

From the DEPARTMENT OF CLINICAL NEUROSCIENCE  
Karolinska Institutet, Stockholm, Sweden

# TONSILLAR CANCER

INCIDENCE, PREVALENCE  
OF HPV AND SURVIVAL

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## ABSTRACT

Since human papilloma virus (HPV) in the mid 1980ies was first observed in squamous cell carcinoma of the head and neck, its role and impact, especially on oral and oropharyngeal cancer, have attracted extensive interest.

The aim of this thesis was to investigate if the clinical impression of increased incidence of tonsillar cancer was true, and if HPV could be linked to this increase. Molecular evidence that HPV could exert its transforming capacity in tonsillar cancer was searched for as well as if the presence of HPV had prognostic value.

The Swedish Cancer Register was used to assess the incidence rates of tonsillar cancer over time. There was a significant increase since the register started, especially pronounced the last 15 years and in men.

Paraffin embedded diagnostic biopsies were analysed to estimate the prevalence of HPV in tonsillar cancer in Stockholm between 1970-2002. Two hundred and three cases could be examined and the HPV prevalence was found to have increased from 23 % in the 70ies to 68 % in 2000-2002.

HPV was found to be a strong favorable prognostic factor and patients harbouring HPV positive tumors fared better. In addition, HPV viral load and expression of the viral oncogenes E6 and E7 were analyzed and in most HPV-16 positive tumors, expression of E6 and E7 was ascertained.

Survival for patients with tonsillar cancer was assessed in a nationwide setting to see if the increased prevalence of HPV had any impact. The Swedish Cancer Register was used to evaluate the relative survival in tonsillar cancer since 1960 and the Stockholm cohort was studied thoroughly to determine if the improved survival was due to diagnostic or therapeutic improvements.

The relative survival has increased, both in men and women, and when analysing the Stockholm cohort this could not be explained by improved treatment or earlier diagnosis. It is feasible that the increased survival of tonsillar cancer the last decades is caused by and closely related to the increase in HPV related tonsillar cancer

This thesis report a parallel and substantial increase in the incidence of tonsillar cancer and the prevalence of HPV in tonsillar cancer in Sweden. HPV positive cases express their oncogenes and patients with HPV positive tumors have a significantly better disease specific survival. Finally, survival in tonsillar cancer improved in all over Sweden the last 40 years.

This should pave way for future trials when planning treatment and also be addressed when discussing vaccination.

## LIST OF PUBLICATIONS

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## LIST OF ABBREVIATIONS

Bp	base pairs
BPV	bovine papilloma virus
CI	confidence intervall
CRPV	cottontail papillomavirus
E	early
E6-AP	E6-associated protein
EGFR	epidermal growth factor receptor
IARC	International Agency for Research on Cancer
ICD	International Classification of Diseases
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	hazards ratio
ISH	in situ hybridization
kDa	kilodalton
L	late
LCR	long control region
ORF	open reading frame
PAD	Pathological Anatomical Diagnosis
RT	reverse transcriptase
PCR	polymerase chain reaction
Rb	retinoblastoma
RT	reverse transcriptase
SCC	squamous cell carcinoma
SEER	Surveillance, Epidemiology and End Results
UICC	International Union Against Cancer
VLP	virus-like particle





# 1 INTRODUCTION

Head and neck cancer is a heterogeneous group of tumours when considering histopathology, prognosis and different incidence rates worldwide. There might even be a great variation in incidence for tumours of different sub sites between regions within one country. These differences can to some extent be explained; for instance are the rates for oral cancer very high in parts of India where Bethel nut chewing is common. Smoking and alcohol are regarded as the main etiological factors for head and neck squamous cell carcinoma (HNSCC) [1] [2], but these tumours do also occur in some 15-20% of patients without these risk factors [2, 3]. Other carcinogenic substances are Bethel and also viruses.

In the literature most reports on head and neck cancer mix sub sites, albeit the histological, prognostic and epidemiologic differences between tumours from one sub site to another. This is why this thesis will describe only tonsillar cancer and its incidence and survival over time, the prevalence of HPV in the disease and the prognostic value of this virus for the disease.

## 1.1 TONSILLAR CANCER

### 1.1.1 Symptoms

Tonsillar cancer is the most common of the oropharyngeal malignancies and belongs to the group of head and neck cancer that originates in the Waldeyer's ring.

Tonsillar cancer is occasionally diagnosed at an early stage but more commonly when regional metastases are present. Quite frequently the patients have called on their GPs due to "a sore throat" and been treated with antibiotics prior to cancer diagnosis. Patients may present with a lump in the neck as the sole symptom and later, after examination, the primary tumour of the tonsil is found. One sided ear pain or swallowing difficulty are other common first symptoms.

### 1.1.2 Descriptive epidemiology

As for other head and neck cancers the incidence rates of tonsillar cancer vary widely around the world and even within populations. The black population in the US tends to have higher incidence rates than whites and hispanics all over the country. In most continents the rates tend to be higher in males than in females with a ratio from 2:1 up to 5:1. [4] The SEER, Surveillance Epidemiology and End Results, covers approximately 14% of the US population and the age-standardized rates of tonsillar cancer in whites was 1.4 for men and 0.4 for women 1993-1997. For blacks the rates were 2.9 and 0.6, per 100 000 person years, respectively. All rates are standardized to the world population between 1993 and 1997.

By contrast, in China, the rates of tonsillar cancer are in general low, for instance in Beijing where the rates were 0.1 for men and 0.0 for women, respectively.

Interestingly, in Hong-Kong and in Taiwan, places with great western influence, the rates were 6 to 12 times higher than in Beijing.

In India, with high rates of oral cancer, the rates of tonsillar cancer were between 0.8 and 2.8 in men and 0.2 and 0.5 in women, respectively.

The only parts of the world where women had higher incidences than men were in the Philippines and in Vietnam. Interestingly, this was true also for the Philippine population in California.

In Europe the incidence rates showed great variations with intra national variability in some countries. The highest rates were seen in parts of France, in Somme, where the rates for men were as high as 6.4 and for women 0.8. In Sweden the age standardized rates were 0.9 for men and 0.3 for women[4].

Few studies have assessed the changes in incidence of tonsillar cancer over time in different parts of the world. Frisch and colleges used the SEER program to assess any changes in tonsillar cancer incidence between 1973-1995 and found a considerable annual increases in men (2.7% in blacks and 1.9% in whites), while no similar increase was seen in other oral cancers [5]. In Finland where they have a nationwide cancer registry which covers all of the population. Syrjanen investigated the time trend and found a 2-fold increase in tonsillar cancer the last 40 years in both men and women [6].

### **1.1.3 Risk factors**

Smoking and alcohol, in combination or separately, are well known risk factors for oral- and oropharyngeal cancers [7] [8]. Since the early 90ies HPV has also been suggested as a risk factor for this disease [9]. Several studies, in line with cervical cancer, have found that sexual behaviour, such as a high number of sexual partners, is a risk factor for tonsillar cancer [10, 11]. Also certain sexual behaviour can be strongly associated with HPV positive tonsillar cancer including a history of performing oral sex and oral-anal contact [12, 13]. Individuals at increased risk of tonsillar cancer include those with a history of HPV associated anogenital malignancy [14] women over the age of 50 years with a history of in situ cervical cancer, and husbands of women with in situ or invasive cervical cancer [15]. A recent published case-control study found that a high lifetime number of vaginal-sex partners (26 or more) was associated with oropharyngeal cancer, as was a high-lifetime number of oral-sex partners (6 or more). However, it can not be ruled out that transmission can be made by mouth to mouth contact or by other means [16].

It has also been shown that patients with Fanconi anemia have increased risk for HNSCC with as much as 500-fold to 700-fold. This may be attributable to an increased genetic susceptibility to HPV mediated tumour genesis [17, 18].

### **1.1.4 Histopathology**

The vast majority of tonsillar carcinomas are squamous cell carcinoma. The second largest is the undifferentiated cancer group, which comprises less than 10 patients a year during 2000-2006 [19]. In addition, there are few cases of salivary gland tumours, melanomas, Kaposi sarcomas, other sarcomas and adenocarcinomas. The lymphomas are classified differently and not included in this group. The tonsillar cancers with squamous cell origin are subdivided into well, moderately and poorly differentiated tumours. The HPV positive tumours are more frequently poorly differentiated than well

differentiated [20-23]. Sometimes the HPV positive cancer cells can present with koilocytosis.

### 1.1.5 Classification

Classification of head and neck cancer is done prior to treatment according to the International Union Against Cancer, UICC, 2002. The classification is based on the size of the primary tumour (T), presence, size, numbers and localization of regional metastatic disease (N) and presence of distant metastasis or not (M). This classification scheme has been revised during the years and the latest is from 2002. During the last years small changes have been made, mainly in subdividing different categories, but since the first edition major revisions have been made [24].

### 2002 (Dark Green back and grey glossy front cover) – “Sixth edition”- Wiley-Liss

#### T-Category

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4a	Tumour invades any of the following: the larynx, deep/extrinsic muscle of tongue (genioglossus, palatoglossus, and styloglossus), medial pterygoid, hard palate, and mandible
T4b	Tumour invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base; or encases the carotid artery

#### N-Category

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a single ipsilateral lymph node, more than 6 cm in greatest dimension

**Note:** midline nodes are considered ipsilateral nodes

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1,T2	N1	M0
	T3	N0,N1	M0
Stage IVa	T1,T2,T3	N2	M0
	T4a	N0,N1,N2	M0
Stage IVb	T4b	Any N	M0
	Any T	N3	M0
Stage IVc	Any T	Any N	M1

**Table 1.** *TNM and stage classification of oropharyngeal cancer according to UICC 2002.*

### 1.1.6 Treatment

Treatment for tonsillar cancer varies over the world, and there have also been changes over time in Sweden. In 70ies and 80ies treatment included not only radiotherapy but also chemotherapy [25] and patients were more often surgically treated at the primary site. Today treatment is based on radiotherapy, either conventionally fractionated or accelerated/hyper fractionated. Neck dissection is performed when neck nodes are present at diagnosis and do not show complete response after radiotherapy, or in the case of extensive neck disease. Surgery towards the primary site is only performed when there is residual or recurrent disease. Patients with advanced disease may participate in a study with induction chemotherapy followed by accelerated radiotherapy with EGFR-blocker.

