing of our 1266 articles was initiated. After filtering out 230 review articles, 30 articles that were not written in English, and 41 articles without an abstract, 965 articles remained for further selections. The abstracts containing information about HPV data were further reviewed by reading the methods and results sections of the articles in order to determine whether to include or exclude the article. Articles reporting HPV data by PCR, ISH, or immunohistochemistry (p16), in "lymphoepithelial" oropharyngeal sub-sites (i.e. tonsillar and base of tongue) and in "non-lymphoepithelial" oropharyngeal sub-sites (i.e. walls of oropharynx, uvula and soft palate) in an unselected cohort (retrospective/prospective, randomized/non-randomized) were included. From each article, the number of patients with HPV$^+$ and HPV$^-$ tumors per sub-site were extracted or calculated, together with what HPV detection method was used. The most common reason for excluding a study, was that the oropharyngeal sub-sites were not specified. Ultimately 64 studies met the inclusion criterion, and from these 58 unique cohorts were identified.

Methodological considerations: A common issue with systematic reviews is that it may be difficult to know if the same patient cohort has been included in several studies, and thus may be reported twice within the same analysis. In our case, we managed by reading the articles and consulting the authors when suspecting the use of overlapping/or identical cohorts, to identify and exclude a couple of studies that were already included in the analysis. Furthermore, we restricted our analysis to patient cohorts included in reports published only during three consecutive years, in order to reduce the risk of double

reporting patients, yet still allowing for the inclusion of more than 11.000 patients. Moreover, it is important to cover all published articles in the study field and therefore the search terms are very important. Here we used very broad search terms, which rendered us with >1000 articles, where the loss of relevant articles hopefully should have been minimal.

3.2.10 Statistical analyses

In most papers (Paper I, II, IV, V) patient characteristics and categorial data were compared and analyzed using the Chi-squared test, or in small samples (n<100) using Fishers exact test. In case that mean values were compared, Student's t-test was used (Papers I, II, III, V). In paper IV, pooled odds ratios (OR) with 95% confidence intervals (CI) across studies using the Der Simonian and Laird random-effects methods were used. Two-tailed p-values were reported for all analyses, where a value ≤ 0.05 was considered statistically significant. For further details, please see each paper separately.

Survival/clinical outcome was measured in days from diagnosis until an event occurred, or until 3 years (Paper I, II), or 5 years (Paper V) after diagnosis, when all remaining patients where censored. Events were defined as death due to any cause (overall survival, OS), death with TSCC or BOTSCC present (disease-specific survival, DSS) or a recurrence in disease (disease-free survival, DFS). Patients who died without a documented TSCC or BOTSCC present were censored at time of death in DSS. Patients, who were never tumor free, were censored at day 0 in DFS. The Kaplan-Meier estimator was used for the estimation of OS, DSS, and DFS. Differences in survival were tested using the log-rank test, and only patients who were treated with the intention to cure were included in the analyses. In papers I and V, multivariate analyses, using the Cox proportional hazards ratio model, were used for calculations of adjusted and unadjusted hazard ratios (HRs). In paper II, the Spearman rank correlations test was used in order to test the correlation of different APM to each other and to HLA class I.

4 RESULTS AND DISCUSSION

4.1 PAPERS I & II

Correlation of LMP10 expression and clinical outcome in human papillomavirus (HPV) positive and HPV-negative tonsillar and base of tongue cancer (Paper I).

Reduced expression of the antigen processing machinery components TAP2, LMP2, and LMP7 in tonsillar and base of tongue cancer and implications for clinical outcome (Paper II).

Aims

To examine the potential role of the immunoproteasome units, LMP2, 7 and 10 and the antigen processing machinery components (APM) TAP1 and TAP2 as potential prognostic biomarkers in HPV$^+$ and HPV$^-$ TSCC and BOTSCC, as well as to study their possible co-dependence.

