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Oral and Oropharyngeal Cancer –

Aspects on Epidemiology and

Prognostic Factors

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Karin Annertz

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Abstract

Head and neck cancer is a heterogeneous group of tumours, although histopathologically, >95% are squamous cell carcinomas (SCC). Some tumours respond better to therapy than others. Not enough is known to predict the outcome and tailor individualised treatment. Although one of the main risk factors, smoking, has decreased over the last decades, some head and neck cancer groups show increasing incidence. Oral and oropharyngeal SCC (OOPHSCC) are amongst them. High risk human papillomavirus (HPV) has been reported to be found in a high proportion of OOPHSCC, especially in oropharyngeal cancers. It is presumed to be the reason for the incidence increase of oropharyngeal SCC, but not for oral SCC. Many studies, but not all, show better survival for patients with HPV positive tumours. Different methods for HPV detection are being used, making it difficult to compare results. Alpha B-crystallin, a small heat-shock protein, has in a previous study been found to be an independent prognostic marker for poor outcome in head and neck SCC.

This thesis aimed to investigate the epidemiological changes for tongue cancer in the Nordic countries and to compare some possible predictive factors for OOPHSCC. During the period 1960-1994, an incidence increase in SCC of the mobile tongue in all ages, except for women aged 65-79, was observed. Data including site and histology was obtained from the national cancer registries. The increase was most pronounced in young adults (20-39 years), with a five-fold increase in men and a six-fold increase in women. Young adults had a significantly better crude, as well as relative, survival compared to older patients. In a follow-up study, the incidence trends were studied, extending the study period by 14 years, extracting data from the NORDCAN registry. The incidence increase continued for all ages, except for in young adult men.

In a consecutive series of 128 OOPHSCC, no difference in five year survival was found between patients with HPV positive and HPV negative tumours. Sample collection method was by cotton tipped swab and mouth wash (SMC). When 91 patients from the same study population were analysed, instead using formalin fixed paraffin embedded tissue from their diagnostic biopsies (FFPE), again no survival difference was found. The rate of inconsistent results between SMC and FFPE was 27%.

In a set of 55 OOPHSCC, alpha B-crystallin was found to be a strong independent prognostic marker for poor prognosis in oral SCC, but not in oropharyngeal SCC. The findings highlight the fact that more and more differences between oral and oropharyngeal SCC are being revealed.

Key words: Tongue cancer, SCC, oral cancer, oropharyngeal cancer, survival, incidence, HPV, human papillomavirus, alpha B-crystallin, sample collection methods, prognostic factor

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Abbreviations

AB	Alpha B-crystallin
ANCR	Association of the Nordic Cancer Registries
BoT	Base of tongue
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
DFS	Disease free survival
DNA	Deoxyribonucleic acid
DoD	Dead of disease
DSS	Disease specific survival
ECM	Extra cellular matrix
EGFR	Epidermal growth factor receptor
ENT	Ear, Nose and Thorat
FFPE	Formalin fixed paraffin embedded
G1	Gap 1 phase of the cell cycle
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
IACR	International Agency for Research on Cancer
IHC	Immunohistochemistry
MDM	Multi disciplinary meeting
NCI	National Cancer Institute
OC	Oral cavity
OOPHSCC	Oral and oropharyngeal squamous cell carcinoma
Ophx	Oropharynx
OS	Overall survival
OSCC	Oral squamous cell carcinoma
PAI-1	Plasminogen activator inhibitor type 1
PCR	Polymerase chain reaction
pRB	Retinoblastoma protein
QIMR	Queensland Institute of Medical Research
QoL	Quality of life
SCC	Squamous cell carcinoma
SEER	Surveillance, Epidemiology and End Results programme
sHSP	Small heat shock protein

SMC	Swab and mouthwash collection
SPARC	Secreted protein acidic and rich cysteine
SPT	Second primary tumour
SweHNCR	Swedish Head and Neck Cancer Register
uPA	Urokinase type plasminogen activator
WHO	World Health Organization

List of papers

Paper I

Karin Annertz, Harald Anderson, Anders Biörklund, Torgil Möller, Saara Kantola, Jon Mork, Jörgen H Olsen, Johan Wennerberg.

Incidence and survival of squamous cell carcinoma of the tongue in Scandinavia with special reference to young adults.
Int J Cancer 2002;101:95-99

Paper II

Karin Annertz, Harald Anderson, Karolina Palmér, Johan Wennerberg

The increase in incidence of cancer of the tongue in the Nordic countries continues into the twenty-first century
Acta Otolaryngol. 2012 May;132(5):552-7. Epub 2012 Feb 19

Paper III

Karin Annertz, Kerstin Rosenquist, Gunilla Andersson, Helene Jacobsson, Bengt Göran Hansson, Johan Wennerberg

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Acta Otolaryngol. 2014; June 16: 1-9

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Submitted June 2014

Paper V

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Alpha B-Crystallin - a validated prognostic factor for poor prognosis in squamous cell carcinoma of the oral cavity
Acta Otolaryngol. 2014; May: 134(5):543-550

Introduction

Head and neck cancer is a very heterogeneous group of tumours, yet in many ways treated similarly. The majority of malignant head and neck tumours arise from the squamous cells in the mucosa, lining the oral cavity, the oropharynx, the hypopharynx and the larynx, which gives them the name of squamous cell carcinomas (SCCs). Examples of other, much less common, tumour types are adenocarcinomas, e.g adenoid cystic cancer (usually emanating from salivary glands) and sarcomas arising from connective tissues.

Patients with squamous cell carcinomas are treated with surgery, radiotherapy and chemotherapy, quite often in combination. For head and neck cancer in general, the smallest tumours can usually be treated with surgery or radiotherapy alone, while more advanced tumours in most cases are treated with a combination of surgery and radio/chemotherapy. There are exceptions though, due to either differences in surgical accessibility or expected response to therapy. One example in the Swedish treatment guidelines, is oropharyngeal cancer, that respond very well to radiotherapy and, thus, small tumours are treated with full dose radiotherapy alone, while surgery is saved for recurrences. Another example, where radiotherapy is used as mono-therapy for small tumours, is hypopharyngeal cancer, where surgery is more difficult to perform.
Side effects can be severe and include xerostomia, dysphagia, trismus, dysphonia, pain and disfigurement, often with severe impact on quality of life (QoL). In general, the more treatment modalities included, the more side effects.

There is a very heterogeneous response to therapy between the subgroups of SCCs, and also between tumours from within the same site. Although much effort has been made to improve survival, no major survival improvement has been reached over the last decades; five-year survival figures for the whole group of head and neck squamous cell carcinomas (HNSCC) still remain below 50% but with great variation between sites.

Overall, HNSCC is the sixth commonest cancer worldwide with about
690 000 new cases diagnosed per year. Within the group of HNSCC, there are epidemiological movements in different directions, some sites with increasing incidence, others decreasing. This is an interesting phenomenon, given the major risk factors for HNSCC are smoking and excessive alcohol intake, which habit changes could be expected to show the same epidemiological response, regardless of the site. This is not the

case, which tells us there must be other factors contributing to carcinogenesis and epidemiological changes.

Over the last decades, research within the field of viral and other infectious diseases has revealed a range of agents capable of causing malignant tumours, the most well known example being the impact on uterine cervical cancer by human papillomavirus (HPV). Over the last ten years, also HNSCC has been reported to have connection to HPV infection and it is now widely accepted that HPV is involved in one way or another in a subset of HNSCC, most commonly the tumours arising from the tonsillar fossa and the base of tongue.

It is important to follow and document epidemiological changes over time to be able to chase down and find what factors might be involved in the incidence changes of malignant diseases. Also, the biology behind the tumourigenesis is crucial to the endeavour to improve treatment and find new therapeutic agents with possible better effect but also better side-effect profile than the ones available today.

This thesis reflects on both epidemiology and prognostic factors in the context of oral and oropharyngeal squamous cell carcinoma (OOPHSCC).

Epidemiology and time trends of head and neck cancer

WHO's organ, International Agency for Research on Cancer (IARC), has launched the Globocan project with the aim to estimate incidence, prevalence and survival of the major types of cancer from 184 countries.

In 2012, about 14.1 million new cancer cases were reported worldwide. About 690 000 of them were cancers arising from the head and neck region (lip and oral cavity, nasopharynx, other pharynx and larynx). This does not include the approximate 300 000 new cases of thyroid cancers. The same year, about 8.2 million cancer deaths were reported (Globocan 2012, www.globocan. c.fr).

In 2000, an average incidence rate of 8.8 and 5.1 per 100 000 for men and women respectively was reported. An average mortality rate during the same time was 7.3 and 3.2 per 100 000, men and women respectively (Shibuya and Mathers 2002). The incidence rates vary greatly, both between regions and between men and women. In 1993-1997 the highest incidence rate for men was found in Somme, France, with 43.1 new cases per 100 000 whereas the highest incidence for women was reported from Bangalore, India, with 11.2 new cases per 100 000. Lowest rates were found in Quito, Ecuador, for men, averaging 2.4 new cases per 100 000, and in Kanghwa county, Korea, for women, with 0.5 new cases per 100 000 reported (Parkin 2002).

The reporting of cancer to national registries varies widely around the world. In Scandinavia, compulsory registries have been in use for a long time, giving population-based epidemiological studies an excellent ground. The Danish cancer registry started in 1942, the Norwegian, Finnish and Icelandic in 1951-1954 and the Swedish in 1958. The Association of the Nordic Cancer Registries (ANCR) was founded in 1984 to facilitate joint research. In 2000, the NORDCAN program started, enabling comparative research between the countries by converting data from each registry into similar format. In other parts of the world, epidemiology data are based on reporting to non population-based registries. Globocan classifies the quality of data (A-G) from countries and regions dependent on their coverage, where the highest classification, A, is given if coverage is >50%. The Nordic countries are all A-classified, covering >95% of all cases.

Smoking and alcohol are the two major risk factors for HNSCC arising from the upper aerodigestive tract. During the first half of the 20th century, the incidence of HNSCC constantly increased. Although the tobacco plant has been known to have grown in the US for millennia (Kim and King 2010), it was not until late 19th century that cigarettes became popular and tobacco reached its greatest popularity during the first half of the 20th century. It was then widely socially accepted amongst both men and women; more then half of all American men smoked and about a third of the women. When tobacco smoking was linked to increased risk of heart disease, lung cancer and head and neck cancer (Wynder and Bross 1957), efforts to decrease smoking resulted in a (still ongoing) decline in numbers of people smoking.

As a result of this, tobacco related cancers started decreasing a few decades later and in the last two decades there has been a continuous incidence decrease in HNSCC. This is not true for individual sites within the heterogeneous group of HNSCC though, oropharyngeal cancer and cancer of the mobile tongue, especially in younger individuals, increasing in some reports from different parts of the world (Shemen and Klotz 1984, Schantz and Byers 1988, Conway and Stockton 2006, Hammarstedt and Dahlstrand 2007, Warnakulasuriya 2009, Annertz and Anderson 2012).

Figures 1-4 show the incidence trends in Sweden, for four different head and neck cancer sites. Lip cancer, which is strongly associated with sun exposure and smoking, decreases rapidly in men but increases in women. Also laryngeal cancer, which is tobacco related, decreases rapidly in men but stays fairly stable in women. Oral and oropharyngeal cancer increases in both sexes. Smoking habits have not changed as much in women as in men, which is reflected in the trends of lip cancer and laryngeal cancer. Table 1 shows the proportion of daily smokers in Sweden during 1980-1996, which should be reflected in today's incidence figures of smoking related cancers. Getting suntanned has for the last few decades been a cosmetic ideal in Sweden, especially for women, which most likely accounts for most of the increase in lip cancer in the female group.
Altogether, the decrease due to declining smoking habits is overridden by the increase seen in oral and oropharyngeal cancers, showing a climbing incidence curve when looking at the HNSCC group as a whole (NORDCAN).

