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DIAGNOSTIC AND PROGNOSTIC MARKERS IN PRIMARILY NON-SMOKING RELATED HEAD AND NECK CANCER

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Diagnostic and Prognostic Markers in Primarily Non-Smoking Related Head and Neck Cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To Dodo, my family, and friends.

A specialist is a man who knows more and more about less and less.

William J. Mayo

POPULAR SCIENCE SUMMARY OF THE THESIS

In Sweden, head and neck cancer accounts for approximately 2 % of all new cancer diagnoses yearly. This may not sound much, but the numbers are rising by approximately 2–3 % yearly and have been increasing for the last couple of decades. If we investigate these numbers and head and neck cancer more specifically, we see that most of the cancers accounting for the recent increase are originating from the middle part of the throat, behind the mouth, in the region called the oropharynx. Furthermore, we know today that they are mostly (but not all) caused by human papillomavirus (HPV) infection and not by smoking and alcohol, which are otherwise common risk factors for the development of other head and neck cancers. These tumours represent approximately 40 % of all head and neck cancer cases in Sweden and it is estimated that their frequency will continue to rise over the next 30-40 years. In the future it is, however, assumed that many of these cancer cases will be prevented by the HPV vaccine! Patients with virus-initiated cancer have better survival rates than patients with non-virus mediated cancers and are effectively treated with radiotherapy or a combination of chemotherapy and radiotherapy. Treatment results are relatively good; 80 % are alive five years after finished treatment. Nonetheless, they struggle with crippling side effects, such as dry mouth, difficulties swallowing, loss of taste and appetite, and jawbone tissue destruction. Finally, we must not forget the other less fortunate 20 % of the patients, who get both post treatment side effects, as well as a tumour relapse. With the information available today we cannot identify the outcome in advance and therefore, we treat all oropharyngeal cancer patients with the same strategy (even the non-virus related). The first two projects in this thesis address this problem and our aim was to come closer to finding the most effective treatment for the right patient and to minimize side effects.

In Project I we compared two ways of defining HPV-positivity in oropharyngeal cancer. Today a protein called p16 is used as a surrogate marker for an active HPV infection. If more than 70 % of the cells within a tumour sample express this protein the tumour is positive for p16 and consequently considered positive for HPV. We however show that this is not a bulletproof method as these cancers sometimes, although positive for p16, do not carry genetic information (DNA) of the actual HPV. Furthermore, in our analyses, we divided oropharyngeal cancers into two groups: those with lymphatic tissue (lymphatic tissue in the tonsil and the base of tongue) and those without it (the remaining part of the throat behind the mouth) and show that p16-analysis only works in cancers with lymphatic tissue. Additionally, we found that the combination of p16-analysis and the presence of actual viral DNA together give a better prediction of the clinical outcome in these patients. We therefore recommend the use of both methods in combination when determining HPV-positivity in cancers of the oropharynx.

In Project II we investigated a possible prognostic role of a protein called psoriasin in a homogenous group of patients with tumours originating from the base of tongue, often originating from the lymphatic tissue. Psoriasin is believed to play a role in cell survival and maturation, and it is believed that its appearance in the tumour tissue could be associated with clinical outcome of the patient. We showed that this was the case in p16 positive tumours in the base of tongue, as those with no or low psoriasin levels had a more favourable survival rate.

In Project III we studied a newly defined cancer, namely HPV-related multiphenotypic sinonasal carcinoma (HMSC). Fewer than 100 cases of this cancer type have been described in the world so far. It is postulated to affect the nose and sinuses, and it also seems to be caused by an HPV infection. We characterised the clinical and microscopical nature of such tumours and were the first to categorize all patients with this diagnosis so far. We propose that these tumours, unlike previously assumed, could also originate from outside the nose and sinuses and that they indeed are related to the presence of HPV. However, more studies are needed to better define this cancer type in the future.

In Projects IV and V we studied another rare cancer, called adenoid cystic carcinoma (AdCC), which can arise from the salivary glands and their associated tissue. These cancers grow slowly, but late relapses are common, sometimes even after 10–15 years. Relapsed tumours may present new characteristics and grow extremely fast. The aetiology of these cancers is still unknown and no specific risk factors are established. There is also no consensus regarding treatment of these patients and how they should be followed up.

In Project IV we studied a possible influence of viral infections in the transformation of normal cells into cancer cells in AdCC. We analysed for the presence of both the previously mentioned HPV, but also another virus, which is included in the human polyomavirus (HPyV) family. We were not able to find any major causative role of HPV or HPyV in these cancers. However, we identified three tumours positive for HPV: one in the nose and two in the tonsil. After thorough analysis, we found that these three tumours were in fact not AdCC but resembled the newly defined cancer type from Project III, HMSC. This strengthens our findings, namely that HMSC could appear outside the nose and sinuses.

Project V is a retrospective descriptive study of AdCC patients who were diagnosed and treated in Stockholm. We investigated how these patients did over time and aimed to find new, or confirm general prognositc factors (such as gender, age, or smoking history) possibly influencing their survival. In summary, we confirmed that these general factors did not influence survival in AdCC cancer patients. However, we showed that patients with AdCC in the major salivary glands survived longer and had fewer relapses. On the other hand, we observed that some previously postulated factors, such as surgery with total removal of the tumour before radiotherapy, did not influence the survival of AdCC patients.

<u>To summarize, with the work of this thesis we found:</u>

- That p16 is a relevant surrogate marker for HPV only in oropharyngeal cancers with lymphatic tissue.
- A more precise way of diagnosing HPV in oropharyngeal cancers by testing both for p16 and HPV DNA.
- That psoriasin is a possible new prognostic marker for p16 positive base of tongue cancer.
- All HMSC cases described in the world so far and found cases described both inside and outside of the nose and sinuses. Furthermore, we found two additional cases that resemble HMSC in the tonsil.
- That general prognostic markers, such as: age, gender, and smoking history are not applicable in AdCC.
- That AdCCs within the major salivary glands have the best clincial outcome and that total removal of the AdCC tumour might not be crucial for the outcome of these patients, if they are later treated with radiotherapy as well.

POLJUDNOZNANSTVENI POVZETEK DOKTORSKEGA DELA

Na Švedskem rak glave in vratu predstavlja približno 2 % vseh novih diagnoz raka letno. To se morda ne sliši veliko, vendar se številke vsako leto povečajo za približno 2–3 % in rastejo že zadnjih nekaj desetletij. Če natančneje pogledamo številke, vidimo, da večina rakov, ki so odgovorni za nedavno naraščanje števila novih diagnoz, izvira iz srednjega dela žrela, predela imenovanega orofarinks. Poleg tega danes vemo, da jih večinoma (vendar ne vedno) povzroča okužba s humanim papilomskim virusom (HPV) in ne kajenje in alkohol, ki sta sicer pogosta nevarnostna dejavnika za raka glave in vratu. Ti tumorji predstavljajo približno 40 % vseh primerov raka glave in vratu na Švedskem in ocenjujeje se, da bo njihova pogostnost v naslednjih 30-40 letih še naraščala. Domneva se, da bo cepivo proti HPV veliko število teh rakov v prihodnosti preprečilo! Bolniki z rakom, ki ga povzroča HPV, imajo boljšo prognozo kot bolniki z rakom, ki ga ne povzroča virus, in se učinkovito zdravijo z radioterapijo ali kombinacijo kemoterapije in radioterapije. Rezultati zdravljenja so relativno dobri; 80 % bolnikov živi pet let po končanem zdravljenu. Kljub temu se bolniki borijo s hromečimi stranskimi učinki, kot so suha usta, težave pri požiranju, izguba okusa in apetita ter nekroza čeljusti. Nazadnje ne smemo pozabiti tudi drugih manj srečnih 20 % bolnikov, ki dobijo tako neželene učinke po zdravljenju kot tudi ponovitev tumorja. Z informacijami, ki so na voljo danes, ne moremo vnaprej napovedati izida, zato vse bolnike z rakom srednjega dela žrela zdravimo z enako strategijo (tudi tiste, ki niso povezani z virusi). Prva dva projekta v tej doktorski dizertaciji obravnavata ta problem. Naš cilj je bil najti najučinkovitejše zdravljenje za pravega pacienta in čim bolj zmanjšati stranske učinke.

V projektu I smo primerjali dva načina določanja HPV-pozitivnosti pri raku srednjega dela žrela. Za določanje aktivne okužbe s HPV-jem se danes uporablja protein p16, ki služi kot nadomestni marker za HPV v rakavnem tkivu. Če več kot 70 % celic v vzorcu tumorja izraža ta protein, velja, da je tumor pozitiven za p16 in posledično pozitiven za HPV. Pokazali smo, da ta metoda ni brez napak, saj ti raki včasih, čeprav so pozitivni za p16, ne nosijo genetske informacije (DNK) dejanskega virusa. Poleg tega smo v naših analizah rak orofarinksa razdelili v dve skupini: tiste z limfnim tkivom (limfno tkivo v mandljih in dnu jezika) in tiste brez njega (preostali del žrela za usti) in pokazali, da p16-analiza deluje samo pri rakih z limfnim tkivom. Poleg tega smo ugotovili, da kombinacija analize p16 in hkrati prisotnost dejanske virusne DNK dajeta boljšo napoved kliničnega izida pri teh bolnikih. Zato pri ugotavljanju HPV-pozitivnosti pri raku orofarinksa priporočamo kombinacijo obeh metod.

V projektu II smo raziskovali možno prognozno vlogo proteina, imenovanega psoriazin, v homogeni skupini bolnikov s tumorji na dnu jezika, kjer tudi najdemo limfno tkivo. Psoriazin naj bi imel vlogo pri preživetju in zorenju celic in domneva se, da je pojav psoriazina v tumorskem tkivu lahko povezan s kliničnim izidom bolnika. Pokazali smo, da to drži pri p16 pozitivnih tumorjih na dnu jezika, saj so imeli tisti brez ali z nizkimi vrednostmi psoriazina v tumourju boljšo prognozo.

V projektu III smo preučevali na novo definirano diagnozo raka, in sicer multifenotipski sinonazalni karcinom povezan s HPV, po angleško HPV-related multiphenotypic sinonasal carcinoma (HMSC). Do sedaj je v literaturi opisanih manj kot 100 primerov te vrste raka na svetu. Domneva se, da prizadene nos in sinuse, vzrok pa naj bi bila prav okužba s HPV. Opredelili smo klinično in mikroskopsko naravo tovrstnih tumorjev in kot prvi kategorizirali vse bolnike s to diagnozo doslej. Predlagamo, da bi lahko ti tumorji, za razliko od prejšnjih domnev, izvirali tudi zunaj nosu in sinusov ter da so dejansko povezani s prisotnostjo HPV. Za boljšo opredelitev te vrste raka je v prihodnosti potrebnih več študij z večjim številom pacientov.

V projektih IV in V smo preučevali še en redek rak, imenovan adenoidno cistični karcinom (po angleško adenoid cystic carcinoma = AdCC), ki lahko nastane iz žlez slinavk in z njimi povezanega tkiva. Ti raki rastejo počasi, vendar pogosto pride do poznih ponovitev raka, včasih tudi 10–15 let po končanem zdravljenju. Ponovljeni tumorji imajo lahko nove in drugačne značilnosti in pogosto rastejo zelo hitro. Izvor teh rakov je še vedno neznan in nevarnostni dejavniki prav tako še niso definirani. Poleg tega tudi ni enotnega mnenja o zdravljenju bolnikov s tem rakom in o tem, kako jih je treba spremljati po končanem zdravljenju.

V projektu IV smo preučevali možen vpliv virusnih okužb na transformacijo normalnih celic v rakave celice v AdCC. Analizirali smo prisotnost tako prej omenjenega HPV kot drugega virusa, ki je vključen v družino humanih poliomavirusov (HPyV) v AdCC. Večje vzročne vloge HPV ali HPyV pri teh vrstah raka nismo mogli najti. Kljub temu smo identificirali tri tumorje pozitivne za HPV, enega v nosu in dva v mandljih. Po temeljiti analizi smo ugotovili, da ti trije tumorji dejansko niso bili AdCC, ampak so bili podobni na novo definiranemu tipu raka iz projekta III (HMSC). To krepi naše prejšnje ugotovitve, in sicer, da se ti raki lahko pojavijo tudi zunaj nosu in sinusov.

Projekt V je retrospektivno opisna študija bolnikov z AdCC, ki so bili diagnosticirani in zdravljeni v Stockholmu. Raziskali smo, kako se je zdravstveno stanje the bolnikov spreminjalo med leti 2000 in 2022. Želeli smo najti nove ali pa potrditi splošno znane negativne dejavnike (kot so spol, starost ali zgodovina kajenja), ki bi lahko vplivali na njihovo preživetje. Potrdili smo, da ti splošni negativni dejavniki niso vplivali na preživetje bolnikov z rakom AdCC. Kljub temu smo pokazali, da so bolniki z AdCC v velikih žlezah slinavk preživeli dlje in imeli manj ponovitev tumorja. Po drugi strani pa smo opazili, da nekateri prej predpostavljeni pozitivni dejavniki, kot je operacija s totalno odstranitvijo tumorja, če je bolnik zatem tudi obsevan, niso vplivali na preživetje bolnikov z AdCC.

<u>Če povzamem, smo z delom te doktorske naloge pokazali da:</u>

• Je p16 relevanten nadomestni marker za HPV infekcijo le pri raku orofarinksa z limfnim tkivom.

• Natančnejšo diagnostiko za HPV infekcijo pri raku orofarinksa dosežemo s testiranjem za p16 in HPV DNK hkrati.

• Je psoriazin možen nov prognostični marker za p16 pozitivne rake baze jezika.

• Se HMSC lahko pojavi tako znotraj kot tudi izven nosu in sinusov, poleg tega pa smo v naši testni skupini našli še dva dodatna primera v mandljih, ki spominjata na HMSC.

• Da splošni prognostični markerji, kot so: starost, spol in zgodovina kajenja, niso uporabni v AdCC.

• Da imajo AdCC v velikih žlezah slinavk najboljši klinični izid in da totalna odstranitev AdCC tumorja morda ni ključna za preživetje teh bolnikov, če se kasneje zdravijo tudi z obsevanjem.

ABSTRACT

Head and neck cancer (HNC) includes cancers of the oral cavity, the pharynx (i.e., the nasopharynx, the oropharynx, and the hypopharynx), the larynx, the nasal cavity, the paranasal sinuses, and the salivary glands. Traditional risk factors are smoking, alcohol, opium, betel chewing, and virus infections, such as human papillomaviruses (HPV) and Epstein-Barr Virus (EBV). These viruses play a major role in some cancers, and it is evident that the aetiology of different HNC types differs. In Sweden, HNC risk profiles have changed in the past decades due to a decrease of smoking in both men and women, while an increase in HPV-related cases has been noted. This increase is anticipated to continue for some decades, until the introduction of the HPV vaccine will hopefully prevent most of them. This thesis has therefore focused on HNC primarily not associated with smoking. It includes studies on oropharyngeal squamous cell carcinoma (OPSCC) and HPV-related multiphenotypic sinonasal carcinoma (HMSC) in a broader context. Finally, we also studied adenoid cystic carcinoma (AdCC), where the aetiology is mainly unknown, and the diagnostics are still very challenging.

In **Paper I** we investigated the relationship between the presence of HPV DNA and $p16^{INK4a}$ (p16) overexpression and prognosis of different OPSCC subsites. We found that the presence of HPV DNA and p16 overexpression were favourable prognostic markers in the tonsillar and base of tongue cancer subsites (TSCC and BOTSCC, respectively) but not in other OPSCC cancer subsites (otherOPSCC). We also showed the importance of testing for both HPV DNA and p16 expression status for better prognostication.

In **Paper II** we investigated a possible prognostic role of psoriasin expression, examined by immunohistochemistry in base of tongue cancer. In this pilot study we could show that low psoriasin expression was a favourable prognostic marker in HPV-positive (HPV^+) BOTSCC.

Paper III includes a systematic literature review of studies on HMSC and the presence of different HPV types in these tumours and their various locations. The data indicated that HPV^+ tumours with such characteristics may not only be located within the sinonasal region.

In **Paper IV** we initially investigated whether the presence of HPV and human polyomaviruses (HPyVs) played a role in the prognosis of adenoid cystic carcinoma (AdCC) where the aetiology is still mainly unknown. In addition, we wanted to examine whether the presence of these viruses could play a diagnostic role. HPyVs did however not have a major role in the aetiology of AdCC, as no case was positive for HPyV. Of 68 patients analysed, there were three HPV^+ AdCC cases, but upon re-examination their pathology was more similar to HMSC. This suggested that HMSC may not be limited to the sinonasal region. Furthermore, our findings indicated that the presence of HPV could be used in diagnostics when distinguishing between AdCC and HMSC.

In **Paper V** we investigated a large cohort of AdCC patients (155 cases) regarding clinical presentation, treatment, and survival. We found that subsite (major salivary glands), early stage (stage I-II), and multimodal treatment were positive prognostic factors, while age, gender, perineural growth, or negative surgical margins did not influence clinical outcome.

In conclusion we were able to show that the presence of HPV DNA and p16 overexpression were favourable prognostic markers in TSCC and BOTSCC but not in otherOPSCC. In addition, low psoriasin expression was found to be a positive prognostic marker for HPV^+ BOTSCC. In the systematic literature review of HMSC we disclosed that this tumour entity could also arise at sites outside the sinonasal area. Furthermore, we could show that neither HPyVs nor HPV played a major role in AdCC, but that presence of HPV could be of differential diagnostic value, especially in the sinonasal area. The final paper describing a large AdCC patient cohort confirmed that commonly used prognostic factors, e.g., gender, age, and smoking history did not correlate with survival, notably in this study neither did perineural invasion nor radical surgery of the tumour primary.

SCIENTIFIC PAPERS INCLUDED IN THE THESIS

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CONTENTS

LIST OF ABBREVIATIONS

AC	Adenocarcinoma
AdCC	Adenoid cystic carcinoma
AJCC	American Joint Committee on Cancer
BKV	BK-virus
BOTSCC	Base of tongue squamous cell carcinoma
bs	Broad spectrum
CD	Cluster of differentiation
CDKN2A/B	Mutated cyclin dependent kinase inhibitor 2A/B
ChT	Chemotherapy
CRT	Chemoradiotherapy
CUP	Cancer of unknown primary
DFS	Disease-free survival
DM	Distant metastasis
DOD	Dead of disease
DR	Distant recurrence
EBV	Epstein-Barr virus
ECOG	Eastern Cooperative Oncology Group
ENE	Extranodal extension
FFPE	Formalin fixed and paraffin embedded
FGFR	Fibroblast growth factor receptor
FIGO	International federation of gynaecology and obstetrics
GP	General primer
HE	Haematoxylin and eosin
HLA	Human leukocyte antigen
HMSC	Human Papillomavirus-Related Multiphenotypic Sinonasal Carcinoma
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HPV-	Human papillomavirus negative
HPV+	Human papillomavirus positive
HPyV	Human polyomaviruses
HR-HPV	High risk human papillomavirus
ICD-10	International Classification of Diseases Version 10
IHC	Immunohistochemistry
JCV	JC-virus
LCR	Long control region
LR	Local relapse
LR-HPV	Low risk human papillomavirus
LRR	Locoregional recurrence
M	Distant metastatic spread
MFI	Median fluorescent index

MST	Malignant salivary gland tumours
N	Lymph node involvement
NED	No evidence of disease
NGS	Next generation sequencing
NKSCC	Non-keratinizing squamous cell carcinomas
NOTCH 1	Neurogenic locus notch homolog protein 1
OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
p16	P16INK4a protein
PAC	Polymorphous low-grade adenocarcinoma
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIK3CA	Phosphatidyl-inositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PML	Progressive multifocal leukoencephalopathy
PORT	Postoperative radiotherpay
PS	Performance status
PV	Papillomavirus
PVAN	Polyomavirus associated nephropathy
PyV	Polyomavirus
Rb	Retionblastoma protein
RRP	Recurrent respiratory papillomatosis
RT	Radiotherapy
SCC	Squamous cell carcinoma
T	Primary tumour size
TILs	Tumour infiltrating lymphocytes
TNM	TNM Classification of Malignant Tumours
TSCC	Tonsillar squamous cell carcinoma
UICC	Union for International Cancer Control
VEGFA	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
WHO	World health organisation

1 INTRODUCTION

Head and neck cancer (HNC) by definition includes cancers of the oral cavity, the nasopharynx, the oropharynx, the hypopharynx, the larynx, the nasal cavity, the paranasal sinuses and the salivary glands, summarizes a very large and diverse group of cancers. In the past in Sweden and many other Western countries, HNC was related to heavy smoking and alcohol consumption, while in other countries also betel chewing, opium and Epstein Barr virus (EBV) were important risk factors. More recently human papillomavirus (HPV) has been added to the causative agents for some HNC types (Marur and Forastiere, 2016, Cohen et al., 2018). Notably, the past decades in Sweden and many other Western countries, the risk profiles for HNC have changed due to a major decrease of smoking in the population, while instead a rise in HPV-related cases has been noted (Nasman et al., 2009, Marur et al., 2010, Attner et al., 2010, Haeggblom et al., 2019b, Hammarstedt et al., 2007, Mourad et al., 2017).

Working as a medical doctor, specializing in oncology as well as having special interest in oropharyngeal anatomy and HNC this recent change of the risk profile in HNC, with an increase of HPV-related cases was intriguing and made me more interested in HNC not related to smoking and alcohol.

The topics of this thesis have therefore all focused on HNC not primarily associated with smoking. They consist of studies of long-term survival and the prognostic role of HPV in oropharyngeal squamous cell carcinoma (OPSCC) at different subsites, such as tonsillar and base of tongue squamous cell carcinoma (TSCC and BOTSCC, respectively) and those at other subsites than the latter, i.e., otherOPSCC (Paper I). In addition, I have studied the prognostic role of the biomarker psoriasin in BOTSCC (Paper II). In the third Project I investigated another HPV-related cancer, Human papillomavirus-related Multiphenotypic Sinonasal Carcinoma (HMSC) and whether it was located only in the sinonasal sinuses (Paper III). Finally, I have also studied a possible role of HPV and human polyomaviruses (HPyVs) in another non-smoking or alcohol related cancer, adenoid cystic carcinoma (AdCC) (Paper IV). Lastly, I have in detail investigated different parameters potentially associated to clinical outcome in a large AdCC cohort in the Stockholm region (Paper V).

