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HUNTING THE END OF THE RAINBOW: PROGNOSTIC BIOMARKERS AND HUMAN PAPILLOMAVIRUS IN TONSILLAR AND BASE OF TONGUE CANCER

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HUNTING THE END OF THE RAINBOW: PROGNOSTIC BIOMARKERS AND HUMAN PAPILLOMAVIRUS IN TONSILLAR AND BASE OF TONGUE CANCER

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family…

A few words about the title:

The end of the rainbow…Some might think that it is impossible to find the end of the rainbow, some may say that it is a physical phenomenon and play of the light…However, there are stories and legends talking about a treasure at the end of the rainbow…Except from the treasure the most important is to try and go together with the colors of it…try and become a part of it and always try to ask yourself if it is worth believing in it or no…I do believe that we always have to use the facts and our imagination in order to be able one day, together with the colors of the rainbow to reach the "treasure" at the end of it… In our case and when it comes to medical science, the end of the rainbow will always be for the benefit of the patients…

ABSTRACT

Aim. The aim of this thesis was to hunt for additional prognostic biomarkers in HPV positive TSCC and BOTSCC to identify patients that could if they wished, be enrolled in clinical trials for de-escalation of treatment.

Background. Tonsillar squamous cell carcinoma (TSCC) and base of tongue squamous cell carcinoma (BOTSCC), which account for most oropharyngeal squamous cell carcinomas (OSCC) have been increasing in the last decades and now comprise almost half of all head and neck cancers (HNSCC) in Sweden. The main risk factors for HNSCC were originally considered to be smoking and alcohol however in 2007 the International Agency for Research on Cancer (IARC) acknowledged HPV infection as a risk factor for OSCC. It has been shown that patients with HPV positive OSCC and especially TSCC and BOTSCC have a much better clinical outcome (80% 3 year disease free survival, DFS) as compared to for patients with the corresponding HPV negative tumors (40%). Due the generally poor prognosis for HNSCC, treatment for HNSCC including TSCC and BOTSCC has been intensified, leading to more serious side effects for the patients. It has been proposed that due to the good prognosis of patients with HPV positive TSCC and BOTSCC such an aggressive treatment might be unnecessary and de-escalation of treatment would be of benefit. However, since 20% of patients with HPV positive tumors do not do well, additional prognostic biomarkers are needed for selecting patients that would be the best responders to less intensive treatment.

Results. In paper I, high numbers of CD8+ tumor infiltrating lymphocytes (TILs) were shown to correlate with a favorable clinical outcome in both HPV DNA+ and HPV DNA- TSCC and BOTSCC. In addition, HPV DNA+ tumors were significantly more infiltrated than the HPV DNA- ones. In paper II, absence of HLA class I in HPV DNA+ OSCC was shown to be associated with increased survival of the patients while the opposite was true for normal expression. In paper III, the HLA-A*02 allele, common in the Scandinavian population, was demonstrated to be a negative prognostic factor for patients with HPV DNA+ TSCC and BOTSCC. Following that study, in papers IV and V, the expression of components of the antigen processing machinery (APM) were examined for nuclear and cytoplasmic staining and the absence of LMP10 nuclear staining as well as low LMP7 expression were disclosed to be correlated with increased survival of patients with HPV DNA+ TSCC and BOTSCC. Finally, in paper VI, biomarkers CD8+ TILs, HLA class I, HLA-A*02 and LMP10 were combined with clinical characteristics of age, stage of the patients with HPV DNA+ TSCC and BOTSCC in order to develop an algorithm allowing for prediction of the outcome of the patients and CD8+ TILs, age and stage of the patients were found to be most significant prognostic contributors.

Conclusion. Investigating and combining our best prognostic biomarkers, we have developed an algorithm which makes use of different biomarker and clinical characteristics allowing for prediction of patients with good clinical outcome that will enable de-escalation of treatment.

ΠΕΡΙΛΗΨΗ

Σκοπός: Η εξεύρεση επιπρόσθετων προγνωστικών βιοδεικτών, σε ογκούς των αμυγδαλών και της βάσεως της γλώσσας σχετιζόμενους με την ύπαρξη του ιού των ανθρωπίνων κονδυλωμάτων (HPV). Η εξεύρεση αυτή των επιπρόσθετων βιοδεικτών μπορεί να οδηγήσει στην εξαγωγή πιο ασφαλών συμπερασμάτων σχετικά με την πρόγνωση της κλινική τους εικόνας. Η ασφαλέστερη αυτή πρόγνωση, θα μπορούσε να χρησιμοποιηθεί για την επιλογή ασθενών που θα μπορούσαν να συμμετάσχουν σε κλινικές δοκιμές, αυτοβούλως, με μοναδικό σκόπο την χρησιμοποίηση μιας πιο ήπιας και με λιγότερες επιπλοκές θεραπείας.

Τι είναι γνωστό μέχρι τώρα: Η συχνότητα εμφανίσεως του καρκίνου των αμυγδαλών και της βάσεως της γλώσσας, που αποτελούν την πλειοψηφία των καρκίνων που διαγνώσκονται στην περιοχή του οροφάρυγγα, έχει αυξηθεί τις τελευταίες δεκαετίες, και σήμερα καταλαμβάνει περίπου το 50% των καρκίνων στην περιοχή του κεφαλιού και του λαιμού, στην Σουηδία. Οι κυριότεροι παράγοντες σχετιζόμενοι με την συγκεκριμμένη μορφή καρκίνου θεωρούνταν και είναι το κάπνισμα και η κατανάλωση αλκοόλ. Εντούτοις, το 2007 η Διεθνής Αντιπροσωπεία για την έρευνα στον καρκίνο (IARC) αναγνώρισε την μόλυνση με τον ιό των ανθρωπίνων κονδυλωμάτων ώς έναν επιπλέον καθοριστικό παράγοντα για την ανάπτυξη του συγκεκριμένου τύπου καρκίνου. Έχει αποδειχθεί από επιστημονικές μελέτες – με έναν από τους πρωτοπόρους στο πεδίο την κ. Δαλιάνη – ότι ασθενείς με καρκίνο των αμυγδαλών ή της βάσεως της γλώσσας που σχετίζοντε με την ύπαρξή του ιού, παρουσιάζουν πόλυ καλύτερη κλινική εικόνα κατόπιν θεραπείας, όταν συγκριθούν με αντίστοιχους ασθενείς των οποίων ο όγκος δεν σχετίζεται με την ύπαρξη του ιού (80% έναντι 40% ποσοστό επιβίωσης το λιγότερο 3 χρόνια μετά την διάγνωση). Εξαιτίας του γεγονότος ότι όγκοι που διαγνώσκονται στην περιοχή του κεφαλιού και του λαιμού σχετίζονται με σχετικά χαμηλά ποσοστά επιβίωσης των ασθενών αυτών (40%) η χορηγούμενη θεραπευτική αγωγή είναι εξαιρετικά αυξημένης έντασης. Όπως μπορεί εύκολα να γίνει κατανοητό, η αυξημένης αυτής έντασης θεραπεία, που κατα κύριο λόγο περιλαμβάνει ακτινοθεραπεία και χημειοθεραπεία, οδηγεί σε μεγαλύτερης εντάσεως βραχυπρόθεσμες και μακροπρόθεσμες παρενέργειες (ξηροστομία, αδυναμία κατάπωσης, προβλήματα ομιλίας). Εντούτοις, όπως προαναφέρθηκε, ασθενείς με όγκους θετικούς στον ιό HPV, παρουσιάζουν πολύ καλύτερη κλινική εικόνα και υπάρχει μια τάση στην επιστημονική κοινότητα σχετικά με την μείωση της έντασης της χορηγούμενης θεραπείας, που θα μπορούσε να οδηγήσει σε μείωση των προαναφερθέντων αρνητικών συνεπειών. Εξαιτίας του γεγονότος όμως ότι ένα 20% των ασθενών με θετικούς στον ιό όγκους δεν παρουσιάζει καλή κλινική εικόνα, επιπρόσθετοι βιοδείκτες είναι αναγκαίοι για την επιλογή ασθενών σε μελλοντικές δοκιμές για μείωση της έντασης της θεραπείας.

Αποτελέσματα: Στην πρώτη μελέτη, επικεντρωθήκαμε στην μελέτη του αριθμού των κυτταροτοξικών Τ λεμφοκυττάρων. Δείξαμε ότι υψηλός αριθμός των λεμφοκυττάρων αυτών όταν έχουν διεισδύσει στην μάζα του καρκινικού όγκου σχετίζονται με καλή κλινική εικόνα των ασθενών με όγκους θετικούς στον ιό αλλά και στους μή θετικούς όγκους. Επιπροσθέτως, οι θετικοί στον ιό όγκοι βρέθηκαν να σχετίζονται με υψηλότερους αριθμούς Τ λεμφοκυττάρων στο εσωτερικό των όγκων. Στην δεύτερη κατα σειρά μελέτη μας επικεντρωθήκαμε στην μελέτη της έκφρασης του μείζονος συστήματος ιστοσυμβατότητας (HLA), το οποίο σχετίζεται με την παρουσίαση ενδοκυττάριων αντιγόνων στο εξωτερικό της κυτταρικής μεμβράνης των κυττάρων. Καταφέραμε να δείξουμε ότι απουσία του συστήματος αυτού από την επιφάνεια των κυττάρων σχετίζεται με εξαιρετικά μεγάλο ποσοστό επιβίωσης των ασθενών με όγκους θετικούς στον ιό, ενώ αντίθετη εικόνα παρουσιάζεται για ασθενείς με μη θετικούς όγκους. Στην τρίτη μελέτη ασχοληθήκαμε με το ευρέως διαδεδομένο στην Σκακδιναβία αλληλόμορφο HLA-A*02, του μείζονος συστήματος ιστοσυμβατότητας. Η ύπαρξη του αλληλομόρφου αυτού σχετίζεται με μειωμένα ποσοστά επιβίωσης των ασθενών με θετικούς στον ιό όγκους. Στην τέταρτη και πέμπτη μελέτη μας επικεντρωθήκαμε στην μελέτη της έκφρασης πρωτεϊνικών υπομονάδων του πρώτεασώματος και του ενδοκυτταρικού μονοπατιού υπεύθυνου για την επεξεργασία και την παρουσίαση των ενδοκυττάρικών αντιγόνων από το σύμπλεγμα που προαναφέραμε. Στις δύο αυτές μελέτες δείξαμε ότι απουσία έκφρασης, όπως εντοπίζεται με την χρήση ανοσοϊστοχημείας, των πρωτεοσωμικών υπομονάδων LMP10 και LMP7, σχετίζεται με αυξημένα ποσοστά επιβίωσης ασθενών με θετικούς στον ιό όγκους. Τελικά, στην έκτη μελέτη μας όλοι οι προαναφερθέντες βιοδείκτες, των προηγούμενων μελετών, συνδιάστηκαν με κλινικά χαρακτηριστικά των ασθενών όπως η ηλικία, το στάδιο του καρκίνου και η διάγνωση με στόχο την δημιουργία ενός αλγορίθμου ικανού να παρέχει στοιχεία σχετικά με την πρόγνωση των ασθενών βασιζόμενο σε όλα τα προαναφερθέντα χαρακτηριστικά. Η ηλικία, ο υψηλός αριθμός των λεμφοκυττάρων στο εσωτερικό των όγκων καθώς επίσης και το στάδιο του καρκίνου βρέθηκαν να είναι οι πιο ισχυροί παράγοντες ικανοί να παρέχουν πληροφορίες σχετικά με την επιβίωση και την κλινική εικόνα των ασθενών.

Επίλογος: Βασιζόμενοι στα στοιχεία και τα ευρήματα των μελετών μας όσον αφορά του προγνωστικούς βιοδείκτες και στην ταυτόχρονη χρήση κλινικών χαρακτηριστικών των ασθενών με θετικούς στον ιό όγκους, καταφέραμε να δημιουργήσουμε έναν αλγόριθμο ικανό να παρέχει στοιχεία σχετικά με την πρόγνωση των ασθενών αυτών, οι οποίοι θα μπορούσαν αργότερα να συμμετάσχουιη σε κλινικές δοκιμές για εφαρμογή μικρότερης έντασης θεραπείας. Φυσικά επιπρόσθετες μελέτες χρειάζονται προκειμένου να αποδειχθεί η ορθότητα το αλγορίθμου και επιπλέον βιοδείκτες θα μπορούσαν να καταστήσουν τον αλγόριθμο αυτό περισσότερο ικανό για ασφαλέστερες προβλέψεις. Η προσπάθεια συνεχίζεται...

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. Nordfors C*, Grün N*, **Tertipis N***, Ährlund-Richter A, Haeggblom L, Sivars L, Du J, Nyberg T, Marklund L, Munck-Wikland E, Näsman A, Ramqvist T, 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*

II. Näsman A, Andersson E, Marklund L, **Tertipis N**, Hammarstedt-Nordenvall L, Attner P, Nyberg T, Masucci GV, Munck-Wikland E, Ramqvist T, Dalianis T HLA class I and II expression in oropharyngeal squamous cell carcinoma in relation to tumor HPV status and clinical outcome. *PLoS One. 2013 Oct 10;8(10):e77025.*

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*Contributed equally

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

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II. Sivars L, Näsman A, **Tertipis N**, Vlastos A, Ramqvist T, Dalianis T, Munck-Wikland E, Nordemar S. Human papillomavirus and p53 expression in cancer of unknown primary in the head and neck region in relation to clinical outcome. *Cancer Med. 2014 Apr;3(2):376-84*

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LIST OF ABBREVIATIONS

APC	antigen presenting cells
APM	antigen processing machinery
ATP	adenosine triphosphate
BOTSCC	base of tongue squamous cell carcinoma
CDK	cyclin dependent kinases
CD	cluster of differentiation
CIN	cervical intraepithelial neoplasia
CRT	chemoradiotherapy
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DFS	disease free survival
DNA	deoxyribonucleic acid
D/R	death/relapse
DSS	disease specific survival
E	early region
E6AP	E6 associated protein
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
EV	epidermodysplasia veruciformis
FFPE	formalin fixed paraffin embedded
GA	Golgi apparatus
HC	heavy chain
HLA	human leukocyte antigen
HNSCC	head neck squamous cell carcinoma
HPV	human papilloma virus
HPV DNA+	human papillomavirus DNA positive
HPV DNA-	human papillomavirus DNA negative
HSV	herpes simplex virus
IARC	International Agency for Research on Cancer

ICD	International classification of diseases
IFN	interferon
IFN-γ	interferon-gamma
IHC	immunohistochemistry
IL	interleukin
IRF	interferon regulatory factor
L	late region
LC	Langerhans cells
LCR	long control region
LMP	low molecular weight polypeptide
HLA	major histocompatibility complex
mRNA	messenger ribonucleic acid
NALT	nasal associated lymphoid tissue
NCR	non-coding region
NK	natural killer cells
ORF	open reading frame
OS	overall survival
OSCC	oropharyngeal squamous cell carcinoma
P16+	$p16^{ink4a}$ overexpression in >70% of malignant cells
P16-	$p16^{ink4a}$ absent or weak in <70% of malignant cells
pA	poly-adenylation
Pap	Papanicolaou
PAMP	pathogen associated molecular patterns
PCR	polymerase chain reaction
PD	programmed cell death protein
PRR	pattern recognition receptor
PSA	prostate specific antigen
Rb	retinoblastoma
RNA	ribonucleic acid
RT	radiotherapy
RSV	Rous sarcoma virus

SCC	squamous cell carcinoma
TAP	antigen peptide transporter
TGF	tumor growth factor
Th	T helper
TIL	tumor infiltrating lymphocytes
TLR	toll-like receptor
TNF	tumor necrosis factor
TNF- α	tumor necrosis factor alpha
Treg	regulatory T cells
TSCC	tonsillar squamous cell carcinoma
UICC	International Union Against Cancer
UCR	upstream control region
VLP	virus like particle
b2m	beta 2 microglobulin

1 INTRODUCTION

The human body has been designed to keep invaders away and to protect itself from exogenous and endogenous enemies. There are different ways of doing this, comprising of natural and physical barriers, such as skin and mucosa as well as internally built in or acquired mechanisms such as innate and adaptive immunity.

One of the "internal" enemies that has been recognized since old times is cancer. Cancer was recognized as a disease several thousand years ago and a lot of different theories regarding the etiology of the disease have been described over the years[1].

Among other theories, infectious agents were linked with cancer development and as early as 1907, an Italian physician named Giusseppe Ciuffo described the viral etiology of human warts. Nevertheless, this finding was not appreciated by the scientific community and it took several decades before scientists realized that infectious agents (viruses) were linked to human malignancies. Before that, already in 1908, two Danish scientists, Vilhelm Ellerman and Olaf Bang showed that a cell-free filtrate of chicken leukemia cells was able to induce the disease in healthy chicken[2].

The big revolution within the field of tumour virology was in 1911, when Peyton Rous showed that the inoculation of cell-free extracts from sarcomas of diseased chickens into healthy chickens led to tumour development, describing for the first time transmission of a tumor virus (Rous Sarcoma Virus, RSV)[3]. This led to a new paradigm in cancer research. Despite the fact that his research was not understood in the beginning, the recognition of the importance of his finding lead to the Nobel Prize award in 1966. After the initial detection and confirmation of the RSV as an agent capable of causing tumour development, the field of tumour virology was expanded and more tumour viruses were discovered between 1930s and 1960s. So, is cancer a deregulation of the homeostasis of the cell, performed by external factors? The question still remains to be answered in the future.

For now let us begin our journey…

As described previously, the physical barriers in our bodies consist of the skin and the mucosa, which covers the openings in our bodies (e.g. mouth, colon, vagina, cervix, penis). Under normal circumstances, these physical barriers cannot be overcome by invaders and we remain healthy and uninfected. However, sometimes micro-injuries and openings can occur which enables the invaders and exogenous factors to enter into our body and cells. Such mechanisms are e.g. used by viruses and in this case by human papillomavirus (HPV).

This thesis will mainly focus on HPV and components involved in immune recognition and presentation and cells of the immune system, in that they may serve as prognostic biomarkers in patients with oropharyngeal cancer and mainly tonsillar and base of tongue cancer.

1.1 HUMAN PAPILLOMAVIRUS (HPV)

1.1.1 HPV and cancer, a brief history

Professor Harald zur Hausen was awarded the Nobel Prize in 2008 for the discovery that the causative factor for cervical carcinogenesis was HPV. However, it had been noted much earlier that prostitutes and married women had a higher frequency of cervical cancer than nuns and virgins in a study performed between 1760 and 1839 in Verona Italy. This higher incidence was assumed to be due to sexual contact and an unknown factor that was causing the cancer[4].

Studies were then initiated in order to identify the unknown factor and in the beginning the carcinogenesis mechanism was mainly attributed to herpes simplex virus 2 (HSV2) but studies failed to prove this assumption. During the 1970's HSV2 was substituted by HPV and after many attempts finally, in 1982, the genome of HPV16 was sequenced from cervical cancer biopsies[5, 6]. In 1983, Southern blot from cervical cancer specimens by zur Hausen and his colleagues demonstrated DNA from HPV16. Following that the genome of HPV was isolated from other cancer types mainly from the anogenital region (e.g. vulva, vagina, penis and anus)[7]. Furthermore, already during this period there was a suspicion of an association between HPV and head and neck cancer[8, 9].

However, it was not before the early 2000s, that an association between HPV and oropharyngeal squamous cell carcinoma and especially tonsillar cancer and base of tongue cancer was disclosed[10-13]. These data were confirmed by our research group and also by others, and in 2007, the International Agency for Research on Cancer (IARC) acknowledged HPV infection as a risk factor for oropharyngeal squamous cell carcinoma (OSCC)[14].

1.1.2 Classification

Human papillomaviruses (HPV) (>170 types have been identified so far) can be divided into two tropism groups: those that that have the ability to infect the keratinized epithelium leading to the development of the common warts; and those able to infect the mucosal epithelium lining of the most exposed cavities of the human body (mouth, throat, respiratory tract and genital and anogenital tracts). Cutaneous HPV infection is very common and highly prevalent worldwide and is mainly transmitted by skin-to-skin contact[14].

On the other hand mucosal HPV is generally transmitted by mucosa-to-mucosa contact and mainly by sexual intercourse. The risk of a HPV infection has been correlated with age, early sexual debut and increased number of sexual partners[15-20]. Mucosal types can also be divided into high risk (HR) and low risk (LR) types, depending on the ability to infect the mucosa and persist, leading to perturbation of the cellular homeostasis and the development of cancer[21].

In addition to the division into two tropism groups, HPVs are also classified taxonomically, based on the sequence of one of their gene called L1 (see 1.1.3.2.1). The L1 gene helps in the classification of HPVs and phylogenetic trees can be constructed based on this[22, 23]. Viruses that share the same structure and organization characteristics belong to the same family, the "*Papillomaviridae*" family. HPVs are divided into genera, which are named after the Greek letters (alpha, beta, gamma, nu and mu). Each of the genera described above present branches indicated as "species" and then these "species" are divided into "types" (Figure 1). The types within each species present a genomic diversity in the L1 sequence of >=10%[21, 23].