### 1.1.7 Prognosis

The prognosis for head and neck cancer are highly dependent on tumour stage at diagnosis and on tumour origin. Regarding tonsillar cancer specifically the 5-year survival rate in Europe was 32 % according to a survey by Sant and colleagues. In Europe, survival was usually lower in France, Italy and Spain when compared to Northern Europe, and these differences were more pronounced in younger than older patients. In Sweden the age-standardized 5-year survival was 46.6 % in men and 56.2 % in women [26].

## 1.2 HPV

### 1.2.1 Taxonomy

HPV was first identified in 1949 [27] and today over 100 different HPV types have been characterized and several sub-genomic sequences are being considered as novel HPV types [28]. HPVs belong to the Papillomaviridae family, and they infect a broad spectrum of vertebrates, including man. They are highly species specific and do not infect over the species barrier. Therefore the different papillomaviridae are named after the species they infect and numbered after the order of their discovery. The most

studied viruses are cottontail rabbit papillomaviruses (CRPVs), bovine papillomaviruses (BPVs) and human papillomaviruses. The taxonomic classification is based on sequence variation in the L1 open reading frame (ORF) and the taxonomic levels within the family are “genus”, “species”, “types”, “sub-types” and “variants”. The criteria for different genera are less than 60 % nucleotide sequence identity in the L1 ORF and a full length sequence identity of 23-43 % [28]. Species within a genus share 60-70 % nucleotide identity and have common biological and pathological properties. For the lower levels, the L1 ORF sequence should differ more than 10 % between types, 2-10 % between sub-types and less than 2 % between variants.

### 1.2.2 Viral particle

Human papillomaviruses are non-enveloped, small circular double stranded DNA viruses with an icosahedral capsid [29]. The capsid of 72 capsomeres is approximately 55 nm in diameter. The DNA of HPV is chromatin-like and covered by histones [30].



**Figure 1** *The viral capsid*

### 1.2.3 Genomic organization

The viral genome consists of 7200-8000 base pairs, and is organized in three different regions; the long control region (LCR) and the two open reading frames: the early (E) and the late (L) region. The LCR is a non-coding region where the regulation of viral gene expression is controlled. The E region codes for regulatory proteins E1, E2, E4-7 and the L region codes for structural proteins L1 and L2.

### 1.2.4 Life cycle of HPV

HPV infection is highly restricted to basal cells in the mucosal or epithelial cell layers. Replication occurs within the nucleus of the infected cell and is dependent on S-phase entry since it requires the cellular DNA machinery [31]. The virus is present in a latent form in proliferating cells, while the productive large scale viral DNA replication, translation, functional activities of the late proteins and viral assembly are restricted to the differentiating layers of skin and mucosa [30]. Regulation of viral gene expression is complex and involves both viral and host factors and is controlled by elements

present in the LCR, and in some cases by elements within the gene itself. In the basal cells of the squamous cell epithelium low levels of early transcripts (E6, E7, E5, E1 and E2) are detected. These transcripts are initiated at the early promoter, located upstream of the E6 ORF, in the LCR. When the cell has divided, one daughter cell migrates towards the suprabasal regions and starts to differentiate. An uninfected cell leaves the cell cycle when it starts to differentiate, while an HPV infected cell re-enters the S-phase after reaching the suprabasal layer [32], giving that both cell cycling and differentiation occur at the same time [33].

The early ORFs (E1, E2, E4, E5, E6, and E7) encode for proteins involved in regulatory functions, DNA replication and activation of the lytic cycle, while the late ORFs (L1 and L2) encode for proteins that build the viral capsid.

#### *1.2.4.1 E1*

The E1 is the largest and a highly conserved region of the HPV ORFs and codes for a 68 kDa proteins, with both ATPase and helicase activities [34]. The E1 protein is expressed at low levels in HPV positive cells and has site-specific DNA binding sequences that bind to the origin of replication and initiate DNA replication[35]. DNA binding is stabilized by complex formation with the E2 protein.

#### *1.2.4.2 E2*

The E2 protein regulates viral transcription and is therefore important for the viral cell-cycle. E2 also assists E1 in viral replication [29] since it directs E1 to its DNA binding site and enhances the binding affinity of E1 to DNA. It is suggested that E1 and E2 control the the copy number of episomal HPV copies/cell since the number of HPV copies increases with increased E1 or E2 expression [33]. The E2 ORF is frequently disrupted when the HPV genome is integrated. This leads to a more malignant phenotype as seen in cervical cancer biopsies and cell lines derived from cervical cancer [30] and has been suggested to be due to the loss of the E2 repression of the E6 and E7 transforming proteins [29].

#### *1.2.4.3 E4*

The 17kDa E4 protein from high-risk types has been described to associate with and destroy keratin networks which facilitates the release of viral particles [32]. Other suggested functions are regulation of gene expression and induction of G2 arrest [35].

#### *1.2.4.4 E5*

The E5 ORF codes for a small highly hydrophobic membrane bound protein which is primarily expressed in the late viral life-cycle. The E5 proteins of high-risk HPVs has weak transforming activities[35].

#### *1.2.4.5 E6*

The HPV-16 E6 protein is a 151 amino acid protein, containing two zinc fingers. Although small, E6 induces several important changes in the host cell with impact both

on the normal viral life cycle and the process of immortalization. E6 is one of the earliest expressed genes during an HPV infection. It provides several functions that alter the cellular environment, making it more amenable to the production of new viral particles. These include blocking apoptosis through p53 degradation and increasing cellular life-span through increased telomerase activity[33].

It is important that viruses avoid cell death, at least until they have replicated. Apoptosis is a natural death process for cells and is normally induced in response to cellular stress. p53 is a transcription factor that stimulates the expression of genes involved in cell cycle arrest and apoptosis, such as the cyclin dependent kinase inhibitor, p21<sup>CIP</sup>. E6 has been shown to bind to p53, induce its degradation via ubiquitin dependent proteolysis of p53 by interacting with the E6-associated protein (E6-AP) [36, 37]. The result is a blocking of apoptosis and accumulation of genomic abnormalities.

#### *1.2.4.6 E7*

The E7 ORF of HPV encodes the major transforming protein. HPV-16 E7 is a nuclear protein of 98 amino acids with casein kinase II phosphorylation sites. Numerous interactions between E7 and cellular proteins have been described, and many of these involve factors associated with the regulation of cell growth. The most important effect is the interaction between E7 and the retinoblastoma tumour suppressor protein family. When bound to the Rb protein, the transcription factor E2F is released and this induces transcription of cyclins and cyclin dependent kinases necessary for progression of the cell cycle from G1 to S-phase [33].

#### *1.2.4.7 L1*

The L1 ORF is highly conserved between different HPV types and is only expressed in terminally differentiated epithelial cells [38]. It codes for the major capsid protein, which is present in 360 copies per virion. The L1 protein self-assembles into pentamers, which are building blocks of the viral capsid and they can also self-assemble to virus-like particle (VLP).

#### *1.2.4.8 L2*

The L2 ORF codes for the minor capsid protein, present in around 12 copies per virion. Expression of the L2 protein is restricted to terminally differentiated cells of the epithelium [38].

### **1.2.5 Integration**

In cervical cancer, the genome is mainly integrated [39]. The integration frequently leads to the disruption of E2 gene regulating the expression of E6/E7. With regard to tonsillar cancer, a few studies have addressed this issue. However, these studies have not been conclusive since some report HPV to be mainly episomal [9, 40] while others report the opposite [41, 42]. All these studies used different methods to address this issue and could explain the discrepancy, but the physical status has to be clarified in further studies.

### 1.2.6 HPV in human cancers

Approximately 15 % of all human cancers are thought to be caused by viruses [43]. Viruses can induce tumours indirectly or directly. Indirect ways involve suppression of the immune system in order to avoid the elimination of virally infected cells. This is the mechanism in for instance some lymphomas. Direct ways include the so called “hit and run” mechanism and insertion of the viral genome into the host cell genome. Hit and run comprise events where the virus initiates something that eventually leads to the development of tumour growth, although the virus itself is no longer present in the affected cell. In addition, the viral genome may carry oncogenes which may, or may not, be a part of the viral life cycle [44]. The studies on viral oncogenes have provided great knowledge, not only related to the virus, but also concerning what controls the normal cell cycle.

The most commonly associated cancer with HPV is cervical cancer. Parkin and Bray reviewed the literature and found 40 % of the anal cancer was attributable to HPV worldwide, 40 % of the vulvar and vaginal cancers and 40 % of penile cancers [45]. Anal cancer is most common in men with a ratio of 2:1 to females. There is a particularly high incidence among male homosexuals in San Francisco and the risk is further increased with HIV. Cigarette smoking, anal intercourse and number of lifetime sex-partners also increases the risk of anal cancer [46].

Cancer of the penis is globally a rare cancer, and in some populations there are particularly low incidence rates. For instance in Israel among the Jewish population the incidence rate is low. The importance of circumcision in decreasing the risk of penile cancer has been evident for many years, and a case-control study have implied a three-fold risk reduction in circumcised men [47]. The geographical correlation between penile and cervical cancer has suggested a common aetiology, as has the concordance these two cancers shows in married couples [48].

