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CLINICAL USE OF PROGNOSTIC MARKERS IN HEAD- AND NECK CANCER

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Clinical Use of Prognostic Markers in Head- and Neck Cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To Johan, I miss you…

POPULAR SCIENCE SUMMARY OF THE THESIS

Head and neck cancer (HNC) is a mixed group of tumors in various parts of the head and neck area. In 2020, approximately 1600 new cases of HNC were reported in Sweden which accounted for 2.3% of new cancers annually. In Sweden, as in other Nordic countries, HNC is a relatively uncommon form of cancer, but in global terms it is a significant group of disease.

The dominant cause of HNC is traditionally smoking and alcohol but in 2007 human papillomavirus (HPV) was recognized as a cause of cancer in the upper part of the throat, the oropharynx. This area includes the tonsils and base of tongue

HPV are best known for its role in cervical cancer and its prevention with HPV-vaccines. But the viruses are also linked to tonsillar cancer and cancer of the base of tongue. There are many different types of HPV, where some can cause cancer, and some are harmless causing for example warts. Cancer of the throat caused by HPV are associated to better prognosis. The patients are younger and non-smoking to a greater extent than patients with other HNC.

Head and neck cancer often metastasize to the lymph nodes in the neck. It is previously known that the metastases in the neck in HPV positive oropharyngeal tumors also are HPV-positive. There is thus likely that if you have HPV in a metastasis of the neck the cancer arises from an HPV-caused cancer in the throat. It is also unclear if HPV appears in other neck masses than metastases.

A type of HNC with worse prognosis is cancer of the lower throat, the hypopharynx. It is not so common accounting for about 4 % of all HNC cases in Sweden. It has a strong connection to alcohol and smoking.

Treatment of head and neck cancers are surgery, chemo- and radiotherapy. Often a combination of two or three of these. There also relatively new treatment methods who are involving the immune system. Regardless choice of treatment it is almost always comes with side effects. After treatment all patients are followed up in some matter. Since a couple of years all patients with cancer in the oropharynx go through a radiological examination called PET-CT 12 weeks after ending treatment. If there are sign of remaining disease surgical removal of several lymph nodes in the neck are performed.

In the first study we tested if you could find HPV in samples taken from lumps in the neck by a fine needle, so called fine needle aspiration cytology. We tested patients without knowing their final diagnosis. It turned out that patients with HPV in their samples from the neck all had cancer caused by HPV. We did not find HPV in any other type of neck masses.

There is a condition called a branchial cleft cyst that is a remnant from the fetal period. This is a harmless condition, but it can be hard to distinguish it from a metastasis of a head and neck cancer. It is especially hard to distinguish from cystic metastasis which is more common in cancer caused by HPV. Therefore, we were interested to see if HPV could be found in branchial cleft cysts. In the second study we collected material from patients that had underwent surgery with removal of branchial cleft cysts and tested it for HPV. We did not find HPV in any of the branchial cleft cysts.

Study 1 and study 2 together suggests that test of HPV in fine needle samples is a good method for finding HPV-positive oropharyngeal cancer and that HPV testing of neck masses should be recommended as part of the investigation before treatment.

In the third study we tested samples from patients with hypopharyngeal cancer for proteins related to the immune system and compared it with normal tissue and in relation to HPV-status. HPV positive tumors contained higher amounts of those proteins compared to HPV negative tumors and all tumors had higher number than the normal tissue. In the future this can be of importance for treatment with drugs related to the immune system. We also tested a certain type of immune cell called CD8+ lymphocyte. Patients with higher amounts of these cells had better prognosis. In the future this can be of importance for selecting patients to different treatment forms.

I the fourth study we evaluated if suspected metastases of remaining cancer, after treatment, from the tonsils and the base of tongue that are found in PET-CT are located in the same place when surgery is performed. This to see if it is possible to reduce extent of the surgery. We found that only a third of patients with suspected metastases on PET-CT actually had active remaining cancer. When there was an active cancer the location of cancer on PET-CT and in laboratorial examination were coherent in most cases.

The main conclusions of the thesis are:

HPV in a neck mass is a strong indicator of oropharyngeal cancer caused by HPV

HPV are not found in branchial cleft cysts

Cancer of the hypopharynx have an immunological activity greater than normal tissue.

High numbers of CD8+ lymphocytes in hypopharyngeal cancer are linked to better prognosis

Reduced surgery may be an option for patients with remaining cancer in the neck after treatment for cancer in the tonsils or base of tongue.

ABSTRACT

Head and neck cancer (HNC) is a heterogeneous group of tumors where squamous cell carcinoma is the most dominant. In 2020, 1640 new cases of HNC were reported in Sweden which accounted for 2.3% of the total cancer incidence. In Sweden, as in other Nordic countries, HNC is a relatively uncommon form of cancer, but in global terms it is a significant group of disease.

The dominant etiological factor for HNC is traditionally smoking but in 2007 human papillomavirus (HPV) was recognized as carcinogenic to oropharyngeal cancer (OPSCC), which include tonsillar- (TSCC) and base of tongue cancer (BOTSCC).

Human papillomavirus (HPV) are small DNA viruses, best known for its role in cervical cancer and its prevention with HPV-vaccines. But they are also linked to the tonsillar cancer and cancer of the base of tongue. Today there are more than 170 known types of HPV, but only a fraction infects the mucosal surfaces and only a few of these types oncogenic. The most common oncogenic types in HNC are HPV 16, 31 and 33.

In the last decades there has been a sharp rise in tonsillar cancer and cancer of the base of tongue (TSCC, BOTSCC). HPV is the dominating cause of this. HPV- induced oropharyngeal cancers (OPSCC) correlates strongly to better prognosis. A disease with worse prognosis is hypopharyngeal cancer (HPSCC), here the role of HPV is more unclear.

In study 1 we wanted to see if HPV can be used as a marker for OPSCC in fine needle aspiration cytology (FNAC) of cervical neck masses. This in a prospective setting where 66 patients with cervical masses were tested for HPV in their FNAC-sample. All 17 patients who had HPV-positive OPSCC as final diagnosis also had HPV in FNAC. No patients with benign neck masses had HPV in FNAC.

The challenge of distinguishing a cystic metastasis of an HPV-positive OPSCC from a branchial cleft cyst, which is a benign condition, is well known. It is also known that the neck metastases of HPV-positive OPSCC are HPV-positive and that HPV-DNA in the FNAC correlates to an HPV positive OPSCC. It has not been investigated if HPV-DNA also can be present in benign neck cysts. In study 2 we wanted to see if branchial cleft cysts could be HPV-positive. From 112 patients diagnosed with branchial cleft cyst under the years 2007-2015 DNA was extracted from formalin fixed paraffin embedded surgically resected material. None of the branchial cleft cysts contained HPV-DNA.

In the third study we evaluated the expression of immune related proteins and tumor infiltrating lymphocytes in tumor samples compared to normal tissue and in relation to HPV-status and clinical outcome in patients with HPSCC. Fresh frozen tissue from 33 patients were analyzed for protein expression by the Proseek immuno-oncology immunoassay. Tumors, especially HPV-positive, had more immunological activity than normal tissue. In

addition to this, 144 formalin-fixed biopsies, from patients with HPSCC, were analyzed for CD8+ TILs in relation to clinical outcome. Patients with high numbers of CD8+TILS hade improved clinical outcome

OPSCC are often diagnosed with presence of metastases in the neck and different strategies has been used for treatment of the neck after radio/chemoradiotherapy, (RT/CRT). The impact of RT/CRT in the tissue of the neck makes surgery of the neck surgically complicated and the risk of long-term side effect increases why more reduced surgery would benefit the patient. I the fourth study all patients diagnosed with TSCC and BOTSCC between 2017 and 2021 in the County of Stockholm were identified through the Swedish Cancer Registry. 217 patients who had post-treatment PET-CT or salvage ND were assessed included for further analysis including HPV and p16 status. Neck dissection was performed in 36 patients due to PET-CT criteria, the result of PET-CT was compared to pathological report from the neck specimen regarding location of metastases with viable cancer.