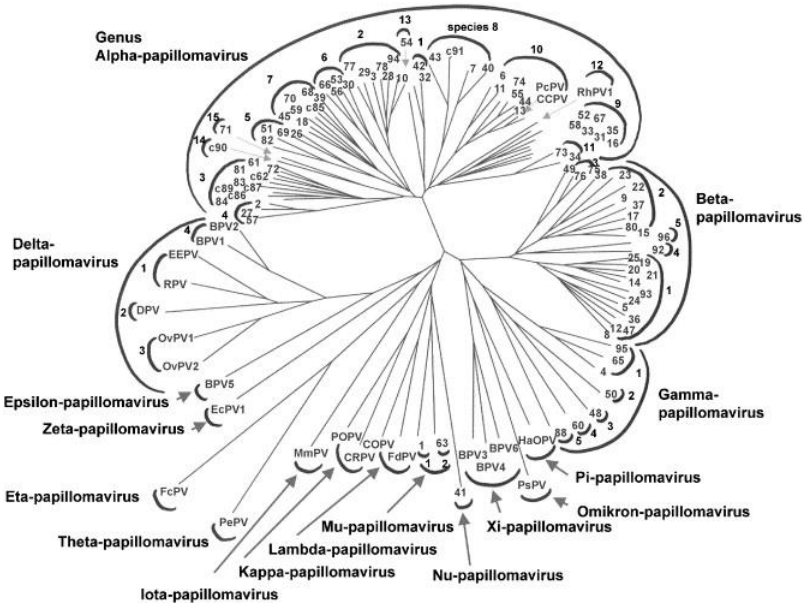


Figure 1: Classification of human papillomaviruses in the different genera and species (adapted from Villiers et al. 2004[24].

1.1.3 Characteristics and genome organization of HPV

HPVs (>170 types, with both potentially oncogenic and non-oncogenic types) are small non-enveloped viruses with a closed, double stranded, circular DNA genome that is packed in an icosahedral capsid of approximately 50-60 nm in diameter[25, 26] (Figure 2A).

The genome of HPV is approximately 8000 bp in length and can be divided into two "coding" regions, the early (E) and late (L) regions, and in between them lies the non-coding regulatory region (NCCR), alternatively called the upstream control region (UCR) or the long control region (LCR). Two poly-adenylation sites, (pA_E and pA_L) separate the three regions of the viral genome[15, 16].

The E region covers approximately 50% of the whole genome and contains the so-called early genes or early open reading frames (ORFs), encoding for proteins responsible for the viral life cycle, invasion and use of the cellular machinery allowing for viral production (see sections 1.1.3.1 to 1.1.3.2 for more details). The early regulatory proteins

are E1, E2, E4, E5, E6, and E7 and in HPV-31 there is also E8, while the L region that covers around 40% of the genome contains the genes that encode the major and minor viral capsid proteins L1, L2[26, 27]. In between the E and L regions mentioned above, lies the LCR with regulatory functions for the viral genome and an illustration of the structure of the HPV genome is given in Figure 2B.

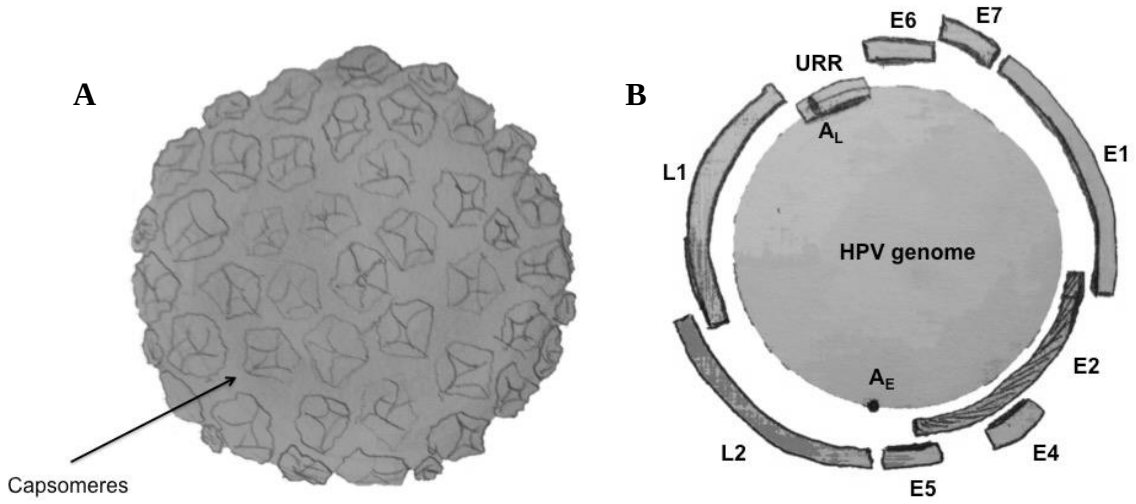


Figure 2: A) Illustration of a HPV viral capsid, which consists of capsomers built up by the structural proteins of HPV L1 and L2. The size of the capsid is approximately 50 to 60nm in diameter. **B)** HPV genome organization.

1.1.3.1 Early proteins and functions

1.1.3.1.1 E1 and E2

E1 has been shown; to be expressed at low levels in an infected cell and its active form is usually considered to be present only upon interaction with the E2 protein. Taken together, E1 and E2 are the two most important proteins during the initial steps of infection. They have the ability to interact with the DNA at specific regions and form complexes that allow for binding of other cellular proteins important and necessary for replication[23, 28].

E2 has the capacity to bind viral DNA sequences that present a palindromic motif and as stated above recruit E1. There, E1 exhibits its helicase activity, thus making the chromatin accessible to the cellular replication machinery. The assembly of the aforementioned complex leads to the disassociation of E2, which then leads to the formation of a double hexameric ring formed by E1. This ring has the ability to mimic similar structures that are normally formed during replication in the host cell[28, 29]. In addition, there is evidence suggesting that E2 has the potential to lead to segregation of the viral DNA by creating a linkage between the replicating episomal molecules of the viral DNA and the mitotic chromosomes of the host[29].

E2 seems to be a multifunctional protein of HPV. In a "dose dependent" manner E2 may exhibit both activating and suppressing properties. At low levels the expression of E2 acts as an activator of transcription which leads to the upregulation and overexpression of the viral oncogenes E6 and E7. In contrast, high E2 levels as seen at the initial phase of the infection keep the expression of the viral oncogenes E6 and E7 low. This characteristic of E2 is believed to be due to the fact that there are differences in the affinity presented by E2 regarding different binding sites[27]. The tight control presented by E2 is of importance since deregulation of E2 can lead to uncontrolled overexpression of E6 and E7 thus leading to uncontrolled cell growth.

The viral genome also has the ability to become a part of the host genome by integrating into it. Often, integration leads to the disruption of the E2 gene, which then leads to loss, or low levels of E2 and high expression of the E6 and E7 oncogenes as the suppressive ability of E2 is lost. However, this only explains a part of the story since it has been observed that integration it is not always crucial for the establishment of an active HPV infection and the development of cancer[25].

1.1.3.1.2 E4

The E4 protein of HPV is expressed at the late phase of the viral infection and it is mainly involved in contacts with the cytoskeleton and has been implicated as a factor in the cytokeratin filament collapse in differentiating keratinocytes[30, 31]. Studies have shown that it is expressed simultaneously with the late region encoded L1 and L2 viral capsid proteins of HPV[32]. However, the role of the E4 protein is still enigmatic and some studies claim that E4 plays a role in viral replication transcription of the viral capsid proteins and in the release of the viral particles[30, 33, 34].

1.1.3.1.3 E5

E5 protein is considered together with E6 and E7 as one of the transforming proteins of HPV though possibly with lower levels of transforming capacity as it has been shown both for human and bovine HPVs[35-38]. The transforming capacity of E5 implicates the EGFR as a key player of the effects caused by E5, especially for the potentially oncogenic high risk (HR) HPV16. In general, the E5 proteins of HPVs are hydrophobic proteins located in the membrane of intracellular compartments of the cell such as the endoplasmic reticulum (ER) and the Golgi apparatus (GA). A low or physiological expression level of E5 is correlated with its localization at the ER, while high E5 levels are associated with its presence at the Golgi apparatus and the nuclear envelope[39, 40]. The E5 of HPV 16 has been shown to be able to enhance growth factor signaling pathways. E5 is able to induce angiogenesis and anti-apoptosis by upregulating COX-2 expression. In addition, it has the potential to downregulate the tumor suppressors p21/p27 through the ET1 receptor[41].

Another important function of E5 and of particular interest for this thesis is its ability to downregulate the human leukocyte antigen (HLA) or major histocompatibility complex (MHC) of class I by accumulating the localization of the molecules in the Golgi apparatus.

The arrest of HLA class I in the Golgi apparatus is mainly due to two function of HPV E5. On the one hand, E5 as a membranous protein has the ability to lead to alkalization of the endomembrane compartment and on the other hand it has the ability to form direct interactions with the heavy chain of the HLA class I complex[42-45]. Through that, it prevents the HLA from translocating to the cellular surface and this may lead to the immune escape of the afforementioned cells, thus avoiding clearance by e.g. cytotoxic T lymphocytes. A schematic representation of the E5 mediated downregulation of HLA class I is given in Figure 3. Other studies also suggest that E5 has the potential to downregulate the HLA class II in keratinocytes that have been treated with interferon gamma (IFN-γ)[42, 43, 46, 47].

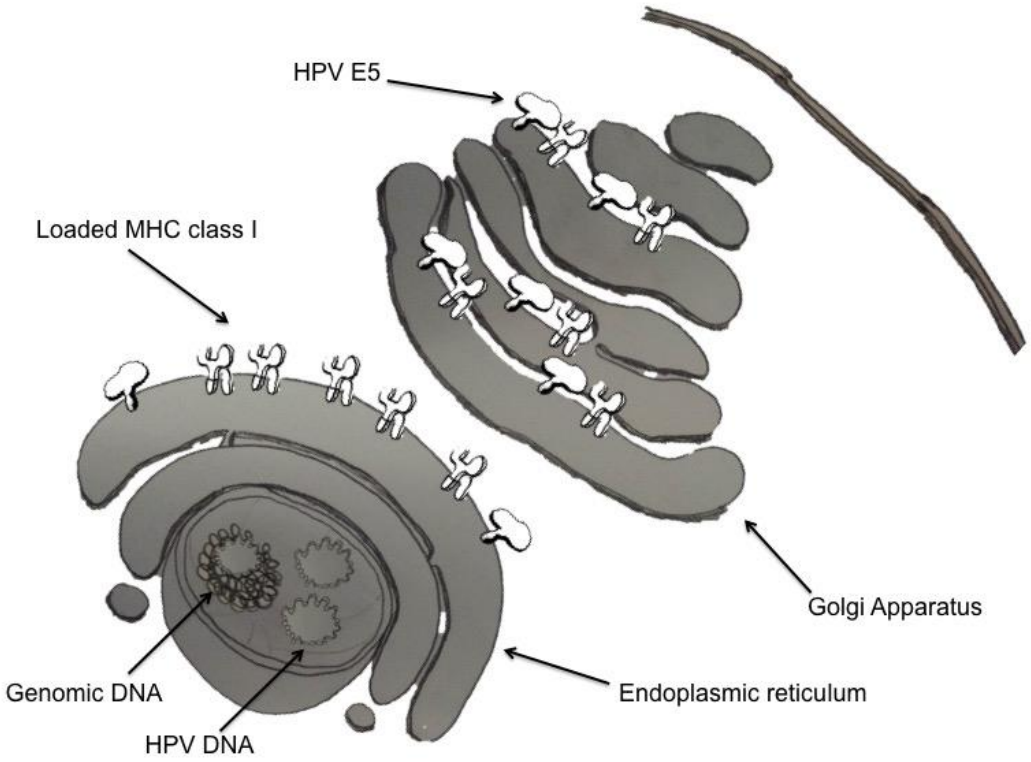


Figure 3: Illustration of the E5 mediated downregulation of HLA class I. As a highly hydrophobic protein, E5 of HPV is usually found in the membrane of interacellular compartments such as the ER and the GA. E5 when accumulating in the GA induces alkalinisation of the endomembrane compartment which leads to the arrest of the HLA class I in the GA. In addition, direct interaction of E5 with the heavy chain of HLA class I complex has been shown. Additionally, when HPV genome is integrated it can lead to disruption of the HLA class I promoter leading in defective or no expression of HLA.

1.1.3.1.4 E6

The most important gene products of HPV with regard to transforming activity, are the two oncogenes E6 and E7. The E6 protein, is a well conserved protein of 151 amino acids. Its most known function is to bind to the tumor suppressor protein p53 and together with

the E6 associated protein (E6AP) leads to the ubiquitination and proteosomal degradation of it. This abrogates both cellular DNA repair and cell cycle control.

E6 of HR HPVs exhibit other functions and also interacts also with many cellular components and proteins implicated in important signalling pathways. Briefly, E6 has the potential to interact with proteins that present a PDZ-domain and by that lead to the degradation of them. Moreover, E6 has been shown to be able to inhibit apoptosis by inhibiting molecules such as Bax and Bak. In addition, due to the afforementioned events E6 leads to cell cycle progression and cellular transformation[48, 49].

When it comes to differences between HR and non-oncogenic low-risk (LR) HPVs, it has clearly been shown that the E6 protein of HR types has the potential to lead to p53 degradation, while this is not the case for LR types. One possible explanation for this, which has not yet been fully elucidated, is the ability of the HR E6 to bind to two positions of p53, thus making strong bonds that lead to the perturbation of the 3D structure of the protein. In contrast for LR types, binding is only seen in one position which is more easily broken[25, 50].

1.1.3.1.5 E7

E7 is an oncogene encoded by the genome of HPV. It can bind the protein retinoblastoma (pRB) and destabilize its conformation. There are also interactions with other related tumor suppressor proteins, such as the p107 and p130. All the proteins of this family, have the ability to regulate and control the e2F family of transcription factors[51, 52].Upon inactivation of pRB e.g. E2F is not inhibited and this leads to abrogation of the control of the cellular cycle (Figure 4). Upon, activation of the cell cycle, cyclin D inhibitors such as $p16^{Ink4a}$ are activated in order to maintain homeostastis and inhibit uncontrolled cell growth and therefore, overexpression of $p16^{ink4a}$ has previously been used as a surrogate marker for presence of HPV[53]. However, upregulation of $p16^{INK4a}$ is not 100% associated with an HPV-positive status of the tumor and in about 10% of the cases there is a discrepancy between HPV and p16 positivity[54-57].

Of course the E7 protein of HPV exhibits more functions. It has the ability to inhibit the CDK-inhibitors p21 and p27 as the E5 does, which in turn leads to chromosomal instability. It has been also associated with interaction that lead to chromosomal alterations and modifications[58]. Another worth-considering function of E7 for this thesis is its ability to exhibit immune modulating functions. For example expression of E7 has been linked to downregulation of the major histocompatibility complex (MHC) which is the molecule responsible for antigen presentation of intracellular antigens[59, 60]. By downregulating HLA, HPV, as with many other viruses has the ability to evade the immune system and persist. Chronic infection and persistence is what leads to cellular alterations and combined with other factors may lead to the development of cancer.

1.1.3.2 Late proteins and functions

1.1.3.2.1 L1

L1 accounts for 80% of the viral capsid with 360 molecules, structured as capsomers. L1 has the natural ability to assemble spontaneously, building virus-like particles (VLPs), which mimic the viral capsid, without L2 and DNA. This is an advantage that has been used in the development of VLP-vaccines against HPV, which serve as prophylactic vaccines against cervical cancer[61, 62].

1.1.3.2.2 L2

L2 is the minor capsid protein and binds to L1 from the inside of the viral capsid, helping in the maturation and formation of the viral capsid as well as in the cellular uptake of the virions. A very nice review has been written by Wang *et al.*[63]

1.1.4 Transmission and viral entry

As described previously, mucosal HPVs have the ability to infect mucosal epithelium. The mechanism by which HPV gains access to the basal layer of the epithelium and enters the cells is not fully understood. There are studies showing that HPV transmission occurs via direct contact, however mucosa and skin without discontinuities are also suggested to be resistant to inoculation and infection by HPV[14, 64-66]. Thus, the main assumption is that HPVs gain access to the basal layers through micro traumas in the epithelium that makes the basal cells accessible to the virus. The mechanism of viral entry has not been elucidated yet, but there are studies showing that the virus is actually using heparin sulphate proteoglycans and a-6 integrins in order to enter and infect the aforementioned cells[67, 68]. Recently, syndecan-1 was also found to play an important role in the infection process[69]. Furthermore, Annexin A2 molecules able to form heterotetrameres have been associated with a mechanism supporting viral entry and currently small molecule inhibitors are being developed for blocking viral entry and infection[70].

1.1.5 Post entry events and characteristics of viral proteins

If we accept sexual intercourse as the route of infection, a sexual intercourse in which one of the two partners already has an HPV infection, could lead to the establishment of a new infection in the other partner due to microablations of the epithelium either in the genital, anal or the oral tract. In this case, viral particles are released and can infect the basal layer of the epithelium. Usually, it is considered that the infection is followed by a genome amplification phase in which the virus is kept at a relatively low copy number[27, 71, 72]. This initial amplification of the viral genome, has been suggested to take place during the cellular S-phase and the viral genome copy number is maintained between 50 to 200 copies/cell[73].

After the internalization of the viral particle, a whole chain of events takes place and the life cycle of the virus begins, for further details see below.

1.1.6 The viral life cycle and cellular transformation

As soon as the virus gets access to the basal level of an epithelium a whole story of events starts taking place. After the internalization the genome of HPV remains episomal and following the S-phase of the host cell it starts replicating until it reaches a copy number of around 10 to 200 copies/cell[73].

During the early phase of infection, E1 and E2 early proteins levels are usually low. In addition, during the differentiation of the epithelia, the p97 promoter of the HPV genome facilitates the expression of the E6 and E7 genes, which are necessary for the entry in the S-phase of the cell cycle. During the epithelial differentiation the genome of HPV is also differentially expressed. As the cells from the basal layer differentiate and move into the higher epithelial layers, the p670 promoter of the HPV genome facilitates the expression of the viral replication proteins, as they have been described in the previous section (E1, E2, E4, E5). In these upper layers, there is an abundance of the aforementioned proteins, which leads to the amplification of the viral genome. When the keratinized epithelium starts to form, E4 is expressed more abundantly and accumulates in the cytoplasm of the cell, and it is also suggested to upregulate the production of the capsid proteins L1 and L2, which allow for viral assembly and release[27, 73]. The life cycle and the expression of the different viral proteins mentioned above is illustrated in Figure 4.

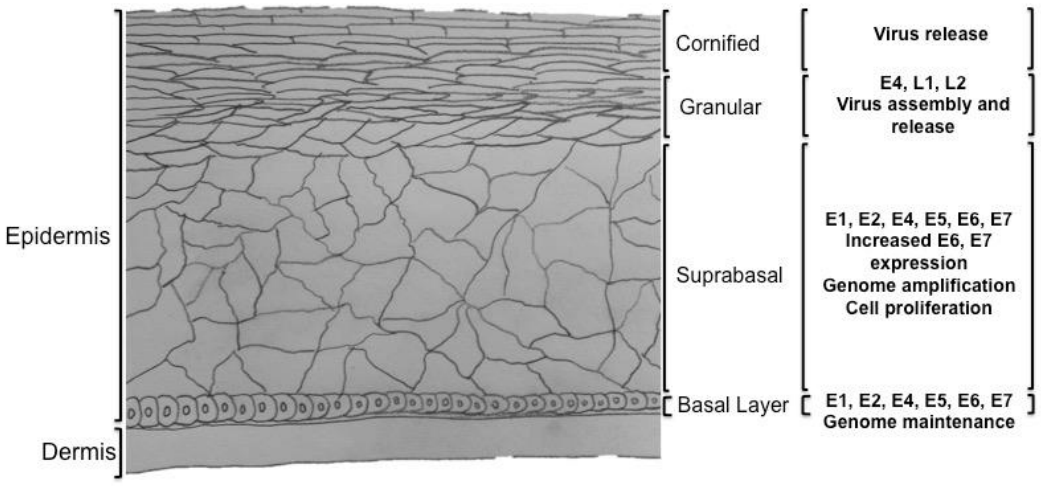


Figure 4: Life cycle of HPV. In this figure the expression pattern of the proteins of HPV are given alongside their functions and the stage of the cycle as well as the part of the epithelium in which they are taking place.

The above-described life cycle takes place whenever we face a HPV infection. Usually, the infections are not persistent and they regress, but in the absence of this regression, lesions may persist and may undergo malignant transformation. The mechanisms underlying this transformation have been described extensively and they are mainly based

on the functions of the oncogenes of HPV. E6 and E7, and to a lesser extent E5 have the ability to complement each other and alter the physiological properties leading to the immortalization of the human primary keratinocytes[74].

E6 and E7 have synergistic effects on transformation by acting on p53 and pRB respectively as described before. Inactivation of pRB by E7 has been shown to induce an increase in the levels of p53, which is then counteracted by the function of E6 that allows for p53 arrest and uncontrolled cell growth.

Normally, during S-phase of the cell cycle, proteins called cyclins and the associated kinases (cyclin-dependent kinases) are induced upon specific mitotic signaling. Such a mitotic stimulation leads to the phosphorylation of pRB by the cyclin-CDK complexes, which in turn lead to the inhibition of the E2F binding. As mentioned above, when E7 inhibits pRb, E2F transcription factors can activate and transcribe genes of the S-phase[27]. As also briefly mentioned above, one well-studied molecule associated with HR HPVs is the upregulation of $p16^{INK4a}$ the natural inhibitor of the CDK4/ CDK6-CyclinD complex that promotes the S-phase[75] (Figure 5). Notably, however in a recent study $p16^{INK4a}$ up-regulation in HPV positive cells has been shown to be due to the histone demethylase KDM6B, which is induced by HPV16 E7[52]. An illustration of the action of E6 and E7 is given in Figure 5.