2 LITERATURE REVIEW

In this literature review, a brief background of head and neck cancer, epidemiology, risk factors and details of different subtypes will be presented. In addition, some data on human papillomavirus, human polyomaviruses and other biomarkers will be included.

2.1 BACKGROUND ON HEAD AND NECK CANCER, AND SOME OF THEIR SPECIFIC SUBSITES

2.1.1. Head and neck cancer – epidemiology – risk factors

HNC accounts for roughly 900.000 new cases and > 400.000 deaths per year worldwide (Chow, 2020). Moreover, it is the 6th most common cancer from a global perspective and accounts for almost 5 % of all cancer diagnoses (Chow, 2020, Bray et al., 2018). Major risk factors for HNC are smoking, alcohol, opium, betel quid chewing, oral infections, radiation exposure, personal history of HNC and furthermore, virus infections, such as HPV and EBV (Figure 1) (Chow, 2020, Sankaranarayanan et al., 1998, Nasman et al., 2020, Bray et al., 2018).

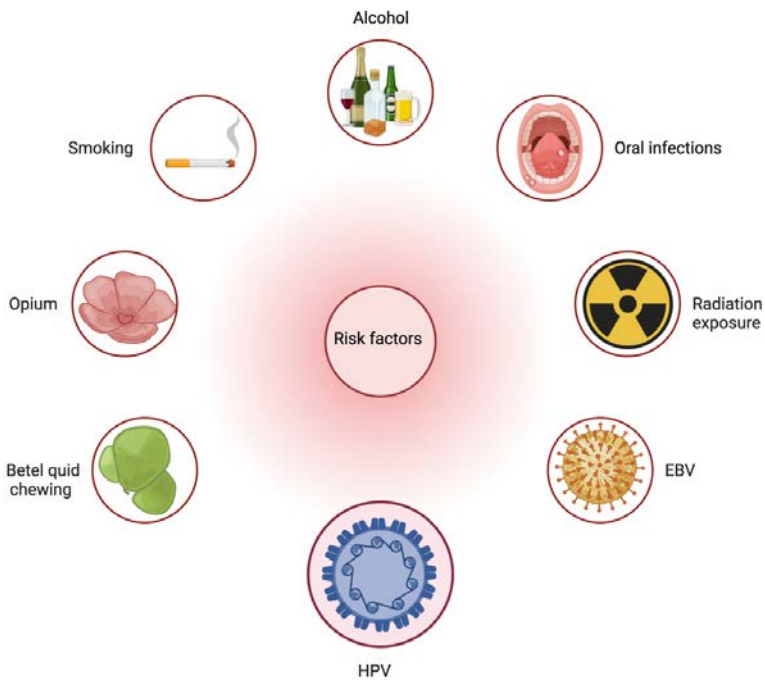


Figure 1. Risk factors for head and neck cancer. Created with BioRender.com.

In Sweden, when compared to the total global proportion, HNC is less common mainly due to a major decrease in smoking the past decades and accounts for approximately 2 % of all new cancer diagnoses yearly ((SweHNCR), 2021). However, although HNC is much less frequent in Sweden, its incidence has been rising 2-3 % yearly the past 40-50 years due to changes in its risk factor profile, with gradual changes in sexual behaviour and a rise in HPV-related cases ((SweHNCR), 2021, Nasman et al., 2020, Ramqvist and Dalianis, 2010). A corresponding picture, with some differences in the proportion of smokers, is observed in the Western world, where in parallel an increase in HPV-related cases has also been observed (Nasman et al., 2020, Zamani et al., 2020, Gillison et al., 2015, Johnson et al., 2020). These latter cases which predominantly affect men are presently anticipated to continue increasing for some more decades, until the HPV vaccine, in Sweden introduced for boys only in 2020, will gradually prevent most HPV^+ cases (Johnson et al., 2020, Nasman et al., 2020, Zamani et al., 2020, Gillison et al., 2015).

HNC accounts for cancers originating from the nasal cavity, the paranasal sinuses, the oral cavity, the pharynx (i.e., the nasopharynx, the oropharynx, and the hypopharynx), the larynx, and the salivary glands (Figure 2) (Chow, 2020, Johnson et al., 2020). In Sweden the most common sites are the oral cavity and the oropharynx ((SweHNCR), 2021) (Figure 3). However, other cancers originating from the same anatomical area, including the eye, thyroid gland, oesophagus, brain, skin cancers or lymphomas are not classified as HNC (Chow, 2020, Johnson et al., 2020). HNC are in 90 % of the cases squamous cell carcinomas (SCC) arising from mucosal surfaces (Johnson et al., 2020, Chow, 2020). HNC usually spreads locoregionally and finding two synchronic malignancies is not uncommon, especially in smokers. That is why a panendoscopy is performed upon diagnosis (Chow, 2020). HNC can also spread to the lymph nodes of the neck and/or via bloodstream. Sometimes SCC can be found in lymph nodes of the neck region, but the primary tumour cannot be detected. These cancers are called metastatic squamous cell carcinoma of unknown primary (CUP) of the head neck region and are counted as HNC (Nasman et al., 2020, Sivars et al., 2016).

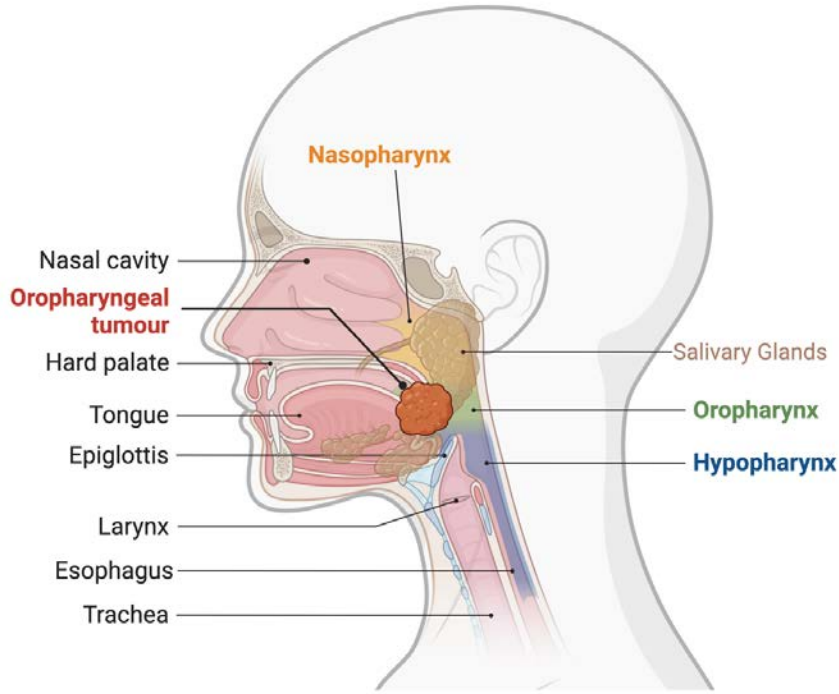


Figure 2. Schematic overview of the head and neck region. Created with BioRender.com.

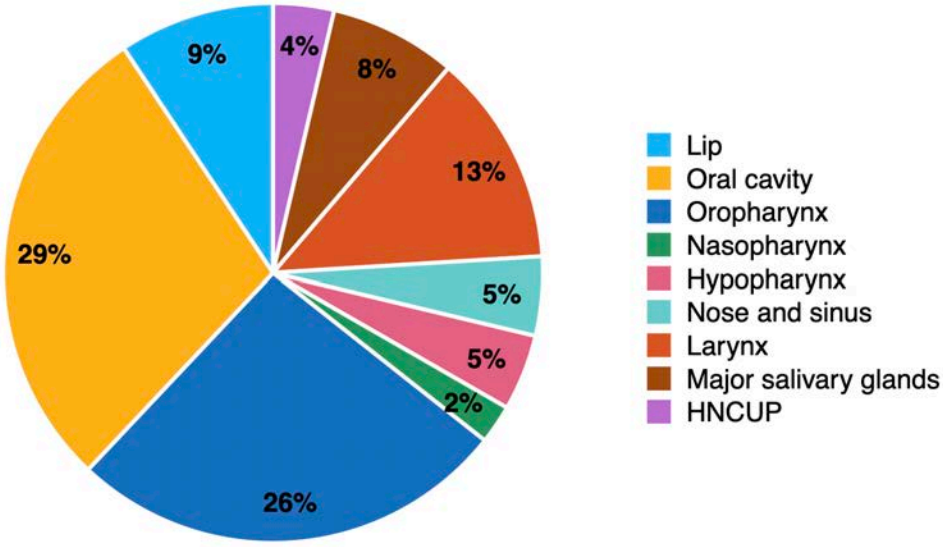


Figure 3. Distribution of head and neck cancer cases in Sweden between 2008 and 2017, Swedish Head and Neck Cancer Register (SweHNCR, 2020). HNCUP: Head and neck cancer of unknown primary.

Clearly, the different HNC types differ considerably. This thesis project deals mainly with HNC primarily not associated with smoking and covers oropharyngeal squamous cell carcinoma (OPSCC) and HPV-related multiphenotypic sinonasal carcinoma (HMSC) in a broader context, where the latter two are associated to infection with HPV (Nasman et al., 2020, Sabatini and Chiocca, 2020, Zupancic and Näsman, 2021). In addition, this project includes studies on malignant salivary gland tumours (MST), and more specifically adenoid cystic carcinoma (AdCC), where knowledge of the aetiological factors is mainly unknown (Pan et al., 2017, Horn-Ross et al., 1997).

This literature presentation will first introduce the above tumours individually. Moreover, in each specific tumour section, aspects of their diagnosis, and when relevant an introduction to some specific biomarkers, to some extent specific or completely specific for each tumour group may be included. In addition, reflections on treatment will be presented for each tumour type in more detail. After that, a presentation of the HPV family with more information on the epidemiology of HPV-related tumours will follow and some words on HPV vaccination will be presented. Finally, a short summary of human polyomaviruses (HPyV) and their pathogenic role will be included.

2.1.2 Oropharyngeal squamous cell carcinoma (OPSCC)

Background and epidemiology

Background. Oropharyngeal squamous cell carcinoma (OPSCC) is a tumour entity including cancers of the tonsils (TSCC), the base of tongue (BOTSCC), the posterior pharyngeal wall, and the soft palate, for details of the anatomy of the oropharynx see Figure 4 (Chow, 2020, Nasman et al., 2020). OPSCC is today the most commonly occurring HNC in Sweden that is not primarily dependent on smoking and alcohol as risk factors ((SweHNCR), 2021). Nevertheless, in the past, this tumour entity was mainly due to smoking and alcohol (Sankaranarayanan et al., 1998).

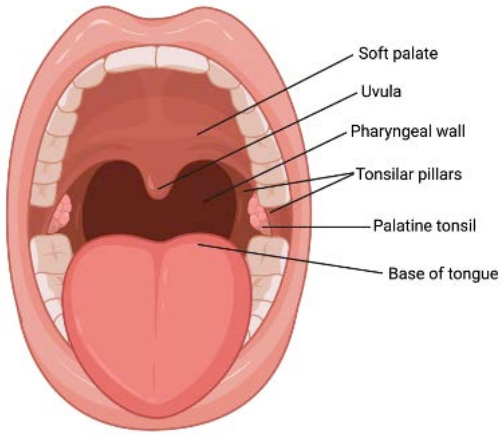


Figure 4. Schematic view of the oropharynx. Created with BioRender.com.

Epidemiology. Notably, however, since the 1970s the proportions of HPV$^+$ TSCC and BOTSCC, have been increasing in Sweden, while this has not been the case for cancers of the posterior pharyngeal wall and soft palate, the latter two are here defined as otherOPSCC (Nasman et al., 2020). For details of this increase between 1970-2021 see data from the Head and Neck Society and the Swedish Cancer Registry in Figure 5 and 6, respectively (SweHNCR, 2016) Today, around 70-80 % of TSCC and BOTSCC are HPV-related in Sweden, and they have increased by 7 % yearly during 2008-2017 ((SweHNCR), 2021). In fact, in Sweden, HPV$^+$ TSCC and BOTSCC are among the fastest increasing cancers and account for approximately 50 % of the total increase of HNC (Socialstyrelsen, 2018). Moreover, this is occurring even though smoking has been decreasing recently (Folkhälsomyndigheten, 2019).

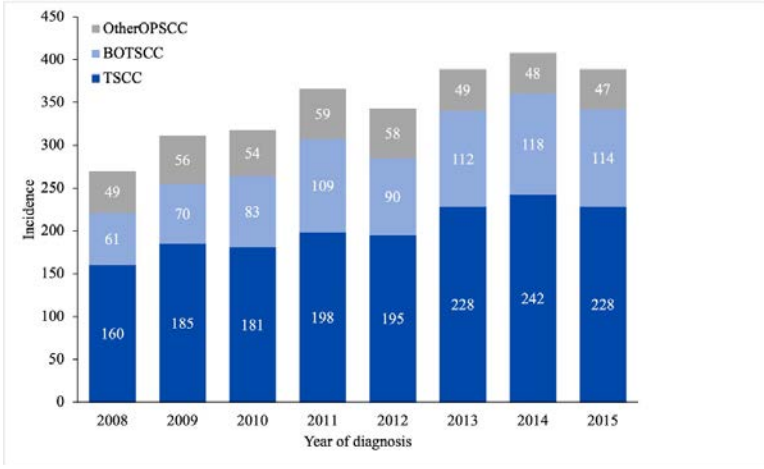


Figure 5. The incidence of OPSCC in Sweden between 2008-2015. Adopted from yearly report from SweHNCR 2016. (SweHNCR, 2016).

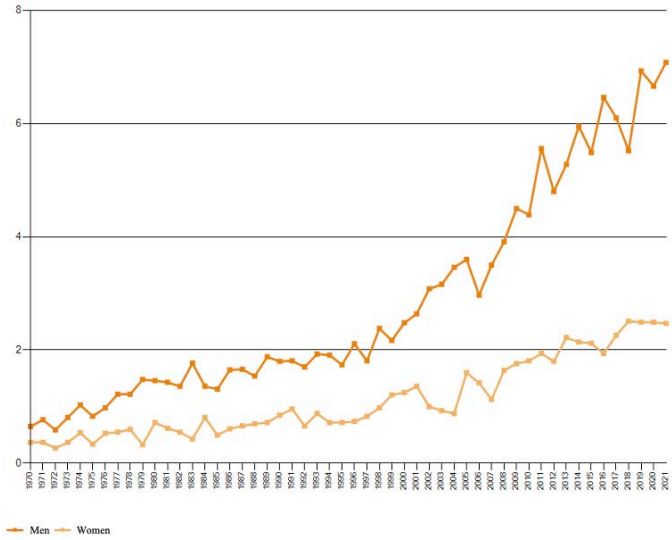


Figure 6. The incidence (number of new cases per 100.000 persons) of TSCC and BOTSCC in men and women from 1970 to 2021 in Sweden. Data from socialstyrelsen.se, Statistical Database, Cancer (Socialstyrelsen, 2022).

Most patients with OPSCC are men (Figure 6). Some studies show that in the HPV^+ group the proportion of men exceeds 80 %, but even in HPV unrelated OPSCC approximately 70 % of the patients are male (O'Sullivan et al., 2016). Patients with HPV^+ disease, infrequently consume alcohol excessively and are often non-smokers, and they have generally been younger than those with HPV-negative (HPV^-) disease (55-60 years of age vs. 65 years of age) (Nasman et al., 2020, O'Sullivan et al., 2016, Gillison et al., 2008, Thompson et al., 2018). However, recently the age difference has been narrowing between patients with HPV^+ and HPV^- HNC (Gillison et al., 2008, O'Sullivan et al., 2016, Thompson et al., 2018, Nasman et al., 2020). Another presumed risk factor for HPV^+ TSCC and BOTSCC is a greater number of different sexual partners at a young age. This has however, not been confirmed by all, nonetheless, changes in lifestyle factors over the past decades likely play an important role in the development of HPV^+ TSCC and BOTSCC (Nasman et al., 2020, O'Sullivan et al., 2016, Gillison et al., 2008, Thompson et al., 2018, Quabius et al., 2020, D'Souza et al., 2009).

It is also assumed that the most likely explanation for the presence of HPV in the tonsil and base of tongue, is their lymphoepithelial morphology, and their specific crypts, which are vulnerable to HPV infection (Johnson et al., 2020, Nasman et al., 2020). HPV is not known to play as an important role for HNC at other sites, with the exception of CUP of the head and neck region, but HPV can also occasionally be observed in e.g., a subset of sinonasal carcinomas, hypopharyngeal cancer and in otherOPSCC (Nasman et al., 2020, Sabatini and

Chiocca, 2020). Moreover, recently, a new emerging entity has been described, multiphenotypic sinonasal carcinoma, which has been linked to HPV infection and mainly HPV type 33 and is here defined as HPV-related multiphenotypical sinonasal carcinoma (Bishop et al., 2013b). Below this potentially emerging entity will be presented in a broader context.

Symptomatology, diagnosis, prognosis, treatment, and studies of diagnostic and prognostic markers

Symptomatology. OPSCC can clinically present with a variety of symptoms, such as odynophagia, dysphagia, tonsillitis, or lumps in the neck. However, the pattern is frequently different in HPV^+ OPSCC, where the primary tumour often does not give many symptoms, but instead early on spreads to the lymph nodes in the neck (Carpén et al., 2018). HPV^+ OPSCC has therefore often lower T-status (primary tumour size), but higher N-status (lymph node involvement) at diagnosis, meaning that HPV^+ primary tumours, even when smaller in size of the primary, more often present a larger nodal disease (Marklund et al., 2020).

Diagnosis. Diagnosis of OPSCC is usually performed by radiology, panendoscopy, histomorphological examination of a tumour biopsy, and immunohistochemistry (IHC). At Karolinska University Hospital, the definition of HPV status is assayed by both p16 overexpression by IHC and the presence of HPV DNA by polymerase chain reaction (PCR), and the reason for this is explained briefly below. HPV type 16 dominates in OPSCC and accounts for 80-90 % of the HPV^+ cases (Nasman et al., 2020).

A widely accepted golden standard for positive HPV status is the presence of HPV E6*1 mRNA, which suggests active expression of the HPV oncogenes (Smeets et al., 2007). However, in the past this analysis was complicated and instead overexpression of $p16^{ink4a}$ (p16) by IHC was used as a surrogate marker for presence of active HPV infection, while in some laboratories the analysis of presence of HPV DNA was performed (Mellin et al., 2000, Nasman et al., 2020). Notably, however it has been shown that neither the analysis of the presence of p16 overexpression nor the presence of HPV DNA have the accuracy of the golden standard, although when using the two in combination the results were almost as accurate as using the golden standard (Smeets et al., 2007). In this thesis we have therefore always attempted to use both HPV DNA and p16 overexpression when defining HPV status.

Prognosis. Several studies have shown that the prognosis of OPSCC, more specifically TSCC/BOTSCC differs depending on their HPV status and that patients with HPV^+ tumours have a significantly better overall survival (OS) and disease-free survival (DFS) compared to those with HPV^- tumours (Näsman et al., 2021, Mellin et al., 2000, Nasman et al., 2020, Sabatini and Chiocca, 2020). Notably, patients with HPV^+ TSCC/BOTSCC have an 80 % curation rate as compared to 40-50 % in patients with corresponding HPV^- TSCC/BOTSCC and otherOPSCC (Nasman et al., 2020, Näsman et al., 2021). Moreover, HPV^+ and HPV^- TSCC/BOTSCC have different genetic characteristics (Nasman et al., 2020, Näsman et al., 2021, Dahlgren et al., 2003, Smeets et al., 2006). HPV^+ and HPV^- OPSCC have therefore been suggested to be two different entities. Today, these are generally treated in a similar way, but in the future the aim is to offer a more personalized treatment to both these groups of patients.

Based on some of the differences between HPV^+ and HPV^- OPSCC the American Joint Committee on Cancer (AJCC) / Union for International Cancer Control (UICC) has now in its 8^{th} edition of TNM classification of malignant tumours (TNM-8) taken this into consideration (Porceddu, 2016, O'Sullivan et al., 2016). For example, the higher N-status of HPV^+ OPSCC is no longer associated to a higher tumour stage, resulting in that some tumours are being downstaged according to their HPV status. This will be explained in more detail in Chapter 4 in the section Material and Methods. Unfortunately, however this edition has two drawbacks. One is that the presence of p16 overexpression is regarded sufficient to diagnose positive HPV status and the other is that it does not differentiate lymphoepithelial TSCC/BOTSCC from the non-lymphoepithelial otherOPSCC. These two factors may lead to that patients with otherOPSCC overexpressing p16 may be diagnosed with a lower tumour stage and potentially be treated in a suboptimal way.

Studies on diagnostic markers (Including studies in Paper I). The drawbacks of TNM-8 are highlighted in Project I of this thesis, but also in other reports (Tham et al., 2019, Hammarstedt et al., 2021). In Project I, long-term survival was followed for 10 years in TSCC, BOTSCC, and otherOPSCC in association to whether the tumours overexpressed p16 and/or were positive for HPV DNA (Wendt et al., 2021). The compiled data in this study showed that patients with $p16^+$ OPSCC had fewer late relapses than $p16^-$ cases. Nevertheless, a clear prognostic value of $p16^+$ was only detected in TSCC/BOTSCC. Importantly, the combination of HPV DNA and p16 status showed a better prognostic value compared to p16 status alone in TSCC/BOTSCC (Wendt et al., 2021).

Therapy. Notably, although we today already know that HPV^+ TSCC and BOTSCC have better prognosis than their HPV^- counterparts and otherOPSCC regardless of HPV status, this information has not yet led to any changes in therapeutic strategy.