In total, 26/36 patients examined by PET-CT and treated with ND had no sign of viable cancer. In 8/36 patients, the localization of metastasis in histopathological examination and the PET-CT was consistent. Thus, in two of the 10 patients with viable tumor the viable tumor cells was found in cervical lymph nodes other than those seen in PET-CT; both tumors were HPV-DNA and p16-positive

Conclusions for the thesis are:

HPV DNA in FNAC of neck masses is a strong indicator of TSCC or BOTSCC and was not present in any benign conditions or other malignant masses

HPV-DNA is absent in FFPEs of branchial cleft cysts.

HPSCC have higher expression of immune related protein than normal tissue, and the expression was even higher in HPV-positive tumors.

High numbers of CD8+ TILs in HPSCC are related to better clinical outcome.

Reduced neck dissection after post treatment PET-CT may be feasible for patients with TSCC or BOTSCC.

LIST OF SCIENTIFIC PAPERS

I. Sivars L*, **Landin D***, Haeggblom L, Tertipis N, Grün N, Bersani C, Marklund L, Ghaderi M, Näsman A, Ramqvist T, Nordfors C, Munck-Wikland E, Tani E, Dalianis T. Human papillomavirus DNA detection in fine-needle aspirates as indicator of human papillomavirus-positive oropharyngeal squamous cell carcinoma: A prospective study. Head Neck. 2017 Mar;39(3):419-426. doi: 10.1002/hed.24641. Epub 2016 Nov 29. PMID: 27898186.

II. Sivars L, **Landin D**, Rizzo M, Haeggblom L, Bersani C, Munck-Wikland E, Näsman A, Dalianis T, Marklund L. Human papillomavirus (HPV) is absent in branchial cleft cysts of the neck distinguishing them from HPV positive cystic metastasis. Acta Otolaryngol. 2018 Sep;138(9):855-858. doi: 10.1080/00016489.2018.1464207. Epub 2018 May 15. PMID: 29764277.

III. **Landin D**, Ährlund-Richter A, Mirzaie L, Mints M, Näsman A, Kolev A, Marklund L, Dalianis T, Munck-Wikland E, Ramqvist T. Immune related proteins and tumor infiltrating CD8+ lymphocytes in hypopharyngeal cancer in relation to human papillomavirus (HPV) and clinical outcome. Head Neck. 2020 Nov;42(11):3206-3217. doi: 10.1002/hed.26364. Epub 2020 Jul 1. PMID: 32613643.

IV. **Landin D**, Näsman A, Jonmarker Jaraj S, Hammarstedt Nordenvall L, Dalianis T, Munck-Wikland E and Marklund L. Post-Treatment Neck Dissection of Tonsillar and Base of Tongue Squamous Cell Carcinoma in the Era of PET-CT, HPV, and p16. *Manuscript*

*contributed equally

CONTENTS

LIST OF ABBREVIATIONS

BCC	Branchial cleft cyst
BOTSCC	Base of tongue squamous cell cancer
CD8	Cluster of differentiation 8
CT	Computed tomography
DNA	Deoxyribonucleic acid
DSS	Disease specific survival
FDG	Fluorodeoxyglucose
FFPE	Formalin fixed paraffin embedded
FNAC	Fine needle aspiration cytology
HNC	Head and neck cancer
HNCUP	Head and neck cancerof unknown primary
HPSCC	Hypopharyngeal squamous cell cancer
HPV	Human papilloma virus
ICD-10	10th revision of the International Statistical Classification of Diseases
IHC	Immunohistochemistry
ISH	In situ hybridization
MRI	Magnetic resonance imaging
ND	Neck dissection
OPSCC	Oropharyngeal squamous cell cancer
OS	Overall survival
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFS	Progression free survival
SweHNCR	Swedish head and neck cancer register
TIL	Tumor infiltrating lymphocyte
TORS	Transoral robotic surgery
TSCC	Tonsillar squamous cell cancer

1. INTRODUCTION

Cancer is a global burden, and this doctoral project aims to improve management of head and neck cancer and other related conditions. A small brick in building knowledge.

1.1 HEAD AND NECK CANCER

Globally, about 14 million cases of cancer are diagnosed every year of which head- and neck cancer (HNC) accounts approximately for 880 000 cases and 453 000 deaths annually.[1, 2]

In Sweden, the total cancer incidence increases, and so does HNC. Approximately 1600 new cases of HNC are reported annually, i.e. 2.3% of the country's total cancer incidence. In Sweden and the Nordic countries, HNC is a rare tumor disease, while globally it is a significant disease group. In the western world, HNC is the 5-6th most common type of cancer as compared to the 2-3rd most common in the developing countries.[2, 3]

Head- and neck cancer includes cancer of the larynx, oropharynx, nasopharynx, hypopharynx, oral cavity, nose, nasal sinuses, salivary glands and cancer of unknown primary in the head and neck region. It is a diverse group of cancers with different etiology, treatment and prognosis between different subsites. About 80-90% of the tumors are squamous cell carcinomas.[3]

Tobacco smoking and alcohol use are well known risk factors for HNC. However, the last 15-20 years it has become evident that human papilloma virus (HPV) also has an important causative role in HNC. In year 2007 Human Papilloma Virus type 16 was added to the list of risk factors for oropharyngeal squamous cell carcinomas (OPSCC) by International Agency for Cancer Research(IARC).[4] Patients with HPV-positive oropharyngeal cancer has better prognoses than patients with corresponding HPV- tumors[5, 6]. For other types of HNC cancer the importance of HPV is unclear. [7] For the majority of HNC we lack clinically useful predictive and prognostic markers.

Depending on tumor localization HNC can be treated with surgery, radiotherapy (RT), chemotherapy (CT), targeted therapy and combinations of these. In recent time also immunotherapy is used but generally when conventional treatment options have been tried. Small tumors are often treated with single therapy while the more advanced tumor stages receive combination therapy. The head and neck are a complex area with vessels, nerves and muscles necessary for important functions such as swallowing, speech, sensory perception,

and cosmetic appearance why treatment for head and neck cancer is a fine balance between cure and side effects from treatment. [8] All patients with cancer are monitored post treatment. For HNC this is, in Sweden, clinical examination every third month for two year and then every sixth month for three years.

1.1.1 Oropharyngeal cancer

The oropharynx is the middle part of the pharynx. It is located behind the oral cavity and consist of the soft palate, the uvula, the tonsils, the base of tongue and the pharyngeal walls. Cancers in the different subtypes are defined by ICD-10 as tonsillar cancer c 09.0, c09.8, c09.9; base of tongue cancer c01.9; cancer in the arch of the soft palate c09.1; cancer in the anterior part of soft palate c05.1, c05.8, c05.9; uvula cancer c05.2; cancer of the posterior pharyngeal wall or lingual surface of epiglottis c10.0, c10.2, c10.3, c10.8, c10.0. All subsites are lined with non-keratinized squamous cell epithelium and approximately 90% of the oropharyngeal cancers are squamous cell cancers.[9]