### 1.2.7 HPV in cervical cancer

zur Hausen proposed already in 1976 that cervical cancer might be caused by HPV and in the early 80ies epidemiological data was presented supporting this hypothesis [49]. Since then, accumulating epidemiological and molecular data have demonstrated a causal relationship between HPV and cervical cancer [50]. Today HPV is accepted to contribute to nearly 100 % of the cervical cancer cases [45]. In a pooled analysis from 11 case-control studies around the world, Munoz and colleagues assessed that the prevalence of HPV types differs between countries and continents. The most prevalent type was HPV 16 with an overall prevalence of 58.9 percent, ranging from 43.9 percent in the Philippines to 72.4 percent in Morocco. HPV-18 was the second most prevalent type with an overall prevalence of 15.0 percent (3.7 percent in Spain and 27 percent in the Philippines). HPV 31 and 35 were more prevalent in Latin America and the prevalence of type 52 was highest in Peru. The third most common type, HPV-45 had its highest prevalence in the Philippines and lowest in Spain and Colombia [51].



## 1.3 HPV IN TONSILLAR CANCER

### 1.3.1 Evidence for HPV in tonsillar cancer

Numerous studies have shown a high prevalence, 45%-75%, of HPV in tonsillar cancer [9, 16, 20, 21, 52]. However, HPV presence in tumour tissue does not necessarily imply viral involvement in the carcinogenesis but may reflect a transient infection [53]. An important epidemiological finding was when Mork and co-workers in a nested case-control study showed that sign of an earlier HPV infection, as measured in sera, was an independent risk factor for the development of tonsillar- or base of tongue cancer. This finding was important since it showed that HPV infection preceded the development of cancer by 9.4 years on average. The odds ratio for developing oropharyngeal cancer was 10.2 (95% CI 2.4-42.9) and base of tongue cancer was 20.7 (95% CI 2.7-160.1)[54].

There are several epidemiological links between HPV and oral and oropharyngeal cancer. In case-control studies oral oncogenic HPV infection has been found to be associated with an increased risk for oral- and oropharyngeal cancer. Risk estimates were stronger when restricted to oropharyngeal cancer [12, 55, 56].

Different molecular findings are also in favour of HPV as a causative agent for tonsillar cancer. When using DNA as well as RNA *in situ* hybridization, the viral genome and its transcription products (performed on HPV-16) have been located in cancer cells, both at the primary site and in the metastasis, but not in the surrounding stroma [22, 57, 58]. HPV 16 E6 and E7 mRNA expression is known to be essential for transformation in cervical cancer [59]. These oncogenes have also been detected in tonsillar cancer, thus also suggestive of a causal role for HPV [60-62].

Smeets and colleagues demonstrated HPV-DNA by PCR in 24/143 oral and oropharyngeal tumours, but could confirm viral involvement by E6/E7 expression analysis in only 12/24 samples [63]. Thus, the presence of HPV-DNA does not per se mean that the virus is biologically active. It was shown that tumours with transcriptionally active HPV exhibited lack of p53 mutations and a limited number of genetic imbalances, whereas their counterpart without transcriptionally active HPV showed p53 mutations in 75% and many genomic imbalances [64, 65]. By considering detection of HPV E6 in frozen samples as a gold standard for valid HPV infection, Smeets and co-workers found p16 immunostaining followed by GP5+/6+ PCR on the p16 positive cases to harvest a 100% sensitivity and specificity for assumed clinically relevant HPV infection [63]

### **1.3.2 Prognostic value of HPV in tonsillar cancer**

When investigating the prognostic value of HPV in HNSCC in general, some studies have shown no effect or worse survival in the HPV positive cases [66-68]. However, when stratifying for tonsillar cancer only, the majority of studies report an overall better prognosis for patients with HPV positive tumours [13, 20, 52, 69-71]. This becomes even more evident when subdividing the HPV positive tumours in “active” vs not “active” HPV in the tumors as measured by p16 over-expression [72]. Some studies have suggested that an increased radiosensitivity is the reason for the improved survival but others have found an increased survival in the surgically treated patients as well [73, 74]. Ragin and Taioli reviewed all reports on tonsillar cancer and prognosis and found that an overwhelming majority of the investigations reported an improved survival for HPV positive tonsillar cancers [75]. One recent published prospective clinical trial showed a strong association between HPV positivity and therapeutic response and survival [76]. All these studies have included less than 100 tonsillar cancer patients.

### **1.3.3 HPV in tonsillar cancer over the world**

The proportion of HPV positive tonsillar cancer may vary depending on the methods used for analysis but also due to geographical differences. There has been a few multi-centre studies conducted on this issue and they showed great variance in the prevalence of HPV in this disease. There was a significantly higher prevalence of HPV-16 in oropharyngeal cancer in North America and Asia when compared with Europe [77]. Also, in Europe there was a great variation ranging from 8.3 % to 100 %, whereas reports from North America showed greater homogeneity, ranging from 33.8 % to 57.1 %. In the IARC multicenter case-control study they showed an overall prevalence of 24.7 % for HPV in tonsillar cancer [78]. Interestingly, Li and colleagues found no HPV positivity among those 16 investigated tonsillar cancer cases from The Jilin Province in northern China [79] but a prevalence of 29 % in cases from Hong-Kong, in China but with a strong western influence [80].

### **1.3.4 Different types of HPV in tonsillar cancer**

In contrast to HPV in cervical cancer, the prevalence of different HPV types in tonsillar cancer does not tend to vary with the region investigated. Herrero and colleagues launched a multi-centre case-control study from nine countries [78]. Overall they found a prevalence of 24.7 % when investigating tonsillar cancer and 18.3 % in patients with cancer of the oropharynx. Similar to other studies [10, 20, 81] they found that the vast majority of HPV positive tumours had HPV-16, around 95 %. Kreimer and colleagues reviewed published articles and also found the vast majority to be HPV-16 positive (95 % CI 82.6-90.1). They also found that the second most common type, HPV-18, was detected in only 2.8 % (95 % CI 1.3-5.3) of the cases [77]. The HPV types tend to be more homogeneously distributed over the world in tonsillar cancer as compared to cervical cancer, where large differences are noted [51].

## 2 AIM OF THE THESIS

The aim of this thesis was to study what influence HPV may have had on the incidence rates of tonsillar cancer in Sweden and the prognostic value of HPV on this disease. More specific:

-To investigate if the incidence of tonsillar cancer has increased over time in Sweden, despite a decrease in other smoking related cancers.

-To investigate if the prevalence of HPV in tonsillar cancer has increased the last decades, indicating HPV as a possible explanation for the increasing incidence.

-To study the presence of HPV, HPV-16 viral load, expression of E6 and E7 mRNA and their possible impact on prognosis.

-To study if the increasing prevalence of HPV in tonsillar cancer has had impact on the prognosis of the disease in a nationwide setting. Finally, using the population for survival analysis and the Stockholm population as a reference to see if earlier diagnosis or different treatment strategies may have influenced outcome.

## **3 MATERIAL AND METHODS**

### **3.1 SETTINGS**

#### **3.1.1 The Swedish Cancer Register**

The Swedish Cancer Register was started by the Swedish Board of Health and Welfare in 1958, coded according to the 7<sup>th</sup> edition of International Classification of Diseases (ICD-7). The register also includes information on basis of diagnosis. A code to distinguish accidental findings during autopsy was introduced around 1971-1975. All newly diagnosed malignant tumours must be notified to the register by physicians, pathologists or cytologists. During the first two years the register has been estimated to be incomplete but since 1960 the register is estimated to be more than 98 % complete, though the completeness may vary by site [82].

The patients in the Stockholm cohort were also recruited from the Swedish Cancer Register.

#### **3.1.2 The Swedish Causes of Death Register**

This registry holds information on the date of death, as well as any underlying contributory causes of death of all deceased residents in Sweden since 1952. The overall completeness of the register is estimated to exceed 99 % [83]. The causes of death are classified according to ICD-7 through 1968, ICD-8 during 1969-1986, ICD-9 during 1987-1996 and ICD-10 thereafter.

#### **3.1.3 The Swedish Population and Migration Register**

The registry is maintained by Statistics Sweden and contains official Swedish census data since 1960 in computerized form, including current address of all Swedish residents alive at the end of each year. Migration has been registered since 1968.

#### **3.1.4 The Swedish Inpatient Register**

The Swedish Inpatient Register was launched by the Swedish Board of Health and Welfare in 1964, collecting data on individual hospital discharges. This register also follows the ICD-7 through 1968, ICD-8 between 1969-1986, ICD-9 between 1987-1996 and thereafter ICD-10. Surgical procedures are coded according to Swedish Classification of Operations and Major Procedures through 1996 and the Nordic medico-statistical committee (NOMESCO) classification of surgical procedures thereafter. The percentage of the Swedish population covered by the Inpatient Register was 60 % in 1969, 85 % in 1983 and 100 % from 1987 and onwards [84].

## **3.2 STUDY POPULATIONS**

### **3.2.1 All tonsillar cancer patients in Sweden (Paper I and IV)**

In paper I, all cases of tonsillar cancer (ICD-7 code 145.0) were retrieved between 1960-2003 from the Cancer Registry, and for study IV from 1960-2001.

Histology codes for paper I was SCC and for paper IV, SCC and undifferentiated cancer.

In total, there were 2165 cases of tonsillar cancer code 145.0 between 1960-2003 and 1848 cases between 1960 and 2001.

In paper I, the trends of tonsillar cancer incidence rates were compared with the trends for oral cancer incidence rates, which are smoking and alcohol induced.

For paper IV, the cohort was linked to The Swedish Causes of Death Register and to The Swedish Population and Migration Register for accurate death data and data on loss to follow-up.

### **3.2.2 All tonsillar cancer patients in Stockholm (Paper II, III and IV)**

Clinical data and tumour characteristics from all cases of tonsillar cancer in Stockholm County between 1970-2002 with squamous cell carcinoma were retrieved. All cases had diagnose-code 145.0. In all, there were 515 cases. Data are summarized in Table I, paper III and in Table III in paper IV.