In TSCC and BOTSCC standard of care therapy is currently radiotherapy and concomitant systemic therapy can be added, according to the stage of the disease. Standard of care today is Cisplatin and if unfit for Cisplatin, e.g., impaired hearing and/or kidney failure, the Epidermal growth factor receptor (EGFR) inhibitor Cetuximab can be considered (Gebre-Medhin et al., 2021). Regardless of HPV status 66-70 Gy accelerated or conventional, is today´s standard dosage (Hay and Nixon, 2018, Golusiński and Golusińska-Kardach, 2019). Surgery can however still be performed in preselected TSCC/BOTSCC patients with early-stage disease (Hay and Nixon, 2018, Golusiński and Golusińska-Kardach, 2019). Furthermore, surgery plays a more important role in the treatment of otherOPSCC (Hay and Nixon, 2018, Golusiński and Golusińska-Kardach, 2019).

Biomarker studies (Including studies in Paper II). Clearly, patients succumbing to OPSCC can have a very different prognosis and this presents a clinical problem since this group is very heterogenous. This becomes especially apparent in those with HPV^+ TSCC and BOTSCC who in general have a very good prognosis and are often younger, and where the given therapy has numerous side effects, such as dysphagia, xerostomia, muscle fibrosis, and osteoradionecrosis of the mandible.

In the past decade, many studies have been performed trying to identify prognostic markers to distinguish patients with even more favourable prognosis, who could potentially receive de-escalated treatment (for a review see (Näsman et al., 2021)). This way such patients could potentially receive lower energy doses of irradiation and lower doses of systemic therapy. Many potentially useful prognostic markers have during the years been identified by others and us, for a review see (Näsman et al., 2021) and for some further details see Table 1. Favourable prognostic markers by IHC are e.g., high numbers of $CD8^+$ tumour infiltrating lymphocytes (TILs), low HLA class I expression, low LRIG1 expression, while others have been identified by molecular methods (Näsman et al., 2021).

Other prognostic favourable biomarkers are e.g., low T-stage, young age, and expression of HPV16E2 mRNA. These can for example, be combined with a high number of $CD8^+$ TILs to predict prognosis (Bersani et al., 2017a). Having 3 out of 4 of these markers distinguished

> 50 % of the patients with a very high probability (> 98 %) to survive at least 3 years after treatment (Bersani et al., 2017a). Nonetheless, this does not identify all 80 % of patients with good prognosis. Prognostic biomarkers will be further discussed separately regarding HPV status in Chapter 2.2.2.

Table 1. Potential prognostic biomarkers for HPV$^+$ TSCC and BOTSCC

Additional prognostic biomarkers[1]	
Tumor infiltrating CD8+ and Foxp3+ lymphocytes (Nasman et al., 2012).	Reduced Expression of TAP2, LMP2 and LMP7 (Tertipis et al., 2015a).
Absent/weak CD44 Intensity (Nasman et al., 2013b).	HPV16E2 mRNA (Ramqvist et al., 2015)
CD8+ and CD4+ TILs (Nordfors et al., 2013).	FGFR3 mutations (Bersani et al., 2017b)
HLA class I and II expression (Nasman et al., 2013a).	FGFR3 overexpression (Bersani et al., 2018)
LRIG1 immunoreactivity (Lindquist et al., 2014).	Psoriarin expression (Zupancic et al., 2021)
LMP10 nuclear expression (Tertipis et al., 2014a).	CDC27 deletion (Ahrlund-Richter et al., 2021).
HLA-A*02 presence (Tertipis et al., 2014b).	FGF11 overexpression (Flon et al., 2023).

[1]Abbreviation list: CD: Cluster of differentiation, Foxp3: Forkhead box p3, TIL: Tumour infiltrating lymphocyte, HLA: Human leukocyte antigen, LRIG1: Leucine rich repeats and immunoglobulin like domain 1, LMP: Low molecular weight protein, TAP: Antigen peptide transporter, FGFR3: Fibroblast growth factor receptor 3, CDC27: Cell division cycle 27, FGF11: Fibroblast growth factor 11.

Studies on biomarkers in this thesis (Paper II). To contribute to the field of predictive biomarkers in OPSCC in Paper II, the role of psoriasin in the prognosis of HPV$^+$ BOTSCC was studied in a pilot study (Zupancic et al., 2021). In this study, we found that psoriasin expression was useful as a significant prognostic factor (Zupancic et al., 2021). In conclusion, psoriasin could potentially, in combination with other markers, be used as a prognostic marker in HPV$^+$ BOTSCC and HPV$^+$ OPSCC.

2.1.3 Human papillomavirus (HPV)-related multiphenotypic sinonasal carcinoma (HMSC)

Background

As mentioned above, the presence of HPV is mainly observed in TSCC and BOTSCC, although it has occasionally been noted in a subset of sinonasal carcinomas and in rare cases also in hypopharyngeal and laryngeal squamous cell carcinoma and others (Sabatini and Chiocca, 2020, Nasman et al., 2020). As previously mentioned, HPV can also be detected in CUP of the head and neck region, but here the main aetiology is likely a primary tumour never found in the tonsils or the base of tongue (Sivars et al., 2016).

Notably, however, in 2013 Bishop and colleagues, described a new tumour entity, with an aggressive histomorphology and morphological similarities to AdCC, but with a more favourable prognosis and an association with HPV. In these cases the presence of HPV type 33 was predominant (Bishop et al., 2013b). This entity was originally denoted as HPV-related carcinoma with adenoid cystic carcinoma-like features and was later named HPV-related multiphenotypic sinonasal carcinoma (HMSC). Examples of its presentation by IHC are shown in Figure 7.

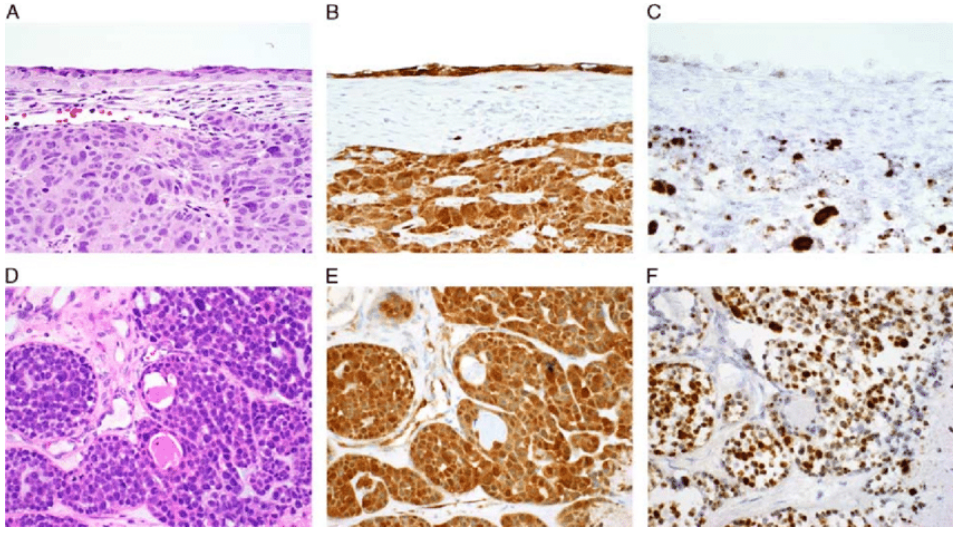


Figure 7. The HPV-related multiphenotypic sinonasal carcinomas **(A and D)** diffusely positive for p16 by IHC **(B and E)** and high-risk HPV by RNA in situ hybridization **(C and F)** in both the surface and invasive components **(A–C)** and in both the ductal and abluminal tumour cells **(D–F)**. The pattern of in situ hybridization signals was both punctate and diffuse **(C and F)**, with the diffuse signals localizing to the tumour giant cells **(C)**. From (Bishop et al., 2017), with permission from the publisher.

Epidemiology, diagnosis, therapy, and prognosis

Epidemiology. The first published reports of this tumour entity limited its presence to the sinonasal tract, but recently new case reports show that this tumour entity may even arise outside the sinonasal area (Hodgson et al., 2021). HMSC is a rare disease, and so far less than 100 cases in total have been described around the world (Zupancic and Näsman, 2021).

Diagnosis and prognosis. Patients with HMSC are rather young, often in their 5th decade at the time of diagnosis and their sex distribution is very even. Nonetheless the aetiology is unknown. Cervical lymph node involvement is uncommon, and prognosis is better than in AdCC or other HNC in the sinonasal area (Zupancic and Näsman, 2021).

Treatment. Treatment is very unspecific; either surgery alone or surgery followed by radiotherapy (Zupancic and Näsman, 2021).

Systematic study on multiphenotypic sinonasal carcinoma (Paper III). Since this diagnosis is rare, and we had some data suggesting that HMSC possibly could be present outside the sinonasal area, we decided to do a systematic review and a meta-analysis of the literature on the subject and summarize the data as a brief report on HMSC (Zupancic and Näsman, 2021). This made up Project III of this thesis (Zupancic and Näsman, 2021). In this study, we identified 127 articles published between 2013 and 2021, of which 18 included unique cases. In these 18 articles, totally 79 unique patient cases were identified, and their tumours had a complex histomorphology and were not found merely in the sinonasal site. In this brief report we concluded that both better clinical follow-up data as well as more thorough tumour characterisation are preferably needed before this tumour type can finally be justified as its own tumour entity (Zupancic and Näsman, 2021).

2.1.4 Malignant salivary gland tumours (MST) a general introduction

Background

General background. As mentioned above, most HNC is due to smoking, alcohol, or viral infections but this is not the case for MST with a mainly unknown aetiology (see review (Young, 2023) and book (Locati, 2017)). There are three paired, so called major salivary glands in the head and neck region: the parotid gland, the submandibular gland, and the sublingual gland (here listed by size). Besides that, there are uncountable small glands distributed in the lips, oral cavity, throughout the throat, and upper digestive tract that are called minor salivary glands. Most salivary gland tumours are benign but approximately 30 % are malignant (Young, 2023). The rate of cancer development increases with the reduction of gland dimension, being about 25 % in parotid, 50 % in submandibular and 80 % in sublingual glands (Locati, 2017, Barnes L, 2005).

MST represents a very heterogenous group with different histomorphology, immune profile, and prognosis and thereby also treatment regimens. In 2005, WHO recognized 24 different MST types (El-Naggar AK, 2017, Barnes L, 2005), where mucoepidermoid carcinoma, acinic cell carcinoma, adenoid cystic carcinoma, and adenocarcinoma (AC) were the most common ones (Boukheris et al., 2009).

Due to the heterogeneity of MST and lack of specific diagnostic markers, finding the correct diagnosis can be a real challenge. In some cases even the differentiation between benign and malignant tumours can be challenging (Young, 2023, Barnes L, 2005). This can lead to prolonged time to diagnosis and delayed start of treatment. Therefore, more knowledge on this subject is warranted (Young, 2023).

Aetiology, epidemiology, and clinical presentation. As mentioned above, the aetiology of MST is mainly unknown. However, past irradiation of the area, chronic inflammation of the gland, and previous cutaneous neoplasm in the area, are possible known risk factors (Young, 2023, Spitz and Batsakis, 1984, Barnes L, 2005). The peak age at diagnosis is 60-70 years and the annual incidence in European countries is around 1.2 cases per 100.000 inhabitants. Paediatric cases are even more uncommon (Locati, 2017). Clinical presentation of MST is not different from benign tumours, usually starting with asymptomatic masses. Spread disease or secondary malignancy at diagnosis are uncommon (Locati, 2017).

Treatment. Although these cancers are heterogenic, their initial treatment protocol is very similar (see review (Young, 2023) book (Locati, 2017)). If possible, radical surgery is the first line treatment. This is usually followed by irradiation in case of high-grade malignancy, high T-stage, uncertain radicality, and perineural or perivascular tumour growth. Inoperable malignancies can be treated with irradiation with or without chemotherapy (ChT) to achieve local control (Carlson et al., 2013). Five years after diagnosis approximately 75 % of patients are alive, but these numbers vary depending on stage of disease at diagnosis ((SweHNCR), 2021, samverkan, 2021). In case of local recurrence, that occurs in 15-85 % of cases, re-surgery or re-irradiation can be attempted (Locati, 2017). Patients with spread disease can also be treated with platinum-based ChT combinations, but here success rates are poor, with only 10-20 % objective response rates. So far neither ChT based treatments nor molecular-driven therapy have been shown to increase overall survival (Young, 2023, Locati, 2017).

Notably, this thesis focuses on AdCC in particular, which will therefore be described in more detail below.

2.1.5 Adenoid Cystic Carcinoma (AdCC)

Background, clinical presentation, diagnosis, treatments, and potential biomarkers for prognostication

Background. AdCC is a rare malignancy with yet unknown aetiology, originating from the secretory glands. It accounts for 10 % of all neoplasms in the major salivary glands and approximately 30 % in the minor salivary glands (Young, 2023). However, AdCC is the most common malignant tumour in the minor salivary glands and the second most common in the major salivary glands (Ouyang et al., 2017). However, this tumour entity is not limited to the salivary glands. It also arises in other parts of the head and neck region and in rare cases even in the secretory glands outside this area, such as in the oesophagus, the breast, the lung, the prostate, and the vulva (Dillon et al., 2016). It can affect patients of all ages but is most common in the 5th or 6th decades of life and around 60-70 % of the patients are female (Dillon et al., 2016, Locati, 2017, Young, 2023).

Clinical presentation. Like other MST, AdCC presents with unspecific symptoms, where clinical differentiation from benign tumours is difficult (Young, 2023, Locati, 2017, Dillon et al., 2016). AdCC is characterised by: slow growth, in many cases difficult diagnostics, lack of good prognostic markers, and perineural invasion (Barnes L, 2005). Initially AdCC is treated aggressively, with surgery, frequently followed by adjuvant radiotherapy. However, despite

the aggressive therapy regimen, late local relapses (15-85 %) and distant metastases (25-55 %) are common, often even more than 5 years after finished primary treatment (Young, 2023). This is believed to be due to the perineural invasion beyond the surgical margins and microscopic haematogenous dissemination, already at early stages of the disease (Li et al., 2012, Jaso and Malhotra, 2011). Due to their slow growing nature, AdCCs often run an indolent course with 5-year survival rates of 80-85 %. However, these favourable numbers decline when observing long-term survival, with 10- and 15-year survival rates of 50-60 % and 30-35 %, respectively (Ouyang et al., 2017, Jang et al., 2017).

Diagnostics. There have been numerous attempts to find better markers for diagnostics, prognostication, and possible targets for molecular-driven therapy, but so far, much is still unknown. As mentioned above the diagnosis of AdCC can be challenging due to its heterogeneity as well as its histomorphological similarities with other MSTs as well as other non salivary gland carcinomas, such as polymorphous low-grade adenocarcinoma (PAC) (Jaso and Malhotra, 2011, Barnes L, 2005).

Diagnostics today mostly rely on histomorphology, IHC and cytogenetics (Barnes L, 2005). AdCC shows three different growth patterns: cribriform, which is the most common, followed by tubular, and solid, where the latter is the most aggressive one, with the worst prognosis. (Young, 2023, Dillon et al., 2016, Barnes L, 2005). Examples of the three growth patterns are presented in Figure 8. AdCC has a biphasic differentiation with both secretory glandular (ductal), as well as myoepithelial elements. Specific staining for both components, even if not specific for AdCC, are an important part of the diagnostics (Figure 8) (Azumi and Battifora, 1987, Dillon et al., 2016). An important, however, nonspecific histological hallmark of AdCC is perineural invasion, seen in 60-70 % of the cases (Figure 8) (Ouyang et al., 2017, El-Naggar AK, 2017).

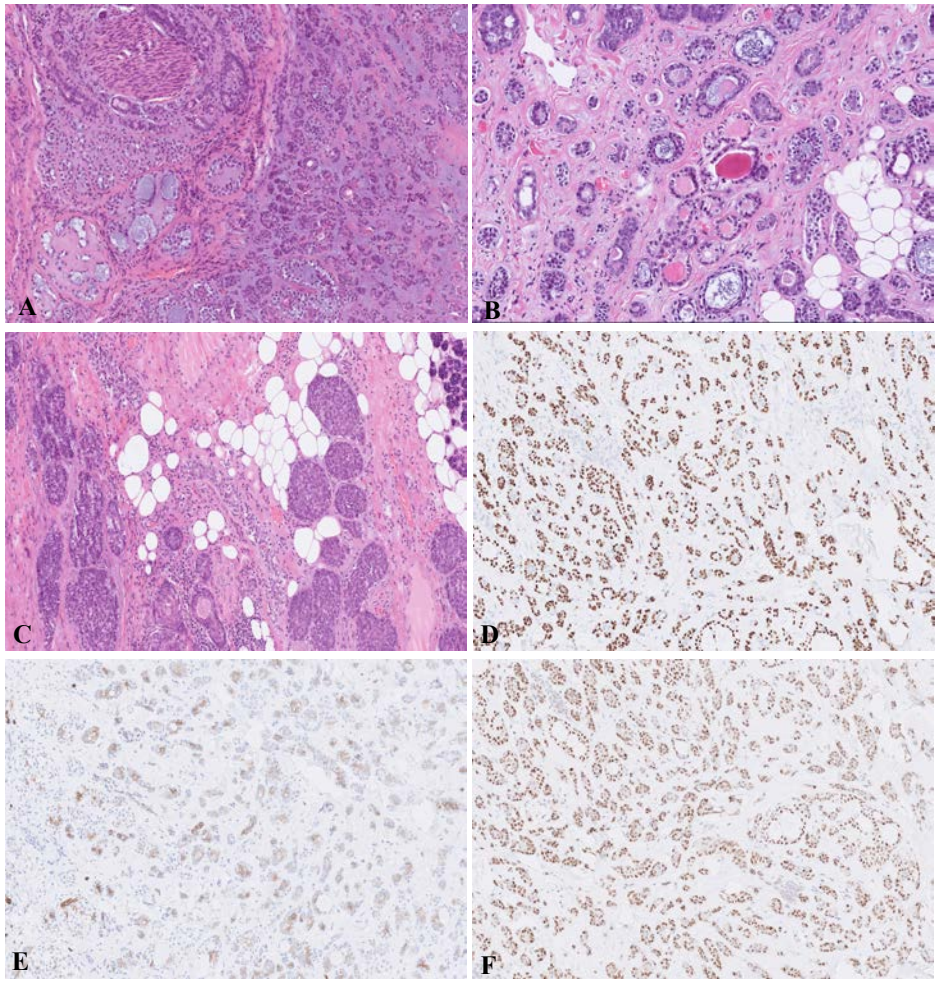


Figure 8. Different presentations of AdCC by IHC. Magnification, x20. **(A)** Haematoxylin and Eosin (HE) staining of a cribriform growth pattern with a typical perineural invasion in the upper left corner **(B)** HE-staining of a tubular growth pattern **(C)** HE-staining of a solid growth pattern **(D)** Positive p63-staining, a myoepithelial marker of the basaloid cells in AdCC **(E)** Positive c-KIT (CD117)-staining of the ductal epithelial cells in AdCC **(F)** MYB-staining, as a surrogate marker for a *MYB:NFIB* gene fusion, sensitive but non-specific biomarker in AdCC.

It has also been shown that a t(6;9) translocation generates a *MYB:NFIB* gene fusion in AdCC, leading to an overexpression of the MYB oncoprotein, which can be detected by IHC (Jaso and Malhotra, 2011, Stenman et al., 2010), see Figure 8. This mutation has been shown to be driving the proliferation of AdCC cells (Andersson et al., 2017, Drier et al., 2016, Wagner et al., 2022). Around 50 % (in some studies around 80 %) of all AdCC have this translocation, which can be a useful diagnostic tool. However, its therapeutic value is still unknown (Dillon et al., 2016, Persson et al., 2012).

A few other biomarkers have also been associated with AdCC, such as receptor tyrosine kinase c-KIT (CD117) (see Figure 8 for an example of a CD117 staining by IHC), vascular endothelial growth factor receptor (VEGFR)−3, Ki-67 (proliferation marker), and p53. These are primarily linked to an aggressive disease and less favourable prognosis (Dillon et al., 2016).

Recently, it was suggested that there could be a way of dividing AdCC patients into two groups with regard to their prognosis (Ferrarotto et al., 2021). The group with the more favourable prognosis was shown to upregulate *TP63* and receptor tyrosine kinases, while the group with worse prognosis had a strong upregulation of *MYC* and MYC target genes, as well as enrichment of *NOTCH*-activating mutations (Ferrarotto et al., 2021). This novel discovery should be further validated in larger cohorts but could potentially be promising.

Treatment. As mentioned above initial treatment of AdCC is surgery. Because of relatively high recurrence rates, 30-75 % (Jang et al., 2017), surgery is often followed by radiotherapy with doses up to 54-71Gy (median 64Gy) (Chen et al., 2006). In smaller tumours, with no nodal metastasis (T1N0), the value of postoperative radiotherapy is debatable, but it has been suggested that it should be considered when perineural invasion and solid histologic subtype are present (Dillon et al., 2016).

Randomized controlled trials are rare in AdCC, but retrospective data have reported that PFS is better when using multimodal therapy regimen (Dillon et al., 2016, Chen et al., 2020). One study showed that patients given a combination of surgery and radiotherapy had superior 5-year local control rates than those treated with surgery alone; 90 % compared to 42 %, respectively (Ali et al., 2017). Similar results have been shown in a recent large cohort study by Chen et al (Chen et al., 2020). Another report compared radiotherapy alone with surgery followed by radiotherapy. Also, here local control rates were better with multimodal treatment. The 5- and 10-year local control rates were both better in the surgery and radiotherapy group compared to the group receiving radiotherapy alone; 94 % and 91 % compared to 56 % and 43 %, respectively (Mendenhall et al., 2004). Even if PFS is prolonged with combination therapies, the effect of postoperative radiotherapy (PORT) on OS is still unclear (Chen et al., 2006, Choi et al., 2022), possibly because of very long follow-up times needed due to slow tumour growth. Furthermore, additional survival benefit of PORT may not benefit all AdCC patient groups, especially in low-risk patients with limited disease the role of PORT needs to be further specified (Chen et al., 2020, Tasoulas et al., 2021).