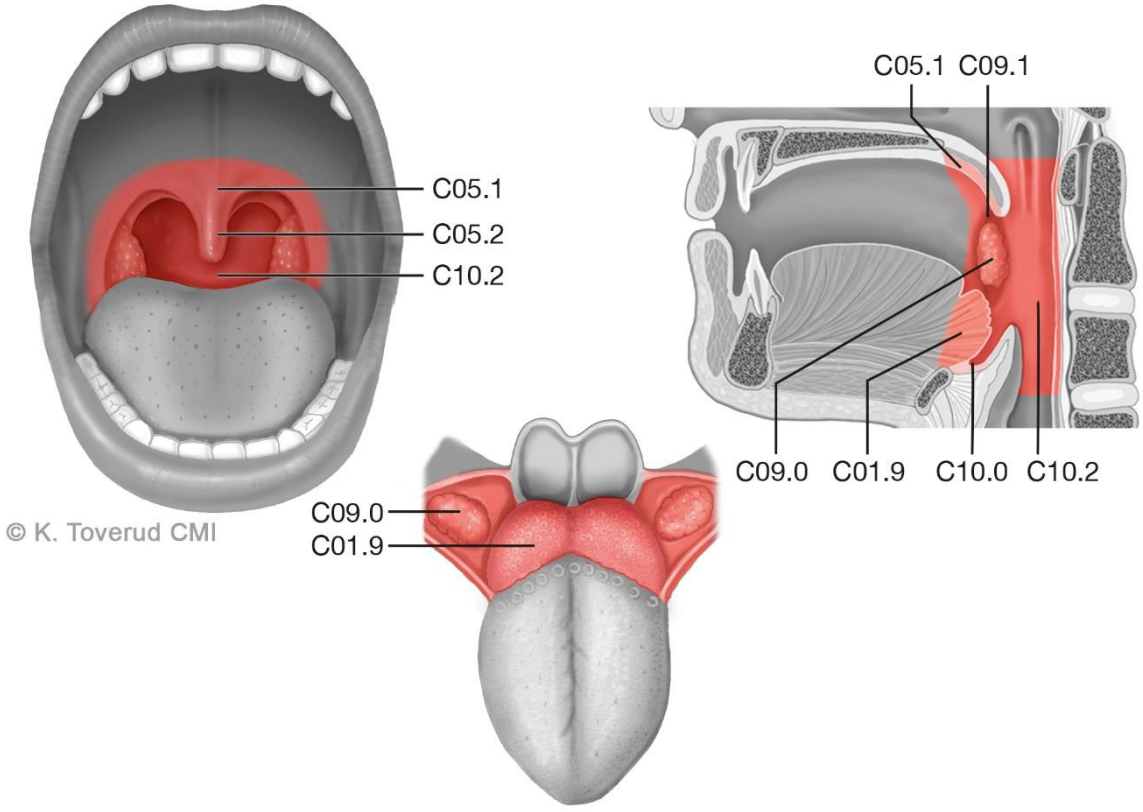

Image 1,2 and 3: *Oropharynx and its subsites with ICD-10 codes. © Kari C. Toverud CMI (certified medical illustrator)*

In Sweden oropharyngeal cancer constitutes of about 27 % of all HNC. Among the oropharyngeal subsites tonsillar and base of togue cancer dominates with around 90% of the cases. OPC is more common in men than in women with a ratio of 3:1.[3]

I the last decades there has been a sharp rise in the incidence of tonsillar squamous cell carcinomas (TSCC) and base pf tongue squamous cell carcinomas (BOTSCC). This due to increase of HPV-related cancers.[10-13]

The overall five-year survival in all oropharyngeal cancers is about 70 %, but earlier research from our group and others has shown that presence of HPV is a favorable prognostic marker for tonsillar and base of tongue cancer both regarding survival and risk of relapse. This was independent of tumor stage. [5, 6, 14-16]

This finding has been confirmed by many studies and in the latest UICC TNM classification TNM8, HPV-mediated OPSCC (defined by p16 overexpression) are downstaged as compared to HPV-unrelated OPSCC.[17]

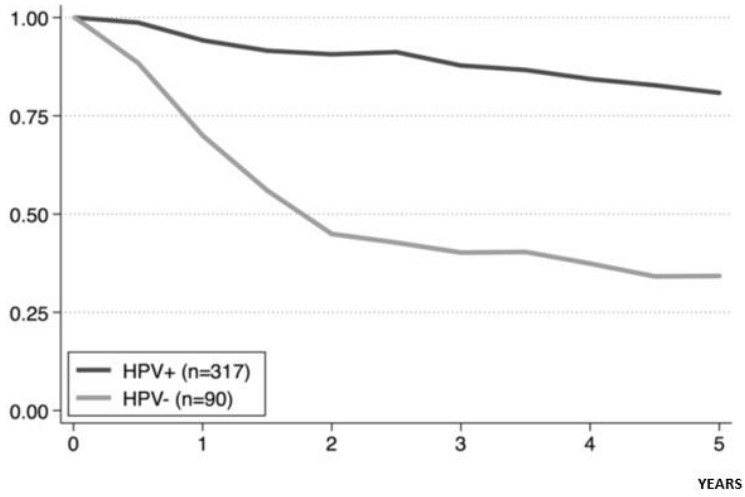

Image 4: *Relative survival of patients with oropharyngeal cancer, comparing HPV- and HPV+ cancer 2008-2016 From SweHNCR*

The prevalence of HPV infection, p16-status, the impact of HPV infection on prognosis and the correlation between the markers vary between the OPSCC sub-sites. Impact of HPV and p16 on survival are different between tumors from lymphoepithelial oropharyngeal sites, ie TSCC and BOTSCC, and cancers from non-lymphoepithelial subsites of the oropharynx for example the soft palate, uvula, posterior pharyngeal wall. HPV-, and p16-status in other oropharyngeal cancer than TSCC and BOTSCC should be interpreted carefully.[18, 19]

TNM 8th edition	T-category p16-negative oropharyngeal cancer	TNM 8th edition	T-category p16-positve oropharyngeal cancer
T1	Tumor ≤ 2cm	T1	Tumor ≤ 2cm
T2	Tumor > 2cm ≤ 4cm	T2	Tumor > 2cm ≤ 4cm
T3	Tumor > 4cm, or extension to lingual surface of epiglottis	T3	Tumor > 4 cm or extension to lingual surface of the epiglottis
T4a	Tumor invading: larynx, deep muscles of tongue, medial pterygoid, hard palate or mandible	T4	Moderately advanced local disease; tumor invades larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible or beyond
T4b	Tumor invading: m.pterygoideus lateralis, pterygoid plate, lateral aspect of nasopharynx, skull base or around carotid artery		

TNM 8th edition	N-category p16-negative oropharyngeal cancer	TNM 8th edition	N-category p16-positve oropharyngeal cancer
N1	Ipsilateral node ≤ 3cm with no extranodal extension	N1	Unilateral node or nodes ≤ 6 cm
N2a	Single ipsilateral node > 3 cm ≤ 6 cm wtih no extranodal extension	N2	Contralateral or bilateral nodes ≤ 6cm
N2b	Multiple ipsilateral nodes ≤ 6 cm wtih no extranodal extension	N3	Node or nodes > 6cm
N2c	Contralateral or bilateral nodes ≤ 6 cm wtih no extranodal extension		
N3a	Node > 6 cm with no extranodal extension		
N3c	Extranodal extension		

The 8th Edition of the American Joint Committee on Cancer (AJCC8) Staging Manual for Oropharyngeal cancer

The diagnostic of OPSCC includes a thorough clinical examination, radiology with CT and MRI and examination including biopsies in general anesthesia.

OPSCC are generally treated with external radiotherapy often in combination with chemotherapy. In Sweden standard treatment is radiotherapy with 68 Gray under 6 weeks with 2 Gray/fraction and day. In more advanced stages of diseases radiotherapy is often combined with concomitant chemotherapy, commonly platinum-based treatment with Cisplatin. When Cisplatin is not tolerated by the patient due to side effects or hearing loss combination of radiotherapy and cetuximab, an epidermal growth factor receptor-inhibitor, can be considered.[20] The treatment is tough and related to several acute and long term side effects for many patients.[21]

Previously all patients with metastasis in the neck were planned for neck dissection after completed radio/chemoradiotherapy. Although only few patients with complete response in

the neck presented viable tumor cells in the neck specimen.[22] Since 2017 FlouroDeoxyGlucose-Positron Emission Tomography with Computer Tomography (PET-CT) is performed 12 weeks post treatment at many medical centers including Karolinska University Hospital. Only patients with suspected remaining metastasis in the neck according to PET-CT criteria are then selected for neck dissection including neck region 1-4/5. This regime was introduced after an article where PET-CT was compared with neck dissection for all patients with nodal disease.[23]

Hence the better prognosis of HPV-positive OPSCC efforts have been made to de-escalate treatment for these patients. So far this has not been very successful, and the recommended treatment regime is still the same for patients with HPV-positive and HPV-negative tumors.[24, 25]

1.1.2 Hypopharyngeal cancer

Hypopharynx is the lower part of the pharynx. In the posterior wall it goes from the level of the hyoid bone to the esophagus. The anterior boundary is the glottis and epiglottis. Hypopharynx includes the following sub sites; the sinus piriformis, the post cricoid area and the posterior pharyngeal wall.

