Human papillomavirus in recurrent respiratory papillomatosis, tonsillar and mobile tongue cancer

Christos Loizou
Human papillomavirus in recurrent respiratory papillomatosis, tonsillar and mobile tongue cancer.

©Christos Loizou 2016
christos.loizou@umu.se
“You don’t develop courage by being happy in your relationships everyday. You develop it by surviving difficult times and challenging adversity.”

Epicurus (Greek philosopher of the 4th century BC.)

To my wife and my children, the true meaning of my life.
Table of Contents

Table of Contents iv
Abstract vii
Abbreviations ix
Original Papers xi
Sammanfattning på svenska xii
1. Introduction 1
  1.1 Human papillomavirus 1
    1.1.1 Epidemiology 1
    1.1.2 Taxonomy 1
    1.1.3 Genomic organization 1
    1.1.4 Viral proteins and viral genome integration 2
  1.2 Human papillomavirus and human disease 5
    1.2.1 HPV and benign lesions 5
    1.2.2 HPV and malignant lesions 6
    1.2.3 Risk factors linked to HPV 7
    1.2.4 HPV and the immune response 7
  1.3 HPV vaccines 8
  1.4 HPV DNA detection methods 9
  1.5 Anatomy of the tongue, oropharynx and larynx 10
  1.6 Voice evaluation and voice production 13
  1.7 Recurrent respiratory papillomatosis (RRP) 14
    1.7.1 Epidemiology of RRP 14
    1.7.2 Clinical features of RRP 15
    1.7.3 Surgical and adjuvant treatment of RRP 16
    1.7.4 Prevention with HPV vaccines 18
  1.8 p16 18
  1.9 Head and neck cancer (HNSCC) 19
    1.9.1 Epidemiology and risk factors of HNSCC 19
    1.9.2 TNM classification and staging of oropharyngeal cancer 19
    1.9.3 Treatment of HNSCC 21
    1.9.4 Prognosis of HNSCC 22
  1.10 Tonsillar cancer 23
    1.10.1 Epidemiology and risk factors 23
    1.10.2 Clinical features of tonsillar cancer 24
    1.10.3 Treatment of tonsillar cancer 24
    1.10.4 Prognosis of tonsillar cancer 25
    1.10.5 HPV vaccines 26
  1.11 Tongue cancer (TSCC) 26
    1.11.1 Epidemiology of TSCC 26
    1.11.2 Clinical features of TSCC 27
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. Acknowledgements</td>
<td>53</td>
</tr>
<tr>
<td>8. References</td>
<td>55</td>
</tr>
</tbody>
</table>
Abstract

This thesis focuses on the effects of the human papillomavirus (HPV) in tonsillar cancer, mobile tongue cancer, and recurrent respiratory papillomatosis (RRP). The purpose was to characterize patients with RRP in northern Sweden in order to identify more care-intensive RRP patients and to describe the voice and quality of life aspects that follow RRP. Further aims were to confirm the expected increase of HPV-positive tonsillar cancer cases in northern Sweden, and to study the correlation between HPV, its surrogate marker p16 and HPV receptor syndecan-1 in both tonsillar cancer and mobile tongue cancer.

A total of 27 consecutive patients with RRP were evaluated at 3 months postoperatively using the voice handicap index (VHI) and SF-36 questionnaires to assess the impact on life and voice in a RRP population. The values were compared to normative data. This report was further extended by examining consecutive data from 21 new patients in order to characterize RRP patients in northern Sweden. In order to study HPV DNA in tonsillar (n= 65) and mobile tongue cancer (n=109), HPV DNA was extracted from paraffin-embedded biopsies and detected by polymerase chain reaction using general primers Gp5+/6+ and CpI/IIG. Expression of HPV surrogate marker p16 and the HPV receptor syndecan-1 was analysed by immunohistochemistry.

Patients that underwent more than one RRP surgery per year were younger than those treated less frequently and they had significantly impaired voice quality as compared to normal subjects. Females, patients with frequent surgical treatment sessions, and patients with the high-risk HPV subtypes scored significantly lower in several domains of the quality of life assessment as compared with normal subjects. Forty-eight RRP patients had a median age of 44.5 years; 71% were men and 29% females, preferentially infected with HPV6. Patients with high surgical treatment frequency/year showed more widespread RRP in the larynx compared to the patients treated less frequently.

A total of 214 tonsillar cancer cases were identified. The vast majority were men. They had a median age of 58 years at diagnosis and expressed HPV as well as p16. The incidence of tonsillar cancer revealed a 2.7-fold increase in men between the years 1990 and 2013. The study demonstrates a strong association between p16 and HPV infection in tonsillar malignancies. These findings are in contrast to the mobile tongue cancer cases, where no evidence of HPV DNA could be detected although one-third showed p16 staining. This demonstrated a poor correlation between HPV and p16 in mobile tongue cancer. There was no difference in the expression of the primary HPV receptor, syndecan-1, between tonsillar and mobile tongue cancer.
In conclusion, the frequency of RRP operations, age at onset, gender and subtype of the HPV may be used as factors to predict voice disability. RRP patients with high surgical treatment frequency were significantly younger and had a more widespread laryngeal disease compared to the low-frequency treated group. This study confirms the existence of a clinical RRP group, not primarily related to HPV subtype, but to a more care-intensive RRP population. Our findings identify a 2.7-fold increase in the incidence of tonsillar cancer, HPV and p16 in men between 1990-2013. We can use p16 to detect HPV in tonsillar cancer but not in tongue cancer.

The introduction of vaccination against HPV may have a role in the prevention of specific HPV-subtype positive head and neck malignancies and recurrent respiratory papillomatosis since the current vaccine protects against HPV6, 11, 16, 18, 31, 33, 45, 52 and 58. Males will definitely benefit indirectly from vaccination of females, though males will still remain at risk of cancers associated with HPV. This highlights the need for sex-neutral vaccination strategy. Our intention is that this thesis will provide scientific data to support a gender-neutral vaccination and to develop simple tools to detect HPV in tonsillar cancer.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>AoRRP</td>
<td>Adult-onset recurrent respiratory papillomatosis</td>
</tr>
<tr>
<td>BSCC</td>
<td>Base of tongue squamous-cell carcinomas</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinases</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CT</td>
<td>Computer tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ENT</td>
<td>Ear, nose, throat</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug administration</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin embedded</td>
</tr>
<tr>
<td>FNA</td>
<td>Fine-needle aspiration</td>
</tr>
<tr>
<td>GERD</td>
<td>Gastro-esophageal reflux disease</td>
</tr>
<tr>
<td>HF</td>
<td>High-frequency group (treated RRP $\geq$1/year)</td>
</tr>
<tr>
<td>HFJV</td>
<td>High-frequency jet ventilation</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Heparan sulfate proteoglycans</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>ICD</td>
<td>International classification of diseases</td>
</tr>
<tr>
<td>IVD</td>
<td>In vitro diagnostic</td>
</tr>
<tr>
<td>JoRRP</td>
<td>Juvenile-onset recurrent respiratory papillomatosis</td>
</tr>
<tr>
<td>LF</td>
<td>Low-frequency group (treated RRP $&lt;$1/year)</td>
</tr>
<tr>
<td>MCS</td>
<td>Mental component summary score</td>
</tr>
<tr>
<td>MGP-PCR</td>
<td>Modified general primer polymerase chain reaction</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MICA</td>
<td>MHC class I chain-related proteins A</td>
</tr>
<tr>
<td>MICB</td>
<td>MHC class I chain-related proteins B</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic acid sequence-based amplification</td>
</tr>
<tr>
<td>NBI</td>
<td>Narrow band imaging</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural killer group 2 member D</td>
</tr>
<tr>
<td>ORFs</td>
<td>Open reading frames</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oropharyngeal squamous cell carcinoma</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCS</td>
<td>Physical component summary score</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PRO</td>
<td>Patient-reported outcome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RRP</td>
<td>Recurrent respiratory papillomatosis</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEER</td>
<td>Surveillance, Epidemiology, and End Results Program</td>
</tr>
<tr>
<td>SF-36</td>
<td>Short Form (36) Health Survey</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Pack for Social Sciences</td>
</tr>
<tr>
<td>TORS</td>
<td>Transoral robotic surgery</td>
</tr>
<tr>
<td>TSCC</td>
<td>Tongue squamous cell carcinoma</td>
</tr>
<tr>
<td>URR</td>
<td>Upstream regulatory region</td>
</tr>
<tr>
<td>VLPs</td>
<td>Virus-like particles</td>
</tr>
<tr>
<td>VHI</td>
<td>Voice handicap index</td>
</tr>
</tbody>
</table>
Original papers


Sammanfattning på svenska

Syftet med avhandlingen är att beskriva effekterna av humant papillomvirus (HPV) vid cancer i halsmandlarna, cancer i tungan och vid luftvägspapillom.


RRP patienter hade en medianålder på 44,5 år; 71% var män och 29% kvinnor, företrädesvis infekterade med HPV6. Patienter som opererades mer än en gång per år var yngre än de som behandlats mindre ofta och hade en statistiskt sämre röstkvalitet än friska kontroller. Kvinnor, patienter med täta kirurgiska behandlingsintervall och högrisk-HPV hade signifikant sämre livskvalitet jämfört med friska kontroller. Patienter med hög kirurgisk behandlingsfrekvens per år var signifikant yngre och hade mer utbredd RRP sjukdom i luftstrupen, jämfört med gruppen med låg behandlingsfrekvens.


Antalet RRP operationer, ålder vid insjuknandet, kön och genetisk variant av HPV kan användas som indikatorer för att förutsäga grad av röststörning. RRP patienter med hög kirurgisk behandlingsfrekvens var signifikant yngre och hade en mer utbredd luftvägssjukdom jämfört med RRP patienter som behandlas mindre ofta. Vi har identifierat en undergrupp av RRP patienter som inte primärt karakteriseras efter HPV virusets genetik utan av ett mer vårdintensivt förlopp. Den aktuella avhandlingen har identifierat en 2,7-faldig ökning av antalet halsmandelscancer hos män och ett starkt samband mellan p16 och HPV infektion i halsmandlar men inte i HPV-negativ tungcancer som inte korrelerar till p16 uttryck. Vi kan använda p16 för att påvisa HPV i tonsillcancer men inte i cancer i mobil tunga.
Idag ingår HPV vaccination i det allmänna vaccinationsprogrammet för flickor. Vi förväntar oss en tydlig profylaktisk effekt avseende insjuknande i HPV-relaterad huvud- och hals cancer samt luftvägspapilom eftersom vaccinet skyddar mot HPV bl.a. 6, 11, 16 och 18. Män kommer definitivt att gynnas indirekt genom vaccination av kvinnor men kommer att ha fortsatt högre risk än kvinnor att insjukna i HPV relaterad cancer vilket understryker behovet av könsneutral vaccination. Vår avsikt med avhandlingen är att ge vetenskapligt stöd för könsneutralt vaccination och enkla metoder att påvisa halsmandelscancer.
1. Introduction

1.1 Human papillomavirus
In the 1970s, Harald zur Hausen detected the human papillomavirus (HPV) in warts and cervical cancer. He published the hypothesis that HPV could play an important role in the pathogenesis of cervical cancer. He isolated and cloned different strains of HPV, and concluded that infection with HPV16 and 18 constituted an increased risk of developing cancer. (1) For these findings, as well as the fact that subsequent to these descriptions, HPV vaccines were developed in order to prevent cervical cancer, Dr. Hausen received the Nobel Prize in Physiology and Medicine in 2008. Currently, HPV is the most common sexually transmitted disease, (2) with close to 200 genotypes identified, and more than 80 genomes which have been completely sequenced. (3) HPV infection is common and most infected individuals are able to successfully eliminate evidence of the virus without presenting any manifestations of clinical disease. The classification of the HPV genotypes has been frequently revised, with current data supporting the idea that 15 genotypes are oncogenic (for example HPV16, 18, 31, 33, 45, 52 and 58), 3 subtypes are considered high-risk, 12 low-risk, and 3 are referred as undetermined risk genotypes. (4) HPV infects the squamous epithelium of the skin (subtypes 1, 2, and 4), the genital mucosa (condylomata acuminata, subtypes 6 and 11), and the upper airways (subtypes 6 and 11).

1.1.1 Epidemiology
The reported prevalence of HPV infection among women around the world ranges from 2% to 44%, probably due to differences in the age range of the population studies and the methods used for the detection of the virus. (5) HPV infection in men has not been studied as extensively as in women, but the prevalence varies between 16.5% (6) and 32.7%. (7)

1.1.2 Taxonomy
Human papillomavirus belongs to the family Papillomaviridae. The HPV viruses are small, non-enveloped, double-stranded, circular DNA viruses that have a particular tropism for the epithelium. (3) They are highly species-specific. The taxonomic classification is based on sequence variations in the L1 open reading frame and the taxonomic levels related to ‘families’, ‘genera’, ‘species’, ‘types’, subtypes’, and ‘variants’. (8) Despite their small size, their molecular biology is complex. The different proteins of the late (L) and early (E) regions have specific functions.

1.1.3 Genomic organization
The human papillomavirus genome contains a double-stranded circular DNA with approximately 8000 base pairs. (8) These are functionally organized into two
regions: Upstream Regulatory Region (URR) and Open Reading Frames (ORFs) (Figure 1). The URR is a non-coding region, which regulates the expression of viral gene. The ORFs can be divided into two coding regions; the late (L, codes for the L1-L2 capsid proteins which comprise the outer coat of the virus) and the early (E, codes for the E1-E2 and E4-E7 proteins which are necessary for the replication, cellular transformation, and the control of viral transcription). (9) The E1 and E2 viral proteins play a significant role in DNA replication. The E1 protein is the largest and most conserved; it is a 68 kDa protein, which is necessary for DNA replication.