Background

Patients with HPV$^+$ TSCC and BOTSCC generally have a much better clinical outcome compared to patients with HPV$^-$ tumors (roughly 80% vs. 40-50% survival) [15, 101, 132]. Since current treatment often leads to severe side effects, de-escalation trials for patients with HPV$^+$ TSCC and BOTSCC with an expected excellent prognosis would be an attractive alternative. Therefore, there is a need for more biomarkers that together with HPV status would help identify patients that would be expected to have an excellent prognosis, and to benefit of de-escalated or targeted therapy. Previous publications had indicated that expression of HLA class I can be used as a prognostic marker in HPV$^+$ and HPV$^-$ TSCC and BOTSCC [200, 201]. Furthermore, a study by Meissner et al. suggested that some APM components and HLA class I had potential prognostic value [199]. Therefore, an important aim of these studies was to investigate markers associated with HLA class I expression and more specifically the molecules involved in preparation/processing of peptides for antigen presentation. The immunoproteasome subunits LMP2, LMP7, and LMP10 together with APM components TAP1 and TAP2 were therefore an obvious choice as potential prognostic biomarkers in HPV$^+$ and HPV$^-$ TSCC and BOTSCC. Another aim was to investigate the correlation of these components to one another as well as to HLA class I expression, and whether downregulation of AMP components follow HLA class I downregulation.

Material and methods

In **paper I**, in total 385 patients with TSCC and BOTSCC were diagnosed between 2000 and 2007 at the Karolinska University Hospital. From these 278 FFPE biopsies, with known HPV DNA status were tested for LMP10 nuclear and cytoplasmic expression by IHC, by studying the fraction of stained cells and intensity. The data was then correlated to clinical outcome.

In **paper II**, in total 151 FFPE TSCC and BOTSCC biopsies, with known HPV DNA and p16 status, were tested for LMP2, LMP7, TAP1 and TAP2 nuclear and cytoplasmic expression by IHC, by studying the fraction of stained cells and intensity. The studied molecules were correlated to each other, and to previously reported data on LMP10 and HLA class I, as well as to clinical outcome.

Main results

Paper I

- In HPV$^+$ TSCC and BOTSCC an absent/low LMP10 nuclear fraction was correlated to a better 3-year DFS.
- In HPV$^-$ TSCC and BOTSCC a moderate/high LMP10 cytoplasmic fraction, and a weak/moderate/high LMP10 cytoplasmic intensity was correlated to a better 3-year DFS.
- An absent/low expression of LMP10 was common in both HPV$^+$ and HPV$^-$ TSCC and BOTSCC.
- Fraction of nuclear LMP10 positive cells and HLA class I intensity were independ predictors of a 3-year DFS in HPV$^+$ TSCC and BOTSCC.
- Fraction of cells positive for cytoplasmic LMP10 and HLA class I were independ predictors of a 3-year DFS in HPV$^-$ TSCC and BOTSCC.

Paper II

- An absent/low expression of TAP2, LMP2 and LMP7 was common in both HPV$^+$ and HPV$^-$ TSCC and BOTSCC.
- TAP1 and TAP2 expression was correlated, as was LMP2 and LMP7 expression.
- LMP2 and LMP7 expression was correlated to HLA class I expression.
- Absence of nuclear LMP7 expression was correlated to increased DFS in both HPV$^+$ and HPV$^-$ TSCC and BOTSCC.

Discussion

By studying components of the APM, we were able to identify immunoproteasome components LMP10 and LMP7 as prognostic markers for both HPV[+] and HPV[-] TSCC and BOTSCC. More specifically, patients with HPV[+] tumors with an absent/low (0-25%) fraction of LMP10 stained nuclei had a better prognosis compared to those with a moderate/high (26-100%) fraction of LMP10 stained nuclei. In HPV[-] tumors, however, a moderate/high expression of LMP10 in the cytoplasm was instead correlated to a better survival. For LMP7, absence of nuclear LMP7 expression was correlated to a better prognosis in both HPV[+] and HPV[-] TSCC and BOTSCC. That a normal expression of LMP10 in the cytoplasm was correlated to a better prognosis compared to low levels, seemed logical, since low levels of LMP10 would suggest that the immune system is compromised in theses tumors, which previously has been shown is dismal for tumor clearance [212]. It is however harder to understand why a lower or absent fraction of nuclei, positive for LMP7 or 10, in HPV[+] tumors (and in HPV[-] for LMP7), would be of survival benefit. One possible explanation could be that the immunoproteasome also has secondary functions to protein processing, and that is the regulation of cell proliferation, differentiation, signaling, and gene transcription [213] A high expression of e.g. LMP10 and LMP7, could thus enhance cell survival and proliferation, thereby resulting in a poorer clinical outcome.

