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OPTIMIZING TREATMENT- TUMOR MARKERS AND SCLEROTHERAPY IN HEAD AND NECK LESIONS

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Optimizing treatment – tumor markers and sclerotherapy in head and neck lesions

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Malin Wendt

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To Vilhelm, Axel and Thea

– to be your mother is the most exciting journey possible

ABSTRACT

Treatment of malignant and benign lesions in the head and neck area is challenging, due to complex anatomy and function. It is thus urgent to find ways to individualize treatment, in order to reduce side-effects, but with preserved efficacy.

Human papilloma virus (HPV) and its surrogate marker p16 are well accepted as a prognostic markers in tonsil and base of tongue cancer, and in the latest TNM oropharyngeal tumors are classified based on p16-status. However, the prevalence and impact of HPV and p16 in head- and neck cancer located outside of tonsils and base of tongue is unclear.

Hypopharyngeal cancer is the subgroup of head and neck tumors with the worst prognosis, and both surgical and oncological treatment have considerable side-effects. Only a few previous studies have focused on HPV in hypopharyngeal cancer, and the results on prevalence and impact on survival are ambiguous.

In oropharyngeal cancer the long-term outcome related to HPV, p16 and subsites is not well studied, but observations have reported late and atypical recurrences in HPV-positive patients. Previous studies have also indicated differences regarding prevalence and outcome related to these markers between the oropharyngeal subsites; tonsil (TSCC), base of tongue (BOTSCC) and otherOPSCC.

Ranula is a benign cystic lesion originating from the sublingual gland in the floor of the mouth. Traditional treatment is surgery, with the risk of side-effects such as nerve damage, and need of general anesthesia. Sclerotherapy OK-432 is a less invasive treatment, used since long in patients with lymphatic malformations, and with promising preliminary results also on patients with ranula.

The research questions this thesis addresses are:
- What is the prevalence of HPV and overexpression of p16, has it changed over time and do these markers have a prognostic value in patients with hypopharyngeal cancer?
- Is there an impact on long-term survival and recurrence in patients with OPSCC related to p16, HPV and subsite?
- Is sclerotherapy with OK 432 an effective and safe way to treat ranula?

In paper 1, 109 patients with hypopharyngeal cancer diagnosed in Stockholm-Gotland region 2000-2007 and treated with intention to cure were analyzed regarding prevalence of HPV and overexpression of p16, and results were correlated to overall and disease-free survival. In paper 2, 82 patients with hypopharyngeal cancer diagnosed in Stockholm-Gotland region 2008-2013 were analyzed in the same manor. Results from both studies regarding HPV and p16 were compared to assess differences in prevalence over time. Survival analysis of 142 patients from both studies, treated with intention to cure and with a follow-up period of three years, was performed. Prevalence of both markers was low, HPV-DNA was found in 3,7% of the patients in paper 1 and in 6,4% of patients in paper 2, overexpression of p16 in 14,6% and 16,5% respectively. The conclusion is that presence of HPV in hypopharyngeal cancer was rare in Stockholm 2000-2013, only 5%, and did not increase over time in this period. Further, p16 is not a suitable surrogate marker for HPV in hypopharyngeal cancer.

In paper 3 529 patients with oropharyngeal squamous cell carcinoma (OPSCC), diagnosed in Stockholm-Gotland 2000-2010, treated with intention to cure and with available data on p16, were included. Clinical data, including localization and time to recurrence, was correlated to HPV and p16. 10-year overall and disease-free survival (OS and DFS) were significantly improved for patients with p16+ TSCC and BOTSCC as compared to p16- cancers ($p<0.05$ and $p<0.05$), while in otherOPSCC p16-status had no impact on either. p16+ OPSCC showed a higher frequency of distant metastasis but did not relapse later than p16- OPSCC at any subsite ($p<0.05$ and p=ns respectively). In TSCC and BOTSCCC, both HPVDNA+/p16+ and HPVDNA-/p16+ correlated to a longer OS in the univariate analysis compared to HPVDNA-/p16- cancer ($p=<0.001$ and 0.0001 resp.), while in the multivariate analysis only HPVDNA+/p16+ correlated to a better OS and DFS. After recurrence, survival was low (5.9%), and did not differ significantly in relation to p16 or HPVDNA status in TSCC and BOTSCC. In conclusion, we find that p16+ TSCC and BOTSCC did not have a higher frequency of late recurrence compared to corresponding p16- tumors. p16+ TSCC and BOTSCC have better long-term outcome than corresponding p16- cancer, but p16 status did not affect OS or DFS in patients with otherOPSCC. The combination of p16 and HPVDNA-status significantly improves prognostic accuracy for TSCC and BOTSCC, compared to p16 alone.

In paper 4 20 patients with a plunging or an intraoral ranula were randomized to two double-blinded injections with OK 432 or saline. Effect on the cystic lesion and evaluation of symptoms and QOL were investigated. 19/20 patients completed the study. 5 patients with intraoral ranulas showed complete response, but only 1/4 of the patients with a plunging ranula. The inflammatory reaction after injection with OK 432 caused a mild to moderate impact on QOL. No serious complications were observed. From these results we conclude that sclerotherapy with OK 432 in ranula is a safe and effective treatment for intraoral ranulas, but possibly less useful in plunging ranulas. The inflammatory reaction after treatment is moderate and well tolerable.

In summary, we find that HPV is rare in hypopharyngeal cancer and that overexpression of p16 is not a suitable surrogate marker for HPV in this group of patients. Further, long-term outcome in TSCC and BOTSCC is affected by p16-status, but not in otherOPSCC, p16+ OPSCC did not relapse later than p16- OPSCC at any subsite, and the combination of HPV/p16 increases prognostic accuracy compared to p16 alone in TSCC/BOTSCC. p16+ status TSCC and BOTSCC did not have a higher frequency of late recurrence compared to corresponding p16- tumors and p16 status did not affect survival after recurrence. Finally, we conclude that sclerotherapy with OK 432 can be recommended as primary treatment in intraoral ranulas.

LIST OF SCIENTIFIC PAPERS

I. **Wendt M**, Romanitan M, Näsman A, Dalianis T, Hammarstedt L, Marklund L, Ramqvist T, Munck-Wikland E. Presence of human papilloma viruses and p16 expression in hypopharyngeal cancer. *Head and Neck 2014;36:107-112*

II. Dalianis T, Grun N, Koch J, Vlastos A, Tertipis N, Nordfors C, Näsman A, **Wendt M**, Romanitan M, Bersani C, Munck-Wikland E and Ramqvist T. Human papillomavirus DNA and p 16 (INK4a) expression in hypopharyngeal cancer and in relation to clinical outcome, in Stockholm, Sweden. *Oral oncology 2015;51:857-761*

III. **Wendt M**, Hammarstedt L, Dalianis T, Landin D, Munck-Wikland E, Näsman A, Marklund L. Long-term survival and recurrence in oropharyngeal squamous cell carcinoma, in relation to HPV and p16-status. *Manuscript*.

IV. **Wendt M**, Papatziamos G, Munck-Wikland E and Marklund L. Sclero-therapy with OK 432 on ranula – a prospective, randomised, double-blinded placebo-controlled study. *ACTA Otolaryngol. 2021; 27:1-6*

LIST OF ABBREVIATIONS

BOTSCC	Base of tongue squamous cell carcinoma
CRT	Chemoradiotherapy
CT	Computerized tomography
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DR	Distant recurrence
DSS	Disease-specific survival
FFPE	Formalin-fixed, paraffin-embedded samples
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IARC	International Agency for Research on Cancer
ICD-10	International Statistical Classification of Diseases and Related
IHC	Immunohistochemistry
IMRT	Intensity modulated radiotherapy
ISH	In situ hybridization
KTA	Karolinska Trial Alliance
LRR	Locoregional recurrence
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
PCR	Polymerase chain reaction
QOL	Quality of life
RT	Radiotherapy
SCC	Squamous cell carcinoma
SweHNCR	Swedish Head and Neck Cancer Registry
TSCC	Tonsillar squamous cell carcinoma

CONTENTS

1 PROLOGUE

The head and neck area is one of the most complex parts of our human body when it comes to both topography and function. In this area an intriguing web of nerves, vessels and muscles supplies important organs to fulfill their purpose; our senses, but also other important functions such as swallowing, speech, sensory impulses of the face etc. All together this means that the functionality of this part of the body has a great impact on aspects of being human; our ability to communicate, to listen to music and see a beautiful sunset, to eat and drink, to be able to enjoy what we eat and drink…. in short crucial aspects for our quality of life.

When treating both malignant and benign lesions in the head and neck area the head- and neck surgeon and oncologists must balance the possible benefit of treatment in relation to the risk of affecting function. An overall goal, both in malignant and benign disease, is therefore finding therapies that benefits or cures the patients, but in an organ preserving way. To achieve this new treatment modalities are evaluated, and we search for predictive factors and/or markers to help us identify each patient's best treatment option.

As a result of this, every doctor facing a patient with a condition in this area must, before recommending a treatment, carefully consider the efficacy and benefit of the suggested treatment, in relation to the complications it might cause.

We are all individuals, with different anatomical predisposition, genetic set-up and personal preferences. The diseases we face have their individual distribution within the head and neck area and unique characteristics on a molecular level. In order to choose the best therapy for each patient we therefore strive at individualizing our treatment protocols based on these differences. We aim at having the possibilities to design treatments in a more tailor-made, and to be able to predict the effect of the measures in relation to the complications that the patient fear. We hope that in the future our patients can be offered treatment that is less harmful but with preserved, or even better, efficacy.

2 INTRODUCTION

This doctoral project aims at gaining knowledge on factors relevant for designing the best clinical management for patients with hypopharyngeal cancer, oropharyngeal cancer and ranula.
In patients with hypopharyngeal cancer we study the prognostic value of human papilloma virus (HPV) and overexpression of $p16^{INK4a}$(p16), to evaluate if these markers could help us predict treatment outcome. (Paper 1-2). For patients with oropharyngeal squamous cell carcinoma (OPSCC), we study long- term survival and pattern of recurrence in relation to HPV, p16 and subsite (Paper 3). In paper 4 we evaluate sclerotherapy with OK-432, as alternative treatment to surgery in patients with ranulas, in a double-blinded randomized placebo-controlled clinical treatment study.
This introduction gives a background on the tumor markers we study, on definitions, diagnosis, epidemiology and treatment of these three conditions, and on OK-432 as a sclerosing agent.

2.1 HEAD AND NECK CANCER AND HPV

2.1.1 General background

Head- and neck cancer (HNSCC) is the sixth most common type of cancer in the western world[1] - half a million new patients are diagnosed each year worldwide[2] and in Sweden about 1600[3].

The complexity in this area of the body is reflected in the heterogenicity of the head and neck cancer panorama; there are over 60 diagnose codes of malignant head- and neck lesions defined in the ICD-10, and there are large variations in clinical presentation and prognosis, even between tumor locations within the same subsite. However, the vast majority, 80-90%, are squamous cell carcinomas[3].

Traditional risk factors for HNSCC are tobacco and alcohol abuse[2] However, already in the 1980´s reports came of a possible connection between HPV and head- and neck cancer[4, 5] and in 1994 our group presented the finding of HPV type 16 DNA in tonsillar cancer[6]. In the decades that followed numerous studies has confirmed the etiological role of high-risk HPV for oropharyngeal cancer[7-11] and 2007 HPV was established as a risk factor of oropharyngeal cancer by IARC [12]. Patients with HPV -positive oropharyngeal tumors were also shown to have a better prognosis[13-17] and a different epidemiological profile with younger age, and a stronger dominance of incidence in males. The incidence trends are also different; whereas the incidence of HPV-negative oropharyngeal tumors in many countries are decreasing (correlated to less use of tobacco), the incidence of HPV-positive tumors show an increase in many countries, including Sweden[18-20]. Figure 1.

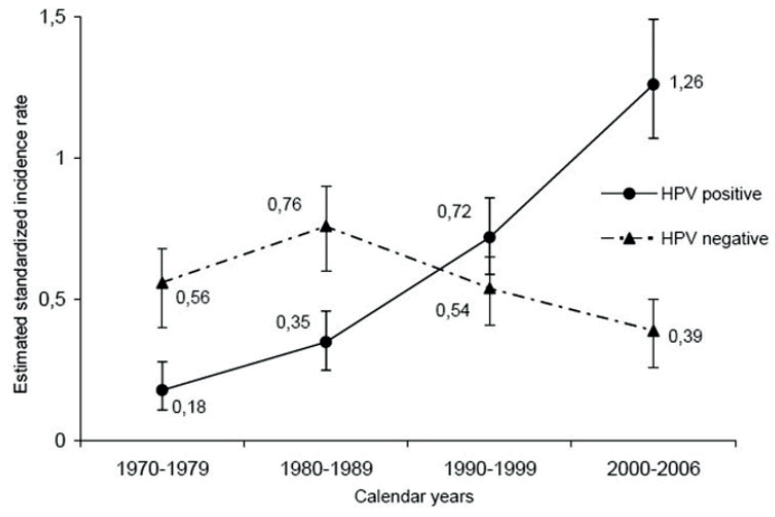


Figure 1: Incidence rate of HPV-positive and HPV-negative tonsillar SCC cases per 100,000 person-years in the County of Stockholm between 1970 and 2006. Näsman A et al, Inj J Cancer Jul 15;125(2):362-6

The number of sex partners throughout life is a strong risk factor of HPV-positive tumors at all locations[21] and in HPV-associated HNSCC connections with oral sexual behavior has been seen[22]. Why the incidence of HPV-related oropharyngeal cancer is higher in men than in women is not entirely clear – it could be related to differences in risk of infection at oral sex, differences in sexual behavior or immunological differences between men and women[10].

Table 1: A comparison of HPV-negative and the HPV-positive head and neck cancers, Rettig, D´Souza et al: Epidemiology of head and neck cancer 2015

HPV-negative compared with HPV-positive cancer:

Parameter	HPV-negative	HPV-positive
Gender	2- 3 fold more common in men than in women	4-5 fold more common in men than in women
Age	Median age late 50´s and 60´s	Median age mid 50´s, with an increasing incidence among younger cohorts
Smoking	>90% have a smoking history; risk increases with increasing tobacco use	50-60 % have a smoking history
Alcohol abuse	Synergistic with tobacco in increasing risk	Not a significant factor
Sexual history	Not a significant risk factor	Number of oral sex partners is a strong risk factor
Site	Larynx and oral cavity most common; other oropharynx, hypopharynx, nasopharynx, nasal cavity and paranasal sinuses	Oropharynx, specifically lymphoid tissue of tonsils and tongue base
Incidence trends	Decreasing	Increasing
Prognosis	All sites; 5-year survival 65%, 5-year recurrence 50% Oropharynx; 5-years survival 20-25 %, 5-year recurrence 50%	Oropharynx; 5-years survival 60-90%, 5-year recurrence 10-15%

In the tumor panorama of HNSCC, with so many different entities both anatomically and on a molecular level, the findings of HPV and its prognostic value has raised the hope that this marker could be the first step towards more individualized treatment protocols. If de-escalated therapy could be introduced without compromising survival, morbidity after treatment could be reduced – and since HPV-positive patients are younger, this would give many years of better quality of life.