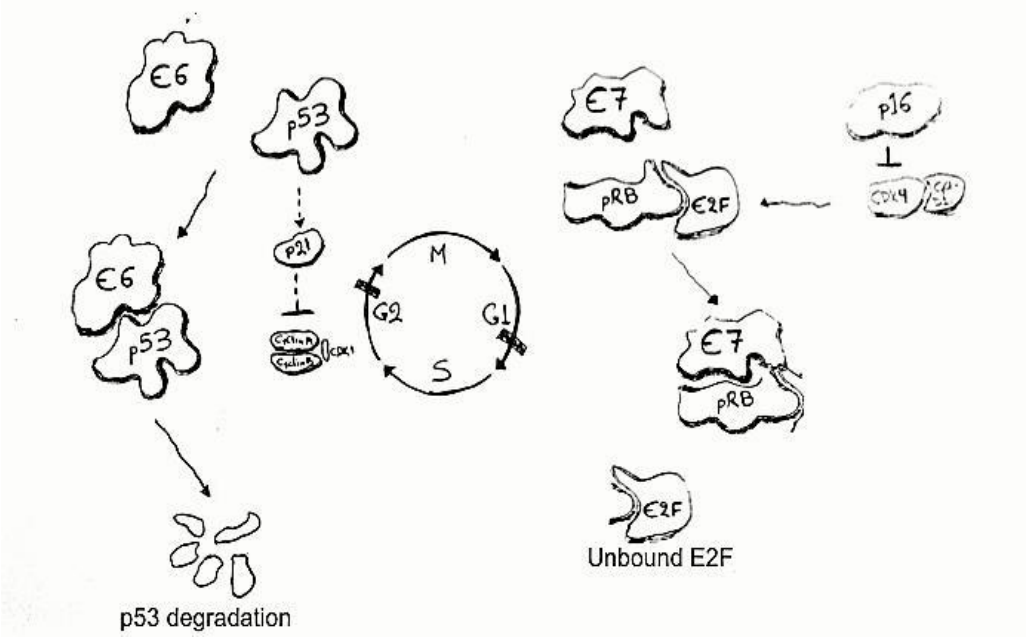


Figure 5: Mechanism of action of E6 and E7 in carcinogenesis. The main action of E6 and E7 as described is downregulation and degradation of p53 and pRB respectively, which allows for cellular transformation and immortalization and also inhibition of apoptosis, which in turn leads to the development of malignancies. p16 is upregulated as a consequence of the uncontrolled gene transcription and cell cycle.

1.1.7 HPV associated diseases and cancer types

As stated, HPV has the natural ability to infect epithelia. It can cause benign warts, condylomas and cancer.

Cellular proliferation and transformation is a result of persistent infections together with other factors. For example, in immune suppressed individuals benign warts as well as HPV induced tumors occur more frequently. Moreover, in the genetic disease epidermodysplasia verruciformis (EV) research has shown that preordained deficiencies presented in the cutaneous immunity of patients with EV makes these patients more susceptible to infections in persistent infections with HPV[76]. These genetic defects, lead to an inability to clear infections. More specifically patients with EV usually have persistent HPV5, HPV8 and HPV14d infections, which are the most common types associated with malignant transformation of EV[77]. In addition, while most HPV 5, 8 and 14d infections are mainly asymptomatic, in some cases they cause verrucae and a minority of these cases may lead to the malignant transformation of the skin and also the anogenital tract[76].

However, best known among the associations of HPV and squamous cell cancer is the association of HPV with cervical cancer, where 90-99% of all cases are found to be positive for HPV[78-80]. Moreover, HPV is also found in other anogenital cancers, such as anal cancer (~ 80% of cases positive), vulvar cancer (~ 40% of cases positive), vaginal cancer (~ 80% of cases positive), and penile cancer (~ 40% of cases positive)[81-83]. Finally, as mentioned above the association between HPV and oropharyngeal squamous cell carcinoma (OSCC) more specifically tonsillar and base of tongue cancer, which is the main focus of this work and will be presented in more detail later (Section 1.5.2).

1.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX

1.2.1 The major histocompatibility complex (MHC) - a general introduction

This thesis deals with components of the major histocompatibility complex (MHC), especially with that of MHC class I antigens, so before going further it renders a short introduction. MHC molecules emerged as a result of large-scale chromosomal duplications, which occurred early in the chordate evolution, according to Susumu Ohno[84-86]. There is also further evidence that MHC-like genes existed prior to the origin of the vertebrates and they might be in existence even before the separation of protostomes and deuterostomes[87].

In 1974, there was a study that at that time resulted in the revolution of the field of immunology, by which Doherty and Zinkernagel managed to show a correlation between cytotoxic T-lymphocytes and MHC molecules[88, 89]. What they actually show is that T cells are activated by a double signal based on the ability of the immune cells to recognize and distinguish between self and non-self-antigens. Based on that, they were able to explain the specificity presented by the immune surveillance. In addition, they proposed that MHC molecules must be of a specific haplotype in order to be recognized by the cytotoxic T

lymphocytes and also that antigens must be presented on MHC molecules. A self-antigen presented on the context of MHC is simply spared by the T cells, while a non-self-antigen such as one coming from a virus infected cell will likely trigger an immune response. MHC molecules are divided into classical and non-classical and the classical ones are implicated in the presentation of molecules triggering the immune response while the non-classical are tolerogenic leading to the inhibition of the immune response[90]. In this thesis we will only focused on the classical molecules MHC class I and II.

To conclude, it can clearly be seen that MHC existed early during evolution and its function seems to be very crucial for organisms for censoring of their environment and the recognition of self and non-self. Below therefore there will be an introduction of the HLA class I and II antigens denoted as human leukocyte antigens (HLA) in human, followed by an introduction of the immune system.

1.2.2 HLA class I and II - a general introduction

1.2.2.1 HLA class I

The HLA class I molecules consist of a polymorphic alpha-chain which is called the heavy chain (HC) and an additional subunit called b2-microglobulin. These two together forms the HLA class I molecule, which has a peptide-biding cleft on the top of the structure. HLA class I molecules are expressed in nearly all cells in the body. In their peptide cleft they mainly present peptides of 8 to 10 amino acids long that are mainly obtained as products from intracellular degraded proteins from the proteasomes. In the cells, there is constant degradation and replacement of proteins. The majority of the degradation is catalyzed by the cytosolic proteasome[91-93].

Proteasomes are ancient enzymes that can also be found in archaebacteria. Because the proteasome is so well conserved it has been said that the entire HLA class I restricted antigen pathway has been evolved in such a way to process antigens and peptides that the proteasome generates. Thus, HLA class I molecules have the ability not only to present peptides from degraded cellular proteins, but also of intracellular pathogens. However, they can also occasionally bind peptides when they are on the surface of the cell[94-96].

When it comes to humans, there are two forms of the proteasome, one that is called the constitutive proteasome, which is always present degrading ubiquitinated proteins into peptides, and the other form is the so-called immunoproteasome. The constitutive proteasome is present in all normal cells, while the immunoproteasome is normally present upon stimulation when subunits of the constitutive proteasome are replaced by others as will be presented in detail below[97, 98]. The two have many similarities, but they also differ when it comes to the subunit composition of the active sites.

The proteolytic subunits of the constitutive proteasome which is a 2.5 megadalton complex are β1 (or PSMB6, Y and δ), β2 (PSMB7, Z and MC14), β3 (PSMB5, X, MB1 and ε) and they are expressed in most cells of the human body[99, 100]. The constitutive

proteasomes can be found in all cells. Peptides arising from the constitutive proteasome are 3 to 22 residues and therefore whole peptides do not always fit into the groove of the HLA class I molecules, which usually has an optimal capacity to fit in 8-10 aa peptides. By having the constitutive proteasome, cells have the ability to degrade proteins from the cytosolic compartment and present them on the context of HLA class I. This way they have the ability to scan for self-peptides and monitor their internal environment[101]. How do cells know which protein should be degraded? One of the most common pathways used by cells to identify unwanted proteins is the ATP-dependent ubiquitination pathway - which will not be presented here - but which marks proteins to be processed and degraded in the proteasome[100, 102].

After stimulation of cells with pro-inflammatory cytokines such as interferon-gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α), which are usually present after pathogen infection the three catalytic/proteolytic subunits of the 20S proteasome (constitutive) are replaced by homologous subunits called LMP2, LMP7 and LMP10. As it can be clearly understood from its name, immunoproteasomes are mainly responsible from processing and degrading foreign proteins rather than self-proteins allowing for initiation of immune defense upon presentation of the antigens on the surface. Interestingly, as it has been found, immunoproteasomes are constitutively found in cells of the lymphoid organs such as, thymus and lymph nodes[97]. These subunits present distinct peptidases sites and cleave proteins in a distinct manner from constitutive particles and generate more peptides capable of binding to HLA class I molecules, thereby serving an important role in antigen presentation[103-105]. In addition, the modification, taking place after the subunit replacement mainly affects specificity, and not in the efficiency of how the two kinds of proteasomes process proteins.

After degradation of the peptides irrespective of which proteasome is presently functioning in the cell, they are transferred and translocated from the cytosol to the endoplasmic reticulum (ER), where peptide loading on HLA takes place. Two molecules called TAP1 and TAP2 facilitate the translocation to the ER. These two molecules for the heterodimer called TAP (transporter associated with peptides), which will allow for translocation and loading of the peptide on to the HLA class I molecules. Both TAP1 and TAP2 are very important for efficient formation of the peptide-HLA complex and it has been shown that in mice if one of these is disrupted there is reduced expression of HLA class I on the surface of the cells. Furthermore, it has been shown that viruses such as cytomegalovirus and herpes simplex viruses 1 and 2 have the potential to block TAP. This leads to a reduced loading of viral peptides on HLA class I and reduced presentation on the cellular surface allowing for evasion of clearance by the CD8+ TILs.

In parallel, the formation of HLA molecules is taking place in the ER and the HLA class I heavy chain (HC) is translocated to the ER by Sec61. Subsequently, HLA class I molecules are through the action of the chaperones called Calnexin and BiP correctly folded and bound to the beta2 macroglobulin chain. Following that Calnexin is no longer

needed and is dissociated from the complex. Subsequently, Calreticulin and Erp53, which are responsible for stabilizing the HLA class I molecules, replace it. Another molecule called Tapasin allows then for the association of the HLA molecule to TAP1 and TAP2 allowing for the loading of the peptides and the formation of the peptide-loading complex. Thereafter, the loaded HLA class I molecules are translocated to the cell surface through the ER and the Golgi complex and any non-completed or incorrect loading leads to their degradation[106-109]. However, in some cases, it has been shown that the aforementioned trimeric complex can disassemble and HC and β2m may appear as free forms on the cell surface[110]. HLA class I molecules are finally translocated to the cell surface, where they, if carrying peptides of pathogens or of mutated or altered host genes, potentially are recognized by CD8+ T cells. A simplified illustration of the antigen processing pathway in the context of HLA class I is given in Figure 6.

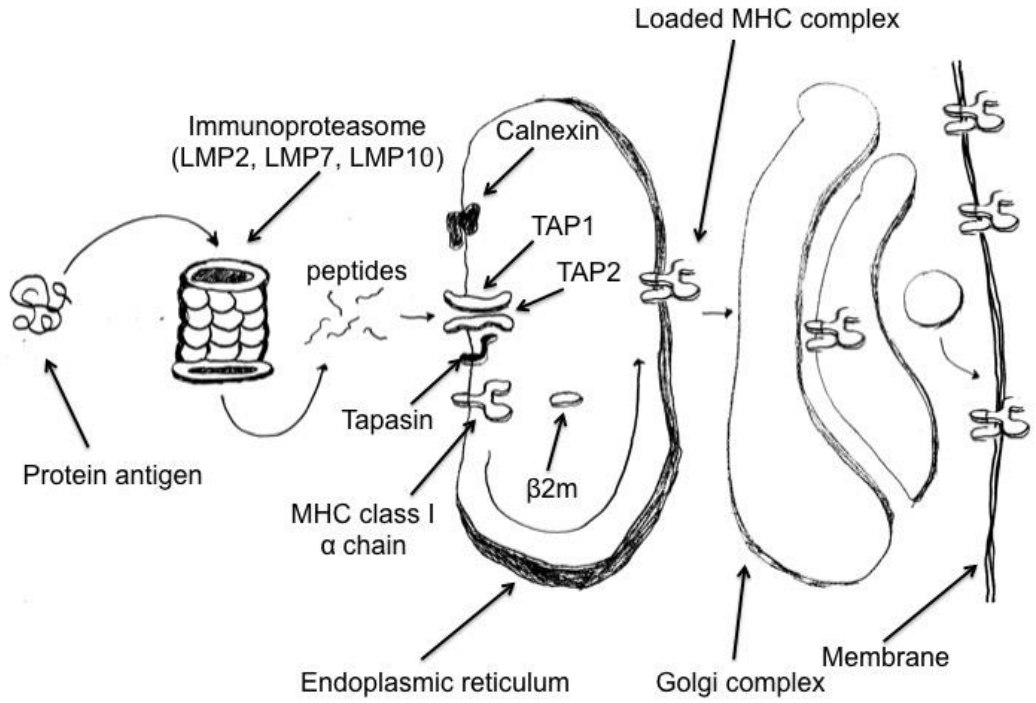


Figure 6: Antigen processing pathway in the context of HLA class I. Only some of the examined molecules in the context of this thesis are presented to give the reader an idea of how the pathway works. In this case we see the immunoproteasome degrading antigens but the same principles are used from the constitutive proteasome as well, as described above.

1.2.2.2 HLA class II

The HLA class II molecules consist of two polymorphic chains called alpha and beta and there is no β2-microglobulin subunit. This makes a slight difference with HLA class I, but the overall structure is very similar. They also present a binding cleft on the top of the structure responsible for binding digested peptides. These peptides are usually obtained from the extracellular compartment. They either bind extracellular peptides directly, or peptides derived from extracellular proteins digested by proteases in the endosomal and lysosomal compartments. The size of peptides binding to HLA class II molecules varies and has been suggested to be larger than the size of those binding to the HLA class I

molecules because the groove that is on the top of the molecule is open at the ends thus enabling the binding of longer peptides. The loading of HLA class II molecules with peptides takes place in specialized vesicles formed into the cytoplasm before their translocation to the cell surface[111-113]. HLA class II molecules are expressed mainly in cells of the immune system and also tissues of the immune system such as DCs, mononuclear phagocytes, B-lymphocytes, endothelial cells and thymic epithelium. Finally, for HLA class II the responsive immune cells are the CD4+ T cells. It has to be mentioned that in some cases, dendritic cells are capable of ingesting whole cells that are e.g. infected with viruses. In these instances, these cells can process the antigens from the engulfed cells and transport their antigens into the cytoplasm. From there they can be transported to the ER where they are loaded on HLA class I molecules that can be presented for recognition by CD8+ T cells[114]. This process is called cross-presentation.

1.3 THE IMMUNE SYSTEM – A BRIEF INTRODUCTION

Host immune defense consists of two parts: the innate immunity, which resembles the first line of defense against infections and the adaptive immunity which is slower but more specific and effective in combating infectious agents. The players of both systems develop in the bone marrow, but afterwards they show different migration patterns.

1.3.1 Innate immunity

The main characteristic of the innate immunity is its presence naturally in the human body prior to any encountering of pathogens. It has been mentioned previously that one of the first lines of defense is the skin, which forms tight junctions and does not allow entry of pathogens. Another form of innate immunity is the normal flora on our skin, which prevents pathogenic bacteria from being introduced and proliferating and probably causing infections and perturbation of homeostasis. Following down from the epithelium there are also other mechanisms such as secretion of mucus which with its colloidal texture does not allow pathogens to adhere and establish an infection and also here the complement system which leads to phagocytosis of pathogens can be included but it will not be presented in detail here[115, 116].

In the innate immunity there is also the “cellular compartment” which is mainly comprised of cells having the capability to phagocytize pathogens and also activate and recruit other immune cells. Granulocytes account for the vast majority of circulating leukocytes, and they can release antimicrobial peptides and enzymes that have the ability to combat invaders[117]. Dendritic cells (DC) are also cells of the innate immune system that have the ability to phagocytize pathogens and after enzymatic digestion and degradation to present peptides on their surface allowing for stimulation and activation of other cell types of the immune system. A big part of their function is the bridging between the innate and the adaptive immunity as will be presented below. In addition, they have the ability to lead to increased migration of cells at the areas of infection[118]. Macrophages, which also have phagocytic capabilities and mainly work on the clearance of cellular debris are another

category in this group, as are NK cells. The function of NK cells is mainly to eliminate cells that are found to be under stress and one of their very specific functions is to eliminate and clear cells that have lost their ability to express HLA molecules on their surface[119, 120]. Of course all of these different cells are not independent of each other and there is interplay, which is also facilitated by the secretion of different types of cytokines and chemokines[121]. In this thesis we mainly focused on cells of the adaptive immunity and thus this part will be presented in more detail below.

1.3.2 Adaptive immunity

Innate immunity, with all of the cells and mechanisms presented briefly above, has the ability to clear and eliminate most of the pathogens infecting the human body. However, in some cases pathogens manage to overcome this line of defense and after that a more specific response is needed in order for the pathogens to be eliminated and homeostasis of the human body to be maintained. This requires the important bridging between the innate and adaptive immune response.

Of course there are many different molecules that can be recognized by the immune system and serve as antigens, but here our focus will be on protein antigens. Conventional DCs are constantly sampling and scanning the epithelial microenvironment for any foreign antigens. Whenever a DC encounters an antigen that is foreign it exhibits its main function, which is to phagocytize it. The recognition is facilitated by receptors that they express on their surface, allowing them to recognize microbial patterns. Upon phagocytosis DCs start losing adhesiveness for epithelia and start migrating to the lymph nodes through the lymphatic vessels. During this process they start to mature and change from phagocytic cells to antigen presenting cells (APC) which are capable of stimulating T cells.

Here, I believe that a short and summarized description of a lymph node is relevant. Lymph nodes are organs of the lymphatic system that are widely distributed through the whole human body. Lymph nodes resemble the main sites in which the activation of adaptive immune responses takes place. In order for an immune response to be initiated, the antigen specific T and B cells have to come into contact with professional antigen presenting cells (APCs) and these are usually the peripheral and the follicular DCs[122]. Thus, the first line is usually interaction and internalization by DCs, which then present the peptides to naïve T cells in the lymph node, thus initiating an immune reaction for the elimination of the antigen or it may become tolerant or die to avoid causing autoimmunity. This decision is mainly facilitated by "information" offered in the lymph nodes[123]. In the lymph nodes, there are separate compartments where T and B cells located. B cells are found in the follicles (follicular B cells) or in the cortex, which are situated in the periphery of the lymph nodes, while T cells are mainly in the paracortex, which is usually called T cell region, adjacent to the cortex, but on the inner side of the lymph node and closer to the medulla of the node which is mainly rich in antibody-secreting plasma cells which are then close to the efferent lymphatic vessel. This separation is mainly facilitated

by chemokines and specific receptors which are expressed by B and T cells[124, 125]. An illustration of the lymph node and the different compartments is given in Figure 7. The lymph node is where B and T cells mainly interact after an antigen has been presented by DCs or after direct contact with their receptors, but prior to that some words about B and T-cell development will follow.

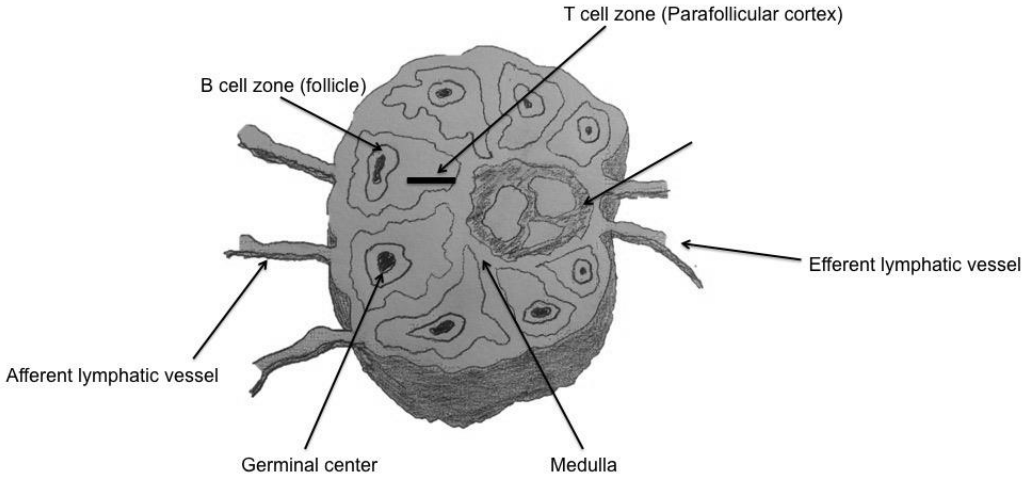


Figure 7: Illustration of the structure and organization of a lymph node. The different compartments are given alongside the location of the immune cells within the structure.

Both B and T cells develop in the bone marrow from hematopoietic precursor cells however, they follow different patterns when it comes to their development. Of course it is difficult for the whole development process to be described here and for that reason; the amazing papers describing the process can be read by someone really interested. For B cells, the development consists of many different stages where they undergo conformational changes and genetic rearrangements with regards to the expression of the heavy and light chains of the immunoglobulins presented on their surface (For review see Pieper *et al.*[126]). After these different steps, the so-called immature B cells undergo selection regarding their self-reactivity and their ability to survive in the peripheral lymphatic system. Immature B cells that bind self-antigens undergo further receptor rearrangements or die or are finally inactivated. All of the steps up to this stage are taking place in the bone marrow and once they finally reach the stage of the mature or naïve B cells, they acquire the ability to migrate in the periphery and come into contact with antigens[126].