The function of the immunoproteasome has been described to take place in the cytoplasm, but whether the immunoproteasome has another function in the nucleus is unclear [160]. There are a few studies that have shown presence of immunoproteasome components in the nucleus, as we also do in this study [161–165]. Moreover, some studies have shown that the proteolytic activity of proteasomes also has been detected in the cell nucleus [214, 215], which could be a similar reason for immunoproteasomes to reside in the nucleus.

AMP components have been studied by IHC in other cancer types, such as bladder cancer and esophageal cancer, and similarly to our studies evaluated by both fraction and intensity of stained cells [216, 217]. These studies did however, not report any prognostic value of the markers examined, but showed, like us, that they often were downregulated.

Reduced expression of APM components, as well as reduced HLA class I surface expression has previously been shown, e.g. in HNSCC, laryngeal squamous cell carcinoma, cervical

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and urethral cancer, and is assumed to be mechanisms of immune evasion [199, 216–219]. In HNSCC and cervical carcinoma, expression of APM components TAP1, TAP2, LMP2, and LMP7 was commonly reduced, although LMP7 reduction was not as pronounced in the latter [199, 219]. In the HNSCC study by Meissner et al., frequencies of absent or low TAP1, TAP2, LMP2, and LMP7 expression were somewhat higher than those presented here, but still similar to our data [199]. Some described mechanisms for immune evasion due to decreased expression of APM components or HLA class I expression have previously been presented in section 1.5.3.

In paper II, we show that TAP1 and TAP2 expression were correlated to each other, as was LMP2 and LMP7 expression. Furthermore, LMP2 and LMP7 expression was also correlated to HLA class I expression. LMP10 expression was however not correlated to any of the studied components. This can possibly be explained because the gene for LMP10 is located on a different chromosome (chromosome 16) while the genes for TAP1, TAP2, LMP2 and LMP7, are located within a narrow region of the class II cluster of the major histocompatibility complex on chromosome 6 and are regulated together [220, 221].

The difference between the nuclear and cytoplasmic staining in regard to HPV status and survival is puzzling. It is also not known if HPV has the ability to influence the expression of the immunoproteasome components. It has however been shown that E5 has the potential to downregulate the expression of HLA class I [51, 174, 222]. Further investigations are needed to study possible interactions between HPV and the immunoproteasome components.

4.2 PAPER III

Effects of irradiation on human leukocyte antigen class I expression in human papillomavirus positive and negative base of tongue and mobile tongue squamous cell carcinoma cell lines.

Aim

To study whether irradiation affects HLA class I expression in HPV$^+$ and HPV$^-$ HNSCC, and primarily to investigate whether HLA class I expression is upregulated in HPV$^+$ cancer after irradiation.

Background

A previous study by our group showed that absent or low HLA class I expression in pre-treatment HPV$^+$ OPSCC was correlated to a good prognosis, whereas the opposite trend was observed in HPV$^-$ OPSCC [200]. The former is paradoxical, since HLA class I expression is important for recognition and killing of tumor cells by CD8$^+$ T cells and the number of CD8$^+$ TILs have been shown to be correlated to clinical outcome. However, it is also known that HPV can downregulate HLA class I expression, and therefore we were interested in examining whether irradiation could increase HLA class I expression, making HPV$^+$ TSCC and BOTSCC more sensitive to the immune system. Therefore, the aim of this study was to test whether radiotherapy increased HLA class I expression in HPV$^+$ and possibly also HPV$^-$ cancer cell lines.

Material and methods

HPV16 positive head and neck cancer cell lines UPCI-SCC-154, UPCI-SCC-090 and UM-SCC-47, and the HPV$^-$ cancer cell line UT-SCC-14, were treated with 2-10 Gray (Gy), or kept untreated as negative controls. Following characteristics were then compared between treated and non-treated cells: HLA class I expression, cell cycle changes, and apoptosis were examined using flowcytometry, and HPV16 E5, E7, and HLA-A mRNA expression was examined using real time PCR.