2.1.2 HPV and its molecular implications

HPV is a small, non-enveloped, double-stranded virus, with the ability to infect mucosal and cutaneous epithelia, and with a special affinity to the reticulated epithelium in the crypts of the tonsillar tissue[23]. There are more than 150 known types, and they are divided into viruses with high and low risk of causing cancer development. High risk viruses are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 ,58 and 59 [24]. In HNSCC the dominant type of high-risk HPV virus found is HPV 16, which accounts for about 90 % of HPV-positive HNSCC tumors[25], but also 18, 31, 33 and 35 are found[26]. The traditional tobacco- and alcohol-related head- and neck cancer consists of keratinized cells, in contrast to the HPV-related cancer with mostly non-keratinized squamous cancer cells[27, 28]. On a molecular level HNSCC: s caused by HPV exhibits a very different profile than HPV-unrelated. Of the HPV-negative tumors 75-85% of cases have alterations of the tumor suppressor gene p 53 [29], and p16, a protein that inhibits pRb-phosphorylation and blocks cell cycle progression, can be lost in up to 60 %[30].In the SCC: s were the oncogenesis is driven by HPV the virus-genome is integrated in the host cell genome and a number of early (E) and late (L) proteins are transcribed. The main factors in the development of HPV-induced squamous cell carcinomas are the oncogenic proteins E6 and E7. E6-protein inhibits and increases degradation of tumor suppressor p 53 and its function as a controller of DNA-damage and inducer of apoptosis, leading to a functional decrease of p 53 even though the protein itself remains wild-type. E6 also activates telomerase which increases the life span of the cell[31]. In addition, E7-protein inactivates phosphorylated retinoblastoma protein (pRb), an important controller of the host cell cycle[32, 33], and other pathways involving factor E2F. The sum of these processes, and possibly epigenetic changes, leads to overexpression of p 16 in HPV-positive tumors[34-36], see figure 2.

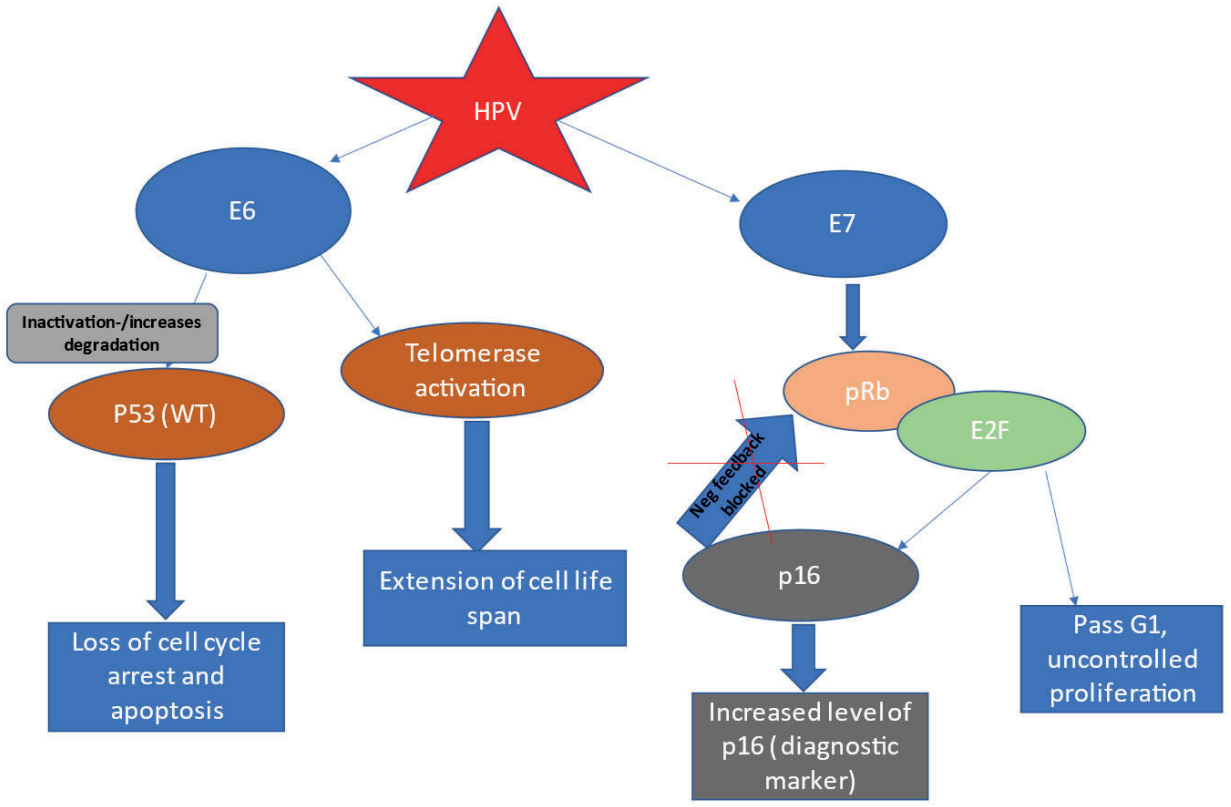


Figure 2: An overview of the oncogenic mechanisms of HPV in oropharyngeal cancer

In conclusion, in important differences between HPV-positive and negative cancers are that in HPV+ p53 is most often inactivated but not mutated, and p 16 is not lost but instead overexpressed.

2.1.3 HPV and p16

p16 is a protein normally involved in the regulation of the cell cycle, by inhibiting the progression from G1 phase to S phase – in its normal function it thus slows the cell cycle down and serves as a tumor suppressor[37, 38]. p16 has been accepted to correlate very well to the presence of HPV-DNA in tonsillar and base of tongue cancers – sensitivity at 80-100% and specificity on 70-90% has been reported[39-41], and p16 also correlate to a better prognosis[40, 42-45]. Since immunohistochemistry is less expensive and easier available than detection of HPV-DNA (see point 2.1.4), overexpression of p16 is widely used as a surrogate marker of oropharyngeal carcinomas caused by HPV.

In the latest 8th TNM-classification, HPV mediated and HPV unrelated oropharyngeal squamous cell carcinomas are separated based on p16-status (see p 2.3.3) [46]. However, recent studies from our group and others, indicate that the high correlation between HPV and overexpression of p16 may not hold true in other locations of the head and neck, for example hypopharynx (paper 1 and 2 in this thesis) and even in other subsites of the oropharynx than tonsils and base of tongue; the soft palate, the uvula and the pharyngeal wall[47, 48]. These findings support the theory that there are substantial differences in prevalence of HPV and its relation to p16 between cancers originating from the lymphoid and non-lymphoid-tissues[49, 50], an issue that is further discussed in paper 3 of this thesis. In addition, there have been indications that patients with discordant HPV/p16-status have an intermediate outcome compared to +/+ or -/-, indicating that HPV and p16 could have their independent prognostic values and that the combination of these markers improve prognostic accuracy[51, 52].

2.1.4 Detection of HPV and P16

2.1.4.1 Direct HPV-testing

Different molecular techniques are available to detect HPV in head and neck cancers. They differ in specificity and sensitivity, and in other characteristics influencing on how they can be utilized in clinical and academical processes.

Southern blot is a method where DNA is extracted and processed into small fragments that are separated by electrophoresis in a gel, then labeled hybrids are used to detect presence of target DNA. This method has the advantage of being able to assess whether DNA is integrated in genome or not, but it cannot be used on formalin-fixed, paraffin-embedded samples (FFPE) and it is less sensitive than for ex PCR and this limits its use of this method substantially.

Polymerase chain reaction (PCR) is a method that has had a major impact of many different types of analyses and is now widely used both in clinical and research work. This process is in short a way of by preset primers amplifying certain signal sequences of DNA or RNA by transcribing an immense number of copies, if the target sequence is present. These copies can then be detected with different techniques (hybridization, fluorescent procedures, enzyme immunoassays etc). For the detection of HPV in head and neck cancers, the advantages of this method is that it can be used on both fresh tissue biopsies and FFPE:s, is it cost-effective and it is highly variable. Disadvantages are that this technique do not distinguish integrated HPV-DNA from DNA present outside of genome, and it cannot differ HPV in tumor cells from HPV in non-cancer cells[53]. The efficacy of the technique is also depending on that the sequence analyzed is not compromised during viral genome integration. A common target, also used by our research group, is the primer set GP5+/GP6+, which is at a proper length (140 basepairs) also for FFPE-samples where DNA can be fragmentary after processing. (Procedure further described in p 4.1.2.)

As mentioned before, PCR can fall out positive based on only a few copies of HPV-DNA in the cell, and cannot assess the quantity/viral load of HPV-DNA, meaning that false positive samples are possible. To solve this **real-time PCR** has been tested, a method where you by special markers keep information on how much target DNA was found in the sample.

In the light of clinical interest, however, the key issue is how strong the impact of HPV is on the host genome; in short determined by the levels of viral transcripts[54]. To assess the level of HPV-genome transcription real-time PCR aiming at **HPV mRNA** for oncogenic proteins **E6 and E7** is used, and is accepted as the golden standard for assessment of HPV-infection of clinical relevance regarding malignant transformation [33, 34, 53, 55]. This method is outstanding regarding sensitivity and clinical relevance; however, it is time consuming and too complicated to be used in routine clinical protocols.

In situ hybridization (ISH) is a method to detect HPV-DNA in place, in the nuclei of the infected cell. It will detect almost exclusively clinically relevant HPV-infections[53]. It has a very high specificity, but a lower sensitivity, by some regarded as a strength in comparison with PCR since false positive tests then are avoided. ISH for mRNA E6 and E7 is under development.

There are also methods used to detect HPV in oral brush-tests, imitating the pap-smears of cervical cancer. One is the **hybrid capture 2 (HCII) HPV-DNA** test, but this is very seldom used for HNSCC

2.1.4.2 Proxy tests correlated to HPV

Immunohistochemistry (IHC) of p 16 is a widely used method for proxy assessment of transcriptionally active HPV in tonsil and base of tongue cancer. As described above, when a cell is infected with a high-risk HPV and HPV-DNA is integrated in the genome the viral proteins E6 and E7 are transcribed, see p 2.3.1. E7 will inhibit the Retinoblastoma protein (pRb), which leads to an increase of p16 (see figure 2). IHC of p16 is a well-accepted method on FFPE-samples, which makes it easy to use in the clinical setting. Earlier there were discrepancies on how IHC on p16 was evaulated, which led to difficulties comparing results[41]. An accepted algorithm set by American college of Pathologists gives that samples with more than 70% staining are considered positive[56] – this assessment is often straightforward since most samples are either strongly stained or not stained at all. The reliability of p16 as proxy of HPV-related OPSCC is debated, (see p 2.3.5), and the combined analysis of HPV DNA and overexpression of p 16 is by many argued as the preferred method [34]. This combination has been found to increase specificity significantly[57].

Biomarkers such as oral rinse samples and E6/E7-antibodies have been suggested for screening of HPV-infection and related risk of cancer. Oral rinse samples have been found to have a poor sensitivity, but HPV16 E6 antibodies up to 88%[58]. In a retrospective pilot study pretreatment E6 antibodies were found to be associated with risk of recurrence[59]but this has not yet been confirmed in prospective studies.

2.2 HYPOPHARYNGEAL CANCER

2.2.1 Definition and classification

The hypopharynx is the area caudal of the pharynx, above the esophagus, behind and lateral to the larynx. The World Health Organization defines four sub-sites in the hypopharynx; the piriform sinus, the hypopharyngeal part of the ary-epiglottical fold, the post-cricoid area and the posterior pharyngeal wall of the hypopharynx[60] (se image 1 and 2).

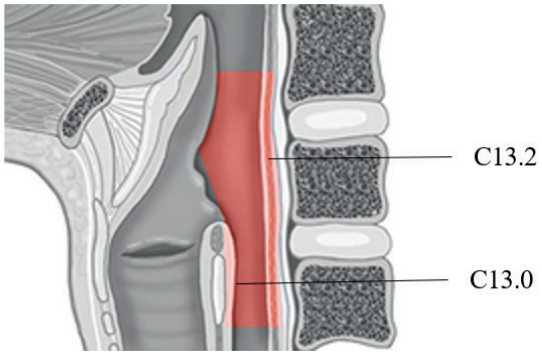


Image 1: Hypopharynx from a lateral view

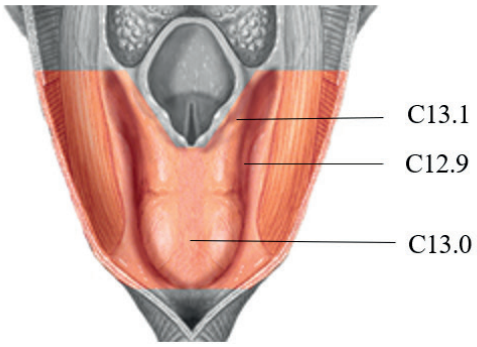


Image 2: from a posterior view

Image by: K. Toverud with permission

ICD-10	
Piriform sinus	C12.9
Post-cricoid area	C13.0
Hypopharyngeal part of ary-epiglottical fold	C13.1
Posterior hypopharyngeal wall	C13.2
Non-specific location, overgrowth	C13.8, C13.9

Hypopharyngeal cancer is a small subgroup of HNSCC:s that represents 3-7 % of the whole group[61]. In Sweden approximately 60 patients/year are diagnosed with hypopharyngeal cancer, there is a clear male/female positive ratio with an incidence of 0,66/100 000 for males and 0,20 for women (SweHNSCCR).

Classification of hypopharyngeal cancer:

(T= tumor, N= regional metastasis in lymph nodes of the neck, M= distant metastasis)

T-categories Hypofaryngeal cancer, AJCC/UJCC 8th edition (2017) *	
T1:	Tumor limited to one subsite of hypopharynx and ≤2 cm in greatest dimension
T2:	Tumor invades more than one subsite or adjacent site or measures > 2 cm but ≤ 4 cm without fixation of hemilarynx.
T 3:	Tumors > 4 cm with fixation of hemilarynx or extension into esofagus
T 4a:	Tumor invades thyroid/cricoid cartilage, hyoid bone, thyroid gland, esophagus or central compartment of soft tissue.
T4 b:	Tumor invades prevertebral fascia, encases the carotid artery or involves mediastinal structures

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

Clinical staging (c TNM 8):				
	N0	N1	N2	N3
T1	I	III	IVA	IVB
T2	II	III	IVA	IVB
T3	III	III	IVA	IVB
T4	IVA	IVA	IVA	IVB
T4b	IVB	IVB	IVB	IVB

(M1 always results in stage IVC)

2.2.2 Epidemiology and prognosis

Malignancies in the hypopharynx are predominantly squamous cell carcinomas (SCC)[62]. The traditional risk factors for HNSCC in general are tobacco and alcohol abuse[2, 63], and in hypopharyngeal cancer this is has not changed over time. Hypopharyngeal cancer is the subgroup of HNSCC: s with the worst prognosis – the 5-year overall survival rate is at 30-35%[61, 64, 65] The disease is often diagnosed at a late stage – up to 75 % are in stage 3 or 4 [64, 66, 67], due to several factors. The anatomical location is "silent", so that lesions give symptoms later compared to other locations[68], and that the hypopharynx has a lymphatic drainage promoting early neck node metastasis [69] - at the time of diagnosis 60-80% of patients have ipsilateral metastases and up to 40 % show contralateral metastasis, leading to advanced tumor stage at diagnosis[63].

The poor overall-prognosis of hypopharyngeal cancer is not only due to diagnosis at a late stage, but also related to the epidemiologic profile of the patients diagnosed with this disease. Patients are often male, with a history of tobacco and alcohol abuse, and often have co-morbidities. This results in a poor performance status which can limit treatment intensity and options, and entails a predisposition to develop secondary malignancies. Recurrence in hypopharyngeal cancer occurs both locoregionally and distant and prognosis after recurrence is, as for primary tumor, not good [70-72].