Table 1. Smoking in Sweden, 1980, 1985 and 1996 as per gender (W=women, M=men) ("Women and men in Sweden 1998", Statistics Sweden)

Daily smokers by age in 1980, 1985, and 1996

Proportion (%) in age group

Age	1980		1985		1996	
	W	M	W	M	W	M
16–24	37	28	30	23	23	16
25–44	40	42	37	33	27	21
45–64	24	37	27	34	27	27
65–74	14	32	13	25	17	18
75–84	4	25	6	21	9	13
Total	29	36	27	30	23	21

Source: Survey of Living Conditions, Statistics Sweden

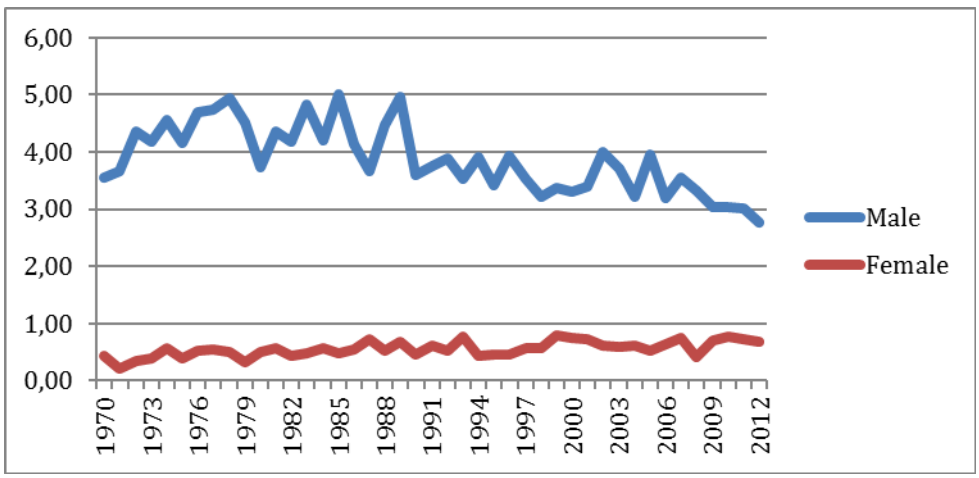

Figure 1. Laryngeal cancer. Incidence per 100 000 in Sweden 1970-2012

(The Swedish National Board of Health and Welfare, statistics database 16-05-2014)

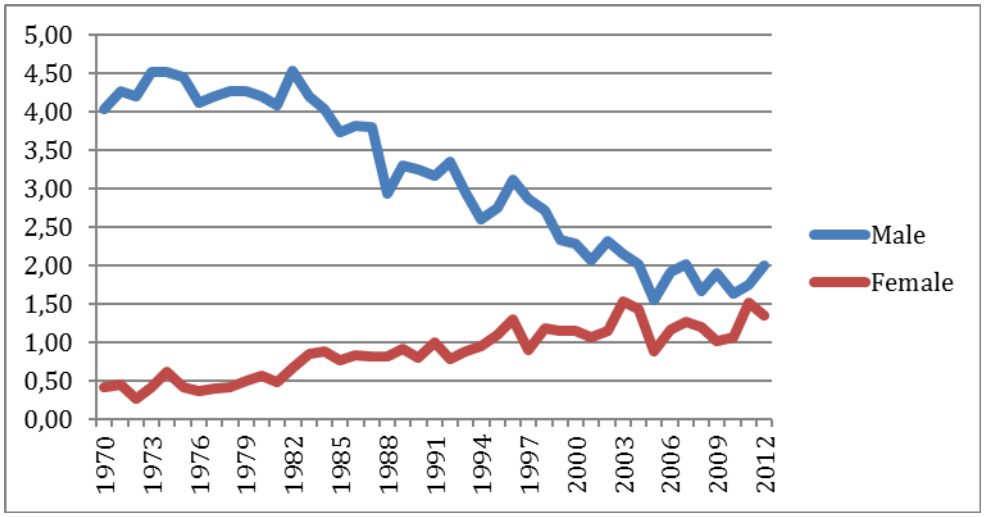

Figure 2. Lip cancer. Incidence per 100 000 in Sweden 1970-2012

(The Swedish National Board of Health and Welfare, statistics database 16-05-2014)

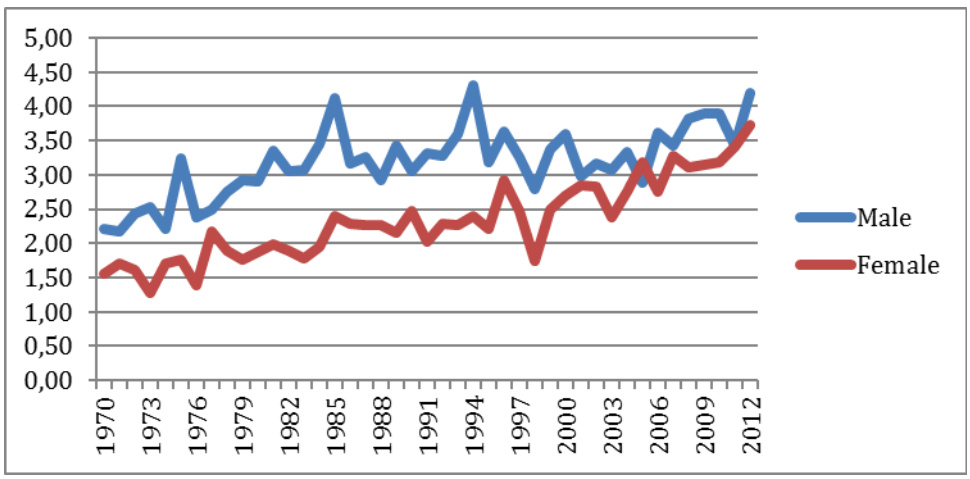

Figure 3. Oral cancer. Incidence per 100 000 in Sweden 1970-2012

(The Swedish National Board of Health and Welfare, statistics database 16-05-2014)

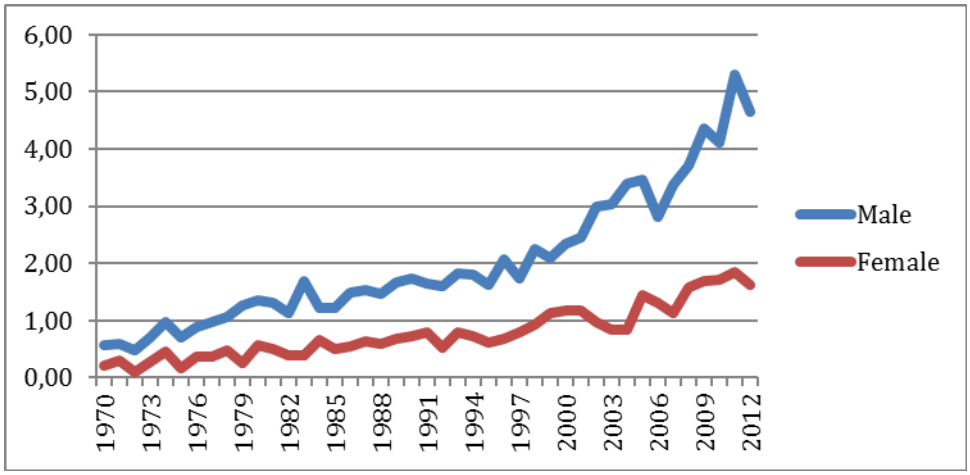

Figure 4. Oropharyngeal cancer. Incidence per 100 000 in Sweden 1970-2012

(The Swedish National Board of Health and Welfare, statistics database 16-05-2014)

The increase in tongue cancer in young adults seems to appear in an age group even younger than the group of young patients with increasing oropharyngeal cancer. The increase in young tongue cancer patients has been reported in the group of patients <45 years, whereas for oropharyngeal cancer the most pronounced increase is seen in patients in their fifties. Also, the increase in oral cancer starts at an older age for women than for men, whereas it starts at the same age for both sexes in oropharyngeal cancer, Figure 5-6. Reports are unequivocal though, and some reports cannot confirm an increase in young adults regarding these sites (Braakhuis and Visser 2009).

Interestingly, oral cavity cancer and oropharyngeal cancer differ in yet other epidemiological aspects; the male:female ratio for oral carcinoma being almost 1:1 whereas it is close to 3:1 for oropharyngeal carcinoma, Figure 7.
Most epidemiological reports on HNSCC lately have focused on the incidence increase in oropharyngeal cancer. This is often referred to as being caused by human papillomavirus (HPV), which first was associated with oral cancer in 1983 (Syrjanen and Syrjanen 1983), and is considered a risk factor for HNSCC, particularly oropharyngeal cancer, especially in young adults and more commonly with no association with smoking or alcohol.
What is more unclear is the incidence increase in cancer of the tongue, since most reports in the literature cannot find at all as high a proportion of these tumours being positive for HPV deoxyribonucleic acid (DNA) as in the oropharyngeal group (Ha and Pai 2002, Dahlgren and Dahlstrand 2004, Hansson and Rosenquist 2005, Liang and Lewis 2008), thus requiring another explanation for its increase. This has not gained as much attention in the literature as the HPV connection to oropharyngeal cancer.

These differences reasonably reflect discrepancies in tumour biology between oral and oropharyngeal squamous cell carcinoma and highlight the theory of tumours arising from these two different sites being two different entities.

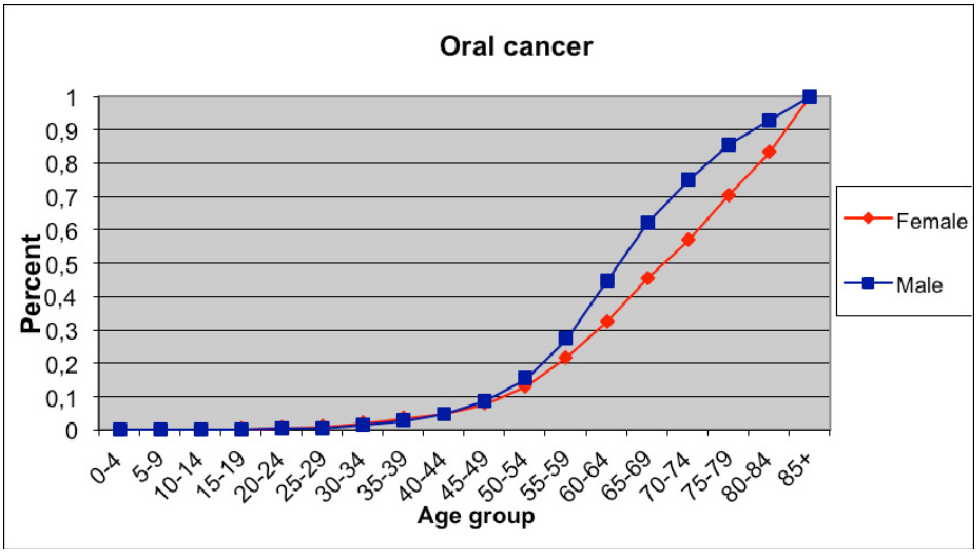

Figure 5. Oral cancer. Relative cumulative incidence, in Sweden, years 2005-2012

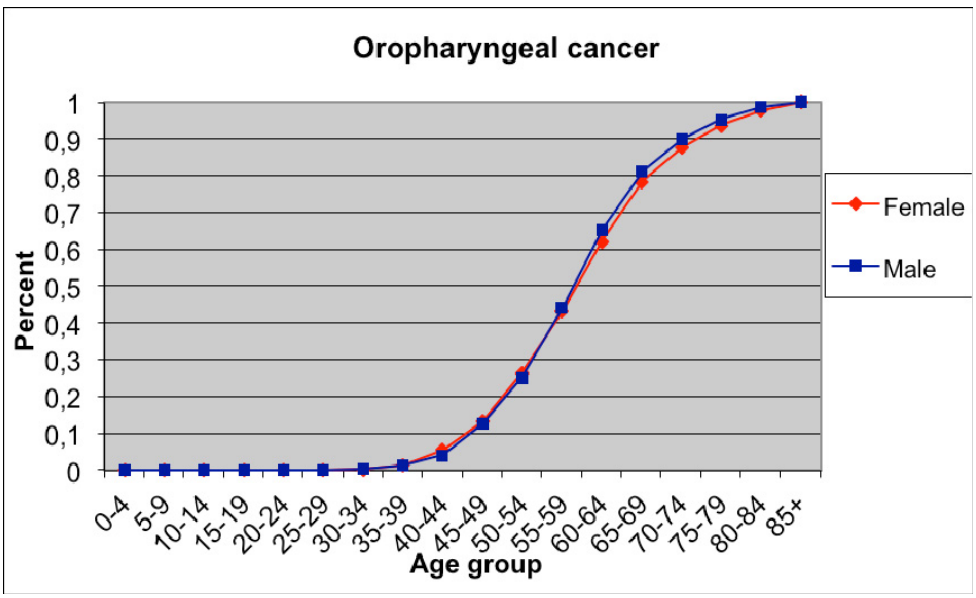

Figure 6. Oropharyngeal cancer cancer. Relative cumulative incidence, in Sweden, years 2005-2012

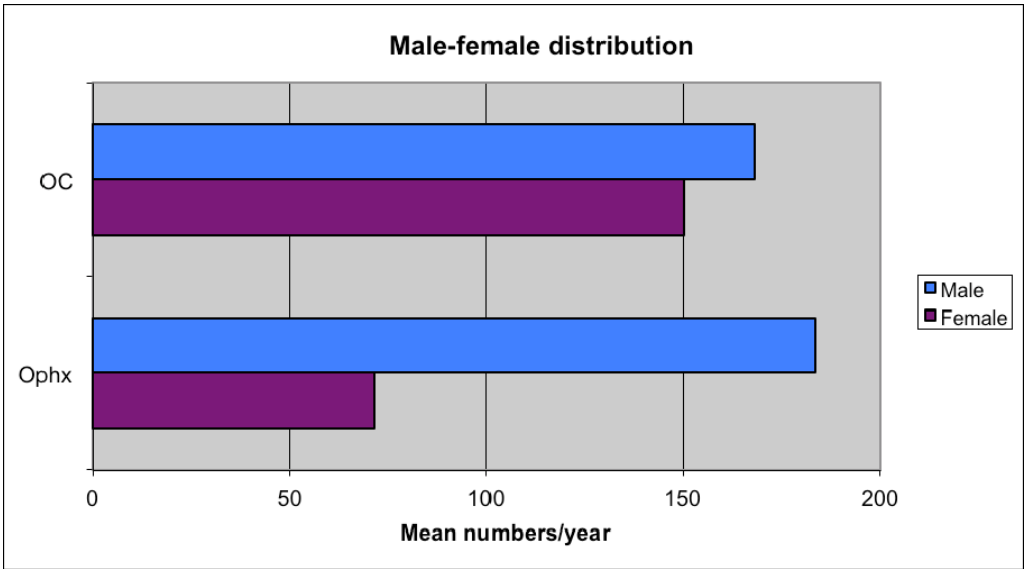

Figure 7. Male to female ratios. OC=oral cavity tumours. Ophx = oropharyngeal tumours. Mean numbers/year in Sweden during the period 2008-2012.

(Figure 5-7 based on data extracted from *The Swedish National Board of Health and Welfare, statistics database*)

Prognostic, predictive and risk factors in OOPHSCC

The terms *prognostic factor, predictive factor* and *risk factor* are used frequently in cancer research. The distinction between them can sometimes appear unclear and the definitions merge into each other.