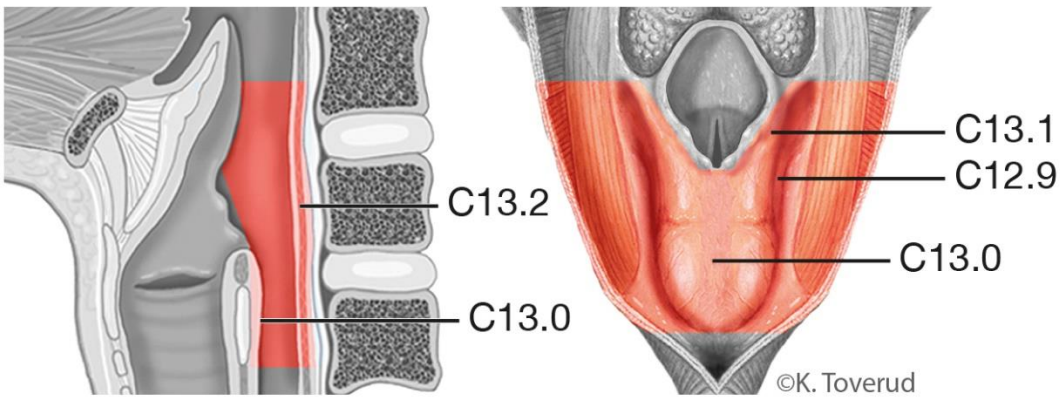

Image 5 and 6: *Hypopharynx and its subsites with ICD-10 codes. © Kari C. Toverud CMI (certified medical illustrator*)

Hypopharyngeal cancer accounts for about 3-5% of all HNC, in Sweden approximately 60 patients/year are diagnosed with hypopharyngeal cancer. The disease is more common in men than in women with an incidence of 0,66/100 000 for males and 0,20/100 000 for

women.[3] Hypopharyngeal cancer is related to tobacco smoking and alcohol [26, 27] and is the HNC with the worst prognosis with an average 5-year overall survival of only 15-30%. Most of the hypopharyngeal cancers are diagnosed at a late stage, due to several factors. The anatomy is such that lesions give symptoms later compared to other locations. For example, the vocal cords which gives hoarseness with very small tumors. The hypopharynx has a lymphatic drainage promoting early neck node metastasis. At the time of diagnosis 60-80% of patients have ipsilateral neck metastases and up to 40 % on the contralateral side.[28] The tumors are also difficult to visualize at most primary care center, probably delaying the diagnosis in some cases.

Hypopharyngeal cancer has the worst prognosis of all HNC, but the prognosis differs slightly within this patient group. If we could predict the response to oncological treatment for patients with hypopharyngeal cancer, we could select patients for primary surgical or oncological treatment and maybe improve survival

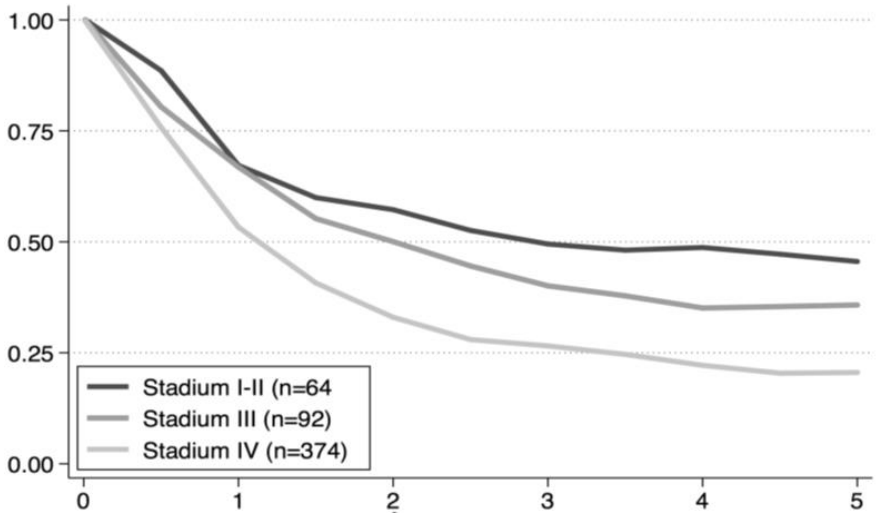

Image 7: *Relative survival of patients with hypopharyngeal cancer, per stage 2008-2016. Year on x-axis, cumulative probability on y-axis. From SweHNCR*

The poor prognosis of hypopharyngeal cancer is not only due to diagnosis at a late stage but also related to the patient's history of tobacco and alcohol abuse as well as co-morbidities. This results in a poor performance status limiting the treatment options and the tobacco and alcohol leads to a predisposition to develop secondary malignances.[29]

Treatment options include radiotherapy or chemoradiotherapy and surgery. In Sweden most patients are primarily treated with radiotherapy (RT) or chemoradiotherapy (CRT) in cases with advanced stage. If the patient has residual regional metastasis or recurrence after oncological treatment, they undergo neck dissection and in the case of residual local disease/recurrence salvage surgery, laryngectomy is performed when possible.

The treatment algorithm for advanced hypopharyngeal cancer has changed over time. Some 30 years ago standard treatment was primary surgery with total laryngectomy, but this has shifted towards more laryngeal preservative methods as chemoradiotherapy. In the early eighties there were good results published with induction chemotherapy with 5-fluorouracil.[30]. Later randomized trials were conducted by the European Organization for Research and Treatment of Cancer (EORTC). This showed similar results for surgical methods and laryngeal preservative method with induction chemotherapy for patient with hypopharyngeal cancer [31]. Also in the early nineties the Department of Veterans Affairs Laryngeal Cancer Study Group compared chemoradiotherapy in patients with advanced laryngeal carcinoma with surgery and post-operative radiation. The survival rates were comparable. After that chemoradiotherapy has become standard first-line treatment in hypopharyngeal cancer. [32]

An emerging area within all treatment strategies of cancer is immunotherapy. The role for these treatments in hypopharyngeal cancer is still unclear. [33]

In later years new surgical techniques with endoscopic laser technique as well as transoral robotic surgery (TORS) offer new opportunities for surgical treatment of laryngeal and hypopharyngeal cancer. [34-36]

Possibly more patients should primarily be treated surgically, but we need markers to identify who these patients are. If we could identify the patients that will respond well to chemoradiotherapy, primary treatment could easier be individualized.

In a previous study, we identified HPV as a predictive marker for radiation sensitivity in hypopharyngeal cancer patients, but since less than 5% of tumors show HPV positivity, more clinically useful markers are indeed needed. [37]

1.1.3 Head and neck cancer of unknown primary

Head and neck cancer of unknown primary (HNCUP) is a cervical lymph node SCC metastasis without a detectable primary tumor despite extensive medical examination including Computed Tomography (CT), Magnetic Resonance Imaging (MRI), clinical examination in general anesthesia with biopsies from the epipharynx, base of tongue and tonsillectomy. HNCUP accounts for approximately 3-5 % of the total HNC. Of the HNCUPs about 80 % are SCC.[38, 39]

Compared to CUP in general HNCUP has better clinical outcome. HNCUP also seems to have more in common with other HNC than with other CUP. The better prognosis of

HNCUP can be related to the fact that some are most likely clinically undetectable HPV-positive OPSCC.[40, 41]

Head and neck cancer of unknown primary was earlier treated with neck dissection followed by radiotherapy. Side effects from this combined treatment is often serious.

Our research group has previously shown that patents with HPV positive CUP have improved prognosis compared to patients with HPV negative CUP and survival mimic survival for TSCC/BOTTSCC. [42] Therefore, if the cytology of the metastasis of the neck shows HPV DNA positive SCC, we regard the lesion as a metastasis from an undetectable tonsillar or base of tongue cancer and the patients are treated with RT/CRT. Patients HPV-negative CUP still are treated with neck dissection followed by RT/CRT.

1.2 BRANCHIAL CLEFT CYSTS

The branchial arches are embryological precursors of the head and neck area. The arches are divided into branchial clefts and branchial pouches. Incomplete obliteration of a branchial cleft during embryogenesis can cause different kinds of cysts, sinuses and fistulas.