There are no standard treatment recommendations for systemic therapy in AdCC patients and specific ChT regimens have not been effective in clinical trials (Dillon et al., 2016). It has been suggested that the lack of effect of cytotoxic agents is due to the slow growing biology of AdCC. Interestingly, however, even upon spread and rapidly progressive disease the treatment responses to ChT are very limited (objective response < 20 %) (Laurie et al., 2011). Although not proven effective, ChT is still being used due to absence of other effective treatment options upon disseminated disease (Dillon et al., 2016, Laurie et al., 2011).

Studies on potential prognostic factors in AdCC patients (Papers IV and V). Clearly AdCC is a rare and heterogenic disease. It is therefore difficult to draw general conclusions, since prospective studies are rare, and cohorts are usually small and heterogenic. For this reason, we have attempted to collect a large cohort (n > 100) of AdCC patients to investigate a possible presence and roles of HPV and HPyVs (Project IV), and to study long-term follow-up data of a large AdCC cohort in more detail (Project V).

In Project IV, in part of the above described AdCC cohort, we studied whether HPV or HPyVs were present, as suggested in some earlier smaller studies (Qian et al., 2016, Hämetoja et al., 2019). Neither HPV nor HPyVs were noted to be present in AdCC in this cohort (for more details see below). However, in the three cases where HPV was found the diagnosis was upon re-evaluation noted as possibly not being AdCC (Zupancic et al., 2022), suggesting that when HPV is disclosed in a suspected AdCC the AdCC diagnosis is likely incorrect.

In Project V, clinical characteristics, such as age, gender, smoking, performance status, tumour stage (TNM-8), perineural growth status, type of treatment, and recurrence status were followed. We could confirm that multimodal treatment resulted in improved survival when compared to single modality treatments. Moreover, we could show that when using multimodal treatment perineural growth or incomplete surgical margins did not confer a survival disadvantage. Furthermore, in AdCC of the head and neck region, subsite was a prognostic factor, with prognosis being best in patients with AdCC in the major salivary glands, especially in the parotid gland, and poorest in those with AdCC in the sinonasal subsites.

2.2 Background of human papillomavirus (HPV) and human polyomaviruses (HPyV)

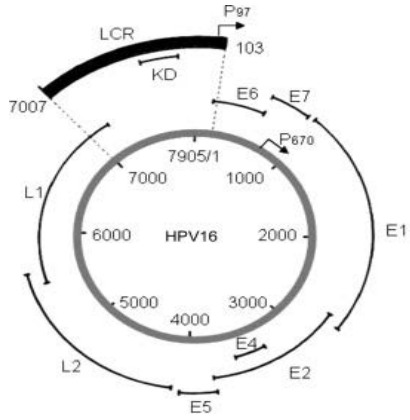
Papillomaviruses (PV) were historically initially categorised together with the polyomaviruses (PyV) into the family *papoviridae,* since both had circular double-stranded DNA genomes, nonenveloped capsids and were present in many animals (Melnick et al., 1974). Later reports disclosed different genome organizations and genome sizes. For reviews see (Van Doorslaer et al., 2017, Dalianis and Hirsch, 2013). In this literature review, more focus will be given to PV, specifically HPV, since HPV is more prominently present in the cancer types studied in this thesis. Some words will also describe PyV since we attempted to disclose possible presence of them in AdCC.

2.2.1 Human papillomaviruses (HPV)

Classification, the genome and its components and products

Classification. PV are grouped into 16 families, with HPV included in five families *Alpha*, *Beta, Gamma, Mu* and *Nu-papillomavirus* (Doorbar et al., 2012, Tommasino, 2014). Classification is done by examining the L1 gene nucleotide sequence, since it is well conserved. A 10 % dissimilarity in this region compared to other HPVs is presented as a new type (Bernard et al., 2010). There are > 200 HPV types, and most are low risk (LR) contained within the alpha, beta and gamma branches and are mainly asymptomatic or cause genital or skin warts (Tommasino, 2014, Doorbar et al., 2012). There are in addition, high risk (HR) mucosal HPVs that are associated with cancer development, although they too mainly cause asymptomatic infections, and these are all members of the alpha branch (Doorbar et al., 2012, Tommasino, 2014).

Genome. All HPVs have circular genomes, comprising of a nearly 8000 base-pairs long double-stranded DNA, divided into three major regions: the early region, that covers about 50 % of the genome; the late region, that covers about 40 %; and the long non-coding control region, that covers about 10 % of the genome (Figure 9) (Tommasino, 2014, Van Doorslaer et al., 2017). The early region includes the genes encoding for the regulatory proteins (E1, E2, E4, E5, E6, and E7), while the late region includes the genes encoding the viral capsid proteins L1 and L2. (Tommasino, 2014).



Viral Protein	Functions and Features
E1	It forms a heterodimer complex with E2 and controls viral replication
E2	It regulates early gene promoter and together with E1 viral DNA replication
E4	It may mediate the viral particle realease by destabilizing cytokeratin network
E5	It stimulates mitogenic signals of growth factors
E6	It inactivates many cellular proteins and is one of the major viral oncoproteins
E7	It inactivates many cellular proteins and is one of the major viral oncoproteins
L1	It is the major capsid protein and is the component of the HPV prophylactic vaccine
L2	It is the minor capsid protein

Figure 9. The double-stranded DNA HPV16 genome is represented by a grey circle annotated with the nucleotide numbers. The positions of the long control region (LCR) and the early genes (E1–7) and late genes (L1 and L2) are also shown. The early and late promoters, P97 and P670, respectively, are indicated by arrows. The main functions and features of the early and late gene products are listed in the table. From (Tommasino, 2014), with permission from the publisher.

The long control region (LCR) is located between the early and late region (Figure 9) and includes most regulatory elements needed for viral DNA replication and transcription. LCR can differ substantially in size in different HPV types (Tommasino, 2014).

The early region (E) proteins, E1 and E2 are essential during early infection, but are also active during initial replication and for establishing the viral infection (Tommasino, 2014). E2 also regulates of E6/E7 abundance (see below).

E4 although coded by the early region is found to be produced during the later viral cycle and it has been shown to be active in viral release (Doorbar et al., 2012, Tommasino, 2014).

The E5 viral protein is suggested to promote genome amplification by modifying the cellular environment and to act as an oncoprotein and to hinder immune recognition by downregulating the expression of proteins of the major histocompatibility complex (Venuti et al., 2011, Doorbar et al., 2012).

The E6 and E7 viral proteins are in HR-HPV types considered oncogenes. E6 binds to p53 and abrogates cell repair, enhances proliferation, and indirectly enhances the number of mutated cells in HPV induced cancers (Doorbar et al., 2012, Tommasino, 2014). E7 attaches to the retinoblastoma protein (Rb) and inhibits control of the cell cycle and promotes proliferation, thereby indirectly activating the $p16^{INK4a}$ (p16) protein which then can result in p16 overexpression (Tommasino, 2014, Doorbar et al., 2012).

The major capsid protein L1 and the minor capsid protein L2 are two structural proteins. Approximately 360 L1 molecules on the outside of the viral capsid, mainly formed as pentamers, together with roughly 20 L2 molecules from the inside make up the viral capsid (Tommasino, 2014). L1 proteins can self-assemble and form virus like particles (VLPs), which are very immunogenic. This is the basis of the present HPV vaccines of today, where L1 are either produced in insect cells or in yeast (Lowy and Schiller, 2012, Harper and DeMars, 2017).

Transmission

HPV is proposed to be spread via microtears in the mucosa or skin (Tommasino, 2014). The most common ways of transmission are sexual contact and kissing (Syrjanen, 2003, Syrjanen, 2004, D'Souza et al., 2009). The risk of anogenital and oral HPV infections have been suggested to be associated to early sex debut, high sexual activity, and high number of lifetime sexual partners (Anaya-Saavedra et al., 2008, D'Souza et al., 2009, Pickard et al., 2012). Also, vertical transmission has been suggested (Syrjanen, 2010). Moreover, HPV can also be found in the blood of cancer patients and assaying for HPV in plasma is being studied for monitoring treatment effects after treatment in HPV-related cancers (Routman et al., 2022), but these approaches need to be followed up.

HPV-related tumours and carcinomas with emphasis of those in the head neck region

As stated above most HPV types are found in the skin and mucosa and are asymptomatic, and do not cause cancer. LR HPV types can induce skin warts, genital warts, and recurrent respiratory papillomatosis (RRP) (Syrjanen, 2010). HR HPV types can induce cancer, dominated by cancer of the cervix uteri and other anogenital cancers as well as OPSCC, more specifically TSCC and BOTSCC, where HPV 16 dominates (Tommasino, 2014). HPV has however also been found in HMSC, where HPV 33 is suggested as the dominant HPV type (Bishop et al., 2017, Zupancic and Näsman, 2021). Below some words will follow about the process from viral infection to malignancy.

The process from infection to a malignant tumour is best studied in cervical cancer. It is assumed to take several years and is presumed to start with dysplasia, a precursor of an invasive cancer, later transforming into a highly malignant cancer (Figure 10) (Doeberitz and Vinokurova, 2009, Dillner, 2019).

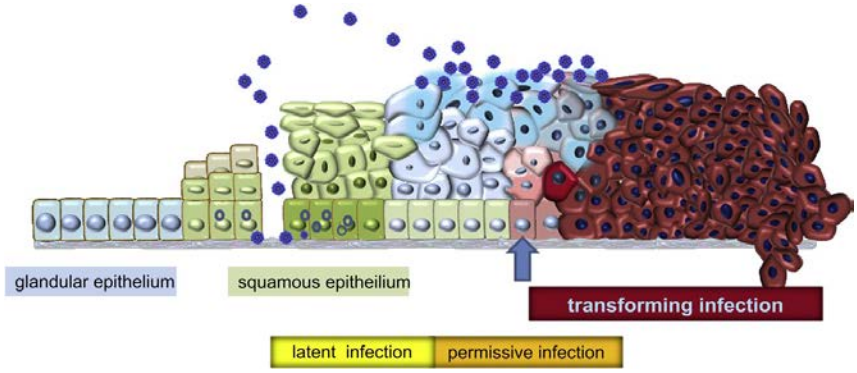


Figure 10. Schematic representation of the various modes of an HPV infection. Minor lacerations of the squamous epithelium permit the virus to meet its natural host cell at the bottom of the squamous epithelium. Upon viral uptake, transport to the nucleus and release of the circular episomal viral genome genetic activity of the virus appears to be blocked (latent infection, dark green cells). Viral gene expression starts in individual cell, and, for unknown reasons, permits the local expansion of the infected cells into a permissive infection that results in viral replication and release of replicated viral particles at the surface of the squamous epithelium (light green basal cells, blue intermediate and superficial cells). In some instances, and, particularly in basal cells at the transformation zone between squamous and glandular epithelial cells, the permissive or replicating mode of viral gene expression may shift into the transforming mode of viral gene expression. The latter is characterised by high-level expression of the E6 and E7 genes (red cells), (for further details please refer to the text of this review.) From (Doeberitz and Vinokurova, 2009), with permission from the publisher.

This long transformation process and accessibility to identify precancerous lesions allows for screening for cervical cancer, while this is not easily the case for OPSCC. Here the tumour is assumed to originate deep inside the tonsillar crypt epithelium, not visible nor detectable by a superficial brush biopsy (Pickard et al., 2012, Nasman et al., 2020).

Furthermore, due to the secretion of 0.5-1.5 litre saliva per day it is not possible to monitor and screen for presence of HPV as one can do in cervical cancer (Nordfors et al., 2014, Pickard et al., 2012).

Finally, it is not yet known how long it takes from the viral infection until the cancer development in the head and neck region, so there are additional knowledge gaps and challenges that need to be addressed, but these are not topics of this thesis (Timbang et al., 2019).

HPV vaccination

HPV vaccination will likely prevent the majority of HPV-related cancer cases, including in the head and neck region. However, it will take several decades to envisage this decrease since HPV vaccination of boys was initiated in 2020 in Sweden and the average age of encountering an HPV^{+} head and neck cancer is usually in the 6^{th} decade (Gillison et al., 2015,

Nasman et al., 2020). It is therefore important not to neglect the increasing numbers of patients with HPV-related head and neck cancer and to be prepared to improve and tailor treatment for these increasing numbers of patients.

Globally, an increase in vaccination coverage will be required in order to be sufficient enough to cover people in many poor areas (Lowy and Schiller, 2012). Moreover, HPV vaccination coverage is also dependent on different global cultural attitudes (Hertzum-Larsen et al., 2020). Therefore, also for this reason, improvements in treatment for patients with HPV^+ head and neck cancer are of importance.

2.2.2 Biomarkers in tonsillar and base of tongue cancer according to HPV status.

The first attempts to find specific biomarkers in OPSCC were concentrated on characterising notable differences between HPV^+ and HPV^- TSCC and BOTSCC, but also to find similarities between different HPV-related cancers. Initial reports revealed various similarities between HPV^+ OPSCC and HPV^+ cervical, as well as vulvar cancer. Some similarities are for example: p16 overexpression, the frequent absence of *TP53* mutations, and chromosome 3q amplifications (for review see (Näsman et al., 2021), and also (Dahlgren et al., 2003), (Crook et al., 1992) and (Wilting et al., 2009)).

When the association between the HPV infection and the development of OPSCC/ TSCC/BOTSCC was finally acknowledged, the scientific community started searching for prognostic and targetable markers. Initial studies mainly used IHC and studied immunological and stem cell markers. It is well established that the counts of $CD8^+$ lymphocytes infiltrating the tumour and/or the adjacent tissue are numerically higher in HPV^+ TSCC and BOTSCC than in their HPV^- counterparts. Higher counts of $CD8^+$ cells in HPV^+ tumours were associated with superior clinical outcome (Nasman et al., 2012, Nordfors et al., 2013, Oguejiofor et al., 2017, Oguejiofor et al., 2015, Tertipis et al., 2015b, Welters et al., 2020). Moreover, low $CD4^+/CD8^+$ ratios, or high $CD8^+/FoxP3^+$ ratios in TSCC and BOTSCC were also associated with longer survival (Nasman et al., 2012, Nordfors et al., 2013). $CD68^+$ $CD163^+$ M2-macrophages have also been analysed and a high infiltration of these macrophages was correlated to poorer survival in head and neck squamous cell carcinoma (HNSCC), with most cases being OPSCC (Balermpas et al., 2014, Cioni et al., 2019, Santegoets et al., 2020)

Molecular methods, such as next generation sequencing (NGS) have also been used to compare HPV^+ and HPV^- HNC (Lui et al., 2013, Sewell et al., 2014, Gaykalova et al., 2014, Chung et al., 2015, Rusan et al., 2015, Bersani et al., 2017b, Cancer Genome Atlas, 2015, Tinhofer et al., 2016) and for review see: (Näsman et al., 2021). In HPV^+ OPSCC/TSCC/BOTSCC mutations were frequently found in the Phosphatidyl-inositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA),* notch homolog 1 translocation-associated *(NOTCH1)*, and Fibroblast growth receptor (*FGFR) 3* genes. HPV^- tumours on the other hand often had mutated *TP53* and cyclin dependent kinase inhibitor 2A/B (*CDKN2A/B)* (Lui et al., 2013, Sewell et al., 2014, Gaykalova et al., 2014, Chung et al., 2015, Rusan et al., 2015, Tinhofer et al., 2016, Bersani et al., 2017b, Cancer Genome Atlas, 2015, Seiwert et al., 2015) and for review see: (Näsman et al., 2021)

The prognostic value of the mutations discussed above in HPV^+ OPSCC/TSCC/BOTSCC varied between different reports, see review (Näsman et al., 2021). However, our group and others have tried to apply additional models for optimizing prognostication, but these prognostic models have their limitations and are not yet used clinically (Tertipis et al., 2015b, Bersani et al., 2017a).

The scientific community has therefore searched for additional biomarkers, such as microRNAs, as well as the presence of various HPV mRNAs and many interesting data have been generated (Hui et al., 2013, Gao et al., 2013, Lajer et al., 2011, Lajer et al., 2017, Miller et al., 2015, Ramqvist et al., 2015, Campo et al., 2010, Li et al., 2006), for review see: (Näsman et al., 2021). There are also some studies on protein profiling and for example it was shown that a high expression of vascular endothelial growth factor A (VEGFA) was associated to worse prognosis in HPV^+ cancer (Sewell et al., 2014, Slebos et al., 2013, Ramqvist et al., 2018a).

More recently, the oral microbiome has also been investigated in HNSCC and one study found a lower diversity of microbiota in HNSCC than in healthy controls (Guerrero-Preston et al., 2016).

Many of these attempts, have added some information to general picture, but they have not been entirely groundbreaking by themselves. It has therefore been suggested that further markers are needed, and this is the reason why we pursued Project II.

2.2.3 Human polyomaviruses (HPyVs)

In 1953, Gross published that cell free filtrates from a mouse leukaemia induced tumour development in the parotid gland after inoculation into new born mice (Gross, 1953). Later Stewart and Eddy produced the corresponding virus in mouse embryo cells and confirmed Gross´s findings, disclosing it induced many different tumours when injected into new born mice, thereby the name polyomavirus (Greek: poly oma meaning many tumours) (Eddy, 1977). The first disclosed primate polyomavirus, Simian Virus 40 (SV40), originated from African green monkey kidney cells used for producing poliovirus vaccine (Sweet and Hilleman, 1960).

In humans, the first two human polyomaviruses (HPyVs) were isolated in 1971 from two immunosuppressed patients (Gardner et al., 1971, Padgett et al., 1971). The first a BK virus (BKV) was detected in the urine of a renal transplant patient and the second a JC virus (JCV) was detected in the brain of a patient with progressive multifocal leukoencephalopathy (PML) (Padgett et al., 1971, Gardner et al., 1971).

Both BKV and JCV only present serious health danger in immunosuppressed patients. BKV is associated with haemorrhagic cystitis post-haematopoietic stem cell transplantation and polyomavirus associated nephropathy (PVAN) in renal transplant patients. JCV can cause PML in immunosuppressed patients (Borriello et al., 2022, Tan and Koralnik, 2010, Dalianis and Hirsch, 2013). Initially, all known polyomaviruses were possible to produce *in vitro*, so in contrast to the later detected HPVs, attempts to disclose them with molecular techniques were not pursued until 2007.

New molecular techniques were needed to detect the first three additional members of the HPyV family, KIPyV, WUPyV and MCPyV, of which the latter was detected in Merkel cell carcinoma (Allander et al., 2007, Gaynor et al., 2007, Feng et al., 2008). Very soon again after using molecular techniques various more HPyVs were detected, leading to that around 15 HPyVs are now known in humans (Dalianis and Hirsch, 2013, Prado et al., 2018, Moens et al., 2022).

Notably, MCPyV was the first HPyV to be associated with a malignancy. This suggests that other HPyVs also could be associated with cancer development, and it was therefore we investigated whether this could be the case for AdCC.

All polyomaviruses have a double stranded circular genome of around 5000 base-pairs, that is organized similar to HPVs, with a non-coding regulatory region, an early coding and a late coding region (Dalianis and Hirsch, 2013). The early region codes for 2-3 early proteins called, small T, middle T (only in some polyomaviruses) and large T antigen, and a late region coding for two capsid proteins the major and minor capsid antigen (VP1 and VP2, respectively) and in some cases the agnoprotein (Prado et al., 2018, Dalianis and Hirsch, 2013, Moens et al., 2022).

3 RESEARCH AIMS

As a medical doctor in the field of oncology, I am continuously in contact with patients suffering from cancer. Some of them are lucky because they have recovered from their disease but are now suffering from the side effects of their treatment. Others have to deal both with their disease, as well as the side effects of their treatment. As you probably might have guessed, the main aim of this thesis is to make the lives of cancer patients better, by improving diagnostics, prognostication, and treatment of oropharyngeal and sinonasal cancer and malignant salivary gland tumours. The specific aims are given below for each project.

In Paper I, entitled "Long-Term Survival and Recurrence in Oropharyngeal Squamous Cell Carcinoma in Relation to Subsites, HPV, and p16-Status," the aim was to follow long-term survival and recurrence in OPSCC in relation to subsites, as well as to study the prognostic role of both HPV DNA- and p16 status.

In Paper II, entitled, "Psoriasin expression is associated with survival in patients with human papillomavirus-positive base of tongue squamous cell carcinoma," the aim was to investigate whether psoriasin could be used as a potential prognostic marker in BOTSCC patients.

In Paper III, entitled "Multiphenotypic Sinonasal Carcinoma – An Even Broader Tumour Entity?" the aim was to accumulate more knowledge on the features and locations of HMSC, as a consequence of findings in Project IV.

In Paper IV, entitled "Analysis of Human Papillomavirus (HPV) and Polyomaviruses (HPyVs) in Adenoid Cystic Carcinoma (AdCC) of the Head and Neck Region Reveals Three HPV-Positive Cases with Adenoid Cystic-like Features," the aim was to study for the possible presence of HPV and HPyV in AdCC and their potential diagnostic and prognostic potential. A secondary aim was to identify and characterise possible HMSC patients previously diagnosed with AdCC.

In Paper V, entitled "Adenoid cystic carcinoma (AdCC): A clinical survey of a large patient cohort" the aim was to study a large cohort of AdCC and to investigate how these patients have performed clinically and whether any prognostic factors could be disclosed.

4 MATERIALS AND METHODS

The aim of this section is to provide the reader with an overview of the materials and methods used in the projects within this thesis, as well as the ethical considerations regarding work with human samples. For any further details please see sections including material and methods of every specific project at the end of this book.

4.1 Study Design

Studies I, II, IV and V are all retrospective cohort studies, and the inclusion criteria are described below. Study III is a brief report, designed as a systematic review and a meta-analysis of the literature and only data from sample material published previously by others are analysed as described below.

4.2 Study Subjects

Study subjects included in Papers I, II, IV and V were identified through the Swedish National Cancer Registry, that has existed since the 1950s and it registers cancer diagnosis and patient identification. It has a > 99 % accuracy and enhances the possibility for us to find all cancer patients, diagnosed with a specific type of cancer during a certain time period. Study subjects included in Paper III were taken from scientific papers previously published by others.