Main results

- A radiation dose of 10 Gy significantly increased HLA class I surface expression in two HPV$^+$ head and neck cancer cell lines, and a similar trend was observed in another HPV$^+$ cancer cell line, and in an HPV$^-$ cancer cell line.
- HLA-A class I mRNA did not change upon radiotherapy.

- HPV16 E5 mRNA expression significantly decreased in one out of three cell lines, whereas HPV16 E7 mRNA did not change upon radiotherapy.

- In all cell lines a shift towards G2/M phase, and increased apoptosis was observed after radiotherapy.

Discussion

The main finding of this study, was that HLA class I cell surface expression could potentially be increased by radiotherapy, at least for some cell lines, as shown significantly for 2/3 HPV$^+$ cell lines but not for the one HPV$^-$ cell line. This would partially explain, without too much emphasis, why some patients with an HPV$^+$ TSCC or BOTSCC with absent/low experession of HLA class I, before treatment, could have a good prognosis. Since, an increase in HLA class I expression may lead to increased presentation of tumor and viral antigens to the immune system, and especially if the tumor has high numbers of cytotoxic T cells (CD8$^+$) to recognize the antigen, and subsequently kill the tumor cell. Our group has previously shown that a high number of CD8$^+$ tumor infiltrating T cells in pretreatment biopsies from patients with TSCC or BOTSCC, which is more common in HPV$^+$ tumors, is correlated to a better survival [192]. A couple of other studies have also observed an increase of MHC class I expression after irradiation, in e.g. melanoma and murine colon cancer cell lines as well as *in vivo* [183], and in human colon and lung cancer cell lines [223].

It has been shown that the HPV viral genes E5 and E7 may be able to downregulate surface HLA class I expression [49–51, 174, 222]. Our results hint of a possible decrease in HPV E5 mRNA expression after radiation, which could therefore have an association to the observed increase HLA class I surface expression. These results are however only indicative and must be tested further in order to establish a correlation. On the other hand, we observed a tendency for increased HLA class I surface expression also in the HPV$^-$ cell line, which would indicate that another mechanism could be behind the increase.

One could further speculate as to why HLA class I expression would be upregulated upon radiotherapy. Under normal conditions the TAP transporters are not fully active, due to that the peptide pool forms the limiting factor in the pathway of antigen presentation [183]. However, stressful situations, such as an acute viral infection, may change that [183]. The rapid generation of viral proteins will lead to a massive increase in the numbers

of generated peptides, thereby enabling increased HLA class I presentation and a swift cytotoxic t cell response to the infection. This is what Reits et al. suggest happens, but instead of an infection, the radiation damage is suggested to increase the intracellular peptide pool, which in turn would lead to an upregulation of surface MHC class I expression [183]. For especially HPV+ tumors this may be extra beneficial, since increasing the peptide pool with both viral and tumor antigens would lead to more diverse peptides presented, which in turn may lead to a greater chance of cytotoxic killing.

In our study, we did not see an increase of HLA-A mRNA expression after radiotherapy, although there was a protein increase of cell surface HLA class I expression. However, increased protein expression does not always have to reflect an increased mRNA expression. It has e.g. been shown that HPV protein E5 can inhibit HLA class I from being expressed on the cell surface, by keeping them in the Golgi apparatus [51, 52]. Moreover, only mRNA expression of HLA-A was tested, while testing for more HLA groups, e.g. HLA-B, and HLA-C which were included in testing of surface HLA class I expression, would show a more representable picture of HLA class I mRNA expression levels.

A shift towards G2/M cell cycle arrest was observed in mainly cell lines UM-SCC-47 and UPCI-SCC-154, whereas a small tendency in shift towards G2/M was observed in the other two cell lines. This could indicate that radiotherapy has a larger effect on UM-SCC-47 and UPCI-SCC-157, whereas the other two are more radiotherapy resistant. Since UPCI-SCC-090 is a tumor recurrence [203], a greater resistance to therapy would not be surprising. UT-SCC-14 is an HPV- cancer cell line and HPV- tumors are in general known to have greater range of mutations compared to HPV+ tumors, and which therefore could maybe explain why this cell line also seems to be affected to a lesser extent by radiotherapy. On the other hand, all cell lines showed a significant and similar increase in apoptotic cells. We were, however, not able to measure exact percentages of apoptosis, since many cells were already fragmented at the time of measurement, thus the total number of cells undergoing/had underwent apoptosis may vary more than that reported between the different cell lines.