Archival paraffin blocks with diagnostic tumour biopsies were collected for all patients for analysis of HPV, study II and III.

All studies were conducted with ethical approval from the Ethical Committee of Karolinska Institute.

## **3.3 MEASUREMENTS**

### **3.3.1 DNA and RNA extraction (Paper II and III)**

DNA extraction from paraffin embedded material was performed using either a standard phenol-chloroform protocol, the QIAamp DNA Mini kit (Merck Eurolab AB) or as in most cases, using the High Pure RNA kit (Roche Diagnostics, Stockholm, Sweden) excluding DNase treatment. RNA extraction of the samples in paper III was performed using the High Pure RNA kit (Roche Diagnostics, Stockholm, Sweden). DNA and RNA content was measured with a spectrophotometer. All samples analysed by real time PCR were extracted with the High Pure RNA kit.

### **3.3.2 General HPV PCR (Paper II and III)**

For detection of at least 27 HPV types, consensus primers GP5+/6+, located within the conserved L1 region, were used [85]. The final volume of 50µl consisted of 5 µl 10xPCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 3.5 mM magnesium chloride (MgCl<sub>2</sub>), 25 pmol of each primer, 0.5 µmol/ul bovine serum albumine (BSA) and 1 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). Cloned plasmids of HPV-16 were used as positive controls and a negative control with no DNA (water) was always included. The program started with 4 min denaturation at 94°C followed by 40 cycles of 94°C for 1 min, 44°C for 1 min, 71°C for 2 min, and finally 71°C for 10 min.

The samples negative for GP5+/6+ were further tested using Cpl/IIG primers, located within the E1 region [86] to avoid false negative samples due to disrupted E1. The 50µl used for this amplification was a solution of 5µl 10xPCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 3mM MgCl<sub>2</sub>, 17 pmol of Cpl, 26 pmol of CpIIG, 0.5µg/µl BSA and 2.5 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). Positive and negative controls were used as described above. This program started with 5 min denaturation at 94°C followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, and finally 72°C for 10 min.

### **3.3.3 Type specific HPV PCR (Paper II and III)**

To determine the HPV type, type specific primers for HPV 16, 18 and 33 were used [87] and in some cases the samples from the general HPV PCR were also sequenced for type verification (see below). The 50 µl PCR mix used for these amplifications consisted of 5 µl 10xPCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 3.5 mM magnesium chloride, 20 pmol of each primer and 1 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). Cloned plasmids of HPV 16, 18 and 33 were used as positive controls and a negative control with no DNA was included. The PCR program started with 4.5 min denaturation at 95°C followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and finally 72°C for 4 min.

### **3.3.4 Verification of amplifiable DNA by PCR (Paper II)**

To check if amplifiable DNA could be obtained from the samples, 127 bp of the ribosomal S14 gene was amplified with S14 sense/anti-sense primers. The 50 µl PCR mix consisted of 5 µl 10xPCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 1.5 mM magnesium chloride, 15 pmol of each primer, 0.5 µg/µl BSA and 1 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). A positive control (human DNA) and a negative control (water) were included in each run. A 40-cycle amplification was run in an automated thermocycler (Perkin-Elmer, Norwalk, CT). The program started with 1 min denaturation at 94°C followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, and finally 72°C for 10 min.

### **3.3.5 Sequencing of HPV positive PCR products (Paper II and III)**

The results from the type specific PCR were in some cases confirmed by direct sequencing of the PCR products from the general HPV PCR. The general HPV PCR was re-run to increase the yield. The products were purified by gel extraction (QIAquick PCR Purification Kit, Merck Eurolab AB) and were then sequenced using the Big Dye Terminator Cycle Sequencing Kit and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Both DNA strands were sequenced and aligned to those available at NCBI BLAST GenBank ([www.ncbi.nlm.nih.gov/blast/Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)).

### **3.3.6 Real time Quantitative PCR (Paper III)**

In paper III, the number of HPV copies per genome equivalent was estimated using a real time quantitative TaqMan PCR. A dilution series of a cloned plasmid of HPV-16 was used in order to create a standard curve with known amounts of viral copies. The primers and the probe used are described in [40]. Ten  $\mu\text{l}$  of sample DNA was run in three different concentrations in triplicates in 25  $\mu\text{l}$  reactions containing 2.5  $\mu\text{l}$  10x PCR buffer II (Applied biosystems), 200  $\mu\text{M}$  of each dNTP, 1.5 mM  $\text{MgCl}_2$ , 10 pmol of each primer, 5 pmol probe and 0.5 U of Taq DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems). A 40-cycle amplification was run in an iCycler iQ (iCycler iQ real-time PCR detection system, BioRad) and the program consisted of an initial step at 50°C for 120 sec, and 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, and annealing and elongation at 60°C for 60 sec. As an internal reference gene, a commercial kit was used according to the manufacturer's instructions to estimate the gene content of the human RNase P gene (TaqMan RNase P Detection Reagents Kit, Applied Biosystems, Stockholm, Sweden). Calculation of viral load/genome-equivalent was performed as described by Si et al [88].

The detection of E6 and E7 mRNA was also performed as a real time PCR with the primers presented in paper III and the same standard dilution series as for the estimation of the viral load. After RNA extraction as described above, cDNA was synthesized from the extracted RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Copenhagen, Denmark). An HPV-16 type specific PCR was run as described above before cDNA synthesis to verify that no contaminating DNA from the RNA extraction was present. Ten  $\mu\text{l}$  of cDNA was run in a SybrGreen protocol in an iCycler iQ with the 25  $\mu\text{l}$  reaction mix containing 12.5  $\mu\text{l}$  iQMN SYBR Green Supermix (BioRad Laboratories) and 10 pmol/ $\mu\text{l}$  of each of the HPV-16 primers. The program started with 50°C for 120 sec, and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and elongation at 74°C for 30 sec, then a melting curve was applied to verify specificity, starting at 40°C and increasing by 0.5°C every 10<sup>th</sup> second until 120°C was reached. The commercial kit used for quantification of DNA (see above) was used as a control to verify presence of cDNA.

## **3.4 STATISTICAL ANALYSES**

### **3.4.1 Paper I**

In paper I direct standardization according to age distribution of the Swedish population in 1970 was used. Linear regression was used to describe changes over years, with age-standardized rate as a dependent variable and calendar year as an independent variable.

### **3.4.2 Paper II**

Chi-square test was used to compare prevalence rates of HPV in the tumours across different calendar periods. Mann-Whitney test was used to compare age in the HPV positive vs negative patients.

The Swedish population of 1970 was used for direct standardization of tonsillar cancer rates over calendar years.

### **3.4.3 Paper III**

The survival analysis was performed with a multivariate Cox proportional hazards model controlling for age at diagnosis, gender and stage. Kaplan-Meier graphs were used to present the data and Log-Rank test was used to assess the differences in survival rates.

HPV status was correlated to clinical data with the Chi-square test.

### **3.4.4 Paper IV**

The relative survival was calculated as the ratio between observed and expected survival, the latter inferred from the survival of the entire Swedish population in the same age, sex, and calendar year structure. Observed survival rates were estimated using the life-table method.

Age-standardized relative survivals were also estimated due to the aging trend in the Swedish population. Poisson regression was performed to evaluate the univariate and multivariate effects of sex, age groups and time periods on survival of tonsillar cancer. Tests of the equality of survival between patients diagnosed in different time periods were performed using the Maximum likelihood test.

In the Stockholm cohort the Kaplan-Meier method and Cox proportional hazards model were used to evaluate death risk in tonsillar cancer patients over the different time periods.

In both the Stockholm and the Swedish cohort five year survivals were compared in different time periods.



## 4 RESULTS

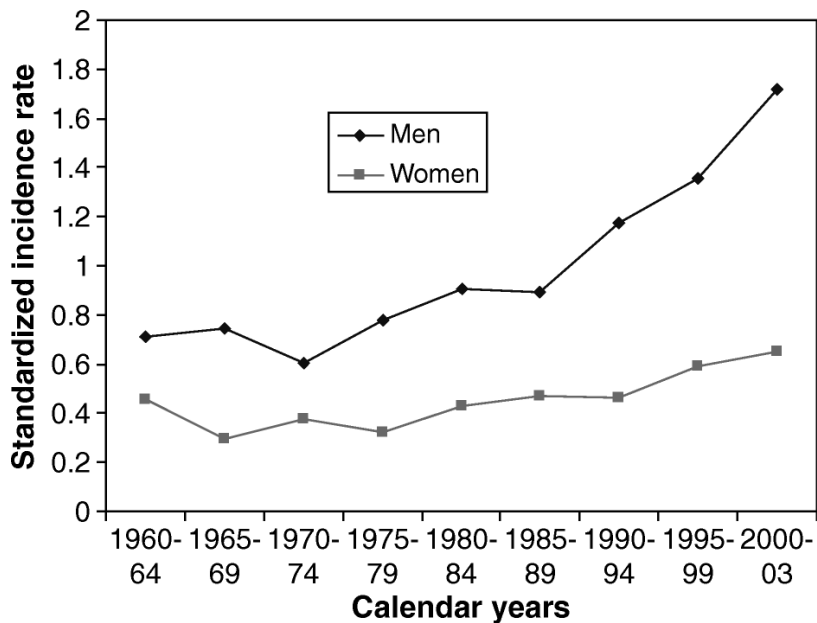
### 4.1 PAPER I

#### 4.1.1 Incidence

During the period 1960-2003 a total of 2165 cases of tonsillar cancer were recorded in the Swedish Cancer Register. The standardized incidence rate of tonsillar SCC in women in Sweden increased from 0.45/100 000 person-years in 1960-1964 to 0.65/100 000 person-years in 2000-2003, with a yearly increase of 1.1%. In men the standardized incidence rate increased 2.6% per year. Table II, figure 2.