"**prognostic factor:** A situation or condition, or a characteristic of a patient, that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring (coming back)."
"**predictive factor:** A condition or finding that can be used to help predict whether a person's cancer will respond to a specific treatment. Predictive factor may also describe something that increases a person's risk of developing a condition or disease."
"**risk factor:** Something that increases the chance of developing a disease. Some examples of risk factors for cancer are age, a family history of certain cancers, use of tobacco products, being exposed to radiation or certain chemicals, infection with certain viruses or bacteria, and certain genetic changes"
(*National Cancer Institute (NCI) Dictionary of Cancer Terms*)

The most useful *prognostic factor* for OOPHSCC, so far, with impact on choice of treatment, is the TNM-classification (see below). Age is another prognostic factor as well as histopathological staging.

In recent years, HPV has been presented as a possible, future candidate for de-escalated therapy (see below) in selected cases. p16 over-expression is another prognostic factor, strongly linked to the HPV, regarding favourable outcome.
Alpha B-crystallin, a small heat-shock protein (sHSP), has been suggested to be a prognostic factor for poor outcome in HNSCC (Chin and Boyle 2005, Annertz and Enoksson 2014,), but further studies are required to determine its possible applicability regarding OOPHSCC assessment (see below).

HPV is additionally considered a *predictive factor* for developing OOPHSCC, which also makes it a *risk factor* for OOPHSCC. Most common *risk factors* associated with OOPHSCC are smoking and excessive alcohol consumption. Another known *risk factor* for OSCC is poor dental hygiene (Rosenquist and Wennerberg 2005).

Prognostic factors in OOPHSCC

TNM-stage classification

For decades, the Tumour, Node, Metastasis classification (TNM), based on tumour size (T), regional lymph node metastasis status (N) and distant metastasis status (M) has been used to estimate the prognosis in HNSCC, thus also for OOPHSCC. It is routinely used in assessment of all OOPHSCC cases. The classification was developed in 1943-1952 by Pierre Denoix (PF 1944, PF 1945, PF 1950, PF 1952) and has been revised many times over the decades ever since.
For example, a T1 (tumour <2cm), N0 (no regional lymph node metastasis) and M0 (no distant metastasis) condition, is usually considered having the best prognosis while a very advanced stage, T4 (large tumour, invading adjacent sites or destroying cartilage or bone structures), N3 (extensive cervical lymph node involvement) and M1 (presence of distant metastasis) is known to have a very poor prognosis and is incurable. However, this classification is rough and the prognosis cannot be predicted with accuracy. For example, there are small primary tumours with advanced neck node metastasising and vice versa.
To address this issue, a staging system I-IV has been invented, taking all the different combinations of the T-, N- and M-status into account (Sobin LH 2002). Although the TNM classification can be useful when looking at a whole group of patients, still, the prognosis is hard to predict in individual cases of OOPHSCC using the TNM classification system; some patients recover from their cancer while others die from it rapidly, although exactly the same TNM-status has been confirmed (Montero and Yu 2014).

Age

Most patients diagnosed with OOPHSCC are in their late fifties or older, most commonly in their late sixties. Survival decreases with increasing age. Camilon et al show that overall survival (OS) as well as disease specific survival (DSS) decreases significantly in patients with oropharyngeal cancer, 65 years and older. Also Jones et al found the same for patients with oral cancer, as well as oropharyngeal and laryngeal cancer (Jones and Beasley 1998). The reason for worse survival in this group of patients might be multi-factorial, as ageing itself might make people more susceptible to developing cancer as well as not tolerating the treatment and its side effects as well, due to a higher rate of other medical conditions compared to younger patients.
While increasing incidence of tongue carcinoma in young adults (<45 years) has been reported, it has been found these young patients have a better survival compared to older patients by some authors (Clarke and Stell 1992, Annertz and Anderson 2002,). Yet others have found the opposite, with worse outcome for young adults (Byers 1975,

Depue 1986, Sarkaria and Harari 1994). Since OOPHSCC is rare in young adults, a problem with many reports is that they are based on small numbers and selected material.

Histopathologic staging

In 1920, AC Broders published the first paper in a series of four, presenting a tumour grading system based on tumour cell differentiation. Percentage of the tumour showing incomplete differentiation was divided into four grades; grade 1 the lowest grade tumour and grade 4 with the highest percentage of incomplete differentiation. This method has been widely used worldwide as a prognostic factor for squamous cell carcinoma (SCC) and is still in use. However, its correlation with prognosis has not shown to be very strong, while it has been modified by several authors (Ivkic M 2002). Anneroth et al presented a refined version for oral squamous cell carcinoma, which is still in use in Sweden. The system takes into account the heterogeneity within the tumour, which is extensively recognised as a feature of HNSCC, using parameters such as structure, degree of keratinization, nuclear polymorphism, number of mitoses, mode and stage of invasion, lymphocytic infiltration and vascular infiltration (Anneroth and Batsakis 1987). This has shown better accuracy with regards to survival and response to therapy.
Brandwein-Gensler et al have recently presented yet another morphological system with prognostic value in oral SCC. The system evaluates three parameters in surgical specimens: pattern of invasion, inflammatory response and neural invasion and showed predictive value for decision-making regarding post-operative radiotherapy (Brandwein-Gensler and Smith 2010).

The benefits of the histological grading systems are equivocal though, (Rodrigues and Miguel 2014) and the TNM-classification remains more reliable, despite its great limitations.

Human papillomavirus (HPV)

Papillomaviridae is the virus family to which all human papilloma viruses belong. The virus comprises a circular DNA and targets the basal cells of the epithelial mucosa. More than 180 different types of HPV have been characterised along with several sub-types (de Villiers and Fauquet 2004). HPVs are divided into cutaneous and mucosal types, based on their tissue tropism. Furthermore, they are divided into high-risk and low-risk types based on their ability to cause malignant transformation. The most well known benign skin lesion caused by HPV is the normal common wart. Other, benign HPV-caused

lesions are mucosal papillomas, e.g. in the larynx or the nasal cavity and chondylomas in the ano-genital region (Krueger H 2010).

HPV was established, since the recognition of its association with precursor lesions in the uterine cervix in the1980s, as the main cause of uterine cervical cancer (zur Hausen 2009). Studies have demonstrated that close to 100% of uterine cervical cancer harbour the virus. More than 50% of the cancers are found to harbour HPV 16 DNA, one of the subtypes covered in the vaccine that has been introduced in the western world in the last decade (Villa and Costa 2005). The second commonest type is HPV 18, also covered by the vaccine. Also, 45-78% of other ano-genital cancers are regarded as HPV-related, still with the HPV 16 as the most common type (Krueger H 2010).

The human body clears most HPV infections spontaneously. About 60% of uterine cervical HPV infections are cleared within a year and 90% within three years (Rodriguez and Schiffman 2008). The remaining 10% stay as persistent infections and might, due to risk factors or potential immune factors, promote the cell to malignant transformation. In contrast to the latent viral infection with viral DNA in an extrachromosomal form, it is thought to require integration of viral DNA into the host cellular genome to cause this transformation (Krueger H 2010). Expression of the viral oncogenes E6 and E7 interact with the p53 and the retinoblastoma protein (pRb) respectively, proteins that are involved in the healthy pathway of cell apoptosis (McMurray and Nguyen 2001). The cell loses its normal life cycle and transforms into a malignant cell. This is an established process regarding HPV infection and uterine cervical cancer.

High-risk HPV infection has also, during the last decade, been confirmed as an involving factor in a subset of HNSCC, especially from the tonsillar fossa and the base of tongue (Hansson and Rosenquist 2005, Ragin and Taioli 2007). One difference, compared to uterine cervical cancer, is a lower prevalence of HPV in OOPHSCC. Study reports of HPV prevalence in OOPHSCC vary greatly, with rates between 2 and 100%. In the oropharynx, the majority of studies present a prevalence of HPV greater than 50% but less in the oral cavity (Hansson and Rosenquist 2005, Bragelmann and Dagogo-Jack 2013). As mentioned, within the oropharynx, the HPV positivity is highest in the tonsillar fossa and base of tongue, and lower in other oropharyngeal subsites such as the soft palate. Also, within the oral cavity there seems to be differences between the subsites, the floor of mouth presenting higher proportions of HPV positive cases than the mobile tongue and the buccal mucosa (Hansson and Rosenquist 2005).

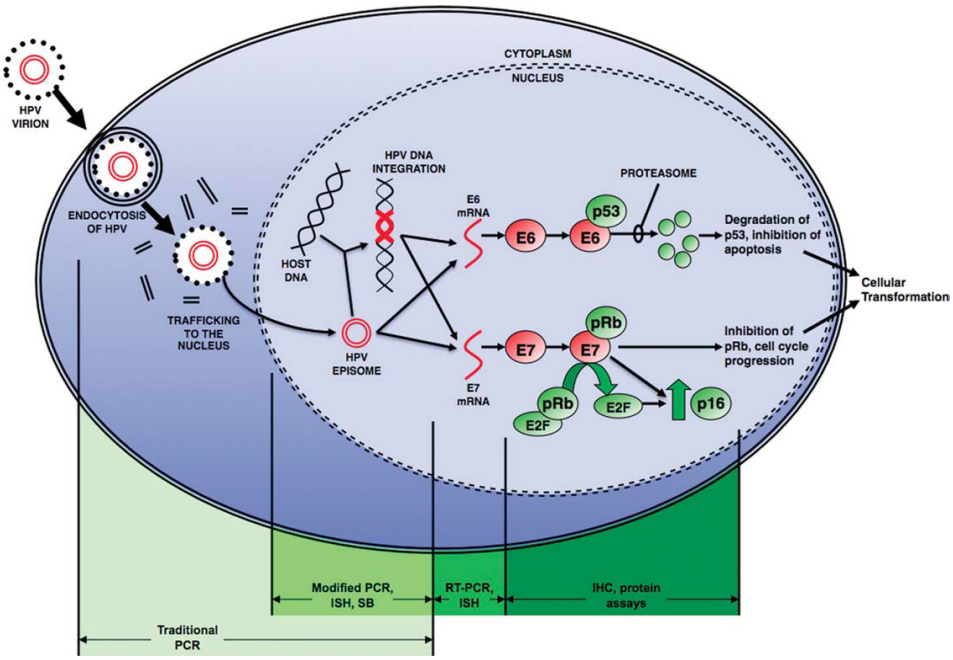

Figure 8. "Schematic of HPV infection of a mucosal cell. After virion entry via endocytosis, the virus establishes a persistent infection as a viral episome or integrates into the host genome. HPV E6 and E7 oncoproteins are expressed from both forms of the viral DNA, which lead to p53 degradation and Rb inhibition, respectively. Methods of HPV, oncogene or p16 detection are depicted with respect to stage of HPV biologic activity." (Allen and Lewis 2010)

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Also in HNSCC, HPV 16 is the predominant type in the majority of studies. However, this does not seem to be applicable in some geographical areas. HPV 18 has been reported as the major HPV type in OOPSCC in reports from Sicily, Greece, India and Taiwan (Balaram and Nalinakumari 1995, Aggelopoulou and Skarlos 1999, Giovannelli and Campisi 2002, Chang and Lin 2003). An interesting remark on this note is that HPV 18 was found to be the predominant type in cervical cancer in an Indonesian study (Bosch and Manos 1995). This shows that the virus, like other infectious agents, behaves contagiously, thus different types and subtypes predominate in different geographical areas.

There are other observed differences between HPV in the uterine cervix and HPV in the oral cavity and oropharynx. In contrast to the uterine cervix, HPV presence increases in the oral and oropharyngeal region with age (D'Souza and Fakhry 2007, Krueger H 2010).

It is clear that HPV in the uterine cervix is a sexually transmitted disease, whereas it is not clear how the HPV makes its way to the oral mucosa. Evidence shows that women with cervical HPV infection have a higher risk of oral mucosa HPV infection. Interesting in this matter though, is the findings of precise HPV types in cervix and oral region are not identical in the majority of cases (Smith and Ritchie 2004, Smith and Ritchie 2004, Rintala and Grenman 2006, Fakhry and D'Souza 2006,).

As mentioned before, expression of the viral oncogenes E6 and E7, through integration of the HPV DNA into the host genome, is required for malignant transformation and maintenance of the malignant phenotype. It is assumed to be valid also for OOPHSCC although not proven. In uterine cervical cancer, the viral genome is almost always found integrated into the host genome. In OOPHSCC HPV positive cancers, the HPV physical status can be found episomal, integrated or in a combined mixed form (Kim and Koo 2007, Allen and Lewis 2010, Deng and Hasegawa 2013) and not all HPV positive HNSCC express E6 and E7 (Ragin and Modugno 2007). With this background, Krueger et al suggest it is a smaller proportion of OOPHSCC being attributable to high-risk HPV infection than the literature sometimes has concluded (Krueger H 2010). In contradiction to this, some authors have found that E6 and E7 also are expressed in some OOPHSCC where the physical status of the virus has been found to be episomal and not integrated, suggesting viral integration is not a requirement for malignant transformation (Mellin and Dahlgren 2002), Figure 8.

A surrogate marker for HPV positivity is detection of the tumour suppressor protein p16, which gets over-expressed via the oncoprotein E7's inactivation of the tumour suppressor protein pRb (Allen and Lewis 2010), Figure 8. p16 is now routinely used in clinical practice to assess the HPV status of HNSCC. It is an easy and low cost immunohistochemical analysis. Important to remember is that p16 over-expression is not pathognomonic for HPV infection although the association with HPV is very high. The sensitivity for p16 regarding HPV infection has been described as high as 100%, whereas the specificity does not get higher than approximately 80% (Rietbergen and Leemans 2013). This means that the method finds about 20% false positive cases, something to consider when discussing de-escalation of treatment to HPV positive patients based on their p16 status.