T cells also originate and start their life span in the bone marrow, from common lymphoid progenitors with the B cells. After that some of these progenitors migrate to the thymus where they start proliferating. There cellular interactions with the thymic stroma allow for T cell development which also takes place in different steps. Here there are also receptor development and gene rearrangements -although quite different to that of B cells- which allow for the positive and negative selection of the T cells in order for them to be reactive against foreign antigens, but not self-antigens. Initially, T cells do not express any of the CD4 and CD8 receptors and that is why they are called double negative. Following this

the double negative cell can give rise to two lineages of T cells and the most common and larger is the so-called alpha-beta lineage, which is at this stage comprised of double positive cells. Most cells then die in the thymus as they undergo selection for interaction with self-antigens and self-HLA molecules and finally the outcome is T cells, which are single positive either for CD4 or for CD8 receptors. They are then called mature T cells and they are exported from the thymus, and migrate to the periphery and the peripheral lymphatic organs and tissues. Indeed this is a very short summary of the developmental steps of T cells and therefore it is highly recommended that one reads the very nice and informative reviews from Koch and Radtke[127] and also from Juan Carlos Zúñiga-Pflücker[128].

As mentioned, the bridging between the innate and adaptive immunity is facilitated by DCs, which are also called professional antigen presenting cells (APC). DCs can after phagocytizing pathogens or antibody or complement covered cells, degrade them in the lysosomes. The degraded antigens are then presented by DCs mainly in the context of HLA class II molecules to the residential CD4+ T helper cells, which then have the ability to initiate effector actions. Intracellular pathogens such as viruses on the other hand are mainly degraded in the cytoplasm and not in the lysosomes as described previously and then the peptides are presented onto HLA class I molecules. These complexes then have the ability to activate CD8+ cytotoxic T cells that can together with other signals eliminate the infected cells[129, 130].

1.3.3 HPV and interactions with the immune system

HPV is an invader having the potential to infect cells in our body and initiate an infection with the only aim of reproducing itself. As an invader HPV should be recognized as a foreign body by our immune system and be cleared by various immune associated mechanisms, and a fine balance is usually the case, since HPV has evolved mechanisms to avoid immune recognition and clearance. However, in some cases, and especially upon immunosuppression or under special conditions, the balance between the presence of HPV and the immune system is disturbed and pathological conditions emerge.

At the initial stages of the infection with HPV, the innate immune system is the one that should sense the presence of a non-self-factor. The first line of defense is the epidermis, which is a natural barrier against pathogens. Upon the presence of micro-wounds as has been already described, HPV gets access to and can infect the cells of the basal layer of the epithelium. Being the main target of HPV, keratinocytes play an important role. Keratinocytes can be considered as a part of the innate immune system, since they have been described as sentinels[131]. It is shown that they can act as “non-professional” antigen presenting cells and have the ability to promote the expression of cytokines of the Th1 and Th2 types and they can also elicit in CD4+ and CD8+ memory T cells[132].

Furthermore, keratinocytes have Toll-like receptors (TLRs) both on their surface and in endosomes that can recognize the presence of pathogen associated molecular patterns

(PAMPs). There are many different types of TLRs that have been described namely TLR-1, TLR-2 etc. that can sense different patterns from double stranded RNA (dsRNA) to double stranded CpG rich DNA. The activation of TLRs by PAMPs leads to signaling cascades related to innate and adaptive immune responses. In addition, activation of the aforementioned receptors leads to the production of cytokines that create a strong pro-inflammatory environment and as an example TLR-9 in human keratinocytes leads to the production of type-1 IFN, TNF-α, CCL2, CCL20 and CXCL9[133, 134]. The main function of interferons (IFNs) is to mediate protection of the cells against viruses through three distinct pathways; antiviral; anti-proliferative and immunostimulatory mechanisms.

Apart from the keratinocytes, at the primary sites of infection there are the professional antigen-presenting cells (APC), called dendritic cells (DC). In the epidermis DCs are mainly comprised of Langerhans cells and in the dermis from 3 other subsets of cells, which are not of importance for this thesis and thus will not be presented[135]. Immature DCs, have the natural ability to continuously sample the extracellular matrix and surroundings for pathogens and microbes that may have entered through pattern recognition receptors (PRR). Upon engulfment of a microbe or in our case viral products, they undergo a change called maturation and they migrate to the regional lymph nodes.

The engulfed products or microbes are then digested into peptides, after which they are presented on the major histocompatibility complex (MHC) or in human, human leukocyte antigen (HLA). Through presentation of the peptides on HLA by DCs they are then able to bind to cytotoxic CD8+ T cells or CD4+ helper T cells and elicit different immune responses in the presence of different cytokine and chemokine environments. Upon activation, CD4+ T cells start to proliferate and they may end up in two different types called Th1 and Th2 depending on the cytokine environment again. IFN-γ favors the Th1 response. In the situation of a viral infection a Th1 response is of importance since secretion of IFN-γ induces maturation of CD8+ T cells and also activates macrophages and natural killer (NK) cells[136].

1.3.3.1 HPV, antigen processing and HLA class I

As has been shown for many viruses, HPV has, through evolution developed the natural ability to interact with various components within the intracellular and cytoplasmic compartment. In contrast to other viruses, which have the ability to interfere or interact with many of the APM components, HPV16 has only been shown to be able to interact with HLA class I[91]. Here in this thesis we have shown similar results. In addition, other reports have shown that HPV E5 proteins that are mainly found in the Golgi apparatus, have the ability to bind the heavy chain of HLA class I and thus retain it in the Golgi and hence not allowing for antigen presentation[42, 47, 137, 138]. Other studies have found that E7 may have the ability to repress the HLA class I promoter leading to decreased presentation of HLA class I on the surface[139].

1.3.3.2 HPV and antibody responses

During natural HPV infection antibody titers are directed mainly against L1, and these can be seen as a sign of past infection. Notably, antibody titers obtained naturally are generally much lower than those obtained through vaccination (see 1.3.4).

1.3.3.3 HPV and T-cell responses

While antibodies are suggested to block viral infection, it has been suggested that the cellular immune system is of ultimate value for clearing an established infection. More specifically, T cell responses are of importance comprising both virus specific T cell responses (CD8+ CTLs) and also CD4+ T cells producing IL2 and IFN-γ producing Th1 cells[140]. Studies have shown that in cervical cancer, the presence of T cells was not associated with the production of granzyme B, which is a mechanism utilized by T cells for inducing apoptosis of the infected cells. Instead it was found that PD-1 was expressed as a sign of T cell exhaustion resulting in ineffective elimination of the infected cells[141].

1.3.3.4 HPV and evasion of immune system

HPV infections are usually cleared by the immune system, or at least they are controlled in most cases. This is demonstrated, in that most of us clear warts, and that in the majority of the cases, young women, who have developed an incident HPV infection will show clearance of this infection within 12 to 30 months[142]. In contrast, immunosuppressed patients present HPV associated lesions, e.g. warts and tumors much more frequently than then normal population and have more difficulties in clearing them.

Cutaneous HPVs can to some extent evade the immune system in that the life cycle is limited to the differentiating keratinocytes[133]. In addition, the HPV life cycle does not present any blood borne phase and there is no lytic cycle and thus no viral particles are released, which could serve as danger signals for the immune system to be activated[143]. Furthermore, HPV is characterized by sub-optimal codon usage and this is something that keeps the viral proteins low, thus avoiding detection from the immune system.

Other studies have suggested that APCs in the epidermis, the Langerhans cells (LC) even upon engulfment of HPV capsid are not able to elicit an immune response and that the cytokine profile in the microenvironment is also very crucial for the establishment of a Th1 or a Th2 response[144]. Most of the time this profile is more leading towards a Th2 response, which as has been stated previously is not optimal for clearance of virally infected cells[145]. HPV has also been suggested to be able to inhibit type 1 interferons (IFN-alpha and beta) through its oncogenes that interfere with components named interferon regulatory factors (IRFs) and are responsible for the interferon pathways[133]. Finally, it has been shown that HPV has the potential to upregulate the cellular deubiquitinase UCH1 in order to suppress the innate immune response elicited from keratinocytes[146].

1.3.4 Vaccines and efficacy

There are preventive vaccines against some HPV types and attempts have also been made to obtain therapeutic HPV vaccines[147, 148], but so far the latter have not been as successful. The fact that the L1 protein of HPV has the natural ability to form viral particles without containing DNA, called virus like particles (VLPs) gave scientists the opportunity to develop vaccines against HPV.

Based on that, today there are two vaccines available in the market which are prophylactic (Cervarix®, GlaxoSmithKline, UK and Gardasil®, Merck, USA) and more are on the way. In addition, there is an FDA approved 9-valent vaccine that has shown increased efficacy and no severe side effects (fever and pain at injection site).

Gardasil is a quadrivalent vaccine covering the two most common HR HPV types in cervical cancer in the Western world, HPV16 and 18, and it also covers two low-risk types (6 and 11)) since they are associated with the development of common warts. Cervarix on the other hand covers only the two HR HPV types stated above. The efficacy of the vaccines is almost 100% with regard to prevention of HPV16 and HPV 18 infection in CINIII lesions after 4 years. There are also studies showing some cross protection against some other related HR HPV types[149-151]. In addition, more recent studies have suggested that the vaccines are indeed very effective and they have also shown that less than the three doses maybe sufficient. Protection is about 86% for HPV16 and 18 in CIN III lesions after 4 years following vaccination of women aged 15–25 years, with one dose, and two doses of the HPV-16/18 vaccine seems to protect against cervical HPV-16/18 infections, similar to the protection provided by the three-dose vaccination program[152]. The main mechanism being activated and responsible for the efficacy of vaccination is mainly attributed to the production of antibodies. It has been shown that Cervarix induces mainly Th1 responses while the opposite is true for Gardasil and despite the fact Th1 responses are more preferable when it comes to viral infections, both vaccines have shown the same efficacy[153, 154].

In addition, there are studies in young adults showing that the oral prevalence of the HPV types covered by the vaccines mentioned above is much lower in vaccinated women as compared to the non-vaccinated ones, indicating the high efficacy of the vaccines. (Grün N, unpublished). Furthermore, in another study looking for oral HPV infections, they show that HPV prevalence 4 years after initiation of vaccination in Costa Rica, oral prevalence of HPV16 and 18 was much lower in the women that had been vaccinated compared to the control group, suggesting that the vaccine offers strong protection also against oral HPV infection. This also offers implications for prevention of the increasing HPV associated oropharyngeal cancer[155].

1.4 HEAD AND NECK CANCER

Head and neck cancer includes a wide range of different cancer types including tumors of the larynx, the hypopharynx the oropharynx, the epipharynx, the oral cavity, as well as the mobile tongue, the lip, the salivary glands, the sinuses, and the ear. Head and neck cancer represents the 6th most common cancer worldwide and 90% of the cases are squamous cell carcinoma (SCC). Traditional risk factors for head neck squamous cell carcinoma (HNSCC) are smoking and alcohol, but also bethel chewing. The outcome for HNSCC is generally quite poor with <50% having a 5-year survival rate and this is not helped by the fact that the tumors are often detected late [156].

In 1983, Syrjänen *et al*. suggested a possible association between HPV and oral squamous cell carcinoma[8, 9]. The kick off from the Syrjänen group led the scientific community to initiate studies on the association between HPV and head and neck cancer, despite the fact that the community was very slow in its reaction and accumulated evidence supported the fact that HPV was mostly present in OSCC. Thus, in 2007, the International Agency of Cancer Research (IARC) acknowledged HPV16 as a risk factor for oropharyngeal squamous cell carcinoma (OSCC), where tonsillar and base of tongue squamous cell cancer (TSCC and BOTSCC) dominate[157]. HPV is found in roughly 10-90% of all OSCC, and then mainly in TSCC and BOTSCC. Furthermore, HPV positive OSCC, especially HPV positive TSCC and BOTSCC, but not OSCC at other sites, have a much better clinical outcome than the corresponding HPV-negative tumors[158-161].

Less than 10% of other HNSCC are HPV positive and the role in the cancers of other sites has still to be investigated further, but there are reports suggesting that although HPV is rare, it may be a positive prognostic factor also in hypopharyngeal cancer[162].

Notably, the prevalence of OSCC has increased in the Western world in the last few decades and what is more intriguing is that prevalence of HPV-positive OSCC has also increased during the last decades, whilst on the other hand the prevalence of HPV-negative cases has decreased. This has been shown for TSCC and BOTSCC since 1970 in Sweden [163]. However, prevalence of HPV in OSCC varies depending on the characteristics of the study population such as country, alcohol consumption, smoking, age and which years the study was conducted. Another factor that has to be mentioned is the difference in the techniques that have been used in order to define HPV positivity.

1.5 OROPHARYNGEAL, TONSILLAR, AND BASE OF TONGUE SQUAMOUS CELL CARCINOMA (OSCC, TSCC AND BOTSCC)

Malignancies located in the oropharynx area represent 3-5% of all the cancers diagnosed each year in the Western world. It is estimated that each year 600.000 cases of HPV associated malignancies arise and of these 10% are due to OSCC, more specifically TSCC and BOTSCC. Historically, HNSCC as well as OSCC have mainly affected males. This is most likely due to the fact that smoking and alcohol consumption has, in the past, been higher among men than women. However, so far, HPV positive OSCC is also mainly a

male disease and around 80% of the cases occur in men[13, 163-165]. It has also recently been suggested that men are more sensitive to oral HPV infection than women, since women more easily mount an immune response in their genital tract. Whether this hypothesis holds true or not, will have to be investigated further. An illustration of the oropharynx is given below in Figure 8.

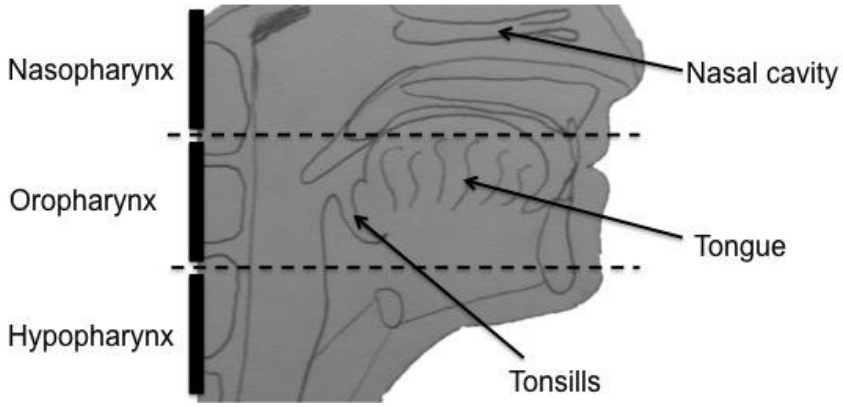
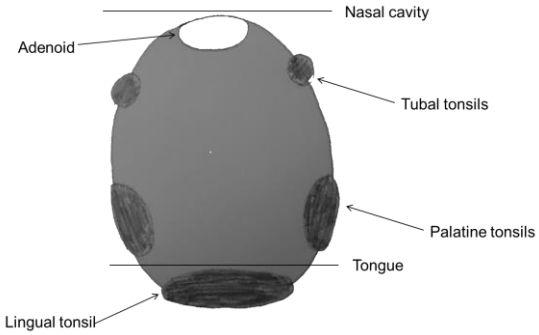


Figure 8: Anatomy of the oropharynx.

1.5.1.1 Anatomy and histology of the oropharynx

The oropharynx is a part of the head and neck region, which is located in the backside of the oral cavity and is in the middle of a region called the pharynx. The oropharynx can be divided into four sub-sites, which are distinct from each other. This includes the palatine tonsils, the base of the tongue, the soft palate and the pharyngeal walls, which are all covered by squamous epithelium.

The histology of the normal oropharynx, also includes areas of "lymphoepithelium". This is due to the fact that the squamous epithelium makes invaginations into the underlying lymphoid tissue and leading to the formation of structures called crypts. The majority of the crypts are found in tonsils with 10-30 per tonsils while their existence is less prominent (one or two) at the base of the tongue[13]. This area in the oropharynx including lymphoepithelium, consists of the palatine tonsils, the lingual tonsil and the nasopharyngeal tonsil including the adenoid pad and is called the Waldeyer's ring or nasal-associated lymphoid tissue (NALT) and for details sees Figure 9.



Notably, due to the structure of the epithelium and the invaginations tumors arising here usually metastasize very early and there are usually metastases in the lymph nodes prior to the discovery of the primary tumor, especially when it comes to small tumors[13].

Figure 9: The Waldeyer's ring.

1.5.1.2 Oropharyngeal cancer classification and staging

OSCC is well-characterized and there are classification systems and codes defining different OSCC sub-types. The standard classification system for specific localization of tumors is the International Classification of Diseases (ICD) system. In the table below a very simplified version of the classification used will be given in order for the reader to be able to follow.

Different cancer types are also categorized according to their stage and based on the stage and other clinical or molecular characteristics of the tumor a treatment schedule can be designed, since tumor stage generally also has great prognostic significance. Clinical staging of the OSCC is made according to the TNM classification system (International Union Against Cancer (UICC)). According to the last updated version of the TNM system the stage of a tumor is based on:

- Size of the primary tumor (T-stage)
- Presence, size, number of metastasis to lymph nodes and localization to regional metastasis to lymph nodes (N-stage)
- Presence of distant metastasis (M-stage)

Finally after the definition of the T, N and M stages of the tumor the TNM-classification is divided into stages I to IV[166]. The different T, N and M stages and the tumor stage arising from the combination of them are presented in tables 1 and 2 in the next page.

Table 1: TNM classification of OSCC according to the UICC, copied and modified from Näsman A.[167].

TNM classification		Definition
T	TX	Tumor cannot be assessed
	T0	No evidence of primary tumor
	Tis	In situ carcinoma
	T1	Size of tumor <2cm in greatest dimension
	T2	Size of tumor >2cm in greatest dimension
	T3	Size of tumor >4cm in greatest dimension
	T4a	Moderately advanced local disease
	T4b	Very advanced local disease
N	NX	Regional lymph nodes cannot be assessed
	N0	No metastasis in regional node
	N1	Metastasis in a single ipsilateral lymph node, <3cm in greatest dimension
	N2a	Metastasis in a single ipsilateral lymph node, >3cm in greatest dimension
	N2b	Metastasis in multiple ipsilateral lymph nodes, <6cm in greatest dimension
	N2c	Metastasis inbilateral or ipsilateral lymph nodes, <6cm in greatest dimension
	N3	Metastasis in a lymph node, >6cm in greatest dimension
M	MX	t metastasis cannot be assessed
	M0	No distant metastasis
	M1	Distant metastasis present

Table 2: Staging (Stage I-IVb) according to TNM staging and classification according to UICC, copied from Näsman A.[167].

	T1	T2	T3	T4a	T4b
N0	I	II	III	IVa	IVb
N1	III	III	III	IVa	IVb
N2a	IVa	IVa	IVa	IVa	IVb
N2b	IVa	IVa	IVa	IVa	IVb
N2c	IVa	IVa	IVa	IVa	IVb
N3	IVb	IVb	IVb	IVb	IVb

1.5.2 OSCC, TSCC, BOTSCC, HPV epidemiology and clinical outcome

Each year in Sweden there are approximately 350 cases of OSCC and more than 90% of these cases are TSCC and BOTSCC[168]. Notably, the incidence of OSCC, more specifically the incidences of TSCC and BOTSCC have increased since the 1970s not only in Sweden, but also in many other Western countries[159, 165]. This is due to an increase of the HPV positive cases especially.

In countries, where smoking has decreased, HPV-negative TSCC and BOTSCC have been shown to decrease, as is the case for HNSCC in general. In some cases there are differential trends with regard to smoking among men and women as well, complicating the picture. Nevertheless, OSCC is mainly a male disease, with male dominance both for HPV positive and HPV negative cancer.

Notably, distinguishing HPV-positive from HPV-negative OSCC is of importance, since patients with HPV-positive TSCC and BOTSCC have higher 5-year disease free survival (DFS) than those with HPV-negative cancer (80% vs. 40% for HPV-positive and HPV-negative cancers respectively).

This has resulted in the scientific community now describing two different OSCC/TSCC/BOTSCC entities that would probably require different treatment strategies. Moreover, patients with HPV-positive TSCC and BOTSCC are frequently not heavy smokers or drinkers, when compared with patients with HPV-negative cancer and they are also younger (with a median age often <60 years of age). In addition, there are several

studies pointing out differences in the biological and molecular level of the two diseases[169-171]. It has been shown that in HPV-positive TSCC the mutation rate is lower compared to the HPV-negative ones[172]. In addition, $p16^{INK4a}$ is generally up-regulated in HPV-positive OSCC compared to the HPV-negative OSCC. Furthermore, p53 is usually mutated in the negative cases while it is been degraded in the HPV-positive cases due to the function of E6 as mentioned previously, and this has been presented for cervical as well as head and neck cancer[173-175]. Differences have also been revealed in other molecules, such as microRNAs[176].

1.5.3 Treatment modalities for HNSCC and OSCC, TSCC and BOTSCC

In the past, standard treatment for HNSCC was radiotherapy and/or surgery and for early stage tumors survival was fairly good[177, 178]. However, most HNSCCs are usually detected late and of higher stage and with poor prognosis, i.e. with a 40% 5-year DFS. This has led to intensification of treatment in the past decades with a somewhat increased survival, but also at the cost of increased side effects for the patients[178, 179]. Intensified treatment includes induction or concomitant chemotherapy and also hyperfractionated radiotherapy. In addition, in some cases Cetuximab is also administered, which is a monoclonal antibody used to block endothelial growth factor receptor (EGFR)[180, 181].

It is doubtful whether all patients with HPV-positive TSCC/BOTSCC need this intensified treatment, since in the past 80% of this category of patients survived with only conventional radiotherapy and surgery. However, to taper therapy, it is necessary to identify which patients will benefit from lesser treatment. To allow for a safer and more accurate selection of patients for de-intensified treatment, it is of importance to combine positive HPV status with additional prognostic biomarkers. The hunt for additional prognostic biomarkers is a main focus of this thesis.