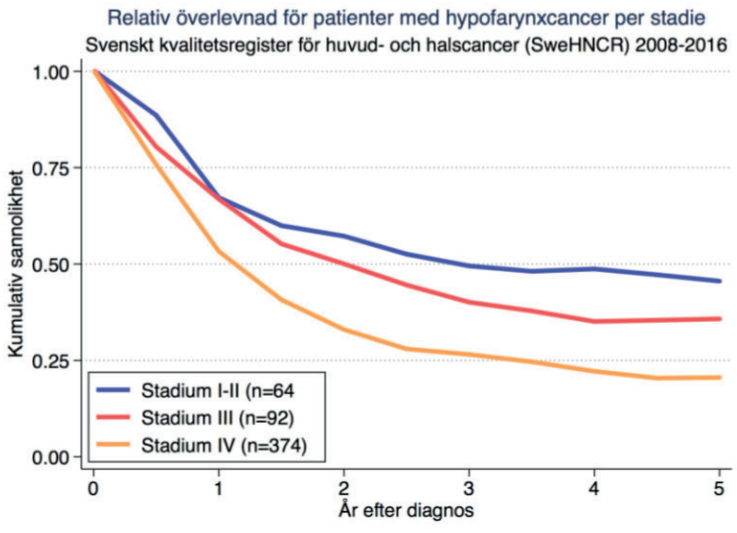


Figure 2: Relative survival of patients with hypopharyngeal cancer, per stage 2008-2016 (SweHNCR 2019)

In hypopharyngeal cancer many different molecular markers have been suggested to have a possible prognostic and predictive value, also HPV and p16. However, hypopharyngeal cancer is rare, study-groups are small and no pioneering results have yet been found, so further work is of essence.

2.2.3 Treatment

Depending on clinical stage and co-morbid conditions the available treatment options are, radiotherapy and/or chemotherapy and surgery. Targeted therapy i.e., EGFR-receptor blockers are also used, and in recent years immunotherapy such as PD-L1 -inhibitors have been added in select cases, often as second-line therapy.

Over time the treatment algorithm of advanced hypopharyngeal cancer has changed. Before the 1990´s standard treatment was often primary surgery with total pharyngo/laryngectomy[73, 74], but in the last decades a shift has taken place towards laryngeal preservation therapies[75, 76]. The shift towards a non-surgical regimen of hypopharyngeal cancers was inspired by the ”game-shifting” randomized trial by Department of Veterans Affairs Laryngeal Cancer Study Group published in 1991[77]. In this study chemoradiotherapy was compared to laryngectomy and post-operative radiation in patients with advanced *laryngeal* carcinoma, and results showed that the outcome was very similar. Chemoradiotherapy has over time become standard first-line treatment in advanced head and neck cancer for many subsites, also hypopharynx[78, 79]. However, in the hypopharynx there are challenges with organ preserving therapy – the submucosal growth implicates difficulties in assessing treatment response, and to perform extensive salvage surgery in patients with failing response is difficult and outcome is poor[70, 80, 81]. In a study from 2019 Tassler et al retrospectively assessed 137 patients treated with primary surgery (in this study total laryngectomy or pharyngectomy) plus adjuvant RT, or organ preserving non-surgical therapy, and found a significantly higher overall survival in the primary surgery-group (p=0.02)[82].

In Sweden most patients with hypopharyngeal cancer are primarily treated with radiotherapy, or chemoradiotherapy in advanced stage patients fit for heavier treatment. In the case of residual disease or recurrence, pharyngo- and/or laryngectomy is performed as salvage if possible. Small tumors can be submitted to primary surgery.

However, new surgical techniques with endoscopic laser surgery, ultrasound knives and, in later years, robotic surgery have opened new windows for surgical treatment of hypopharyngeal cancer, also with partly preserved larynx, and with reports on improved survival compared to RT/RCT. Already in 2001 Steiner reported of a series of 129 patients with carcinoma in sinus piriformis treated with transoral laser microsurgery, with a 5-year survival of 71% in stage 1 and 2, and 47% in stage 3 and 4 subgroup[83]. In a large review from 2011 Ambrosch et al states that in selected T1, T2 and T3 hypopharyngeal cancers transoral microsurgery gave less morbidity and significantly higher survival than both open surgery and RT[84].

Since hypopharyngeal cancer is a relatively rare disease, there are few studies that gives level 1 evidence regarding treatment. Many studies on non-surgical laryngeal preservation treatments contain a heterogenous population of both laryngeal and hypopharyngeal carcinomas[62]. In a review from 2018 by A Habib, two randomized and 11 observational studies were evaluated[85]. A meta-analysis of the two randomized trials showed a small difference with better overall survival after surgical treatment, the observational studies showed no survival advantage with either treatment. In 2002 Godballe et al analyzed 110 patients diagnosed from 1965-1998, and the only factor that significantly had impact was the increased radiation dose that what introduced 1985- following this alteration patients had a better disease specific survival (DSS) than earlier years[70].

Immunotherapy is a rapidly emerging field in treatment for head and neck cancer in general, and also in hypopharyngeal cancer. Suggested targets for immunotherapy are the death ligand pathway (PD-1, PD-L1)[86], also in combination with tumor infiltrating lymphocytes such as CD8+ [87]. There are also molecular targets such as p53 and ERCC1 – expression of interest when studying treatment response [88].

2.2.4 Previous data on HPV and hypopharyngeal cancer

In hypopharyngeal cancer, the prevalence and prognostic impact of high-risk HPV remains uncertain.

Only a few previous studies have focused on HPV and hypopharyngeal cancer, and the results on prevalence and impact on survival are ambiguous.

Regarding prevalence of HPV in hypopharyngeal cancer the results are diverging, but mostly reported as relatively low, between 10 – 17%[89, 90]. Ernoux-Neufcouer reported in 2011 a very high rate, 82%, but very few of these where p16-positive[91]. A few studies have indicated that HPV may be a favorable prognostic factor[90], and it has been suggested that HPV-positivity could serve as a selection factor between organ preserving chemoradiotherapy and laryngectomy[89]. There have also been observations that HPV-positive tumors often are located in the pyriform sinus and had a characteristic gross feature with exophytic and granulomatous, whart-like growth, whereas the HPV-negative tumors tended to be ulcerative[90, 92].

On the other hand, there are studies where no prognostic impact of HPV in hypopharyngeal SCC was seen[93] and even indications of higher risk for recurrence[91] . Regarding overexpression of p16 the results also diverge regarding impact on outcome[91, 94].

Since most studies on hypopharyngeal cancer includes a relatively small number of patients and the prevalence of HPV-positivity is relatively low, more studies are needed to answer the question on the predictive value of HPV in hypopharyngeal cancer, possibly to establish whether HPV-status could help selecting patients to different primary therapy regimens.

2.3 OROPHARYNGEAL CANCER

2.3.1 Definition

The oropharynx includes the base of the tongue and the tonsils, and also the uvula, posterior pharyngeal wall and the soft palate. These subsites are defined by the ICD 10 /World Health Organization as: tonsillar: C 09.1,.8,.9, base of tongue: C01.9 and formerly C02.4, and for other oropharynx C 10.0-C10.9 and C05.1- C 05.9.

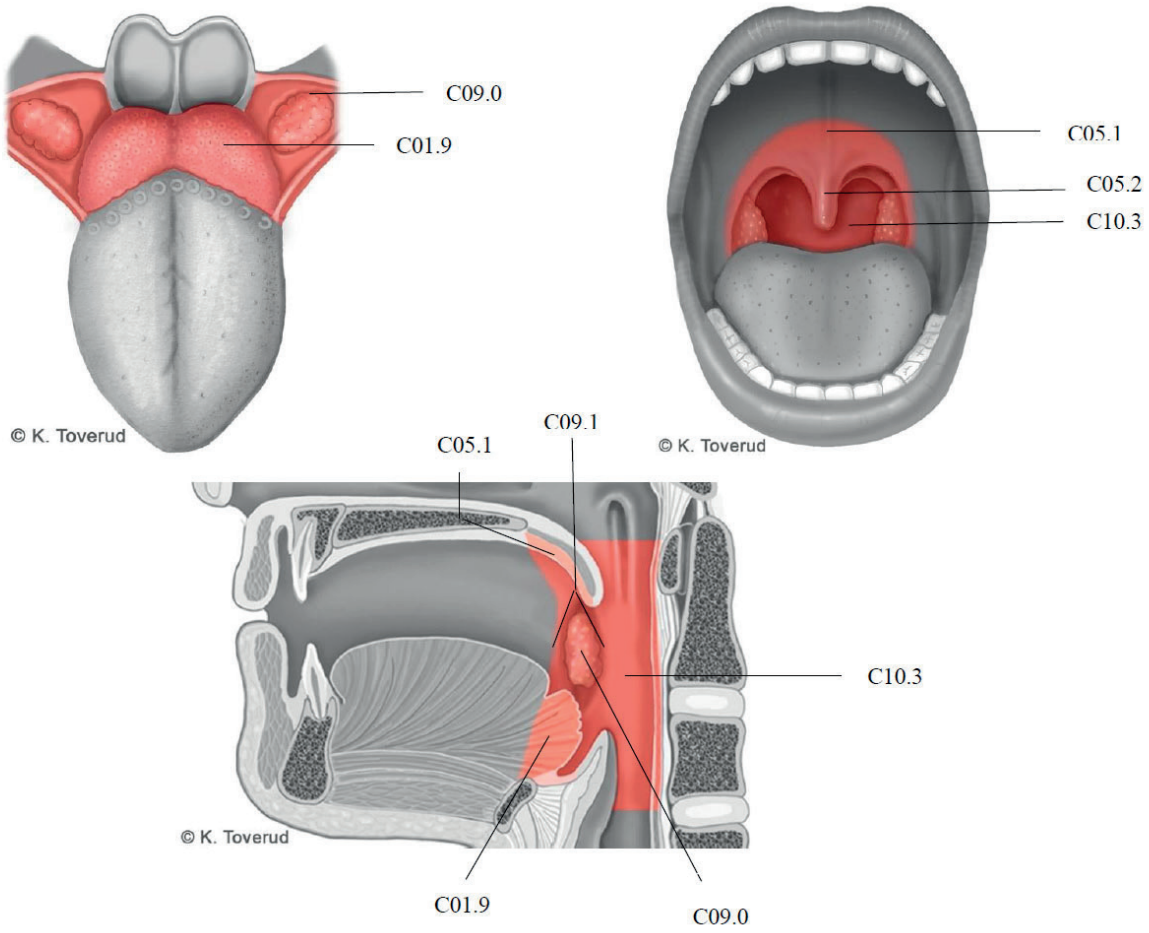


Image 3, 4 and 5: Subsites of the oropharynx. Image by: K. Toverud with permission

ICD-10	
Tonsil	C 09.0, C 09.8, C 09.9
Base of tongue	C 01.9
Arch of the soft palate	C 09.1
Soft palate surface to oral cavity	C 05.1, C 05.8, C05.9
Uvula	C 05.2
Posterior pharyngeal wall, lingual surface of epiglottis	C 10.0, C10.2, C10.3, C10.8, C10.0

90% of oropharyngeal cancers are squamous cell carcinomas, but also salivary gland tumours and hematolymphoid tumors occurs. The tonsils and base of tongue are a part of the mucosa-associated lymphoid tissue and forms, together with the adenoid, the Waldeyer´s ring. The tonsillar crypts are lined with a specialized reticular epithelium, important for the immune system[95], while the other sites of the oropharynx origins from classic mucous membrane. Tonsillar and base of tongue cancer dominates oropharyngeal cancer with roughly 90% of all cases.

2.3.2 Epidemiology

As mentioned already, the epidemiology of oropharyngeal cancer is dominated by HPV as a "new" risk-factor [2, 14, 96, 97], a fact that has been correlated to an increase in high-risk HPV-related tumors ; see point 2.1.1.Simultaneously, the prevalence of smoking has decreased, resulting in a shift towards a larger proportion HPV-positive tumors in the oropharyngeal subsites[11, 98]. Data from our group indicated a plateau in the increase of tonsillar cancers positive for HPV in Stockholm 2000-2008, however, the increase has recurred after 2009 and currently around 70% of TSCC and BOTSCC in Stockholm are HPV+[99, 100]

In OPSCC there is a male/female dominance of 3:1. The far most common localizations are tonsil and base of tongue, which accounts for 90% of all OPSCC.

The prevalence of HPV has been found to be significantly higher in the lymphoepithelial sites (tonsils and base of tongue) than in the non-lymphoepithelial sites (otherOPSCC)[47, 101, 102], and as shown in image 5, the increasing incidence of OPSCC in Sweden depends fully on the increase of tonsillar and base of tongue cancer, while the other oropharyngeal sites have remained at a low level.

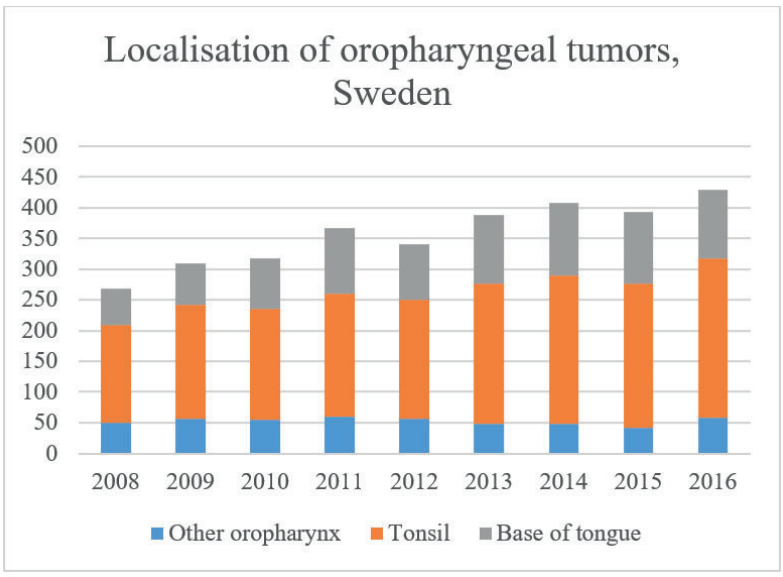


Figure 3: Incidence of OPSCC, subsites, in Sweden 2008-2016, data from SweHNCR

2.3.3 Classification

Since HPV is a well-accepted risk factor of oropharyngeal cancer, and affects prognosis, the WHO recommends to separate OPSCC into HPV+ and HPV- tumors. The TNM-classification for oropharyngeal cancer was altered in the latest edition, the 8th, now separating HPV+ (p16+) and HPV negative (p16-) tumors[46]. The new classification is based on p16-status as a surrogate marker for presence of HPV, and has foremost an impact on nodal status:

TNM 8th edition*:	T-category p16-negative oropharyngeal cancer	T-category p16-positive oropharyngeal cancer
T1	Tumor≤ 2cm	Tumor≤ 2cm
T2	Tumor > 2 cm ≤ 4 cm	Tumor > 2 cm ≤ 4 cm
T3	Tumor > 4 cm, or spread to lingual surface of epiglottis	Tumor > 2 cm ≤ 4 cm
T4a	Tumor invading: larynx, deep muscles of tongue, medial pterygoid, hard palate or mandible	T4: Tumor invading: larynx, deep muscles of tongue, medial pterygoid, hard palate or mandible, m. pterydoideus lat., pterygoid plate, lateral aspect of nasopharynx, skull base, around carotid artery
T4b	Tumor invading; m. pterydoideus lat., pterygoid plate, lateral aspect of nasopharynx, skull base, around carotid artery	

TNM 8th edition*:	Clinical N-category p16-negative oropharyngeal cancer		Clinical N-category p16-positive oropharyngeal cancer
N1	One ipsilateral node ≤ 3cm with no extranodal extension (ENE)	N1	Unilateral node/nodes ≤ 6 cm
N2a	One ipsilateral node > 3cm ≤ 6 cm and no ENE	N2	Contralateral or bilateral nodes ≤ 6 cm
N2b	Multiple ipsilateral nodes ≤ 6 cm and no ENE		
N2c	Contralateral or bilateral nodes,≤ 6 cm and no ENE		
N3a	Node > 6 cm no ENE	N3	Node or nodes > 6 cm
N3b	Extranodal extension, clinical or radiological		

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

In addition there are pathological N categories, based on histopathological examination of neck dissection specimens, where pN1 is metastasis in 1 to 4 lymph nodes and pN2 in metstasis in 5 or more lymph nodes.

The split of p16-positive and negative oropharyngeal tumors has yet no implications on treatment in Sweden, but a clear aim to separate HPV+/- patients is to de-escalate oncological treatment for the HPV-positive patients, in order to reduce side-effects but without impairing their good prognosis.