To measure the prevalence of HPV in OOPHSCC, many different methods are being used, which might account for some of the great variability in prevalence figures. Methods in use are, for example, polymerase chain reaction (PCR), immunohistochemistry (IHC) and *in situ* hybridization (ISH) (Allen and Lewis 2010, Mirghani and Amen 2014). The methods detect the HPV in different stages of the HPV's biological activity, as shown in Figure 8. Also different consensus primer sets for HPV DNA PCR are used, e.g. MY09/11 and GP5+/6+. Methods for sample collection is yet another variable, including swabs, mouth rinses, brush and biopsies (Lawton and

Thomas 1992, Furrer and Benitez 2006, Mirghani and Amen 2014). Exfoliated cells or tissue are also treated in different ways, i.e. freshly frozen or in formalin for paraffin embedding. All this taken into account makes it difficult to compare results from different studies while a more uniform approach is warranted for future studies.

Alpha B-crystallin

Alpha B-crystallin is a protein that belongs to the sHSP, also known as the sHPS20 family. For a long time, it has been known that crystallins are found in the eye lens where they maintain the transparency and the refractive index (Augusteyn and Parkhill 1992, Arrigo and Simon 2007,). Three crystalline types, alpha, beta and gamma, are found in the mammalian eye lens. Recently, crystallins have been found presented also in other tissues of the human body, such as heart, skin, brain, spinal cord, and lung tissues (Bhat and Nagineni 1989).

Alpha B-crystallin is an extremely stable protein and is found in very low levels in tissues under normal conditions (Parcellier and Schmitt 2005), except for in the eye lens where the crystallins constitute about 40% of the cytoplasmic proteins (Gruvberger-Saal and Parsons 2006).
During stress, such as heat shock, radiation or exposure to cancer drugs, alpha B-crystallin level increases and it acts as a chaperone by preventing denatured proteins, from the shock, to aggregate. Thus, it preserves intracellular architecture and the integrity of the cell membrane. Additionally, it is strongly anti-apoptotic, by interacting with different apoptotic proteins at the apoptotic key regulator points and, therefore, has gained interest in the carcinogenesis and anti-cancer drug research.
Alpha B-crystallin has been detected with constitutive expression in prostate cancer, gliomas, renal cell carcinomas and in head and neck cancer (Arrigo and Simon 2007). Also in breast cancer, alpha B-crystallin has been shown to have a pathogenic role, suggesting it acts as an oncoprotein(Gruvberger-Saal and Parsons 2006).

SPARC/osteonectin, uPA and PAI-1

SPARC, also known under names of osteonectin or BM-40 (basement membrane), is a glycoprotein that binds calcium and collagen. Both normal and malignant cells derived from primordial germ cells release this protein. It can increase the levels of certain enzymes, such as collagenase and stromolysin as well as some extracellular matrix proteins (fibronectin and laminin) in fibroblasts. This leads to degradation of membranes and increases endothelial permeability, facilitating extravasation of malignant cells. Over-expression of SPARC has been associated with neoplastic progression in colorectal cancer and poorer outcome in malignant melanoma (Chin and Boyle 2005).

Urokinase type plasminogen activator (uPA) plays an essential role in generating plasmin from plasminogen, which leads to proteolysis of the extracellular matrix (ECM). Plasminogen activator inhibitor type 1 (PAI-1) is the most potent of natural inhibitors to balance uPA's enzymatic activity (Pasini and Brentani 2001). The urokinase plasminogen activator system has occasionally been found to be involved in metastasis and the system has been studied in relation to HNSCC, suggesting it might be of value as a prognostic marker (Pasini and Brentani 2001).
Chin et al found the combination of SPARC, uPA and PAI-1 a marker for poor prognosis in a set of 62 patients with HNSCC (Chin and Boyle 2005). The combination outperformed other known prognostic factors, such as nodal involvement and tumour size.

Other prognostic factors in OOPHSCC

Many other prognostic factors are associated with HNSCC, e.g. the proteins *cyclin D1* and *p53* and *epidermal growth factor receptor (EGFR).*
CCND1 is a proto-oncogene, encoding for the protein *cyclin D1,* which, when over-expressed, shortens the gap 1 phase of the cell cycle (G1). Amplification of CCND1 and/or over-expression of *cyclin D1* correlate with advanced stage in HNSCC. Amplification or over-expression has been found in 17-79% of HNSCC specimens.
The protein *p53* is encoded by the TP53 gene and, like *p16*, is involved in the cell cycle control. It initiates G1 arrest as a response to DNA damage and apoptosis. TP53 mutation is found in 50-69% of HNSCC.
EGFR is a transmembrane tyrosine kinase receptor that, when phosphorylated, activates multiple signalling pathways that are oncogenic regulators. This leads to, for example, cell-cycle progression and tumour cell motility. EGFR over-expression is common in HNSCC, seen in 34-80% of the tumours, more commonly in pharyngeal and oral tumours than in other HNSCC sites. It is reported that EGFR over-expression is associated with worse outcome.
(Thomas and Nadiminti 2005).
These factors are not being addressed further in this thesis.

The thesis and its aims

In the 1980s, several hospital based reports indicated an increasing incidence of carcinoma of the mobile tongue in young adults (<40 years)(Shemen and Klotz 1984, Davis and Severson 1987, Schantz and Byers 1988,). That led to the first of the papers in this thesis (paper I), and to the later follow up (paper II), paper I being the first comprehensive population based study of carcinoma of the tongue in young adults, and older patients. The study compared incidence rates and changes over a 35-year period in a Nordic population > 20 million between young adults and older patients and also the survival rates between the groups. This study was then followed up ten years later with paper II, to answer the question whether the incidence increase in SCC of the tongue continued.

During the years of collecting data for papers I-II, reports of a possible impact of HPV on HNSCC become more and more frequent (Gillison and Koch 2000, Ragin and Taioli 2007, Krueger H 2010,). Also, reports on increasing incidence of oropharyngeal cancer, more commonly seen in slightly younger patients than earlier, were published. The age profile, though, was different from that seen in tongue cancer; the oropharyngeal cancer incidence increase was seen more notably in patients in their 50s and very rarely in patients under the age of 40 (Hammarstedt and Dahlstrand 2007, Braakhuis and Visser 2009,). There was then a possibility to use the material from a former population based study of the Swedish southern health care region (Hansson and Rosenquist 2005, Rosenquist and Wennerberg 2007), with regards to HPV in oral and oropharyngeal cancer, to investigate the possible difference between oral cancers and oropharyngeal cancers with respect to aetiology and outcome (paper III). That paper could then be followed by a methodological study of the same material (paper IV) to see how sample collection could impact on the results.

At the same time, we had the option, in co-operation with the University of Queensland, Brisbane, Australia, to validate some potentially prognostic markers, in a Swedish study population, and look at if and how they differed between oral and oropharyngeal cancers (paper V).

The aims of the separate papers

Paper I

To determine whether an incidence increase in SCC of the mobile tongue in young adults, over a 35-year period, could be verified in a Nordic population of over 20 million. A second aim was to compare survival rates between young adults and older patients with SCC of the tongue.

Paper II

To investigate whether the trend of increased incidence of tongue cancer in general, and especially in young adults, continued into the twenty-first century in a population-based study in the Nordic countries (with 25.2 million inhabitants in 2009).

Paper III

To do a five-year follow up of the population based cohort, covering more than 80% of incident cases, from the case-control study carried out in the Swedish southern health care region between 2000 and 2004, with regards to disease specific survival (DSS), in relation to high-risk HPV status, and oral versus oropharyngeal tumour sites.

Paper IV

To compare the outcome of HPV detection and HPV related survival between the method of fresh exfoliated cells taken with cotton tipped swab and a mouth wash and detection on paraffin embedded tissue taken from the same patients' tumours at time of diagnosis (diagnostic biopsies).

Paper V

To validate the role of alpha B-crystallin as an independent prognostic marker and SPARC/osteonectin, PAI-1 and uPA as prognostic markers in patients with oral and oropharyngeal SCC, by testing the described findings on a new set of tumours.

Material and Methods

Papers I and II

The material consists of all SCCs of the mobile tongue, reported to the Swedish, Danish, Norwegian and Finnish cancer registries during the period 1960-1994 (paper I). For Denmark, the numbers of SCCs prior to 1978 was calculated from the numbers of all tongue malignancies, due to lack of histology reports included. Cases were divided into three age groups: 20-39, 40-64 and 65-79. Patients under 20 years of age were excluded, being perceived as a different entity since different mechanisms in malignant transformation during childhood and adolescence can be expected. In all, 5024 cases were included, 276 of them in the 20-39 group, subsequently referred to as young adults.
In the following paper (paper II), all tongue malignancies reported to the same registries and also the Icelandic cancer registry during the period 1960-2008, extracted from the NORDCAN database, were collected. Histology and subsite data cannot be obtained from NORDCAN. A separate analysis of the Swedish data was therefore performed, separating trends for mobile tongue and base of tongue as well as differentiate between SCC and other histological subtypes. The analysis showed the expected proportion of SCC (95%) of all tongue tumours and the relationship of base of tongue tumours versus mobile tongue tumours remained constant over the study period, Figure 9. 12 280 cases of tongue malignancies were reported to the NORDCAN registry during the study period 1960-2008. Of them, 673 were young adults.

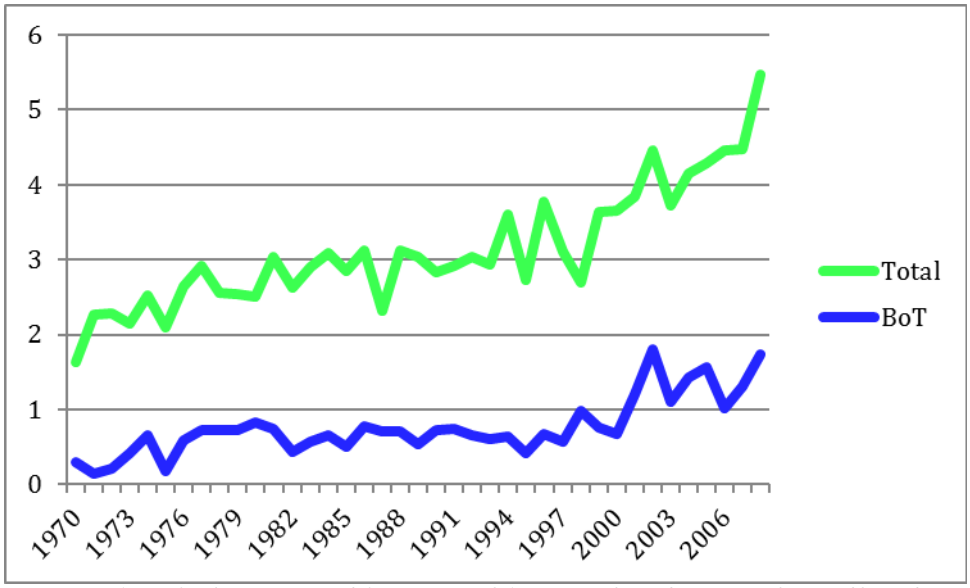

***Figure 9**. Relationship between SCC of the tongue (mobile tongue + base of tongue) and SCC of base of tongue, Sweden, 1970-2008. Total = mobile tongue + base of tongue. BoT = base of tongue. Incidence per 100 000.*

Statistical methods

In both papers, cancer incidence was standardised for age using the standard European population as reference, to adjust for age differences between countries and time periods. The same relative weights were also used for the age-standardised incidence rates for the age groups 20–39, 40–64, and 65–79 years.

Survival probability was estimated using life table methods. Log-rank tests were used to compare different groups. Crude as well as relative survival was calculated. Nation specific mortality data was used to calculate expected survival.

Papers III and IV

One hundred and twenty-eight, consecutive patients, with previously untreated oral or oropharyngeal SCC, diagnosed between September 2000 and January 2004 were included (paper III). Site distribution was 76 and 52, oral cavity and oropharynx respectively. All patients were assessed and discussed at the multi-disciplinary meeting (MDM) run by the ENT and Oncology departments at the Lund University Hospital, Sweden. Samples for HPV detection were collected by getting exfoliated cells from cotton tipped swabs taken from the tumour surface and from the oropharynx. An additional mouth wash was performed. The method will subsequently be referred to as swab and mouth wash collection (SMC). Samples were handled freshly frozen.

For paper IV, 91 of the 128 patients were included, from whom formalin fixed paraffin embedded (FFPE) tissue from the patients' biopsies at the time of diagnosis were HPV assessed. The site distribution was 50 and 41, oral cavity and oropharynx respectively.

The reasons for not including the remaining 37 patients were (*i)* paraffin embedded tissue not stored in biobanks (n=17) and (*ii*) lack of amplifiable DNA (n=20) (Beta-globin gene).

HPV analysis on the exfoliated cells from SMC was made by nested PCR; HPV typing made using outer primers MY09 and MY11 followed by inner primers GP5+ and GP6+ (paper III). MGP-PCR and subsequent Luminex analysis for identification of 38 mucosal HPV types were used on the FFPE samples (paper IV).

Statistical methods

Kaplan-Meier analyses were performed to calculate Disease Specific Survival (DSS), evaluated by the log rank test. Fisher's Exact Test and Mann-Whitney U Test were used to analyse difference between HPV negative and HPV positive patients. Cox regression analyses were carried out to assess the relationship between age, stage, high-risk HPV status and DSS. A p-value < 0.05 was considered statistically significant.