**Table II.** *Number of cases of tonsillar cancer, code 145.0, in Sweden per period and gender.*

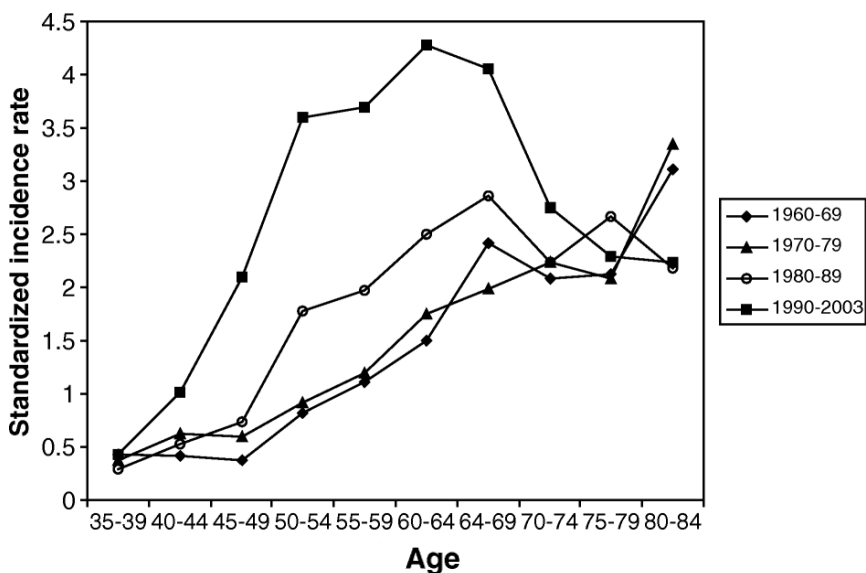
<b>Period</b>	<b>Men</b>	<b>Women</b>
1960-1964	86	34
1965-1969	99	27
1970-1974	92	56
1975-1979	134	44
1980-1984	158	76
1985-1989	163	75
1990-1994	223	88
1995-1999	272	124
2000-2003	299	115



**Figure 2.** Age-standardized incidence rate (to the 1970 Swedish population) of tonsillar cancer, ICD-7 code 145.0, by gender in Sweden during 1960-2003.

#### 4.1.2 Age-specific incidence

For men, the age-specific incidence rates were similar between the periods 1960-1969 and 1970-1979. In later years, particularly after 1990, there was a significant increase of incidence rates in the group aged 40-70 years. However, the incidence rates during the periods 1980-1989 and 1990-2003 in older age groups tended to decrease and converge to the levels during 1960-1969 and 1970-1979. In women there was a similar trend but it was not as pronounced as for men. Figure 3.



**Figure 3.** Age-specific incidence rates of tonsillar cancer, ICD-7 code 145.0, by period among men

#### 4.1.3 Smoking prevalence

Smoking prevalence data are available on a yearly basis since 1980 and smoking has gradually decreased during this time period. Prior to 1980 there are sporadic reports in 1946, 1963 and 1977 indicating a higher prevalence of smoking among men at that time [89].

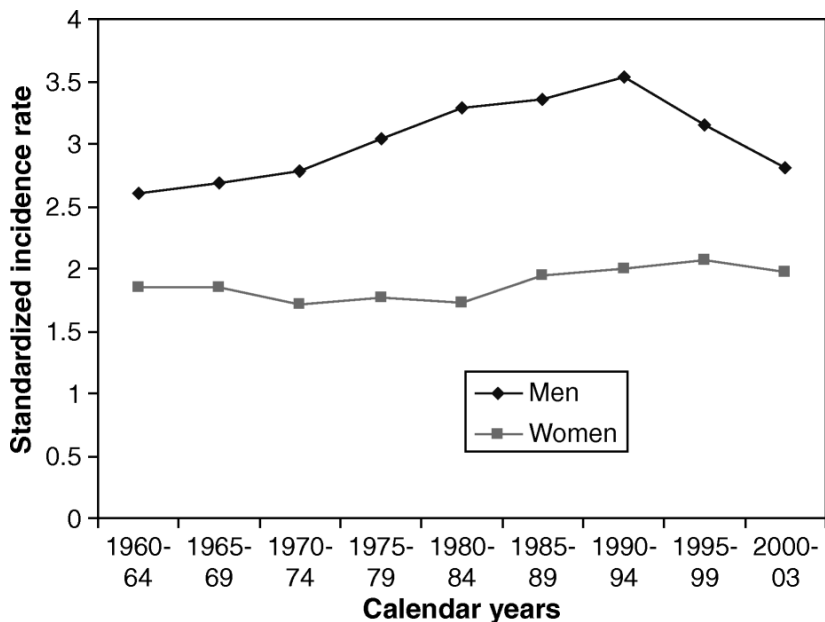
#### 4.1.4 Incidence of other smoking induced cancers

The lung cancer we investigated was coded as 162, tracheal, bronchial and pulmonary cancer. Secondary lung cancer and primary cancers of the pleura were not included in this code. There has been a small decrease in the incidence of lung cancer in men in the last 30 years; however, it is still increasing for women.

Most tonsillar cancers are reported as code 145.0 but some are reported as codes 145.7 or 145.9. The code 145.8 is rarely used. We decided to present these codes separately to show that the increase was mostly due to code 145.0. There were 391 cases of tonsillar cancer coded as 145.7-9 during the last 30 years. There was an increase also in the 145.7-9 group over the whole time period but this increase was not as large as for the 145.0 group, 0.60% per year for men and 0.2% for women. In fact, there has been a leveling off and a small decrease over the last 10 years in this group, for both men and women (data not shown). Translation of site codes in ICD-10 to ICD-7 and 8 are presented in Table I, paper I.

We also investigated the incidence rates of other oral cancers for comparison and we used the codes 141 (tongue cancer), 143 (cancer of the floor of mouth) and 144 (unspecified oral cancer). In the tongue cancer group we excluded code 141.0 since this

is coded for carcinoma of the base of the tongue. This tumour is in the oropharynx and may be related to HPV as well. The rest of the code 141 group was included and it is coded for oral tongue. There has been an increase also in this group compared to 30 years ago, 1.8% for men and 0.3% for women, although the increase has leveled off in women and there has been a decrease in men in the last 10 years. Figure 4



**Figure 4.** Age-standardized incidence rate (to the 1970 Swedish population) of oral cancer, ICD-7 codes 141 (excluding 141.0), 143 and 144, by gender in Sweden during 1960-2003

## 4.2 PAPER II

### 4.2.1 Prevalence of HPV in tonsillar cancer

Ninety-nine out of 203 (49%) of the samples were positive for HPV by either PCR protocol. Eighty of these samples were positive with the broad spectrum HPV primers, and an additional 19 were detected using HPV-16-specific primers. Eighty-six (87%) of the HPV positive samples were HPV-16 positive. Direct sequencing of the products from the general PCRs revealed that 3 samples were HPV-33 positive, 1 sample HPV-35 positive and 1 sample HPV-45 positive. The remaining 8 HPV-positive samples were not possible to type verify by sequencing. The distribution of HPV-positive tonsillar cancer biopsies over time was as follows: 7/30 (23%) from the 1970ies, 12/42 (29%) from the 1980ies, 48/84 (57%) from the 1990ies, and 32/47 (68%) from 2000-2002. The distribution of samples with amplifiable DNA per number of patient samples

over time was 30/39 (77%) from the 1970ies, 42/46 (91%) from the 1980ies, 84/86 (98%) from the 1990ies and 47/47 (100%) from 2000-2002. Hence, there was an increase in the proportion of HPV-positive tonsillar cancer in the period 1970-2002 (Table II). More specifically, there was a significant increase in the proportion of HPV-positive tonsillar cancer 1990-1999 and 2000-2002 compared to 1980-1989 2.0-fold ( $p = 0.0045$ ) and 2.4-fold ( $p < 0.001$ ), respectively. Furthermore, the increase in the proportion of HPV-positive tonsillar cancer 1990-1999 and 2000-2002 compared to 1970-1979 was 2.45-fold ( $p = 0.003$ ) and 2.9-fold ( $p < 0.001$ ), respectively. Table III

**Table III.** Presence of HPV DNA in Pretreatment Tonsillar Squamous Cell Carcinoma Biopsies Obtained between 1970 and 2002

Calendar years	Cases of tonsillar SCC	Pretreatment biopsies retrieved	Excluded <sup>1</sup>	Presence of HPV DNA <sup>2</sup>	$p$ -Value <sup>3</sup>
1970-1979	84	39	9	7/30 (23)	
1980-1989	133	55	13	12/42 (28)	0.79
1990-1999	168	95	11	48/84 (57)	0.0025
2000-2002	92	48	1	32/47 (68)	<0.001
Total	477	237	34	99/203 (49)	

<sup>1</sup>Blocks excluded due to unamplifiable DNA.

<sup>2</sup>Values in parentheses indicate percentage values.

<sup>3</sup> $\chi^2$  test, compared to the frequency of HPV in the 1970-1979 calendar period.

#### 4.2.2 Age in patients with HPV positive and negative tumours

There was a significant difference in median age between patients with HPV positive and HPV negative tonsillar cancer ( $p < 0.001$ ). Patients with HPV positive cancer, had a median age of 55 years, 70 patients were males between 30-84 years of age (median age 55 years), while 29 patients were females between 43 and 78 years of age (median age 55 years). Patients with HPV negative cancer had a median age of 65, 75 patients were males between 37 and 88 years of age (median age 65 years) and 29 patients were females between 42 and 78 years of age (median age 65 years).