1.1.4 Viral proteins and viral genome integration
The E1 protein binds to specific DNA elements during the viral replication process and assembles into hexameric helicases with the aid of the 50 kDa E2 viral protein. The resultant complex provides the template for subsequent DNA syntheses through an initiation of origin DNA unwinding. (9, 10) Furthermore, E1 protein binds to DNA polymerase during basal replication and interacts with cyclins A and E, (11) while E2 protein acts as a transcription factor whose functions can be disrupted by mutation or integration of the viral genome. As a consequence, the expression of E6 and E7 genes are increased, enhancing carcinogenesis and malignant progression. (12, 13)

Figure 1. Schematic presentation of the HPV genome. Reproduced by kind permission of Rightslink/Elsevier. (14)
E4 is a 17 kDa protein contributing to genome amplification efficiency and viral synthesis. This is done through the induction of G2/M arrest function and with a particular role in the latest phase of the viral cycle life. Furthermore, it can serve as a biomarker of active virus infection and disease severity, especially in the case of high-risk HPV genotypes. (15, 16)

E5 protein is one of the oncoproteins expressed by the HPV genome, enhancing the activation of the epidermal growth factor receptor (EGFR) after stimulation by EGF in human keratinocytes. (17) E5 can augment the function of E6 and E7 proteins in the transforming activities of HPV viruses, contributing to tumor progression when expressed alone. E5 has a weak transforming activity. (18) Another suggested function is the inhibition of localization of the major histocompatibility complex (MHC) class I and II proteins to the plasma membrane. (19)

E6 protein is expressed early in the viral cycle and, along with E7, is one of the main oncogenic proteins. This acts by stimulating the growth and transformation of the host cell through the inhibition of protein p53’s normal tumor suppressing function. The cell proliferation activity is suggested to be the result of the formation of a trimeric complex including E6, p53, and the cellular ubiquitination enzyme E6-AP. The formation of this complex leads to degradation of the tumor suppressor p53 protein. (20) The complex consists of 158 amino acid residues and contains two zinc-finger binding motifs. (21) Another suggested function is the increase of telomerase activity in epithelial cells, a feature observed in normal proliferating tissues and malignancies. (22)

E7 protein is also expressed early in the viral life cycle, as mentioned above. It is a small protein consisting of 98 amino acids, and is thought to have the major transforming effects. More specifically, E7 acts by binding to members of the retinoblastoma (Rb) tumor suppressor protein family, and induces cellular proliferation by release of the E2F transcription factor. (23) Several other functions of E7 have been proposed; the promotion of HPV replication by direct activation of cyclin-dependent kinase 2, (24) activation of hypoxia-inducible factor 1-mediated transcription by inhibiting binding of histone deacetylases, (25) and suppression of cadherin-mediated cell adhesion via the ERK and AP-1 signalling pathways. (26)

The L1 major capsid protein supports the viral capsid, together with L2. It self-assembles into pentameres, and 72 pentameres self-assemble into virus-like particles (VLPs). The ability of VLPs to elicit neutralizing antibodies has been used for the development of prophylactic vaccines that can prevent persistent HPV infection-associated malignancies. L2 is the minor capsid protein, not required for VLP formation and in accordance with L1. It is only expressed in terminally differentiated epithelial cells. Proposed functions of this protein are the interaction
with the viral genome and encapsidation of viral DNA. (27) Furthermore, it facilitates the infection of cells by HPV through an interaction with an unknown cell surface receptor. (28)

**Viral genome integration**
The virus is easily transmitted, possibly through microscopic tears in the surface of the epithelium of the skin or mucosa. An HPV infection is strictly limited to the basal cells in the mucosa or skin. The virus interacts with the surface of the cell via interaction of the L1 major capsid protein with heparan sulfate proteoglycans (HSPGs) on segments of the basement membrane. There is also accumulating evidence for the role of secondary receptors such as alpha-6-integrin and the L2 minor capsid protein during the binding process. (29, 30) In the majority of human cervical carcinomas, the HPV genome is integrated in the host genome, which frequently leads to disruption of the E2 gene regulating the expression of E6 and E7. (31) There has been discordance between different studies that evaluated the integration of HPV virus in oropharynx/tonsillar cancer, identifying the presence of integrated, (32, 33) episomal, (32-35) or mixed (32) HPV DNA. The results from those studies have not been widely confirmed though; the question whether the virus is integrated or episomal remains unanswered with conventional consensus primer-based PCR.

Replication occurs within the nucleus of the infected cells, and requires DNA mechanisms that are partly controlled by E1 and E2 proteins. (36) The differentiation of the squamous epithelial cells takes place as they move from the basement membrane towards the epithelium of the surface. Replication of the viral DNA in high numbers occurs only in terminally differentiated cells near the surface layers. Similarly, the expression of the L1 and L2 proteins that form the virus particle is also encoded in these highly differentiated cells by the late viral genes. (37, 38)

**HPV and cancer development**
High-risk human papillomaviruses produce the oncoproteins E6 and E7, which play a major role in malignant transformation through their proliferation-stimulating activity. The E6 protein binds and induces the degradation of the p53 tumor suppressor protein through ubiquitin-mediated proteolysis, leading to substantial loss of p53 activity and uncontrolled cell cycle progression. (39) On the other hand, the E7 oncoprotein binds and inactivates the retinoblastoma protein (pRb), allowing the transcription of E2F-dependent genes. This causes the cell to enter S phase and leads to loss of cell cycle control. The inactivation of pRb results in overexpression of the p16 protein, which is encoded by the CDKN2A tumor suppressor gene. The latter explains why p16 overexpression is associated with high-grade precancerous lesions and carcinomas. This demonstrates the value of immunohistochemical
evaluation of p16 as a surrogate marker in identifying HPV infection in cancer cells. (40, 41)

The correlation between p16 and HPV in malignant tumors appears to be site-specific, and must therefore be charted individually. (42, 43) Furthermore, the oncoproteins E6 and E7 can lead to DNA mutations of the host cell through alterations of DNA repair mechanisms. This can explain how specific HPV genotypes can induce malignancy without any other co-factors. (44) The presence of the oncoproteins E6 and E7 is not enough to explain the HPV-induced carcinogenesis that often requires decades to develop. Additional cellular changes and risk factors previously named are often present during the process of initial hyperplasia of the normal epithelium, which can proceed to dysplasia, carcinoma in situ, and ultimately development of invasive cancer.

1.2 Human papillomavirus and human disease

1.2.1 HPV and benign lesions

*Recurrence respiratory papillomatosis (RRP)*

The association between RRP and HPV infection was first postulated in the 1920s. (45) It is now well-recognised that persistent HPV infection, mainly HPV6 or HPV11, is the primary cause of RRP. The presence of additional co-factors, some yet unknown, may be required.

*HPV in trachea*

Tracheal spread of RRP has been proposed in 16% of adult-onset RRP (AoRRP) and in 30% of juvenile-onset RRP (JoRRP). (46) RRP affecting the larynx has a higher tendency of remission than RRP localized in trachea and/or bronchi. (47)

*Non-visible HPV in larynx*

Although RRP is thought to be rare, the airway is constantly exposed to HPV throughout an individual’s life. HPV6 has been detected in healthy laryngeal mucosa in 25%, and the amount of HPV6 DNA in RRP patients is consistently higher in the papilloma lesion than in healthy adjacent mucosa. (48-50)

*HPV in oral cavity*

HPV is identified in the oral cavity in less than 7% of the general population. This colonization rate is much higher in patients with AoRRP and their long-term sexual partners. (51) The oral HPV prevalence in Sweden (2009-2011) among HPV-unvaccinated young people aged 15–23 years was 9.3%. (52)
HPV in genital warts
Condylomata acuminata is a benign disease, sexually transmitted and caused by HPV6 and 11. Women with genital warts have an increased risk of cervical carcinoma and the lesions are associated with local clinical symptoms such as burning, bleeding and pain. (53)

1.2.2 HPV and malignant lesions
The high-risk subtypes of the HPV virus are more likely to cause malignancies. Some individuals develop a long-lasting HPV infection that causes changes leading to precancerous lesions or cancer.

Cervical cancer
Cervical cancer in a global perspective is the 4th most common form of cancer and the 4th most common cause of female cancer death. (54) An estimated 528 000 cases of cervical cancer occurred in 2012, with an approximate 266 000 patients deaths. This means that cervical cancer constitutes approximately 8% of all cancer-related death in women. (54) HPV16 and 18 account for 70% of all cervical cancer, with some regional variations worldwide. HPV16 has been detected in 24% of women infected with HPV, while 9% are infected with HPV18. (55, 56)

Head and neck and oral cancer
The majority of HPV infections are asymptomatic and are cleared spontaneously. It is uncertain why some HPV infections lead to permanent infections, thus creating conditions for developing carcinoma, and in specific cases requiring lifelong treatment. In Finland, HPV-related oropharyngeal cancer incidence has almost tripled over the last 30 years. (57) The increase in oropharyngeal and oral malignancies is noted worldwide, affecting young non-smokers and non-drinkers. Smoking and drinking have been considered as the main risk factors for head, neck and oral cancers. Besides tonsillar cancer, HPV has been associated with cancer of the base of the tongue (BSCC) in 40% to 75% (58, 59) and laryngeal carcinomas with a lower incidence. (60, 61) Certain molecular profiles of HPV-related head and neck squamous cell carcinomas (HNSCCs) are different from HPV-negative cancers. These can be biologically distinct entities where the HPV-positive HNSCC cancers have a better prognosis. (62) Most HPV-associated HNSCCs are caused by HPV16, and tend to present mostly at an early T stage and advanced nodal stage. (63)

Anogenital cancer
The etiological role of high-risk HPV has also been studied in penile cancer (23%-48%), (64, 65) anus (90%), vagina (40%) and vulva (40%). (66) The full extent of the association between HPV and these cancers in terms of age and onset of diagnosis is not well known. Further studies are needed in order to clarify this issue. The introduction of vaccination against HPV has been shown to have utility in the
prevention of HPV-related anogenital cancers. (66)

**Lung cancer**
Syrjänen first reported (1979) that HPV could possibly be involved in bronchial squamous cell carcinoma. (67) Several studies have reported a role of HPV in the development of squamous cell carcinoma of the lungs. The mean incidence of HPV in lung cancer is 24.5%. (68) The incidence is heterogeneous and the diversity related to the “home country” is about 17% in Europe and 15% in the U.S., while HPV was present in 35.7% of the Asian lung cancer samples. (68)

**1.2.3 Risk factors linked to HPV**
Behaviourally-based risk factors linked to HPV include an increasing number of lifetime sexual partners and partner characteristics such as age, ethnicity and smoking, (69) the use of oral contraceptives, (70) alcohol and illicit drug use, (71) and diet. (72) On the other hand, biologically-based risk factors include age <15 years at first sexual intercourse and age at first menarche, (71) immunosuppression, concurrent HIV infection, and occurrence of other sexually transmitted infections. (73, 74)

**1.2.4 HPV and the immune response**
HPV-infected individuals develop an ineffective HPV-specific T-cell immune response. Recent studies have shown that HPV infections create a Th2-like immune response in which CD4+ T-cells induce expression of immunosuppressive cytokines (e.g. IL-4 and IL-10) that suppress Th1 (cytotoxic) immunity. (75) The RRP lesions have been found to express a parallel decrease in IFN-γ, IL-12 and IL-18. (76) Elevated Th2-like cytokine response has been suggested to correlate with disease severity. (77) This imbalance of Th1/Th2-like cellular response could explain why RRP patients fail to prevent the recurrences of the disease. The expression of major histocompatibility complex (MHC) antigens seems to play a significant role in patients with RRP through altered function of cell-mediated immunity. MHC class I chain-related (MIC) A and B molecules, ligands of the activating NK-cell receptor NKG2D, and stress-induced molecules were shown to be up-regulated and exhibited differential expression among HPV-infected and non-infected cell lines. (78) Dysfunctional natural killer (NK) cells are present in RRP lesions, but are unable to clear the infected HPV keratinocytes. (79) A summary of the immune responses to HPV is shown in Figure 2 below.
The NKG2D receptor-ligand system constitutes an important cytotoxic effector mechanism in elimination of infected, foreign, stressed and/or transformed cells. The engagement of NKG2D with a ligand could bypass inhibitory signals from other NK receptors, and lead to destruction of the NK-cell target. (81) The deficiency of the system NKG2D receptor-ligand has been shown to promote the development of spontaneous tumors in mice. (82) The NKG2D ligands serve as antigens that can induce cell proliferation, cytotoxicity, and cytokine production after linking to the appropriate NKG2D receptor. (83) NK-cell triggering by NKG2D ligands induces CD25 expression, NK-cell proliferation, cytokine production, and perforin-mediated cytotoxicity. This results in killing of target cells that express these ligands. (81, 84)

1.3 HPV vaccines
Recently, the introduction of vaccination against HPV-associated anogenital cancers in non-infected subjects has revealed a promising amount of HPV protection. These prophylactic HPV vaccines contain the major capsid protein L1 that self-assembles into immunogenic virus-like particles similar to authentic virions though they are non-infectious. (85) Currently, two HPV prophylactic vaccines have been successfully developed: the bivalent Cervarix® (GlaxoSmithKline, Rixensart, Belgium) and Gardasil® (Merck & Co., Whitehouse Station, NJ). Both are highly efficacious for the prevention of persistent infection with HPV16 and 18 in cervical
lesions. (86) Two-dose schedule is currently recommended by WHO (2014) and applies in Sweden since January 1, 2015 for girls aged 9-13 years. For women 14 years and older (Cervarix® 15 years and older), a three dose schedule is recommended. The vaccines are given as intramuscular injections. Gardasil 9® is a 9-valent recombinant vaccine against human papillomavirus, and a further refinement of Gardasil®. The major change in Gardasil 9® is the inclusion of boys for the prevention of diseases caused by HPV genotypes 6, 11, 16, 18, 31, 33, 45, 52, and 58. The vaccine has the ability to protect against 90% of cancers of the cervix, vulva, vagina, and anus. It is approved in the United States against these forms of cancer, and against genital warts in females 9-26 years and boys 9-15 years. (87, 88)

A growing number of countries recommend or permit HPV vaccination for males. Cost-benefit analyses have concluded a clear benefit of expanding HPV vaccination programs including males. (89) Prophylactic vaccination against high-risk HPV genotypes has been proven effective in the prevention of genital HPV infection, and consequently genital HPV-related malignancies. Despite this, there is no proven efficacy in the prevention of HPV-related tonsillar cancer or recurrent respiratory papillomatosis. (90, 91)

Despite the effectiveness of these vaccines, several issues still need to be addressed. The main challenge is delivering these vaccines to patients in low- and middle-income countries, owing to the high cost of the vaccines. Furthermore, immunity to several high-risk HPV genotypes is not provided by these vaccines. Additionally, whether or not a booster dose is needed is not known.