Paper V

Histological sections from 55 patients, diagnosed with oral or oropharyngeal cancer (N=35 and 20), at the Lund University Hospital, Sweden, between the years 1990-1999 were collected. All patients had complete and thorough follow-up, curatively intended treatment and verified cause of death. Patients were followed to date of death or December 2008, whichever came first. Immunohistochemical staining for alpha B-crystallin, SPARC/osteonectin, PAI-1 and uPA was performed at The Queensland Institute of Medical Research (QIMR), Brisbane. Australia, as previously described by Chin et al(Chin and Boyle 2005).

Patients' stage was divided into T1-T2/T3-T4 and N+/N-. Distribution of sex, T- and N-status between oral cavity and oropharynx is seen in Table 2.

Table 2. Distribution of sex, T- and N-status between oral cavity and oropharynx. Numbers and (%) of the study population of 55 patients in paper V.

	Male	Female	T1-T2	T3-T4	N-	N+
Oral cavity	26 (74.3)	9 (25.7)	15 (42.9)	20 (57.1)	20 (57.1)	15 (42.9)
Oropharynx	17 (85.0)	3 (15.0)	10 (50.0)	10 (50.0)	8 (40.0)	12 (60.0)
Total	43 (78.2)	12 (22.8)	25 (45.5)	30 (54.5)	28 (50.9)	27 (49.1)

Statistics

The overall percentage scores for staining were divided into quartiles. The significance was found to be due to the fourth quartile, thus, the first three quartiles were combined, and considered the cut-off for negative and positive staining. This is in accordance with the previous study by Chin et al (Chin and Boyle 2005). Fisher's exact test and Mann-Whitney U test were performed for analysing differences between sites, and Kaplan Meier analysis to calculate survival differences for proteins and T- and N-status. For alpha B-crystallin, an additional Cox regression was performed to adjust for nodal status and tumour staging. A p-value < 0.05 was considered statistically significant.

Results and discussion

Papers I and II

In the 1980s, the first reports of increasing incidence in cancer of the mobile tongue in young adults were published (Shemen and Klotz 1984, Davis and Severson 1987, Schantz and Byers 1988,). The condition in this age group is very rare and it is hard to get substantial numbers for significant findings. In the Nordic countries, compulsory reporting to the national cancer registries since the 1950s has made population based epidemiological studies possible. The reporting to the registries has been shown as high as >95%.
We decided to extract data from the Swedish, Danish, Norwegian and Finnish registries for our first study.

Main results for paper I:

- In all, 5024 cases of SCC of the mobile tongue in ages 20-79 were reported to the registries.
- 276 (5.5%) were 20-39 years, referred to as young adults.
- During the study period, incidence increased in all age groups except for in women 65-79 years where it remained constant.
- The incidence in young adults increased 5 times in men and 6 times in women. For both sexes aged 40-64 and for men aged 65-79, the incidence about doubled, Table 3.
- Crude as well as relative survival rates were better for young adults compared to older patients, Table 4.

Table 3. Incidence of SCC of the mobile tongue in Sweden, Denmark, Norway and Finland, per 100 000 person-years.

	Start of study period 1960-1964	95% Confidence interval (CI)	End of study period 1990-1994	95% Confidence interval (CI)
20-39 years male	0.06	0.02-0.11	0.32	0.25-0.41
20-39 years female	0.03	0.00-0.06	0.19	0.12-0.26
40-64 years male	0.86	0.71-1.01	1.90	1.68-2.12
40-64 years female	0.58	0.46-0.70	1.01	0.85-1.17
65-79 years male	2.37	1.89-2.85	4.05	3.54-4.56
65-79 years female	2.29	1.88-2.70	2.28	2.04-2.52

Table 4. Crude and relative survival of SCC of the mobile tongue in Sweden, Denmark, Norway and Finland, 1960-1994, per age group.

	5-year crude survival %	95% Confidence interval (CI)	5-year relative survival %	95% Confidence interval (CI)
20-39 years	65	59-71	66	59-71
40-64 years	45	43-48	48	46-51
65-79 years	33	31-35	43	40-45

It was possible to get histology reports for all cases except for the Danish material prior to 1978, since histology was not included in the Danish registry during that period. The proportion of SCC in the Danish material prior to 1978 was therefore estimated by using the corresponding overall SCC percentage for the other countries included in the study. The method for this is described thoroughly in paper III. Since SCC constitutes more than 90% of all malignancies of the mobile tongue, and the population of Denmark constitutes only 22 % of the study population, any bias due to this estimation would be extremely small. Also, the populations in the different Nordic countries are considered homogenous, compared to many other regions in the world, not only ethnically, but also socio-economically.

In paper II, we extended the study period by 14 years, to also include the years 1995-2008. Data this time was extracted from the NORDCAN registry, which is based on the cancer registries in all the Nordic countries. The differences regarding data extraction from NORDCAN compared to extracting data from each national cancer registry separately were two:

1. Histology was not reported to the NORDCAN registry. Thus, all tongue malignancies were included, not only SCC.
2. Base of tongue was included, since NORDCAN does not differ between mobile tongue and base of tongue.

In addition to this, there is another difference compared to paper I, namely the inclusion of data from Iceland. We chose to also include Iceland, since data was easily obtainable and gave an even larger dataset and thus more reliable results.

Main results for paper II:

- During the study period, a total of 12 280 cases of tongue cancer were reported, including base of tongue.
- 673 (5.5%) were young adults, 20-39 years
- Incidence increased in both sexes and all ages, except for women aged 65-79, between the years 1960-1994
- The trend of increasing incidence persisted 1995-2008, except for in males 20-39, but with the addition of women 65-79

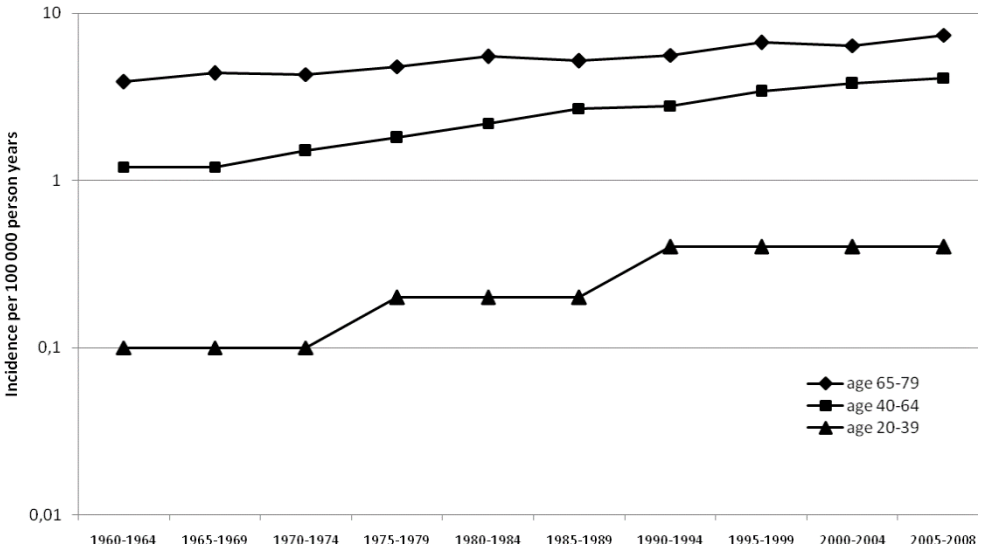

Figure 10. Age-standardised incidence of cancer of the tongue (mobile tongue and base of tongue), men in the Nordic countries, 1960-2008 (logarithmic scale)

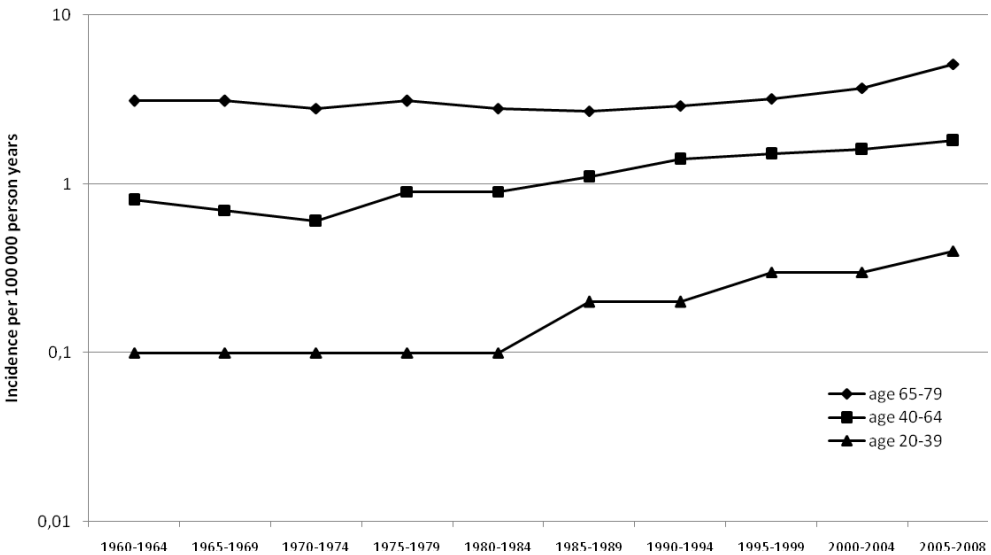

Figure 11. Age-standardised incidence of cancer of the tongue (mobile tongue and base of tongue), women in the Nordic countries, 1960-2008 (logarithmic scale)

SCC of the mobile tongue is a rare condition in young adults. That creates study problems, since not many countries can provide population based, comprehensive data

regarding incidence and survival. Davis et al presented a report in 1987, based on data from the Surveillance, Epidemiology and End Results programme (SEER) of the National Cancer Institute in the US. Their SEER material was based on nine population–based cancer registries, covering < 13% of the US population, during 1973-1984. They showed an increased incidence of mobile tongue cancer, most pronounced in men aged 30-39 (Davis and Severson 1987). Other studies presenting incidence increase in tongue cancer, especially in young adults, prior to our first paper, were based on small numbers and selected hospital material (Shemen and Klotz 1984, Schantz and Byers 1988,). Our first study clearly shows an incidence increase in SCC of the mobile tongue in all age groups, except for older women, in the four Nordic countries, Sweden, Denmark, Norway and Finland, during the study period 1960-1994. The study population at the time was 23.2 million.

Our second paper based on yet a larger population (25.2 million) also showed an increased incidence of tongue cancer, this time continuing until 2008. However, no incidence increase was seen for young adult males. The data in our second paper is not as accurate since we could not extract histological type from the NORDCAN registry. Also, the inclusion of base of tongue has made us estimate the numbers of mobile tongue tumours. We do believe the possible bias in this regard is of minor importance due to (*i*) SCC constitute about 95% of all tongue malignancies, (*ii*) base of tongue tumours represent a smaller proportion of the tongue malignancies than mobile tongue tumours and (iii) we could get the accurate figures for SCC and site proportions for the Swedish part of the material which showed proportions of SCC and base of tongue tumours in line with what was expected.

We concluded in paper II that the incidence increase was attributed to SCC of the mobile tongue. When looking at the results, there is also an increase in base of tongue tumours, but as stated in the results, the relationship between mobile tongue and base of tongue does not change. This means that there is also an increase in base of tongue tumours, thus part of the total increase is due to base of tongue tumours.

Base of tongue belongs to the oropharynx and not the oral cavity. The reason for the increase in numbers of base of tongue tumours might therefore be different than that for mobile tongue tumours. Since high-risk HPV infection has been proven to cause a subset of HNSCC, especially from the oropharynx, this virus might account for changes in base of tongue epidemiology. High-risk HPV infection has not at all been found to the same extent in tumours of the mobile tongue, compared to the tonsils or the base of tongue, and we do not believe it has caused the incidence increase of mobile tongue tumours presented. To say this for sure, it would have been of interest to know the HPV status of the material upon which these papers are based.

In paper II, we did not present any calculations of significances regarding incidence changes, since we were only looking for trends. It would have been required to analyse the significance of the incidence increase changes if the aim had been to establish the exact magnitude of the changes.

What causes the incidence increase in SCC of the mobile tongue is still unclear. As mentioned before, high-risk HPV has not been found to be associated with tongue cancer to a great extent. The most well known risk factors for HNSCC, smoking and high alcohol consumption, have continuously decreased in the western world and have had a favourable impact on other smoking and drinking related cancers and, therefore, are less likely to be the cause of increasing numbers of tongue cancers. Since we have no data of smoking and alcohol consumption for the patients included in papers I-II, this can only be an assumption for these studies.
Since the incidence increase is not as great after 1994, and no longer seen in male young adults, we cannot, from our studies, conclude whether there is still an existing active factor accounting for this increase in the Nordic countries or if it has been of temporary character.

It is still debatable whether young adults with SCC of the tongue have better or worse outcome compared to older patients and the reports keep varying (Park and Sun 2010, Soudry and Preis 2010, Thomas and Moore 2012). The survival was only studied in paper I. Our results of both crude and relative survival were presented with 95% CI. None of the intervals overlap, and a significant difference in survival figures between the age groups can therefore be considered quite clear.

We do not know why the young patients in our study have better survival than older patients. Young patients do not usually suffer from co-morbidities to the same extent, are considered stronger and withstand tougher treatment better and, thus, might be suitable for more comprehensive treatment regimens.

We cannot say if this has been the case in our study since we did not look at possible treatment differences between the age groups. As mentioned above, we have no data on smoking and alcohol habits for this group and cannot say if that influences outcome. We do not know if our young group came earlier to diagnosis and do not know their TNM-status or stage at time of presentation.