4.2.1 Patients with oropharyngeal squamous cell carcinoma

All cancer patients diagnosed with OPSCC (International Classification of Diseases 10th Revision (ICD-10); TSCC: C09.0-9 and C02.4; BOTSCC: C01.9; otherOPSCC: C10.0-9, C05.1-9) from 2000 and on, depending on paper, in the Region of Stockholm and Gotland, treated at Karolinska University Hospital were identified using the Swedish National Cancer Registry.

In Paper I, we included OPSCC patients, treated with curative intent and with previously known HPV DNA and p16 status, obtained from previous studies done by our group (Hammarstedt et al., 2021, Nasman et al., 2015). Patients without to us available tumour material, either due to diagnosis through cytology only, or loss of tumour material, and with unknown HPV DNA and/or p16 status, were excluded from further analysis (n = 25). In total we could include 529 OPSCC patients in Paper I.

In Paper II, the same OPSCC cohort was used as when identifying study subjects for Paper I, except that here only patients diagnosed with BOTSCC between 2000 and 2007 were studied.

The included samples from these patients had also been tested for HPV DNA and p16 status beforehand (Nasman et al., 2015, Hammarstedt et al., 2021).

Patient data were collected from patients' records and assessed for certain parameters, e.g., stage (TNM-7 (Paper I) and TNM-8 (Paper II)), gender, age, smoking status, Eastern Cooperative Oncology Group (ECOG) performance status (PS), type of treatment, recurrence, time to recurrence and location of recurrence, and survival.

The studies were conducted according to ethical permissions from the Stockholm Regional Ethical Review Board: 2005/431-31/4 (Paper I); 2009/1278-31/4 (Paper I and II); 2017/1035-31/2 (Paper I and II).

4.2.2 Patients with HPV-related multiphenotypic sinonasal carcinoma

In Paper III, we have not included any patients of our own. In accordance with PRISMA guidelines we searched PubMed for key terms: *""multiphenotypic" OR "multiphenotypic sinonasal" OR "adenoid cystic like""* in May 2021 and could identify 18 original articles, including 79 unique patients diagnosed with HMSC.

Patient and tumour data (age, sex, tumour localization and size, treatment, outcome and follow-up time in months after treatment), pathological parameters (morphology, IHC data and HPV status (defined by several different methods: p16, HPV DNA PCR, HPV DNA ISH and HPV RNA ISH status) were collected when available in the papers.

For survival analysis outcomes were classified as: "No evidence of disease" (NED), "Local relapse" (LR), "Distant metastasis" (DM) or "Dead of disease" (DOD). Event-free survival was calculated and was defined as the time from first diagnosis (months) until the first reported event (LR, DM, DOD). Patients with NED were censored at the last day of follow-up.

4.2.3 Patients with Adenoid cystic carcinoma

In Paper IV and V, as described above we identified all patients diagnosed with AdCC within the head and neck region in Stockholm and Gotland Region. AdCC does not have its own ICD-10 code and can arise in different sites with salivary gland tissue. In order not to miss any patients and have as complete cohort as possible we have even double checked our cohort with the pathology database in SymPathy, programme used in the Stockholm and Gotland region.

The included diagnose codes according to ICD-10 were: C00.5, C01.9, C04.9, C05.9, C06.9, C07.9, C08.0, C09.9, C11.9, C30.0, and C31.9.

In Paper IV, we identified 94 patients diagnosed between 2000 and 2012, and since the diagnosis HMSC was first described in the year 2013, we also wanted to investigate whether any of the allegedly diagnosed AdCC cases in fact were more fitting for the diagnosis of HMSC. From the 94 patients, FFPE tumour material with sufficient amount and quality for further analysis was obtained from 73 samples. More specifically, these samples were obtained from 68 different patients and included 66 primary tumours, an additional two local recurrences and three distant metastases, as well as one local recurrence and distant metastasis each, without the corresponding primary tumour.

In Paper V, 155 patients diagnosed between 2000 and 2022 were included and characterised according to the following criteria: stage according to TNM-8, age, gender, smoking status, ECOG PS, perineural growth pattern, type of treatment, recurrence, as well as time to recurrence and location of recurrence, and survival.

Papers IV and V were conducted according to ethical permissions from the Stockholm Regional Ethical Review Board: 99-237 (Paper IV and V), 2005/431-31/4 (Paper IV and V), 2009/1278-31/4 (Paper IV and V), 2012/83-31/2 (Paper IV and V), 2017/1035-31/2 (Paper IV and V), 2019-0521 (Paper IV and V), and 2022-05287-02 (Paper V).

4.2.4 Considerations regarding study subjects

Head and neck oncology is centralized in the Stockholm and Gotland Region, which implies that all head and neck cancer patients are diagnosed and treated at Karolinska University Hospital. Previously, head and neck cancer patients were treated at two different hospitals in the region, but these were closely collaborating, and the patient journal systems gave us access to the follow-up history of all patients in the region. The organisation of our pathology department is also centralised, in that all head and neck cancer diagnostics are done at the Department of Clinical Pathology at the Karolinska University Hospital. The study subjects included in Papers I, II, IV, and V were all cancer patients diagnosed at the Department of Clinical Pathology at Karolinska University Hospital, and they were treated either at Karolinska University Hospital or Södersjukhuset in Stockholm. Paper III is a systematic review and includes only previously published patient data.

4.3 MATERIALS

4.3.1 Cancer Tissue

Papers I, II and IV all included human material from cancer patients described above. All primary tumour samples and samples from recurrences or distant metastasis from patients with OPSCC or AdCC were pre-treatment samples, collected for diagnostic purposes. All their samples were evaluated by at least two pathologists to verify the initial diagnosis. Once the diagnosis was secured, the left-over material was stored, as formalin-fixed paraffin-embedded (FFPE) tissues, for further diagnostic purposes and if patients consent also for possible future research purposes. Storing human material fixed in formalin and embedded in paraffin blocks is a widely used technique and considered the gold standard for archiving human material that can potentially be used many years later both for clinical diagnostics as well as for research purposes.

4.3.2 Considerations regarding the obtained cancer tissues

Using FFPE materials allows convenient storing in room temperature, keeps cells intact, and preserves tissue from degrading. It has however some disadvantages, e.g., the preserved protein structures are denatured and are not suitable for certain IHC studies, moreover upon very long-time storage nucleic acids can also be damaged and therefore not optimally suitable for all genetic analysis.

All tumour samples of patients diagnosed and treated in the above defined time spans were not available to us due to different reasons: Firstly, not enough tumour material was secured after the diagnosis, and since a certain amount of tissue needs to be stored for future diagnostics in case of recurrence or any other clinical reasons, we could not obtain material from such patients. Secondly, some FFPE blocks were just not found at the pathology department. Thirdly, the diagnosis was secured by cytology only.

The first two reasons occurred randomly and therefore most likely had no influence on the outcomes in the studies included in this thesis. The last reason was more common in patients with recurrent disease for reassuring the diagnosis, and for patients treated with palliative intent, the latter however, were for obvious reasons not included in most of the survival analyses performed in this thesis. We therefore conclude that the fact that we did not obtain samples from all patients likely did not affect the outcomes of our studies.

Notably, all studies in this thesis were retrospective and include material collected from patients diagnosed and treated in Stockholm and Gotland Region from 2000 and onward. No new human material was obtained, as the material was either already in the possession of our research group or stored in the in the biobank as previously described, see above.

All work was done according to our previously and recently obtained ethical permits and the research individuals were not further informed about our studies. The reason for this was that many of the patients were cancer-free and reminding them of their previous disease would cause additional damage and of the patients with older samples many had died and contacting their relatives would also cause additional emotional damage.

Practically all patients however, had already initially at diagnosis agreed to their samples being saved and used for research purposes. Individuals diagnosed prior to 2000 were not included in the studies in this thesis for two reasons; before the 2000s the clinical data was less complete and more difficult to come by as patient's journals were not yet digitalised, and moreover since the quality of the FFPE samples degrades with time we found it better to work within the time frame from 2000 and on.

4.3.3 TNM Classification of Malignant Tumours

TNM ("Tumour", "Nodes", "Metastases") classification is the most commonly used classification system for staging of solid tumours. The system was developed by Dr Denoix and is in use since 1953 by the Union for International Cancer Control (UICC) (UICC, 2021). The same staging system is even used by the American Joint Committee on Cancer (AJCC) as well as International Federation of Gynaecology and Obstetrics (FIGO).

UICC does regular updates according to the newest standard and in 2016 the current 8th edition, where important changes regarding OPSCC staging were introduced (Zanoni et al., 2019) For this thesis relevant changes will be presented below. For easier understanding of the TNM classification please see Table 2.

Table 2. TNM classification*

T	**Extent of invasion and/size of the primary tumour**
Tx	Tumour cannot be assessed
T0	No signs of primary tumour
Tis	Carcinoma *in situ*
T1, T2, T3, T4	Size and/or extension of the primary tumour
N	**Degree of spread to regional lymph nodes**
Nx	Regional lymph nodes cannot be assessed
N0	Tumour cells absent from regional lymph nodes
N1	Regional lymph node metastasis present; (at some sites: tumour spread to closest or small number of regional lymph nodes)
N2	Tumour spread to an extent between N1 and N3 (N2 is not used at all sites)
N3	Tumour spread to more distant or numerous regional lymph nodes (N3 is not used at all sites)
M	**Presence/absence of metastasis**
M0	No distant metastasis
M1	Metastasis to distant organs (beyond regional lymph nodes)

**Classification of tumour stage in these papers was done according to the International Union against Cancer. The mandatory parameters are listed above.*

4.3.3.1 Changes in the staging of oropharyngeal cancer in the TNM-8 compared to TNM-7

The T staging for HPV^+ OPSCC remains the same as for the HPV^- counterparts, however the TNM-8 combines all T4 stages into one. The biggest alterations in TNM-8 have been done in the staging of the nodal disease, due to that many studies showed that nodal metastases did not have the same negative impact on outcome when comparing HPV^+ and HPV^- OPSCC (O'Sullivan et al., 2016).

Please see Table 3 and 4 for details about the new HPV^+ OPSCC nodal staging with only patients who have distant metastases being assigned to stage IV.

Table 3. Differences between HPV^- and HPV^+ OPSCC in the TNM-8**

	Clinical N-category in $p16^-$ OPSCC		**Clinical N-category in $p16^+$ OPSCC**
N1	One ipsilateral node ≤ 3cm with no extranodal extension (ENE)	**N1**	Unilateral node/nodes ≤ 6 cm
N2a	One ipsilateral node > 3cm ≤ 6 cm no ENE	**N2**	Contralateral or bilateral nodes ≤ 6 cm
N2b	Multiple ipsilateral nodes ≤ 6 cm no ENE		
N2c	Contralateral or bilateral nodes ≤ 6 cm no ENE		
N3a	Node > 6 cm no ENE	**N3**	Node or nodes > 6 cm
N3b	ENE (clinical or radiological)		

Table 4. Changes in TNM-7 and TNM-8 in OPSCC-staging according to p16-status**

Stage	TNM-7 / TNM-8 HPV$^-$ (p16$^-$)	cTNM-8 HPV$^+$ (p16$^+$)
I	T1 N0 M0	T1/T2 N0/N1 M0
II	T2 N0 M0	T1/T2 N2; T3 N0/N1/N2 M0
III	T3 N0 M0 T1, T2, T3 N1 M0	T1/T2/T3 N3; T4 Any N M0
IV	IVa: T1, T2, T3 N2 / T4a N0/N1/N2 M0 IVb: T4b Any N / Any T N3 M0 IVc: Any T Any N M1	Any T Any N M1

***Adopted by Brierly JD, Gospodarowicz MK, Wittekind C. AJCC/UJCC TNM classification of malignant tumours, 8th edition. 2017 Wiley-Blackwell.*

In Paper I we used the TNM-7 staging, since it was in clinical use during the inclusion time period, and we wanted to study whether p16 status alone was sufficient in distinguishing prognostic differences between HPV$^+$ and HPV$^-$ OPSCC. For this reason, we did not want any additional differences between the groups.

In Paper II we restaged the BOTSCC patients according to the TNM-8, since here psoriasin was tested as biomarker for its prognostic value in addition to HPV status according to p16.

Patients included in Papers IV and V were also restaged according to the TNM-8 since AdCC occurs in many different head and neck locations and most of the changes did not influence the staging of these tumours. Paper III did not include any parameters regarding the staging of tumours.

4.3.4 Considerations regarding the study materials

No additional considerations, to what has been stated above regarding FFPE are included here (see above), since other material types, e.g., fresh frozen samples were not used in this thesis.

4.4 METHODS

Of note, some experiments described in this thesis were performed by members in the group before I joined it in 2018 and the methods have been described in several theses written by other colleagues in the past. Nevertheless, all methods included in this thesis that have previously been established / adopted by our group will still be summarized below. However, for more details please read the specific chapters in the papers included in this thesis, as well as the cited papers of our previous publications.

4.4.1 DNA and RNA extractions

As mentioned above only previously taken FFPE samples were included in this thesis, thus no new samples were collected form any of the patients. In Paper I and II, we dissected tumour and adjacent normal control (from the same patient) from 10-25µm FFPE tissues sections from the same FFPE tissue block. In Paper IV, we received 1 mm core biopsy cylinders taken from FFPE blocks from the tumours and the adjacent normal controls. The sample selection was in all cases done by an experienced head and neck cancer pathologist.

Two different kits were used for DNA and RNA extractions during the years by our group. In the early 2000's the group used the "High Pure RNA Paraffin Kit" (Roche Diagnostics, Basel Switzerland). In more recent years however we have used the "AllPrep DNA/RNA FFPE Kit" (Qiagen, Hilden Germany). Both kits were used according to the manufacturer instructions. The main differences were that the "High Pure RNA Paraffin Kit" was primarily designed for extraction of RNA, so in that kit we had to skip the step including the DNase treatment in order to extract for DNA. These modifications were however not necessary with the kit from Qiagen, that we use today, since it is developed for the extraction of both RNA and DNA.

In Paper I and II, DNA was extracted with the "High Pure RNA Paraffin Kit" and in Paper IV we used the "AllPrep DNA/RNA FFPE Kit" to extract both RNA and DNA. RNA and DNA concentrations in the samples were evaluated for their amount and purity to ensure that the extractions were successful prior to further analysis. Initially we used the NanoDrop Microvolume Spectrometer (Thermo Fisher Scientific Waltham, MA, USA), but shortly after we switched to the Qubit 4 Fluorometer, where for RNA we used the Qubit RNA Broad Range Assay Kit and for the DNA the Qubit DNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.4.2 HPV and HPyV DNA analyses

In this thesis positive HPV status was determined by the joint presence of HPV DNA assayed by PCR and p16 overexpression assayed by IHC. In addition, we aimed at defining HPV-type. Immediately below only the determination of presence of HPV DNA will be described. The description of p16 overexpression will be described further below.

For presence of HPyV, the importance was to define if an HPyV was present and in that case, which type of HPyV was present.

Determination of presence of HPV DNA and HPV-typing

In this thesis HPV DNA detection is based on multiplex PCR followed mostly by a bead-based multiplex assay on a MagPix instrument (Luminex Corporation, Austin, TX, USA) with broad-spectrum GP5+/6+ (bs-GP5+/6+) primers targeting the L1 region of the HPV genome developed by Schmitt et al in 2006 (Schmitt et al., 2006). When initially set up in our lab, this assay detected 24 different HPV types, however, a few years later we added three more less common types, thereby including 27 different HPV-types (Nordfors et al., 2014, Ramqvist et al., 2011). Both assays include the 10 clearly HR-HPV types and the majority of possibly HR-HPV types ((IARC), 2009). As an internal control of presence of amplifiable DNA, the housekeeping gene β-globin was used.

Of note, earlier, in the 1990s and the early 2000s, we used only a PCR with general HPV primers GP5+/6+ and/or CPI/IIG (Dahlgren et al., 2004, Mellin et al., 2000). Upon a positive result then the samples were run for HPV16, 18, and 33 specific PCRs. Samples positive with general primers but negative for HPV16, 18, and 33 were sequenced. However, when the HPV DNA detection based on multiplex PCR was introduced in our lab, we additionally re-ran many of the previous samples together with the newer samples to assay for the reproducibility of the different assays. We could show that the reproducibility was very good. Furthermore, all samples with divergent HPV DNA and p16 status were re-analysed.

The more recent assay, bs-GP5+/6+ primers, and more about this will be explained below. The PCR based Luminex multiplex assay step by step and is illustrated in Figure 11.

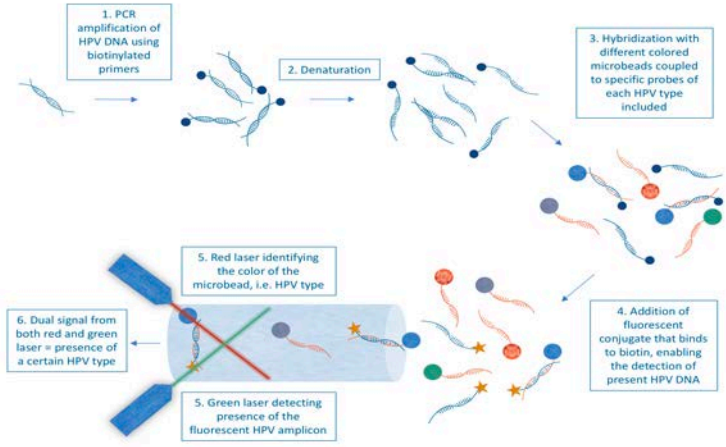


Figure 11. HPV genotyping by the PCR based Luminex multiplex assay. Created by Dr. Linnea Haeggblom, used with permission.

1. First a PCR amplification of HPV DNA was done using the biotylinated primer set: bs-GP5+/6+ detecting the L1 region of HPV-DNA. This set of primers is designed to get equal amplification strength for all the 27 HPV-types included in the assay, with a sensitivity of 10-100 gene copies.
2. Second a denaturation step was included.
3. This was then followed by a hybridization step with coloured microbeads with 27 specific probes binding to each type of HPV DNA and one additional probe for the internal control of β-globin.
4. Thereafter, a fluorescent conjugate that binds the biotinylated primers, enabling the detection of precent HPV DNA was added.
5. The samples were then put into the Luminex MagPix where each sample was evaluated by two lasers: Red identifying the colour of the microbead, determining which HPV types are present; Green identifying the presence of fluorescent HPV amplicon, giving us the amount of HPV DNA.
6. From the MagPix a Median Fluorescent Index (MFI) value is obtained which is transformed into an index according to protocol by Schmitt et al (Schmitt et al., 2006). As cut-off for HPV positivity 1.5 x background + 15 was used (Schmitt et al., 2006, Cuschieri and Wentzensen, 2008). Samples with an MFI value for β-globin < 30 were considered not to have amplifiable DNA to be properly assessed for HPV and were excluded from the study.

Determination of HPyV status

For the analysis of presence of HPyV DNA and specific HPyV type, a similar bead-based multiplex assay based on multiplex PCR was used, therefore only the main differences will be presented below. This assay includes 42 primers, targeting the small T-antigen (ST) and the viral protein 1 (VP1) of the following 10 HPyVs; BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, TSPyV, HPyV6, HPyV7, HPyV9 and HPyV10, as well as the two primate PyVs; SV40 and LPyV simultaneously. β-globin was used as a positive control for the presence of amplifiable human DNA as described previously by Ramqvist et al (Ramqvist et al., 2014), for further details of this assay please see even (Gustafsson et al., 2013). The detection of PCR amplicons was done on a MagPix instrument, similarly, as described above. The output was assayed as MFI, values above 2 × background + 300 were regarded as a positive. For MCPyV, this value corresponded to approximately 5 genomes, lower values were considered not significant (Ramqvist et al., 2018b). MFI values for β-globin below 30 were considered to have poor DNA quality and were excluded from the study (Ramqvist et al., 2018b). No cross-reactions between different viruses were observed.

Methodological considerations

As mentioned above different kits for extracting of the tumour DNA have been used during the years. The "High Pure RNA Paraffin Kit" is particularly designed for preparation of the RNA from FFPE material. However, it works well for both RNA and DNA preparations, when skipping the DNase treatment. The DNA amount and purity were evaluated for every sample, as described above to ensure that the DNA was prepared correctly.

Considering the primers used for HPV DNA analyses, it is important to add that bs GP primers exclusively assay for the L1 region in the HPV DNA. It is therefore possible, although relatively unlikely, that some tumours may lack the L1 region upon a total viral integration and would not be detected with this method. In order to prevent the latter, we included E6 primers and E6 specific probes for HPV 16 and 33, that cause the vast majority of OPSCC/HMSC. The cases negative for L1 but positive for E6 were very rare during the many years the above assays have been used in our lab, it is therefore unlikely that this has affected our HPV data considerably.

Regarding the analysis of members of the HPyV family, primer sets were used for both the ST and the VP1 region, so here this was not an issue.

4.4.3 Immunohistochemistry and immunostaining evaluations

Immunohistochemistry has an important role in modern head and neck pathology and is consequently used in several experiments included in this thesis. IHC represents a widely used technique to identify the protein in cells, the principles are simple and include an antibody that binds a specific antigen (marker) in a tissue sample. IHC is a relatively cheap laboratory technique allowing to search effectively for one specific antigen/protein at a time.

IHC was used in several experiments in this thesis, the performed immunostainings include: p16, S100A7/psoriasin, p63, CD117, and S100. Below the principles of the technique will be explained, including some details on p16 and S100A7 staining. Staining protocols for p63, CD117, and S100 are very similar and were only performed on three samples included in this thesis and will therefore not be presented further, for details see the respective papers.

All immunostainings were performed on 4µm FFPE tumours sections. For specific concentrations please see Material and Methods of the corresponding papers.

IHC, the main steps:

1. Deparaffinization with Xylene and rehydration in decreasing concentrations of Ethanol (100-95-70-30 %) 5 minutes per container. Wash with distilled water.
2. Heat-induced antigen retrieval with citric acid buffer (pH6). High effect in the microwave until boiling, after that another 20 min on low effect.
3. Peroxide treatment for blocking the endogenous peroxidase, incubation time 30 min. Wash with phosphate-buffered saline (PBS).
4. Incubation with horse serum for blocking the unspecific sites for antibodies.
5. Overnight incubation with a primary antibody diluted in PBS at +8 °C. For specific dilutions of antibodies please see the papers including the method and for p16 / S100A7 this will be explained below.