Currently, efforts are directed towards strategies that could make therapeutic HPV vaccination possible. These strategies can elicit a cytotoxic CD8+ T-cell response against high-risk HPV E6 and/or HPV E7 oncoproteins in both humans and mice. (92) In mice, transplantable tumors that express these viral antigens have been successfully decreased in size by this treatment strategy, but this approach is not yet applicable in humans. (93, 94)

1.4 HPV DNA detection methods

It is not possible to propagate or isolate HPV in tissue culture, which is a common method for virus detection. HPV can be detected by identification of proteins of HPV genomic sequences in the infected tissue, or indirectly by measuring specific IgG antibodies in the serum against virus-specific antigens from earlier infections. The most commonly used detection method is based on direct hybridization or DNA amplification techniques, polymerase chain reaction (PCR). Additionally, detection of HPV E6/E7 mRNA can be performed by reverse-transcriptase PCR.
**Polymerase chain reaction, PCR**

PCR is a biochemical technology used to amplify a piece of DNA, generating multiple copies of a particular DNA sequence. It is currently regarded the most sensitive technique allowing tests on samples with only small amount of tissue available.

**Quantitative PCR**

Q-PCR is also referred as real-time PCR, and is used to quantify the target DNA molecule. Q-PCR uses a DNA binding dye, causing fluorescence. An increase in the DNA product during PCR leads to increased fluorescence intensity, measured at each cycle, allowing DNA concentration to be assessed.

**PapilloCheck®**

The DNA array PapilloCheck® (Greiner Bio-One GmbH, Frickenhausen, Germany) is certified in the European Union (CE) as an in vitro diagnostic method (IVD) for the qualitative type-specific identification of human papillomavirus. It has a sensitivity of 98%. PapilloCheck® is a test probe which is utilised in conjunction with a DNA array analysed in a computer-controlled, high-resolution optical microarray scanner. This ensures reproducible and objective results of samples. With this test, a total of 6 low- and 18 high-risk HPV subtypes can be detected. (95)

1.5 Anatomy of the tongue, oropharynx and larynx

**Tongue**

The tongue is a muscular organ that forms part of the floor of the oral cavity. The tongue is essential in functions such as taste, deglutition (swallowing), articulation (speech), mastication (chewing) and oral cleaning. The lingual septum divides the tongue into the left and right side. The tongue is furthermore divided into anterior (oral/mobile tongue) and posterior (base of tongue) parts. The anterior is the mobile part of the tongue that is located in the oral cavity. This constitutes two thirds of the tongue, which is further divided by the terminal sulcus. The posterior part of the tongue is located in the oropharynx. The foramen cecum is a remnant of the thyroglossal duct, and is located at the tip of terminal sulcus. The base of tongue contains the lingual tonsils, the inferior most portion of Waldeyer’s ring.
The tongue consists of eight muscles, classified as either intrinsic or extrinsic. The four intrinsic muscles (superior and inferior longitudinal muscles, vertical and transverse muscles) change the shape of the tongue and are not attached to bone. The four extrinsic muscles (genioglossus, hyoglossus, styloglossus, palatoglossus) act to change the position of the tongue, and are anchored to bone. The lingual papillae cover the surface of the body of the tongue and are projections of the lamina propria covered with epithelium. There are 4 types of lingual papillae: vallate (circumvallate), foliate, filiform, and fungiform. The fungiform, foliate, and circumvallate papillae are known as the gustatory papillae. They contain taste buds and work as sensory organs.

The main artery that supplies blood to the tongue is the lingual artery, a branch of the external carotid, along with accompanying lingual veins. The different veins of the tongue drain into the internal jugular vein. Concerning sensory innervation, the anterior two thirds of the tongue are supplied by (1) the lingual nerve (of the mandibular nerve) for general sensation and by (2) the chorda tympani (a branch of the facial nerve that runs in the lingual nerve) for taste. The glossopharyngeal nerve supplies the posterior third of the tongue, including the vallate papillae for both general sensation and taste.

**Oropharynx**
The oropharynx is the middle part of the pharynx that is localized posteriorly of the oral cavity, extending from the uvula to the level of the hyoid bone. It consists of the
base of tongue and the epiglottic vallecula inferiorly, the inferior surface of the soft palate and uvula superiorly, the palatine tonsils, tonsillar fossa and tonsillar pillars laterally and the posterior pharyngeal wall posteriorly. It constitutes both part of the digestive system and the conducting zone of the respiratory system. It is lined by non-keratinized squamous stratified epithelium.

The *palatine tonsils* (frequently referred to as the “tonsils”) are located in the lateral walls of the oropharynx, between the palatoglossal and palatopharyngeal arches. The tonsils contain lymphoid tissue, and are part of the Waldeyer’s tonsillar ring. This also includes the adenoid tonsil, the lingual tonsil and the lymphoid tissue in the posterior pharyngeal wall. The tonsils are largest relative to the diameter of the throat in young children, where they sometimes cause upper airway obstruction. They reach their largest size near puberty. In adults, the tonsillar tissue gradually undergoes atrophy.

The tonsils are lined with non-keratinized squamous stratified epithelium, and contain four lymphoid compartments: the crypt epithelium, the follicular germinal centre with the mantle zone and the interfollicular area. These all participate in the immune response.

**Figure 4.** The parts of the oropharynx are presented. This image has been released as part of an open knowledge project by Cancer Research UK.

**Larynx**

The larynx is located in the upper part of the airway, and it holds a strategic position in the crossover between the respiratory and gastrointestinal tracts. It combines three
functions: 1) respiration (open airway to the lungs), 2) sound and speech generation, and 3) protective function, by preventing foreign objects, fluids and foods, from entering the trachea, bronchial tree and lungs. The larynx is subdivided anatomically into a supraglottic, glottic, and subglottic compartment (Figure 5). The supraglottic larynx encompasses the epiglottis, the false vocal cords, the arytenoids and the ventricles. The glottis consists of the true vocal cords including the anterior and posterior commissures, and it extends approximately one cm below the vocal cord into the paraepiglottic space. The subglottic larynx starts below the glottis and includes the cricoid cartilage. Below the cricoid is a transit zone towards the trachea with its first tracheal ring. The larynx is lined by squamous epithelium.

Figure 5. The anatomy of larynx. This file has been identified as being free of known restrictions under copyright law, including all related and neighbouring rights.

The muscles of the larynx are divided into intrinsic and extrinsic muscles. The intrinsic laryngeal muscles are responsible for controlling sound production, while the extrinsic muscles support and position the larynx within the trachea. Innervation of the larynx is provided by branches of the vagus nerve on each side of the neck. The external branch of the superior laryngeal nerve innervates the cricothyroid muscle and provides sensory innervation to the glottis and laryngeal vestibule. On the other hand, the inferior (recurrent) laryngeal nerve provides motor innervation to all other muscles and sensory innervation to the subglottic area.

1.6 Voice evaluation and voice production

Recurrent respiratory papillomatosis (RRP) is associated with a high grade of voice handicap due to first, the papilloma lesions affecting the voice and resonance source in the larynx, and second, the recurrent surgical outcomes. Surgical intervention aims at radically removing the lesion with optimal organ preservation. However, this
does not always result in voice function preservation. The pathophysiological vocal fold effects of the lesion itself, as well as the postoperative consequences, can alter local microcirculation, and lead to fibrosis, chronic inflammation as well as oedema of the cords and its surrounding tissues. (96, 97)

Voice can be measured using patient experiences, acoustics, phonetograms, electroglottography, electromyography and perceptual analysis of the voice. In this thesis (Study I), the voice was evaluated using patient-reported outcomes in order to assess the impact on life and voice in a RRP population and encompass any report stemming from the patients themselves regarding his or her condition. In this way, the reporting should be free from interpretation by clinicians.

Voice production involves a three-step process. First, air pressure is transmitted from the lungs towards the vocal cords by the coordinated action of the diaphragm, abdominal muscles, chest muscles and rib cage. Second, the air pressure change sets the vocal cords into an oscillatory motion when there are pressure differences above and below the vocal cords. The pressure conditions make the membranous (phonatory) portion of the cords move in a semi-cyclic vibration pattern. The vibrations are age- and gender dependent. Third, the sounds generated by the vocal cords are filtered through the resonance tube and come out of the mouth as voice that can be perceptually described from different observation dimensions as distinctive qualities of each person’s voice.

1.7 Recurrent respiratory papillomatosis (RRP)

Recurrent respiratory papillomatosis (RRP) is a neoplastic disease of infectious origin that is caused by specific genotypes (6 and 11) of human papillomavirus (HPV). RRP is characterized by benign, wart-like lesions that cause hoarseness and airway obstruction. RRP accounts for extensive morbidity due to its influence on breathing, voice and recurrence in surgical sessions. (98)

1.7.1 Epidemiology of RRP

Two types of RRP are recognized: juvenile-onset RRP (JoRRP) and adult-onset (AoRRP). There is some confusion in reporting of these since the age proposed as cut-off points for the JoRRP and AoRRP vary between 12 and 20 yrs. The most frequently used cut-off point is the age of 18 years, that is, JoRRP includes patients<18 years while AoRRP patients≥18 years. (99) Only a few population-based studies are published. The overall incidence rates of JoRRP and AoRRP are suggested to be somewhere between 0,17 and 0,54 per 100 000. (100) RRP is the most common benign neoplasm of the larynx in children, and the second most frequent cause of childhood hoarseness. (101) In contrast to AoRRP, JoRRP seems to have a gender-neutral distribution. (102) HPV is often present in healthy, unaffected vocal cord mucosa, and it is not possible to distinguish infected from
non-infected epithelial cells. (103) In AoRRP, the peak incidence is around the age of 30 years, with a predominance of males. (103, 104) The cause of the male predilection is unknown, however also reflected in HPV-related HNSCC incidence, (62) suggesting growing evidence for viral transmission during oral sex and kissing. (105, 106)

Another possible explanation of AoRRP could be a reactivation of a latent HPV infection acquired in childhood. JoRRP tends to be more aggressive and is most likely caused by vertical (intrapartum or perinatal) transmission between mother and child for children delivered vaginally, especially if the labor was prolonged. (107) Caesarean section has been associated with reduced risk in epidemiological studies. (108) However, there is no clear-cut evidence for the protection against RRP using caesarean section when the mother has genital warts. It has been suggested that there is a 200-fold increased risk of JoRRP when the mother has a history of genital warts during pregnancy. (109) Despite the fact that HPV DNA was recovered from 30% of the nasopharyngeal secretions of newborn children exposed to HPV perinatally, only a small proportion of those children developed RRP. (110) This indicates the likelihood of necessary co-factors for developing JoRRP.

Although the disease can occur in almost any part of the respiratory tract, it affects the larynx and the voice source in the majority of infections. (111) Despite the benign nature of RRP, there is significant morbidity due to the multiple recurrences of the lesions. The disease burden is high, and numerous hospital admissions and surgical procedures are required to improve voice quality, and in serious cases to keep the airway patent. RRP tends to affect patients from families with low socioeconomic status at a higher rate. (112)

1.7.2 Clinical features of RRP
The main clinical characteristics of the disease are hoarseness, and in advanced cases even respiratory distress. (113) Less common presenting symptoms include dysphagia, recurrent pneumonias, failure to thrive, and chronic cough. (98) The clinical course of RRP is highly variable. Some RRP patients experience a spontaneous remission after a relatively short period of symptoms, while others require repetitive surgical sessions due to the aggressive type of the disease and sometimes tracheotomy. (114) Notably, HPV DNA (both low- and high-risk genotypes) has been detected in 7-58 % in laryngeal carcinomas without pre-existing clinical RRP. (115) Previous radiotherapy, (116) high-risk HPV genotypes, and smoking (117) are co-factors associated with higher risk for malignant transformation. The aggressiveness of RRP is associated with low age and HPV11 genotypes. (111, 117)
1.7.3 Surgical and adjuvant treatment of RRP

Surgical treatment
At present, there is no definite “cure” for RRP. Currently available treatments aim at reducing symptoms through surgical removal of the RRP lesions and preserving the normal structures and function of the larynx.

High-frequency jet ventilation (HFJV) is the preferred method of ventilation during surgery, since it provides full access to the larynx and thus improves the operative field for radical surgery with preserved functionality. (118) It has been speculated that distal spread of RRP can be facilitated by open ventilation systems such as HFJV with CO2 laser. (119) It may be argued that optimal access to the surgical area improves radical scope of the resection, reduces postoperative complications such as scarring, and promotes fewer numbers of surgical sessions requiring general anaesthesia. At the same time, HFJV RRP surgery should be performed in operating rooms with high air exchange/minute, optimized local air extraction, and capture (and eliminated) exhaled patient air, to protect surgery and operating room staff and avoid transmission of virus, since we don’t know how the virus is transmitted.

Surgical excision is usually accomplished using the carbon dioxide (CO2) laser, which has replaced cold instruments. This method can accurately vaporize the lesions with minimal bleeding. The CO2 laser must be managed carefully. (98) An emerging technique is debulking of the lesions using microdebrider (“shaver”) with improved outcomes for voice quality and reduced operation time, less tissue injury, and an important cost benefit. (120, 121) Attempts have also been made to surgically remove the RRP lesions with radiofrequency cold ablation (coblation), with reports of longer symptom-free periods between surgeries. (122) It is generally recommended to leave residual papilloma tissue during surgical removal of an
extensive disease, if it is judged that removing all papilloma will jeopardize postoperative functionality, due to risk for glottis scarring and/or web formation. (98)

Tracheotomy is the last surgical measure in aggressive RRP cases that cause critical airway embarrassment. Despite this, some authors recommend avoiding tracheotomy unless absolutely necessary since it has been associated with distal spread of the disease to the trachea and lungs. (123) If the procedure becomes unavoidable, decannulation should be attempted as soon as possible, since the disease can be managed by other techniques.