1.5.4 HPV positive status in OSCC/TSCC/BOTSCC

The definition of HPV positive status has varied immensely in the literature with 4 or 5 different definitions being used since the beginning of the HPV era and especially, with regard to OSCC/TSCC and BOTSCC. Initially, this was not given much importance. However, when the prognostic importance of HPV for TSCC and BOTSCC emerged then exactness has gradually become more important.

Initially, e.g. HPV positive OSCC/TSCC/BOTSCC were mostly defined as overexpressing $p16^{INK4a}$ and later on a definition of the p16 positivity was defined as overexpression in >70 % of the malignant cells (p16+) or as being HPV DNA+. Since $p16^{INK4a}$ was overexpressed in the majority of the HPV positive tumors, p16 overexpression was used as a surrogate marker for the presence of HPV in numerous studies. This was also the case with the presence of HPV DNA. HPV DNA alone is a very good indication of HPV positivity of a tumor but it does not show active HPV infection.

The presence of HPV E6/E7 mRNA is of course more secure as being the sign of an active HPV infection and is the golden standard if one wants to assess HPV as a causative factor in a tumor[182]. However, monitoring mRNA has been more difficult and has been dependent on what samples possible to obtain. Originally, fresh frozen samples were necessary for determining presence of RNA.

Throughout the years, many different detection methods have been used for identifying HPV in a tumor. The fact is that today neither HPV DNA nor $p16^{INK4a}$ can alone stand as markers for active HPV infection. It has relatively recently been shown that the combination of positive HPV DNA (HPV DNA+) status alongside overexpression of $p16^{INK4a}$ (p16+) easily determined in formalin fixed paraffin embedded (FFPE) samples, has almost the same sensitivity and specificity as determining presence of the golden standard i.e. HPV E6/E7mRNA[183, 184]. In this thesis, HPV-positive status has been determined as being both HPV DNA+ and overexpressing $p16^{INK4a}$, i.e. HPV DNA+/p16+. That the definition of having an HPV positive status is not consistent throughout this thesis, which in retrospect is unfortunate reflects in many ways changes in the field so far. Hopefully, in the future the definition of HPV positive status will become more convergent and consistent throughout the scientific community.

1.5.5 Prognosis and biomarkers: Where have we come? What do we miss?

An important aspect in medicine is to be able to prevent a disease and another one is to be able to predict what the outcome of it will be. It is very important that the prognosis is robust enough so that the clinicians have the ability to decide on treatment regimes. In this direction, many prognostic biomarkers apart from the HPV and p16 status of the tumor have been studied for their importance in OSCC and many different ones have been proposed.

Different tumor types overexpress different molecules and they are used in the clinic as prognostic biomarkers. Relatively specific biomarkers are e.g. $p16^{INK4a}$ for cervical and oropharyngeal cancer[55, 185], HER-2 for breast cancer[186] and PSA for prostate cancer[187].

There are also more universal biomarkers that have been used and studied in many different cancer types. One such example is the use of counting tumor infiltrating lymphocytes (TILs). The immune system plays an important role in combating tumors and infiltration of lymphocytes and especially CD8+ and CD4+ TILs have been used as biomarkers in various tumor types such as anal[188], breast[189], lung[190], urothelial carcinoma[191], head and neck carcinoma[192, 193] and others. Furthermore, different models have been proposed and immunoscore, where various types of TILs are investigated with regard to their number and their location in or out of the tumor and an algorithm has been used to calculate the prognostic significance of the findings[194].

HLA class I expression has also been investigated in various tumor types such as breast, lung, ovarian, cervical and head neck cancer and in many tumor types HLA class I expression has been found to be downregulated[195-200]. Moreover, downregulation of HLA

class I expression has also been linked with a poor outcome in various tumors such as ovarian and head neck cancer[198, 201]. The investigation of specific HLA alleles has also been performed and it has been shown e.g. that presence of the HLA-A*02 allele is associated with worse prognosis in ovarian cancer of stage III and IV and also a correlation with prognosis has been demonstrated in prostate cancer and malignant melanoma[202, 203].

Evaluating protein expression by immunohistochemistry on formalin fixed and paraffin embedded (FFPE) tissues has the advantage of easily being performed in the pathology unit at the clinic and is also a reliable and straightforward technique. Recently, several studies by others and us have focused on finding prognostic markers for TSCC and BOTSCC. Well-studied and confirmed biomarkers from different studies are p16, which is often used as a surrogate marker of HPV infection and co-expression with Ki-67, EGFR, LRIG1[204-206].

In this thesis, the focus is on biomarkers related to the immune system. Studying the infiltration of TSCC and BOTSCC biopsies by CD8+ and CD4+ TILs was an obvious choice, since this has been extensively studied on other tumor types as described previously.

Furthermore, since both E5 and E7 mRNA have the capacity to regulate HLA class I, studying expression of HLA class I was also intriguing in relation to prognostic significance. The fact that we also looked at the expression of HLA class II was based on findings that it can be observed in cervical cancer, despite the fact that it is normally not expressed in epithelial cells[207-209]. In addition, its expression has been linked with both good and poor prognosis for many malignancies [210-212] and because there was no such study on TSCC/BOTSCC, it was an obvious choice as well.

HLA-A*02, was also an interesting candidate, since as mentioned above HLA-A2 has shown prognostic significance for other malignancies and is a very common allele in the Scandinavian population. Finally, based on our findings on HLA class I we decided to look at some of the components of the antigen processing machinery (APM) and examine if they were in any way related to HLA expression, to each other and examine their prognostic significance.

After accumulating information on many markers, it was obvious that using single biomarkers were not optimal to predict clinical outcome for all patients. For this purpose we attempted to combine different biomarkers, which have high specificity and sensitivity in a single algorithm in order to be able to identify the best combination that could lead to a safer and more accurate prognosis for individual patients. This way it might be easier for the clinicians to decide on a different regime of treatment and a possible de-escalation of it.

2 AIMS

- To investigate the role of HLA class I and II antigen expression in a large cohort of OSCC in relation to the HPV status of the tumor and the clinical outcome. (Paper I)

- To study the role and the prognostic significance of CD8+ and CD4+ tumor infiltrating lymphocytes (TILs) in relation to HPV and the clinical outcome of patients with TSCC and BOTSCC. (Paper II)

- To investigate the role of the HLA-A02* allele in relation to HPV an clinical outcome of patients with TSCC and BOTSCC. (Paper III)

- To investigate molecular biomarkers mainly associated with antigen processing such as TAP1, TAP2 and the immunoproteasomal subunits LMP2, LMP7 and LMP10 in TSCC and BOTSCC and their expression in correlation to HPV status and clinical outcome of the patietns. (Paper IV and V)

- To combine information and prognostic significance of some of the aforementioned biomarkers together with clinical characteristics of the patients in one single algorithm allowing for prediction of the clinical outcome of patients with HPV-positive tonsillar and base of tongue cancer. (Paper VI)

3 MATERIALS AND METHODS

3.1 PATIENTS, MATERIALS AND DESIGN OF THE STUDIES

3.1.1 Patients and biopsies and ethical permissions

The patients included in the studies of this thesis will be presented separately for each study below. All the studies conducted and included in this thesis were conducted according to the ethical permissions 2003/507, 2005/431-31/4, 2005/1330-3, and 2009/1278-31/4 from the Regional ethical committee at Karolinska Institutet, Stockholm, Sweden.

Paper I. In this study, 385 patients were diagnosed with TSCC (ICD-10 C09.0-9) or BOTSCC (ICD-10 C01.9) at Karolinska Institutet in Stockholm between 2000 and 2007. 203 with TSCC and 77 with BOTSCC were treated with curative intent and had available pre-treatment biopsies to be included in the study.

Paper II. In this study, all patients with OSCC defined by the ICD-10 codes C09.0-9; C01.9; C05.1-9; C10.0-9 and diagnosed between 2000 and 2009 were included. Patients with C09.0-9 and C01.9 were diagnosed between January 2000 – September 2009 and patients with C05.1-9 and C10.0-9 between January 2000 – January 2009. All patient records were examined and patient characteristics and clinical outcome were obtained to be used for the study.

Paper III. Between 2000 and 2009, 445 patients were diagnosed with TSCC and BOTSCC (ICD-10 codes: C09 and C01.9 respectively) at the Karolinska University Hospital. Of these 425 patients with biopsies previously tested for HPV DNA and with sufficient material to test for HLA-A*02 were included. 383 of these patients were treated with the intention to cure and were included in the survival analysis.

Paper IV. During 2000-2007, 385 patients were diagnosed with TSCC (ICD-10 C09.0-9) or BOTSCC (ICD-10 C01.9) at Karolinska Institutet in Stockholm. 278 patients with available pre-treatment biopsies previously tested for HPV DNA and with sufficient material included in the study.

Paper V. In this study, two patient cohorts were included. One with TSCC and one with BOTSCC, with 151 patients in total, all treated at the Karolinska University Hospital. The first sample set consisted of 78 TSCC (ICD-10 C09.0-9) from patients diagnosed from 2000 to 2006 and pre-treatment biopsies available. This set included 48 HPV DNA-positive and 30 HPV DNA-negative TSCC derived from a set of 83 TSCC samples included in the analysis for HLA class I, but with 5 tumours excluded due to lack of material. The second set consisted of all 73 BOTSCC samples, 53 HPV DNA-positive and 20 HPV DNA-negative diagnosed between 2000 and 2007 and with available pre-treatment biopsied. 66 of these were treated with curative intent.

Paper VI. Data were collected on patients diagnosed during 2000–2011 with HPV DNA+ tumour biopsies, 308 with TSCC (ICD-10 code: C09.0-9) and 112 with BOTSCC (ICD-10 code C01.9), at the Karolinska University Hospital. The study was initiated on a training cohort of 197 patients diagnosed during 2000–2007, and validated on a cohort of 118 patients from 2008–2011. Only curatively treated patients with HPV DNA+/p16+ FFPEs were included.

3.2 METHODOLOGY AND TECHNIQUES

In this thesis, the techniques and methods utilized were DNA extraction, PCR and immunohistochemistry. Below each one of these will be explained and presented in more detail. It has to be mentioned that the determination of the HPV status of the tumors was conducted earlier (2000-2007) by using conventional PCR and later (2007 onwards) mainly by a bead-based multiplex assay.

3.2.1 DNA extraction

DNA was extracted for all the applications in this thesis by using the High Pure RNA extraction kit (Roche, Molecular Biochemicals, Mannheim, Germany). The procedure followed was the one suggested from the manufacturer with the exception that the DNAase treatment step was excluded. The reason for extracting DNA with an RNA extraction kit was that we wanted to maintain the possibility to also examine for RNA from the same sample. Blank controls were used in order to excluded cross-contamination of HPV DNA and HLA-A*02 between samples.

3.2.2 Conventional and Multiplex PCR for HPV detection

Briefly, for the conventional detection of HPV DNA the commonly used consensus primer sets GP5+/GP6+ and CPI/CPIIG and also the primers specific for HPV 16 were used as has been described previously[165]. The coverage of these primers includes all known HR-HPV types. The GP5+/GP6+ primers amplifies the L1 ORF giving rise to an amplicon of approximately 130 to 150bp while the other set of primers recognizes the E1 ORF and the amplicon is around 190bp. The HPV type specific primers target the HPV 16 E6 Open Reading Frame (ORF). The samples that were positive for HPV other than HPV 16 were subsequently subjected to sequencing for type determination. The amplicons were with this method evaluated by gel electrophoresis.

For samples from 2008 and onwards a Luminex bead-based multiplex HPV assay was used for the HPV status determination of the tumors and this technique is described in detail elsewhere[179]. This assay allows for increased sensitivity and has the ability to detect and identify up to 27 different types at once.

Furthermore, the Multiplex PCR was used to reevaluate some of the samples, earlier tested with conventional PCR, both for having consistency and also for validating the method itself.

In this assay, a set of variants of the GP5+/GP6+ primers were designed[213], to allow for a similar sensitivity for all the included HPV types. With this primer set, a more equal amplification was obtained for all the HR HPV types and the sensitivity was 10 to 100 copies[213]. In addition, primers for human DNA b-globin were included as a control for amplifiable cellular DNA. All the reverse primers were biotinylated, which allowed for a detection step later on in the assay as described below.

So, how does the Luminex assay work? In the MagPix instrument from Luminex up to 50 different magnetic beads can be used, all with different colors that can be identified by lasers in the MagPix machine. To each of the specific beads, which in our case are 28 (27 HPV types and b-globin and E6 of HPV16), a specific probe is coupled. Each of these different probes is specific only for one of the analyzed HPV types. In the assay, the PCR amplicons are denatured and mixed with the bead mixture at a specific hybridization temperature, in order for the probes that are coupled on the beads to be hybridized with the PCR amplicons. After this incubation, all primers and PCR amplicons that have not bound to the probes are washed away and the hybridized products are then subjected to a step where fluorescent streptavidin binds to the biotinylated primers. After all these steps, the beads are analyzed on a magnetic plate. As mentioned before two lasers are used and each bead is identified by its color and the amount of amplicons bound to it is measured by analysis of the fluorescent streptavidin. The output is given as Median Fluorescent Intensity (MFI) for each bead type. HPV DNA positive is below denoted HPV DNA+, while HPV DNA negative denoted as HPV DNA-.

3.2.3 PCR for HLA-A*02 amplification from FFPE

Conventional PCR was used for the detection of the HLA-A*02 allele in paper III. The method for amplifying the allele from FFPE biopsies is described in detail elsewhere[214, 215]. HLA-A*02 primers (forward A*577LL and reverse A*503invLL) were designed in order to amplify the HLA-A*02 exon 2, with exception of the A*020109, 0248, 0250, 0255 alleles, which have been found to be uncommon in the Swedish population, and have been described previously[202]. This method has the advantage of being able to perform in FFPE material and blood samples from the patients are thus not necessary to obtain. It thus allows for retrospective studies without the need for additional samples.

The PCR reaction and program used for the amplification of the allele are given in the next page:

PCR reaction

- 5ul 10X PCR Buffer PE
- 8ul DNTPs (1.25 mM/dNTP)
- 3ul $MgCl_2$ (25mM)
- 2ul forward primer (10pmol/ul)
- 2ul reverse primer (10pmol/ul)
- 0.4ul Taq polymerase
- 2.5ul sample DNA (50ng)
- dH_2O until total volume of 50ul

PCR program

• 94°C	4 min	1 hold
• 94°C	30 sec	40 cycles
56°C	30 sec	
72°C	45 sec	
• 72°C	5 mis	1 hold
• 4°C	on hold	

3.2.4 Immunohistochemistry

As mentioned above, testing of all the different biomarkers in this thesis, except for HLA-A*02, was mainly done by immunohistochemistry (IHC). Different antibodies were used for each marker, but the immunohistochemistry protocol was similar for each biomarker.

For detection of CD8+ and CD4+ TILs the streptavidin-biotin peroxidase method was used on 4µm formalin fixed, paraffin embedded (FFPE) sections. The mouse monoclonal antibodies anti-CD8 (clone 4B11, dilution 1:40) and anti-CD4 (clone 1F6, dilution 1:40) both from Novocastra Laboratories were used. All sections were subsequently incubated with biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA, U.S.A.). This was followed by incubation with the avidin-biotin-complex-PO using the VECTASTATIN® Elite® ABC kit (Vector Laboratories) and developed with DAB.

For detection of HLA class I and II, heavy chains the mouse monoclonal antibodies (mAb) HCA-2 and HC-10 were utilized. HCA2 recognizes most HLA-A and HC-10 most HLA-B and -C heavy chains, with some overlaps- The latter was obvious in paper IV and correlation to clinical outcome was as evaluated for both HC10 and HCA2 expression. HLA class II antigens were analyzed using mAb LGII-612.14 which recognizes HLA-DR –DQ and DP, but not other HLA class II antigens. The protocol used for immunohistochemical staining was similar to as that described for CD8+ and CD4+ TILs.

HLA-DR –DQ and DP, but not other HLA class II antigens). The protocol used for staining was similar to as that described for CD8+ and CD4+ TILs.

For the different APM components that were analyzed in this thesis, the following antibodies were used: for TAP1, rabbit polyclonal H-300; for TAP2, rabbit polyclonal

H210, both from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); for LMP2, rabbit polyclonal antibody ab3328; for 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 and the protocol was the same as described above.

3.2.4.1 Evaluation of immunohistochemistry slides

Despite the fact that the IHC protocol was similar for all the studies included in the thesis, the evaluation criteria were different in some cases and here this will be described in more detail.

For the evaluation of CD8+ and CD4+ TILs counts, 5 microscopic fields in a magnification of 40X were used and at least two independent researchers counted the infiltrating lymphocytes.

For the evaluation of HLA class I and II expression, both intensity of staining and percentage of the stained malignant cells were evaluated in comparison to the surrounding normal tissue, stroma and immune cells. The intensity of staining was categorized as "absent", "weak" or as "normal/strong" staining. In addition, the percentage of stained malignant cells was defined on a four tier scale and this was 0%, 1-25%, 26-50%, 51-75% and 76 -100%. Notably, in some cases HLA staining could vary within tumors from weak to normal or strong. In that case the part that resembled the most was used as an intensity score. This could be a result of different cell clonally selected populations within the same tumor or HPV could have something to do with that. However, it is difficult to comment on that. One could argue that staining is not a safe way to go here but an absent HLA expression was of absolute absence and weak was always in between the two categories.

The APM components that were examined as potential prognostic biomarkers in this thesis were evaluated both for nuclear and cytoplasmic compartments. The reason for the discrepancy in evaluation between different markers was mainly due to the fact that different staining patterns were observed between the tumors. Thus it was reasonable to evaluate both staining patterns. Thus, intensity and percentage of staining was also evaluated but separately for each of the compartments. The intensity was scored as "absent", "weak", "medium" and "strong/normal". The percentage was scored on a four tiered scale: 0%, 1-25%, 26-50%, 51-75% and 76 -100% of stained malignant cells. Of course, one could still argue about the evaluation and the uncertainty but it is quite easy for someone to distinguish the differences in the figures presented in papers IV and V.

3.2.5 Statistical analysis and data interpretation

Statistics and analysis of the data is very useful because it gives us the mathematics and values to evaluate the possible significance of the accumulated data. The statistical analysis and tests that were used throughout this thesis are presented below.

The Wilcoxon-Mann-Whitney test was used in order to test for differences in continuous and ordered categorical variables as the ones presented in paper I. Fisher's exact test and Pearson Chi-square test were used for unordered categorical variables. Student t-test or independent samples t-test was used for comparison of mean values between groups. Spearman rank correlation test was used for the comparison of expression of the different APM components.

Clinical outcome and survival of patients was measured in years after diagnosis until the occurrence of an event or 3 years after diagnosis where censoring of patients was applied. An event was defined as death due to any cause even non-related to disease (Overall survival, OS), death of patients by disease (Disease Specific Survival, DSS) or relapse of the disease (Disease Free Survival, DFS). Patients that died without any obvious signs of the disease were considered censored in DSS and patients that died without any prior relapse were censored at day 0 in DFS. Kaplan Meier curves were used for survival estimates of OS, DSS and DFS and differences in the survival were calculated by using the log-rank test. The Cox-proportional hazards model was used for calculation of the unadjusted and adjusted hazard ratios. More advanced statistics were used for the development and building of the prediction model in paper IV and these are analyzed in detail in the aforementioned paper.

4 RESULTS AND DISCUSSION

4.1 CD8+ AND CD4+ TILS IN HPV POSITIVE AND NEGATIVE TSCC AND BOTSCC AND PROGNOSIS (PAPER I)

Aim: To study the role of the CD8+ and CD4+ TILs in TSCC and BOTSCC in relation to HPV status of the tumor and the clinical outcome of the patients.

Results: In total 280 patients diagnosed 2000-2007 with TSCC (203 patients) and BOTSCC (77 patients) that had available biopsies and treated with curative intent were included in the study. All of the samples had previously been tested for HPV DNA and p16 expression. Immunohistochemistry was applied on sections for detection of CD8+ and CD4+ TILs.

Of the 280 tumors, 79% were HPV DNA+, with HPV 16 being the dominant type (94%). In addition, patients with HPV DNA+ TSCC were generally younger and had lower T-stages when compared to those with HPV DNA- tumors. Furthermore, the majority of the patients were males without any difference in sex distribution between TSCC and BOTSCC. Patients with HPV DNA+ BOTSCC had higher nodal stage and T-stage when compared with patients with HPV DNA- tumors.

HPV DNA+ tumors were found to be more frequently p16+ as compared to the HPV DNA- ones (82% vs. 8%). The numbers of the CD8+ and CD4+ TILs were possible to evaluate in 98% and 96% of the tumors respectively, and the loss of adequate samples was due to the fact that there was very little material left. There were significantly higher numbers of CD8+ TILs in the HPV DNA+ tumors compared to the negative ones (median was 36.0 and 6.2 respectively, MWW test, $p<0.001$). The same was true for the CD4+ TILs (median 13.3 and 7.3 respectively, MWW test, $p=0.001$).

A high CD8+ TIL count but not CD4+ count was found to be a favorable prognostic factor for patients with HPV DNA+ and HPV DNA+/p16+ tumors. To be able to evaluate the samples, patients were divided into four quartiles depending on their TIL counts. Upon evaluation patients within the 3 quartiles with the highest counts had a significantly better prognosis with regard to 3-year OS and DFS as compared to those in the quartile with the lowest counts (Figure 10). The same trends were observed for HPV DNA- tumors but statistical significance was obtained only for OS and the 4^{th} quartile with the highest counts. Notably, also the numbers of TILs in the different quartiles were lower for the HPV DNA- samples.