Next day

1. Wash with PBS.
2. Secondary antibody incubation diluted in PBS as well as 1.5 % horse serum, for 40 min at room temperature (specific for the primary antibody type e.g., mouse, rabbit antibody) followed by avidin-biotin-complex (ABC-HRP) incubation, for 40 min at room temperature (Vectastain; Vector Laboratories, Inc.). Wash with PBS.

3. The immunostainings were developed in chromogen 3´ diaminobenzyne (DAB) until brown (ABC emzyme turns brown when in contact with DAB) and counterstained by hematoxylin.
4. At the end, step 1.) and 2.) were repeated backwards for fixation of the stainings.
5. Mounted sections were later evaluated by light microscopy.

Considerations regarding IHC as a method.

IHC is evaluated by a researcher / pathologist and is an optical evaluation, where discrepancies are possible. In the case of p16 expression and OPSCC the cut-off at 70 % is very clear as most of the cases are either 100 % or 0 % strongly stained. But such is not the case in other immunostaining, and it is important to keep this in mind, when trying to implement a new method in the lab or in the clinic. To come around this problem every staining has been evaluated by several researchers and we have in uncertain cases even asked one or two trained pathologists for external help.

4.4.3.1 p16Ink4a antibody and evaluations of the immunostainings

In Paper I we used a p16Ink4a primary monoclonal mouse antihuman antibody, clone JC8 by Santa Cruz Biotechnology, diluted 1:100. For an example of this staining see Figure 12. All stained slides were evaluated by light microscopy by at least two independent researchers, blinded for the clinical outcome. Slides, where the evaluations were inconsistent, were evaluated by a third researcher or a trained pathologist. We considered brown colour in both the nuclear and cytoplasmatic compartment in over 70 % of the tumour cells as a positive staining. This is in accordance with the American college of Pathologists (Lewis et al., 2018), DAHANCA recommendations for scoring of oropharyngeal cancer (Lassen et al., 2011), and also in accordance with recommendations by El-Naggar (El-Naggar and Westra, 2012).

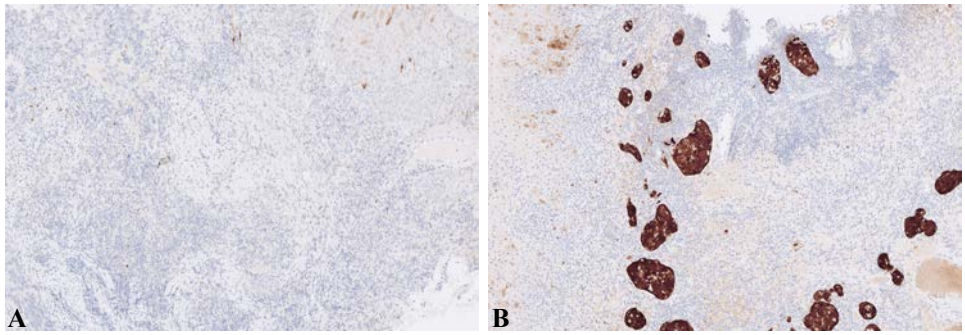


Figure 12. Representative images of p16 immunostaining by IHC. Magnification, x20. **(A)** Low p16 expression in HPV^- TSCC **(B)** High p16 expression in HPV^+ TSCC.

4.4.3.2 S100A7/psoriasin and evaluation

The antibody used in Paper II was a S100A7 mouse antihuman antibody, more specifically, clone 47C1068 (Santa Cruz Biotechnology) diluted 1:100. For an example of this staining see Figure 13. The immunostainings were evaluated by three independent researchers, blinded for the clinical outcome and average values were used in the analysis. The staining was considered positive when the proportion of immunostained cells cytoplasmatic and nuclear was > 30 %. This cut-off value was used since it was used by others, and we wanted to compare our data with studies done by others (Tripathi et al., 2010). It is however important to note that a cut-off value of 30 % is not yet validated or recommended for the clinical use.

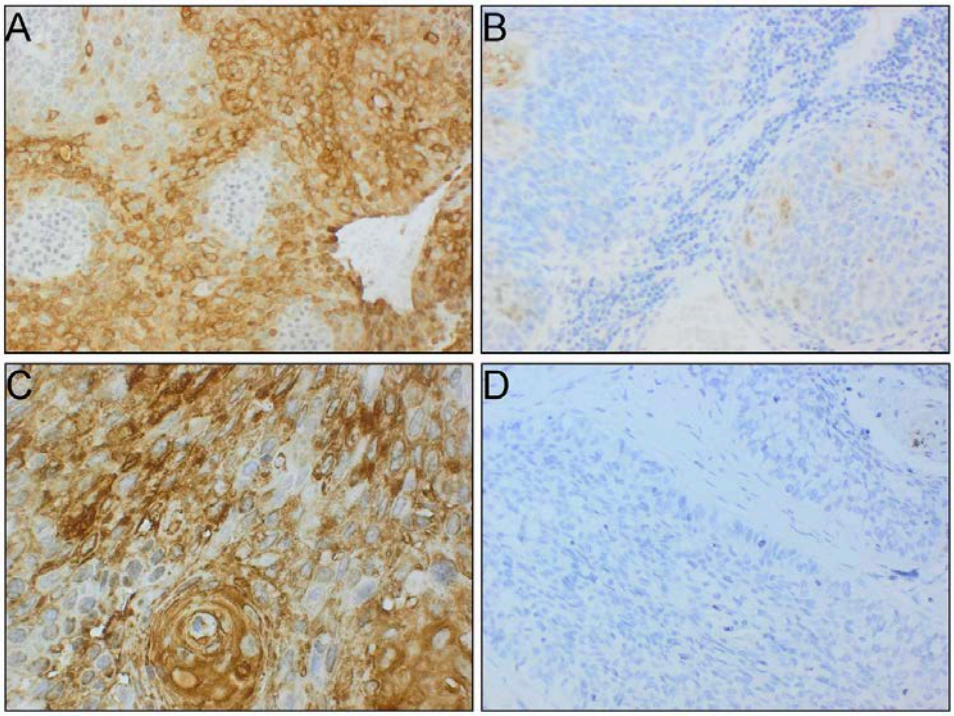


Figure 13. Representative images of psoriasin immunostaining by IHC. Magnification, x400. **(A)** High expression in HPV+ BOTSCC. **(B)** Low expression in HPV^+ BOTSCC. **(C)** High expression in HPV^- BOTSCC. **(D)** Low expression in HPV^- BOTSCC. From (Zupancic et al., 2021) with permission from the publisher.

4.4.4 Fusion Transcript Analysis

In Paper IV three patient samples were analysed for fusion transcript. This work was done outside our group, but the principles are described below. The RNA was extracted similarly as mentioned previously, however using different instruments; Maxwell® 16 MX3031 instrument with Maxwell® 16 FFPE LEV RNA Purification Kit from Promega (Fitchburg, WI, USA). The amount and purity of the RNA was measured as previously described with Nanodrop technology. cDNA was, then synthesized using the High-Capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA, USA) and RT-PCR and done in duplicates for targeting the fusion gene transcripts corresponding to *MYB-NFIB* variants on the LightCycler® 480 II instrument (Roche Diagnostics), according to Fehr et al (Fehr et al., 2011). Samples from previously identified AdCC cases with present *MYB-NFIB* translocation were used as positive controls and the reference gene HPRT1 was included to correct as a positive control.

4.4.5 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)

PRISMA is an acronym for: Preferred Reporting Items for Systematic Reviews and Meta-Analyses and consists of checklists and flow diagrams, that help researchers when conducting a meta-analysis or a systemic review. When complying by PRISMA statement reviews / meta-analyses can be replicated, maintain certain quality, structure and format as well as demonstrate a quality standard. For details about PRISMA statement see: (Page et al., 2021).

4.4.6 Statistical analyses

The data presented in the papers included in this thesis are presented with descriptive statistics. To evaluate differences between categorical variables we used Chi-^{2} test and for continues variables we used the independent two-tailed t-test.

OS was defined as the time from diagnosis until the time to death of any cause, or until the end of follow-up period in which all patients were censured. DFS was defined as the time from diagnosis until the event of recurrence of any kind. Study subjects that never became tumour-free after treatment were excluded from further analyses or censored at day 0. Also, patients with recurrence within 6 months after finished treatment were considered “never tumour-free” and treated as such. In Paper III we calculated event-free survival and that was defined as time from diagnosis until first event reported (LR: local relapse, DM: distant metastasis, DOD: dead of disease).

The results, i.e., differences in survival were assessed with the log-rank test and presented with the Kaplan-Meier survival curves. The Cox proportional hazard model was used for regression analysis and for the calculation of hazard ratios (HR) with 95 % confidence intervals (95 % CI) in the univariable and multivariable analysis.

P-values of < 0.05 were considered statistically significant.

Data management and statistical analyses were performed with IBM SPSS Statistics, (Version 25.0) and R (Version 3.4.1, R-project.org).

5 RESULTS AND DISCUSSION

5.1 PAPER I

Long-Term Survival and Recurrence in Oropharyngeal Squamous Cell Carcinoma in Relation to Subsites, HPV, and p16-Status

Aims

To follow long-term survival and recurrence in OPSCC in relation to different subsites, as well as to study the prognostic role of both HPV DNA and p16 status.

Background

The fact that HPV infections contribute to the development of HNSCC was suspected already in the 1980s (Syrjanen et al., 1983). This is today well-established, especially in OPSCC (Chaturvedi et al., 2011, Marklund and Hammarstedt, 2011, Mork et al., 2001, Nasman et al., 2009, Dahlgren et al., 2004, Rietbergen et al., 2013) . Recent data by others and us also showed that HPV^+ OPSCC, especially TSCC and BOTSCC had better prognosis than their HPV^- counterparts (Fakhry et al., 2008, Dahlstrand et al., 2008, Nygard et al., 2012). Despite that, long-term survival of HPV^+ and HPV^- OPSCC has still been sparsely studied.

In the Western world, although HNSCC as a diagnosis group is decreasing in prevalence due to the decrease in smoking and alcohol consumption, a clear continuous increase of HPV^+ OPSCC since 1970s has been observed. This mainly has to do with the epidemic increase of the HPV infections (Nasman et al., 2009, Sturgis and Cinciripini, 2007, Marur et al., 2010).

It has also been suggested that the prevalence of HPV infection, its correlation to p16 status and the impact of HPV on prognosis differs between OPSCC subsites. This can probably be explained by different tissue of origin within OPSCC; TSCC and BOTSCC counting to lymphoepithelial sites in comparison to otherOPSCC that are considered non-lymphoepithelial (Haeggblom et al., 2017). However, it has also been proposed that HPV^+ OPSCC develop different recurrence patterns, with different locations and later recurrences, compared to the HPV^- tumours (Trosman et al., 2015, Huang et al., 2013, Guo et al., 2016, O'Sullivan et al., 2015).

Nevertheless, the above has not been studied extensively and long-term follow up studies are few. To better understand the prognostic value of HPV DNA and p16 status in OPSCC and obtain more knowledge about the recurrence pattern of different OPSCC subsites, we studied

long-term follow up in a large cohort of OPSCC patients diagnosed between 2000 and 2010 in the Stockholm and Gotland Region.

Material and Methods

In this retrospective cohort study, we included 529 OPSCC patients treated with curative intent in Stockholm and Gotland Region between 2000 and 2010 and with known HPV DNA and p16 status (Nasman et al., 2015, Hammarstedt et al., 2021). We performed long-term survival analysis (OS and DFS) separated for different OPSCC subsites and analysed the recurrence frequency as well as the location (locoregional vs. distant). For further details, see Chapter 4 above or the section regarding Material and Methods in Paper I.

Main results

Figure 14 illustrates that long-term survival of OPSCC patients correlated with p16 overexpression, and that patients with $p16^+$ cancer had significantly better DFS in comparison to those with $p16^-$ cancer, log rank: $p < 0.0001$. This trend was still obvious when separated for TSCC and BOTSCC. However, a similar survival benefit was not observed in the group separated for otherOPSCC (non-lymphoepithelial subsites), where there was no significant difference in DFS between patients with $p16^+$ and $p16^-$ tumours, log rank: $p = 0.9$. Analogous data were obtained for OS for the above groups.

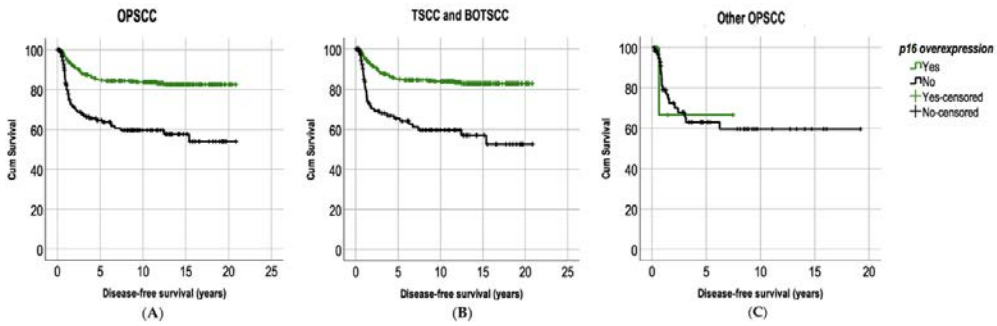


Figure 14. Kaplan–Meier figures with disease-free survival (DFS) in patients with **(A)** OPSCC and separated for subsites: **(B)** TSCC/BOTSCC and **(C)** otherOPSCC. **(A)** Patients with $p16^+$ OPSCC had a significantly better DFS compared to patients with $p16^-$ OPSCC (log rank: $p < 0.0001$). **(B)** Patients with $p16^+$ TSCC/BOTSCC had a significantly better DFS compared to patients with $p16^-$ TSCC/BOTSCC (log rank: $p < 0.0001$). **(C)** No significant differences in DFS between patients with $p16^+$ and $p16^-$ otherOPSCC (log rank: $p = 0.9$). (Paper I, with permission from the publisher).

Since the prognostic role of p16 overexpression was confirmed in the lymphoepithelial TSCC and BOTSCC, but not in the non-lymphoepithelial otherOPSCC, the analysis of HPV DNA status as a potential additional prognostic marker was performed only in TSCC/BOTSCC. Using both the presence/absence of HPV DNA and/or p16 we could further divide the TSCC and BOTSCC patients into three groups, for details see Figure 15.

More specifically, patients with HPV DNA positive and p16 positive (HPVDNA$^+$p16+) tumours had the most favourable OS. Patients with HPV DNA negative and p16 positive (HPVDNA$^-$p16$^+$) tumours had an intermediate OS, while patients with HPV DNA negative and p16 negative (HPVDNA$^-$p16$^-$) had the worst OS. The DFS data were similar, with a significant difference between patients with HPVDNA+p16+ tumours compared to those with HPVDNA$^-$p16$^-$ tumours ($p < 0.05$) (Figure 15). Furthermore, although trends towards differences in DFS in the HPV DNA and p16 convergent groups were observed, these were not statistically significant (HPVDNA$^+$p16$^+$ vs HPVDNA$^-$p16$^+$, HPVDNA$^-$p16$^-$ vs HPVDNA$^-$p16$^+$ (log rank test); $p = 0.1$ and $p = 0.05$, respectively).

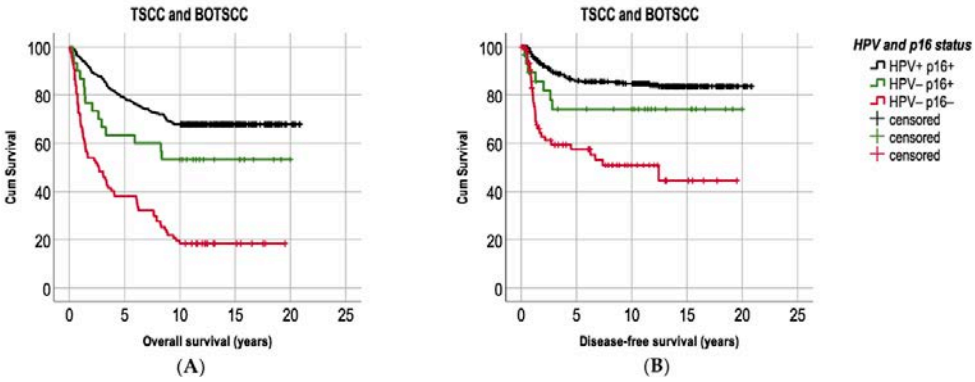


Figure 15. Kaplan–Meier figures with **(A)** overall survival (OS) and **(B)** disease-free survival (DFS) in patients with TSCC/BOTSCC. HPVDNA$^+$p16$^+$ TSCC/BOTSCC had a significantly better OS and DFS compared to HPVDNA$^-$p16$^-$TSCC/BOTSCC (log rank: $p < 0.0001$ and $p < 0.0001$, respectively) (**A, B,** respectively). HPVDNA$^-$p16$^+$ TSCC/BOTSCC presented a tendency towards an intermediate OS and DFS compared to patients with double positive or double negative HPV/p16 status. (HPVDNA$^+$p16$^+$ vs. HPVDNA$^-$p16$^+$ (log rank test): OS: $p = 0.047$; DFS: $p = 0.1$, and HPVDNA$^-$p16$^+$ vs. HPVDNA$^-$p16$^-$ (log rank test): OS: $p = 0.001$; DFS: $p = 0.05$). (Paper I, with permission from the publisher).

Recurrences of any kind were seen in 21 % of patients (112/529). The distribution between locoregional recurrences (LRR) and distant recurrences (DR) was equal in TSCC and BOTSCC, whereas LRR were more common in otherOPSCC (Figure 16). Notably, $p16^+$ cancers (TSCC/BOTSCC/otherOPSCC) had significantly more frequently DR in comparison to the $p16^-$ cancers (log rank test $p < 0.04$).

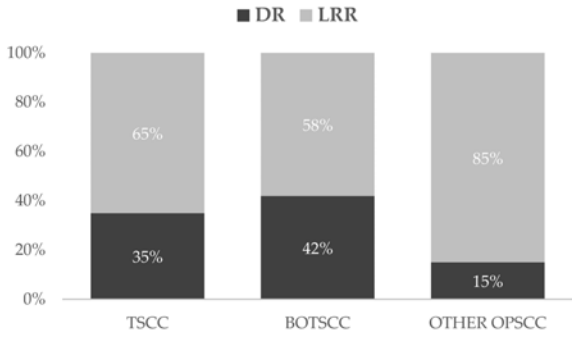


Figure 16. Proportions of loco-regional (LRR) and distant (DR) recurrences separated per OPSCC subsite. (Paper I, with permission from the publisher).

Moreover, HPV DNA and p16 status did not prove to be prognostic for survival upon recurrence of any kind, and the prognosis was poor irrespective of HPV status, (Figure 17).

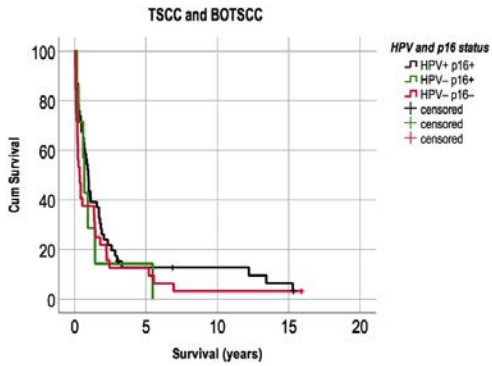


Figure 17. Kaplan–Meier figures with survival after a loco-regional or distant recurrence separated for HPV DNA and p16 status. No significant differences were observed in survival between patients with TSCC/BOTSCC after recurrent disease independent of HPV and p16 status. ($HPVDNA^+p16^+$ vs. $HPVDNA^-p16^-$ (log rank test): $p = 0.17$). (Paper I, with permission from the publisher).

Discussion

In this large OPSCC cohort, we confirmed previous data by us and others, strengthening the hypothesis that the favourable prognosis of HPV^+ OPSCC only holds for the lympho-epithelial tumours arising in TSCC/BOTSCC, but not in otherOPSCC (Haeggblom et al., 2017, Marklund et al., 2020, Hammarstedt et al., 2021, Tham et al., 2019, Gelwan et al., 2017, Ljokjel et al., 2014, Garnaes et al., 2015, Haeggblom et al., 2018). More specifically, we found that $p16^+$ was not a prognostic factor for survival in patients with otherOPSCC and that $p16^+$ alone was not reliable for the determination of an active HPV infection in otherOPSCC similar to previous data (Marklund et al., 2012). In addition, when further scrutinizing the presence of HPV $DNA^{+/-}/p16^{+/-}$ in TSCC/BOTSCC we found some additional differences in prognosis, although upon late recurrence the prognosis was poor for all OPSCC subsites, independent of HPV $DNA^{+/-}/p16^{+/-}$ status.

The fact that the combination of HPV DNA and p16 status divided TSCC/BOTSCC patients into three groups with different prognosis further implies that some patents, with convergent HPV DNA and p16 status (intermediate prognosis), might run a risk of being undertreated, when staged according to TNM-8. Considering these data, we propose that value of p16 is inferior to the combination of HPV DNA and p16 as a prognostic marker in TSCC/BOTSCC (Marur et al., 2010). This has previously been suggested by others, but in those studies, they did not differentiate between different subsites of OPSCC (Wagner et al., 2020, Rasmussen et al., 2019).

In this large OPSCC cohort, we could not show that patients with $p16^+$ TSCC/BOTSCC had a higher incidence of late relapses compared to those with $p16^-$ TSCC/BOTSCC, as previously reported by others in smaller cohorts (Marklund et al., 2012, Lewis et al., 2018). Moreover, we observed in contrast to some earlier reports, that survival upon recurrence was poor and that there was no difference in survival between patients with $p16^+$ and $p16^-$ TSCC/BOTSCC (Fakhry et al., 2014, Argiris et al., 2014).