Our results should be interpreted with caution, mainly because all experiments were performed *in vitro* on a limited amount of cell lines. *In vitro*, many other cell types and molecules are missing, that would be present *in vivo*. E.g. the interplay with the immune

cells as well as a possible cytokine release by these, as a result of irradiation, would be missing *in vitro* [224]. One example of this is INF-γ, a pro-inflammatory cytokine, that is assumed to trigger the immunoproteasome activity [160]. Nonetheless, we observed a consistent tendency for increased surface HLA class I expression after radiotherapy in all cell lines, thereby suggesting that HLA class I expression could indeed increase after radiotherapy.

As already mentioned briefly in section 3.1 it would have been preferred to include more both HPV+ and HPV- cell lines that were exclusively derived from TSCC or BOTSCC that had a suppressed HLA class I expression. However, cell lines with these naturally occurring, specific properties do not exist, and it is also very difficult to establish OPSCC cell lines that retain their HPV infection. In this study at least one cell line was not derived from TSCC or BOTSCC, and was instead derived from the mobile tongue. Another cell line used is described as originating from the lateral tongue, however it is not published whether it is derived from a base of tongue or mobile tongue cancer. On the other hand, since this cell line expresses HPV E6 and E7 mRNA, this indicates that it is HPV-driven.

In conclusion, our results show that surface HLA class I expression may be increased by radiotherapy. This could explain why patients with an HPV+ TSCC or BOTSCC with an absent or low HLA class I expression before treatment, still have a good prognosis, where radiotherapy would lead to more cytotoxic killing through an increase in HLA class I expression.

4.3   PAPER IV

Time to change perspectives on HPV in oropharyngeal cancer. A systematic review of HPV prevalence per oropharyngeal sub-site the last 3 years.

Aim

To investigate HPV prevalence per OPSCC sub-site by performing a systematic review and meta-analysis.

Background

HPV is a well-established risk factor in OPSCC, however accumulating data suggest that grouping all OPSCC together is too unspecific in regard to HPV prevalence, and to its clinical importance [77, 185, 208, 225]. Our group has previously published that HPV is mainly observed in TSCC and BOTSCC, and less frequently found in OPSCC sub-sites outside the tonsil and the base of tongue [185]. Furthermore, in the same study, HPV only had prognostic influence in TSCC and BOTSCC and not for other OPSCC sub-sites [162]. To, validate this study, it would be useful to perform a systematic review and meta-analysis on a large number of patients, since they would likely provide us with more robust data. This was therefore the aim of this study.

Material and methods

The database PubMed was searched, and studies reporting HPV data (p16/HPV DNA/HPV RNA) in both "lymphoepithelial" and "non-lymphoepithelial" sites were included. HPV prevalence was compared between histological sites and pooled odds ratios by HPV detection method were analyzed using a random effects model.

Main results

- HPV prevalence was 56% and significanlty higher in the "lymphoepithelial" sites (TSCC and BOTSCC) compared to 19% in the "non-lymphoepithelial" sites (cancer of the soft palate and posterior walls).
- HPV prevalence was 59% in TSCC, 40% in BOTSCC, 19% in cancer of the posterior wall, and 12% in cancer of the soft palate.
- Significant association of HPV to "lymphoepithelial" vs. "non-lymphoepithelial" sites was observed independent of HPV detection method.
- Statistical homogeneity was only observed when data from studies using p16 overexpression together with presence of HPV DNA as detection method were

pooled. Statistical homogeneity was not observed for studies reporting p16 or HPV DNA only.

Discussion

It did not entirely come as a surprise that this review showed that HPV was more prevalent in the "lymphoepithelial" sites compared to "non-lymphoepithelial" sites, since other studies have suggested these differences before [185, 226]. However, by summarizing, for the first time, a large amount of data in a systematic review, we now emphasize and suggest that we have great support that the concept of OPSCC is too unspecific in regard to HPV prevalence. Moreover, several studies show that the prognostic value of HPV in "non-lymphoepithelial" sites is more uncertain [77, 185, 208, 225]. These findings are important for understanding the differences in these diseases, and for the planning of future treatment strategies of HPV$^+$ and HPV$^-$ OPSCC.