Some studies show young adults present with more advanced TNM-status whereas other studies show no difference in TNM-status at time of presentation. Park et al found no stage differences between patients under and over the age of 45 in a retrospective study of 105 patients with SCC of the tongue. However, the recurrence rate was significantly higher in the young group. No significant difference could be seen on survival, though. Mallet et al report 52 patients under 35 years with SCC of the oral tongue, 58% Stage I-

II (72% T1-T2 and 76% N0) at time of diagnosis. Despite the relative high numbers of small tumours, DFS at five years did not reach higher than 52% (Mallet and Avalos 2009). No older patients were included for comparison. Soudry et al report 11 patients under the age of 30, with SCC of the oral tongue, with significantly worse N-stage and higher perineural invasion rate, but no difference in OS or DSS compared to 74 older patients.

To get an idea of TNM differences between age groups with SCC of the tongue, we extracted data from The Swedish Head and Neck Cancer Register (SweHNCR). The registry started in 2008 and has coverage of > 97% of all incident new cases. Data extraction from 2008-2010 of SCC of the mobile tongue, divided into three age groups (<40, 40-64 and > 65) showed 467 patients, of which 5.5% were <40 years of age. No significant difference was seen between any age group regarding T-status, N-status or Stage I-IV at time of presentation. It is, thus, likely no major difference in TNM-status or Stage between age groups is present in the materials of papers I-II, given the homogenous populations within the Nordic countries, but cannot be ruled out.

Health care in the Nordic countries is primarily public. Only a small proportion of the population hold private health insurance. One possible explanation for better survival figures for young adults in the Nordic countries might be the access to health and dental care and the relatively homogenous populations. Socio-economically, the differences within the populations are not as big as in some other parts of the world. Dental care has been free, or subsidised for school children and young people for many decades, which has seen most people accustomed to seeing a dentist once a year. One could speculate that this could lead to earlier detection with favourable impact on outcome.

Limitations

We had to estimate the proportion of SCC in the Danish material prior to 1978. As mentioned before, we do not find a great risk of bias in that respect, given the homogenous populations and the small proportion of Danish tumours in the whole study group.

Also, the proportion of mobile tongue versus base of tongue had to be estimated for paper II. The accurate figures for Sweden were extracted from the Swedish cancer registry and we feel confident that no major bias exists from calculating the corresponding figures for the other countries.

In paper II, we present trends rather than statistically verified incidence changes.

Strengths

In a rare condition, like SCC of the tongue in young adults, many reports are based on limited numbers and selected material. One strength of these papers is their population-based, large dataset.

The coverage of the Nordic cancer registries is estimated to >98%, adding to the comprehensiveness of the material.

Our studies also cover a long period, 35 and 49 years respectively, which gives a good picture of long term trends. To find the time period when an incidence increase starts and/or finishes might be helpful in finding and understanding what factor or factors are involved in the development of the condition.

Papers III and IV

The material in these papers had previously been part of a case-control study in the Swedish southern health care region where a strong association between high-risk HPV and oral and oropharyngeal cancer was found. Healthy controls, examined with cotton tipped swab of the oropharynx and a mouth wash, for HPV detection, carried high-risk HPV DNA in only 0.94% of the cases (Hansson and Rosenquist 2005).

We could then, based on the same material, show a higher risk for recurrence or second primary tumour (SPT) in the high-risk HPV positive group (Rosenquist and Wennerberg 2007). Since more and more reports appeared, indicating a better survival for high-risk HPV positive patients, we found it useful to see whether, in that context, our contradictory higher rate of recurrence/SPT for high-risk HPV positive patients, would correspond to worse survival, while we collected data on the same material for a five-year follow up (paper III).

Main results for paper III:

- 20 (26%) of patients in the oral cavity group and 27 (52%) in the oropharyngeal group were high-risk HPV positive in SMC.
- HPV 16 was found in 38 (81%) of the positive cases
- No statistically significant difference was found in DSS, on univariate analyses, between high-risk HPV positive and negative patients, neither in the whole study

population, nor when analysed by site.

- DSS was statistically better, on univariate analyses of the whole study population, for
    - T-status 1-2, compared to T-status 3-4
    - N-negative compared to N-positive status
    - lower compared to higher Stage (Sobin LH 2002)
- Stage had significant impact on DSS for *oral cavity cancer* on Cox regression multivariate analyses
- Age had significant impact on DSS for *oropharyngeal cancer* on Cox regression multivariate analyses
- No association of high-risk HPV with DSS on Cox regression multivariate analyses was found in *any group*.

In contrast to the majority of studies of oropharyngeal SCC, we could not find any significant survival difference between high-risk HPV positive and negative patients. The literature results are not completely consistent and, additionally, the association between high-risk HPV and oral cancer seems to be even less clear. With this background and the knowledge that many different HPV detection methods are used, which complicates the comparability of the studies, we decided to take our material for further investigation. We wanted to compare our method of taking samples, using SMC, with taking tumour biopsy tissue (FFPE) from the same patients, study the impact on HPV prevalence and determine if the SMC method used could be the reason for the lack of DSS difference in our paper III.

Main results for paper IV:

- High-risk HPV prevalence seemed to be higher using SMC than FFPE, most pronounced in the oral cavity. Distribution of HPV status according to site is shown in Table 5.
- HPV 16 was the dominating HPV-type in both methods
- The rate of inconsistent results between SMC and FFPE was 27% (N=25)
- No statistically significant difference in DSS was found, when adjusted for age and stage

Table 5. High-risk HPV positive cases of 50 oral cavity SCCs and 41 oropharyngeal SCCs. Frequency per site according to sample collection methods SMC and FFPE.

	High-risk HPV positive No (%)	
	SMC	**FFPE**
Oral cavity	15 (30%)	8 (16%)
Oropharynx	24 (59%)	23 (56%)

HPV and sample collection methods

Statistically significant better DSS in univariate analyses was seen for high-risk HPV positive cases in FFPE with oropharyngeal tumours (p=0.039 and 0.048). This did not remain significant when adjusted for age and stage in the multivariate analyses. No other significant differences in DSS were found. The sample collection methods thus did not impact the survival results in our material.

Since a tumour biopsy, rather than a superficial swab or a mouthwash, could be presumed to represent the actual tumour cells, we had expected a possibly higher prevalence of HPV in FFPE compared to SMC. To our surprise we found the opposite. Lawton et al described in 1992 a comparison of three sample collection methods from the normal oral mucosa; a mucosal scrape, a biopsy and a mouthwash were collected from each subject in a cohort of 60 test persons. HPV DNA was found in 51% of mouthwashes, 45% of mucosal scrapes and 12% of biopsies. They found that mouth wash was the best single screening method for studies of HPV DNA in the oral mucosa, but a combination of multiple sampling techniques detected even more positive cases (Lawton and Thomas 1992). One could argue that collecting cells with SMC might be a too sensitive a method, showing too many transient, superficial infections with no impact on malignant transformation. However, our material in paper III has previously been part of a case-control study, where controls (320 healthy controls matched by sex, age and region) were examined with the same cotton-tipped swab from oropharynx and a mouthwash (Hansson and Rosenquist 2005). HPV DNA detection was performed in the same way as for the cases. HPV DNA found in samples from one or both of the collection techniques (cotton tipped swab of the oropharynx or a mouthwash) was considered positive. Only 0.94% of the controls were high-risk HPV positive which, most likely, disaffirms the hypothesis of a too sensitive method. The high-risk HPV prevalence in the Swedish

control group diverges greatly from Lawton's et al findings of 60% high-risk HPV positive cases in their group of 60 healthy, Caucasian, test subjects in Queensland, Australia.
Another factor that might impact on the results is the difference in handling of the samples; SMC, where fresh frozen cells were analysed compared to formalin fixation in FFPE. The fixation process in FFPE is said to cause fragmentation of the DNA to lengths shorter than 200 base pairs and reproducibility from fresh frozen results has been difficult (Mirghani and Amen 2014). This could potentially be an explanation of a higher detection rate in our SCM samples.

In addition, there were a substantial proportion of discordant HPV-status results between the two methods (27% for the whole study population). When studied by site, the discordance was even higher in oropharynx (32%) but lower in oral cavity (24%). Again, an explanation of these divergent findings could be too sensitive methods, this time regarding the PCR methods. They can detect as few as five DNA copies. If the viral load is low, it might be detectable in one sample but not in another from the same patient. Also, heterogeneity within the tumours might play a role in the discrepancies in the two methods. It has been shown for the precancerous lesions in the uterine cervix, cervical intraepithelial neoplasias (CIN II and III), that different HPV types can be found in different areas of a lesion, mapped by laser capture microdissection, followed by PCR (Quint and Jenkins 2012).

HPV prevalence

The incidence of high-risk HPV positive tumours in oropharyngeal cancer is reported in the literature as increasing and given the role as the cause of the increasing incidence of oropharyngeal cancer. The proportion of high-risk HPV positive tumours varies greatly and figures between 45 and 95% (Marklund and Hammarstedt 2011) and 14-72% (Braakhuis and Visser 2009)are described. Our paper III shows a proportion of 52% high risk HPV positive tumours in the oropharyngeal group. In paper IV, the proportions are 59 and 56% for SMC and FFPE methods respectively. Our figures are comparable to those in the literature and cannot account for divergent survival results. Interestingly though, they are much lower than the HPV rate in oropharyngeal cancer reported from another Swedish region, only about 500km further north. Näsman et al in 2009 reported increasing HPV prevalence figures in tonsillar cancer, from 1970-2007, in the county of Stockholm, with almost doubling figures each decade, reaching 93% at the end of the study period (Nasman and Attner 2009). Attner et al in 2010 reported similar results for the same region regarding base of tongue cancer, HPV being positive in 58% of the cases in 1998-2001, reaching 84% in 2004-2007 (Attner and Du 2010). There is most likely no demographic discrepancy to explain our diverse findings, whereas methodological differences should be considered. Näsman and Attner performed HPV PCR on FFPE like

we did in our paper IV, but with thicker slices (30µm versus 5µm). They used general primer pairs GP5+/GP6+ CPI/IIG. In addition, they tested all samples with an HPV-16 type-specific PCR. Regarding our FFPE samples, we used MGP-PCR and subsequent Luminex analysis for identification of 38 HPV types, of which HPV-16 is one. These PCR assays are both considered very sensitive and can detect as few as five DNA copies. Whether this can explain our discrepancies remains to be elucidated.

It is also reported that high-risk HPV prevalence is lower in other oropharyngeal subsites than the tonsils and the base of tongue (Marklund and Nasman 2012). Our material has not been further analysed divided by oropharyngeal subsites. Tonsillar cancer is the far most common oropharyngeal cancer, followed by base of tongue. Also in our material, these two subsites dominated with 33 (63%) and 12 (23%), tonsil and base of tongue respectively, of the 52 oropharyngeal cases in paper III. Two of the remaining seven cases were positive for high-risk HPV. Our lower HPV prevalence therefore cannot be due to a higher proportion than expected of subsites other than tonsil and base of tongue.

Our results show that high-risk HPV prevalence in the oral cavity was, as expected, lower than in oropharynx; 26% in paper III, 30 and 16% (SMC and FFPE respectively) in paper IV. Lower HPV rates in the oral cavity are consistent with most other reports (Gillison and Koch 2000, Pintos and Black 2008, Bragelmann and Dagogo-Jack 2013, Grimm and Iftner 2014,)

HPV and survival

The finding of no impact of high-risk HPV status on survival for oral cancer is consistent with most previous reports, whereas the same finding for oropharyngeal cancer is not (Ragin and Taioli 2007). In our study, there is an impression of better survival for high-risk HPV positive patients in the oropharyngeal group when looking at the Kaplan-Meier curves in paper III, although we could not find any statistical significance. On the contrary, in paper IV, the Kaplan-Meier univariate survival analyses show statistically significant better DSS in the oropharyngeal group when HPV was tested on FFPE (p=0.039) and there was a statistically significant association of high-risk HPV positivity and DSS in the univariate Cox regression (p=0.048) in the same group. However, that significance disappears when adjusting for age and stage in the Cox regression multivariate analyses. It is not likely that larger numbers could reach statistically significance since the p-values are high.

Age and survival

The median age difference between oral cavity and oropharynx is significant in our study with a higher median age in the oral cavity group. This difference is not seen between high-risk HPV positive and negative patients in either group. Most studies show that high-risk HPV positive patients with OPHSCC in general are younger. Näsman et al 2009 found, in a Swedish cohort of tonsillar cancer, significantly lower mean age in the male group of HPV positive patients (Nasman and Attner 2009). A generally lower age in HPV positive patients might account for part of the favourable survival, considering a younger age predisposes for less co-morbidities and better conditions to tolerate more challenging treatment. Since we found no difference in age between high-risk HPV positive and negative patients, there is no age factor in the high-risk HPV positive oropharyngeal group to possibly impact in favour of better survival in that group. This could be a plausible explanation to the absence of survival difference in relation to HPV status in our study.

In uni- and multivariate analyses, age had a significant impact on DSS in the group of oropharyngeal cancer patients. Younger patients had better DSS. This difference was not seen in the oral cavity cancer group. The proportion of mobile tongue in paper III was 27 of 76 (36%), and can therefore not be compared to our results in papers I and II where only mobile tongue was included, and age had significant impact on OS and DSS.

Limitations

We cannot, from our results, say whether HPV has been transcriptionally active. We can only show its presence. E6/E7 mRNA detection and/or IHC detection of p16 would have added information to our results. It should be kept in mind though, that p16 is a surrogate marker for HPV infection, with a specificity of 80%. However, if used in combination with HPV PCR, the accuracy for potential transcriptionally active HPV is as high as 98% (Smeets and Hesselink 2007, Rietbergen and Leemans 2013,).