#### 4.2.3 The incidence of tonsillar cancer in the Stockholm area

The age-standardized incidence of tonsillar cancer increased from 1.3 to 3.6 (2.8-fold) per 100,000 between 1970 and 2002 in the Stockholm area. In men, the increase was

2.6-fold (1.077-2.81 per 100,000), while in women the increase was 3.5-fold (0.232-0.829 per 100,000)

### 4.3 PAPER III

#### 4.3.1 Patients and tumor features

Two hundred and three patients, see paper II, were included in this study. Clinical data and tumor characteristics are presented in table I, paper III. Complete case reports were available for 192 patients. HPV-positive tumors were more often poorly differentiated ( $p=0.02$ , Chi-square test) and patients with HPV-positive tumors had more often regional lymph node metastasis at the time of diagnosis ( $p=0.03$ , Chi-square test), but no correlation between stage and HPV distribution could be found (TNM stages I and II, compared to TNM stages III and IV) ( $p=0.63$ , Chi-square test).

#### 4.3.2 HPV and prognosis

Only patients receiving treatment with curative intent and with reliable survival data were included in the survival analysis which is why 150 patients could be included. HPV positive patients had a significantly better 5-year survival ( $p<0.001$ , Log-rank test) independent of age, gender and tumor stage, as determined with a multivariate Cox proportional hazards regression model.

The improved survival was consistent over time during the 1980s, the 1990s and 2000-2002, but not during the 1970s, when the number of patients was limited.

Eighty-two patients had a local or regional relapse and patients with HPV positive tumors (24 patients, 29%) had significantly less often relapses compared to patients with HPV negative tumors (58 patients, 71%) ( $p<0.01$ , Chi square test). Table IV.

**Table IV.** Patient and tumour characteristics and HPV status correlated to survival<sup>a</sup>

Characteristics	HR <sup>b</sup> Cox univariate (95% CI <sup>c</sup> )	HR <sup>b</sup> Cox multivariate (95% CI <sup>c</sup> )
HPV	0.17 (0.10–0.30)	0.17 (0.09–0.32)
TNM stage <sup>d</sup>	3.82 (0.93–15.69)	4.06 (0.99–16.71)
Age	1.03 (1.01–1.06)	1.01 (0.98–1.03)
Gender	1.05 (0.63–1.76)	1.15 (0.64–2.06)

<sup>a</sup> Survival hazard ratio according to Cox proportional hazards uni- and multivariate regression.

<sup>b</sup> Hazard ratio.

<sup>c</sup> 95% confidence interval.

<sup>d</sup> TNM stage, tumor stage according to International Union Against Cancer 1997 and TNM stage I–II compared to III–IV.

### 4.3.3 Viral load and survival

The viral load was estimated for the 86 HPV-16 positive tumours analyzed in paper III and it varied between 0.08-130 copies/genome equivalent. Using quartiles as cut-offs for dividing the patients in comparable groups, no clear correlation between survival and viral load could be observed. The cut-offs measured in copies/genome equivalent and the patients with intermediate viral load had the best survival, whereas the patients with the highest and the lowest viral load had lower survival rates.

### 4.3.4 Expression of E6- and E7 mRNA in HPV-16 positive cancers

Fifty-four of the 86 HPV-16 positive tumours analyzed in paper II were available for further analysis of mRNA expression. The tumours not available for analysis were either not available from the archive (20 cases) or contained too little material on the paraffin block (12 cases). Presence of mRNA and successful cDNA synthesis could be ascertained in 53/54 cases, determined by the presence of the housekeeping gene RNase P. Fifty of the 53 tumors (94%) contained E7 mRNA and 42/50 of the E7 expressing tumors also expressed E6 mRNA Table 3. Thus, in three samples no expression of the viral oncogenes could be found despite high levels of the housekeeping gene and presence of HPV-16 DNA. To make sure no DNA was present after RNA extraction, but prior to cDNA synthesis, an HPV-16 type specific PCR was performed. When DNA could be detected in this PCR, the RNA was purified again, using the same kit and performing the steps included DNase treatment and the subsequent washing procedure, Table V.

**Table V** Number of HPV-16 positive tonsillar cancer biopsies also positive for HPV-16 mRNA and E7 mRNA.

	E6 positive	E7 positive	E6/E7 positive	E6 and E7 negative	Total
Number of cases	42 (79 %)	50 (94 %)	50 (94%)	3 (6 %)	53 (100 %)

## 4.4 PAPER IV

### 4.4.1 Patients

We identified a total of 2893 records of tonsillar cancer in the Swedish Cancer Register between 1958-2005. We excluded patients due to non-squamous cell or

undifferentiated carcinoma, patients lost to follow-up due to emigration, cases diagnosed on autopsy or between 1958-1959. A complete follow-up was not possible for cases diagnosed after 2001. During 1960-2001, we identified 1848 cases of primary tonsillar cancer (ICD-7 code 145.0 and PAD code 146 or 196), out of which 2 cases were excluded due to invalid personal registration number and 46 cases excluded due to accidental finding during autopsy. Thus our final cohort comprised of 1800 patients.

Among the patients 70.6 % were males and 29.4 % were females. Mean age at diagnosis was 62.5 years in 1960-1969, 63.6 years in 1970-1979, 62.2 years in 1980-1989 and 60.0 years in 1990-2001. Basic characteristics for the Swedish Cohort are presented in Table VI.

**Table VI.** Patient characteristics for tonsillar cancer cases diagnosed in Sweden during 1960-2001

Period of diagnosis	Total		Men		Women	
	No. of patients	Mean age at diagnosis	No. of patients	Mean age at diagnosis	No. of patients	Mean age at diagnosis
1960-1969	194	62.5	152	62.6	42	62.0
1970-1979	277	63.6	201	63.5	76	63.8
1980-1989	449	62.2	303	62.4	146	61.7
1990-2001	880	60.0	615	59.5	265	61.3
Total	1800	61.4	1271	61.2	529	61.8

#### 4.4.2 Survival in the Swedish cohort

Among men, both the relative and observed survival improved significantly from the 1980ies to the 1990ies. There was a decrease in both relative and observed survival from 1960-1969 to 1970-1979 but thereafter survival improved. The 5-year observed survival was 25.7 % in 1960-1969, 22.4 % 1970-1979, 29.4 % 1980-1989 and 52.7 % 1991-2000.

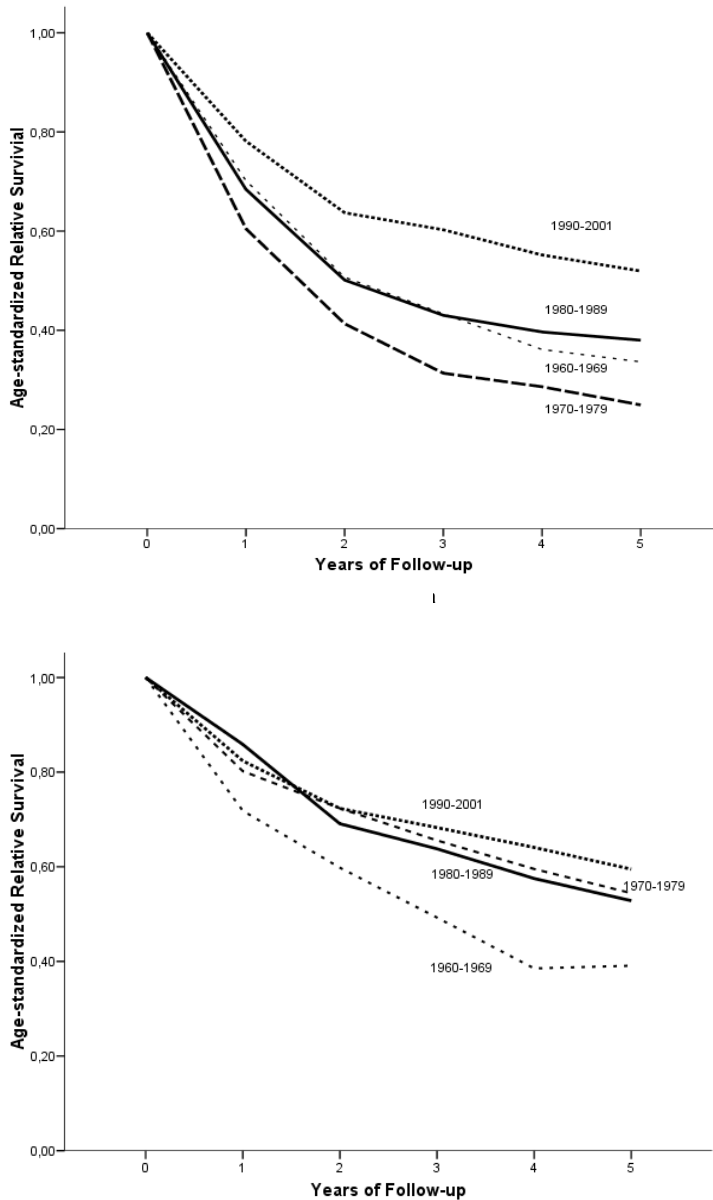
The age standardized relative 5-year survival was 32.0 % 1960-1969, 27.8 % 1970-1979, 35.7 % 1980-1989 and 53.4 % in 1990-2001.

Among women, both the observed and relative survival rates have increased, however a more moderate increase was observed and the survival rates were better to start with compared to for men. In 1960-1969 the observed survival was 40.5 % whereas the age standardized relative survival was 45.1 %. In the following decades the observed and age standardized relative survival was 50.0 % and 59.1 % in 1970-1979, 46.6 % and 49.8 % in 1980-1989, 56.2 % and 59.8 % in 1990-2001, respectively. Figure 5.



When stratifying for gender in the Swedish cohort the relative hazard of dying were significantly lower in 1990-2001 in both genders.