For CD4+ TIL counts, there was no effect on the clinical outcome of patients with HPV DNA+ and HPV DNA+/p16+ tumors. There was a tendency for better outcome of patients with HPV DNA- and HPV DNA-/p16- tumors but no statistical significance was obtained.

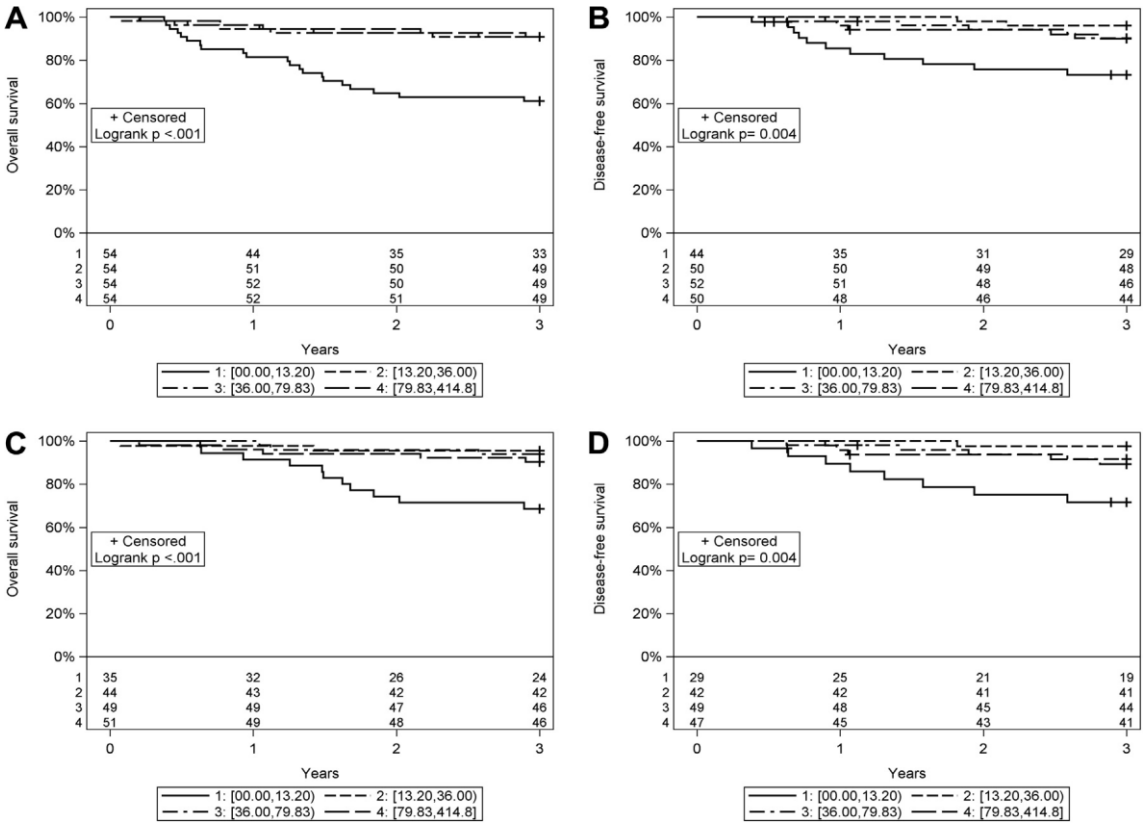


Figure 10: Kaplan Meier curves showing OS and DFS of patients with HPV DNA+ TSCC (A and B respectively) and HPV DNA+/p16+ TSCC (C and D respectively). Data have been stratified for counts of CD8+ TILs and they have been divided into 4 quartiles (The figure from Paper I, with permission from the publisher).

Discussion: In this study CD8+ and CD4+ TIL counts were investigated with regard to their potential as prognostic factors for clinical outcome of patients with HPV DNA+ and HPV DNA- TSCC and BOTSCC. We found that 79% of the samples were HPV DNA+ and that in general HPV DNA+ tumors were more infiltrated and displayed higher counts of CD8+ TILs than the HPV DNA- ones. Since HPV status of tumor defines two different tumor entities the two groups were studied separately and notably the mean/median CD8+ TIL count was different for the two groups. For patients with HPV DNA+ and HPV DNA+/p16+ tumors, the three highest quartiles of CD8+ TIL counts correlated with an increased survival of patients, while for HPV DNA- tumors this was only true for the highest quartile. In the latter group the CD8+ count of the highest quartile was similar to that of the first quartile of the ones with HPV DNA+ tumors.

CD4+ TIL counts were not correlated with survival in any of the groups although, there was a tendency for better clinical outcome of patients with HPV DNA- tumors and HPV DNA-/p16- tumors and high CD4+ TIL counts.

The fact that a high CD8+ TIL count was correlated with a high survival of patients was in line with a pilot study that had previously been performed by our group[193]. In addition, there are also other studies showing the same difference obtained between the HPV DNA+

and HPV DNA+/p16+ and the HPV DNA- and p16- groups with regard to survival in other malignancies such as cervical cancer and OSCC. The higher numbers of CD8+ TILs in HPV DNA+ tumors and/or HPV DNA+/p16+ tumors are also in line with *in vitro* studies indicating the importance of T-cell responses for HPV positive OSCC tumors[140]. Recent studies have also confirmed the significance of CD8+ TILs with regard to survival of patients with HPV positive HNSCC and they have also shown the increased infiltration that we show here[216]. They have looked at CD8+ TILs in head and neck cancer in general and the result of this study was similar to what we found, showing that CD8+TILs is indeed a very good biomarker for prognosis of the patients with clinical outcome and for possible stratification regarding treatment. Apart from the tumor infiltrating CD8+ TILs people have looked at the existence of these cells in the tumor microenvironment and they have shown in a recent study by Oguejiofor et al. that infiltration of the surrounding stroma by CD8+ TILs is associated with increased survival of patients with OSCC[217]. Thus, it becomes obvious that the immune system indeed plays a major role and infiltrating lymphocytes contribute to the better survival of patients.

Having high counts of CD8+ infiltrating the tumors suggests that these cells have the potential to be active against the tumor, despite the fact that we have pretreatment biopsies. It is worth assuming that upon treatment the pool of intracellular peptides being presented is increasing and the fact that we have more infiltration in the HPV DNA+ group is probably due to the fact that we have foreign antigens being presented. This allows for easier recognition by CD8+ TILs allowing for increased activity. This assumption can be strengthened further from a study showing that a higher proportion of CD8+ TILs deriving from HPV DNA+ HNSCC samples have the capacity to produce IFN-gamma and IL-17 upon in vitro stimulation[218].

The number of CD4+ TILs did not show any significant correlation to survival of patients with HPV DNA+ tumors but in patients with HPV DNA- tumors and HPV DNA- p16- tumors a higher infiltration with CD4+ TILs tended to result in a better clinical outcome of these patients. Other studies testing for CD3+ TILs which is a marker of T cells in general (thus including both CD4+ and CD8+ TILs) have shown similar data with regard to survival of patients with HPV DNA- and HPV DNA-/p16- tumors but no association has been revealed with HPV positive OSCC[216].

Conclusion: Patients with HPV DNA+ and/or HPV DNA+/ p16+ tumors with a high infiltration of CD8+ TILs generally have a better clinical outcome than those with tumors with low infiltration of CD8+ TILs. The data therefore suggests that CD8+ TILs are an additional prognostic factor that could be used together with the HPV status of the tumor in order for better prediction of the outcome of the patients when selecting for studies on more individualized therapy.

4.2 HLA CLASS I AND II EXPRESSION IN OSCC IN RELATION TO HPV STATUS AND CLINICAL OUTCOME (PAPER II)

Aim: To extend a previous study on HLA class I expression and also investigate the role of the classical HLA class I and II antigens in a large cohort of OSCC in relation to HPV status and the clinical outcome of the patients.

Results: In total 439 patients diagnosed with OSCC that were treated with curative intent and had available pre-treatment biopsies were included in the analysis and of these, 303/439 (69%) were HPV DNA+. The analysis of HLA class I and class II expression was performed both for HPV DNA+ and HPV DNA- and for HPV DNA+/p16+ and HPV DNA-/p16- tumors.

Patients with HPV DNA+ tumors had in general a better OS, DSS and DFS when compared with HPV DNA- and this was true in a multivariate analysis as well where age, stage and tumor localization were included and were therefore analyzed separately. Patients with HPV DNA+ OSCC were more often younger ($p<0.001$), non-smokers ($p<0.001$) and they generally presented smaller tumors ($p=0.009$), greater nodal stage ($p<0.001$) and a higher tumor stage ($p=0.009$).

In this study, in patients with HPV DNA+ tumors we showed that absence of HLA A, B and C, expression, measured as intensity of membranous staining by IHC with the antibodies HC2 and HC10 was correlated with a high OS, DFS and DSS and this was irrespective of the treatment regime used. More specifically, patients with absence of HLA class I and HPV DNA+ tumors had a very high survival, while the opposite was s true for strong intensity and weak intensity presented an intermediate survival (HC10: OS $p=0.009$, DFS $p=0.003$, DSS $p=0.021$) (Figure 11). Survival data were also analyzed in a multivariate manner adjusted for sex, age, stage and tumor localization and still patients with HPV positive OSCC and absence of HLA class I staining presented a better DFS, DSS and OS (HC10: all disease free, $p=0.040$ and $p=0.024$ respectively).

Similar data were obtained for HPV DNA+/p16+ tumors, but here statistical significance was obtained only for DFS.

In contrast, in patients with HPV DNA- tumors, absent HLA class I expression was correlated to a worse survival of these patients, while high HLA class I correlated to a better clinical outcome.

HLA class II expression, was not found to be correlated to survival in patients with HPV DNA+ tumors, but a high HLA class II expression was found to be correlated with increased DSS and OS, but not DFS in patients with HPV DNA- tumors.

In general, HLA class I expression was generally lower in HPV DNA+ OSCC and HLA class II was more often higher as compared to the HPV DNA- cases and these differences were significant. ($p=0.009$ and $p<0.001$ for intensity of staining).

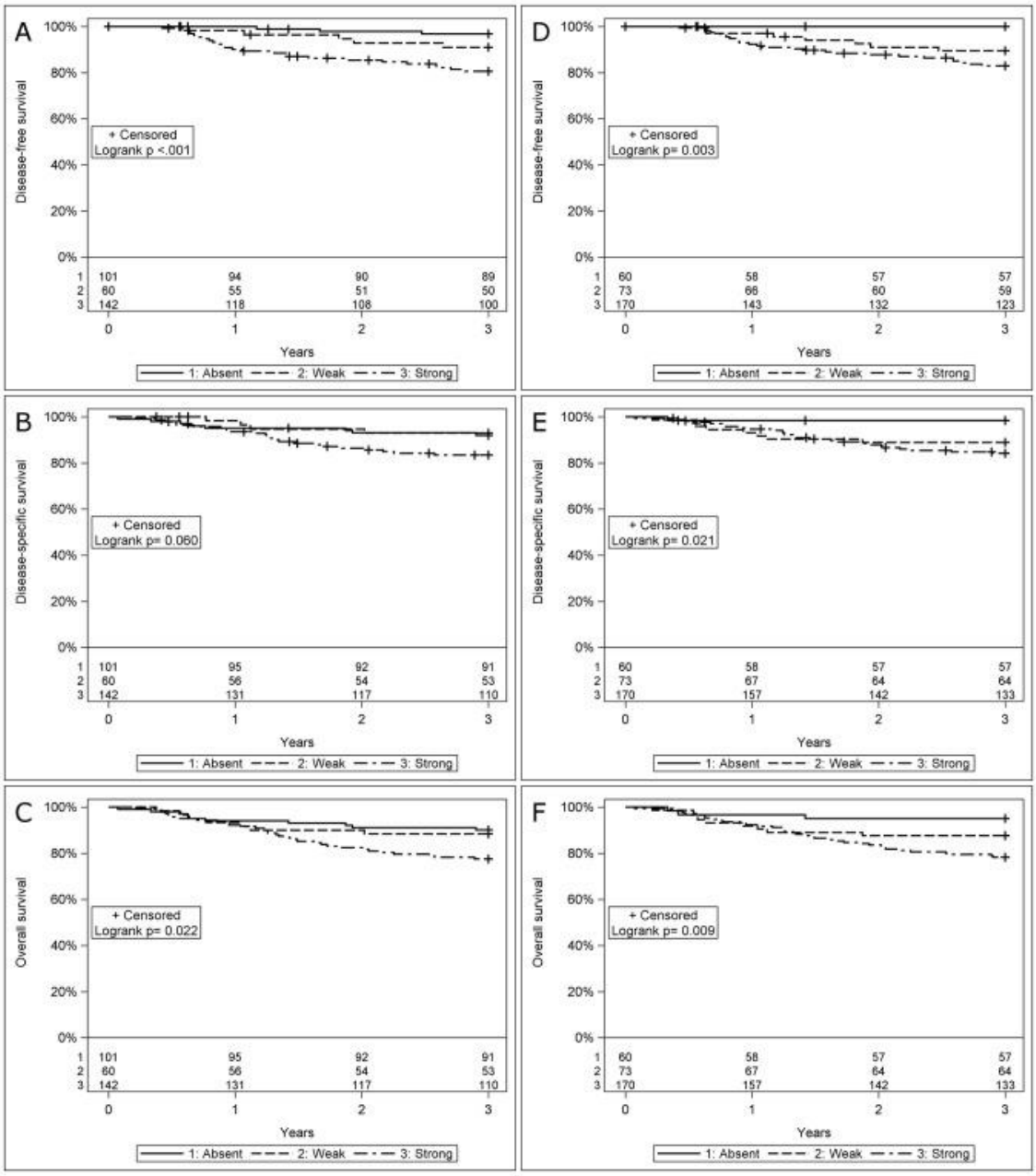


Figure 11: Kaplan-Meier curves showing disease-free survival (DFS), disease-specific survival (DSS) and overall survival (OS) for patients with HPV DNA+ oropharyngeal squamous cell carcinoma (OSCC) in relation to HLA class I expression. (With permission of the publisher) (A) DFS, (B) DSS, (C) OS, all stratified for HCA-2 intensity (D) DFS, (E) DSS, and (F) OS, all stratified for HC-10 intensity. HPVDNA+ OSCC and absence of HLA class I showed a significantly better clinical outcome than tumors with strong HLA class I intensity., Weak intensity staining revealed an intermediate survival (HCA-2: DFS p<0.001; DSS p=0.060; OS p=0.022; HC-10: DFS p=0.003, DSS p=0.021 and OS p=0.009,log-rank test).

Discussion: The fact that absence of HLA class I expression was associated with a very high survival of patients with HPV DNA+ OSCC was in line with previous data from the pilot study where similar findings were obtained in a smaller cohort of TSCC, but paradoxically from an immunological point of view. In addition, it is in contrast with what others have shown and a recent study looking at penile carcinoma and HPV, showed that

partial loss of HLA class I expression was associated with poor survival[219]. Why absence of HLA is associated with increased survival of patients with HPV DNA+ OSCC remains to be elucidated. There are however differences in our tumor model and those of others. In HPV positive tumors, E5 has the potential to repress HLA expression and the absence of HLA class I expression in some of the HPV DNA+ tumors could be a result of a prominent viral action. Also E7 has been demonstrated to down regulate HLA class I expression. HLA class I down regulation could be of benefit for the virus in this way avoiding immune recognition during infection.

The finding that patients with HPV DNA+ tumors with absent HLA class I expression do very well, we hypothesize may depend on the fact that upon radiotherapy (RT) the pool of antigenic peptides is increased and an abrogation of the suppression of HLA class I expression. The presence of increased HLA class I expression and presentation, leading to improved immune recognition and better prognosis. Experimentally, it has been previously shown that RT can cause upregulation of HLA class I expression in cervical and myeloma cell lines[220], and recently, data by Linnea Haeggblom in our group suggest that HLA class I expression is increased in HPV positive OSCC cell lines upon RT with doses around 10 Gy (unpublished). There are of course other studies showing that HLA class I downregulation may be due to viral integration within the HLA loci[221]. Thus, there are a number of possibilities on why absence of HLA class I still can be associated with increased survival of patients with HPV DNA+ OSCC, and this still has to be elucidated further.

Clinical outcome was also examined according to which type of therapy was given. For patients with HPV DNA+ tumors and absent/weak of HLA class I expression, there was no difference in OS, DFS or DSS, irrespective of whether the patients had received RT alone or chemoradiotherapy (Figure 12). However, for patients with HPV DNA+ tumors with strong HLA class I expression, there was a significantly better survival (OS, DFS and DSS) for patients that had received chemoradiotherapy (CRT) when compared to RT alone. However, there may have been selection bias for more patients receiving only RT than CRT and having poor prognosis, which could possibly be the answer for the difference presented in the group of patients with strong HLA class I staining.

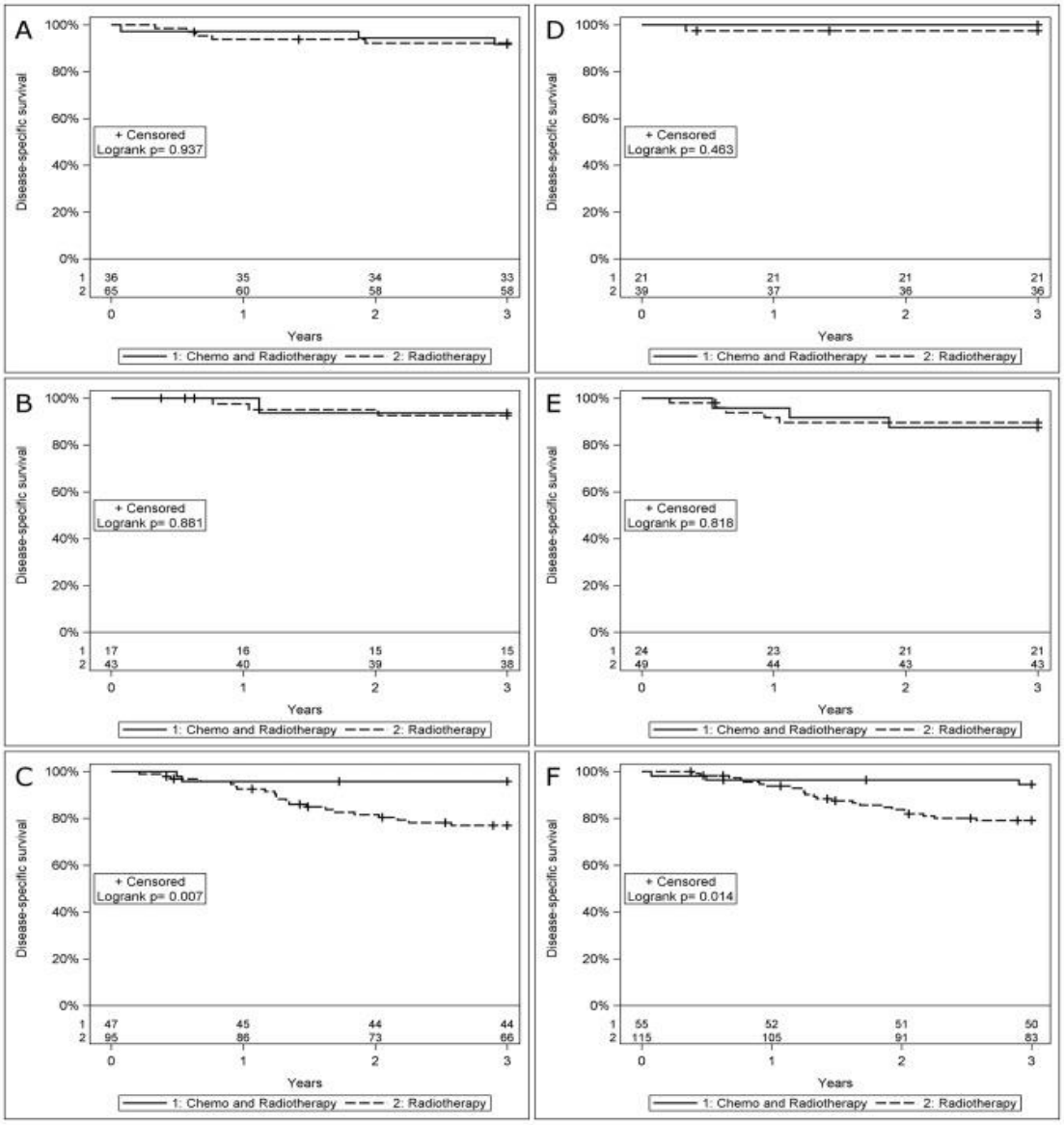


Figure 12: Kaplan-Meier curves by using the log rank test, for DSS of patients with HPVDNA+ OSCC and known HLA class I expression stratified for different treatment modalities. (A) DSS in HPVDNA+ OSCC, absent HCA-2 intensity and stratified for RT and CRT, (B) DSS in HPVDNA+ OSCC, weak HCA-2 intensity and stratification for RT and CRT, (C) DSS in HPVDNA+ OSCC, strong HCA-2 intensity stratified for RT and CRT (D) DSS in HPVDNA+ OSCC with absent HLA class I (HC-10) intensity stratified for RT and CRT (E) DSS in HPVDNA+ OSCC with weak HLA class I (HC-10) intensity stratified for RT and CRT, (F) DSS in HPVDNA + OSCC with strong HLA class I (HC-10) intensity stratified for RT and CRT.

Conclusion: Patients with HPV DNA+ tumors and absence of HLA class I expression have a better DFS, DSS and OS independent of what treatment they have received when compared to those with strong HLA class I intensity staining or those with HPV DNA- tumors. In contrast, for patients with HPV DNA- tumors strong HLA class I intensity expression was associated with increased survival. HLA class II expression did not show any correlation to survival.