2.3.4 Treatment

In Sweden the baseline treatment is radiotherapy (RT) by external beam up to a curative dose, given locally and regionally. There have been fast advances in the field of external radiation, including the development of intensity-modulated RT technique (IMRT), a method with high precision and less effect on adjacent normal tissue[103]. For base of tongue cancer, locally administered brachy boosts are used is selected cases.

Concomitant chemotheraphy (CT) with platinum-based treatment, mainly cisplatin is used in more advanced stages, and is internationally widely recommended. Cisplatin has been shown to improve survival in patients with advanced stage [104, 105]. There have been several de-escalation studies investigating whether cetuximab (Erbitux) could replace cisplatin in order to reduce toxicity in HPV+ patients, but data has shown that RT+cisplatin provides a better overall and disease-free survival than RT+ cetuximab[106, 107]. However, for patients not suitable for platinum (renal failure, impaired hearing) cetuximab can be considered.

Surgery is sometimes used as primary modality for small tumors (T1) of the uvula and soft palate. For tonsillar and base of tongue cancers surgery of primary tumor with adequate margins is can be technically challenging and associated with morbidity, but the development of transoral robotic surgery and transoral microsurgery is expected to widen the opportunities. Wilkie et al reported equal survival but less morbidity regarding swallowing with transoral laser microsurgery +postoperative RT compared to CRT[108], but Huang et al compared transoral surgery with IMRT ablation in HPV+ patients and found similar outcome both on survival and morbidity[103]. Oropharyngeal cancers are in Sweden still most often treated with (chemo)radiotherapy but it is possible that a shift towards more surgery is ahead.

Historically patients with oropharyngeal cancer with advanced regional spread were planned for neck dissection after completed radiotherapy, if the primary tumor was considered in complete response. However, studies have showed that in patients with a complete response locally after RT very few have viable cancer cells in the neck dissection specimen[109], and that surveillance with imaging such as PET-CT can provide a safe way so assess the need for surgery, saving costs and morbidity [110, 111]. Based on this now only patients with a clinical or radiological suspicion of remaining tumor in the neck will undergo neck dissection.

Treatment response is evaluated clinically and radiologically, with PET-CT after 3 months. After treatment patients are clinically controlled every third month for 2 years, and then every six months for another 3 years, in total 5 years.

The knowledge of the prognostic impact of HPV-positivity in oropharyngeal tumors has resulted in an intense search for ways to de-escalate treatment without affecting the good prognosis in these patients[112]. Many randomized trials are ongoing, but to find de-escalation protcols that does not compromise survival has shown to be difficult and no results are yet translated into clinical practice. Consequently, the treatment protocols in Sweden are yet the same regardless of HPV(p16) +/-.

2.3.5 Prognosis

Globally there are geographical differences in survival in patients with oropharyngeal cancer, correlating with prevalence of HPV and level of health care infrastructure. In Sweden the relative 5-year overall survival for the whole group of OPSCC is 70 % and for patients treated with curative intent 75%[3].

As mentioned before, survival in patients with tonsillar or base of tongue cancer varies substantially in relation to HPV-status; patients with HPV-positive cancers have a better both overall and disease-specific survival than HPV-negative tumours[17, 33, 113], a prognostic advantage that is shown to be independent of age, gender and tumour stage, see figure 4.

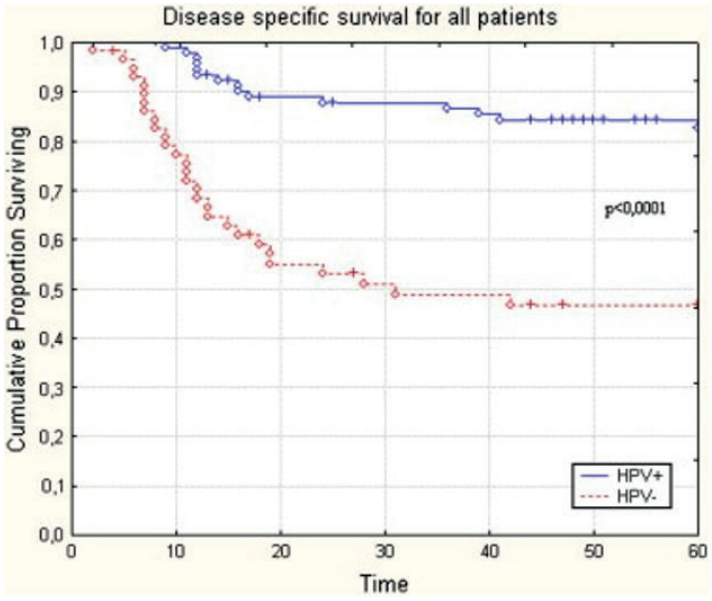


Figure 4: DSS for patients with HPV positive vs negative tonsillar cancer in Stockholm 1970-2002. Lindquist et al, Mol Oncol. 2007;1(3):350-5

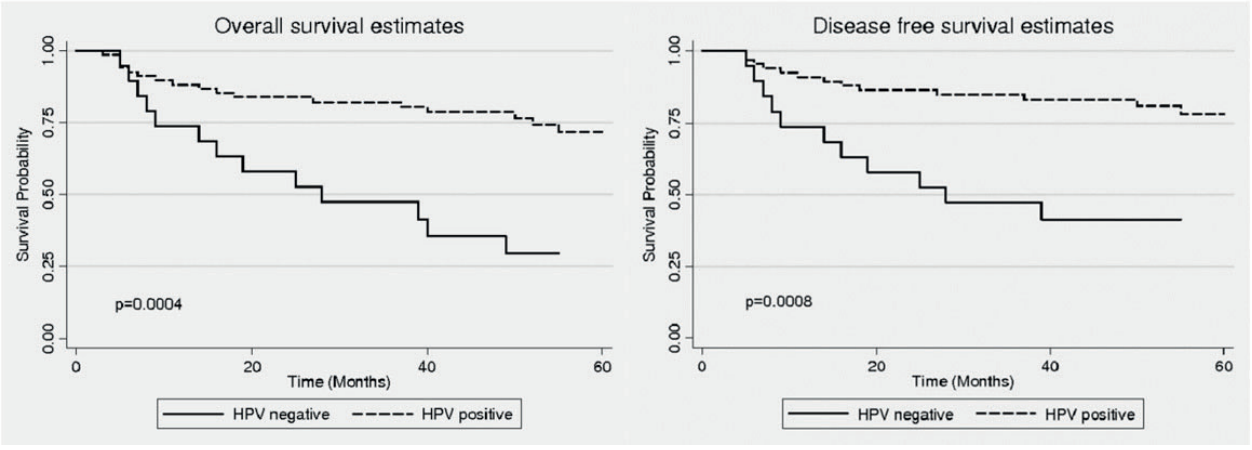


Figure 5: Overall and disease-free survival in patients with base of tongue SCC in Stockholm, Sweden, diagnosed 1998-2007 (Attner et al, Int J of Cancer 2010, vol128;12.p.2892-2897)

There is however increasing evidence of differences in survival in relation to HPV and p16 between the large group of tonsillar and base of tongue cancer (TSCC and BOTSCC), and the other oropharyngeal cancers (otherOPSCC) located in the soft palate, uvula and pharyngeal wall. Our research group have found that the sensitivity of p16 as a surrogate marker in otherOPSCC is low[47], and that p16+ otherOPSCC have a significantly worse survival compared to p16+ TSCC/BOTSCC[48]. That the cancers arising from the non-lymphoepithelial tissue of the oropharynx have different characteristics and relation to HPV than those from the lymphoepithelial tonsils and base of tongue, and in fact behave more like oral cancers, are consistent with the findings of both Gelwan et al[114] and Tham et al[49] .

Most recurrences in oropharyngeal cancer occur within 2 years after end of treatment, and far most common is loco-regional recurrence. In a Danish population-based study from 2018 23 % of patients treated for oropharyngeal cancer had recurrence, less common for HPV -positive (15%) than negative (35%) patients[115] . There has been reports on HPV-positive patients presenting with atypical patterns of recurrence; late failures and aggressive disease in unusual locations has been described[116, 117], but also results indicating that HPV-positive patients have a better outcome after recurrence[118, 119] However, further studies are needed to investigate the long-term pattern of the relatively new panorama of HPV-influenced oropharyngeal cancer.

In conclusion the "epidemic" of viral-induced HPV-positive tonsillar and base of tongue carcinomas[18, 19, 33] is widely accepted, but in other subgroups of HNSCC: s, including the hypopharynx and otherOPSCC:s, the role of HPV, and connection to prognosis, is inconsistent and this needs to be studied further, which is the background of paper 1-3 in this thesis.

2.4 RANULA AND SCLEROTHERAPY OF OK 432

2.4.1 Background

Ranula means "little frog" in latin and originates from the latin word "rana" (frog). A ranula is a pseudocyst originating from the sublingual gland in the floor of the mouth, causing a swelling superficial to the gland- *intraoral ranula* (image 1)- or the cyst can herniate caudally through the mylohyoid muscle to the submandibular space and appear as a cervical swelling - *plunging ranula* (image 2 and 3)[120].

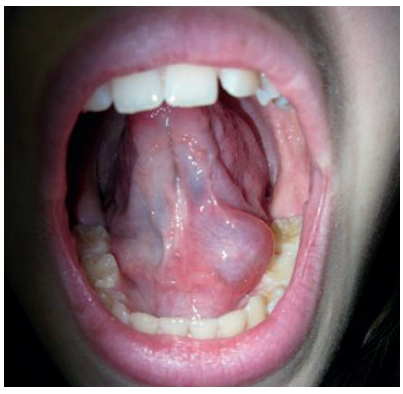


Image 6: Intraoral ranula

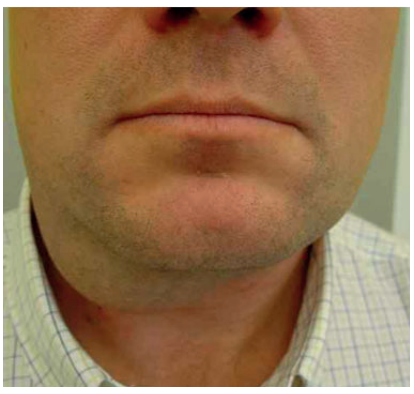


Image 7: Plunging ranula

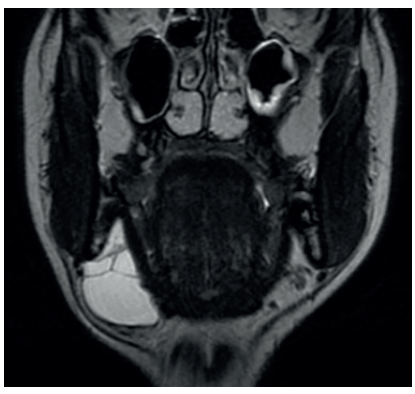


Image 8: MRI plunging ranula, coronal view

Ranulas are not true cystic lesions[121], i.e. their origin is not congenital, instead caused by acquired extravasation of saliva into the surrounding connective tissues. This can occur due to blockage of secretion from a part of the sublingual gland or after trauma [122, 123]. A ranula is thus *pseudocystic*, meaning it has no true epithelial lining. Since this is an acquired lesion, ranulas most commonly present in early adulthood, but can occur at all ages. The prevalence is not well studied but in 1976 Axell et al reported a prevalence of 0,2 per thousand individuals in a cohort study of oral lesions in Sweden [124]. Studies from New Zeeland indicate that genetic factors play a role in the etiology of ranulas – the condition is more common in Maori and Polynesians than in Kaukasians [125].

Intraoral ranulas can cause difficulties to speak and in more advanced cases eating difficulties, but in most cases the lesion is moderately disturbing. Plunging ranulas can cause pressure problems and cosmetic disturbance, and case-reports of plunging ranulas with extensive spread causing more severe problems, amongst others breathing difficulties, have been reported[126, 127].

2.4.2 Diagnosis

The diagnosis of ranula is based on patient's history, clinical examination, radiology and fine-needle aspiration cytology. Radiology can be ultrasound and/or CT /MRI. The viscosity of the cystic fluid in a ranula is of a very typical thread-like mucous type. (Image 9). With higher age the suspicion of malignancy is higher and a more thorough investigation is needed. It is common that patients with plunging ranulas are misdiagnosed since they can mimic many other conditions: abscesses, lymphatic malformations, thyroglossal or branchial cleft cysts or cystic metastases of head- and neck cancer[128-130].

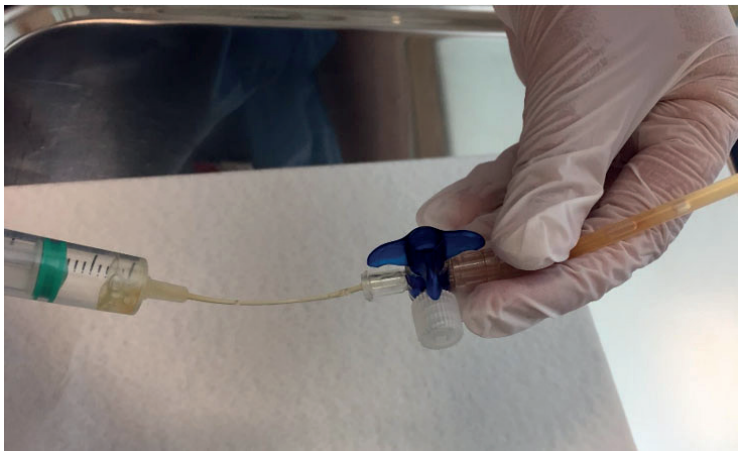


Image 9: Typical thread-like aspirate from a plunging ranula

2.4.3 Treatment

Surgery has been the traditional treatment of ranulas[120]. For intraoral ranulas various surgical approaches have been practiced; marsupialization, excision of the ranula pseudocyst, and excision of the sublingual gland. Both marsupialization and excision of the ranula pseudocyst have a high rate of recurrence[122, 131, 132]. Extirpation of the affected sublingual gland is accepted as the treatment of choice to achieve a definite cure for an intraoral ranula, and to treat patients with plunging ranula [120, 122, 123, 133, 134]. Earlier a transcervical excision of the plunging ranulas pseudocyst was often performed, but studies have shown that a properly performed excision of the gland itself is sufficient; the pseudocystic sac will be resorbed once the sublingual gland is removed[134, 135]. This procedure, however, can cause complications such as injury to the lingual nerve, and is performed under general anesthesia[136].

Apart from full surgery, several less invasive methods have been evaluated. The method of micro-marsipualization has been suggested as a minimal-invasive treatment of intraoral ranulas[137]. This procedure involves a technique in which silk-sutures sutures covering the dome of the ranula are placed, in order to create new epithelium-lined tracts that evacuate saliva and allows healing with maintained new openings from the sublingual gland (method described in detail by Kwon et al[138]).

Another method suggested to treat both types of ranula is to chemically ablate the salivary gland with 98% dehydrated alcohol (EtOH). Brannan et al treated 24 patients with 1-3 injections at a success rate of 87%[139]. However, this method requires general anesthesia or at least sedation since the procedure is painful.

Different sclerosing agents have been also been tried, and OK 432 (Picibanil) is the most frequently used, see point 2.4.4.

2.4.4 OK 432 and ranulas

Sclerotherapy is a non-surgical fibrosis-inducing treatment used for many years in the management of vascular malformations. Advantages of sclerotherapy is the avoidance of surgical complications, but since the effect of all types of sclerotherapy is mediated by an inflammatory response to the drug injected, the patient will experience some level of discomfort after treatment- usually swelling and light pain, but sometimes also fever and more severe discomfort[140].

OK 432 is a lyophilized streptococcal preparation derived from group A hemolytic streptococcus Su strain[141]. It is heated and pretreated with benzylpenicillin. OK 432 was originally developed as an adjuvant immunological cancer therapy - the antitumoral effect was not satisfactory, but it proved to be useful in treating ascites and other cystic conditions related to cancer[142].