Unfortunately, today most studies do not specify the oropharyngeal sub-sites (not separating by oropharyngeal sub-site was the most common exclusion criterion in this study). This is problematic, since obtained data will become more difficult to compare between studies, and this way some valuable information may be lost. In Sweden for example, only cancers of the "lymphoepithelial" sites are increasing, whereas the incidence of cancers of the "non-lymphoepithelial" sites have remained stable, according to the Swedish Head and Neck Cancer Register (SweHNCR). This difference in incidence could easily have been missed if the incidence for all OPSCC had been reported together, especially since the "non-lymphoepithelial" cancers are much fewer than "lymphoepithelial" cancers.

The histology of cancer of the "non-lymphoepithelial" sites is more similar to the histology of the oral cavity [227], moreover, HPV prevalence is also lower and more similar in these sites compared to the "lymphoepithelial sites". This indicates that the etiology of cancer of the "non-lymphoepithelial" sites may be similar to oral cancer.

Our findings show that HPV was significantly more common in the "lymphoepithelial sites", irrespective of what HPV detection method was used. However, statistical homogeneity was only observed when studies using algorithm-based HPV detection were pooled. This finding suggests that data produced by the algorithm-based HPV detection method is more consistent as compared the data presented using only HPV DNA or p16 as

detection method. Since a proportion (ca 10-15%) of HPV DNA$^-$ OPSCC are p16$^+$, data produced by testing p16 only, will especially for OPSCC sites with a low HPV prevalence include a large number of p16$^+$/HPV DNA$^-$ tumors. This does however not explain the inconsistency in data from studies using HPV DNA only as detection method.

Since countless studies have shown that patients with HPV$^+$ OPSCC have great survival rates, suggestions have been made to reduce therapy for this patient group [228]. However, cancer of the "non-lymphoepithelial" sites are a minority of OPSCC (<20% in Sweden [72]), and HPV prevalence is also lower in this site. This may have led to the publication of survival studies on HPV$^+$ OPSCC, where the high survival rates of HPV$^+$ cancer of the "lymphoepithelial" sites overshadow the survival rates of cancer from the "non-lymphoepithelial" sites. We suggest that this implies that the latter patient group may not benefit from reduced therapy. Therefore, it is important that before de-escalation trials are introduced, minor subpopulations, such as the one just described, are identified. Moreover, the introduction of the new staging system for p16$^+$ OPSCC may classify cancer from the "non-lymphoepithelial" sites with a lower tumor stage than would be accurate. This is notably not the only issue with the new staging system. As already mentioned, some p16$^+$ OPSCC cases are HPV DNA$^-$, and as shown by e.g. Rietbergen et al., patients with p16$^+$/HPV DNA$^-$ tumors have a worse prognosis than those with p16$^+$/HPV DNA$^+$ tumors, and also p16$^+$/HPV DNA$^-$ tumors are genetically more similar to p16$^-$/HPV DNA$^-$ tumors [113, 114].

There is a small risk for misclassification of tumors of the oropharynx, which could have influenced the reported HPV prevalence of the different sub-sites. Especially, large mobile tongue cancers can be mistaken for BOTSCC and vice versa. Therefore, we performed a second analysis comparing HPV prevalence from only the TSCC sub-site to the "non-lymphoepithelial" site, which showed an even more pronounced difference in prevalence. However, if possible misclassifications are included in our analysis, these have likely only decreased the difference in HPV prevalence between sites, and since the differences are significant this does not impact our main finding.

A question that remains answering is, however, if even further sub-classification of OPSCC could be of importance, and this will in part be answered in Paper V.

4.4 PAPER V

Human papillomavirus (HPV) and survival of patients per histological sub-site of tonsillar squamous cell carcinoma (TSCC).

Aim

To study the impact of histological context on HPV prevalence and survival in TSCC, by sub-classifying TSCC into specified and non-specified TSCC (STSCC and NSTSCC).