4.3 HLA-A*02 IN RELATION TO HPV STATUS AND CLINICAL OUTCOME IN TSCC AND BOTSCC (PAPER III)

Aim: To examine HLA-A*02 as a prognostic factor in TSCC and BOTSCC in relation to HPV status

Results: Of the 445 patients that were diagnosed with TSCC and BOTSCC between 2000 and 2009 at the Karolinska University Hospital, 425 patients (307 TSCC and 118 BOTSCC) had available pretreatment biopsies and received curative treatment. These patients were included in the analysis.

Patients with HPV DNA+ tumors were younger than those with HPV DNA- ones. HLA-A*02 was present in almost 50% of the samples with 47% in the HPV DNA+ group and 53% in the HPV DNA- group. There were no correlation between the frequency of having an HLA-A*02 allele depending on HPV status of the tumor or the gender of the patients ($p=0.326$ and $p=0.744$, respectively). In addition, there was no difference in the frequency of the HLA-A*02 allele with regard to tumor stage, nodal stage and distant metastasis for the whole group of patients irrespective of the HPV status.

Absence of HLA-A*02 was correlated with a better 3-year DFS for the whole group of patients ($p=0.046$) and also for the HPV DNA+ group ($p=0.016$) but not for the HPV DNA- group ($p=0.968$) (data not shown and Figure 9). When the analysis was conducted separately for males and females, absence of HLA-A*02 was correlated to a better survival for males in the whole group ($p=0.004$) and only for males in the HPV DNA+ group ($p=0.003$). In a multivariate analysis by using Cox regression model, separately for HPV DNA+ and HPV DNA- groups, in the HPV DNA+ group absence of HLA-A*02 and lower age was still correlated with increased DFS of the patients ($p=0.036$ and $p=0.003$). There was no correlation in the HPV DNA- group and no correlation was found with regard to OS for none of the groups (Figure 13).

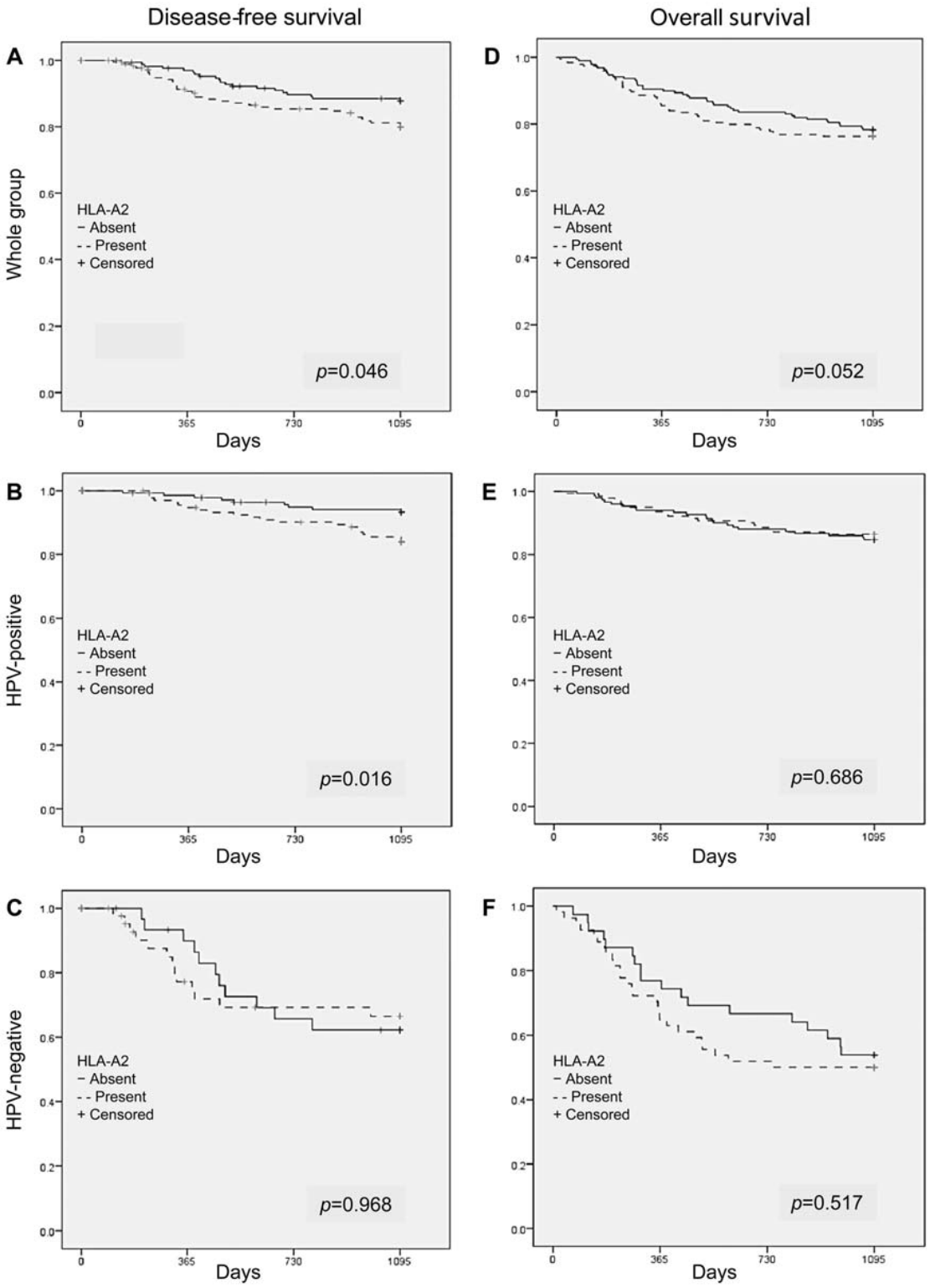


Figure 13: Kaplan Meier curves for DFS (A-C) and OS (D-F) of patients with TSCC and BOTSCC, presented for the whole group (A,D), HPV DNA + cases (BE) HPV DNA- cases (CF).

Discussion: In this study presence of HLA-A*02 was around 50% and similar in patients with HPV positive and negative tumors, indicating that there is no specific sensitivity to the development of HPV positive tumors depending on HLA-A*02 status. Furthermore, the frequency of HLA-A*02 was high in this study, which could be expected since in Scandinavian population it has been reported to be 58% for the phenotype.

Absence of HLA-A*02 was correlated with a significantly better DFS for the whole group and for the HPV DNA+ group of patients and in males with HPV DNA+ tumors. This was in concordance with what has been previously shown for ovarian cancer, prostate cancer and malignant melanoma[202, 203]. That this was not the case for the HPV DNA- group and for females with HPV positive and also HPV DNA- tumors is probably mainly attributed to the low numbers of patients in these categories. Furthermore, there were no differences with regard to OS and this is probably due to the fact that OS is influenced by other factors apart from the disease itself or other causes that may lead to death.

Conclusion: Presence of HLA-A*02 was similar in frequency between patients with HPV DNA+ and HPV DNA- TSCC and BOTSCC. In addition, presence of HLA-A*02 was a negative prognostic factor for patients with HPV DNA+ tumors.

4.4 LMP10 EXPRESSION IN RELATION TO HPV STATUS AND CLINICAL OUTCOME IN TSCC AND BOTSCC (PAPER V)

Aim: To examine LMP10 expression in HPV DNA+ and HPV DNA- TSCC and BOTSCC in relation to clinical outcome.

Results: In total 278 patients with TSCC and BOTSCC that were diagnosed between 2000 and 2007 and had available biopsies and were treated with curative intent were included in the study. Of the tumor biopsies, 207/278 (74%) were HPV DNA+. As described previously patients with HPV DNA+ tumors were younger than those with HPV DNA- tumors ($p<0.001$). LMP10 was evaluated with regard to fraction of the cells stained as well as intensity staining in the cytoplasm and in the nucleus. The fraction of cells stained in the cytoplasm was relatively higher in the HPV DNA+ group as compared to the HPV DNA- group of tumors ($p=0.033$) apart from that no major differences with regard to LMP10 cytoplasmic staining were observed between HPV DNA+ and HPV DNA- tumors.

Absent/ low fraction of positively stained cells for LMP10 nuclear staining was associated with a better 3-year DFS of patients with HPV DNA+ cancer but this was not true for patients with HPV DNA- cancer ($p=0.005$, $p=0.688$, respectively). No significant difference was observed with regard to OS in any of the patient groups. Moreover, absence as compared to presence of nuclear LMP 10 staining was also associated with a better DFS for individuals with HPV DNA+ cancer but not for those with HPV DNA- cancer ($p=0.037$, $p=0.473$, respectively) (Figure 14). Performing a multivariate Cox-regression analysis patients with HPV DNA+ or HPV DNA- tumors, including either fraction of stained positive cells in the nucleus, or intensity of nuclear staining together with age and stage, revealed that in patients with HPV DNA + cancer an absent or low fraction of LMP10 nuclear intensity staining was associated to better DFS ($p=0.027$).This was not true for OS. For this group LMP10 nuclear intensity staining, did not show statistical significance neither for DFS nor for OS.

LMP10 staining in the cytoplasm was evaluated again both for the fraction of positive cells but also for the intensity of staining and correlated to survival. A moderate/high as compared to low fraction of positively stained cells in the cytoplasm was associated to DFS for individuals with HPV DNA- cancer but not for those with HPV DNA+ cancer ($p=0.003$ and $p=0.086$ respectively). For OS the association was only found to be significant for those with HPV DNA- cancer and not for those with HPV DNA+ cancer ($p=0.001$ and $p=0.159$, respectively).

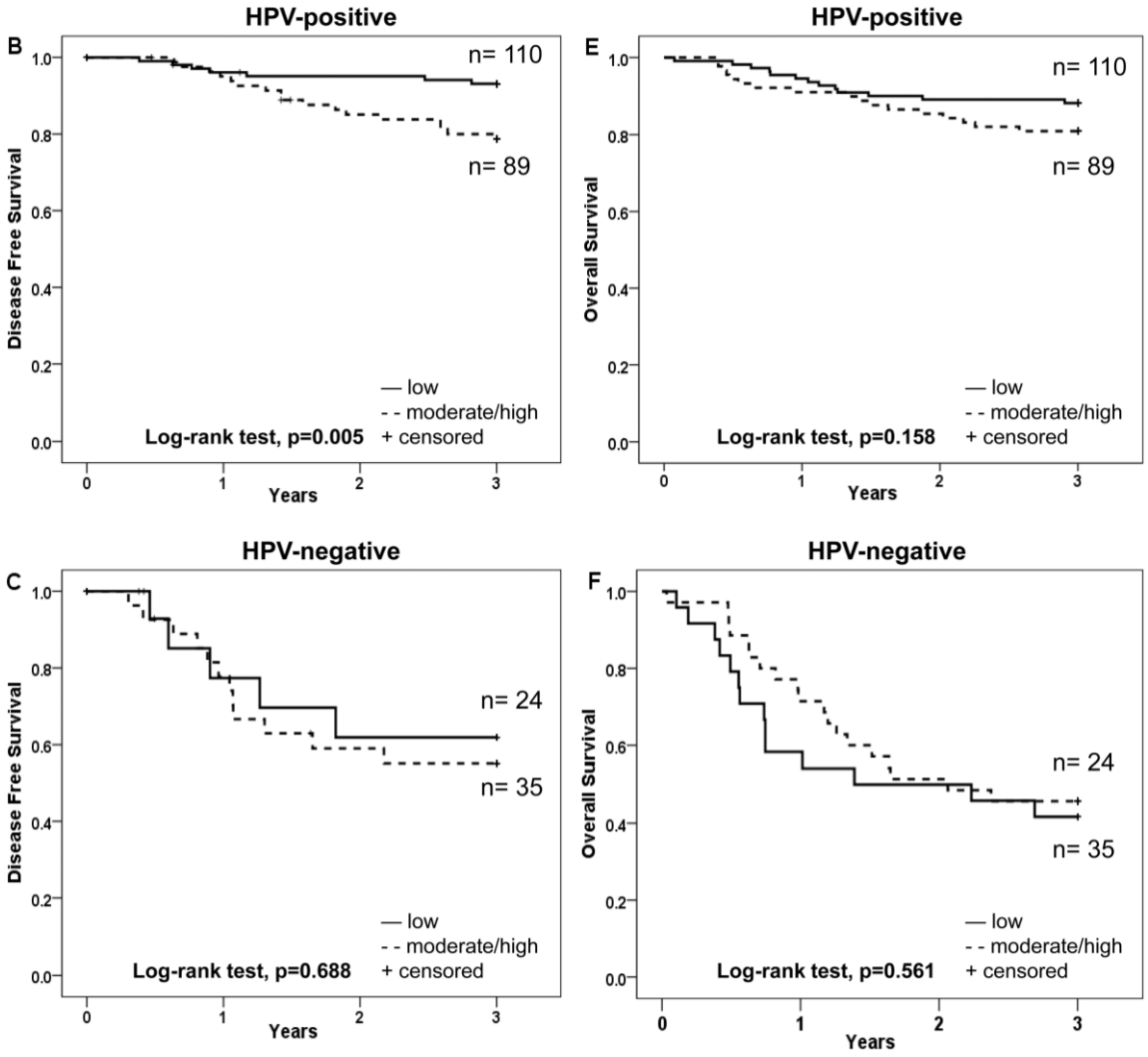


Figure 14: Kaplan-Meier curves by using Log-Rank test, for disease free survival (DFS) and overall survival (OS) of patients with TSCC or BOTSCC and stratified according to fraction of cells positive for nuclear expression of LMP10. B and E: DFS (B) and OS (E) of patients with HPV-positive TSCC and BOTSCC. C and F: DFS (C) and OS (F) of patients with HPV-negative TSCC and BOTSCC.

LMP10 cytoplasmic intensity staining when compared to absence of staining was correlated to a better DFS for patients with HPV DNA- tumors but not for patients with HPV DNA+ tumors (p=0.001 and p=0.368, respectively). The same was found for OS and the corresponding values were p=0.009 and p=0.334 (Figure 15).

In a multivariate Cox- regression analysis, separately performed for r HPV DNA+ and HPV DNA- groups, including either the fraction of positively stained cells in the cytoplasm or the intensity of the cytoplasmic staining and also age and stage of the tumor with regard to DFS and OS, revealed that inpatients with HPV DNA- tumors, both a moderate/high fraction of positively LMP10 stained cells, and presence of LMP10 cytoplasmic staining were correlated to better 3-year DFS (p=0.014 and p=0.006, respectively) (Table 3). Regarding the corresponding figures for 3-year OS, these were p=0.010 and p=0.051 respectively (Table 3). Age and stage at this point, did not reach statistical significance in the analysis for patients with HPV DNA- tumors (data not shown). The corresponding analysis for the HPV DNA+ group suggested that cytoplasmic

LMP10 expression had no association with clinical outcome (3-year DFS and 3-year OS) (Table 3).

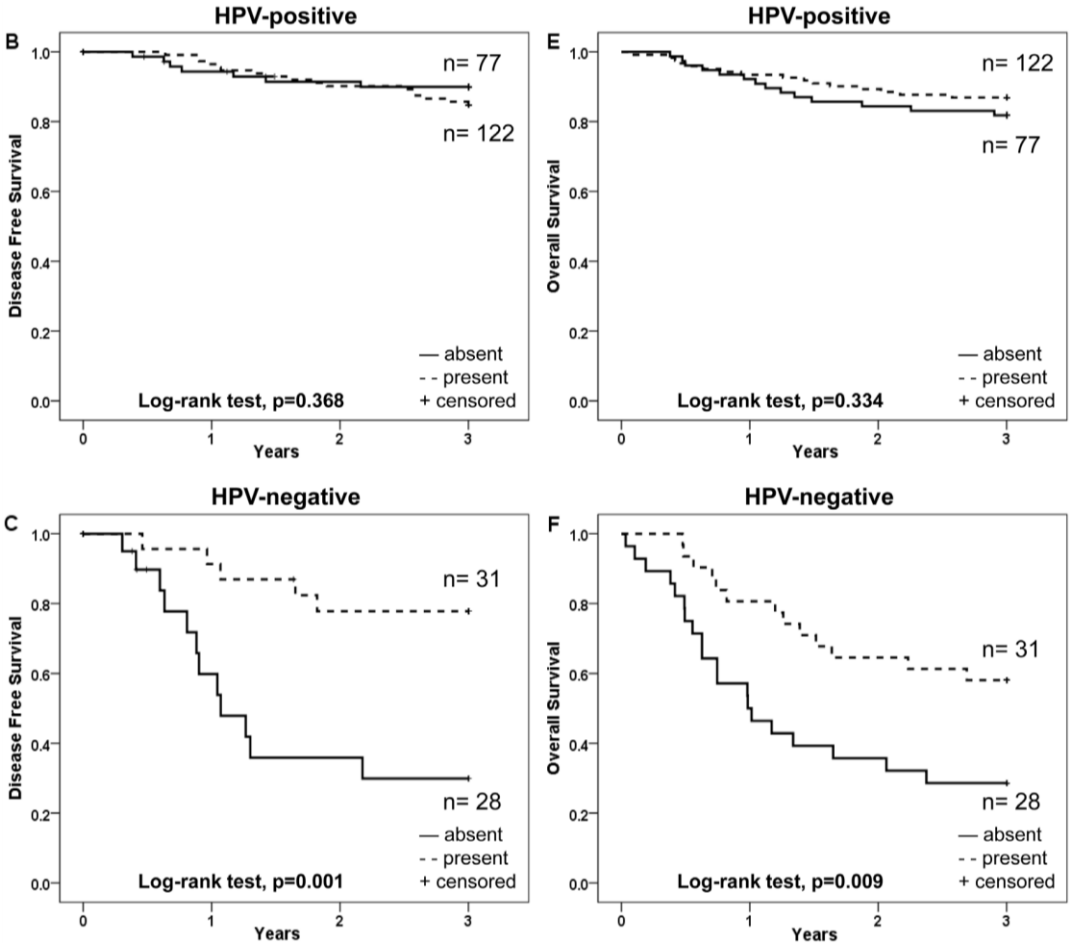


Figure 15: Kaplan-Meier curves showing disease free survival (DFS) and overall survival (OS) of patients with TSCC or BOTSCC, treated stratified according to intensity of cytoplasmic expression of LMP10. B and E: DFS (B) and OS (E) of patients with HPV-positive TSCC and BOTSCC. C and F: DFS (C) and OS (F) of patients with HPV-negative TSCC and BOTSCC.

Table 3: Univariable and multivariable analyses on LMP10 staining of TSCC and BOTSCC in relation to DFS and OS.

		DFS						OS					
		Univariable			Multivariable[§]			Univariable			Multivariable[§]		
		HR	95% CI	p-value	HR	95.0% CI	p-value	HR	95.0% CI	p-value	HR	95.0% CI	p-value
HPV-positive													
Nucleus	Fraction*	3.254	1.349–7.850	**.009**	2.755	1.121–6.767	**.027**	1.673	0.813–3.445	.162	1.340	0.640–2.805	.438
	Intensity**	2.729	1.019–7.310	**.046**	2.253	0.827–6.135	.112	2.228	0.956–5.194	.063	1.792	0.754–4.261	.187
Cytoplasm	Fraction	2.070	0.886–4.838	.093	1.869	0.797–4.382	.150	.590	0.281–1.240	.164	.521	0.247–1.098	.086
	Intensity	1.494	0.620–3.604	.371	1.346	0.557–3.254	.509	.703	0.343–1.441	.336	.617	0.300–1.269	.189
HPV-negative													
Nucleus	Fraction	1.238	0.436–3.518	.688	1.274	0.447–3.631	.650	.815	0.408–1.627	.562	.958	0.474–1.935	.905
	Intensity	1.573	0.452–5.481	.477	1.645	0.461–5.871	.443	1.092	0.474–2.519	.836	1.194	0.516–2.764	.679
Cytoplasm	Fraction	.143	0.033–0.631	**.010**	.154	0.035–0.684	**.014**	.234	0.090–0.610	**.003**	.281	0.107–0.739	**.010**
	Intensity	.204	0.071–0.584	**.003**	.219	0.073–0.653	**.006**	.405	0.201–0.818	**.012**	.487	0.236–1.004	.051

[§]Adjusted for age and tumor stage,
*absent/low vs moderate/high,
**absent vs weak/moderate/high.
Abbreviations: HR, Hazard Ratio; CI, Confidense Interval.
doi:10.1371/journal.pone.0095624.t003

Discussion: In the present study LMP10 was evaluated and scored both for the nuclear and cytoplasmic staining including both fraction of positively stained malignant cells and intensity of staining in both compartments of the cells. An absent/low fraction of LMP10 positively stained cells in the nucleus, as well as absent staining intensity in the nucleus, was associated to a better DFS for patients with HPV DNA+ cancer, but not for those with HPV DNA- cancer. The opposite was observed for cytoplasmic LMP10 staining, where a moderate/high fraction of positively stained cells and weak/moderate/high intensity was associated to a better DFS and OS in patients with HPV DNA- cancer, but not in those with HPV DNA+ cancer.

The reason for the difference described above is not clear but the separation and evaluation of both fraction and intensity of staining has been investigated before for other tumor types such as bladder and esophageal cancer but did not show any prognostic significance. However, in these studies there was no separation in nuclear and cytoplasmic staining which in our case revealed extra information. There are no prior studies on LMP10 expression in relation to survival in HNSCC and more specifically in OSCC. Meissner *et al.*[201] demonstrated a correlation with survival in HNSCC for both LMP2 and LMP7, which is in accordance with what we found in paper IV. However, the tumors were not analyzed for the presence of HPV in any of these studies.