Adjuvant medical treatment
Since surgery is not curative, numerous therapies have been used in combination with surgical debulking, in an effort to reduce the RRP lesions. Adjuvant treatment options have applied in well-defined subgroups of RRP, and more specifically, in the case of frequent recurrence of the disease. (124)

Cidofovir (Vestide, Heritage Pharmaceutical, Edison, NJ, USA)
Cidofovir is an antiviral agent first used in 1995 for adjuvant treatment of severe RRP, which has since become one of the mainstays of adjuvant therapy. (46, 125, 126) The use of Cidofovir for RRP is off-label, since the compound is approved by the US Food and Drug administration (FDA) only for treatment of cytomegalovirus (CMV) retinitis in patients with AIDS without renal dysfunction. Since 1995, several case series have been published reporting beneficial effects of Cidofovir in severe RRP. Large, randomized, placebo–controlled, prospective trials the last five years have not been able to confirm the previous carcinogenic concern. (127, 128) However, more high quality data, especially using randomized controlled trials, are still required to provide evidence of the efficacy of Cidofovir.

α-Interferon
α-Interferon has been used since 1988. The medicine significantly reduces the severity of the disease, however at discontinuation, disease severity rapidly returns to pre-treatment levels. (129) Systemic α-interferon is less popular due to its unfavourable side effects.

Bevacizumab (Avastin)
Prospective investigations and retrospective cohort studies, as well as case series have been conducted both in JoRRP and AoRRP. Avastin was administrated intralesionally as an adjunct to angiolytic KTP laser treatment. Promising results are reported regarding efficacy without significant complications. (130) Long-term results and larger blinded randomized studies are warranted.

Indole-3-carbinol
Indole-3-carbinol, a dietary supplement, has been suggested in the treatment of RRP and has showed promising results when used in animal experiments (131) or small clinical groups. (132) Previous reports have claimed successful treatment outcomes. (132)

**HPV vaccine**

The HPV vaccine procedure is described in section1.3. A two-dose schedule is recommended and applied in Sweden since 2015 for girls aged 9-13 years. The currently used Gardasil 9® has the ability to protect against 90% of cancers of the cervix, vulva, vagina, and anus. The use of Gardasil® in therapy has been presented with promising results in selected cases. (133)

Treatments with mumps vaccine, (134) ribavirin, (135) and photodynamic therapy (PDT) (136) have also been in use. Nevertheless, longer follow-up and trials in larger groups are warranted. Overall, viral persistence seems to occur following all these adjuvant treatment methods, as with surgery.

**1.7.4 Prevention with HPV vaccines**

There is no proven efficacy in the prevention of recurrent respiratory papillomatosis. (90, 91) However, as with anogenital warts, the low-risk genotypes HPV6 and 11 account for the majority of RRP cases. It is therefore conceivable that the 9-valent recombinant HPV vaccine will have impact on the future incidence of RRP. (137) There is a clear predominance of men in RRP as well as oropharyngeal cancer urging the gender-neutral vaccination strategy. The expected vaccination-induced reduction of anogenital HPV infections might additionally decrease the incidence of RRP in future generations. (138, 139) This preventive and perhaps even therapeutic HPV approach seems to be promising, however future trials with larger material in order to test the feasibility of vaccination are warranted.

**1.8 p16**

p16 is a 156-amino-acid protein and it is codified by a gene localized on chromosome 9p21 within the INK4a/ARF locus. (140) It is suggested that this protein plays a significant role in cell cycle regulation by decelerating cells progression from G1 phase to S phase. It acts therefore as a tumor suppressor protein that is implicated in the prevention of malignancies. It is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6 that phosphorylate retinoblastoma protein (pRB), which eventually results in progression from G1 phase to S phase. (141) The way p16 acts as a tumor suppressor is by binding to CDK4/6 and preventing its interaction with cyclin D. This leads ultimately to the inhibition of the downstream activities of transcription factors, such as E2F1, and arrests cell proliferation. (142)
Moreover, p16 is overexpressed in HPV-positive oropharyngeal carcinomas due to the degradation of pRb by the viral oncoprotein E7, which normally is a negative regulator of p16. (143) As a result, p16 overexpression correlates to HPV positivity in oropharyngeal cancer and can therefore be used as a surrogate marker for HPV positivity in these carcinomas. (43, 144) However, the correlation between p16 and HPV status in the oropharynx is complex and organ-specific. Prognostic advantage for p16-expressing oropharyngeal tumors is well-established, and could indicate a significant step in overall and disease-free survival as well as locoregional tumor control. (145) p16 is identified by immunohistochemistry.

Beside this, homozygous deletion of p16 is frequently found in oesophageal and gastric malignancies. (146) Hypermethylation of p16 is also being considered to be a potential prognostic biomarker for prostate cancer. (147)

1.9 Head and neck cancer (HNSCC)

1.9.1 Epidemiology and risk factors of HNSCC
Approximately 650 000 new cases of squamous cell carcinoma of the head and neck (HNSCC) are diagnosed each year worldwide. (148) The incidence in Sweden is approximately 1 400 new cases each year, (149) accounting for 3% of all new cancer diagnoses in the country. In general, this incidence is twice as high in men compared to women. (150) Smoking and alcohol are well-established risk factors for developing HNSCC. Other well-known factors are betel nut and tobacco chewing. (151) Viruses have also been implicated as causative factors; Epstein-Barr virus (EBV) has been associated with nasopharyngeal cancer (152) and HPV with oropharyngeal cancer. (153) High lifetime number of vaginal and oral sex partners, high-risk HPV and seropositivity for HPV16 have been reported in the context of development of oropharyngeal cancer. (106)

1.9.2 TNM classification and staging of oropharyngeal cancer
The most widely used classification system for head and neck cancer is the TNM system developed by the international Union Against Cancer. (154) The classification is based in the size and extension of the tumor (T), presence, size and localization of regional lymph node metastasis (N), and presence of distant metastasis (M). Staging is the process of classifying a primary tumor depending on the extent of the cancer, including the presence or absence of metastases. It aids in treatment planning, prognosis determination and communication between healthcare centres.
### Table 1. Primary tumor (T) (oropharynx).

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tx</strong></td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td><strong>T0</strong></td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td><strong>Tis</strong></td>
<td>Cancer in situ</td>
</tr>
<tr>
<td><strong>T1</strong></td>
<td>Tumor ≤2 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>T2</strong></td>
<td>Tumor &gt;2 cm but ≤4 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>T3</strong></td>
<td>Tumor &gt;4 cm in greatest dimension or extension to lingual surface of the epiglottis</td>
</tr>
<tr>
<td><strong>T4a</strong></td>
<td>Moderately advanced local disease (tumor invades the larynx, deep/extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible)</td>
</tr>
<tr>
<td><strong>T4b</strong></td>
<td>Very advanced local disease (tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases the carotid artery)</td>
</tr>
</tbody>
</table>

### Table 2. Regional lymph nodes (N).

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nx</strong></td>
<td>Regional nodes cannot be assessed</td>
</tr>
<tr>
<td><strong>N0</strong></td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td><strong>N1</strong></td>
<td>Metastasis in a single ipsilateral lymph node ≤3 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>N2</strong></td>
<td>Metastasis in a single ipsilateral lymph node &gt;3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none &gt;6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none &gt;6 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>N2a</strong></td>
<td>Metastasis in a single ipsilateral lymph node &gt;3 cm but not more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>N2b</strong></td>
<td>Metastasis in multiple ipsilateral lymph nodes, none &gt;6 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>N2c</strong></td>
<td>Metastasis in bilateral or contralateral lymph nodes, none &gt;6 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>N3</strong></td>
<td>Metastasis in a lymph node &gt;6 cm in greatest dimension</td>
</tr>
</tbody>
</table>

### Table 3. Distant metastasis (M).

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M0</strong></td>
<td>No distant metastasis</td>
</tr>
<tr>
<td><strong>M1</strong></td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
Table 4. Anatomic stage/prognostic groups.

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>II</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>III</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>IVA</td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4a</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td>IVB</td>
<td>TAny</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4b</td>
<td>NAny</td>
<td>M0</td>
</tr>
<tr>
<td>IVC</td>
<td>TAny</td>
<td>NAny</td>
<td>M1</td>
</tr>
</tbody>
</table>

*TAny*: any of the available classifications of primary tumor size.
*NAny*: any of the available classifications regarding the location of lymph node metastasis.

### 1.9.3 Treatment of HNSCC

The oncologic treatment is primarily aimed at survival. Due to the important communicative and digestive aspects of the affected organs, functional preservation is increasingly desirable. Surgery and radiotherapy (RT) were considered to be the only curative treatment of patients with squamous carcinoma of the head and neck back in the 1960-1980’s. Today, surgery may be used as single therapy in low-stage disease. RT is the mainstay of treatment, and it can be used as single modality treatment or combined treatment. In the majority of cases, a combination of treatments is needed for optimal results. The resection of locally advanced tumors has been made easier due to microsurgical free tissue transfer for reconstruction of surgical defects. Chemotherapy can be administrated as *induction therapy*, that is, given prior to radiotherapy, or *concurrent therapy*, administrated simultaneously as radiotherapy, (155) or as *adjuvant* chemotherapy after surgical resection and in cases of residual disease. (156, 157) The benefits of chemotherapy must be balanced with adverse effects due to its increased toxicity, especially among patients with medical comorbidities and reduced performance status. Organ preservation does not equal functional preservation, making the choice of therapy in specific combinations challenging. The combination of surgery and radiotherapy is more commonly used; RT can be given either pre-operatively or post-operatively depending on the treatment protocols of each hospital in different countries.
Radiotherapy (RT) is an integral part of primary or adjuvant treatment for HNSCC. It results in high tumor control and cure rates for early stage HNSCC including glottic, base of tongue, and tonsillar cancer. Prior to radiotherapy start, a plastic mask is fitted to the patient’s face using a vacuum cushion, aiming to keep the head in the same position for each treatment session. A computed tomography (CT) scan is performed in the RT treatment position. A dosimetric plan is established, and treatment can commence. Treatment of HNSCC employs fractionated radiotherapy protocols. This implies that the total radiation dose is subdivided into smaller doses given over a certain time.

1.9.4 Prognosis of HNSCC
Survival rates are dependent on stage and site of tumor. Cure rates decrease in locally advanced cases and regional node involvement. The overall five-year survival for all stages of HNSCC is estimated to be between 35% to 50% due to late disease presentation. Up to 50% of head and neck cancer patients present with advanced disease. (158) This 5-year mortality rate has not improved significantly in the last few decades, despite advances in treatment modalities, and reduced exposure to traditional risk factors. (159) Figures 7 and 8 depict the percent of cases and 5-year relative survival by stage at diagnosis according to SEER (Surveillance, Epidemiology, and End Results Program) of the American National Cancer Institute, 2005-2011.

![Percent of Cases by Stage](image)

**Figure 7.** Percent of cases at diagnosis: oral cavity and pharynx cancer.
1.10 Tonsillar cancer

HPV-positive tonsillar cancer is considered to be a different tumor entity compared to HPV-negative carcinomas. The former arise in the tonsillar crypts while the latter originates from the epithelium of the tonsillar surface. (160) As previously reported, many of the HPV-positive tonsillar cancer cases lack the traditional risk factors associated to the disease. The patients tend to be younger and generally have a better performance status. (159) Most HPV-positive tonsillar cancers are associated with the high-risk HPV16 (in approximately 95% of the HPV-positive cases), (161) while other subtypes (31, 33 and 35) are relatively infrequent. Overall, patients affected by HPV-positive tonsillar cancer have a better prognosis and improved survival, regardless of treatment strategy when compared to HPV-negative cases. (162-164) Despite this, the biological and molecular mechanisms behind the difference in survival have yet to be revealed. Treatment modalities have so far not been adjusted to the presence or absence of HPV. Previous studies have established an inverse relationship between HPV tumor status and the presence of p53 mutations in head and neck cancer. (165)

1.10.1 Epidemiology and risk factors

The incidence of tonsillar cancer appears to be increasing over time during recent years in several western countries, preferentially in men. (166-168) Tonsillar cancer is the most common form of oropharyngeal cancer, (150) which develops in the base of the tongue and palatine tonsils, posterior pharyngeal wall, and soft palate. Heavy tobacco smoking and alcohol consumption are known traditional predisposing factors and seem to act synergistically in the development of the disease. (169) Despite a decline in smoking and alcohol abuse (170) during the last years, the
Incidence of tonsillar cancer appears to be increasing worldwide. HPV has been regarded an important aetiological agent. (166, 167, 171, 172) Recent studies report a high prevalence of HPV and EBV co-infection in base of tongue and tonsillar cancers. (173)

Demographically, patients with HPV-positive tonsillar malignancies are often white males, with high socioeconomic status, non-drinkers and non-smokers. (106, 159, 174) Changes in sexual behaviour and increased practice of oral sex could account for some of the increased incidence of HPV-associated cancer in younger generations. (167) Other factors that could be implicated are higher number of lifetime sex partners and earlier age at sexual debut. (106, 175) In addition to this, increased risk for tonsillar cancer among women with cervical lesions and husbands of women with cervical dysplasia or cancer has been identified. (176)

1.10.2 Clinical features of tonsillar cancer
Squamous cell carcinoma of the head and neck (HNSCC) is a collective term for malignant disease in the ear, nose, and throat region. It is the sixth most common malignancy, with a significantly varying incidence in different world regions. (148) Typical symptoms are unilateral sore throat and earache, swallowing difficulties, neck lump that turns to be a nodal metastasis, unexplained weight loss, and fatigue. Unfortunately, many patients are asymptomatic initially and therefore, diagnosed in later stages of the disease. It has been suggested that HPV-positive tonsillar carcinomas should be considered different tumor entities from HPV-negative tonsillar tumors based on epidemiology, genetics, response to therapy, and prognosis. (62) Therefore, patients could benefit from less aggressive treatment. (160, 177)

1.10.3 Treatment of tonsillar cancer
Adequate and proper investigation is necessary for planning optimal treatment with curative intentions, organ as well as functional preservation. (178) Current recommended management of patients with HPV-induced tonsillar malignancies do not differ from those who are HPV-negative. (160) As standard of care, radiotherapy is considered to be the best and most effective treatment option today, combined with concurrent chemotherapy in cases of advanced (stages III and IV) tonsillar cancer. Clinical trials evaluating new surgical approaches and especially, transoral robotic surgery (TORS), are now underway. (179, 180) Unresectable disease is usually treated with concurrent chemoradiotherapy. This has been shown to have superior survival rates compared to monotherapy with radiation alone. (181-183) The mortality rate is high due to advanced disease at diagnosis, the need for aggressive treatment strategy, high risk for locoregional recurrences, and comorbidities. The single most debilitating side effect of RT is oropharyngeal mucositis with subsequent sore throat and mouth sores. Long-term sequelae include
dysphagia, xerostomia, weakness and fatigue, candidiasis and osteoradionecrosis. (184, 185)

Surgery vs. radiotherapy during the last decades
Surgical resection of tonsillar tumors is technically challenging, even when properly performed, since it takes place in a complex anatomical area. Currently, surgical resection is reserved as ‘salvage surgery’ in cases of residual or recurrent tumor after oncological treatment. Recent results using transoral robotic surgery (TORS) as single modality treatment have shown promising results, especially for small tumors of the tonsillar fossa that do not extend toward the lateral pharyngeal wall or the base of the tongue. (179, 180) Studies have reported excellent locoregional control rates, ranging from 80% to 90%. (179, 186) Neck dissection is performed in case of nodal cervical metastases that are either extensive or do not respond to radiotherapy.