The exact mechanism of how OK 432 works is not known, but it triggers a strong inflammatory reaction with elevated levels of IL-6, IL-8, VEGF and TGF-beta for weeks after an injection with OK 432[143], without the toxic effect normally seen from streptococcal infections. OK 432 has been used for more than 30 years in sclerotherapy of macrocystic lymphatic malformations, with reported cure rates of 60-90%[144-146]. There have been several studies on the efficacy also on other benign cystic cervical lesions with promising results[147-149].

Even if many patients with lymphatic malformations respond well to treatment, an entity of patients do not respond at all[150]. Studies have indicated that the response to treatment could be correlated to expression of Toll-like receptor expression[151].

Previous studies on the usage of OK 432 sclerotherapy on ranulas have reported various efficacy, with results ranging from 33% to 87% success rate[152-155]. Several studies with OK 432 has shown good results in treating intraoral ranulas[155, 156], but for plunging ranulas the results diverge[154, 157]. Sclerotherapy is done as an ultrasound-guided procedure in an office-based setting, without general anesthesia or sedatives.

3 RESEARCH AIMS

- To study prevalence of and whether HPV and overexpression of p16 has a prognostic value in hypopharyngeal cancer (paper 1 and 2)

- To study long-term survival and recurrence in oropharyngeal cancer, in correlation to p16, HPV and subsites. (paper 3)

- To assess the safety and efficacy of OK 432-sclerotherapy on patients with ranula (paper 4)

4 MATERIALS AND METHODS

4.1 STUDY SUBJECTS, MATERIALS AND METHODS PAPERS 1-3

4.1.1 Study subjects and study design:

Karolinska University Hospital is a tertiary center responsible for the care of advanced ENT diseases in the Stockholm-Gotland region of Sweden, including approximately 2,4 million inhabitants. The studies were conducted under ethical permits Dnr 2009/1278-31/4, amendment Dnr 2010/1117 32 (paper 1-3) and Dnr 2013/553 31-1, amendment No 2017-1007-32-1 (paper 4).

4.1.1.1 Study subjects paper 1:

Patients diagnosed with hypopharyngeal cancer at Karolinska University Hospital 2000-2007 were included, in total 142. The diagnosis covered were set according to the International Classification of Diseases (ICD) system, version 10 [158], including the pyriform sinus (C12.9), the postcricoid area (C 13.0), the aryepiglottical fold (C13.1), extended growth (C13.8) and hypopharynx unspecified localization (C13.9).

All patient charts were evaluated for patient characteristics and clinical data. Only patients treated with intention to cure, n=96, were included in survival analyses.

4.1.1.2 Study subjects paper 2:

93 patients diagnosed with hypopharyngeal cancer between 2008-2013 in the Stockholm-Gotland region were included. From this cohort 46 patients treated with intention to cure and with at least three years follow-up available were included in survival analyses.

Survival analyses of the patients treated with intention to cure in the whole group (2000-2013) were performed, in total 96+46= 142 patients.

4.1.1.3 Study subjects paper 3:

529 patients diagnosed with oropharyngeal cancer (OPSCC) in Stockholm-Gotland, with available data on overexpression of p16 and treated with intention to cure between the years 2000-2010 were identified. Of these 525 had available data also on presence of HPV DNA.

ICD-10 code C09.0-09 and C02.4 were categorized as tonsillar squamous cell carcinoma, TSCC, C01.9 as base of tongue, BOTSCC, and C10.0-9, C05.1-9 as other oropharyngeal squamous cell carcinoma, otherOPSCC.

Patient charts were assessed for survival, date of recurrence and localization of recurrence if such occurred.

4.1.2 DNA extraction and PCR:

As described under p 2.3.2, there are different methods used to detect oncogenic active HPV-DNA. The method used in paper 1-3, was described by Schmitt et al in 2006[159].

Tumor samples were formalin-fixed paraffin embedded pre-treatment biopsies (FFPE). To extract DNA in paper 1 and 2 we used the High Pure RNA paraffin kit, Roche Diagnostics, but excluded the DNase step. This procedure was originally designed for RNA -extraction but is widely used also for DNA extraction.

The Luminex multiplex assay detects 24 different HPV-types including all 15 high-risk HPV and as an internal control of presence of amplifiable DNA the housekeeping gene β-globin was used.

This type of assay consists of four steps:

1) *PCR amplification of HPV-DNA*. This is performed with a primer set of broad spectra of reverse biotylinated GP5+ and GP6+ primers, detecting the L1 region of HPV-DNA. This set of primers is designed to get equal amplification strength for all the 24 different HPV-types of the assay, with a sensitivity of 10-100 gene copies.

2) *Incubation with colored magnetic beads* with 24 specific probes binding to each type of HPV-DNA, and one for the internal control of β-globin/S14.

3) *Incubation with fluorescent streptavidin* binding to the biotinylated primers

4) *Analysis of color and fluorescence*, and thereby presence of HPV-DNA for each type respectively, by a Luminex MagPix instrument. From the MagPix we get a Median Fluorescent Index (MFI), which is transformed into an index according to protocol by Schmitt et al[159].

Samples with a median fluorescent index for β-globin or S14 below 30 were considered to have to poor DNA quality to be properly assessed for HPV and were excluded.

For earlier patients in paper 3 (year 2000-2006) HPV was analyzed with PCR using general primers GP5+/6+ and CPI/IIG. All samples were also tested with HPV16-specific primers, and those positive with general primers but negative for HPV16 were sequenced. These older samples were at a later stage re-analyzed with the Luminex MagPix bead-based assay, so effectively all samples from patients included in paper 3 were analyzed also by this method.

4.1.3 Immunohistochemistry of p16:

All immunohistochemistry was performed on 4 μm-sections from the formalin-fixed paraffin embedded pre-treatment biopsies. In order to reduce the chemical modifications caused by the formaldehyde, the process for all markers was initiated with a heat-mediated epitope retrieval.

In paper 1 and 3, immunohistochemistry with the p16Ink4a primary monoclonal mouse anti-human antibody, clone JC8, was conducted according to manufacturer (Santa Cruz Biotechnology):s instructions, with dilution of antibody 1:100. After epitope retrieval and blocking the sections were incubated with primary antibody, then with secondary biotylinated anti-mouse antibody, an avidin-biotin-complex-PO (VECTASTATIN-kit), developed in DAB and counterstained in hematoxylin. For negative control we used the monoclonal mouse antibody A2.

In paper 2, we used the primary E6H4 mouse monoclonal antibody from CINtec p16 histology (Ventana, Tucsonm Arizona), followed by a secondary Biotinulated Horse Anti-Mouse IgG antibody (Vestor Laboratories, California) diluted 1:200. Procedure of staining was carried out according to manufacturer's instructions.

In all three studies the results of immunohistochemistry were evaluated by light microscopy. Samples with dark brown staining in 70% or more of tumor cells were considered positive, in accordance with the American college of Pathologists[56] DAHANCA recommendations for scoring of oro-pharyngeal cancer[160], also in accordance to recommendations by El-Naggar[161]. Notably, the absolute majority of samples had either <10 % or >90 % of stained cells.

4.1.4 Statistical analyses paper 1 and 2

The statistical analyses were calculated in STATA 10. In paper 2 a comparison between the early and the late cohort was made to evaluate changes over time regarding HPV-DNA and p 16 positivity, and overall and disease-free survival was assessed for both cohorts together.

Fischer´s exact test was used to correlate presence of HPV -DNA with overexpression of p 16.

Overall and disease-free survival related to p 16 and HPV was calculated for the patients treated with intention to cure, in paper 1 96 patients and in paper 2 for all 142 patients from both cohorts, and presented in Kaplan-Meier curves. A log-rank test was used for comparison of the different survival rates.

Uni- and multivariate analysis was performed and there we used the Cox proportional hazard model to adjust for covariates. Significance level was set at p=<0.05.

4.1.5 Statistical analyses paper 3:

In paper 3 the analyzes were made in SPSS (version 25 for Mac).

Differences in categorical data were assessed with Chi2 test. Overall survival (OS) and disease-free survival (DFS) was calculated in relation to p16-status for the whole cohort (OPSCC) and for TSC-C+BOTSCC and otherOPSCC separately.

A sub-group analysis for TSCC/BOTSCC with performance status WHO 0 (as a surrogate for ful-filled treatment) was performed, in which we analyzed uni-and multivariate hazard ratios for OS and DFS related to HPV and p16-status, age, smoking and dichotomized stage. We also analyzed overall survival after recurrence for TSCC/BOTSCC, in relation to both HPV and p16-status. Significance level was set at p=<0.05.

4.1.6 Methodological considerations paper 1-3:

The Luminex bead-based assay is considered to have a high sensitivity and is the method we use in our laboratory. In our experience, 5-10 ng of DNA was enough to obtain a strong signal in HPV 16 -positive tonsillar cancers. When we performed our analyses, we thus started with analyzing 10 ng of DNA from our hypopharyngeal tumor samples. As further described in our results, we found very few samples positive for HPV in the hypopharyngeal cancers, why we re-did the analysis with 50 ng DNA, with consistent results.

The Luminex assay only includes primers testing presence of the L1-region, so if this region is lost when HPV-DNA is integrated it will fall out negative. In later studies by our group, we have included primers aiming at both E6 and L1. Our experience is that is it very rare that L1 is negative but E6 is positive, so we judge it not likely that this would have influenced the results of paper 1 and 2 in any major way.

In paper 3 we excluded patients where diagnosis was based only on cytology, since this material is not enough to make analyses of HPV and p16. The patients diagnosed only in this way are mostly in a palliative state or at a high-performance status, thus not likely to be given a full curative treatment. Therefore, it is not likely that their exclusion had any impact on survival analysis of patients treated with full curative intent.

IHC is a method with many challenges; results are depending on cut-offs, which often differs between studies, and on inter-individual differences when interpreting results. In other tumor-types and other parts of the body IHC of p16 can show an unstable patchier pattern[162], but in head- and neck SCC

it is considered stable, is well tested and cut-off levels are set and internationally accepted, both by researchers and in clinical use[56, 160]. Our experience is that most often the staining for p16 is either strong, or weak, with only a very few samples near the cut-off.

To perform subgroup analyses in a retrospective setting such as in paper 3, gives a limited scope of information. A prospective setting, with strict follow-up on treatment protocols, would be preferable.

In OPSCC there are large differences in numbers between the dominating group of tonsil and base of tongue cancer and the relatively few otherOPSCC. This entails statistical challenges, and the risk of missing characteristics of the minority when evaluating the whole OPSCC-group together.

A general fact is that our study-groups are based on the diagnosis set by the clinician according to ICD-10. Since it is not always clear where a tumor has its origin, some level of uncertainty is involved in this assessment.

Major weaknesses are that the studies are retrospective, with clinical data collected in same cases many years after death, from different chart systems, which could entail missing data.

4.2 STUDY SUBJECTS, MATERIALS AND METHODS PAPER 4:

4.2.1 Study subjects and study design:

This study was approved by the Swedish Medical Agency[161] and registered in the EUDRA-CT clinicaltrials.eu (study 2012-004540-29).

20 patients with ranula, oral or plunging, were included in a double-blinded placebo-controlled randomized trial, in which sclerotherapy with OK 432 was evaluated regarding efficacy and safety.

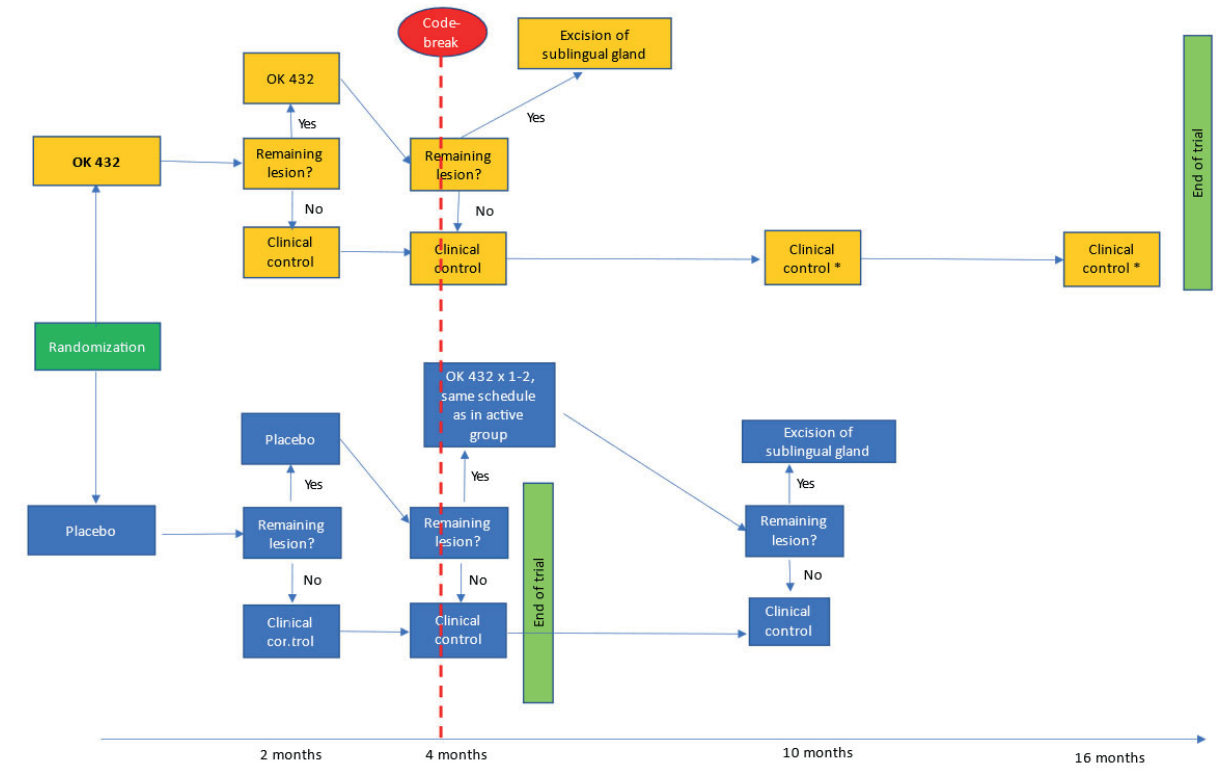
43 patients were assessed for participation, 20 of these were included in the study. The main reason for exclusion was autoimmune disease and patients own wish for immediate treatment, inclusion and exclusion criteria are fully described in paper 4.

Patient characteristics such as age and gender were similar in the included and excluded groups.
The assessment of correct diagnosis was for plunging ranula based on typical appearance on radiology (ultrasound or CT, in some cases MRI) and benign cytology, for oral ranulas a typical clinical appearance and cytology was considered sufficient.

Included in the study were 10 patients with plunging and 10 patients with oral ranulas.

The study thus comprised two injections with a follow-up after approximately 2 months, and after this first part the placebo-group was offered active treatment according to the same protocol.

Flow chart of the study:



4.2.2 Intervention:

The injections were given in our outpatient clinic, by ENT-surgeons experienced in ultrasound.

Patients were randomized by a non-blinded study nurse. All patients in the active group got the same fixed dose, 0,1 mg OK 432, diluted in different volumes NaCl depending on size of the cyst, in accordance with the protocol used for lymphatic malformations at Karolinska. For detailed description of treatment procedure see paper 4.

Patients were handed QOL-questionnaires; EQ5L-Sweden[164] and a patient diary for recording inflammatory response, to be returned at next visit.

4.2.3 Outcome measures:

Patients with both plunging and oral ranulas were assessed by clinical examination and ultrasound at the follow-up, QOL-forms and patient diaries were collected.

Primary outcome measure was the response to treatment. This was assessed in a dichotomized way; complete responder (CR) or not responder (NR). Any remaining cystic lesion clinically or on ultrasound entailed NR-result. *Secondary outcome* measure was the patients experience of the inflammatory reaction after injection, assessed through the documents.