The different outcome in HPV prevalence between the two sample collection methods has not been validated statistically. The impact of the fact that in as many as 27% of the samples, different HPV status was found can however, not be understated.

The material consists of composite, yet site divided, groups. Further division into subsites might have given too small numbers for statistical validity and was not contemplated.

The cells from the two different sample collection methods were handled differently; fresh frozen versus formalin fixed. To have also had the biopsies handled fresh frozen would

have added strength to the outcome and could potentially have impacted on the results. This was not possible, since paper IV was a follow up of results in paper III and not designed when paper III was prepared.

Not all cases included in paper III could be analysed in paper IV.

The age groups <60, ≥60-<70 and ≥70 were used, which is not a common age classification in the literature. With regards to the size of the material, we chose these age groups to get comparable numbers in each group. The material was not big enough to get substantial numbers with a cut-off for the youngest group at a younger age, without accepting an extensive CI. Only 6 cases under the age of 40 were found in the whole material.

The HPV DNA detection was made using different primers in the two papers. This was due to the analyses being made at the WHO HPV testing reference laboratory in Malmö, which updates their accredited methods regularly.

The fact that the previously described higher rate of recurrence/SPT in the high-risk HPV positive group in our material does not correspond to worse outcome could potentially be due to very low frequency of recurrences/SPT. Greater numbers might be required to find a more reliable recurrence/SPT rate. On the other hand, the numbers in the whole material (128 in paper III and 91 in paper IV) cannot be considered low.

Strengths

The original material is a prospective, consecutive series of oral and oropharyngeal SCCs, without any conscious selection bias.
SMC and FFPE were taken from the same patients.

The material is substantial, comprising 128 consecutive cases (paper III) of which 91 could be further analysed in paper IV. All were assessed at the MDM at the same centre, had complete data and follow up, and received treatment with curative intent at the same centre as where the MDM was held. This makes the material homogenous.

We conclude that we do not get the same HPV status using two different methods of sample collection in this study. In clinical practice, HPV detection must be made upon a biopsy or a complete tumour sample, whereas studies on HPV prevalence in healthy controls or populations are easier to both perform and get consent for with a non-invasive sample collection method. For future clinical use of HPV detection, in means of tailoring treatment, a standardised assay, based on greater understanding of HPV's mechanism regarding malignant transformation and assessment of its physical status, is warranted.

Paper V

Histological sections from 55 patients with OOPHSCC diagnosed during the period 1990-1999, at the University Hospital in Lund, Sweden, were analysed with IHC for alpha B-crystallin, SPARC/osteonectin, uPA and PAI-1. These proteins can all be expressed also in non-malignant cells and there had to be a cutoff level determined for positivity. As previously described by Chin et al, we also found the overall significance was due to the fourth quartile, and the first, second and third quartiles were subsequently grouped together to form the cutoff for positive and negative staining.
Due to damaged slides and/or insufficient SCC represented, only 51 tumours were stained for SPARC/osteonectin and PAI-1. All 55 were stained for alpha B-crystallin and uPA.

Main results for paper V:

- 15 (27%) showed positive staining (=high over-expression), for alpha B-crystallin, 10 (28.6%) in the oral cavity group and 5 (25%) in the oropharyngeal (p=1.00).
- DSS was significantly shorter for alpha B-crystallin with high over-expression, in the oral cavity group (p=0.012), but not in the oropharyngeal (p=0.95).
- Risk of death of disease (DoD) was significantly higher in oral cavity tumour patients with high expression of alpha B-crystallin and became even higher when adjusted for T- and N-status.
- No difference in DSS was seen between tumours with high over-expression and lower expression regarding SPARC/osteonectin, uPA and PAI-1.

Median survival figures and Cox regression hazards ratios are shown in Table 6-9.

Table 6. Median disease specific survival (DSS) and overall survival (OS) in months in relation to SPARC/osteonectin (n=51), uPA (n=55) and PAI-1 (n=51) IHC staining. (+) = high over-expression (within the fourth quartile), (-) = lower expression (within quartiles 1-3). Kaplan-Meier univariate analyses evaluated by log-rank test

	SPARC-	SPARC+	*p*-value	uPA-	uPA+	*p*-value	PAI-1 -	PAI-1 +	*p*-value
DSS	21.4	63.5	*0.32*	27.3	63.5	*0.91*	25.0	6.2	*0.59*
OS	17.9	60.4	*0.22*	20.4	6.9	*0.84*	19.4	4.1	*0.017*

Table 7. Median disease specific survival (DSS) in months in relation to alpha B-crystallin (AB)(n=55) IHC staining, as per oral cavity (OC) and oropharynx (Ophx). (+) = high over-expression (within the fourth quartile), (-) = lower expression (within quartiles 1-3). Kaplan-Meier univariate analyses.evaluated by log-rank test.

	OC			Ophx		
	AB-	AB+	*p*-value	AB-	AB+	*p*-value
DSS	27.3	7.5	*0.012*	33.8	34.1	*0.95*

Table 8. Cox regression hazard ratios for alpha B-crystallin (AB), N+ and T+ (=T3-T4) tumours. Oral cavity.

Cox regression. Oral cavity.			
		Disease specific survival	
		Hazard ratio (95% CI)	P-value
Univariate			
	AB +	3.0 (1.2–7.5)	0.016
	N +	6.5 (2.2–18.8)	0.00056
	T +	2.7 (1.1–7.1)	0.038
Multivariate			
	AB + [a]	6.1 (1.7–21.3)	0.0046

[a] Adjusted for N + and T +.

Table 9. Cox regression hazard ratios for alpha B-crystallin (AB), N+ and T+ (=T3-T4 tumours.. Oropharynx.

Cox regression. Oropharynx.			
		Disease specific survival	
		Hazard ratio (95% CI)	P-value
Univariate			
	AB +	1.0 (0.27–4.0)	0.95
	N +	1.2 (0.36–4.3)	0.73
	T +	1.6 (0.46–5.4)	0.48
Multivariate			
	AB+ [a]	1.0 (0.24–4.4)	0.96

[a] Adjusted for N + and T +.

This study was aimed to confirm, if possible, the previous findings of SPARC/osteonectin, uPa and PAI-1 as prognostic markers for poor outcome in HNSCC and alpha B-crystallin as an even stronger, independent prognostic marker in the same condition. It is important to confirm findings of new prognostic markers, in new sets of tumours. Our collaboration with the authors from Australia made it possible to transport tumour samples from the Swedish study population, for IHC analyses, at the same laboratory in Australia, where the previous studies were performed (Chin and Boyle 2005, Chin and Boyle 2005).

DSS versus OS

DSS and OS were calculated. We consider DSS of much greater importance since it shows the survival with regards to the cancer condition. DSS can be a difficult end-point to assess, requiring comprehensive cause of death information. In Sweden, all deaths are reported to the National Death Registry, including cause of death. We were able to extract data on all our cases from the registry and then did a thorough comparison with the patients' records for accurate cause of death confirmation, which made DSS a useful end-point in our study.
Loco-regional control is used as end-point in many studies. By giving over-treatment, a high loco-regional control can be achieved, but also lead to higher mortality. Survival, preferably disease specific, with good QoL is a more relevant end-point from patients' perspective.

Alpha B-crystallin and survival

The term "positive" we used in the publication, refers to the tumours whose staining rates were found within the fourth quartile, thus having the highest over-expression of the protein. Using the term "high over-expression" would probably be more accurate, and will subsequently be used in this discussion.

We found a significantly shorter DSS for tumours with high over-expression of alpha B-crystallin in the whole study group (p=0.046%). The OS difference was not significant (p=0.094).
Interestingly, we found that, divided by site, DSS significance was only found in the oral cavity group. The rates of tumours with high over-expression of alpha B-crystallin in the two groups were similar; 28.6% in oral cavity and 25% in oropharynx (p=1.00). Although the numbers are small, we cannot, in this setting, presume that the rate of high over-expression impacted on the results, since there is no major rate difference between the groups.

Chin et al showed a much higher rate of tumours with high over-expression of alpha B-crystallin, (49/62 = 79%) in their study. Their material consisted of 15 oropharyngeal and 47 oral cavity tumours, thus a greater proportion of oral cavity tumours. Part of the much higher prevalence of alpha B-crystallin positive tumours in their material might be explained by this discrepancy. They also found alpha B-crystallin over-expression to be a strong prognostic marker, while all 13 patients, who stained negative (= below the fourth quartile) for alpha B-crystallin had no recurrences and no deaths.

T- and N- status and survival

TNM status has, for a long time, been the most commonly used factor in clinical practice, to predict outcome. As discussed before in this thesis, although it is still the most valid and strong predictor, its limitations are substantial, including low accuracy in individual cases. We studied also T- and N-status and its hazard ratios for dead of disease (DoD). As displayed in Table 8, a significantly higher risk of DoD for patients with T3-T4 tumours and/or N+ was found in the oral cavity group but not in the oropharyngeal group. This was also found in paper III, where, similarly, stage (according to Sobin and Wittekind) had significant impact on DSS in the oral cavity group but not in the oropharyngeal group.

When adjusted for T- and N- status in Cox regression multivariate analyses, alpha B-crystallin became an even stronger marker for DoD, (p=0.0046) in the oral cavity group. The strongest marker, however, was N+ (p= 0.00056).

SPARC/osteonectin, uPA, PAI-1 and survival.

Although great difference in survival figures between high over-expression and lower expression of these three proteins, in our study, were seen, no significant differences in survival, other than a significantly shorter OS for patients with tumours showing highly over-expressed PAI-1, were found. Again, we do not consider OS a great end-point compared to DSS, since it does not say anything about whether the deaths are caused by the cancer itself.

There were very few tumours with high over-expression; 7 (13.7%) for SPARC/osteonectin, 7 (12.7%) for uPA and 5 (9.8%) for PAI-1. Our study population of 55 cases might be too small to reach significance for these proteins, and further studies are needed before any conclusions regarding their prognostic potential can be made.

Limitations

Some limitations with this study should be acknowledged.

The material is limited (n=55). Divided by site it becomes even smaller. It is a retrospective study and, although we had a larger material to start with, it decreased, due to our high demands on detailed clinical data, follow-up and cause of death, until the final study group was defined.
No power analyses were made to calculate the numbers needed to possibly find significant statistical differences.

The cutoff is arbitrary and based on the results in the paper by Chin et al and on the results in this paper. Thus, reproducibility might be difficult to achieve.

Therapy modality was not included in the multivariate analyses, which could have been of interest, since alpha B-crystallin, theoretically, could cause resistance towards radiotherapy/chemoradiotherapy (Arrigo and Simon 2007, Parcellier and Schmitt 2005). Again, our material is too small to divide even further into more subgroups.

Smoking and high alcohol consumption as well as high-risk HPV are well known risk factors that were not included in this study. Retrospectively (and also prospectively to some extent), a reliable smoking and alcohol history is very difficult to obtain.

Also in this paper, we used the age groups <60, ≥60-<70, ≥70 (see *Limitations* paper III and IV).

SPARC/osteonectin, uPA and PAI-1 were only analysed separately and not as a combination. Again, the rates for high over-expression were very low. Greater numbers than we had are probably required to assess these proteins' influence on survival in HNSCC.

Strengths

We have included only OOPHSCC in this study, which makes the material more homogenous than many other studies regarding HNSCC, where multiple sites are grouped together.

All patients were previously untreated, diagnosed and assessed at an MDM and received treatment at the same centre. Assessment and treatment followed the Southern Swedish Health care region's guidelines at the time, with uniform treatment based on TNM-status at time of presentation.
All patients had treatment with curative intent. Long and uniform follow-up was realised.

Alpha B-crystallin was a strong marker for poor prognosis in oral SCC in this study. There was a clear difference between oral cavity and oropharynx with regards to DSS and alpha B-crystallin over-expression. Again, this reinforces the complex picture of HNSCC, where more and more differences between different sites and subsites are being revealed.
Further studies are needed to clarify alpha B-crystallin's role and mechanism in HNSCC. It is possible it might function as a complement to TNM-staging in the future.

General discussion and future perspectives

During the work with this thesis, differences between oral and oropharyngeal cancer have been highlighted more and more; not only by our own results but also by new findings reported in the literature.

Head and neck cancer is a very heterogenous group of tumours, although the vast majority is of the same histopathological type, SCC. Treatment has not, to date, been able to be tailored, other than based on the very rough TNM classification and to some extent on histopathological classification. This thesis has illuminated, if not necessarily explained, some differences found between oral and oropharyngeal cancer, which reinforces the need for more individualised treatment options in the future.

The incidence increase in tongue carcinoma in the Nordic countries, most pronounced in the young population up until 1994, needs to be further monitored as well as investigated. It is unlikely that high-risk HPV accounts for the increase noted, given the low prevalence of high-risk HPV infection found in oral cancer in our studies, as well as in most other publications. Smoking and alcohol is also not the likely cause for two reasons; firstly, smoking habits have decreased, showing decreasing incidence figures for other smoking related cancers, and secondly, the time of exposure to smoking and alcohol has most likely not been long enough for malignant transformation in patients under 40 years of age. To our knowledge, no other external factor for this increase has yet been identified, while the future researchers will have to embrace a wider field in the search for a possible causative factor or factors.