OPSCCs are regularly treated similarly with RT or CRT regardless of subsite. Based on previous and recent data by others and us, disclosing differences in prognosis between HPV^+ and HPV^- TSCC/BOTSCC and otherOPSCC irrespective of HPV status, we suggest that treatment regimen should be reconsidered. This applies especially to otherOPSCC that indeed have poor prognosis. Clearly, more treatment studies will be needed to determine the best possible treatment option for the latter group. With recent advances in robotic surgery,

upfront surgery could be considered as first line treatment in resectable otherOPSCC, histologically resembling oral SCC.

This study had its limitations, e.g., the relatively small group of otherOPSCC, the retrospective study design (with prospectively collected clinical data) and that we did not correlate survival analyses to the actual given treatment. However, it is important to state that in our centre the treatment has generally been consistent for the OPSCC group over time. Further studies are needed, especially for otherOPSCC, to better tailor their treatment, and these should preferably be multi-centre studies in order to gain knowledge of even larger cohorts.

Conclusion

In this large retrospective cohort study, we confirmed that the combination of HPV DNA^+ and $p16^+$ was the best prognostic factor for lymphoepithelial OPSCC (TSCC/BOTSCC), although $p16^+$ was relatively useful for TSCC/BOTSCC. However, neither HPV DNA nor p16 status were optimal for otherOPSCC. We also showed that late recurrences occurred more often in HPV^- than in HPV^+ TSCC/BOTSCC, but HPV status was not corrrelated to survival upon recurrent disease, as all groups had poor survival with no significant differences.

5.2 PAPER II

Psoriasin expression is associated with survival in patients with human papillomavirus-positive base of tongue squamous cell carcinoma

Aim

To investigate whether psoriasin could be used as a potential prognostic marker in BOTSCC patients.

Background

HPV is a well-established risk factor for the development of OPSCC. It is also known that HPV^+ OPSCC, especially HPV^+ TSCC and BOTSCC have a better prognosis than corresponding HPV^- cancers, see background of Paper I. In recent years, it has therefore been proposed that the treatment of HPV^+ OPSCC, especially HPV^+ TSCC/BOTSCC could be tapered (Nasman et al., 2020). However, around 10-20 % of all HPV^+ TSCC/BOTSCC relapse, so additional biomarkers are needed to detect patients who could potentially receive less aggressive oncological treatments.

Others have shown that psoriasin / S100A7, an important cell mediator, playing part in cell maturation and cell survival, could potentially be correlated to clinical outcome. Moreover, Tripathi et al. (Tripathi et al., 2010) have shown that psoriasin expression was correlated to worse prognosis in HNSCC. For this reason, we wanted to examine the role of psoriasin in a homogenous cohort of BOTSCC, that had previously been tested for both $p16^+$ and presence of HPV DNA.

Material and Methods

In total 72 patients diagnosed with BOTSCC between 2000 and 2007 in Stockholm Region and treated with curative intent were included. For all 72 available tumour samples the HPV DNA and p16 status were known from previous studies. The samples were stained for psoriasin / S100A7 by IHC. A cut-off value of 30 % immunostained cells was defined as positive staining according to a previous publication (Tripathi et al., 2010). Psoriasin expression positive vs. negative results were linked to OS as well as DFS and compared to other typical prognostic factors (disease stage, age, and smoking status) in BOTSCC. For further details, see Chapter 4 in this book or the section regarding Material and Methods in Paper II.

Main results

In this cohort of 72 BOTSCC patients, 36 tumour samples did not express psoriasin in invasive tumour (0 % positive staining), 18 tumour samples were considered positive and defined as having high psoriasin expression (above 30 % positivity), whereas the remaining 18 samples were considered having low psoriasin expression (below 30 % positivity).

Patients with tumours with negative and low psoriasin expression were grouped into the same group (low psoriasin expression, 54 patients) and compared to 18 patients with high psoriasin expression in their tumours. Notably, patients with tumours with low psoriasin expression were significantly more often HPV DNA$^+$ and p16$^+$ ($p = 0.005$) and non-smokers ($p = 0.02$).

As we were especially interested in patients with HPV$^+$ tumours we performed a survival analysis of this group. HPV$^+$ BOTSCC with high psoriasin expression had a significantly worse OS and DFS compared to those with HPV$^+$ BOTSCC with low psoriasin expression, (log rank test: OS $p < 0.001$; DFS $p = 0.02$), see Figure 18 (based on HPV DNA).

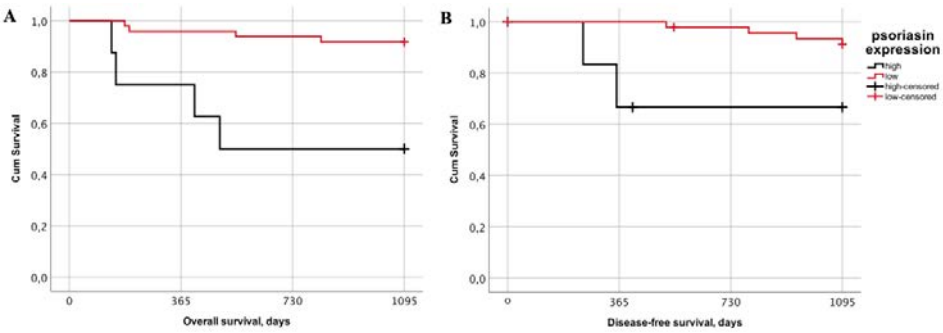


Figure 18. Kaplan-Meier curves of **(A)** OS and **(B)** DFS in patients with HPV$^+$ BOTSCC, stratified into a high (≥ 30 %; 'high') and a low (< 30 %; 'low') fraction of psoriasin-positive tumour. High tumour cell expression of psoriasin was significantly associated with **(A)** a worse OS ($p < 0.001$) and **(B)** a worse DFS ($p = 0.02$) in patients with HPV$^+$ BOTSCC. (Adapted from Paper II, with permission from the publisher).

The three-year OS was 91.7 % in patients with low psoriasin expression compared to 50 % in patients with high psoriasin expression. The three-year DFS was similar, 91.7 % in patients with low psoriasin expression, compared to 66.7 % in patients with high psoriasin expression. Analogous results were obtained when using p16 status alone or in combination with HPV DNA as a definition of HPV status (all differences were significant, data not shown).
Furthermore, high psoriasin expression was independently correlated to a significantly worse OS and DFS both in uni- and multivariable analysis. Previously known prognostic factors, such as: disease stage, age, smoking status, were included in the analysis.

Discussion

In this pilot study we investigated a possible prognostic role of psoriasin in a homogenous cohort of BOTSCC, especially HPV^+ BOTSCC. It is well known that HPV^+ TSCC and BOTSCC have a more favourable prognosis than the corresponding HPV^- tumours. However there are still patients in this group that encounter a more aggressive disease and further prognostic markers would be useful. In this study, we demonstrate a correlation between high psoriasin expression in invasive tumour tissue and a less favourable clinical outcome in HPV^+ BOTSCC. Psoriasin could therefore, together with other biomarkers, be a part of a prognostic algorithm in the future. As this is a pilot study, only including BOTSCC patients, more clinical data, including other OPSCC subsites, is needed to further validate prognostic value of psoriasin. In contrast, we could not show any prognostic value of psoriasin in HPV^- BOTSCC. However, this could possibly be due to that only 16 such patients were included in this study.

Nevertheless, before psoriasin can be used clinically the cut-off values for positive psoriasin expression should be further validated. In this study we used a cut-off of 30 %, since we wanted to compare our data with the data in a previously published HNSCC paper (Tripathi et al., 2010). One could argue that our approach was debatable, as the optimal cut-off values according to Youden´s index were different (15 % for OS and 7.5 % for DFS in our specific cohort) and other trials including tumours, such as breast or bladder cancer have used different cut-off levels (Liu et al., 2020a, Emberley et al., 2004). Notably, however we considered the possibility of comparing our data with the previously mentioned HNSCC cohort of crucial importance for this pilot study.

This short report also further confirms the assumption that psoriasin expression is higher in cancer in situ as compared to invasive tumours, previously shown by others and us (Emberley et al., 2004, Alowami et al., 2003, Haeggblom et al., 2019a). The latter could also explain why only 18 cases in this cohort presented high psoriasin expression, as OPSCC more often are invasive tumours, rarely showing in situ components (Haeggblom et al., 2019a).

Conclusion

In this study in a BOTSCC cohort we could show that high psoriasin expression was correlated with significantly worse survival (both OS and DFS) in HPV^+ BOTSCC. We suggest that psoriasin could potentially be considered as a prognostic marker, in combination with further prognostic markers, when deciding on treatment for HPV^+ BOTSCC.

5.3 PAPER III

Human Papillomavirus-Related Multiphenotypic Sinonasal Carcinoma-An Even Broader Tumor Entity?

Aim

To accumulate more knowledge on the features and locations of HMSC.

Background

In 2013 Bishop et al. published a case series, describing a new tumour entity then called HPV-related carcinoma with adenoid cystic-like features (Bishop et al., 2013b). It was a tumour, assumed to be homogenous, limited to the sinonasal area, caused by an HPV infection, with uniform histomorphology, showing characteristics both from the squamous epithelium and salivary gland tissue (Bishop et al., 2013b, Bishop et al., 2017).

In 2017 HMSC was included in the 4th Edition of the WHO classification of tumours as an emerging tumour entity (Andreasen et al., 2019). However recent case reports have suggested that clinical and histopathological characteristics of HMSC might be broader (Oramas et al., 2020). In the most recent, 5th Edition of the WHO classification of tumours, however, HMSC is characterised as its own entity (Bishop, 2022). In order to better understand this new tumour entity, we wanted to perform a systematic review and a meta-analysis of all HMSC described in the literature.

Material and Methods

The systematic review was performed according to PRISMA guidelines, where we searched the PubMed for "multiphenotypic" OR "multiphenotypic sinonasal" OR "adenoid cystic-like" in May 2021. We found 18 publications with unique cases of HMSC, published since the first report in 2013 (Bishop et al., 2013b). All together we identified and summarized clinical and pathological characteristics of 79 unique HMSC patients. We calculated event-free survival for these patients, with events being: "No evidence of disease" (NED), "Local relapse" (LR), "Distant metastasis" (DM) or "Dead of disease" (DOD). Patients with NED were censored at the last day of reported follow-up. For further details, see Chapter 4 in this book or section regarding Material and Methods in Paper III.

Main results

In total 78 cases were located in the head and neck region with the nasal cavity being the most common. Notably however, HMSC was not restricted to the sinonasal area, since one case was described in the tonsil and additionally one was diagnosed in the female breast tissue (n = 79). The gender distribution was even and the median age at diagnosis was 53 years. Tumour size was available in 57/79 cases and the mean tumour size was 3.5 cm. The treatment modality was specified in 61/79 cases and the most common treatment modality was surgery alone (n = 34), followed by the combination of surgery and postoperative radiotherapy (n = 21). Other combinations including chemotherapy, were less common. The mean and median event-free survival for patients with defined outcome (59/79) were 81.7 months (95 % CI: 52.1–111) and 72 months (95 % CI: 41.1–103), respectively.

The pathology reports described the HMSC having a basaloid proliferation with mostly presence of solid (76/79 cases) as well as cribriform areas (53/79 cases, 20 without cribriform areas and 6 not defined). Focal squamous differentiation was less common and seen in 15/79 cases, and an associated dysplastic squamous epithelium was however described in 50/79 cases. Perineural and lymphovascular invasion were rare and observed in only five and one cases, respectively.

IHC is an important part of HMSC diagnostics and was performed often. Positive IHC immunostainings used for defining a squamous differentiation (e.g., p40, p63) were identified in 53/79 cases (20 without and 6 not defined), the luminal marker CD117/c-kit was identified in 40/79 cases (19 without and 20 not defined).

The authors used several different methods to define HPV status, with p16 overexpression as a surrogate marker for an active HPV infection, defined by IHC, being the most common method. Presence of HPV DNA defined by PCR was only done in 31 cases, and in these cases even HPV type was available. The most common HPV type was HPV33 (17 cases), followed by HPV35 and 56 in four cases each and HPV16 in three cases.

Discussion

To our knowledge, this systematic report of all patients diagnosed with HMSC between 2013 and 2021 summarizes all available clinical and morphological data about this new tumour entity from the literature so far. HMSC seems to have a rather non-aggressive clinical course compared to other tumours arising from the sinonasal area, despite its aggressive

histomorphology. It is however of note, that the follow-up data were not complete and, in many cases, short (31.3 and 23 months mean and median follow-up time). We therefore encourage that the survival data is interpreted with caution. Notably, we could not confirm female prevalence of HMSC, previously suggested in smaller cohorts, but we could confirm that HMSC patients were younger than other HNC patients (Bishop et al., 2017).

Other reports state that HPV33 is dominant in HMSC (Bishop et al., 2017). However, we show here that HPV typing is often lacking in the literature and therefore we suggest awaiting data from larger cohorts before drawing such definite conclusions.

Some differential diagnoses of HMSC are AdCC and SCC with basaloid appearance and it can often be challenging to differentiate between these three. In order to rule out AdCC we suggest performing a gene fusion analysis as HMSC is, in contrast to AdCC, expected to be negative for fusions in *MYB-NFIB* genes (all 79 cases presented here were negative, data not shown). HMSC also mostly lacks perineural invasion, which is common in AdCC (Chen et al., 2020, Atallah et al., 2020, Liu et al., 2020b, Dantas et al., 2015, Brayer et al., 2016, Frerich et al., 2018). Basaloid SCCs are predominant in men and are rarely found in the sinonasal area. Furthermore, in contrast to HMSC, SCC often presents with nodal metastases and is less often positive for HPV (Ishida and Okabe, 2013, Chernock et al., 2010).

Finally, this systematic report showed that HMSC was not limited to the sinonasal area, suggesting that HMSC or HMSC-like morphology can be found in other areas within as well as outside the head and neck.

Conclusion

HMSC is a tumour entity arising mostly in the sinonasal area, with an even sex distribution. Patients often have a relatively good prognosis in comparison to those with other carcinomas in the head and neck area ane are also often on average younger at the time of diagnosis. The main differential diagnoses of HMSC were AdCC and basaloid SCC. All HMSC so far were HPV positive. However more studies are needed in order to better characterise this tumour entity.

5.4 PAPER IV

Analysis of Human Papillomavirus (HPV) and Polyomaviruses (HPyVs) in Adenoid Cystic Carcinoma (AdCC) of the Head and Neck Region Reveals Three HPV-Positive Cases with Adenoid Cystic-like Features

Aim

To search for a possible presence of HPV and HPyV in AdCC and their potential diagnostic and prognostic potential. A secondary aim was to identify and characterise possible HMSC patients previously diagnosed with AdCC.

Background

AdCC is a rare salivary gland malignancy, with varying clinical outcome and yet unknown aetiology (Li et al., 2015). It accounts for 10 % of all neoplasms in major, and 30 % of all minor salivary glands (Young, 2023, Dillon et al., 2016, Ammad Ud Din and Shaikh, 2022). AdCCs are not limited to salivary glands and can arise in other areas of the head and neck, as well as in secretory gland tissue outside this area (Dillon et al., 2016, Young, 2023, Ammad Ud Din and Shaikh, 2022). AdCC can occur at all ages but is more common in the fifth to sixth decade (Chae et al., 2015). As there are no known specific risk factors for AdCC we decided to look for HPV and HPyVs in a large AdCC cohort (Young, 2023, Dillon et al., 2016).

Viral infections play, as mentioned previously, a major role in several different cancers within the head and neck region. HPV is a well-known risk-, as well as a prognostic factor in TSCC and BOTSCC (Chow, 2020, Näsman et al., 2021). HPV is also a known risk factor in a subset of sinonasal non-keratinizing squamous cell carcinomas (NKSCCs), although its prognostic role in these carcinomas is still unclear (Bishop et al., 2013a). Another virus family where at least one member is oncogenic (MCpyV), is the HPyV family (Prado et al., 2018). Both HPV and HPyV have been described in AdCC previously (Chen et al., 2017, Boland et al., 2012, Mohamed et al., 2021, Qian et al., 2016, Hämetoja et al., 2019). Nevertheless, their role in AdCC still remains unclear.

Recent reports, including Paper III of this thesis have suggested that HMSC, a differential diagnosis to AdCC, proposed to be limited to the sinonasal area, may occur outside this specific area (Hodgson et al., 2021, Zupancic and Näsman, 2021). HMSC was first described in 2013 and as it can resemble AdCC closely, it is possible that some HMSC have previously been diagnosed as AdCC (Shah et al., 2018). Today, differentiating between these two diagnoses remains challenging, but we do know that the *MYB-NFIB* gene fusions, common in AdCC do not occur in HMSC (Bishop et al., 2017, Zupancic and Näsman, 2021, Jang et al., 2017, de

Almeida-Pinto et al., 2019). As *MYB-NFIB* gene fusions are not present in all AdCC, additional diagnostic possibilities could be of use (de Almeida-Pinto et al., 2019). For this reason, the potential role of HPV and HPyV in AdCC was investigated further.

Material and Methods

We identified 94 patients diagnosed with AdCC between 2000 and 2012 (before HMSC was first described in 2103). FFPE tumour material and HE-stained slides, as well as the adjacent normal tissue controls, were available from 68 patients, and 68/94 patients were included in the analyses. DNA was extracted using the "AllPrep DNA/RNA FFPE Kit" as described previously. Presence of HPV DNA was based on using multiplex PCR followed by a bead-based multiplex assay for 27 different HPV types, including all HR-HPV types, on a MagPix instrument. A similar bead-based multiplex assay was used for analysing for HPyVs. HPV^{+} samples were further analysed by IHC for p63, CD117, S100, and p16 expression, as well as for the *MYB-NFIB* gene fusion. IHC and fusion transcript analysis was not performed by us. For further details see section on Material and Methods in this thesis or in Paper IV.

Main results

Altogether 73 AdCC tumour samples from 68 patients (66 primaries, one LRR and one DM without adjacent primary tumour and additional two LRR and three DM) were analysed for HPV DNA and p16 expression and HPyV DNA. None of the tumours were positive for HPyV, whereas four samples, from three unique patients were positive for HPV (three primaries and one corresponding LRR).

The three patients with tumours positive for HPV DNA were analysed further; one had a sinonasal tumour (HPV33) and two had a tonsillar primary location (one HPV16 and one HPV33). In all three cases defining the final diagnosis was challenging and the pathology reports were considering a non-keratinizing squamous cell carcinoma as well as a basaloid squamous cell carcinoma as potential differential diagnoses. Nevertheless, at the time of diagnosis, considering both the histomorphology and IHC analyses, the consensus was that all three tumours represented AdCC with solid growth pattern.

To further scrutinize the tumours of these three patients, the histomorphology of the tumours was reviewed, and in addition, we performed IHC for p63, CD117, S100, and p16, as well as a fusion transcript analysis for the *MYB-NFIB* gene fusion. The three tumours had similar morphology with basaloid appearance, showing mostly solid growth pattern with islands of tubular- and cribriform-like growth. No presence of associated dysplastic epithelium was

observed. All tumour samples (three primaries and one corresponding LRR) overexpressed p16, were positive for p63, and were mixed-positive for S100. Focal minimal areas with CD117 were observed in all three primaries. However, none of the four samples showed *MYB-NFIB* gene translocation and the adjacent normal tissue controls were all negative for HPV DNA.

The three patients with HPV^+ tumours all presented an early-stage disease (stage II) with no nodal disease. The patient (Patient 1, Table 2 in Paper IV)) with AdCC in the sinonasal area was treated with CRT (50Gy + Paclitaxel 60mg/m^2 once weekly) and the residual tumour was radically removed. This patient was tumour-free at the last check-up, 73 months after finished treatment. Patient 2 (Table 2 in Paper IV) with a tumour in the tonsil was initially diagnosed and treated as having an HPV^+ TSCC and received conventional RT. However, this patient presented with a locoregional disease 5 months after initial treatment. The tumour was then re-assessed, and the patient was diagnosed as having an AdCC. He underwent several extensive surgeries, as well as brachytherapy, but was never disease-free. He passed away two years after the preliminary TSCC diagnosis. The third patient (Patient 3, Table 2 in Paper IV) was diagnosed with AdCC in the tonsil and was treated with radial surgery followed by CRT (68Gy + Cisplatin 30mg/m^2 once weekly). Patient 3 was disease-free 90 months after initial diagnosis.

Discussion

In this large AdCC cohort we could not find any HPyV positive tumours, however three patients initially diagnosed with AdCC had HPV^+ cancers.

Some studies have previously revealed presence of HPyV in AdCC. Hämetoja et al. tested 68 AdCC tumours for HPyV (JCPyV, BKPyV, and SV40) and showed that 10.3 % of AdCCs harboured JCPyV (Hämetoja et al., 2019). However, all these positive samples had low viral loads. We have also previously tested a large MST cohort (n = 91) including 11 AdCCs for HPyVs and in total three of these MST cases were MCPyV-positive of which one case was an AdCC (Ramqvist et al., 2018b). Even in our study, the HPyV DNA amounts were low in all three cases. Moreover, HPyV infections (MCPyV in our case) were not overrepresented in any specific histological subtype, suggesting that HPyVs do not play any major role in AdCC (Ramqvist et al., 2018b). The fact that none of the AdCCs in this large cohort were HPyV positive strengthens the probability that HPyVs are not an important causative factor for the development of AdCC.

However, in contrast to Hämetoja et al., whose cohort included no HPV$^+$ tumours, in this study 3/66 patients presented with HPV$^+$ AdCC (Hämetoja et al., 2019). After additional assessment of these tumours, we concluded that they all histomorphologically resembled basaloid SCC, rather than AdCC.

The differentiation between a solid variant of AdCC, which was the final diagnosis of these three patients in the clinic, and basaloid SCC is often challenging (Jaso and Malhotra, 2011). Considering the fact that basaloid SCC can be related to HPV infections both in the head and neck area, as well as the anogenital area (Chernock et al., 2010, Thariat et al., 2010, de Sanjose et al., 2014) and that such a relationship is not yet established in AdCC, we suggested that it is possible that these tumours presented a basaloid SCC as opposed to an AdCC.