1.10.4 Prognosis of tonsillar cancer
The overall 5-year survival rate for SCC tonsillar cancer is related to the stage of the disease; approximately 90% for stage I tumors, whereas patients with stage IV tonsillar cancer have a survival rate of less than 20%. (187, 188) It has been reported that non-smoking patients with HPV-positive tumors have a better prognosis than smokers, (159) and HPV-positive males have improved survival rates over HPV-positive females. (189) Studies indicate that p16 can be used alone as a prognostic test for tonsillar cancer survival but combined p16 and HPV testing seems to be superior in predicting survival. (190, 191)

As mentioned before, patients with HPV-associated tumors in the tonsillar region are younger and exhibit better prognoses than their HPV-negative counterparts, regardless of treatment modality. (163, 164, 169) This poses the question of whether the aggressive chemoradiotherapy and surgery applied to patients with HPV-positive tumors could be de-escalated in an effort to reduce toxicity, and by doing this improve the long-term quality of life while maintaining treatment efficacy. The suggested treatment modification due to presence or absence of HPV in malignancies might be achieved by the following: 1) reducing the total dose of radiotherapy, 2) using radiotherapy alone without chemotherapy, 3) using radiotherapy with less toxic chemotherapeutic agents (for example, EGFR inhibitors), and 4) using TORS as single-modality treatment with selective neck-dissection when appropriate. (177)

The reasons for the better response in HPV-positive cases are so far unknown. Some studies might suggest immunological factors related to HPV infection (192) while others focus on the inverse relationship between HPV status in the tumors and the presence of mutations in HNSCC (intact p53-mediated apoptotic response). (165)
1.10.5 HPV vaccines

The HPV vaccine procedure is described in section 1.3. Gardasil 9® is a 9-valent recombinant vaccine against human papillomavirus and a further development of Gardasil®. The vaccine has the ability to protect against 90% of cancers of the cervix, vulva, vagina, and anus. If 90% of females were vaccinated, this could theoretically reduce the number of HPV associated oropharyngeal cancer in men by 66%. (89) Thus, the current vaccination of females positively affects the rates of oral HPV infection in the males. However, it would still be an unsatisfied HPV-related increase in oropharyngeal cancer in men. (89) The numbers of HPV infections in the oropharynx and subsequently, HPV-associated tonsillar cancer cases, are expected to outstrip the rate of cervical malignancies by 2020. This highlights the importance of performing a gender-neutral vaccination strategy in the future. (193)

1.11 Tongue cancer (TSCC)

The tongue is the most common site for cancer presentation in the oral cavity worldwide, (194) and constitutes a major public health problem in a number of countries, causing significant morbidity and mortality. Malignant tumors of the mobile (oral) tongue constitute a challenge due to their unique behaviour, requiring aggressive treatment to minimize the risk of locoregional spread.

1.11.1 Epidemiology of TSCC

Squamous cell carcinoma of the mobile tongue (TSCC) is considered to be one of the most commonly presented head and neck cancers, with an estimated 12 770 new cases in the USA in 2012. (194) In Sweden, tongue cancer constitutes about 12% of all HNSCCs. (195) Increasing incidence trends over the last decades have been reported, (196) particularly in young white females aged 25-44, (195) Afro-Americans, Hispanics and Asians. (197) According to Scandinavian tumor registries, the general incidence of oral cancer SCC increased 5-fold among young men and 6-fold among young women (years 1960-1994) compared to just a 2-fold increase in older patients. (195) In general, malignant tumors of the anterior two-thirds of the tongue are detected earlier than cancers of the oropharyngeal posterior one-third; the mobile tongue cancers are usually better differentiated. (198)

The most commonly named etiological factors for cancer of the mobile tongue are tobacco and alcohol abuse. (199) However, these particular risk factors are not commonly present among the majority of the young patients. Additional risk factors have been discussed including nutritional deficiencies, poor dentition, viruses, (200, 201) and genetic factors. (202) Additionally, the unique gender- and age-specific incidence trends suggest a possible role for bacterial infections, lifestyle, and environmental factors. (197) Squamous cell carcinomas are estimated to constitute about 90-92% of all tongue carcinomas. The remaining tumors are
adenocarcinomas, lymphomas and sarcomas, and they differ from the former in terms of aetiology and carcinogenesis.

1.11.2 Clinical features of TSCC
Leukoplakia, erythroplakia, and chronic glossitis are lesions that appear in the oral cavity with the potential for malignant transformation. (203) Symptoms are usually present in tumors larger than 1 cm, and more commonly involve swallowing, articulation difficulties, articulation disabilities and pain, when the tumor involves the lingual nerve. In such cases, referred pain to the ipsilateral ear may be present. Additionally, dysphagia may lead to aspiration as well as malnutrition.

1.11.3 Treatment of TSCC
In general, superficial lesions are best treated with curative intention and single-modality therapy with surgical excision. Multiple modalities (combination of surgery and radiotherapy) are required for larger tumors and cervical metastasis. As inadequate excision of the primary tumor is considered to be the most common cause of tumor-related death, efforts should be made to obtain clear and wide margins of resection during surgery.

The two main techniques of radiotherapy that are currently being used are external beam radiotherapy (204) and brachytherapy. (205) The role of chemotherapy in the treatment of mobile tongue cancer still remains unclear. Tumors of early stage are not treated with chemotherapy. Advanced primary lesions or the presence of distant metastases and generally poor prognosis constitute an indication for chemotherapy treatment. (206)

The treatment of choice of TSCC aims primarily at survival, but the digestive aspects, with high risk of aspiration, make an organ preservation treatment choice desirable especially in patients with poor general health status. The healthy tissue is highly affected in the surgical, chemotherapy, and radiation process, with major side effects that have to be manageable for the patient. Once radiation has been used in the affected tumor area, the possibility to use it again in case of disease recurrence is reduced.

1.11.4 Prognosis of TSCC
Several reports describe squamous cell carcinoma of the mobile tongue as an aggressive disease among young adults. Some of these reports have demonstrated lower survival rates for young patients compared to older individuals. (207, 208) At the same time, a smaller number of studies indicate better survival rates among younger patients. (209) The overall 5-year survival among patients with TSCC has been reported as between 37% and 85%. (210, 211) The survival rate is highly dependent on nodal status at the time of diagnosis with 50% 5–year overall survival.
in patients with N0 neck compared with 11% in patients with nodal metastasis. (212) Tumor thickness is also an important factor for the prognosis, and has been positively correlated to nodal disease. (213)

Recurrence rates have been estimated between 27 and 40%; (211, 214) significant prognostic factors for local recurrence include T-stage, histopathological grade, time interval between surgical treatment and RT, age, sex and total dose of radiation. (215-218) It is generally accepted that patients should undergo monitoring for a minimum period of at least 5 years after treatment because of the high recurrence rates at both primary and neck sites as well as the increased frequency of distant metastasis and second primary malignancies.
2. Purpose and aims

2.1 Overall purpose
The overall purpose of this thesis was to describe the human papillomavirus (HPV) in recurrent respiratory papillomatosis, tonsillar cancer and tongue cancer.

2.2 Specific aims
- To measure the effects of RRP on voice and quality of life, and to assess the relation of early manifestations of the disease to long-term morbidity.
- To assess the incidence of tonsillar cancer in northern Sweden and study the impact of HPV virus and its surrogate marker p16 in tonsillar cancer.
- To determine clinical characteristics and possible predictor factors affecting the therapeutic needs of RRP patients in northern Sweden, and to identify a potentially high-risk RRP group.
- To assess the clinical and prognostic importance of HPV, p16 and HPV receptor syndecan-1 in mobile tongue cancer and tonsillar cancer.
3. Patients and methods

3.1 Study design

All studies employed quantitative methodology; data was retrieved from population-based descriptive prospective longitudinal trials (Studies I and III) and a retrospective observational cohort study (Studies II and IV). Table 5 summarizes the study design, population and sample size of included papers.

Table 5. Study design.

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Subjects (n)</th>
<th>Male/female</th>
<th>Time interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Prospective</td>
<td>27</td>
<td>17/10</td>
<td>2004-2012</td>
</tr>
<tr>
<td>II</td>
<td>Retrospective</td>
<td>214</td>
<td>155/59</td>
<td>1990-2013</td>
</tr>
<tr>
<td>III</td>
<td>Prospective</td>
<td>48</td>
<td>34/14</td>
<td>2004-2014</td>
</tr>
<tr>
<td>IV</td>
<td>Retrospective</td>
<td>109</td>
<td>54/55</td>
<td>1997-2012</td>
</tr>
</tbody>
</table>

All studies included in this thesis were approved by the Regional Ethical Review Board in Umeå (Dnr. No2012 276 32M, 2010 277 31, 08-003M and 03-201). To access the accurate retrospective paraffin-embedded samples from the BioBank North, we used data from the Swedish Cancer Registry database. The application was approved by the Biobank North (472-13-08 in 2013-03-26). All patients in the prospective studies gave their informed consent after receiving information on the details of the study according to the Helsinki declaration. In Study II, a detailed outline of the patients included and excluded is provided in Figure 6. In Study III, 21 consecutive RRP patients were added to the 27 patients from Study I. All 48 patients were assessed for eligibility, fulfilling the in-, and exclusion criteria (Table 6), none discontinued participation.

Study I

This study was a prospective questionnaire-based cohort study that included 27 consecutive, non-smoking patients (age 21–71 years, median 47 years) that were presented, diagnosed and treated for RRP at the Department of Otorhinolaryngology, University Hospital of Umeå, Sweden, between 2004 and 2012. In-, and exclusion criteria for participants in Studies I and III are provided in Table 6.

Preoperatively, the larynx was examined using transnasal flexible endoscopy or flexible videendoscopy with high definition technique, stroboscopy and narrow band imaging (NBI). The purpose was to visualize the RRP lesions and assess the voice source function. Intraoperative biopsies were obtained for histopathological studies and HPV analysis. An outpatient visit was planned within 8 weeks after surgery and the patients received the questionnaires VHI and SF-36 within 3 months.
after the last treatment session.

Table 6. In-, and exclusion criteria for participants in Studies I and III.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• cognitive abilities</td>
<td>• previous tracheotomy</td>
</tr>
<tr>
<td>• adequate Swedish language competency enabling the patient to independently answer the questionnaires</td>
<td>• vaccination with HPV vaccines</td>
</tr>
<tr>
<td></td>
<td>• treatment with adjuvant therapies (a-interferon, indole-3-carbinol, cidofovir)</td>
</tr>
<tr>
<td></td>
<td>• previous treatment of the disease with radiofrequency coblation or microdebridement during the last 10 years</td>
</tr>
<tr>
<td></td>
<td>• no smoking</td>
</tr>
<tr>
<td></td>
<td>• no occurrence of allergy, gastroesophageal reflux disease (GERD) and asthma</td>
</tr>
<tr>
<td></td>
<td>• no prematures in juvenile onset (JoRRP)</td>
</tr>
<tr>
<td></td>
<td>• non responders of the questionnaire, n=6</td>
</tr>
</tbody>
</table>

**Study II**

This is a retrospective observational study, which included all patients diagnosed with tonsillar cancer during 1990-2013 at the University Hospital of Umeå. Information was extracted by the Swedish Cancer Registry database in order to identify cases of tonsillar cancer. 65 tonsillar cancer biopsies obtained between 2000 and 2012 were analysed. The ICD-7 code used for tonsillar cancer was 145.0. A detailed outline of the eligible patients is provided in Figure 7.

Pre-treatment tumor samples were collected by biopsy or surgical resection and paraffin-embedded tumor blocks were retrieved from the archives of the Department of Laboratory Medicine/Pathology at the University Hospital of Umeå.
Study III
This prospective cohort study included all 48 patients diagnosed or treated with RRP at the University Hospital of Umeå, between 2004 and 2014. We used the same in- and exclusion criteria (Table 6), preoperative examination and biopsy procedures as in Study I. The 48 included patients were categorized for age, disease duration, onset (JoRRP or AoRRP), profile of disease development, and number of surgical sessions in relation to disease duration, localization of papilloma, gender and HPV subtypes. According to the frequency of operations, patients were divided into two groups: low-frequency (LF, <1 surgical treatment/year) and high-frequency (HF, ≥1 surgical treatment session/year) groups. Surgery was performed using CO2 laser. All patients were treated by the same surgeon and were evaluated approximately 8 weeks postoperatively and as needed afterwards.

Study IV
The study included formalin-fixed, paraffin-embedded samples from 109 patients
with primary TSCC; 96 of them were retrieved from the Clinical Pathology lab, Umeå University Hospital, Sweden and 13 from the Second University of Naples, Multidisciplinary Department of Medical, Surgical and Dental Specialties, Naples, Italy. Both patient groups received treatment during a 15-year period. Based on their age at diagnosis, patients were grouped into three groups: ≤40, 41-65 and >65 years. 66% were treated with preoperative radiotherapy followed by surgery while 31% received single modality treatment with surgery. The mean follow-up post-treatment was 45.5 months (range: 1-179) and survival was measured as: alive disease free, alive with disease, dead of disease, dead of other disease or dead with disease but not with oral cancer as first cause of death. 65 patients with tonsillar cancer (participants from Study II) were included in the analysis of syndecan-1 expression.