An additional overall outcome measure was *safety*, and adverse events were assessed at every visit and telephone follow-up. In the study we also calculated the costs per patient compared to surgery (excision of the sublingual gland as a day-surgery procedure) with one series of two injections and the following control visits. The study was continuously monitored by KTA (Karolinska Trial Alliance)

4.2.4 Statistical analyses paper 4:

The study groups were compared through a student's T-test using a two-tailed significance and assuming equal variance.

4.2.5 Methodological considerations paper 4:

The number of patients in the study is small, with an obvious impact on the statistical strength (we can barely show a weak significance active treatment vs placebo). We misjudged the effect based on excellent results in our own small pilot-study, results we could not repeat here. That plunging ranulas could respond differently should possibly have been anticipated based on varying results in other studies, but was not observed in our pilot-study. To some extent these problems reflects the difficulties in performing randomized studies in rare conditions such as ranula. To summon a larger number would take a long time, and the fact that two patients in the placebo-group were cured by NaCl was also surprising to us.

Another somewhat disappointing observation is that included patients showed a low compliance when it comes to filling in and returning QOL-forms and patient diaries. This could reflect that this condition has a moderate impact on patients' daily life; generating a lower compliance in fulfilling study documents than in clinical trials on more serious conditions. Or the documents were too time-consuming; a digital solution could have generated a higher rate of obtained information.

The intent of paper 4 was good, but in retrospect it is hard to avoid the thought that the ambition was too high. We could have set up a simpler treatment-study, entailing a lower level of evidence, gathered a larger volume of patients and the study had been easier to carry out.

5 ETHICAL CONSIDERATIONS

Regarding study 1-3, carried out under ethical permit Dnr 2009/1278-31/4, amendment Dnr 2010/1117 32, and permit 2005 /431 31/4, 2005/1330-32, studies are carried out retrospectively, on existing biopsy material and patient charts. The main ethical issue here is to store data and analyses in a safe way, and to anonymize data so that no participating patient can be identified.

All patient data are coded according to routine (given numbers with a pass-word secured code) and the data sets are stored in the Karolinska system in looked firesafe facilities.

Regarding study 4, ethical permit No 2013/553 31-1, amendment No 2017-1007-32-1, the ethical considerations are mainly:

1. The safety aspect of evaluating OK 432, a well-established treatment of lymphatic malformation, in a new group of patients
2. That the placebo group will be given two injections with only saline with 2 months of observation time between, and thus have their treatment delayed for 4 months.
3. That the placebo group will be subjected to two unnecessary injections, with related pain and discomfort.

Regarding these considerations our research group have made the following conclusions:

1. OK 432 has been used for over 30 years without any known serious complication. The study on ranula means testing the drug on a new indication, but in the same dose and in the same procedure as earlier usage. We saw no alarming risks in this.
2. Ranula is a benign disease that generally causes a mild or moderate discomfort, mainly cosmetical. We therefore judged that the delay of treatment with 4 months for the placebo-group was acceptable, considering the possible benefit of finding a less invasive treatment option.
3. The pain related to injections was judged mild to moderate, and short-term. We considered it was acceptable.

6 RESULTS AND DISCUSSION

6.1 RESULTS AND DISCUSSION PAPER 1 AND 2

6.1.1 Presence of human papilloma viruses and p16 expression in hypopharyngeal cancer:

6.1.1.1 Aim:
In paper 1 we investigated whether HPV is a risk factor and a favorable prognostic factor for patients with hypopharyngeal cancer and assessed the correlation to overexpression of 16 in this subgroup.

6.1.1.2 Main results:
109 out of 142 identified patients had available tumor samples of sufficient quality for analyses of HPV DNA, and were further assessed for overexpression of p16 by immunohistochemistry and included in the further analyses. The overall 5-year survival was 17 %, (19/109 patients), disease-free survival 55 %. 78/109, 72 % of the patients were diagnosed in stage 3 or 4. 7 out of 109 samples were positive for HPV-DNA; 4 samples of HPV 16 and 3 other types (see table 1)

18 patients had tumors positive for overexpression of p16 (for definition of p16-positivity see point 4.1.3). The correlation of p16-positivity and HPV in total was low, with only 4/18 (22%) of p16-positive tumors being also HPV-positive. However, all patients with HPV 16 were also p16-positive, see table 1.

Table 1. HPV and p16 in patients with hypopharyngeal cancer 2000-2007 in Stockholm

	p 16+ No of patients	**p 16-** No of patients
HPV 16+	4	0
HPV 51, 53, 56+	0	3
HPV -	14	88
Total no of patients	18	91

Only patients with curative intent were included in the survival analysis n=96

Neither HPV or overexpression of p16 showed a significant correlation to disease-free survival. Regarding overall survival, the univariate analysis showed no significant correlation of HPV to better overall survival, but in the multivariate analysis the presence of HPV all types and HPV 16 did (p= .015 and .031).

Overexpression of p16 showed no significant correlation to a better overall survival but a possible tendency (see figure 6).

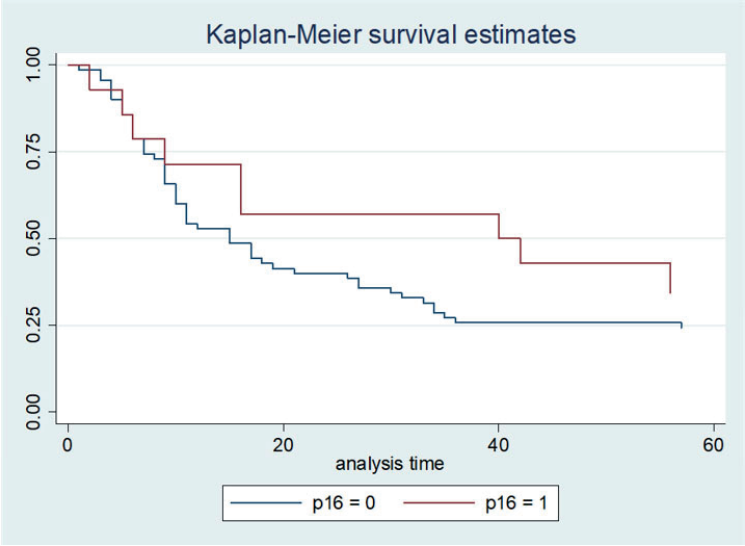


Figure 6: 5-year overall survival for patients with hypopharyngeal cancer stratified for tumor expression of p16

Three of the four patients with tumors positive for HPV 16 and p16 lived tumor free for more than 5 years.

6.1.2 Human papillomavirus DNA and p 16 (INK4a) expression in hypopharyngeal cancer and in relation to clinical outcome, in Stockholm, Sweden

6.1.2.1 Aim:

In paper 2 we seeked to assess whether the prevalence of HPV and overexpression of p16 in hypopharyngeal cancers has enhanced over time and the value of these markers in relation to clinical outcome.

6.1.2.2 Main results:

82 out of 93 identified patients diagnosed with hypopharyngeal cancer between 2008-2013 had available tumor biopsies and were analyzed for HPV and overexpression of p 16 (for definitions and details see point 4.1.2 and 4.1.3, and the papers).

Of these 82 patients, 3 were positive for HPV (3,7%), all HPV 16, and all three were also p16-positive.

12 of the 82 patients (14,6%) were p16-positive, i.e., the correlation to HPV was poor.

These results from HPV and p16-analyses were very similar to the results in paper 1, see table 2:

Table 2: comparison of study 1 and 2.

Tumor HPV DNA and p16 status			
	Paper 1	Paper 2	Total
No of patients analyzed	82	109	191
p16-positive (%)	12 (14,6%)	18 (16,5%)	30 (15,7%)
HPV-positive (%)	3 (3,7%)	7 (6,4%)	10 (5,2%)
HPV 16-positive (%)	3 (3,7%)	4 (3,7%)	7 (3,7%)
HPV 16 and p16-positive (%)	3	4	7 (3,7%)

In the survival analyses all patients from 2000-**2011** (based on the need of a follow-up period of at least three years) were included (96 from 2000-2007 and 46 from 2008-2011). For illustration, see Fig 1 paper 2.

In the survival analyses there was a significantly better 3-year overall survival for the patients HPV16+/p16+ (p=.0185), figure 7 A. Disease specific survival showed a tendency, but is not significant, figure 7 B. p16 was not significantly correlated to OS or DSS, but there was a tendency towards better OS for patients with p16+ tumors, figure 7 C and D.

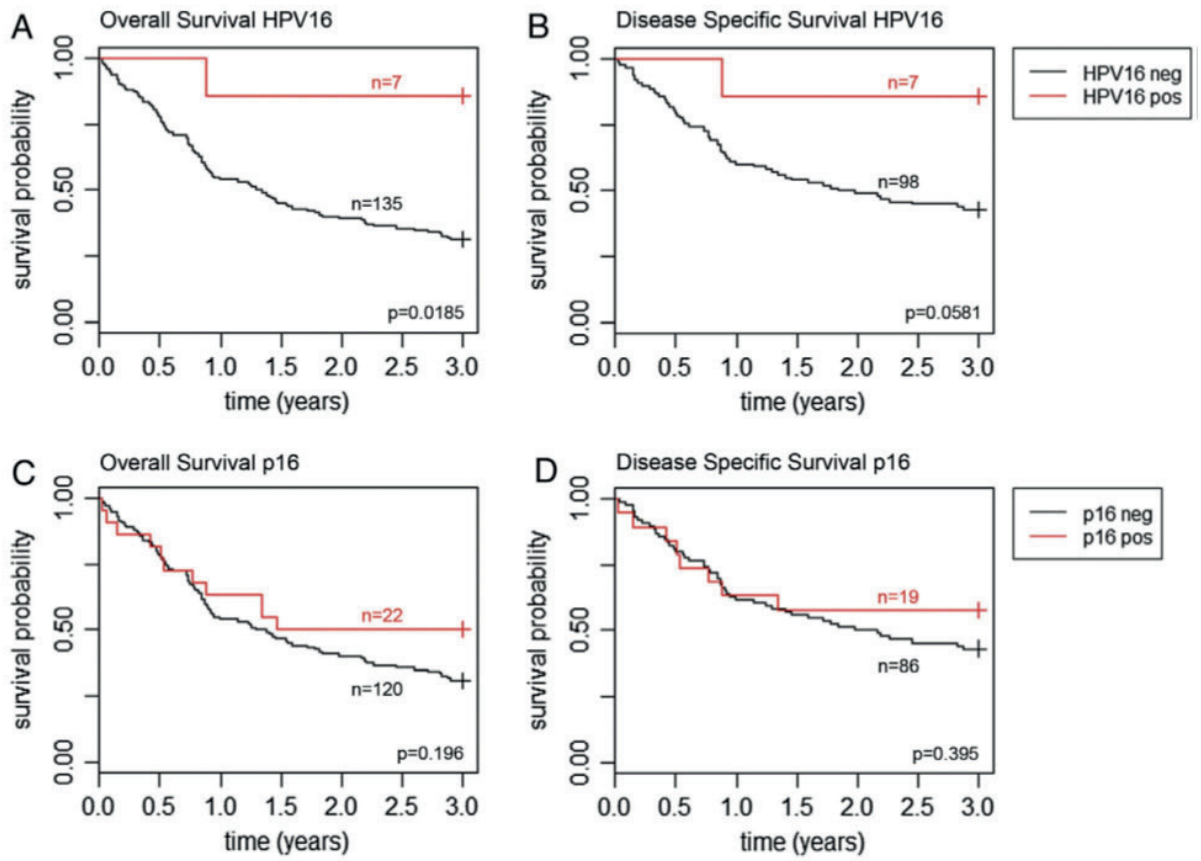


Figure 7: Kaplan-Meier curves of all 142 patients with hypopharyngeal cancer 2000-2011 included in survival analysis. A: OS and B: DSS of patients with HPV16+/p16+ vs other hypopharyngeal cancer, C: OS and D: DSS of patients with p16+ hypopharyngeal cancer vs p16-. A=significant, p=.0185

6.1.2.3 Discussion Paper 1+2:

Our results show that the prevalence of HPV in hypopharyngeal cancer in the years 2000-2013 in Stockholm was very low, only 10/191, 5,2%, and stable over time, in contrast to the high portion of HPV/p16-positive tumors in the oropharynx during the same period of time[18, 97].

When our studies were initiated a study by Ernoux-Neufcouer et al had shown a very high prevalence of high-risk HPV in a group of 61 stage 4 hypopharyngeal cancer, 82%[91] . However, in their study only 7 patients showed overexpression of p16, which could indicate that only a few of the HPV+ patients had clinically relevant transcriptionally active DNA. In the Ernoux study presence of high-risk HPV was not correlated to a better prognosis, rather a worse, but all 7 patients with hypopharyngeal cancer overexpressing p16 were disease free after 3 years. The latter was not significant (low number of p16+ patients) but is an interesting observation.

In the years that followed other studies confirmed that HPV does not show a high prevalence in hypopharyngeal cancer, with HPV found in 10-20% of cases[34, 89, 90, 165, 166]. Since hypopharyngeal cancer is rare, and the prevalence of HPV in this group of tumors is low, multicenter-studies would be very useful in order to gain more knowledge on the issue.

Our results suggests that in this subgroup of head- and neck cancers overexpression of p16 is not well correlated to HPV, and thus not a reliable surrogate marker to oncogenic active HPV. This is in line with the study by Ernoux et al, and for the whole group of non-OPSCC by Lechner[167].

However, there are indications that patients with p16+ hypopharyngeal cancer show a tendency of better survival regardless of HPV-status[168]. Here, in the survival analysis of the merged cohort from paper 1 and 2 we find a tendency that p16-positive patients have a better outcome compared to p16-negativ; 3-year-OS was 50% vs 31% respectively, 3-year DSS was 58% vs 43%. These differences were not significant, but the findings are in line with Chung et al[168] and Ernoux et al[91], and the role of p16 as an independent tumor marker deserves further attention.

All of the seven patients in paper 1+2 with HPV16+/p16+, of which six had a survival for more than three years, had their tumors located in the aryepiglottical fold or the pyriform sinus; thus, they were "true" hypopharyngeal cancers and not overgrowth from tonsils or base of tongue. Naturally no conclusions can be drawn from such a small number, but it is an interesting observation considering the general bad prognosis in this group of patients. It is also interesting related to the finding by Joo et al that HPV-positive tumors often were located in the pyriform sinus and had a characteristic gross feature with exophytic and granulomatous growth[90].

Hypopharyngeal cancer is a rare disease, so many previous studies include a small number of patients – a strength of these two papers is the large material, which includes all patients diagnosed with hypopharyngeal cancer in the population of Stockholm-Gotland region in the actual period of time. This constitutes in comparison to many previous studies a large cohort of patients with a rare diagnosis. Summing patient characteristics give important knowledge of this group; the panorama regarding age, TNM stage etc. remains stable over time, in contrary to the epidemiological changes in oropharyngeal cancers related to the impact of HPV.

Regarding treatment of hypopharyngeal cancer the findings of Steiner and Ambrosch [83, 84]that primary surgery in selected cases gives a more favorable outcome is important. Since the prognosis in this group is so bad, every possibility to improve treatment choices must be carefully assessed, and the development of surgical techniques widens the boundaries of what is technically possible.

6.1.2.4 Conclusions Paper 1+2:

- Precence of high-risk HPV was rare, only 5%, and did not increase over time in Stockholm during the years of 2000-2013
- p 16 is not a suitable surrogate marker for HPV in hypopharyngeal cancer.
- HPV 16 in combination with p16-positivity is a possible prognostic factor in hypopharyngeal cancer, but the numbers in our studies are too few to confirm this.

6.2 RESULTS AND DISCUSSION PAPER 3

6.2.1 Long-term survival and recurrence in oropharyngeal squamous cell carcinoma, in relation to subsites, HPV and p16-status:

6.2.1.1 Aim:

The aim of paper 3 was to investigate the long-term outcome in oropharyngeal cancer, both regarding overall survival and recurrence, in order to gain better knowledge of recurrence pattern in relation to p16, HPV and subsites.