Background

Current data advocate that OPSCC should be divided into sub-sites, when evaluating the presence of HPV and prognosis [77, 185, 208, 225]. More specifically, TSCC and BOTSCC have much higher HPV prevalence compared to other OPSCC, as presented in Paper IV, and the prognostic value of HPV in oropharyngeal sub-sites besides TSCC and BOTSCC is unclear [185, 208, 225]. In a recent report from Denmark, TSCC was further sub-classified into specified TSCC (STSCC) and non-specified TSCC (NSTSCC), with HPV significantly more prevalent in STSCC [208, 225].

Material and methods

In total, 203 FFPE TSCC biopsies (ICD-7 145.0), stained with hematoxylin and eosin, from patients diagnosed between 1970 and 2002 in Stockholm, were evaluated for presence of both tumor and adjacent normal tissue. From these 139 samples met the inclusion criteria, and were separated into STSCC and NSTSCC, and together with HPV status correlated to clinical outcome.

Main results

- HPV was significantly more common in STSCC compared to NSTSCC (75% vs. 20%).
- Patients with HPV$^+$ STSCC had a better DSS and OS as compared to patients with HPV$^+$ NSTSCC.
- No survival differences were observed in patients with HPV$^-$ STSCC and NSTSCC.

Discussion

We were able to confirm that HPV was more significantly common in the STSCC compared to NSTSCC, as previously published by Garnaes et al. [208, 225], and that patients with an HPV$^+$ STSCC had a better clinical outcome compared to HPV$^+$ NSTSCC. On the contrary,

HPV was significantly less prevalent in NSTSCC, and the histomorphological context (STSCC or NSTSCC) of HPV⁻ TSCC did not affect patients' prognosis.

As already discussed and shown to some extent in Paper IV, the oropharynx is a heterogenous site, and HPV is more commonly found the "lymphoepithelial" sub-sites of oropharynx, comprising TSCC and BOTSCC, as compared to the "non-lymphoepithelial" sites, comprising cancer of the soft palate and posterior walls [128]. However, with the findings of this study, we show that even the sites classified as "lymphoepithelial" sites may not be specific enough in regard to HPV prevalence and clinical outcome.

Since the tumor material used for this study is quite old (from 1970-2002), ICD-7 was used for classifying these tumors. Here patients with ICD-7 code 145.0 were included. ICD-7 code 145.0 translates into ICD-10 codes C02.4 (lingual tonsil), C09.0 (tonsillar fossa), C09.9 (tonsil, unspecified), and C14.2 (Waldeyer's ring) [229]. This indicates that different tonsillar sub-sites were included in the studied cohort. We do not know whether these different tonsillar sites would separate into different groups when dividing by STSCC and NSTSCC, however this could be a possible explanation to why we observed differences in histology surrounding the tumors. Therefore, it would be interesting to repeat this study on a newer TSCC cohort using ICD-10 codes.

One can also speculate whether tissue fractions and sections taken from a specimen of the studied tumor is representable for the entire tumor. Notably a number of patients had to be excluded from this study when no surrounding normal tissue or tumor was found. For this reason, this method is not optimal, but for the HPV⁺ STSCC patient group, this type of histological evaluation may still be of benefit and clinically applicable. Moreover, there are some described histological differences for HPV⁺ and HPV⁻ TSCC and BOTCC [230, 231]. HPV⁺ tumors often present with a lack of keratinization, growing in distinct areas with pushing borders, together with a basaloid morphology. Whereas, HPV⁻ TSCC and BOTSCC often present with keratinization, cytoplasm-rich cells with distinct cell borders that grow more infiltrative, without the distinct basaloid morphology found in HPV⁺ tumors [230, 231]. These differences may also affect how our samples have been classified, due to that the "normal tissue" may be more affected by an HPV⁻ tumor with e.g. a desmoplastic stroma, whereas the "normal tissue" in HPV⁺ tumors may retain more original features, such as e.g. germinal centers.

Another limitation to this study was that HPV positivity was only defined by HPV DNA due to that no tumor sections were available for p16 evaluation, however in previously published cohorts, that partly overlap with this one, we have shown that p16 overexpression was significantly associated and concordant with HPV$^+$ DNA status [79, 200]. On the other hand, because the studied cohort was diagnosed before treatment was intensified in OPSCC in the Stockholm region, the cohort was more homogenously treated, in that patients only received radiotherapy, surgery, or both.