3.2 Study participants
A total of 48 patients, diagnosed or treated for RRP between 2004-2014 and 65 tonsillar cancer samples obtained between 2000-2012 were analysed at the tertiary referral centre for northern Sweden, University Hospital of Umeå. Northern Sweden was defined as the part of Sweden consisting of the counties Västerbotten, Norrbotten, Västernorrland and Jämtland, which include a total population of 882 563 (2015). In Study IV, 109 cases of TSCC available at both Clinical Pathology, Umeå University Hospital, Sweden and the Second University of Naples, Multidisciplinary Department of Medical, Surgical and Dental Specialties, Naples, were included.

3.3 Voice handicap index (VHI) and Short Form (36) Health Survey (SF-36)
As a result of the complex nature of the voice, multimodal assessment approaches are often advocated. (219) These include acoustics and perceptual measurement as well as patient-reported experiences. However, objective and subjective measures do not always agree. Patient-reported outcomes (PRO) encompass any report stemming from the patients themselves regarding his or her condition and should hence be free from the interpretation by clinicians. Observers often underestimate or make incorrect judgement of patient experiences. It can therefore be argued that the patient is the most reliable source of information for this purpose and also the freest from bias. The fact that acoustic analysis can be influenced by recording equipment, mouth-to-microphone distance, the software used for analysis as well as the choice of vowel, thereby hampering inter-study comparison, made this measurement technique less interesting. (220) We also chose not to perceptually analyse the voice due to the fact that the listener’s profession can bias the perceptual measurement method. (221)

Voice handicap index (VHI)
The RRP patients underwent pre- and postoperative voice analysis. The patients
vocalized sustained /i/ and /m/ as close as possible to habitual speaking pitch and intensity. The patients received the VHI questionnaire within 3 months after having their last surgery; a reminder was dispatched after 6 months in case the questionnaire had not been returned.

The VHI questionnaire evaluates the effect of voice disorders on daily life. More specifically, it measures the patient’s perception of voice quality, before and after treatment for laryngeal disorders. The survey includes and examines 30 items, grouped into three subscales related to voice disorders: physical, emotional and functional domains. (222) Every aspect or domain is represented by 10 items, formed as statements. Each of the 30 questions will elicit a response and corresponding score: 0 for ‘never’, 1 for ‘almost never’, 2 for ‘sometimes’, 3 for ‘almost always’ and 4 for ‘always’.

The total obtained score (VHI\text{total}) ranges from 0 to 120 points and each of the three subscales from 0 to 40 points. The higher the score, the greater negative impact on daily life due to the voice disorder. A voice handicap score from 0 to 30 is considered to be ‘minimal’ handicap, from 31 to 60 ‘moderate’ handicap and from 61 to 120 points ‘serious’ voice handicap. A validated Swedish translation of the voice handicap index (Sw-VHI) (223) was reported in 2009 and concluded that 20 points or less in the total VHI score can be used as a normative value when the questionnaire is applied to the Swedish population. Moreover, a difference above 13 points for the VHI\text{total} and more than 6 points for each subscale regarding the same individual and between two measurements, was considered to be a true change in the quality of voice experienced. (223)

**Short Form (36) Health Survey (SF-36)**

We used the SF-36 questionnaire as a complementary module to VHI in order to assess quality of life aspects in relation to RRP and VHI. As with the VHI survey, the SF-36 questionnaire was given to the patients approximately 3 months postoperatively with a reminder after 6 months in the case of no return. The SF-36 questionnaire is a 36-item, patient-reported survey of quality of life. (224) It consists of eight scaled scores, related to eight different domains of health-related quality of life: physical functioning, role limitations due to physical health and emotional problems, vitality (energy and fatigue), emotional well-being, social functioning, body pain and general health status. The scores in each domain are the weighted sums of the questions in their section and each patient receives a score for each domain, ranging from 0 (worst) to 100 (best). Combining selected domains generates a further two generalized subscales; the physical (PCS) and mental (MCS) component summary scores. Lower scores mean more disability. Two disadvantages with this survey that are well recognized are that the survey does not take into consideration a sleep variable and furthermore, it has a low response rate in the >65
population. (225)

The SF-36 survey was also applied in the generalized Swedish population (1995), in order to receive the corresponding normative values. (226) The disadvantage of this project is the lack of knowledge about the change over time in the SF-36 measurements.

**Videoendoscopy and stroboscopy**

Functional evaluation of the larynx was initially performed using the Olympus ENF P4 transnasal flexible endoscope and when updating the equipment the later recordings were performed using the Olympus ENF VH flexible videoendoscopy system. Initially the stroboscopic equipment was a Wolf type 5052 and the camera a Wolf endocam 5502. After an update of the system, the later recordings were based on the Olympus CV-170 light source system and the Olympus CLL-SI stroboscope unit. The rhino- and oropharynx and larynx were examined. The localization of the RRP lesions was carefully described and documented in the running hospital's documentation system, Picsara. In contrast to Derkay et al., (227) the goal was not to streamline the prediction of treatment intervals based on anatomical and symptom scores, but rather to identify associations of the other factors to the observations of the HPV deposit in the larynx. No Derkay score was therefore calculated.

### 3.4 HPV DNA extraction and PCR analysis

Biopsy specimens from RRP and tonsillar cancer cases were analysed by an experienced head and neck pathologist as a histopathological basis for the inclusion in the study. The outline of included and excluded specimens is provided in Figure 7. Seventy-four tonsillar cancer specimens were extracted from the archives of the Department of Pathology, University Hospital of Umeå (from 2000 to 2012). Four additional specimens that either contained too little, or completely lacked tumor tissue were excluded. Five additional samples were excluded, as they were duplicates of already registered patients. The RRP fresh tissue sample was kept in saline and the HPV genotypes were settled at the Clinical Microbiology Laboratory at Skåne University Hospital. The laboratory is accredited according to the ISO15189 standard for analysis of 14 oncogenic types and for HPV6 and HPV11.

#### 3.4.1 DNA extraction in RRP lesions

A 2- to 3-mm biopsy from RRP lesion was immersed in 1 mL saline solution before extraction. The saline was removed and the biopsy was digested in 500 μL lysis buffer (10mM Tris-HCl; 10 mM NaCl; 10 mM EDTA; pH 7.8, 4% sodium dodecyl sulfate; and proteinase K [200 lg/mL, Roche]) at 37°C overnight. Then, DNA was extracted with the help of the Total NA-kit (Roche) using MagNA Pure LC (200 μL input and 100 μL output).
3.4.2 Identification of HPV DNA in RRP lesions and tonsillar cancer samples
In Study II, all samples included were from formalin-fixed paraffin embedded (FFPE) diagnostic biopsies. DNA was extracted with QIAamp DNA FFPE Tissue kit (Qiagen, CA, USA) or QIAamp Mini kit (Qiagen) according to the manufacturer’s instructions and a general HPV PCR was run with 100 ng extracted DNA from each patient with general primers GP5+/6+. (228) The process of detection of HPV DNA by PCR is mentioned in details in Study II. S14 primers were used to confirm the presence of amplifiable DNA. S14 was positive in all HPV-negative tonsillar cancer samples. In the RRP lesion biopsy, simultaneous identification of 39 genital HPV types was carried out by modified general primer polymerase chain reaction (MGP-PCR) and subsequent Luminex analysis in a 25 μL reaction containing 5 μL of extracted material. (229) The Luminex assay included probes for HPV types 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68 (a and b), 69, 70, 73, 74, 81, 82, 83, 86, 87, 89, 90, 91 and 114.

3.4.3 Quantitative RT-PCR in RRP lesions
Quantitative RT-PCR assay was performed using the QuantiTect Probe RT-PCR Mastermix (Qiagen, Stockholm, Sweden) and the OligoTex Direct mRNA Mini Kit, (Qiagen, Stockholm, Sweden) and PCR was performed by the use of ABI 7500. (230) The RRP biopsy sample adequacy was assessed by testing 5 μL of the sample for the human beta globin gene with a real-time polymerase chain reaction (PCR).

3.4.4 HPV16 in situ hybridisation in tongue cancer samples
A HPV16 plasmid DNA was amplified, purified and labelled by nick-translation in the presence of digoxigenin-16-dUTP, followed by purification and ethanol precipitation. After dewaxing, endogenous peroxidase activity was blocked in H2O2 in methanol and tissue digested with varying concentrations of proteinase K (Sigma). Hybridisation was performed overnight followed by immunohistochemical detection, with mouse anti-digoxin and avidin–biotin peroxidase complex. A positive control from cervix was included with each batch.

3.5 p16 analysis (immunohistochemistry)
In Studies II and IV, p16 immunohistochemistry was used in order to identify the relationship of the protein marker p16 to HPV virus in mobile tongue and tonsillar cancer samples. A Ventana staining machine was used for detection of p16. For this purpose, an antibody (monoclonal mouse anti-human; cat. no. sc-56330) (Santa Cruz Biotechnology, Dallas, Texas) was used and diluted 1:200. Pre-treatment of slides in Tris-EDTA pH 8.0 was performed before staining. Scoring percentage of tumor cells and staining intensity was calculated with the Quick score system. (231) The expression of p16 in tumor cells was divided in 6 categories: 1=0-4%, 2=5-19%, 3=20-39%, 4=40-59%, 5=60-79% and 6=80-100%. A scale was used for
calculating staining intensity: 0=negative, 1=weak, 2=intermediate and 3=strong staining and a quick score (range 0-18) was obtained by multiplying percentage of tumor cells expressing the protein with intensity.

3.6 Syndecan-1 analysis (immunochemistry)

Immunochemistry was additionally used for identification of the presence of HPV receptor syndecan-1 in selected cases of mobile (oral) tongue cancer and tonsillar cancer samples. The specimens were stained with an antibody detecting syndecan-1 (SP152; Abcam, Cambridge, UK) diluted 1:100, after pre-treatment in citrate buffer pH 6.0 and staining was performed in a Ventana staining machine. The intensity of staining was calculated in the same way (Quick score system) as reported before for p16 analysis. (231)

3.7 Swedish Cancer Registry and BioBank North

When retrieving cases of tonsillar neoplasms (Studies II and IV) and mobile tongue cancer (Study IV), the Swedish Cancer Registry was used. The patients were identified in the database by using the ICD-7 codes for tonsillar cancer (145.0) and tongue cancer (141.7, 141.8, 141.9). The registry was established in 1958 and covers the whole Swedish population; 50 000 cases of different cancer forms are registered each year. Health care providers in Sweden are required to report to the registry any detected new cancer cases diagnosed at clinical-, morphological-, and laboratory examinations, including cases identified during autopsy. Currently, there are six regional registries in Sweden which update the National Cancer Registry with data regarding registration, coding and major check-up of newly detected cases. Three different types of information are included in the Swedish Cancer Registry. 1) Patient data (personal identification number, sex, age and place of residence). 2) Medical records (site of tumor, histological type, stage, basis of diagnosis, date of diagnosis, reporting hospital or department, identification number for each specimen). 3) Follow-up data (date and cause of death or date of migration).

We received approval from the BioBank North to access the accurate samples for the study (472-13-08 in 2013-03-26). Biobank North is administrated by the Laboratory Medicine in Västerbotten and it is responsible for all research sample collections and storage within the unit and that the material is available for each specialty in laboratory medicine. The BioBank North also promotes new collection and increase accessibility to existing tests.

3.8. Statistical analysis

All analyses were performed using SPSS (Statistical Pack for Social Sciences, Inc., Chicago, IL, USA). Descriptive statistics were provided as means with standard deviation (SD). Non-parametric two-tailed tests were used and p<0.05 was considered to indicate a statistically significant difference.
3.8.1 Study I
The Mann-Whitney u-test was used in Study I when correlating the patient ages between different subgroups to VHI scores, frequency of treatment sessions, gender, HPV subtype and onset of the disease. In order to assess how well the relationship between VHI questionnaire and the subscales of SF-36 could be described, we used the non-parametric measure of statistical dependence between two variables, the Spearman's rho correlation coefficient. The non-parametric Kolmogorov-Smirnov test was used to compare the VHI results with a reference (published normative values) probability distribution. The Wilcoxon signed-rank test was applied to compare the results of the SF-36 survey, since the normality assumption was not verified.

3.8.2 Study II
The population of northern Sweden as of 2000 was used for age standardization of tonsillar cancer over the period 1990-2013. To compare patient ages between different genders and HPV status (positive and negative), the Mann-Whitney u-test was performed. The Chi-square test was applied to determine the differences between HPV-status and gender.

3.8.3 Study III
The non-parametric Kolmogorov-Smirnov test was used to assure the normal distribution. An expected normal distribution justified the use of the Student’s t-test; if not a normal distribution was likely we used the non-parametric Wilcoxon signed-rank test. The limited subpopulation sizes at specific comparisons are urging caution in the statistical interpretation.

3.8.4 Study IV
For the calculation of p values, Chi²-test was used. For survival analysis, 2- and 5-year survival was used.

3.9 Ethical considerations
The study design was approved by the Regional Ethical Review Board of Umeå University (2012 276 32M, 2010 277 31, 08-003M and 03-201). To access the accurate retrospective paraffin-embedded samples from the BioBank North, we used data from the Swedish Cancer Registry database. The application was approved by the Biobank North (472-13-08 in 2013-03-26). In the prospective Studies I and III, written information was provided to all study participants. The studies were conducted in accordance with the Declaration of Helsinki (2014). (232) The ethical assurance for the retrospective Studies II and IV lies in approval from both the Regional Ethical Review Board of Umeå and the BioBank North.
4. Results

4.1. Study I
A total of 27 patients (82% response rate) completed the questionnaires. No significant differences were observed when comparing the patients included in the study with those excluded. Fifteen patients with RRP (56%) received less than one treatment session per year (low-frequency group – LF), while 12 patients (44%) had more than one or more operations per year (high-frequency group – HF).

In terms of voice quality and certain aspects of quality of life, the RRP group highly deviates from vocally healthy controls. Results are summarized in Table 7.

Table 7. Summary of findings in voice and quality of life aspects in RRP patients.