Anomalies in the four different clefts give rise to different symptoms, where anomalies of the second branchial cleft are the most common ones. Usually, they appear as a cyst of the lateral neck, but they can also present as fistulas or sinuses First branchial cleft fistulas, sinuses or cysts are rare, account for 1–4 % of the cases, and often misdiagnosed. This can delay proper management and increase surgical risks. Surgery often includes partial parotidectomy with facial nerve dissection. Third and fourth branchial arch anomalies are extremely rare In children second branchial cleft cysts accounts for about 20 % of all cervical masses.[43]

Even though branchial cleft cysts are embryological remnants they can present also in adulthood. Often after and upper airway tract infection. In children and young adults branchial cleft cysts are treated with surgical removal. The diagnosis in children is based on clinical examination and fine needle aspiration cytology often together with radiology.

In adults above 30 the situation is different. Since head and neck squamous cell carcinoma (HNSCC) often presents as a neck mass. However, in human papillomavirus positive (HPV+) oropharyngeal squamous cell carcinoma (OPSCC), the metastasis in the neck is often cystic in its character. Therefore, it is clinically challenging to differentiate between a cystic metastasis originating from a primary HNSCC malignancy, i.e. an HPV+ OPSCC, mimicking a BCC and a benign BCC, especially in patients over 40 years of age. Metastasis from HPV-negative OPSCC is rarely cystic. The incidence of unsuspected squamous cell carcinomas (SCCs) in routinely excised BCCs in adults has been reported to be 3–24% and even more frequent in elderly patients. [44, 45]

Patients above the age of 40 years with BCCs are subjected to an extensive diagnostic workup to exclude a malignancy. At Karolinska University Hospital, these patients undergo computed tomography (CT) followed by panendoscopy with biopsies of the base of tongue and nasopharynx and bilateral tonsillectomy in order to minimize the risk of missing a possible primary tumor before surgery of the BCC.

Image cytometry DNA analysis of fine needle aspirated material is also a method to make diagnostics more precise. Aneuploidy indicates malignant disease and diploidy a benign condition.[46]

There is also a condition described as Branchial Cleft Cyst Carcinomas. In theory this cancer arises from the cyst wall. Although today it´s thought that almost every described cases of this entity actually is a cystic metastasis from a HPV-positive OPSCC.[47] Case reports about cystic metastasis of thyroid cancer mimicking branchial cleft cysts has also been published.[48] But this must be considered extremely rare conditions.

Except for branchial cleft cysts there are many other benign conditions that present as cystic lesions in the neck like colloid nodules, Warthin tumors, thyroglossal duct cyst. They are generally easier to differentiate from SCC in FNAC. However, it is not known if these lesion, or other malignant solid or cystic lesions can contain high risk HPV. [49]

However, since branchial cleft cyst is the most common differential diagnosis to cystic metastasis it is of outmost importance to investigate if also these benign lesions may show HPV positivity.

1.3 HUMAN PAPILLOMAVIRUS

Human Papillomaviruses are small DNA viruses that belongs to the Papillomaviridae family. There are more than 200 of different known subtypes of Human Papillomavirus and some of these infect mucosal surfaces. These mucosal HPV types are roughly divided into low risk and high-risk types, depending on their ability to induce cancer. The today known13 high risk types are 16, 18, 31, 33, 35, 39, 45, 51, 56, 58, 59, and 68. In HNSCC HPV 16 is the absolute dominant subtype, but also HPV 33 and 35 are identified. Low risk HPV types can cause warts or papilloma in other places, for example laryngeal papillomatoses.[50]

The virus genome is built up by a circular double stranded DNA of roughly 8000 base pairs. It consists of an early region (E) that encodes the six early proteins (E1, E2, E4, E5, E6 and E7) , the late (L) region that code for the two structural capsid proteins (L1 and L2) and a non-coding region (NCR). The early proteins interact with the cellular machinery and are involved in viral replication and transcription. For the development of cancer E6 and E7 are the most interesting proteins, which cause cell transformation by interfering with the normal cell cycle by binding to the tumor suppressor genes p53 and pRb respectively.[51]. In high-risk HPV these viral proteins are regarded as oncogenes.

1.3.1 HPV in head and neck cancer

In the early eighties the role of HPV16 in cervical cancer was confirmed. [20] Later on, the virus was found also in head and neck cancer.[52-54] Meanwhile the clinical impression of a rise in the incidence of tonsillar and base of tongue cancer in non-smoking younger individuals was seen and these observations were confirmed. [10, 55] The last decades the incidence of HPV-related TSCC and BOTSCC has increased in many western countries, including Sweden and a parallel increase was seen in the prevalence of HPV in these tumors. Today xx% of the TSCC and BOTSCC are related to HPV.[12, 13, 56-58]

In a study at youth clinics in Stockholm they saw that approx. 7% had high risk HPV I their oral cavity.[59] So it is obvious that not all high risk HPV infections leads to a cancer disease.

As mentioned before, HPV related tumors is linked do better prognosis in both tonsillar- and base of tongue cancer, independent of age, gender and tumor stage. Small studies have also shown promising results for hypopharyngeal SCC, although the prevalence of HPV is low.[37]. For the other HNC subsites, including oropharyngeal cancer outside the tonsillar fossa and base of tongue, the prevalence of HPV is low, and HPV do not seem to have any impact on survival. [18, 19]

1.3.2 HPV - diagnostics

There are different methods to determine HPV-positivity. The most commonly used is detection of HPV DNA or RNA and p16ink4a overexpression.[60]

1.3.2.1 P16ink4a

$p16^{ink4a}$ -overexpression has been proposed by many to occur as a feed-back mechanism of the binding and inhibition of the tumor suppressor protein Rb of E7. However, others have suggested that E7 induced p16 overexpression may be Rb independent. Nevertheless, p16 has been shown by many as a reliable surrogate marker for HPV infection in OPSCC/TSCC/BOTSCC.[61]

In 2010, Ang and colleagues were the first to demonstrate the prognostic role of HPV in OPSCC in a randomized controlled study. In that study HPV was assessed by both in situ hybridization (ISH) and by p16 overexpression by immunohistochemistry (IHC). The authors did not find any differences in survival, on group level, when comparing p16 overexpression by IHC and HPV ISH. Moreover, in that study they also used a cut-off of 70% positivity in cytoplasm and nuclei for p16. Therefore, since this study, a standard of 70% as cut off for p16 positivity has been adopted by many and is now also recommended by College of American Pathologists (CAP) and AJCC. Furthermore, since p16 is easy and cheap to perform, it has been recommended by many.[62] However, it is important to remember that the correlation of HPV and p16 is not absolute, some p16 positive OPSCC are HPV DNA negative[63] and there are HPV DNA positive tumors without p16 overexpression. Furthermore, recent studies have indicated that the correlation HPV DNA and p16 overexpression is relatively low in OPSCC subsites other than the tonsils and base of tongue, i.e. the uvula, the soft palate and the pharyngeal walls [18, 63]

1.3.2.2 HPV DNA and RNA

Presence of HPV DNA can easily be shown by in situ hybridization or by Polymerase Chain Reaction (PCR).

In situ hybridization is a common method used in pathology and the method is very similar to IHC, but instead of a primary antibody a probe is used instead. The advantage of the method when assessing HPV infection is that the HPV DNA can be visualized within the epithelial cells.