6.2.1.2 Main results:

We found that both overall and disease-free long-term survival was significantly higher in p16-postive than negative patients with OPSCC (p=<0.0001 and < 0.0001 respectively). When analyzing subsites separately, the difference holds in TSCC/BOTSCC: s, but in the group of otherOPSCC there is no significant difference in relation to p16-status regarding neither OS or DFS, see figure 8.

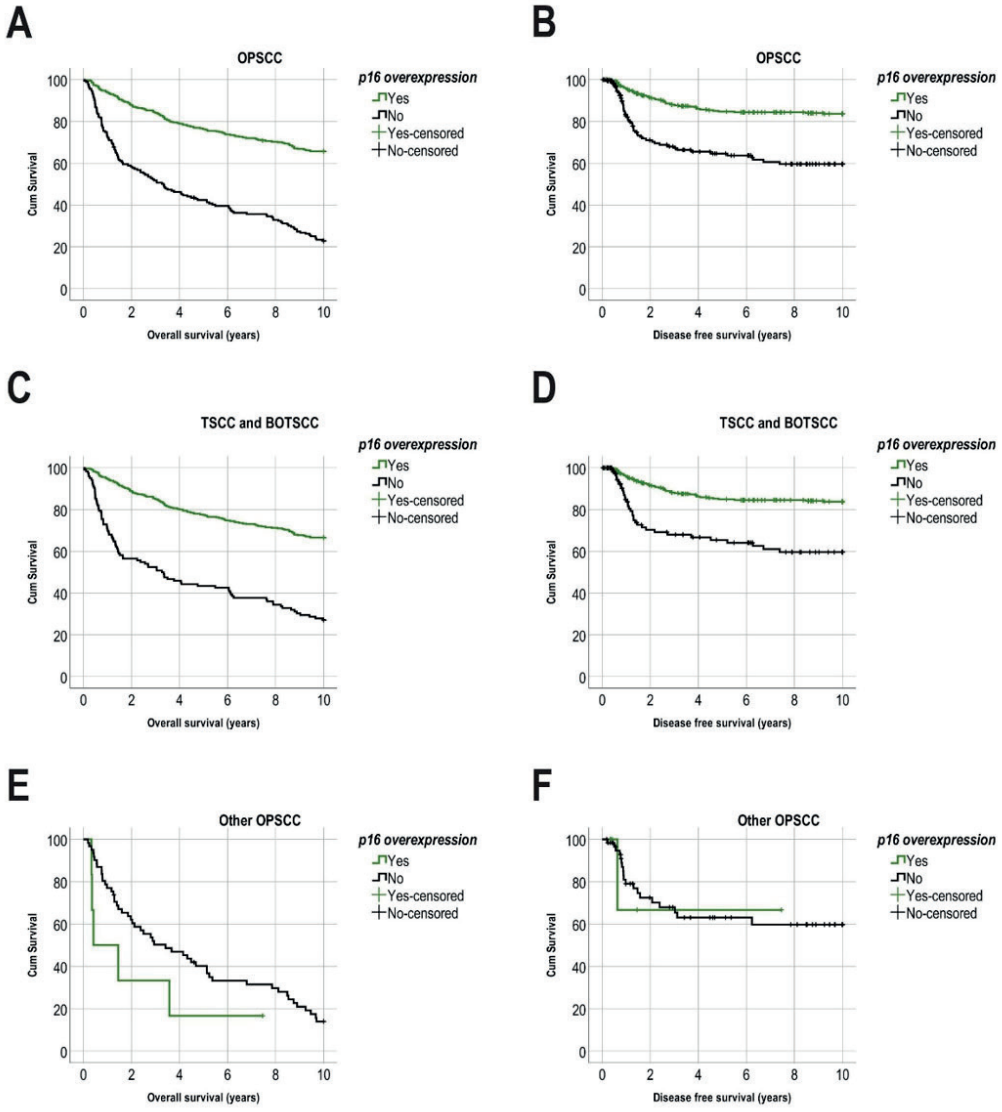


Figure 8: Kaplan Meier figures with 10-year overall survial (OS) and 10-year disease-free survival (DFS) in patients with OPSCC (A-B) and separated on subsite with TSCC/BOTSCC (C-D) and otherOPSCC (E-F). A-B) Patients with p16 positive OPSCC had a significantly better OS and DFS as compared to patients with p16 negative OPSCC (log rank: p<0.0001 and p<0.0001 respectively). C-F) Patients were separated into lymphoepithelial sub-sites (TSCC/BOTSCC) and non-lymphoepithelial subsites (otherOPSCC) and analysed separately. C-D) Patients with p16 positive TSCC/BOTSCC had a significantly better OS and DFS as compared to patients with p16 negative TSCC/BOTSCC (log rank: p<0.0001 and p<0.0001 respectively). E-F) No significant differences in OS and DFS between patients with p16 positive and p16 negative otherOPSCC were observed (log rank: p = 0.13 and p = 0.9 respectively).

In the whole cohort 110/529, 21%, of patients had recurrence. In TSCC/BOTSCC there was an equal distribution between locoregional recurrence (LRR) and distant recurrence (DR), but in otherOPSCC most had LRR (see figure 9). OPSCC patients with p16-positive tumors had a significantly higher portion of distant recurrences (DR) compared to p16-negative, (p=<0.04).

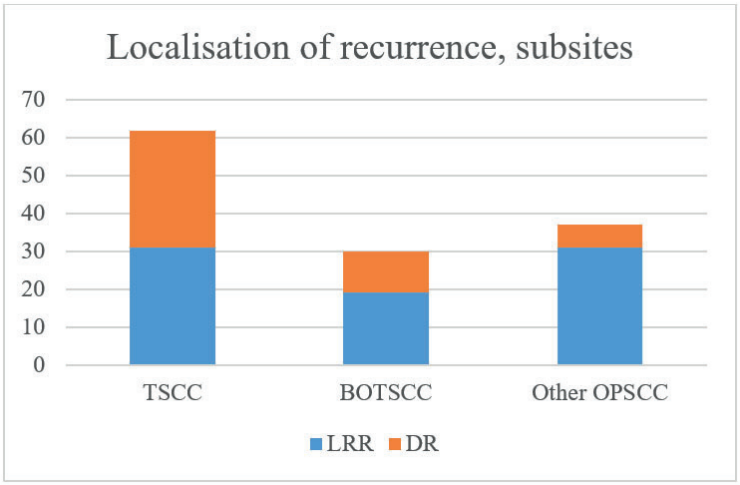


Figure 9: Distribution (count) of LRR and DR separated per OPSCC sub-site

From the subgroup analysis of TSCC/BOTSCC WHO 0 we learn that patients with HPV+/p16+ have a significantly higher long-term OS and DFS compared to HPV-/p16-, (p=<0,0001 and 0.0001), but in addition to this we also find that discordant status, HPV-/p16+, have a significantly worse OS than patients with double positive HPV/p16 (p=<0,029, figure 10), and likewise that HPV+/p16- had an intermediate survival (data not shown).

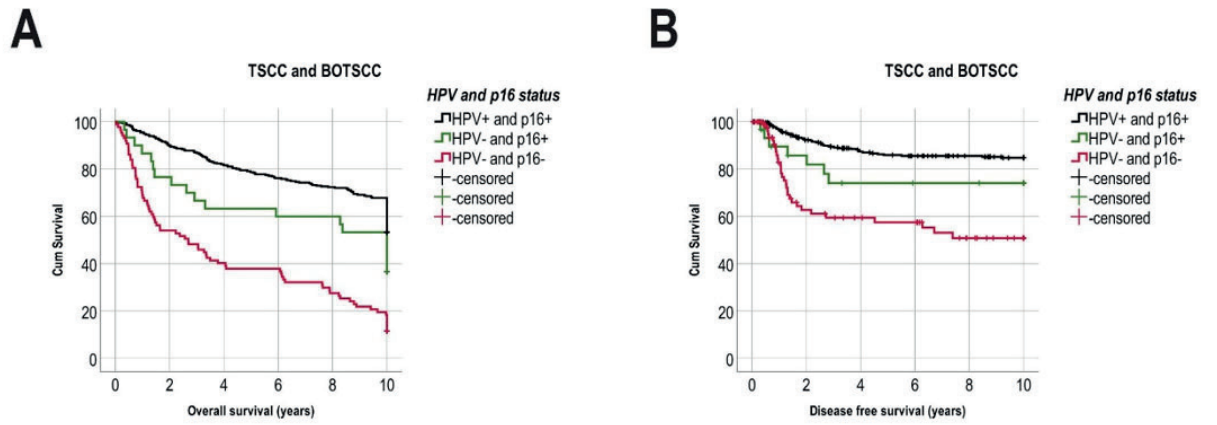


Figure 10: Kaplan Meier figures with 10-year overall survival (OS) and 10-year disease-free survival (DFS) in patients with TSCC/BOTSCC (A-B) A) Patients with HPV DNA positive and p16 positive (HPV+/p16+) TSCC/BOTSCC had a significantly better OS and DFS, as compared to patients with HPV DNA negative and p16 negative TSCC/BOTSCC (log rank: p<0.0001 and p<0.0001 respectively). However, patients with p16 positive but HPV negative (HPV-/p16+) TSCC/BOTSCC presented an intermediate OS and DFS as compared to patients with double positive or double negative HPV/p16 status. (HPV+p16+ vs HPV-p16+ (log rank test): OS: p=0.029; DFS: p=0.086 and HPV-p16+ vs HPV-p16- (log rank test): OS: p=0.001; DFS: p=0.072)

Beyond this, we find that in the multivariate only HPV+/p16+ was correlated to a better long-term OS and DFS compared to HPV-/p16- (not HPV-/p16+), (see table 2 paper 3).

Survival after recurrence in TSCC/BOTSCC was low, 5,9%, and HPV/p16-status was in this material not significantly correlated to prognosis at this point forward. see figure 11:

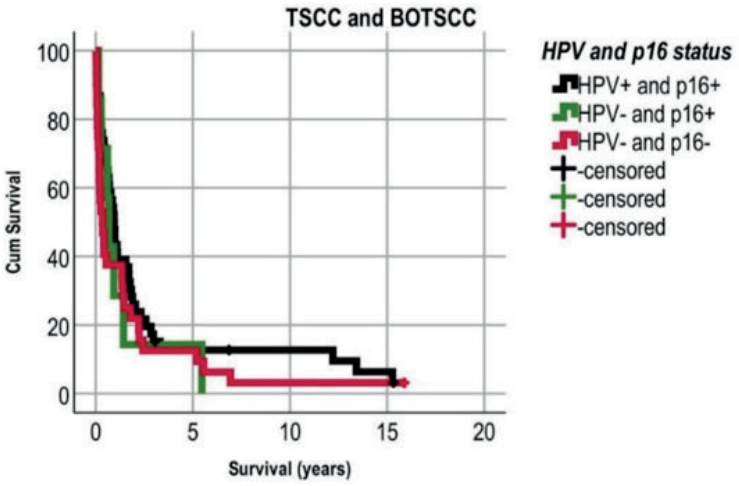


Figure 11. Kaplan Meier figures with 10-year survival after a locoregional or distant recurrence (LRR/DR) separated for HPV and p16 status. No significant differences were observed in survival between patients with TSCC/BOTSCC after recurrent disease independent of HPV and p16 status. (HPV+p16+ vs HPV-p16- (log rank test): p=0.12)

6.2.1.3 Discussion Paper 3:

From paper 3 we gained important knowledge on the long-term recurrence panorama of OPSCC, both for the tumors associated to HPV and those that are not.

The origin of this study was an eager to investigate whether the case reports and observations of HPV-positive tumors with late and atypical recurrences could be verified to represent a difference in recurrence pattern in this relatively new entity of tumors. Secondly, we wanted to continue the assessment of the prognostic value of p16 regarding recurrence and long-term survival separating the subsites TSCC/BOTSCC and otherOPSCC.

The result that patients with p16-positive tumors are at less risk of developing recurrence was expected, several studies have reported this[115, 169]. However, in this study we find no results supporting the observations of p16-positive patients presenting with late relapses[116, 170, 171].

Two findings in this study are important in relation to the new TNM 8-staging separating p16+/p16- OPSCC.

1. In the present study we can confirm that the prognostic value of p16 in OPSCC derived from the non-lymphoid tissue in otherOPSCC differs from TSCC/BOTSCC, also regarding long-term outcome and recurrence.
2. HPV-/p16+-TSCC/BOTSCC patients has a significantly lower OS compared to HPV+/p16+ patients, why de-escalations studies and/or future treatment protocols should not be based on p16-status alone.

In this and previous studies the subgroup of otherOPSCC resembles more the tumors of the oral cavity, regarding both correlation of p16-status to HPV, and long-term prognosis. This entails a need to take precaution in using the new p16-based TNM 8 staging system of oropharyngeal tumors for de-escalation of treatment in this subgroup. There is an obvious risk of under-treatment if the other OPSCC are blindly merged with TSCC/BOTSCC.

Regarding the combination of HPV/p16 it is an interesting observation that TSCC/BOTSCC-patients with HPV-/p16+-status significantly differs in OS from HPV+/p16+. Firstly, this implicates that de-escalation selection based only on p16-status entails a risk to undertreat the group HPV-/p16+, who according to these results do not have the same good prognosis as HPV+/p16+. Secondly, this raises the question of what this combination actually stands for regarding tumor biology. p16 is a tumor suppressor who has its own role in cell cycle and DNA-repair, independently of HPV-infection. Others have found similar results; Worsham et al found that HPV-/p16+ patients had a worse survival

that HPV+/p16+, but still clearly better than HPV-/p16-, and draw the conclusion that HPV-/p16+ are a distinct subgroup where HPV is not involved[51]. This is supported by Rasmussen et al, who in a large cohort-study show that HPV-/p16+ patients have a higher risk of DR than HPV+/p16+[52], and further Klussman et al found that 5,5% of p16-positive OPSCC patients had no HPV-DNA, and in the majority of these patients survival was worse than +/+[172]. Their study does not separate subsites in the oropharynx, but they conclude that p16 as a single marker is insufficient.

Similarly, in our study TSCC/BOTSCC-patients with tumors positive for HPV but negative for p16 (HPV+/p16-) also presented with an intermediate survival compared to double-negative or double-positive. In that case the effect of the new TNM is the reverse; they will be classified as p16-, but possibly have a better prognosis than expected, and thus risk a more intense treatment than necessary.

In our study we did not proceed with subgroup analysis of otherOPSCC and both markers HPV/p16, since the volume of patients ends up too low when separating a small subsite-group into another level of division. It could however be interesting to gather a larger multicenter study, in order to further evaluate the role of p16 as a marker independent of HPV.

Our findings support the theory that SCC arising in lymphoid and non-lymhoid tissue are different entities; even within the tonsils, studies indicated differences between tumors arising in the epithelium of the crypts and epithelium on the tonsil surface, with different survival in relation to HPV[173, 174]. With the new TNM 8 we are approaching different treatment regimens based on HPV by proxy; p16-status. In our opinion, this is not sufficient; HPV status, subsite and possible even histopathological features within subsite should be taken into account.

In these studies, we have not made any correlation of outcome to actual given treatment. Treatment protocols have changed over time, but treatment have been consistent within each group and time-frame.Finally, our result support the theoretical view of otherOPSCC as more alike oral cancers. Together with the recent advances made in robotic surgery and transoral microsurgery this entails that a shift towards more primary surgery in selected patients possibly should be evaluated. Since the number of patients is small, this should be done in a multi-center setting.