In all, there was a 50 % deficit in relative hazard of dying in the first 5 year period after diagnosis for those diagnosed in 1991-2001 compared to those diagnosed in 1960-1969, when controlling for effects of age for men and 46 % for women. Table VII.



**Figure 5.** Age standardized relative survival in tonsillar cancer for men and women per decade of diagnosis

**Table VII.** Relative hazard of mortality during the first 5-year period after diagnosis of tonsillar cancer in Sweden, 1960-2001, stratified for gender.

	<i>Male</i>		<i>Female*</i>	
	Relative hazard	95% confidence interval	Relative hazard	95% confidence interval
<i>Period of diagnosis</i>				
1960-1969	Referent		Referent	
1970-1979	1.20	0.92-1.56	0.57	0.32-0.99
1980-1989	0.96	0.75-1.23	0.70	0.43-1.15
1990-2001	0.50	0.39-0.63	0.54	0.34-0.86
<i>Age at diagnosis</i>				
<50	Referent		Referent	
50-59	1.88	1.39-2.52	1.49	0.87-2.51
60-69	2.85	2.14-3.79	2.64	1.63-4.29
≥70	3.49	2.59-4.71	4.44	2.77-7.14

\* The multivariate regression model included all variables listed in the table.

#### 4.4.3 Survival in the Stockholm cohort

In the Stockholm cohort we could obtain information on 439 patients of 484 cases reported to the Swedish Cancer Registry. Of these, 102 patients were excluded due to palliative treatment, lack of data, other diagnosis than 145.0 or other histological diagnosis than squamous cell carcinoma. The distribution of the excluded patients over decades were 45.9 % in the 1970-1979, 30.6 % in 1980-1989, 23.5 % in 1990-2001. Basic characteristics for the Stockholm Cohort are presented in Table VIII.

In the Stockholm cohort there was a gradual increase in survival over the decades, most notably between the 1980-1989 and 1990-2001. Figure 6.

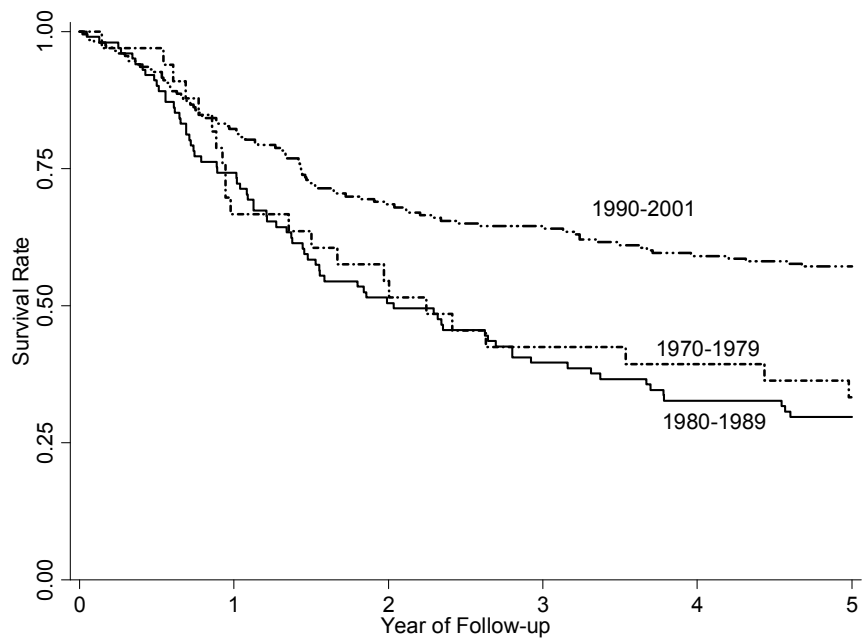
In the multivariate analysis, after adjustment for age, gender, tumour stage, and treatment, the hazard ratio of mortality 5 years after diagnosis for cases diagnosed during 1990-2001 was 0.54 (95% CI 0.32-0.88), as compared with those diagnosed during 1970-1979. As expected, age and tumour stage were another two significant prognostic factors. Treatment with radiotherapy alone conferred a close to 4-fold excess risk of mortality (HR= 3.98, 95% CI 2.76-5.73), as compared to radiotherapy together with neck-dissection. Table IX.

**Table VIII.** Basic characteristics on patients and tumours from the Stockholm cohort, 1970-2001

	1970-1979	1980-1989	1990-2001	Total
Number of eligible cases	96	143	245	484
Number of included cases	33	101	203	337
Males	28	66	144	238
Females	5	35	59	99
Mean age at diagnosis (range)	61.0 (43-76)	63.0 (24-90)	60.0 (29-87)	61.0 (24-90)
Males	60.6 (43-76)	64.8 (37-88)	59.8 (30-87)	61.2 (30-88)
Females	62.8 (56-68)	59.5 (24-90)	60.6 (29-87)	60.3 (24-90)
Tumour stage*				
I+ II	7 (21.2 %)	22 (21.8 %)	19 (9.4 %)	48
III	13 (39.4 %)	37 (36.7 %)	62 (30.5 %)	112
IV	13 (39.4 %)	42 (41.6 %)	122 (60.1 %)	177
Regional lymph nodes at diagnosis	20	54	151	225
Treatment				
Pre-op radiotherapy	7	20	96	103
Post-op radiotherapy	3	7	16	26
Only surgery	0	0	3	3
Only radiotherapy	18	60	58	136
Combined therapy	4	10	21	35
Outcome†				
Death, from				
Tonsillar cancer	21	59	68	148
Other causes/unknown	10	32	48	90
Alive, no evidence of disease	2	10	87	99

\* According to UICC, 2002

† Patients were followed until death occurred, loss to-follow-up or 2007-12-31 whichever occurred first



**Figure 6.** Overall survival rates for tonsillar cancer patients diagnosed in Stockholm between 1970-1979, 1980-1989, 1990-2001.

**Table IX.** Hazard ratios (HRs) and their corresponding 95% confidence intervals (CIs) for mortality during the first 5-year period after diagnosis among tonsillar cancer cases in Stockholm, 1970-2001.

<i>Variables</i>	<b>Univariate</b>		<b>Multivariate*</b>	
	<i>HR</i>	<i>95% CI</i>	<i>HR</i>	<i>95 % CI</i>
<b>Age</b>				
<50	Referent		Referent	
50-59	1.27	0.72-2.24	1.08	0.60-1.95
60-69	2.11	1.21-3.65	1.70	0.96-3.02
≥70	3.03	1.74-5.27	2.11	1.18-3.79
<b>Gender</b>				
Female	Referent		Referent	
Male	1.43	1.02-2.00	1.27	0.89-1.81
<b>Period of cancer diagnosis</b>				
1970-1979	Referent		Referent	
1980-1989	1.11	0.69-0.79	0.95	0.58-1.57
1990-2001	0.55	0.34-0.87	0.54	0.32-0.88
<b>Stage</b>				
I-II	Referent		Referent	
III	1.15	0.68-1.93	1.97	1.15-3.39
IV	2.06	1.28-3.32	4.18	2.50-6.98
<b>Treatment</b>				
RT/neck	Referent		Referent	
Only RT	4.60	2.55-5.07	3.98	2.76-5.73
Combined therapy	1.36	0.78-2.36	1.35	0.76-2.36

\* The multivariate regression model included all variables listed in the table.

## 5 DISCUSSION

The aim of this thesis was to investigate the incidence of tonsillar cancer in Sweden since 1960, and if the prevalence of HPV could have contributed to any change. We examined whether HPV could have a causal role in tonsillar cancer development, and any impact on survival for the patients. Finally, we sought to determine survival in a nationwide setting, to indirectly assess whether HPV could have influenced survival.

We found a striking increase in tonsillar cancer incidence and a parallel increase in the proportion of HPV positive tumours.

The HPV positive cases expressed E6 and/or E7 in 94 % of the cases, indicative of a causal infection. Survival for patients harbouring HPV positive cancer was better independent of age, gender or tumour stage. Finally, in a nationwide setting, we found that the relative survival increased over time, both in men and women, and that stage of the disease at diagnosis has not affected those data. Interestingly, there seems to be a spatial relationship between the increased incidence in tonsillar cancer, increased prevalence of HPV and increased survival all together suggesting a causal role of HPV.

### 5.1 PAPER I

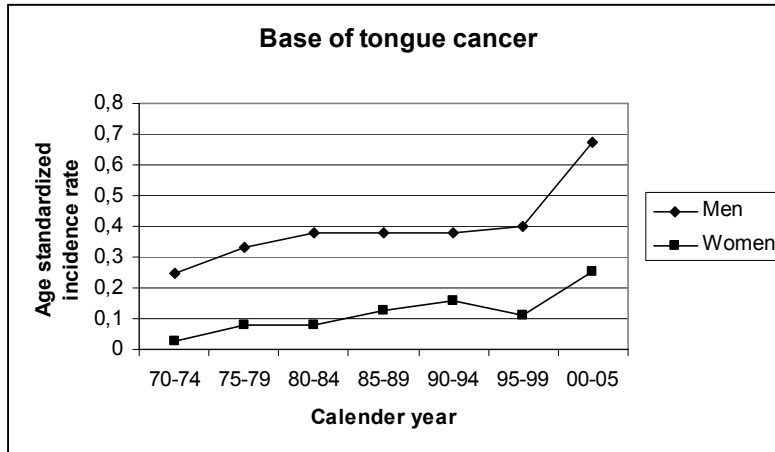
The Swedish Cancer Register was used to assess the incidence rates of tonsillar cancer in Sweden from 1960-2003. We found that the incidence of tonsillar cancer in Sweden has increased, especially the last 20 years and in men, where the incidence rates have increased by 80 %. This in spite of a decreased incidence of other smoking related cancers in men.