In addition, however, in this context, HMSC should also be mentioned, since it histomorphologically resembles AdCC as well, and is HPV-related. Thereby HMSC was suggested as another possible differential diagnosis for our three HPV$^+$ cases.

In Paper III, we presented two cases with HMSC outside the sinonasal area. Here, we add two additional HMSC-like cases within the tonsil: one HPV33 positive and one HPV16 positive. The fact that HPV$^+$ basaloid SCCs resemble HMSC, shown here and by others (Jaso and Malhotra, 2011) and that HMSC-like histomorphology appears outside the sinonasal area, suggests that further studies are required in order to better characterise HMSC before it can be considered an own diagnosis outside the sinonasal area.

Even though we did not show any major role of HPV in AdCC here, we consider HPV status an important question, particularly due to the recent increase of HPV$^+$ malignancies, both in- and outside the head and neck region (Graham et al., 2016, Rahimi, 2020).

Conclusion

In this study, HPV and HPyV did not to play a major role in AdCC. This report, however, indicated that when HPV is detected in cases diagnosed as AdCC the diagnosis could be incorrect. Moreover, since we here detected two additional cases with an HMSC-like morphology outside the sinonasal area we suggest that a better characterisation of HMSC may be necessary before it can be justified as its own diagnosis.

5.5 PAPER V

Adenoid cystic carcinoma (AdCC) a clinical survey of a large patient cohort

Aim

To study a large cohort of AdCC and to investigate how these patients have performed clinically and whether any prognostic factors could be disclosed.

Background

AdCC often runs an indolent clinical course. Many patients present with a disease at an advanced stage with no specific symptoms (for further details on presentation of disease please see Paper IV above). AdCC is however characterised by perineural growth and is often surgically challenging to remove radically (Calzada et al., 2012, Locati, 2017, Young, 2023, Dillon et al., 2016). Furthermore, its diagnostics can be difficult, since AdCC histomorphologically resembles other malignant and benign tumours. Furthermore, specific prognostic markers are lacking (Zupancic et al., 2022, Zupancic and Näsman, 2021, Young, 2023, Dillon et al., 2016, Locati, 2017) and general clinical prognostic markers, such as gender, age, or smoking status, are less reliable in AdCC (Ellington et al., 2012).

Nevertheless, since AdCC is often characterised by slow growth, the 5-year survival rates are relatively high (80-85 %), but late relapses are common and occur independent of treatment modality. Locoregional relapses and distant metastasis are observed in 15-85 % and 25-55 % of the cases, respectively (Young, 2023, Ouyang et al., 2017, Jang et al., 2017). Due to these later incidences, long-term survival numbers are less impressive, and 10- and 15-year survival rates are as low as 50–60 % and 30–35 %, respectively (Jang et al., 2017, Ouyang et al., 2017).

Most studies in the field are retrospective and show that multimodal treatments (including combinations of surgery and postoperative RT or CRT) are better than single modality treatments (Dillon et al., 2016, Miglianico et al., 1987, Khanani et al., 2022, Iseli et al., 2009, Mendenhall et al., 2004). Nevertheless, the effects of the benefit of multimodal treatment on long-term survival is still unclear (Mendenhall et al., 2004, Chen et al., 2006) and therefore more long-term follow-up studies are needed in order to better understand this often slowly progressing disease.

Of note is on the other hand that recurrent disease can run a much more aggressive course and then ChT and other systemic oncological treatments are not impressive, with response rates not exceeding 20 % (Laurie et al., 2011).

Material and Methods

In total 155 patients from the Stockholm and Gotland region, with AdCC of the head and neck were included. All patients' charts were assessed for clinical characteristics, e.g., age, gender, smoking, performance status, tumour stage (TNM-8), perineural growth status, type of treatment, and recurrence status. The survival analyses were performed for 142 patients, who were treated with curative intent and were disease free six months after finished treatment. For further details, see Chapter 4 in this book or the section regarding Material and Methods in Paper V.

Main results

In our cohort female patients dominated (F:M ratio 1.6:1) and tumours were most often arising in the major salivary glands (55.5 %). The majority of the patients presented with limited disease (94.8 %), yet stage IV was most common, with 35.3 %, followed by stage II (28.8 %), stage I (22.2 %), and stage III (13.7 %) disease. Stage IV disease was most common in nasal cavity and paranasal sinuses and the least common in the submandibular gland. Perineural growth was present in 64.5 % of the cases and overrepresented in AdCC in the major salivary glands. Out of 155 patients 142 were eligible for curative treatment and 118 received multimodal treatment, here defined as surgery followed by RT or CRT. In addition, 24 patients were treated with single treatment modality, here defined as RT/CRT (14 patients), or surgery alone (10 patients). In Figure 19, survival (DFS and OS) of the entire cohort treated with curative intent is presented irrespective of treatment modality, and more specifically, 5-year DFS was 64.9 %, whereas the 15-year DFS was only 37.7 %.

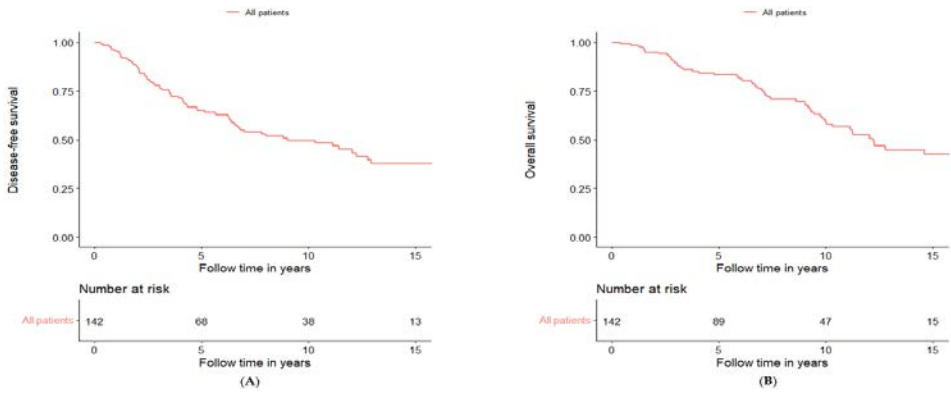


Figure 19. Kaplan-Meier figures with **(A)** DFS and **(B)** OS of all patients treated with curative intent independent of treatment modality. (Paper V, with permission from the publisher).

Patients treated with multimodal treatment had significantly better survival rates compared to those treated with single treatment modalities. The 5-year DFS was 70.7 % vs. 37 %, respectively, and the same trend was observed for OS, 82.8 % vs. 60.9 %, respectively.

In this large AdCC cohort gender, age, and smoking status were assessed as general clinical prognostic factors but none of them had any significant influence on DFS. However, being younger (< 58.5 years) did have a significant effect on OS, which was not the case for gender and smoking status.

Perineural growth pattern was observed in 66.9 % of patients treated with curative intent and did not corelate with a higher disease stage. Likewise, there were no significant differences in survival when dichotomizing for perineural growth according to the pathology reports.

When separating the survival analyses according to the disease stage, it became obvious that lower disease stages had significantly better DFS and OS. We were able to cluster the patients into two groups, Stage I and II vs. Stage III and IV, and in Figure 20 the DFS data are presented.

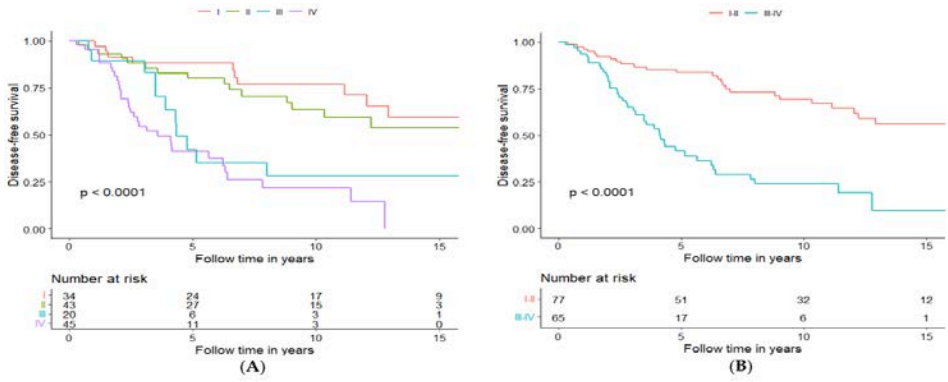


Figure 20. Kaplan-Meier figures with DFS of **(A)** patients with tumours staged I-IV and **(B)** patients with tumours staged I and II vs. III and IV. (Paper V, with permission from the publisher).

Further differences in survival could be seen when separating for the four most common anatomical sites, namely “parotid gland”, “submandibular gland”, “nasal cavity and paranasal sinuses”, and “oral cavity”. Primaries from the parotid gland had the most favourable survival with a 5- year DFS of 81.1 %, followed by those of the submandibular gland 68.3 % and the oral cavity, 61.2 %. AdCC in the nasal cavity and paranasal sinuses had the least favourable survival with the 5-year DFS 42 %. Similar trends were observed for OS (5-year OS were: 94.3 %, 82.4 %, 82.5 % and 73.7 %, respectively).

When analysing the Kaplan-Meier figures for DFS and OS in relation to the different anatomical sites (not shown here) it was clear that the prognosis of patients with AdCCs within the major salivary glands was better than for those with AdCC in other subsites. DFS and OS analyses were therefore performed separating the major salivary glands from other subsites. Figure 21 illustrates that 5-year DFS and OS were 73.5 % and 88.7 %, respectively for patients with AdCC in the major salivary glands as compared to 53.7 % and 76.8 %, respectively for patients with AdCC in other sites. This trend was even obvious at 10 and 15 years after diagnosis.

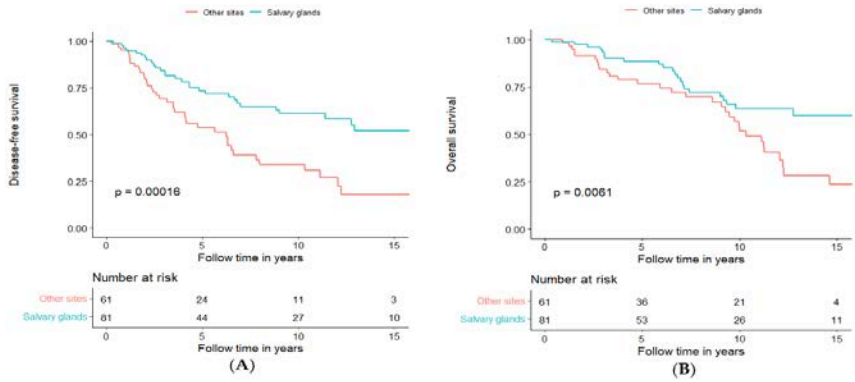


Figure 21. Kaplan-Meier figures with **(A)** DFS and **(B)** OS in patients treated with curative intent, independent of treatment modality, in relation to all tumour subsites, separated for major salivary glands and all other sites together. (Paper V, with permission from the publisher).

Multivariable analyses for DFS and OS including gender, age, smoking status, stage of disease (I + II vs. III + IV), perineural invasion, and dichotomizing surgery and RT/CRT vs. surgery or RT/CRT alone, as well as salivary glands vs. other subsites, were performed. Here, we could confirm that general clinical prognostic markers, such as age, gender, and smoking were not applicable in AdCC. However, it was clear that multimodal treatment was better than treatments of single modality and that both subsite and tumour stage could be used as prognostic markers in AdCC.

Discussion

In this study, AdCC patients were assessed for clinical characteristics and long-time survival. We observed that DFS and OS were better upon multimodal therapy vs. single treatment modalities and that cases with tumour stage I - II had a better outcome than those with stage III - IV disease and that patients with major salivary gland AdCC had a better outcome than those with AdCC at other subsites.

Not unexpectedly, the clinical characteristics of the AdCC cohort in this study were similar to AdCC cohorts presented by others before (Dillon et al., 2016, Ouyang et al., 2017, de Morais et al., 2021, Atallah et al., 2020). AdCC was overrepresented in the female patients (here F:M ratio 1.6:1) and most commonly diagnosed within the fifth and sixth decade (younger than other HNC) (SweHNCR, 2020).

Comparable to other reports most patients were treated curatively with multimodal treatment consisting of surgery followed by RT (Dillon et al., 2016, Ammad Ud Din and Shaikh, 2022, Chen et al., 2020, de Morais et al., 2021, Cantu, 2021). In line with the literature, we could in our large AdCC cohort confirm that neither gender, age, nor smoking status influenced the survival significantly (Chen et al., 2020, de Morais et al., 2021, Atallah et al., 2020, Ko et al., 2016). Likewise, OS data showed a similar trend, with the exception of age, since older patients expectedly have a shorter expected survival (da Cruz Perez et al., 2006, Jang et al., 2017).

Multimodal treatment was superior to single treatment modalities and in line with current literature it correlated significantly with fewer recurrences (Chen et al., 2020, Mendenhall et al., 2004, Chen et al., 2006, Garden et al., 1995). PORT seems to play an important role in longevity of recurrence free survival. However, it has been suggested that the role of PORT needs to be further evaluated in patients with low-stage disease (Chen et al., 2020).

Since AdCC is a rare malignancy most of the study cohorts usually do not include enough patients treated with surgery alone to be able to draw strong conclusions. Here, based on 10 patients with limited disease (Stage I-II), we show that surgery alone was a good treatment strategy and achieved good locoregional control, nevertheless we still suggest considering PORT to achieve the best possible disease control.

Surgery achieving negative surgical margins is often considered as a general positive prognostic marker in many solid tumours and this is no different in AdCC (Cantu, 2021, Amit et al., 2017, Luksic et al., 2016). Nonetheless, this was not the case in our cohort showing no significant difference in DFS and OS between radical and non-radical surgery in all patients treated with curative intent. This in our opinion underlines the importance of the PORT, as our cohort did not have worse survival outcome than comparable cohorts in the literature.

Regarding tumour stage, we could confirm that it is important also in AdCC, since lower stage diseases similar to that in other solid tumours had a more favourable survival (Cantu, 2021,

Spiro and Huvos, 1992, Dubal et al., 2016). This is no news, but it is of note that AdCC in contrast to other HNC rarely presents with nodal metastases, therefore the tumour size is the most important part of the staging (Xu et al., 2017, Ning et al., 2018, DeAngelis et al., 2011, de Morais et al., 2021).

Another important fact was that different anatomical subsites could be used as prognostic markers in AdCC. We show here, in line with some other reports that AdCCs in major salivary glands, especially in the parotid gland have a better clinical outcome. The latter was conversely not in line with some other reports, showing no difference in survival in different anatomical subsites (de Morais et al., 2021, Cantu, 2021, Luksic et al., 2016, Tasoulas et al., 2021, Marcinow et al., 2014). AdCC in the nasal cavity and paranasal sinuses on the other hand showed the least favourable DFS, with the highest incidence of LRR. Nonetheless, this did not influence the OS, as these relapses usually could be treated and achieve good locoregional control (Lupinetti et al., 2007, Husain et al., 2013).

Perineural growth was common and observed in 66.7 % of all curatively treated cases, in line with other reports (Chen et al., 2020, Atallah et al., 2020, Dantas et al., 2015, Liu et al., 2020b). It did however, not correlate with a higher disease stage and was more common in AdCC in major salivary glands, which in our cohort had a better clinical outcome. Some similar studies have linked perineural tumour growth with positive surgical margins and a higher incidence of LRR / distant metastases and less favourable outcome (Jang et al., 2017, de Morais et al., 2021, Garden et al., 1995, Liu et al., 2020b, Ju et al., 2016, Fang et al., 2022, Persson et al., 2009). The latter could not be shown in this AdCC cohort. Opposing to that, here, LRR and distant metastases were more common in the cases without perineural invasion. The reason for this discrepancy we do not presently know, but we have found another report in the literature coming to similar conclusions (Luksic et al., 2016). One possible explanation could be that even though our cohort is fairly large, this still could be explained due to chance or other yet unknown factors.

More studies like ours are needed to better characterise AdCC. Some limitations in our study are that even though the clinical data is collected prospectively in the patients´ journals, this still is a retrospective study. In addition, it is important to add that some recently diagnosed patients have not been followed up for > 15 years, but were still included, as we aimed to have as large cohort as possible. Furthermore, the standard follow-up regimen was not the same during the last 20 years and some patients were followed up longer than the others. This could

to some extent influence the DFS data in this study, but many other reports in the literature have had similar limitations. Nevertheless, the OS data is 100 % complete since it was obtained from the Swedish death registry.

Conclusion

General clinical prognostic markers, e.g., age, gender, smoking status are less reliable in AdCC and we suggest that these should not be used clinically for AdCC. Moreover, previously used prognostic markers, such as radical surgery and perineural invasion need further evaluation and are potentially less reliable than suggested previously. Tumour stage, anatomical subsite and multimodal treatment were the most reliable prognostic factors in this study and the literature shows similar data. The combination of surgery and PORT was the most promising therapy regimen and should, if possible be used to achieve the best clinical outcome. Finally, we suggest that AdCC follow-up regimen should be longer than for other HNC, as no plateau in survival curves is seen after 10 and 15 years of follow-up.

6 CONCLUDING REMARKS

This thesis focuses on HNCs that are not primarily related to smoking. More specifically we studied OPSCC and all its different subsites, HMSC, and AdCC. For AdCC in particular, the field is in desperate need of better biomarkers for both diagnostics, prognostics, and possible targeted therapy. Below some details of our data and conclusions are presented.

I. During a 10-year follow-up period we disclosed that TSCC and BOTSCC, but not otherOPSCC, overexpressing p16 had much better prognosis than their $p16^-$ counterparts. Notably, when combining HPV DNA and p16 status even better precision for prognostication was obtained. Furthermore, we disclosed that late recurrences were more common in the $p16^-$ TSCC/BOTSCC group but that survival was poor after recurrence regardless of p16 status or OPSCC subsite.

II. To better stratify and individualize treatment for preselected OPSCC patients more prognostic markers are needed. In this pilot study the presence of psoriasin was investigated in BOTSCC. We could show that psoriasin was a useful prognostic marker in HPV^+ BOTSCC and that it should be studied further in larger cohorts.

III. HMSC has recently been described as a rare tumour with an association with HPV. Here we performed a systematic review and noted that these tumours were also found outside the sinonasal region. We conclude that more studies will be needed to reveal the true nature of this tumour.

IV. We investigated whether HPV or HPyVs were present in AdCC and possibly could be used for prognostication. However, they were not commonly found and thereby they do not have a prognostic role for these tumours. We did on the other hand find HPV DNA in three tumours. Moreover, they had a morphology more alike HMSC but where found outside the sinonasal region. We conclude that HPV status should be analysed when diagnosis of AdCC is uncertain.

V. In this long-term retrospective study, we confirmed that general clinical prognostic markers, e.g., age, gender, and smoking status are less reliable in AdCC. Furthermore, we disclosed no significant correlation between radical surgery, perineural tumour growth and survival. Finally, early disease stage and major salivary gland subsite were the strongest favourable prognostic factors in our AdCC cohort.

7 FUTURE DIRECTIONS

HPV$^+$ cancers are rising in general and will continue to do so in the near future. More and more countries in the world are already vaccinating girls and the vaccination of boys is now also being implemented. It is expected that general vaccination against HPV will put a stop to this pandemic rise of HPV$^+$ cancer cases. It will, however, take another 30–40 years until we see full effects of these vaccines. For this reason, we need better treatment options for this growing group of patients. HPV$^+$ OPSCC have a good prognosis in general, but better treatments are needed upon recurrent disease, especially where no additional local treatment is possible. Moreover, better prognostic markers, that could be used together with p16 status for more precise stratification of different OPSCCs, are needed. The goal should be to give every patient the best possible, individualized therapy that would lead to the best possible tumour response with a combination of the lowest side effect rate.

In Papers I and II we worked towards this goal. In Paper I we showed that p16 status alone was not enough to define prognosis in the OPSCC patient group and that anatomical subsite was important. Our work should continue with larger, possibly multinational studies to validate these findings in more heterogenic cohorts, where the proportions of other risk factors, potentially confounding the outcome, are different.

In Paper II we detected that psoriasin could be a novel prognostic marker in BOTSCC. We now suggest that HPV DNA and p16 status could be tested in combination with psoriasin in larger cohorts, including both BOTSCC and TSCC.

In Paper III we contributed to the field of HPV$^+$ tumours with a thorough review of all cases of HMSC published in the literature until May of 2021. We suggest that further studies are needed to better characterise this tumour entity and its relation to the HPV. This could initially be done by a multinational collaboration, re-evaluating AdCCs located outside the major salivary glands and in other cases, where the diagnoses were uncertain. A study organised in such a way would allow for standardised follow-up data, where we could get more confident data regarding the HPV types and prognosis of this tumour entity.

In the Papers IV and V some important findings regarding the clinical prognostic factors in AdCC have been confirmed. Others have been challenged, but the most important message should be that a lot is still unclear and that further studies are warranted. We disclose that HPV and HPyV did not play any major role in AdCC. We, however, suggest that these tumours should be tested for HPV when the diagnosis of AdCC is unclear, as HPV$^+$ cases

might be HMSC or basaloid SCC. No IHC or molecular biomarkers were included in this thesis concerning AdCC. However, some of the experimental work regarding this matter has already been performed in our group and our work continues beyond this book. We are aware that we possess a uniquely large AdCC patient cohort with long clinical follow-up data (Paper V) that can be paired with possible new diagnostic and prognostic markers.

8 ACKNOWLEDGEMENTS

I remember having a conversation with my mother, still being in the kindergarten, asking her what a PhD is. Her explanation was simple yet so precise: “A person who knows a great deal about something very specific can obtain a PhD, imagine studying a fall of a raindrop for several years and then writing a book about it.” I understood little back then, but the explanation makes a lot of sense today.

A few of you who did not start reading this book on page 79 probably want me to finish, so you can get on with more important things in your lives, but I promise, I will not be much longer.

I would like to start by expressing my gratitude to the **Karolinska Institutet** for creating such a thriving environment, collaborating with the **Karolinska University Hospital**, where I could grow professionally, both as a researcher and a future oncologist.

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