<table>
<thead>
<tr>
<th></th>
<th>VHI_{total}</th>
<th>SF 36 survey</th>
<th>VHI survey</th>
<th>VHI survey</th>
<th>VHI survey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Social</td>
<td>Voice</td>
<td>Minimal</td>
<td>Moderate</td>
</tr>
<tr>
<td>Social</td>
<td></td>
<td>functioning</td>
<td>dysfunction*</td>
<td>voice</td>
<td>handicap</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>handicap</td>
<td></td>
</tr>
<tr>
<td>RRP</td>
<td>p&lt;0.001 vs. normative value</td>
<td>p=0.029 vs. normative value</td>
<td>78%; n=21</td>
<td>41%; n=11</td>
<td>59%; n=16</td>
</tr>
</tbody>
</table>

*According to the normative value of Sw-VHI (voice dysfunction when VHI_{total} >20 points).

Patients that underwent more than one operation per year were younger (41 years vs. 51 years, p=0.028) than those treated less frequently. Females, patients with frequent surgical treatments and patients with the high-risk HPV types scored significantly lower in several domains of the quality of life assessment compared with normal subjects. The results are interpreted with caution due to the limited number of subjects. Two patients were positive for HPV16 genotype, both with histopathological squamous dysplasia, and one developed a malignancy with a T1bN0M0 classification.

4.2. Study II
There were 214 cases of tonsillar cancer identified, 155 (72.4%) men and 59 (27.6%) women. The total incidence of tonsillar cancer in northern Sweden (age-standardized to the population of northern Sweden as of 2000) doubled from 0.69/100 000 (year 1990) to 1.38/100 000 (year 2013). More specifically, a 2.7-fold increase (0.83 to 2.25 per 100 000) was noticed in men, while the female group showed only a small rise (from 0.46 to 0.48 per 100 000).
65 biopsy specimens obtained between 2000-2012 (median age 58 years, mean 59.3 years, range 45-87) were analysed; 48 (74%) belonged to males (median age 57.5 years, mean 57.6 years, range 45-74) and 17 (26%) were females (median age 65 years, mean 64.1 years, range 45-87). Female subjects were significantly older \((p=0.016)\). 91% of the specimens (59/65) were positive for HPV DNA and 62 (95%) expressed p16. Of the p16-positive samples, 7 (11%) received the highest score of 18 points, 49 (79%) a score of 12 (intermediate intensity) and 6 (10%) samples received between 2-6 points.

4.3. Study III

Twenty-seven of the 48 eligible patients in Study III were also analysed in Study I. The median duration of the disease was 7.2 years (men 8 years, females 6.5 years). A detailed outline of the resulting demographics is provided in Table 8, sample characteristics are presented in Table 9, prominent data are highlighted in bold lines. RRP patients with high surgical treatment frequency were significantly younger and had a more widespread laryngeal disease compared to a low-frequency treated group.

Table 8. Outline of resulting demographics of RRP in Study III.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number, n=48 (median age 44.5 years)</td>
<td></td>
</tr>
<tr>
<td>Females (median age 48 years)</td>
<td>14 (29%)</td>
</tr>
<tr>
<td>Males (median age 43.5 years)</td>
<td>34 (71%)</td>
</tr>
<tr>
<td>Juvenile-onset</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>Adult-onset</td>
<td>42 (88%)</td>
</tr>
<tr>
<td>HPV6</td>
<td>32 (67%)</td>
</tr>
<tr>
<td>HPV11</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>HPV16*</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>HPV31</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Not determined HPV subtype</td>
<td>8 (17%)</td>
</tr>
<tr>
<td>Glottic papilloma</td>
<td>48 (100%)</td>
</tr>
<tr>
<td>Supraglottic papilloma</td>
<td>7 (15%)</td>
</tr>
<tr>
<td>Subglottic papilloma</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Vocal fold web</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Cancer development</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

*One out of two patients developed malignancy, receiving radiotherapy.
Table 9. Sample characteristics of RRP in study III.

<table>
<thead>
<tr>
<th></th>
<th>High-frequency (HF)</th>
<th>Low-frequency (LF)</th>
<th>p-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Median 37 years</td>
<td>Median 46 years</td>
<td>0.04</td>
</tr>
<tr>
<td>Number of surgeries/year</td>
<td>Median 1.6</td>
<td>Median 0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Glottic papilloma (n=48)</td>
<td>17</td>
<td>31</td>
<td>NS</td>
</tr>
<tr>
<td>Supraglottic papilloma (n=7)</td>
<td>5</td>
<td>2</td>
<td>0.03</td>
</tr>
<tr>
<td>Subglottic papilloma (n=5)</td>
<td>5</td>
<td>0</td>
<td>0.02</td>
</tr>
<tr>
<td>Juvenile-onset (n=6)</td>
<td>3</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Adult-onset (n=42)</td>
<td>13</td>
<td>29</td>
<td>0.002</td>
</tr>
<tr>
<td>Web (n=5)</td>
<td>3</td>
<td>2</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS=non significant.

Adult-onset of the disease was more common in the LF group ($p=0.002$). Without scientific support for the efficacy of therapeutic vaccination, eight patients chose to be vaccinated, and seven of them belonged to the HF group. We observed a trend towards reduced necessity of surgery in the vaccinated subgroup of patients. Figure 9 reflects this conclusion by demonstrating the benefit of the 3-step vaccination process (Gardasil®) in one single individual patient.
4.4. Study IV

109 patients with TSCC were eligible for participation in the study; 54 were men and 55 were women (mean age 63.5 years, range 19-93 years). The sample characteristics are presented in Table 10. Two-thirds of the samples were obtained from the lateral border of the mobile tongue, 20% from the ventral side and 2% from the dorsal side. Lower survival and disease-free rate was noticed in patients in the young age group (≤40 years) compared with the older (<65 years) patients.

Table 10. Sample characteristics in mobile tongue cancer, Study IV.

<table>
<thead>
<tr>
<th>Age at diagnosis (years)</th>
<th>Number</th>
<th>Male/female ratio</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>N0</th>
<th>N+</th>
<th>M0</th>
<th>M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤40</td>
<td>16</td>
<td>7/9</td>
<td>2</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>4</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1,3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41-65</td>
<td>38</td>
<td>26/12</td>
<td>14</td>
<td>11</td>
<td>9</td>
<td>4</td>
<td>27</td>
<td>11</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,2:1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;65</td>
<td>55</td>
<td>21/34</td>
<td>15</td>
<td>19</td>
<td>8</td>
<td>13</td>
<td>41</td>
<td>14</td>
<td>54</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1,6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
<td>31</td>
<td>40</td>
<td>19</td>
<td>19</td>
<td>80</td>
<td>29</td>
<td>107</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

M: distant metastasis; N: nodal metastasis; T: tumor size.
67% of the tumor samples were negative for the expression of p16 marker, independent of site of lesion. HPV was not detected in any patients. All samples analysed for syndecan-1 expression were positive while there was no correlation between p16, syndecan-1 expression or HPV in mobile tongue cancer.
5. Discussion

5.1 Specific aims and findings
This thesis aims to increase our understanding of HPV in recurrent respiratory papillomatosis (RRP), tonsillar cancer, and mobile tongue cancer. These are diseases with care-intensive treatments, sometimes life-long treatment courses, and in most cases, major functional complications affecting voice, speech, swallowing and quality of life. We want to provide a scientific basis for an introduction of gender-neutral vaccination, by studying patients with recurrent respiratory papillomatosis and the longitudinal increasing development of HPV-related tonsillar cancer in northern Sweden.

5.1.1 Impact of HPV in malignant and benign lesions
RRP and tonsillar cancer are HPV-related in contrast to cancer in the mobile (oral) tongue. The HPV-related airway disease affects mainly young/mature men and involves mainly the genotypes HPV6 and HPV16. There is a correlation between HPV and its surrogate marker p16 in tonsillar cancer but not in the HPV-negative mobile tongue cancer cases.

In Study II, we noted a gender-neutral 2.7-fold increase in the incidence of HPV-related tonsillar cancer cases in males between 1990-2013. This is in accordance with results of previous studies that suggested a global increase of the incidence of HPV-positive tonsillar cancer, preferentially in males. (153, 233-235)

Recent changes in sexual patterns, such as increasing numbers of sex partners, practice of oral (mainly) or vaginal sex, and early debut of sexual activity have been proposed as possible explanations for the increase in HPV-positive tonsillar cancer. (236) The increase of HPV-related tonsillar cancer cases in men is unclear, but we propose that there is a possible gender difference in sexual-, alcohol- and smoking behaviour. The hypothesis has found scientific support in previous studies reporting a connection between reduced clearance of oral HPV in men associated to heavier tobacco and alcohol use. (237) Females seem to have a stronger immune response due to exposure to genital HPV earlier in life. (237) Furthermore, HPV has been suggested to be more easily transmitted from women to men than the opposite way during sex, though why this might be is unknown. (238)

The majority (95%) of the tonsillar tumor specimens indicated an overexpression of the protein marker p16, consistent with previous findings. (162, 239) Thus, p16 can be used as a surrogate marker and a prognostic guide in specific HPV-related malignancies. The lack of correlation between p16 and HPV in mobile tongue cancer shown in Study IV indicates that HPV-induced squamous cell cancer is a
site-specific disorder in the upper aerodigestive tract. This is in contrast to Begum and Gillison (2003) suggesting that p16 could be used as a marker to determine whether cervical lymph node metastasis originates from the oropharynx, since they found a credible correlation between p16 and HPV. (240) The HPV-related tonsillar malignancies have shown a better prognosis regarding overall survival and locoregional control compared to HPV-negative cases. (241, 242) De-escalation of the aggressive treatment offered to HPV-positive tonsillar cancer cases has therefore been proposed, in order to preserve organ functionality. (243, 244) It is important to note that improved survival rates of HPV-infected tonsillar cancer cases have not been observed in patients diagnosed with HPV malignancies originating from other sites of the head and neck. (245)

In Study III, a cohort of RRP patients recruited from the northern Sweden catchment area was studied prospectively. The predominance of males in the RRP disease is consistent with the gender profile of tonsillar cancer, a fact that needs to be addressed in light of the current vaccination strategy. The division of patients into low-frequency (<1 treatment sessions/year) and high-frequency groups (≥1 treatment sessions/year) was applied to help elucidate an aggressive disease profile characterized by more widespread airway lesions and a higher frequency of treatment sessions. In contrast to some previous reports, no subpopulation was identified with an aggressive course related to juvenile-onset and HPV11. (100) This conflict in outcome could be explained by the small numbers of study patients and thus, reduced reliability. HPV colonisation in the oral mucosa is much higher in AoRRP than JoRRP with a prevalence of less than 7%. (193) This could also indicate different transmission routes depending on age. (51)

As previously reported, HPV-positive tonsillar carcinomas are currently considered to be clinically and biologically different tumor entities as compared to HPV-negative cases. (160) The role of HPV in development of laryngeal cancer has not been definitely established. However, referring to a population-based cancer registry study, HPV may be involved in the development of a subset of laryngeal cancers, especially in females. (60) The HPV16 genotype is undoubtedly considered a high-risk virus linked to cancer development in the airway, and has been identified in 4% of our RRP patients. Today, the existence of HPV infection in laryngeal cancer does not affect the current treatment regime.

Treatment of RRP currently relies on surgery and aims for symptom reduction since curative interventions are still lacking. In light of the fact that we are not able to offer curative treatment options for RRP, we have identified clinical guidelines to focus on particularly care of specific high-risk RRP patients (Study III) and moreover, to understand the impact of the disease regarding patients’ quality of life, social network and affected ability to work full time in voice-profiled professions.
In contrast to Studies I, II and III, HPV could not be detected in mobile tongue cancer (TSCC) (Study IV), despite the use of the highly sensitive in situ hybridization. This finding could either indicate that HPV is absent in TSCC, or present at extremely low levels; despite this, it could be possible that other high-risk subtypes of the HPV virus could be present. We do not believe that cancer of mobile tongue is HPV-induced, supported by low numbers of HPV infection in normal oral mucosa (246) and the outcomes of previous studies. (247, 248) It seems unlikely that HPV constitutes a significant factor in the pathogenesis and rising trends of TSCC in the young population in contrast to the geographically adjacent tonsillar cancer cases; the HPV virus simply prefer tonsils.

5.1.2 VHI and SF 36
RRP patients have been reported to have inferior health-related quality of life and voice deterioration compared to non-RRP matched data. (249) VHI has been considered as an appropriate instrument to validate vocal impairment. It is designed to measure the effects of social and psychological damage due to voice disorders. (222, 250) SF-36 was employed since it is a widely accepted instrument to assess daily activities and measure several aspects of the quality of life. (251)

In Study I, age and the predominance of men were strongly correlated with the frequency of treatment results in accordance with other RRP studies. (99) 11% of patients in Study I exhibited the high-risk subtypes 16 and 31. These patients reported a significant deterioration regarding several aspects of health-related life quality compared to the majority of the patients with the low-risk subtypes 6 and 11. One of the patients with HPV16 converted to malignancy, highly affecting the quality of life and voice standards in the small subgroup analysed. Another gender disparity was that females reported lower scores in different subscales of the SF-36 questionnaire, revealing an overall deterioration of life quality. These outcomes have to be analysed in the light of low numbers of females of which one developed a malignancy, highly affecting the quality of life. However our results are not contradicted by previous reports. (252)

The correlation between voice disorders and quality of life, and more specifically social functioning, is reflected in Study I where significantly lower values were obtained in RRP patients as compared to the normal population. Similar findings were established by other researchers. (249, 253) The impact of voice regarding the quality of life is more extendedly studied in AoRRP compared to JoRRP; the latter was characterized by a quality of life similar to that of children with other chronic diseases. (254) These findings are in line with those in Study I, where significant
impairment of voice quality was shown among small number of juvenile-onset RRP patients.

The majority of RRP patients experienced voice deterioration. Moderate to severe dysfunction was common in the RRP patients, while 22% reported normal voice quality. The perception of normal voice quality is complex, but may be explained by a low vocal burden of the disease affecting the vocal cords, patient's expectation effect, low active RRP disease profile, voice quality in relation to voice load in daily life, and of course the effect of small-sized population analysed, undermining the reliability. The heterogeneous voice outcome in RRP patients is supported by Ilmarinen et al. (255) The scientific diversity is consistent when studying voice outcome and quality of life in the RRP patients. We were unable to detect any association between the frequency of surgical treatments and voice quality. This was in agreement with Lehto et al., reporting that age but not number of procedures correlated with poor voice quality among patients. (256) However, Ilmarinen et al., reported a clear-cut connection between high number of laryngeal procedures and deterioration of voice quality, (255) Lehto et al. concluded that voice quality in RRP patients is not significantly worse than it is in healthy control subjects, even if parameters like roughness and breathiness were observed in the perceptual assessment. (256) This is a reasonable conclusion since the RRP disease and the voice quality over time will be integrated as a normal state of being and sounding for the individual and its social context. This diversity highlights the need for studies with larger sample sizes and higher statistic power to detect consisting reproducible results.