There is a potential for misclassification of disease, probably greater in the 1960ies and 1970ies when treatment of this cancer disease was given at a large number of hospitals as opposed to now when treatment of head and neck cancer is restricted to a small number of cancer centres in Sweden.

There is also a risk that the Cancer Register itself does not hold valid data. On this issue there have been investigations showing that the Register is valid to over 98 %, but that the validity is varying dependent on tumour site [82]. It should be stressed that the most important factor in the deficit of notification to the Register was basis on diagnosis. Non-notification was significantly more common when basis of diagnosis was solely clinical, a fact that should be of minor importance for tonsillar cancer because the accessibility to direct sample, i.e. it is easy to get a biopsy for PAD.

We tried to assess the tonsillectomy rate in the population using the Inpatient Register. This Register however only holds valid data from the mid 1970ies from which point we could see an increase in the frequency of tonsillectomies. To assess the possible impact on the incidence of tonsillar cancer we would need longer follow-up.

However, there is an indirect way to assess whether the tonsillectomy rates have not influenced tonsillar cancer incidence, i.e. by investigating the incidence of base of tongue cancer. This disease has also a high prevalence of HPV [90] and should not be affected by tonsillectomy rates. Since the incidence pattern for base of tongue cancer resembles that of tonsillar cancer, one could expect that tonsillectomy is a less plausible explanation for the increased incidence of tonsillar cancer. Figure 7.



**Figure 7.** Age standardized incidence rates of base of tongue cancer, ICD-7 code 141.0, to the population of Sweden in 2000.

We used only SCC and excluded all other histology codes. HPV positive cancers are frequently poorly differentiated, why a proportion of the tumours could be classified in the undifferentiated group, leading to a differential misclassification. However, the group of undifferentiated cancers is very small with around 5 cases per year and has not increased over the decades, so it seems unlikely that this possible misclassification has changed our results.

Since we used only 145.0 for our study, another potential differential misclassification could be that more cases were diagnosed as 145.7-9 in the early study period as compared to the late study period. However, this is not the case since the curve for 145.7-9 is similar to the 145.0, even if it has levelled of since the mid 1990ies. Another possibility is that better diagnostic tools and more specialized clinical care would result in fewer cancers with unknown primary. This does not seem to be a source of misclassification since secondary cancers to the neck, with unknown primary tumour, have also increased over the decades [19].

Few countries can present such complete registers for the whole population over more than 40 years of follow-up, making it possible to illustrate the increasing trend of tonsillar cancer with a minimized influence of biases. Another advantage with Sweden as the setting for this study is its health care system. Every Swedish resident has the

same right to health care greatly subsidized by the county, which decreases the risk of selection bias due to socioeconomic status.

## 5.2 PAPER II

In parallel to the increased incidence of tonsillar cancer we found that the prevalence of HPV positive cases has also increased from 1970-2002, especially from 1980ies to 1990ies when it increased from 28 % to 57 %.

Patients with HPV positive cancer were also found to be on average 10 years younger than the patients with HPV negative tumours. This confirms earlier studies where a difference of 5-10 years have been described [52, 91, 92]. The inclusion of all tonsillar cancer cases in Stockholm was not possible which could be a source of selection bias if the non-included cases differed in HPV status to the included.

However, no difference with regard to age distribution and gender distribution was found between the included and non-included cases, indicating that there was no selection bias.

The classification over time has changed and this is a possible source of error. The Cancer Register has used ICD-7 over the years and has reclassified cases when clinicians have started to use ICD-9 or ICD-10. The ICD-9 and ICD-10 codes are more specific. If those codes are translated to ICD-7 without caution a differential misclassification could be introduced. To avoid this misclassification we used only 145.0 in ICD-7 which refers to C09.0 in ICD-10.

When screening for HPV in this paper, we used the consensus primers GP5+/6+ and CPI/IIG targeting the two conserved regions L1 and E1. The main reason for introducing a second general HPV protocol was due to the risk of not having the L1 gene retained in the cells. However, the E1 gene is also sometimes lost, for example when the viral DNA is integrated in the host genome [93]. For this reason we performed a HPV-16 type specific PCR in all samples investigated in paper II and III. With this method we discovered 19 more cases in a group of 203. One reason for this could be that type specific PCR has been proven to be more sensitive than the general primer methods.

This might theoretically introduce a selection bias because we did not run type specific analysis towards other HPV types. However, worldwide data show consistent frequencies close to 90 % of HPV-16 in HPV positive tonsillar cancers [77] and if the same relation is transferred to our study, that would imply that we would miss only 2 or 3 cases of other HPVs.



No one has previously succeeded to perform such a study, and despite the inability to include all cases we were able to investigate a large number of cases and indirectly control for selection bias.

These findings have recently been confirmed by Ernster et al. They also verified that HPV positive cases were significantly younger at diagnosis and had a better disease specific survival than the HPV negative ones [94].

Interestingly, a study performed in Sweden in the late 1990s were the seroprevalence of HPV in pregnant women were investigated, an increase from 16 % in the 1960s to 22 % in the 1980s was assessed [95].

### **5.3 PAPER III**

The same patient data was used as in paper II, thus we had 203 cases with pre-treatment biopsies. We used only HPV-16 positive cases to assess the E6/E7 expression indicating an HPV-infection of significance for cancer development.

Ninetyfour percent of the HPV positive tumours expressed mRNA, which confirms previous pilot studies including only a small number of patients [9, 60-62]. These results in paper III strongly support an oncogenic role of HPV in tonsillar cancer.

We also analyzed disease specific survival, thus included only patients treated with curative intent. The better survival for the HPV positive cases could not be explained by a different stage at diagnosis, since lymph node metastasis were more often present in the HPV positive cases. We also found, similar to previous reports [73] that the HPV positive cases relapsed less often. There was no difference when the group treated with palliative intent was included, with regard to HPV status and difference in survival.

### **5.4 PAPER IV**

We used the Swedish Cancer Registry to assess all tonsillar cancers in Sweden from 1960-2001. We also assessed all tonsillar cancer cases in Stockholm between 1970-2001 in order to relate any change in survival to clinical data. The advantages of this study include the nationwide design, the completeness of follow-up and precision due to the large number of cases. We could also relate this improvement to stage-specific survival, further strengthening these data.

We included only the first primary malignant tumour to avoid influence on survival from other cancers. However, we started our survey 1960 and the Swedish Cancer Registry started in 1958. This could mean that in the early 1960ies we could include patients that have had a previous cancer that was not registered to the Registry. Since we could identify all other cases 2 years before the start of inclusion this source of error should be of minor importance and in the beginning of the 1960ies in that case.

We report the observed and relative survival in this paper. Although the observed survival is of interest in clinical practice, in any comparison between different time

periods it is more accurate to study the relative survival rates, since it takes into account the generally increasing survival noted in the Swedish population.

In the Stockholm cohort we analyzed only patients treated with curative intent. This could introduce bias if the palliatively treated patients were unevenly distributed over the decades. However, when analyzing the total number of cases per decade that was treated with palliative intent, we found 6.25 % of the cases in 1970-79, 8.4 % in 1980-89 and 8.2 % in 1990-2001. Interestingly, more advanced disease at diagnosis was more common during the later years, and it is therefore unlikely that stage explains the improved survival during the last decade.

## **5.5 CLINICAL IMPLICATIONS**

Today's treatment of head and neck cancer is standardized with radiation and/or surgery, sometimes with induction-or concomittant chemotherapy. Head and neck cancer constitutes of a large heterogeneous group of cancer with differences in histopathology, aggressive growth pattern, radiosensitivity and prognosis but yet no clinically useful prognostic markers are available. Such markers could be of uttermost interest to tailor treatment, enough for cure but avoid unnecessary sequele. In this thesis we have focused on one site specific disease and one prognostic factor. Our data provides evidence for HPV as responsible for the increased incidence and improved survival of the disease. Today HPV is considered to be a causative agent for tonsillar cancer. However, the magnitude and probable different distribution around the world is still to be shown. In Sweden, as well as in many other Western countries, tonsillar cancer is increasing [5, 6, 76, 94] and in the US the prevalence of HPV in tonsillar cancer have also increased over time [76, 94]. Our studies, as well as others, have shown that HPV has a favorable impact on prognosis [13, 52, 70, 72, 75, 76, 91, 94, 96]. This should be investigated for when examining the patient in the clinical setting, and should possible affect treatment. Some studies have implied that even the HPV positive cases can be further sub-divided into p16 positive/negative, a factor with a different prognostic value [72] This should be further investigated, preferably with a prospective trial with different treatment strategies to clarify which treatment option that is the most beneficial.

In the Western world the HPV vaccines are also on the agenda. In Sweden the vaccine Gardasil<sup>®</sup> has gained access to the market. The proposal is to vaccinate all females between the age of 12 and 17 years of age. There are around 400 new cases of cervical cancer in Sweden each year [19] and approximately 170 cases of tonsillar cancer and 50 cases of base of tongue cancer. In the two latter diseases there are 70 % men making approximately 110 cases a year among men attributed to HPV. The majority, over 90 %, have consistently been shown to be HPV-16 positive [77, 78] which would make 100 cases among men attributable to HPV-16 and hence preventable with vaccination. This, in line with the increasing trend of tonsillar and base of tongue cancer speaks in favor of a debate regarding the value of vaccination for men as well.



## 6 CONCLUSIONS

The main findings in this thesis were the following:

-the incidence of tonsillar cancer in Sweden has shown a remarkable increase between 1960-2003 while other smoking induced cancers have decreased

-the prevalence of HPV in tonsillar cancer has shown a parallel increase between 1970-2002

-in the HPV-16 positive tumours, expression of E6/E7 was ascertained in the vast majority and a better disease specific survival was noted for the HPV positive cases

-since 1960 there has been a significant increase in relative survival for patients with tonsillar cancer in Sweden that can not be explained by earlier diagnosis

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