Another very commonly used method to detect HPV is the PCR method. Here the extracted DNA from e.g. tumor cells is amplified in cycles consisting of thermal steps with denaturation, annealing of primers and elongation. Primers used can be type-specific (only amplifying a selected HPV type) or general primers (amplifying many different HPV types in the same reaction). Commonly used general primers are the GP5+/6+ primer pairs. The

advantage with this method is that it is sensitive and that it can be used in both histological and cytological samples. However, some authors claim that a disadvantage with PCR is that a tumor can be HPV DNA positive by a secondary infection and thus not related to the etiology/carcinogenesis of the tumor. Therefore, it has been suggested by others that p16 overexpression and presence of HPV DNA should be combined in an algorithm to determine HPV status with a higher sensitivity and specificity.[64]

Nevertheless, many authors claim that presence of E6*I RNA transcripts is the golden standard to define presence of active HPV infection.[64]

To ensure an etiologic role of HPV instead of contamination E6 and E7 mRNA analysis can be performed by either qPCR or RNA ISH. Though this is not commonly used in clinical practice.[65]

A new method under development is detection of circulating tumor HPV-DNA in the blood stream which can be used in post treatment surveillance and maybe also for diagnosis in the future. There are some promising results but only in small studies so far.[66]

1.3.3 HPV- vaccine

There are three different kinds of prophylactic vaccines against HPV available in the market. Nowadays the most used vaccine protects against HPV, 6, 11, 16, 18, 31, 33, 45, 52 and 58. Since 2010 all girls in Sweden are offered vaccination in fifth grade. Since 2020 all fifth graders are offered the vaccine.[67] To see a decrease of OPSCC due to the vaccination program is obviously too early, but effect in viral burden and development of antibodies has already been shown.[68]

Therapeutic vaccines against HPV-induced cancer are a rapidly evolving area. There are several candidates under development. But so far none has made to clinical practice.[69]

1.4 BIOMARKERS OF HEAD AND NECK CANCER

There is a need and search for predictive and prognostic markers in HNC and many markers has been studied and others are still under evaluating. These markers are often involved in different pathways in cancer development which also makes them potential targets for therapy. Antibodies, Cetuximab, for Epidermal Growth Factor Receptor(EGFR) is already widely used for treatment of HNC.[70] In this thesis we have focused on Tumor infiltrating lymphocytes together with HPV in HPSCC.

1.4.1 CD8+ Tumor infiltrating lymphocytes

In later years the immune system has gained focus regarding prognosis and treatment for different types of cancer. For several tumor types, tumor infiltrating lymphocytes, and especially CD8-positive T-cells, have been recognized as a prognostic marker, also in HNSCC. For HPSCC this has not been investigated earlier.[71, 72] In previous studies our group has shown that increase in CD8+ TILs is a good prognostic factor in OPSCC and HNCUP.[73, 74]

1.4.2 Other biomarkers

An important growth factor pathway is the **PI3K/Akt/mTor** pathway which is involved in **EGFR** signaling. It can activate the mechanism of anti-cell apoptosis, glucose metabolism and protein synthesis. The PI3K/Akt /mTor pathway is involved in most HNC. This pathway is a possible target for therapy.[75] Cetuximab is an epidermal growth factor receptor (EGFR) inhibitor which is already extensively used in HNC-treatment, but its role is disputed.[76]

PTEN is an inhibitor for the PI3K/AKt/mTor pathway and loss of PTEN expression can be associated with worse prognosis.[77-79]

Psoriasin, s100A7, is a protein part of the S100 family and is an important cell mediator for e.g. cell survival and maturation. Increased expression of Psoriasin has been correlated with clinical outcome and seen in malignant and premalignant lesions. High psoriasin expression is an poor prognostic factor in patients with HPV+ BOTSCC.[80]

cMET is a tyrosine kinase receptor which is overexpressed in many cancers so also in HNC. Overexpression of cMET is linked to poor survival rates. [81, 82]

2 RESEARCH AIMS

To evaluate if presence of HPV in FNAC can predict HPV-positive TSCC or BOTSCC and if HPV were present in benign neck masses.

To study if HPV are present in FFPEs of branchial cleft cysts.

To study expression of immune-related proteins in HPSCC in relation to normal tissue and tumor HPV-status.

To investigate if CD8+ TILs in FFPEs from HPSCC has a role in relation to HPV-status and clinical outcome.

To evaluate if reduced neck dissection was feasible in patients TSCC or BOTSCC with signs of residual nodal disease in PET-CT.

3 MATERIALS AND METHODS

3.1 STUDY SUBJECTS

Karolinska University Hospital is a tertiary referral center for all ENT and Head and Neck care in Region Stockholm and Region Gotland, an area with approximately 2,4 million inhabitants. For people living in Region Stockholm, approximately 2,35 million people, Karolinska University Hospital is the only provider of other than outpatient ENT-care. In the different papers we have used local registries from the hospital and the Swedish Head and Neck Cancer Register (SweHNCR).

3.1.1 Study subjects, paper I

Patients who were referred to the clinic for pathology and cytology at Karolinska University hospital for fine needle aspiration cytology (FNAC) with suspected HNC and other neck masses were prospectively included. This during a period from 2013 to 2016. In total 66 samples were collected where it was enough leftover material to perform analysis.

3.1.2 Study subjects, paper II

By using the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) 138 patients with branchial cleft cysts diagnosed 2007–2015 at the Karolinska University Hospital were identified through electronic health record Take Care. The code used was sinus, fistula and cyst of branchial cleft, Q18.0. Of these 112 were included in the study. Of the 26 excluded patients, 22 patients did not have surgery and, therefore, lacked material for analysis, and four patients did not have representative cystic material upon evaluation by immunohistochemistry (IHC). Only patients above 18 years and with Q18.0 as final diagnosis were included

3.1.3 Study subjects, paper III

Tumor biopsies and adjacent normal tissue from 43 patients diagnosed with HPSCC between 2002 and 2013 at the Karolinska University Hospital were collected before treatment, ICD-10 codes C12.9 (pyriform sinus), C13.0 (postcricoid region), C13.1 (aryepiglottic fold, hypopharyngeal aspect), C13.2 posterior wall of hypopharynx), C13.8 (overlapping sites of hypopharynx), and C13.9 (hypopharynx, unspecified location),. The samples were fresh

frozen at temperature -70° Celsius. Nine patients with to small tumor material were later excluded. One sample failed in the final analysis failed and were therefore excluded. Of the fresh frozen material finally samples from 33 patients were analyzed.

Pretreatment formalin-fixed paraffin-embedded (FFPE) were collected from 149 patients with HPSCC that received treatment from year 2000 to 2013 at the Karolinska University Hospital. The samples had been included in earlier studies of the research group. Of the patients with fresh frozen biopsies 22 were also included among the FFPE biopsies.

3.1.4 Study subjects, paper IV

All patients diagnosed with oropharyngeal cancer in the Stockholm and Gotland counties between 2017 and 2021 were identified through SweHNCR. We identified those with TSCC and BOTSCC using ICD-10 (TSCC: ICD-10 C09.0, C09.8, and C09.9; BOTSCC C01.9). In total 439 patients, of these 217 patients with post treatment PET-CT or salvage neck dissection were included.

3.2 METHODS

Almost all laboratory work in this thesis have been made at Cancer Centrum Karolinska together with the Tina Daliani´s Group at the institution oncology and pathology. The exception is the Proseek Immuno- oncology assay that was carried out by Olink in Uppsala Sweden.

Collection of raw data on patients were made through the electronic health care registry Take Care (Combu Group Medical, Solna) and processed in Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA)

3.2.1 HPV extraction and detection

In paper I, DNA was extracted from fresh-frozen aspirates from neck masses using the QIAmp DNA micro kit (Qiagen, Hilden, Germany). When the aspirate was HPV DNA-positive, RNA was extracted using the RNeasy Micro kit (Qiagen, Hilden, Germany). For practical reasons the material was frozen only to -20° Celsius.

In paper II we had 15 μm fixed paraffin embedded (FFPE) tissue from branchial cleft cysts and DNA was extracted using the Roche High Pure FFPET DNA Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. The extraction process was similar in paper I for the FFPEs that was analyzed there.

HPV DNA was analyzed in the extracted DNA (10 ng/sample) using a multiplex bead-based assay for 27 HPV types (HPV 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73 and 82) DNA detection was performed using a bead-based multiplex assay on a MagPix instrument[83] All known high risk types and putative high risk types where included, and also an number of low risk types.