6.2.1.4 Conclusions Paper 3:

- Patients with p16+ TSCC/BOTSC have a better long-term OS and DFS than patients with corresponding p16-negative tumors
- p16-status did not affect long-term outcome in otherOPSCC, entailing that this markers is not a suitable prognostic marker in this subgroup of patients.
- The combination of HPV and p16-status seemingly give a more correct prognostic information than p16-status alone in TSCC/BOTSCC
- Late recurrences were unusual, did not occur more often in p16+ TSCC/BOTSC vs p16-, and after recurrence p16-status was not related to outcome.

6.3 RESULTS AND DISCUSSION PAPER 4

6.3.1 Sclerotherapy with OK 432 on ranula – a prospective, randomised, double-blinded placebo-controlled study:

6.3.1.1 Aim:

In paper 4, our aim was to investigate sclerotherapy with OK-432 on ranula, intraoral and plunging, regarding efficacy, safety and the impact on QOL of the inflammatory reaction following injection.

6.3.1.2 Main results:

19 out of 20 participants finished the study.

Primary outcome was effect on cystic lesion, assessed as complete response (CR) or non-responder (NR). (For details regarding assessment of outcome see point 4.2.3and paper 4.)

In the randomized groups 6/9 patients treated with OK-432 and 2/10 patients treated with NaCl were cured, see table 3. There is a significant effect of treatment at p= 0,041.

Results of the randomized groups:

Table 3. Summary of results in the randomized groups. p=.041

Double-blinded randomized treatment:		
	Responder	**Non responder**
OK-432	6	3
Placebo	2	8

After the first part of the study, the patients treated with placebo were offered OK-432 or surgery. All patients in the placebo group with remining lesion (n=8) chose treatment with OK-432.

Summarizing both groups (9 patients randomized to OK-432 plus the 8 patients with remaining lesion after placebo treated with OK in a non-blinded setting) gives in total 17 patients treated with OK-432, 8 intraoral and 9 plunging ranulas. After this “second round” of non-blinded active treatment in the placebo-group, all the intraoral ranulas were cured, but only 2 out of 9 of the plunging see table 4.

Table 4. All patients treated

	Responder	**Non responder**
Intraoral	8	0
Plunging	2	7

Secondary outcome was the impact on quality of life after sclerotherapy with OK-432, based on EQ-5D-Sweden and patient diaries. Only 12 out of 20 randomized patients handed in the documents, but from the obtained material we learned that the reaction lasted 2-9 days, with a mild to moderate impact on quality of life. For detailed information see paper 4. A non-validated observation is that the patients that were treated in the non-blinded setting, after placebo, seemed to experience a worse impact on QOL by OK-432 sclerotherapy than the patients treated double-blinded.

Results from the calculations regarding costs per patient show that in Karolinska University Hospital the total sum of surgery (excision of the sublingual gland) was ten times higher than one injection series of OK-432 (including staff, material, drug related costs etc).

No serious adverse event was noted. Adverse events noted were one local infection after placebo injection (the one drop-out from the study) and one patient got a common cold after injection.

6.3.1.3 Discussion Paper 4:
In this study we find that sclerotherapy with OK-432 is safe, very effective in intraoral ranulas, but seemingly less effective in plunging ranulas. We can also conclude that the inflammatory reaction after injection causes an impact on QOL that the patients find acceptable.

In addition, sclerotherapy is cost-effective with a 1:10 rate versus surgery.

Part of the results in this study are somewhat surprising. First, our pilot study indicated a success rate of 80%, and that was the rate we used for the preparatory power analysis, where we also assumed no patient in the placebo group should be cured. Second, we did not anticipate there would be a difference in treatment response between plunging and intraoral ranulas.

In this study the success rate in the group randomized to OK-432 is 67 % (6/9). If we look at all patients including those treated with OK-432 after placebo, all 8 patients with intraoral ranulas were cured but only 2/9 of the plunging, thus a clear indication of difference in treatment response between the two localizations.

Regarding success rate the definition of effect in this study can be discussed. We only considered patient as a responder if total shrinkage had occurred, and no cystic lesion was present. In our experience any remaining cystic lesion, even very small, entails a recurrence eventually. But – patients can sometimes be satisfied with the decrease in volume and voluntarily postpone further injections and/or surgery.

The low response to OK-432 in plunging ranulas compared to intraoral could is interesting and needs to be studied further. Several groups have reported longer series of injections of OK 432 for lymphatic malformations, and also higher doses up to 0,3 mg OK-432/injection [175, 176]. Our choice of 2 injections is based on our pilot-study, in which average number needed for cure was 2,1, in combination with the ethical issue of delaying active treatment further for the placebo group. In Karolinska our experience has been that O,1 mg OK-432/injection (according to original protocol established by Ogita 1987 [144]) is sufficient for lymphatic malformations, but it is possible that a higher dose and/or multiple injections would yield a better outcome on plunging ranulas.

Another possible explanation is that the more complex anatomical set-up of a plunging ranula could lead to difficulties reaching the whole cyst. Or, the mechanism of sclerotherapy on ranulas could be mediated by inflammation of the sublingual gland itself? The good results in the study of chemical ablation of the sublingual gland by Brannan et al[139] indicates that could be a possible explanation. In that case, the OK-432 should be injected directly into the gland, not into the cyst, and it would explain why injection in a cyst located intraorally is more successful than injection into a cyst that has transcended into the submandibular space. For intraoral ranulas the approach of micromarsupialization is an interesting option. In Karolinska we have no experience of this, but as it is described as a simple procedure with seemingly god results and few disadvantages[138] it could be an alternative to consider.

In summary, we find that sclerotherapy with OK 432 is an easily available, safe and effective treatment for intraoral ranulas, but on plunging ranulas further studies are needed.

An overall observation is that many patients are very eager to avoid surgery; and since ranula is a benign condition, often with moderate symptoms, efforts should continue to develop minimal-invasive options further.

6.3.1.4 Conclusions Paper 4:

- Sclerotherapy with OK-432 is a safe and effective way to treat intraoral ranulas, but for plunging ranulas possible less effective
- The inflammatory response after sclerotherapy with OK-432 is well tolerable for the patient.
- Sclerotherapy is cost-effective compared to surgery with excision of the sublingual gland.

7 SUMMARY OF CONCLUSIONS

The results from paper 1-4 in this thesis suggests that:

- The prevalence of high-risk HPV and overexpression of p16 in hypopharyngeal cancer in Stockholm during the years of 2000-2013 was low, 5 %, and did not increase over time

- p16 is not a suitable marker for HPV in hypopharyngeal cancer.

- The very rare combination of HPV 16 in combination with overexpression of p16 is a possible, but not confirmed, prognostic factor in hypopharyngeal cancer.

- In patients with tonsil and base of tongue cancer, long-term overall survival and disease-free survival is correlated to p16-status, but the combination of both HPV and p16 give a more detailed prognostic information in this group. However, in patients with otherOPSCC p16-status is not a suitable prognostic marker.

- In patients with oropharyngeal cancer late recurrences were unusual, did not occur more often in patients with p16+ than p16- tumors, and after relapse p16-status was not related to outcome.

- Sclerotherapy with OK-432 is an effective way to treat intraoral ranulas, but for plunging ranulas possible less effective.

- The inflammatory response after sclerotherapy with OK-432 is well tolerable for the patient, and sclerotherapy is cost effective compared to surgery with excision of the sublingual gland.

8 POINTS OF PERSPECTIVE

Despite efforts regarding chemoradiotherapy, targeted therapy and optimized surgical methods the outcome for patients with hypopharyngeal cancer is stable and discouragingly low.. In this light, the fact that new conquests in robotic and transoral laser surgery show promising results also in patients with advanced hypopharyngeal cancer is interesting, but extensive surgery comes with a price for the patient.

HPV seems, based on the evidence to date, not to be of major importance in hypopharyngeal cancer - but the search for clinically relevant tumor markers must and will go on. If we, on the diagnostic biopsy, could identify those patients that will respond well to radiotherapy/chemoradiotherapy and those who will not, choice of primary treatment could be individualized - so, the need for further search for predictive markers in hypopharyngeal cancer is clear.

Regarding HPV and oropharyngeal cancers, we have an interesting shift ahead. Vaccination against high-risk HPV, in Sweden also offered to boys, will over time have a substantial effect not only on the main target cervical cancer, but also on oropharyngeal cancers. Will HPV-associated oropharyngeal cancer become an almost eradicated disease? Regardless, more focus should be put on the HPV-negative patients and their bad prognosis. Here prevention measures, aiming at less use of tobacco and alcohol, early detection efforts, and further search of predictive markers should be continued.

It is an interesting observation that even within subsites there could be different histopathological features (crypt/surface epithelium in tonsils for example). Our diagnostic system is based on a rough separation mainly based on clinical judgement. With the new molecular knowledge, we are in many areas moving towards precision medicine, where tumors will be mapped based on markers and more detailed histopathological features. In the future, we will probably be increasingly dependent on our pathologists, and possibly re-evaluate the way we separate subsites in the head and neck region.

HPV and its surrogate marker p16 are the first tumor markers in clinical use in head and neck cancer practice. The discovery of HPV: s role in oropharyngeal cancer is an example of how how large epidemiological studies identified a major shift, that through extensive work could be linked to viral and molecular explanations, and a causative chain could be established. However, the work to translate this knowledge into clinical practice is a complex and challenging task. The fact that the combination of HPV and p16-analyzes seemingly gives better prognostic information than either alone, should be taken into careful account in the design of de-escalation studies.

It is of great importance that a de-escalation of treatment, with a benefit regarding morbidity and side-effects for one group of patients does not entail a deterioration for another group, in the enthusiasm of having found a clinically relevant tumor marker.

Serum antibodies against E6/E7 have been suggested for screening of HPV-infection and related risk of cancer, and also as predictive markers of risk of recurrence. This is a very interesting field where prospective studies are ongoing.

Regarding ranula, it is a benign disease with moderate symptoms, and no known risk for malign transformation. The treatments we can offer our patients range from the most invasive method, excision of the sublingual gland, providing a definite cure but entailing risk of complications of surgery and anesthesia, to less invasive but possible less effective non-surgical methods. This is truly a field where patients own perception regarding the level of discomfort and preferences, can help us find the best alternative for each individual.

9 POPULAR SCIENCE SUMMARY OF THE THESIS

The head and neck area is one of the most complex parts of our human body when it comes to both topography and function. It holds many of our senses, but also other important functions such as swallowing and speech. This brings challenges when treating both malignant and benign lesions. The head- and neck surgeon and oncologists must balance the possible benefit of treatment in relation to the risk of affecting function. An overall goal, both in malignant and benign disease, is therefore finding therapies that benefits or cures the patients, but in an organ preserving way. To achieve this, new treatment modalities are evaluated, and we search for predictive factors and/or markers to identify each patient's best treatment option.

This thesis involves two quite different areas of the disease panorama in the head and neck; cancer in the throat, in two different locations, and a benign cystic lesion arising from a salivary gland in the floor of the mouth. All four papers aim at finding ways to treat patients in a more individualized manner, were individual aspects of the patients and their disease can help clinicians to choose treatment wisely.

Head- and neck cancer (HNSCC) is the sixth most common type of cancer in the western world, in Sweden about 1600 new cases are diagnosed each year. Traditional risk factors for head and neck cancer are tobacco and alcohol. However, since the 80`s it has been noted that the number of new patients diagnosed with tonsil and base of tongue cancer is increasing, and that many patients are now younger and without the "regular" history of tobacco and alcohol. Studies by our research group and others have established that this increase is correlated to human papilloma virus (HPV) – a virus since long recognized to cause cancer in the cervix (sv: livmoderhals) - also in the tumors in tonsils and base of tongue. Furthermore, tumors that are caused by HPV have a better survival than those without HPV.

One of the mechanisms involved when HPV causes cancer includes an increased level of a protein named p16 in the affected cell, and abundance of this protein has been found to correspond very well to HPV-positive tumors. Since p16 is easier to analyze than HPV-DNA it is sometimes used as a surrogate marker of cancer caused by HPV.

The knowledge about HPV and p16 in relation to survival are mostly based on patients with tumors in the dominating subsites tonsillar cancer and cancer in the base of the tongue. However, the relationship between HPV, p16 and the development of cancer is not so clear in other subsites of the throat such as the back wall and the uvula, and in the lower part, the hypopharynx.

Patients with cancer in the lower part of the throat, the hypopharynx, are the subgroup of patients with head- and neck cancers who has the worst prognosis, only around 30% of patients are still alive 5 years after diagnosis. Treatment is most often oncological treatment with radiotherapy and sometimes chemotherapy, and few patients go to primary surgery. Some patients respond very well to radiotherapy, and some not at all- if we could find markers already at diagnosis that we could connect to treatment response it would help us chose treatment for each patient in a better and individualized manner.

Since HPV has been established as a risk factor of oropharyngeal cancer, interest has grown to investigate if HPV has an impact also in other subsites of the head- and neck area. In the first two papers of this thesis, we investigate HPV and p16 and the relation to cancer in the lower part of the throat, the hypopharynx. In these papers we analyze data from tumor samples and the patients' chart retrospect. We find that HPV is uncommon in hypopharyngeal cancer, that the correlation to elevated levels of p16 is low, and that this situation did not change over time 2000-2011. From this we draw

the conclusion that HPV is not an important biomarker in hypopharyngeal cancer, and that p16 is not to be used as a surrogate marker for HPV-positivity in this subsite.

There have been observations reporting that tumors caused by HPV show a tendency to recur later than HPV-negative, but the long-term results of patients with cancer in the upper part of the throat – the oropharynx - have not been well studied. In the third paper we analyze the long-term survival and pattern of recurrence in patients with oropharyngeal cancer. We find that late recurrences (after more than 5 years) were rare, both in HPV + and HPV – patients, and that prognosis after recurrence was bad regardless of HPV-status.

In addition, we find that in patients with tonsillar and base of tongue cancer an elevated level of p16 clearly corresponds with less risk of developing recurrence . However, in patients with cancer in non-tonsillar, non-base of tongue oropharynx (back wall, uvula etc) we find no such correlation. In the group of tonsillar and base of tongue cancers, we find that the combination of testing both HPV and p16 gives a more detailed prognostic information than testing only p16. From this we conclude that it is important to take into account which subsite the tumor is found in, and to analyze both p16 and HPV, when tailoring future treatment protocols and clinical studies.

Ranula is a benign disease in which a pseudocyst is arising from the sublingual gland. Ranula can be located either within the mouth, called intraoral ranula, or pass out to the neck, then named plunging (diving) ranula. Traditional treatment of ranula is surgery, which is performed under general anesthesia and entails risk of complications such as nerve damage and infection.

Paper No 4 aims at testing a less invasive treatment, sclerotherapy with OK 432– a method where we, instead of using surgery, inject a substance in the cyst in order to cause inflammation, and consequently shrinking and processes resulting in that the tissue adhering together. This study was conducted in a “looking-ahead”, randomized, double-blinded fashion, meaning that either patient nor doctor knew if the patient received active agent or placebo. The aim was to evaluate the efficacy, but also to assess how difficult the inflammatory reaction was for the patients. The results showed that intraoral ranula responded very well to the treatment, but on plunging ranulas the treatment was seemingly less successful. The inflammatory response was well tolerated by the patients, and no unexpected side effects were noted. In addition, sclerotherapy is cost effective compares to surgery. Since ranula is a rare condition, it is difficult to gather many cases and thus harder to show convincing statistical proofs, but the method is less invasive than surgery and patients have been very interested to participate in the study, despite the risk of first going through placebo-injections and thus delay of active treatment.

Our opinion is that these results, supported by other researchers’ findings, should be enough to establish sclerotherapy with OK 432 as an alternative primary treatment for patients with intraoral ranula, and we judge it wise to conduct further studies of this minimal-invasive method on plunging ranula.

In conclusion, his doctoral project includes studies aiming to gain more detailed knowledge on factors relevant for choosing the best treatment regimen for patients with hypopharyngeal cancer, oropharyngeal cancer and ranula. Hopefully some pieces of the complex puzzles at issue have been added by this work, to be continued……

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