5.1.3 Importance of p16 and syndecan-1

In Study II, we observed an overexpression of protein marker p16 in the tonsillar cancer specimens, demonstrating a strong association between p16 and HPV infection in tonsillar carcinomas and the use of p16 as a prognostic guide. In Study IV, p16 was expressed in some cases of mobile tongue cancer (TSCC), however HPV was undetectable. Therefore, p16 should not be used as a reliable surrogate marker for high-risk HPV infection in mobile tongue cancer, despite the presence of the HPV-receptor syndecan-1. HPV simply prefers the tonsillar environment. Lack of p16 is associated with worse prognosis primarily in young patients with TSCC. (257) Molecular alterations in the p16 pathway, independent of HPV infection, could explain the p16 overexpression. Alternatively, the presence p16 in HNSCC could be the truly important prognostic marker, independent of HPV status, and could alone constitute an important prognostic marker in HNSCC. Some researchers support the argument of using p16 than direct HPV-testing in tonsillar carcinomas. (258)
Expression of the HPV receptor syndecan-1 did not exhibit any difference between the TSCC group and tonsillar cancer (Studies II and IV). The initial binding of HPV virus to the cellular surface of both tongue and tonsillar tissues could theoretically take place in a similar way. However, as 91% of the tonsillar cancer cases were HPV-positive, we could conclude that the virus presents with a preference for tonsillar environment. We assume that this tonsillar predilection of the virus could enlighten the value of co-infection with other viruses such as HSV and EBV. (259, 260) There are conflicting studies concerning the expression of syndecan-1’s role in a tumor recurrence and tumor-specific death in oral carcinomas. (261, 262)

5.1.4 HPV and vaccines
The clinical utility of HPV vaccination as a prophylactic measure for cervical cancer in women is well established. Our hope is that through the on-going vaccination strategy towards cervix cancer we will end up with a reduced incidence of tonsillar cancer and RRP. (89) The incidence rates of tonsillar cancer are rising worldwide. We can probably expect a further increase of HPV-related tonsillar cancer in parallel with a further decline of cervical cancer due to preventive HPV vaccination in females. (263)

HPV vaccines constitute a great opportunity to potentially prevent tonsillar cancer in future generations. In light of the growing incidence of male HPV-related tonsillar cancer and the predominance of males in RRP, the argument for using HPV vaccines in boys becomes more forceful. A study group has conducted a ‘Safety Study of HPV DNA Vaccine to Treat Head and Neck Cancer Patients’. The study was completed in April 2015 but no results have been published to date. (264) Previous studies have reported that men will benefit directly from vaccination of girls, but men remain at an unfortunately high risk of developing HPV-related cancer. (89) The efficacy of HPV vaccine against oral HPV infection has been studied by Herrero et al., reporting a 93,3% reduction in the rate of oral HPV16/18 infection. (265)

The outcome of the vaccine-preventable RRP burden has not been thoroughly studied. Freed and Derkay (2006) stated the expected positive outcome on reduced incidence of RRP in relation to vaccination with Gardasil® vaccine. (137) A first attempt to take advantage of the preventive role of HPV vaccination against RRP on a case level was published by Förster et al. using Gardasil® in order to reduce the aggressive course of RRP in a 2-year old boy. Successfully, the disease became stable after the third immunization and no surgical treatment was necessary for the following ten-month period. (266) Hocevar-Boltežar et al. studied the efficacy of Gardasil® vaccine in a population of 11 patients (aged 13-46 years) with an aggressive RRP course. The results showed extended treatment intervals and consequently, a reduction of necessary surgical sessions. (138) One of the patients in
that study showed complete remission of the disease after vaccination. The effectiveness of vaccination with increased treatment intervals and lower treatment needs has been reported in small-scale studies. (91, 133, 139)

In Studies I and III, we identified a more care-intensive subgroup of RRP patients. In light of the on-going therapeutic vaccination evaluation, we advocate an initial selection of patients for HPV vaccination based on male gender, younger age, more widespread airway disease, and oncogenic HPV genotypes (Studies I and III). Eight of our RRP patients in Study III chose to receive HPV vaccine, and seven of them belonged to the high-frequency group. We observed a trend towards less surgical treatment in this small cohort, results consistent with previous reports. (91, 133, 137-139, 266)

In Study II, we reported a 2.7-fold incidence increase of tonsillar cancer in male patients. In northern Sweden, a male incidence polarization was also obvious in our RRP material (Study III). Therefore, it could be beneficial to evaluate the efficacy of HPV vaccination as a prophylactic measure against both tonsillar cancer and RRP in the male population.

Finally, we briefly want to address awareness of the difficulties with small size studies. A study with low statistical power has an obvious reduced chance of detecting a true effect. The consequence of this is a tendency to overestimate effect size and a low reproducibility of results. There are also ethical dimensions of the problems with small population studies such as unreliable reproducibility as a major methodological principle.

5.2 Clinical implications
RRP patients experience a significant voice dysfunction, affecting quality of life as well as social habits and working capacity. Measuring the influence of RRP on voice and quality of life offers the possibility to identify valuable prognostic factors of the RRP disease. The frequency of RRP surgical interventions, age at onset, gender, and subtype of the HPV may be used as factors to predict voice disability. Patients with the high-risk HPV genotypes are especially vulnerable. The high-risk HPV genotypes do not seem to affect their treatment frequency, but their perception of reduced quality of life and voice.

We have identified a more care-intensive RRP subgroup defined by frequent need of treatment sessions, younger age, male gender and more widespread disease in the airway. This subgroup is not primarily associated with JoRRP or HPV11. The study outcomes can be used in clinical practice in order to inform the RRP patients about the disease upon diagnosis, predict the course of RRP based on clinical characteristics, and become a basis for vaccination discussion.
The increasing trend in the incidence of tonsillar cancer in northern Sweden by 2.7 fold in men, as well a worldwide increase of increase of HPV-positive tonsillar cancer cases, highlights the need of clinically applicable simple tests to detect HPV-related malignant tonsillar disease. There is a strong association between p16 and HPV infection in tonsillar cancer. The overexpression of p16 is highly suggestive of HPV infection in tonsillar cancer, and can be used in clinical practice as a surrogate marker and a prognostic guide of tonsillar cancer but not in mobile tongue cancer. HPV was undetectable in mobile tongue cancer while p16 is expressed in a few cases. Therefore, p16 is not a reliable surrogate marker for HPV infection in mobile tongue cancer. It may serve alone as a prognostic marker, since the lack of p16 is associated with worse prognosis.

This thesis has contributed scientifically with simple tools to detect HPV in tonsillar cancer. It has also presented findings supporting a gender-neutral vaccination in defined RRP subgroups and in tonsillar cancer, but not in mobile tongue cancer.

5.3 Limitations
This thesis was limited by several factors. Firstly, although all patients with RRP in the catch-up area were eligible, the general number of patients was low. This was even more noticeable when statistically analysing the high-risk HPV subtypes, supra- and subglottic HPV distribution and juvenile-onset. There were not enough observations to support confident conclusions. Secondly, an important limitation, but also an asset which applies to Studies I, II and III, is the fact that our patient material was restricted geographically in the area of northern Sweden.

Thirdly, our patient material in Study I was compared to the general, historical Swedish normative values for VHI and SF-36, where no data on the variation of the normative values was available over time. Additionally, the patients’ vocal ability was not evaluated pre-operatively, for comparison purposes. A multimodal assessment would have been desirable. Due to the care-intensive life conditions, and in order to reduce the possibility of discontinuation to participate, we wanted to minimize the documentation as much as possible. This was balanced with the need to preserve validity in terms of the relevant level of detail for the registrations performed.

Finally, there was a lack of a comparable cohort including patients from different regions and assessing subject characteristics at specific time points of their disease. This can be considered an additional limitation in all studies, except Study IV where patients from another country (Italy) were recruited.
5.4 Future perspectives

In order to increase the statistical power, the true effect, the reproducibility of the results and the conclusions of this thesis, further long-term multicentre studies with larger intervention groups are required. In light of the reported efficacy of the HPV vaccine, we could study the effects of vaccination in selected RRP patients longitudinally and in a multicentre design, with effects on surgical intervals and local aggressiveness of RRP lesions. A further purpose with an expanded study design is to investigate the routes of HPV transmission in the airway and the mechanism of mucosal RRP lesion penetration in the larynx of RRP patients.

Additionally, it would be interesting to study patient cohorts with nasal inverted papillomas, given the lack of knowledge around the cause of the disease. It is relevant to understand why, despite its benign histopathology, this disease entity has a malignant clinical course. If we could establish that HPV is present in inverted nasal papilloma then this could have impact on vaccination and affect the protective environment in the operation theatre. Moreover, it would be interesting to study the presence of HPV and other possible viral co-infecting agents in non-malignant tonsils, since we are facing a major increase in HPV-positive tonsillar cancer. This would potentially help to understand the relation between the “time of infection” and the “onset of malignant tonsillar disease”. Finally, high-quality, randomized control trials could be initiated in order to address the most effective treatment methods for both HPV-positive and HPV-negative tonsillar cancer patients.
6. Conclusions

- The frequency of operations, age at onset, gender and genotype of human papillomavirus (HPV) may be used as factors to predict voice disability in patients with recurrent respiratory papillomatosis (RRP). The majority of the patients with RRP experience a significant self-reported voice dysfunction and in specific domains, a clear-cut significant impact on quality of life. A subgroup of RRP patients represented by low age, females, high-risk HPV genotype and high frequency of treatment sessions seems to be more vulnerable for morbidity in terms of quality of voice and life.

- There is a parallel increase in the incidence of tonsillar cancer, HPV infections and expression of p16 among patients from northern Sweden, confirming the strong association between p16, HPV infection and tonsillar cancer.

- RRP patients with high surgical treatment frequency are younger men, infected with HPV6 and have a widespread laryngeal disease. This indicates a clinical subgroup of RRP patients, not primarily related to HPV subtype, but to a more care-intensive course. The majority of RRP patients in northern Sweden are men, with an adult onset and infected with HPV6.

- HPV is undetectable in mobile tongue cancer; p16 is expressed occasionally, indicating poor correlation between HPV and p16 in tongue cancer. p16 is not a reliable surrogate marker for HPV infection in mobile tongue cancer but may serve alone as a prognostic marker since the lack of p16 is associated with worse prognosis in younger patients (≤40 years).
7. Acknowledgments

The completion of my PhD thesis has been a long journey. I would like to express my sincere gratitude to everyone that has helped, encouraged and supported me on my scientific journey during the last four years.

In particular, I would like to thank:

Katarina Olofsson, my main supervisor, for your gentle and positive academic and scientific guidance. Many thanks for your friendship, support and encouragement in times of doubt, for your great experience in research and for teaching me what research is all about: to understand how things really work. You have been a steady influence throughout my thesis; you have oriented and supported me with promptness and care. Thank you for believing in me and your help on generating and managing the projects of this thesis. Without you, nothing of all these could be accomplished.

My co-supervisors, Karin Nylander, David Lindquist and Göran Laurell. Thank you for your scientific skills and your wide knowledge, your patience during our constructive and productive meetings and discussions. Big thanks for helping me solving problems every time a problem showed up.

Charlotte Öfverman and Kristina Stefansson, for all your excellent work and assistance. Thank you for all your help with discussing different lab methods and other interesting issues. Your help in putting numbers into words has been invaluable, many thanks!

Andreas Arvidsson, dear friend and colleague. Thanks for your help, knowledge and wise comments, despite a high work load in the clinic.

Anna Holm, colleague and co-author. Many thanks for being a patient co-researcher and for boosting the project with your positive energy and everlasting enthusiasm. Good luck with your thesis!

Professor Michael Haney. Thank you for the critical analysis and comments on the linguistic aspects of the thesis.

Anders Lundquist, for your important statistical advices and encouragement.

My gratitude is also extended to all the colleagues and friends at the ENT department, University Hospital of Umeå and especially, my ‘roommate’
Mohammad. Thank you for a great working atmosphere, stimulating help and professional attitude. You made work such a nice place for me.

Our fantastic patients, for their patience and willingness to participate in these studies.

Costas and Marios, my fellow countrymen and great friends in Umeå. Big thanks for your friendship, support and endless optimism and for cheering me up with your company and jokes all these years.

My parents for their support and everlasting love; I am eternally grateful for everything you have done for me all these years! You have instilled many admirable qualities in me and given me a good foundation with which to meet life. You’ve taught me about hard work and self-respect, about persistence and about how to be independent.

My siblings, Marilena and Louis. Thank you for your infinite support throughout everything.

Many thanks to all the other members of my big, fantastic family.

My two little angels, Elin and Alexander. Thank you for your encouragement through looking at your beautiful smiles. You’re all the bright stars in my sky, the music in my life, the air I breathe!

Last but not least, my wife and partner in life, Linda. Thank you for your energy, love and support. For sacrificing your time in taking care of our children all those days and nights I was away preparing this PhD thesis. Through your patience and unwavering belief in me, I’ve been able to complete this long dissertation journey. You are my biggest fan and supporter. I am endlessly blessed for you being in my life!

This thesis was supported by grants from Lion’s Cancer Research Foundation at Umeå University, CFF, LP12-1945, CFF LP13-1998, CFF, LP14-2048 and CFF AMP 15-772 and Umeå University Medical Faculty, Sweden.
8. References


46. Schraff S, Derkay CS, Burke B, Lawson L. American Society of Pediatric Otolaryngology members' experience with recurrent respiratory


major histocompatibility complex I-related chain A (MICA) protein and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions. BMC Cancer. 2008;8:16.


230. Letsolo BT, Faust H, Ekblad L, Wennerberg J, Forslund O. Establishment and characterization of a human papillomavirus type 16-