I paper III the result of HPV from the FFPE samples were analyzed in previous studies and not performed again. [37, 84]

3.2.2 Immunohistochemistry

3.2.2.1 p16ink4a

In paper II p16-analysis was performed by IHC using the mouse monoclonal antibody CINtec® p16 Histology ((Ventana CINtec® p16 Histology, clone E6H4, Roche AB, Stockholm, Sweden). As positive control an OPSCC with p16 overexpression was used. Assessment was made by two different examiners. A strong diffuse cytoplasmatic/nuclear staining in ≥70% of the epithelial cells of BCC was considered as positive for p16 overexpression, and <70% as negative.[16, 85]

3.2.2.2 CD8+ tumor infiltrating lymphocytes

CD8+ cells were stained with mouse monoclonal antibody anti-CD8 (clone 4B11; Novocastra Laboratories, UK) and number of CD8+ TILs were counted in 10 randomly selected high-power fields (×40)/tumor and for each tumor the mean value of CD8+ TILs was calculated. This was made by two examiners blinded for clinical outcome.[74]

3.2.3 Analysis on the Proseek immuno-oncology panel

Samples were prepared for protein analysis. Different thickness of cuts was made, frozen, and embedded in optimal cutting temperature compound. The first and last slides were used for evaluation of tumor content by an experienced pathologist and only tumors with ≥40% tumor cells were included in the protein evaluation and 28/33 samples included in the analysis had ≥70% tumor cells. All normal samples were checked to be free from tumor tissue. The method has been more thoroughly described in previous articles.

The samples were analyzed for the presence of 92 proteins with the Immuno-Oncology Proseek multiplex immunoassay (Olink Bioscience, Uppsala, Sweden), at the Clinical Biomarkers facility, Science for Life Laboratory, Uppsala University. For each protein concentration were reported as normalized protein concentration (NPX) in a 2-log scale, and limit of detection was defined as three standard deviations above background.[86]

3.2.4 Statistical methods

All descriptive statistics, calculations and analyses was made using IBM SPSS Statistics, version 26.0. (IBM Corporation, Armonk, NY, US).

In paper III we used progression-free survival (PFS), disease-specific survival (DSS), and overall survival (OS). Kaplan-Meier estimator was used for evaluation of PFS, DSS, and OS. Using the log-rank test. differences in survival were calculated. For calculation of hazard ratios, in both univariate and multivariate analysis, Cox proportional hazards regression analysis was used.

3.2.5 Ethical considerations

Study 1 was carried out under ethical permission Dnr 2005/431-31/4 and amendment 2015/0157-32. Informed consent was collected from every patient at the time of the fine needle aspiration. All patients had planned follow up since before. Our results didn´t interfere with this. The aspiration didn´t cause any more pain than it would have done without the research material collected.

Study 2 is carried out under ethical permission Dnr 2005/431-31/4 and 2009/1758-31. The research didn't interfere with treatment of the patient. There is always a problem when you read patient records and it's a violation of the integrity. Of course, only relevant part of the records was read.

Study 3 is carried out under ethical permission Dnr 2009/1278-31/4. Fresh frozen material was collected from patients who had signed an informed consent. The biopsies from tumors were not different related to the study. The biopsies from normal tissue adjacent to the tumor did probably not cause any mor pain than the tumor biopsies. Treatment or follow up were not altered for any of the patients.

Study 4 is carried out under ethical permission Dnr 2009/1278-31/4. Studies were carried out retrospect, on existing biopsy material and patient charts. Keeping the patients anonymous during the process has been of great importance. The research has not interfered with treatment or how the patients are followed.

4 RESULTS AND DISCUSSION

4.1 PAPER I AND PAPER II

Main results

In paper I, 66 FNACs were analyzed for presence of HPV DNA and 17 of these contained HPV DNA. No other subtype of HPV than HPV 16 were found. All 17 patients with HPV-DNA identified in their FNAC, were finally diagnosed with HPV positive TSCC or BOTSCC. Three patients with HPV negative OPSCC were also HPV DNA-negative in FNAC – two TSCC and one otherOPSCC originating in the posterior wall. There were seventeen cancers that were HPV DNA-negative, none of those were OPSCC. We found 29 cases of benign conditions, 18 of these were branchial cleft cysts. None of those 29 were HPV DNA-positive. HPV status of corresponding histopathological specimens and FNACs showed perfect concordance.

In paper II presence of HPV DNA were analyzed in 112 BCC FFPE samples. All samples were HPV DNA- negative. In 105 of the samples the expression for p16 were evaluated and no samples overexpressed p16. Among the BCCs a weak degree of p16 expression was showed, with the majority of samples having 0–20% staining

Discussion

Paper I suggest that HPV DNA in FNACs collected neck masses could predict an HPV-positive TSCC or BOTSCC as the final diagnosis.

In this setting the specificity and sensitivity for predicting an HPV+ TSCC or BOTSCC was 100%. This should of course be interpretated careful since it is a relatively small number of patients tested. A strength of paper I is the unselected cohort. But there is a risk of selection bias with an experienced clinical cytologist. An interesting finding in the paper was that all branchial cleft cysts were HPV DNA -negative. This led to the second paper where we saw that none of the BCCs were HPV-DNA-positive. Since the difficulty to differ branchial cleft cysts from cystic metastases of HNC is well known this is a very interesting finding. Cystic metastases from HNC also more often are HPV-positive.[49, 87, 88] The finding that BCCs are HPV-negative are strengthened in later studies.[89]

Since some years head and neck cancer of unknown primary with HPV-DNA-positive SCC in FNAC are treated with chemoradiotherapy without any prior neck dissection. If HPV were found in BCCS, or other benign conditions, there could have been a risk of overtreating. In

that case a treatment with significant side effects. These two studies further justify the current treatment of HPV DNA-positive CUP. A limitation is that the HPV-DNA analysis the second study was performed on histological sections and not in material from fine needle aspiration

Currently patients above 40 with suspected BCC undergo a relatively intense diagnostic work up including radiology, tonsillectomy and panendoscopy with biopsies The finding I our studies does suggest that this should change. Though, testing for HPV-DNA in FNAC should absolutely be performed in adults with cervical neck masses including suspected branchial cleft cysts.

4.2 PAPER III

Main results

Of the fresh frozen material, the final analysis included 33 samples out of which 4 were HPV-positive. 66/89 of the evaluated proteins had significantly different expression I tumor cells compared to normal samples. 53/89 were significantly higher in the tumors. A few proteins had a significantly lower expression in the tumor samples.

Among the HPV-positive tumors there were 18 proteins that had significant differences compared to HPV-negative tumors. Out of these 16 proteins had higher expression in the HPV-positive cases, many of those related to tumor infiltration of immune cell indicating a strong immune response.

Survival related to protein expression was not evaluated since only six patients in the analyzed group survived more than 3 years. Of these three were HPV-positive and three HPV-negative.

All together 149 FFPE samples of HPSCC were analyzed for tumor infiltrating CD8+ lymphocytes (CD8+ TILs). Seven were from HPV-positive HPSCC. The HPV-positive samples had more CD8+ TILs than HPV-negative. This difference was not significant (0.085), probably because the low number of HPV positive samples.

In the analysis of CD8+ TILs in relation to survival, only the HPV-negative samples were included. This since we know that HPV-positive patients have better clinical outcome, and notably all patients with HPV-positive tumors that received curative treatment survived.[37, 84] We also know that HPV-positive tumors have higher number of CD8+ TILs and were therefore excluded from survival analysis.

In total 128 HPV-negative tumor samples from patients receiving curative treatment were analyzed. They were divided into four quartiles based on the number of CD8+ TILs. The three lowest quartiles were combined and compared to those in the highest quartile. Patients in the highest quartile of CD8+ TILs had a significantly improved PFS, DSS, and OS.