To conclude, HPV$^+$ status was more commonly found in STSCC, whereas the opposite was observed in NSTSCC, where HPV$^-$ status was more common. Moreover, HPV-positivity was correlated to a good prognosis in the context of STSCC, but not in NSTSCC. Our findings indicate that the context of the tumor is important for tumor prognostication and evaluating HPV prevalence. Lastly, the concept of OPSCC is still too unspecific when sub-grouping patients into groups with HPV$^+$ and HPV$^-$ tumors.

5 CONCLUSIONS

- The immunoproteasome component LMP10 can potentially be used as an independent prognostic marker in both HPV$^+$ and HPV$^-$ TSCC and BOTSCC. In patients with HPV$^+$ tumors, low or absent nuclear LMP10 expression was correlated to better DFS. In patients with HPV$^-$ tumors moderate or high cytoplasmic intensity was correlated to a better DFS (Paper I).

- Expression of antigen processing machinary components TAP2, LMP2, LMP7, and LMP10 is commonly suppressed in both HPV$^+$ and HPV$^-$ TSCC and BOTSCC (Papers I and II).

- Absence of nuclear immunoproteasome component LMP7 expression is correlated to a better DFS in patients with HPV$^+$ or HPV$^-$ TSCC and BOTSCC (Paper II).

- Our results indicate that radiotherapy has the ability to increase cell surface HLA class I expression in HPV$^+$ head and neck cancer cell lines. Although further studies are needed, our findings suggest that radiotherapy may possibly potentiate the immune response to HPV$^+$ tumors, where viral antigens may contribute to immune recognition (Paper III).

- When studying HPV in OPSCC, HPV prevalence was found significantly higher in "lymphoepithelial" sites, which include TSCC and BOTSCC, as compared to the "non-lymphoepithelial" sites, which include cancer of the soft palate and posterior walls. This suggests and supports that the concept of OPSCC is too unspecific in regard to HPV prevalence. (Paper IV).

- HPV is significantly more common in STSCC compared to NSTSCC, and patients with HPV$^+$ STSCC have a better clinical outcome compared to patients with HPV$^+$ NSTSCC. No survival differences were observed in patients with HPV$^-$ STSCC and NSTSCC. This suggests that the histological context is important for evaluating HPV prevalence and tumor prognostication in TSCC and that the concept of OPSCC is too unspecific in regard to HPV prevalence and clinical importance (Paper V).

6 FUTURE PERSPECTIVES

There is great promise that the vaccines against HPV will drastically decrease the number of HPV-related cancers in the future. However, there is a long way to go before the majority of the world population will be vaccinated and herd immunity can be achieved. In addition, there is a long lag period (20-40 years) between HPV-infection and detection of cancer. In the meantime, the number of patients with HPV related OPSCCs will likely continue to increase, as will the need for improved prevention, prognostication and treatment. Moreover, patients with OPSCC, caused by factors other than HPV, is a patient group with poor prognosis, where the need for new treatment options is large. The target is that each patient is given an optimal treatment with a high probability of tumor clearance in combination with minimized side effects.

Papers I and II have led to the discovery of new prognostic markers in HPV$^+$ and HPV$^-$ OPSCC that could potentially be used in combination with other markers to predict patient outcome, and thereby personalize the treatment accordingly. Moreover, these studies may contribute to a deeper understanding of the immunological aspects in HPV$^+$ and HPV$^-$ OPSCC, and this way potentially be helpful in the development of immunotherapies.

Paper III demonstrates how radiotherapy may affect tumors in regard to HLA class I expression, which may trigger the immune system and induce tumor clearance. Since paper III only presents *in vitro* data, performing similar studies on patient material would be very interesting. Future experiments could include studying immunological components pre- and post-treatment, in a patient-derived xenograft mouse model, or preferentially directly in human biopsies, which ethically however, could be more difficult.

Papers IV and V highlight the importance of studying cancer of the oropharyngeal sub-sites separately, and that also the tissue surrounding the tumor may provide additional information. These studies can potentially lay ground for improving ways of grading OPSCC and increase the understanding of where HPV driven tumors arise.

The studies included in my thesis will hopefully, in one way or other, contribute to my goal of improved prevention, prognostication, and treatment, in both patients with HPV$^+$ and HPV$^-$ OPSCC.

7 ACKNOWLEDGEMENTS

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