The difference between the nuclear and cytoplasmic staining with regard to survival suggests many implications. It is not known if HPV has the ability to influence the expression of LMP10 expression. It has however been shown that E5 has the potential to downregulate the expression of HLA class I. If that is the case for LMP10 has to our knowledge not been investigated.

Conclusion: LMP10 expression was in different ways correlated with DFS for both patients with HPV DNA+ TSCC and BOTSCC as well as in the HPV DNA- group of patients. Thus differential LMP10 expression may have the potential to be used in a clinical setting for both groups of patients.

4.5 EXPRESSION OF APM COMPONENTS IN RELATION TO HPV STATUS AND CLINICAL OUTCOME IN TSCC AND BOTSCC (PAPER IV)

Aim: To investigate the potential of APM components as prognostic markers in HPV positive and negative TSCC and BOTSCC and potentially correlate with HLA class I expression especially in HPV positive OSCC.

Results: The study cohort included 151 tumor samples in total, of which 78 were TSCC and 73 BOTSCC. In total, 67% of the tumors were HPV DNA+ with 48% being HPV DNA+ for TSCC and 52% for BOTSCC. DFS, DSS and OS of patients were analyzed in relation to the cytoplasmic and nuclear expression of TAP1, TAP2, LMP2 and LMP7 separately for patients with HPV positive and HPV DNA- tumors.

Nuclear expression of LMP7 was correlated to DFS for both groups, p=0.023 for HPV positive and p=0.049 for HPV DNA- tumors. Absence of nuclear staining for LMP7 was correlated to increased survival, whereas strong expression correlated with poor prognosis for patients with HPV DNA+ tumors, while weak expression was in the intermediate. In addition, absence of LMP7 nuclear expression correlated with DSS and OS in patients with HPV DNA- tumors (p=0.016 and p=0.042 respectively) (Figure 16).

LMP2 and its nuclear expression showed a similar trend to that of LMP7 but did not reach statistical significance (Figure 15).

Cytoplasmic LMP2 and LMP7 expression was not associated to survival of the patients. Similarly, no significant correlation was found for TAP1 and TAP2 in relation to the clinical outcome of the patients.

Notably, expression was measured and evaluated by intensity of cytoplasmic or nuclear staining for TSCC and BOTSCC separately and together and according to HPV status. Cytoplasmic expression of LMP10 was reduced in 89% of the tumors, and a similar trend was observed for TAP2, LMP2 and LMP7 also reduced in a substantial fraction of tumors (33-59%), while TAP1 was only reduced in 9% of the tumors. Similar results were obtained for TAP1, TAP2, LMP2, LMP7 and LMP10, nuclear expression with absence in 18-58% of the tumors.

Cytoplasmic and nuclear expression of the different APM components was examined separately for TSCC and BOTCC and was in general similar for both. The expression of APM components was also evaluated in relation to the HPV status of the tumors. There was no difference in TAP1, TAP2, LMP2, and LMP7 expression in HPV DNA+ and HPV DNA- tumors, while, as presented in Tertipis *et al.*, in paper IV there was a minor difference with regard to cytoplasmic LMP10 expression.

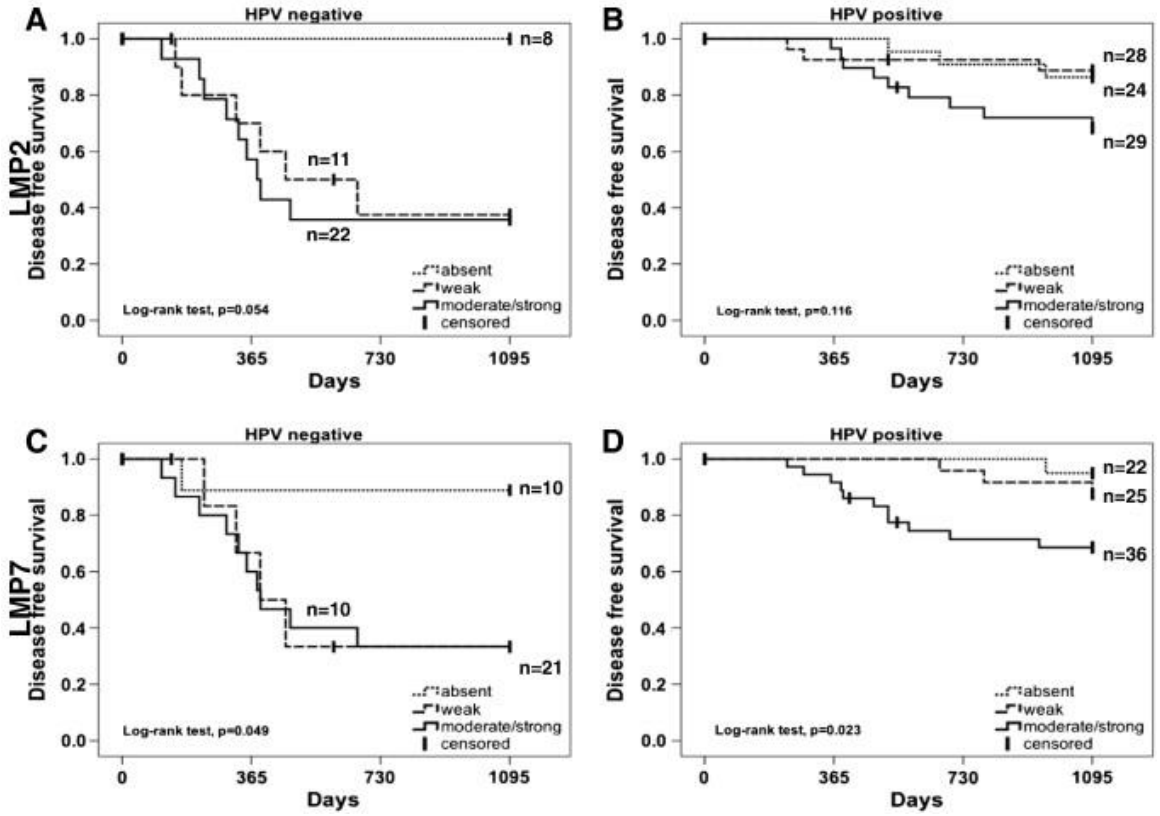


Figure 16: Kaplan-Meier curves, by suing Log-Rank test, for DFS of patients with TSCC and BOTSCC stratified by the intensity of staining in the nucleus for LMP2 in HPV-DNA+ (A) and HPV DNA- (B) cases and for LMP7 for HPV DNA+ (C) and HPV DNA- (D) cases; n shows the number of patients in each of the groups.

Expression of the APM components was also correlated to each other to investigate whether there was any association between them. This was conducted separately for nuclear and cytoplasmic staining. Several significant correlations were found and they are presented in Table 4.

Table 4: Spearman Rank Correlation of cytoplasmic (left) and nuclear expression (right) of APM components

Cytoplasmic Intensity

		TAP1	TAP2	LMP2	LMP7	LMP10	HCA2	HC10	
TAP1	Rho*	1.000	.384**	.040	.137	.003	.054	.030	HPV positive
	p-value		.000	.721	.210	.980	.608	.772	
TAP2	Rho	.563**	1.000	.174	.308**	.064	.129	.057	
	p-value	.000		.113	.004	.532	.219	.587	
LMP2	Rho	-.008	.072	1.000	.724**	.013	.132	.190	
	p-value	.957	.641		.000	.907	.243	.092	
LMP7	Rho	.135	.136	.508**	1.000	-.115	.150	.164	
	p-value	.378	.374	.000		.293	.179	.140	
LMP10	Rho	-.046	-.079	-.062	-.294*	1.000	-.128	-.136	
	p-value	.753	.589	.690	.048		.210	.183	
HCA2	Rho	-.150	-.185	.149	-.125	.249	1.000	.846**	
	p-value	.324	.224	.351	.435	.100		.000	
HC10	Rho	-.229	-.277	.047	-.221	.175	.678**	1.000	
	p-value	.131	.066	.771	.166	.249	.000		
		HPV negative							

*Spearman correlation coefficient
* Correlation significance at the 0.05 level — Positive correlation
** Correlation significance at the 0.01 level — Negative correlation

Nuclear Intensity

		TAP1	TAP2	LMP2	LMP7	LMP10	HCA2	HC10	
TAP1	Rho*	1.000	.456**	.156	.168	-.090	-.009	.053	HPV positive
	p-value		.000	.159	.124	.380	.928	.609	
TAP2	Rho	.700**	1.000	.204	.304**	.182	.081	.045	
	p-value	.000		.062	.005	.074	.442	.672	
LMP2	Rho	.220	.449**	1.000	.721**	.177	.188	.252*	
	p-value	.152	.002		.000	.107	.095	.024	
LMP7	Rho	.446**	.566**	.753**	1.000	.117	.266*	.273*	
	p-value	.002	.000	.000		.285	.016	.013	
LMP10	Rho	.160	.133	-.133	.054	1.000	.008	.090	
	p-value	.273	.363	.391	.719		.939	.382	
HCA2	Rho	-.278	-.345*	-.039	-.127	.073	1.000	.846**	
	p-value	.065	.020	.808	.430	.636		.000	
HC10	Rho	-.271	-.341*	-.043	-.180	.057	.678**	1.000	
	p-value	.072	.022	.791	.260	.710	.000		
		HPV negative							

*Spearman correlation coefficient
*Correlation significance at the 0.05 level — Positive correlation
**Correlation significance at the 0.01 level — Negative correlation

Discussion: In this study, absence of LMP7 nuclear expression was correlated to increased DFS of patients with HPV positive tumors, with a smiliar trend for LMP2. There was some intercorrelations between APM components. For example it has to be mentioned that especially the expression of TAP1 and TAP2 molecules were found to be strongly correlated and the fact that they are known to be regulated together and are regulated by the same promoter, further strengthens our study and implies an indirect proof of the validity of the results of our study. There was a general reduction of their expression in the tumors and in addition nuclear LMP2 and LMP7 expression was correlated to HLA class I expression in HPV DNA+ tumors.

Reduced expression of the APM components as well as of HLA class I antigens is common and has often been shown in many different malignancies and is generally regarded as a mechanims of immune evasion. More specifically, it has been shown for HNSCC, laryngeal squamous cell carcinoma, cervical and urothelial cancers and also malignant melanoma[198, 200, 201, 222, 223]. In HNSCC and cervical carcinoma, TAP1, TAP2, LMP2, and LMP7 expression was frequently reduced, although LMP7 reduction was not as pronounced in the latter[200, 201]. The frequencies of absent or reduced TAP1, TAP2, LMP2, and LMP7 expression in the HNSCC study by Meissner *et al*. were also somewhat higher than those presented here, but in line with our data, TAP1 was less frequently reduced than TAP2.

APM component expression was evaluated for both cellular compartments since different staining patterns were observed. The cytoplasm is assumed to be the active compartment for these proteins, but the significance of nuclear staining for peptide presentation by HLA class I antigens especially in tumors as well as their effects on the immune response has not been investigated thoroughly. Variations in cytoplasmic and nuclear localization of APM components have been observed previously. In one report, the thymus presented pronounced LMP2 and LMP7 nuclear localization, while in liver cells the distribution of LMP2 and LMP7 between the cytoplasm and nucleus was more even[224].

Correlations between APM component expression and clinical outcome are generally interpreted to be associated to effects on the ability of the immune system to elicit an immune response to the tumor. However, LMP2, LMP7, and LMP10 also play a role with regard to cell survival and proliferation and protect cells against oxidative damage[225]. A high nuclear expression of LMP2 and LMP7 would thus enhance cell survival and proliferation, this way resulting in a poorer clinical outcome.

Conclusion: In HPV DNA+ TSCC and BOTSCC, absent/low nuclear LMP7 expression was correlated to better clinical outcome in line with earlier data on absent/low HLA class I and/or nuclear LMP10 expression. TAP2, LMP2, and LPM7 expression was, similar to HLA class I and LMP10 frequently absent or reduced in many TSCC and BOTSCC, and correlations between the analyzed APM components were noted. In addition, for HPV-positive tumors, LMP2, LMP7 and HLA class I expression were correlated.

4.6 A MODEL FOR PREDICTING CLINICAL OUTCOME OF PATIENTS WITH HPV POSITIVE TSCC AND BOTSCC (PAPER VI)

Aim: To combine our so far best predictive molecular markers and clinical characteristics of the patients into a single algorithm for predicting the clinical outcome of individual patients with HPV DNA+ and p16+ TSCC and BOTSCC.

Results: The study was initiated on a training cohort of 197 patients diagnosed during 2000–2007, and validated on a cohort of 118 patients from 2008–2011. A regularized logistic regression was used and coefficients were generated for each of the parameters included in the model. The model provided specific coefficients for the different markers and in this way, also individual patient risk scores and predicted probabilities for 3 year D/R (Death or relapse of the patient). Individual risk scores for each patient are obtained after the multiplication of the coefficient for each variable multiplied by the value of each patient for the corresponding predictor and finally by summing all the products from all the predictors (Table 5).

Table 5: Predictor variables and summary scores for the regularized logistic regression model of patients included in the training cohort.

Variable	Coefficient	Patient 1	Patient 2	Patient 3
Age at diagnosis	3.727	61	51	60
CD8+TILs[a]	−18.848	1.10	4.69	1.31
Stage	51.086	2	1	2
HLA class I (HC10-intensity)	27.429	2	0	1
BOTSCC	−2.653	0	1	0
CD8+ TILs[a] Stage	−10.773			
Risk score		339.9	99.6	300.3
Probability of event[b]		0.40	0.06	0.31
[a]CD8+ TIL counts are measured as $\log_e$ count +1.				
[b]Event is defined as 3-year D/R.				

High scores after the aforementioned multiplications correspond to high risk while low scores correspond to low risks. For that as it is presented in Table 5 above, a low risk for 3-year D/R is associated with younger age, lower stage, absent HLA class I as it is given by HC10 intensity and high CD8+ TIL counts, while minor contributions were offered by BOTSCC diagnosis. Also, in the model there was an interaction term as it is called and this is the CD8*Stage term which shows the fact that the total effect of the CD8+ TILs is dependent on the patients' tumor stage. The discriminative power of the model gave an AUC of 0.77 which is acceptable and was not diminished after calibration of the model.

The risk score (x) for a specific patient is given by multiplying all the different scores for each individual component of the model as presented in Table 5. Thus,

x= 3.727*(age) – 18.848*(CD8+TILs) + 51.086*(stage) + 27.429*(HC10 intensity) – 2.653*(Diagnosis) – 10.773*(CD8+TILs*Stage).

The probability (p) of an event is then calculated by the equation below:

$$p = \frac{1}{1 + \exp(-\left(-3.787 + \frac{x}{100}\right))}$$

As mentioned previously, the model was generated based on an initial cohort of 197 patients and was validated on an additional cohort of 118 patients. Both of the cohorts were very similar with the only exception being that the proportion of patients treated with conventional treatment was lower in the validation cohort reflecting the recent change in the trends for treatment of HNSCC. In the validation of the model the discriminative power remained almost the same (AUC=0.77, 95% CI). High CD8+ TIL counts retained a positive influence, but the significance of HLA class I expression was no longer prominent.

Discussion: In this study, a prediction model was generated for 3-year D/R based on clinical and biomarker data from patients with HPV DNA+ and p16+ TSCC and BOTSCC. The predictor variables included in the model were age, stage, diagnosis HLA class I expression, and CD8+ TIL counts. The predictive model effectively separated patients who died or relapsed within 3-years from those who had a 3-year progression-free survival. It also allowed for predicting risk of an event for individual patients. The model showed that a low risk is associated with younger age, lower stage, absent HLA class I as it is given by HC10 intensity and high CD8+ TIL counts. All the aforementioned variables have been previously shown to have an impact on favorable clinical outcome. It can be clearly seen that from the molecular markers included in the model that CD8+ TILs and HLA are both associated with immunity and we hypothesize that the downregulated HLA class I is due to HPV and upon treatment expression is restored allowing for immune recognition and elimination.

The model has its benefits and limitations. One difficulty in the development of the model is that the overall survival of patients with HPV positive tumors is very high and the numbers of events, i.e. death, or recurrence, are very few. This limits the numbers of biomarkers and clinical markers that can be included into the model when using a restricted number of patients.

There were also some revelations during this exercise in that CD44 and smoking, two biomarkers previously suggested to be of prognostic importance did not add to the predictive value of the model. For smoking it may be due to that the information given in the case reports is incomplete. However, it is also possible that some of the findings revealed here are because we only examine data for patients with HPV DNA+ p16+ tumors, and in these patients survival is generally slightly better than for those with only HPV DNA+ tumors.

It is well known in our field of head neck cancer and especially OSCC that smoking is a risk factor. The impact of smoking including package years has been studied extensively for patients with HPV DNA+ or p16+ tumors separately, but not specifically for those positive for both HPV DNA/p16[56, 226].

In our case, smoking was not included in the model due to the fact that it is a variable that is not well documented in our cohorts and it is not possible to calculate number of pack years.

The fact that treatment was not included in the model may suggest that the prognosis for a patient with HPV DNA+/p16+ TSCC or BOTSCC given conventional treatment might not differ much from the prognosis for the same patient with intensified treatment. The latter is supported by a study from Attner *et al.*, 2012[227] but it still remains to be elucidated and additional data and studies are in need to explore if this is indeed the case.

Conclusion: The present study demonstrates that combined information from several markers and clinical variables can be used in order to predict outcome with high accuracy, also on the individual patient level. Secondly, patient-specific probabilities of 3-year D/R were produced and the data could be validated on a new cohort. The generated model can be further refined with additional biomarkers and can also be evaluated in larger patient groups.

5 CONCLUSIONS

- ✓ Patients with HPV DNA+ (including those with HPV DNA+/p16+) and TSCC and BOTSCC have in general higher numbers of CD8+ TILs than those with HPV DNA- TSCC and BOTSCC.

- ✓ High numbers of CD8+ TILs are associated with a better clinical outcome in TSCC and BOTSCC patients in general and especially in patients with HPV DNA+ (including those with HPV DNA+/p16+) tumors (Paper I).

- ✓ Patients with HPV DNA+ OSCC with absent/low expression of HLA class I intensity staining present a very high DFS, DSS and OS (independent of the treatment regime) as compared to DFS, DSS and OS of patients with HPV DNA+ OSCC with strong HLA class I intensity (Paper II).

- ✓ Patients with HPV DNA- OSCC with a strong HLA class I intensity and/or HLA class II intensity present a better clinical outcome that those with HPV DNA- tumors and absence of HLA class I and/ or HLA class II (Paper II).

- ✓ The presence of the HLA-A*02 allele which is common in the Scandinavian population is not increased in OSCC, but is a negative prognostic factor for patients with HPV DNA+ OSCC and absence of it is correlated with increased DFS of patients with HPV DNA+ tumors (Paper III).

- ✓ Components of the antigen processiong machinery (APM) such as LMP7 and LMP10 and their expression are associated with increased survival of patients with HPV DNA+ TSCC and BOTSCC. More specifically, absence of LMP7 and LMP10 nuclear staining is correlated with increased DFS of patients with HPV DNA+ tumors (Papers IV and V).

- ✓ Cytoplasmic expression of LMP10 is associated with increased survival of patients with HPV DNA- TSCC and BOTSCC (Paper IV).

- ✓ Expression of APM components is often downregulated in TSCC and BOTSCC. Their expression is often intercorrelated. (Paper V).

- ✓ A model comprising information from clinical and molecular marker characteristics of the patients was able to predict the individual patient clinical outcome and produce specific probablities for 3-year D/R for the patients. Predictors included in the model were age, tumor stage, CD8 TIL counts and HC10 intensity staining (Paper VI).

6 FUTURE PERSPECTIVES AND GOALS

In this thesis, the potential of different immunological molecules implicated in antigen presentation and immune recognition, to serve as prognostic biomarkers for clinical outcome of patients with HPV positive and negative OSCC and more specifically TSCC and BOTSCC were examined.

A handful of biomarkers associated with the antigen processing machinery and antigen presentation were identified as good prognostic biomarkers. Some of these e.g. CD8+ TILs and the HLA class I molecules, have been studied in many different malignancies showing prognostic significance.

The specificity and sensitivity of each biomarker differed, and some biomarkers identified patients with good clinical outcome with high accuracy, but selected few patients. Other markers were less specific and selected out a larger number of patients. Therefore we needed to combine and investigate the potential of building up a prediction model, which utilized and combined several molecular biomarkers and clinical characteristics in a way that serves as a prediction tool. This was performed in paper IV, resulting in the individual prediction and probabilities of events with high accuracy.

To strengthen our model even further, more biomarkers and larger patient cohorts would be useful, to develop an even more robust model to identify patients with good prognosis and select out patients that respond to less intensive treatment. The latter is of specific importance in the future to be able to conduct randomized trials with de-escalated treatment.

The main focus of future studies would in my opinion be to identify additional biomarkers that could strengthen our model and also an inclusion of more detailed information on smoking and snuff would be of importance. Furthermore, more extensive studies should be conducted on the basis of combining several markers, and at the same time randomizing into conventional and accelerated treatment.

Additional biomarkers could be investigated on the RNA and DNA level and combined with the current ones. Although IHC is routine in clinical pathology, other technologies are already on their way allowing for the application of more advanced techniques in the pathology units, e.g. genome or transcriptome sequencing, has the potential to in short time (assay time) generate prognostic information.

Last but not least, mechanisms of HLA downregulation should be elucidated and explored on the molecular level to disclose a possible influence of HPV in this respect on several of the molecular markers investigated here.

Questions will always require an answer, but that is how science goes forward… New answers bring new question and so on… Keep on asking yourself …

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