The incidence of oropharyngeal cancer is also increasing, reported from many parts of the western world. The differences to tongue cancer are, amongst others, the predominant age for the increase, and the possible cause. In contrast to tongue cancer, the main incidence increase in oropharyngeal cancer starts at a roughly ten years older age. There is lots of evidence pointing towards high-risk HPV causing a subset (tonsillar and base of tongue) of oropharyngeal cancers, with much higher high-risk HPV prevalence figures compared to tongue cancer and oral cancer as a group. There is also a majority of studies showing better survival figures for high-risk HPV positive oropharyngeal cancers.

Yet another factor that differs is the male-to-female ratio. Approximately as many women as men are found in the oral cavity group, while there is nearly 3 times as many men as women diagnosed with oropharyngeal cancer. In the Swedish National Board's of Health and Welfare statistics, these ratios are consistent over all age groups. From the same statistics, it is found that the proportions have stayed the same since 1970, that is before the major incidence increase in oropharyngeal cancer started and before the most pronounced prevalence increase of high-risk HPV in tonsillar cancer started in the Stockholm county (Nasman and Attner 2009).

Differences between oral and oropharyngeal cancer are also found within other possible prognostic markers, such as we have found regarding alpha B-crystallin. This marker showed prognostic value for oral cancer but not for oropharyngeal. While HPV status assessment is now indicated for oropharyngeal cancers and the moves towards de-escalating treatment for high-risk HPV positive patients are getting closer, alpha B-crystallin might be a future complement to TNM classification for justifying more aggressive treatment for tumours with low TNM stage but high over-expression of alpha B-crystallin. More studies are needed to evaluate alpha B-crystallin's potential role in the clinical setting. It also requires greater understanding and explanation of the mechanism behind alpha B-crystallin's biological action that gives it its prognostic value.

The many reports within the field of oropharyngeal cancer and high-risk HPV are building the picture of not all high-risk HPV positive oropharyngeal cancers being actually caused by the virus. The diversity in assessment methods has to steer towards a more uniform method to get an accurate HPV status confirmed regarding its malignant transformation role in every individual case. De-escalation of treatment to high-risk HPV-positive patients should be an aim, but has to be carefully monitored within the setting of clinical trails, after evidence of the virus having caused the tumour has been established.

HPV-vaccines against HPV 16 and 18 (as well as HPV 6 and 11 in the quadrivalent vaccine) will hopefully decrease or slow down the incidence increase of not only cervical cancer, but also oropharyngeal cancer. Since men as well as women, and indeed men to a greater extent than women, are affected by oropharyngeal cancer, it seems reasonable to vaccinate both boys and girls. Australia, USA, Canada, Austria and parts of Germany and Italy have included boys in their national immunisation program since 2011-2014. High-risk HPV, in particular HPV 16 and HPV 18, are also known to cause about 50% of anal and penile cancers; including boys in the vaccination programs would most likely impact favourably also for these diagnoses. The main reasons for not doing so have been the high costs for the vaccines and the low cost-effectiveness. The high costs make it a challenge to reach out to the developing countries, even if vaccinating only girls. The majority of cervical cancers occur in the developing world while it is of high priority to find ways to lower the costs for HPV vaccines. New vaccines with lower production costs are being

sought. If a successful global vaccination can be achieved, cervical cancer could theoretically be eradicated, and approximately 50% of anal, penile and oropharyngeal cancers prevented.

In terms of prophylaxis, it is important to remember that a great proportion of oral and oropharyngeal cancers are still caused by smoking and high alcohol consumption. The future has to include reinforcement of actions to promote life-style changes in relation to these agents.

Conclusions

- The incidence of SCC of the mobile tongue increased in the Nordic countries during 1960-1994, most notably in young adults (20-39 years).

- Young adults had significantly better relative survival compared to older patients.

- There was a trend of continuing incidence increase of SCC of the mobile tongue in the Nordic countries, including Iceland, 1994-2008, in all ages except for in young adult males.

- In a consecutive series of 128 patients with oral and oropharyngeal SCC, there was no difference in five-year DSS between high-risk HPV positive and high-risk HPV negative patients, neither in the whole study population, nor when analysed by site.

- Using another sample collection method on the same patient cohort (91 out of 128) did not change the outcome of the survival analyses.

- Comparing the two different sample collection methods SMC and FFPE, for HPV DNA analyses, on the same patients, showed inconsistent high-risk HPV status in 27%.

- Alpha B-crystallin was an independent prognostic marker for poor prognosis in oral SCC, but not in oropharyngeal SCC, in a study material of 55 patients with oral and oropharyngeal cancer.

Populärvetenskaplig sammanfattning på svenska

Huvud-halscancer är den sjätte vanligaste cancerformen i världen, med ca 650 000 nya fall diagnostiserade per år. Tumörer i denna grupp uppstår i munhåla, svalg, näsa, bihålor och struphuvud. Slemhinnorna som utkläder dessa delar av kroppen utgöres till stor del av s.k skivepitelceller. Från dessa celler utgår mer än 90% av cancertumörerna inom gruppen huvud-halscancer. Trots att så stor andel av tumörerna är av samma slag, s.k skivepitelcancer, kan de uppföra sig olika. De kan se likadana ut i mikroskopet, vara av samma storlek och ha spridit sig i lika stor omfattning men ändå svara helt olika på samma behandling, varför det är svårt att i varje enskilt fall kunna förutsäga om patienten kommer kunna bli botad eller ej. Vissa patienter blir därför ”överbehandlade” medan andra möjligen skulle behövt ännu mer omfattande behandling för att botas från sin tumörsjukdom. Det behövs därför mer kunskap om vad som skiljer de enskilda tumörerna åt.

Trots att mycket forskning inom området huvud-halscancer bedrivits det senaste halvseklet har ingen nämnvärd förändring i överlevnad uppnåtts. Huvud-halscancer som en enda grupp uppvisar fortfarande en genomsnittlig femårsöverlevnad omkring 50%, men med mycket stora variationer. Behandlingen kan bestå av kirurgi, strålbehandling och, i vissa fall, cellgiftsbehandling. Många gånger kombineras behandlingarna, företrädesvis vid större tumörer och vid spridning till halslymfkörtlarna. Bieffekterna av behandlingarna kan bli svåra och påverka livskvaliteten för många av patienterna. Strålbehandlingens bieffekter kan ge olika grader av svårigheter att svälja, tala och andas. Kirurgisk behandling kan orsaka ett förändrat utseende och också påverka förmågan att svälja, tala och tugga. Det är därför av stor vikt att försöka karaktärisera tumörerna bättre, för att på så sätt välja den bäst lämpade behandlingen, med så lite bieffekter som möjligt, för varje enskild patient.

De vanligaste riskfaktorerna för att utveckla huvud-halscancer är rökning och högt intag av alkohol. I takt med att rökningen minskat i västvärlden har man sett en minskning av rökrelaterade cancerformer. Trots detta ser man, inom gruppen av huvud-halscancer, en ökning av antalet tumörer inom vissa subgrupper och inom vissa åldrar. Den vanligaste åldern för att insjukna i huvud-halscancer som hel grupp är 60-70 år.

I slutet av 80-talet kom några rapporter från USA, att man sett en ökning av antalet fall av cancer i den rörliga delen av tungan hos unga vuxna. Det ledde till det första arbetet i denna avhandling. I det påvisas en ökning av insjuknandet i tungcancer i de Nordiska länderna, i alla åldrar mellan 20 och 79 år, utom för äldre kvinnor, under åren 1960-1994. Mest uttalad var ökningen hos unga vuxna (20-39 år). Det är svårt att göra studier på tungcancer hos unga, då det är mycket ovanligt att insjukna i denna unga ålder och fallen därför är få. Vår studie baseras på rapporter till de olika cancerregistren i Norden, till vilka det är obligatoriskt att rapportera alla nyupptäckta fall av cancer. Registren har varit i bruk sedan 1950-talet. Detta ger en fantastisk möjlighet till s.k populationsbaserade studier, vilket inte är möjligt på de flesta andra ställen i världen. Av 5024 fall av tungcancer under de 35 åren som studerades, var 275 personer under 40 år.

Andra rapporter i litteraturen avseende tungcancer har visat olika resultat gällande överlevnad hos unga jämfört med äldre personer. En del studier har visat på bättre överlevnad medan flera andra visat sämre överlevnad för de unga patienterna. Problemet med många studier har varit deras underlag av få fall och selekterat patientmaterial, dvs inte populationsbaserat, utan utgjorts av de patienter som blivit remitterade till ett visst sjukhus. I vårt populationsbaserade material kunde vi visa att det var signifikant bättre överlevnad för unga vuxna jämfört med äldre patienter.

Det första arbetet följdes upp med en senare insamling av data från samma länder, men nu också inkluderande data från Island. Studie II ämnade följa utvecklingen avseende tungcancer under ytterligare 14 år. Vi kunde visa på en trend av fortsatt ökning av incidensen av tungcancer 1995-2008 i samtliga åldersgrupper, förutom bland unga män.

En annan subgrupp av huvud-halscancer som ökat över de senaste decennierna är svalgcancer, framförallt utgången från halsmandlarna (tonsillcancer) och från tungroten (tungbascancer). Den åldersgrupp där ökningen är mest framträdande är också den, liksom gällande tungcancer, hos yngre patienter. Det som skiljer tungcancer och svalgcancer åt i detta avseende är dock att den ökande unga gruppen av svalgcancerpatienter är äldre än den unga tungcancergruppen. Svalgcancer ökar markant i åldern 50-60 år.

Man vet idag att det är en relativt stor del av tonsillcancrar och tungrotscancrar där man kan påvisa humant papillomvirus (HPV). HPV typerna indelas i högrisk- och lågrisk-typer, baserat på deras förmåga att omvandla friska celler till tumörceller. De indelas också i hud- och slemhinnetyper, beroende på vilken typ av vävnad de infekterar.
Exempel på hudförändringar som orsakas av lågrisk-HPV är vanliga vårtor. Exempel på slemhinneförändringar som orsakas av lågrisk-HPV är kondylom. Mest känt är HPV för att man kunnat påvisa att högrisktyperna HPV 16 och 18 orsakar i stort sett alla fall av livmoderhalscancer. I många länder erbjuds nu vaccin mot HPV 16 och 18 (samt

lågrisktyperna 6 och 11 i vissa länder) till alla unga flickor. En del länder har också börjat vaccinera pojkar.
Jämfört med livmoderhalscancer, där i det närmaste alla fall visar sig vara orsakade av livmoderhalscancer, är det dock betydligt oklarare, gällande huvud-halscancer, exakt vilka fall som med säkerhet kan vara HPV-orsakade.
Mycket pekar på att en del av ökningen när det gäller svalgcancer orsakas av HPV-infektion, medan tungcancerökningen sannolikt inte kan förklaras av HPV-infektion i samma utsträckning.

Många studier pekar mot att patienter med svalgcancer, där man kan påvisa HPV, har en bättre prognos. Det är dock inte alla studier som påvisar bättre överlevnad för HPV-positiva patienter, och det föreligger svårigheter att jämföra resultat då olika typer av analysmetoder, för påvisande av HPV, används i olika studier.

I det tredje arbetet i avhandlingen studerades överlevnaden hos 128 patienter med munhåle- och svalgcancer. De delades in efter om de var HPV-positiva eller HPV-negativa, samt efter om tumören satt i svalget eller i munhålan. Från varje patient samlades celler in genom att stryka med bomullspinne över själva tumörytan och också över själva svalget och gombågarna (eftersom förekomsten av HPV visat sig vara störst just i svalget). Dessutom gjordes en munsköljning och celler från den samlades in efter centrifugering. Till skillnad från de flesta, men inte alla, tidigare publikationer kunde vi inte påvisa någon statistiskt signifikant skillnad i överlevnad mellan HPV-positiva och HPV-negativa patienter, varken för munhålecancer eller för svalgcancer.

Vi funderade då över om metodvalet att samla celler för analys kunde påverka utfallet av undersökningen. I arbete IV jämförde vi därför resultaten av HPV-analys från det föregående arbetet med HPV-analys gjord på tumörvävnad från de vävnadsprover (biopsier) som tagits på samma patienter vid tidpunkten för diagnos. Inte heller vid denna metod fick vi fram någon säker skillnad i överlevnad, men en tendens mot bättre överlevnad för den HPV-positiva gruppen med svalgcancer kunde anas.
Vi fann i denna undersökning också att i 27% av fallen var utfallet av HPV-analysen olika mellan de båda provtagningsmetoderna. Detta belyser vikten av att försöka komma fram till en standardiserad metod för bedömning av HPV-förekomst för att kunna jämföra resultaten mellan olika studier.

I det femte arbetet studerade vi överuttryck av några proteiner som vid överuttryck har visat sig ha samband med en del olika cancerformer. I ett material på 55 tumörer från munhåla och svalg sattes det i relation till överlevnad. Ett kraftigt överuttryck av alpha-B-crystallin visade sig vara förenat med betydligt sämre överlevnad avseende munhålecancer men inte svalgcancer.

Sammanfattningsvis styrker avhandlingen den alltmer komplexa bilden av huvud-halscancer, med påvisande av skillnader mellan munhålecancer och svalgcancer. Det är sannolikt att vi inom en snar framtid kommer kunna använda oss av HPV-påvisning som en faktor för att styra behandling vid svalgcancer men förmodligen inte när det gäller munhålecancer. På motsvarande sätt skulle alpha B-crystallin möjligen vara ett komplement till dagens tumörbedömningar när det gäller valet av terapi vid munhålecancer men inte vid svalgcancer. Betydligt mer klargörande av alpha B-crystallinets roll behövs innan det kan bli aktuellt. För både HPV och alpha B-crystallin krävs fler, om möjligt prospektiva, studier innan de kan komma att användas för terapistyrning i klinisk praxis.

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