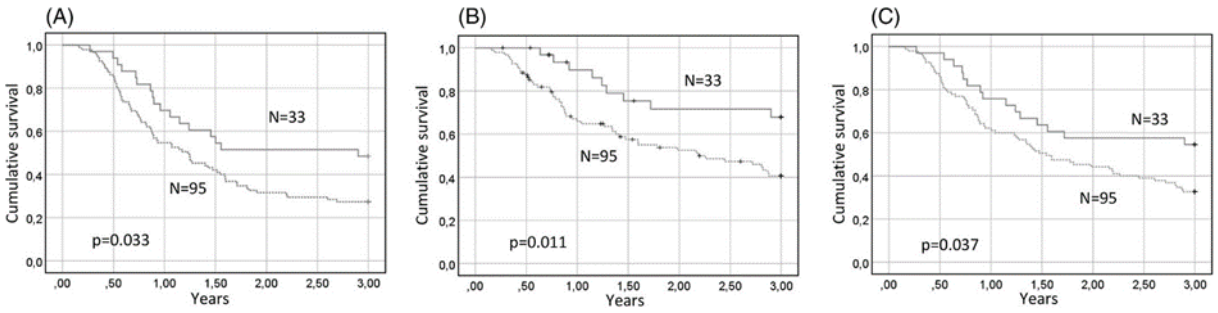

Image 8: *Kaplan-Meier curves presenting patient survival in relation to CD8+ tumor infiltrating lymphocytes, dichotomized between human papillomavirus negative hypopharyngeal cancer with the highest quartile of CD8+ cells (solid line) and the three lowest quartiles combined (dotted line). Notches denotes censored. A, Progression-free survival. B, Disease-specific survival. C, Overall survival*

Discussion

The vast majority of the proteins included in the Immuno-Oncology panel were upregulated in tumor tissue compared to normal tissue in patients with HPSCC. This reflects the high activity of the immune defense in the tumors. The exact significance of each protein in the assay is hard to evaluate.

There was more immune activity in the HPV-positive tumors than in the HPV-negative. Both regarding surface proteins and chemokines.

Higher infiltration by CD8+ TILs in HPV-positive compared to HPV-negative HPSCC shows a more active immune response in the HPV-positive tumors. This is in line with earlier studies showing a more active immune response in HPV-positive OPSCC, the increased number of CD8+ TILs have been suggested as one reason for better survival in patients with HPV-positive OPSCC.[73, 90-92]

The significant better survival for patients with high number of CD8+ TILs in HPV-negative HPSCC are very interesting son it indicates that immune defense plays a major role also for patients with HPV-negative tumors.

Also, for head and neck cancers other than OPSCC and HPSCC the correlation between high numbers of CD8+ TILs and better clinical outcome has been shown. [93-95]

4.3 PAPER IV

Results

Of the 207 patients with TSCC or BOTSCC that were evaluated 202 (93%) were HPV DNA-positive, HPV 16 was the most common subtype followed by HPV 33. A slightly smaller number, 187 (86%), were p16-positive.

39 of the 217 had a post treatment neck dissection, of these 36 were selected for ND due to PET-CT results. In total, 26/36 of patients examined with PET-CT and treated with ND had no sign of viable tumor cells by histopathological examination of cervical lymph nodes. In 8/36 patients, the localization of metastasis in histopathological examination of the neck specimen and the PET-CT was consistent. Two patients had viable tumor cells in other cervical lymph nodes than those seen in PET-CT. Both these tumors were HPV-DNA and p16-positive.

Discussion

At time of diagnosis many OPSCCs already have metastases in the neck. Different strategies have been used for treatment of the neck before or after radio/chemoradiotherapy, (RT/CRT). The impact of RT/CRT in the tissue of the neck makes surgery of the neck surgically complicated and the risk of long-term side effect increases why more reduced surgery would be beneficial for the patient.

Two patients in our material did not have consistent localization of metastasis in PET-CT and histopathological examination so reducing regions for ND should be done with big caution. In one case the metastasis was found in a neck region without known metastasis prior to the treatment, it is clinically extremely rare that HPV-positive tumors develop new neck metastasis during treatment. The metastasis was found on the contralateral side which did not receive therapeutic radiation doses, so maybe it was initially wrongly staged. In the other case the viable tumor cells was found in a metastasis identified in the pretreatment CT but did not fulfill the criteria for neck dissection according to PET-DT. This study implicates that maybe not all neck regions must be included, lumpectomy or alternatively the neck regions containing metastases in the pre-treatment CT.

Compared with surgery for all patients, as was the case in many cancer centers earlier PET-CT has benefits and spare many patients unnecessary surgery.[96] Still, barely one-third of all patients that undergo post treatment ND after PET-CT have viable cancer in histopathologic examination.

A method to improve accuracy could be a repeat PET-CT 16 weeks post treatment if there are signs of metastases in the PET-CT made 12 weeks post treatment. Or maybe you should prolong the first PET-CT to 16 weeks post treatment in HPV-positive OPSCC.[97-99]

5 CONCLUSIONS

HPV DNA in FNAC of neck masses is a strong indicator of TSCC or BOTSCC and was not present in any benign conditions.

HPV-DNA is absent in FFPEs of branchial cleft cysts.

HPSCC have higher expression of immune related protein than normal tissue, and the expression was higher in HPV-positive tumors.

High numbers of CD8+ TILs in HPSCC are related to better clinical outcome.

Reduced neck dissection after post treatment PET-CT may be feasible for patients with TSCC or BOTSCC.

6 FUTURE PERSPECTIVES

Testing for HPV-DNA in FNAC of head and neck masses are such a feasible method that it should be implemented on a lager basis in clinic. FNAC is already a part in the in clinical work up for neck masses and adding HPV DNA-status to this does not require much work.[100, 101] PCR of HPV DNA is a standardized procedure which is not depended on the experience of the cytologist, this makes it suitable for widespread implementation in the Swedish setting. A part of science is to spread your results, this is absolutely needed for our results regarding HPV in FNAC.

It would be interesting to see a prospective study of HPV-status in branchial cleft cysts, where you test HPV both in FNAC and surgical specimens. Such a study can be hard to carry out and an alternative can be a retrospective multicenter study of patients with BCC. In our study the sensitivity and specificity for HPV as a marker for HPV-positive TSCC or BOTSCC was 100%, this should of course be interpreted with caution, but it would be of interest to see the results in a larger study population. And so far, no HPV-positive BCCs are found in the Stockholm material.[89] If larger studies confirm our results patients over 40 years with HPV-negative branchial cleft cyst could be spared the thorough investigation prior surgery performed at many medical centers today.

Hypopharyngeal cancer is a complex disease with several challenges. There have been many efforts to improve therapy for patients with HPSCC, so far not any has been a total success. Our study that shows better survival for patients with higher number of CD8+ TILs can be a piece of the puzzle to improve treatment. If the more immunologically active tumors have better response for radiotherapy, the not so immunologically active tumors should be treated with more intense primary surgery. There are also methods developed where surgery is not that mutilating.[102-104] Immunotherapy and molecular targeted therapy are of course of great interest and there are many ongoing studies for different treatment options.[105]

Maybe the biggest challenges, and those with best effect, regarding HPSCC are early detection and preventive measures. In the hospital setting it is of course easy to focus on optimizing treatment, but at major problem for patients with HPSCC is the advance stage of disease at diagnosis. An accessible somatic health care for people with substance use or psychiatric disorder by strengthening the network of somatic specialists, primary care, psychiatry and addiction medicine could be a successful path forward.

Smoking prevention are maybe the most important part in prevention of HPSCC along with many other diseases. Daily smokers in Sweden today quite few nowadays, but globally the problem is huge with an increasing number of smokers.[106, 107]

Tonsillar and base of tongue cancer in Stockholm today are almost entirely an HPV-driven disease, here we found that 93% of the cases were HPV-DNA-positive and 86% p16-positive. Since 2020 all fifth graders in Sweden are offered HPV-vaccination, so with an increase in

vaccination we could assume that we will see far fewer cases of TSCC and BOTSCC in the future.[108] A decrease in smoking will probably also have a positive effect on the incidence of cancer. The effect of HPV-vaccination lies many years in the future so there is still a need for optimized treatment for these patients.

Post treatment follow up for patients with OPSCC have changed in later years with the introduction of PET-CT 12 weeks after finished radiotherapy. Our aim was the see if there is a possibility to reduce the extent of neck dissection, and there may be such an option. For post treatment surveillance circulating tumor HPV DNA(ctHPV DNA) is a very interesting area. And since PET CT have some obvious limitations in HPV-positive OPSCC this is very promising. Maybe a combination of ctHPV-DNA and PET CT are the future here?[99, 109, 110]

We in the western world focus a lot on advanced methods, like PET CT and biomarkers, for post treatment follow up. In most countries this is not available options. It would be very interesting to compare clinical exam by an experienced head and neck surgeon with PET-CT in finding residual cancer. If the results are comparable, it would be of great interest for many patients worldwide.

In conclusion it is important that research continue but we always need to have a wide perspective when interpretating our results.

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