HUMAN PAPILLOMAVIRUS INFECTION IN HEALTHY YOUTH AND IN HYPOPHARYNGEAL CANCER

Nathalie Hou Grün

Stockholm 2016
Human Papillomavirus infection in healthy youth and in hypopharyngeal cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Nathalie Hou Grün

Principal Supervisor:  
Prof. Tina Dalianis  
Karolinska Institutet  
Department of Oncology-Pathology

Opponent:  
Prof. Peter L Stern  
University of Manchester  
Paterson Institute for Cancer Research

Co-supervisor(s):  
Associate Prof. Torbjörn Ramqvist  
Karolinska Institutet  
Department of Oncology-Pathology

Examination Board:  
Prof. Sonia Andersson  
Karolinska Institutet  
Department of Women and Children’s Health

MD, PhD Anders Näsman  
Karolinska Institutet  
Department of Oncology-Pathology

Adjunct Prof. Ingrid Uhnoo  
Uppsala University  
Department of Medical Sciences  
Division of Infectious Diseases

Associate Prof. Lars Sand  
Uppsala University  
Department of Surgical Sciences  
Division of Oral & Maxillofacial Surgery
In the hope of a better, brighter future;
This is for my sister Nea. The world is yours.
ABSTRACT

The aim of this thesis was to follow the prevalence of human papillomavirus in the oral cavity and in the cervix in youth during the period of 2008-2015, a period when HPV vaccination was gradually introduced to young girls in Sweden. In addition, we explored the prevalence of HPV in hypopharyngeal cancer during the period 2000-2012 on the basis that there has been an epidemic of HPV positive tonsillar and base of tongue cancer, which arise in locations with close physiological proximity to the hypopharynx.

The main questions addressed were whether HPV prevalence is similar within different cohorts of Swedish youth, how the prevalence changes over time and after the introduction of the HPV vaccines, and if the increased proportion of HPV positive oropharyngeal cancer is mirrored also in hypopharyngeal cancer. Since the prevalence of oral and genital HPV previously observed at a Stockholm youth clinic was high during the period of 2008-2011, there was an opportunity to compare these data to the HPV prevalence in different geographical locations and at other time points. The strong Swedish tradition of biobanking granted access to a relatively large sample of hypopharyngeal cancers, and the high prevalence of HPV in oropharyngeal cancers in Sweden made it credible that HPV would also be present at detectable levels in hypopharyngeal cancer and make it possible to detect changes occurring in the HPV prevalence in this cancer type.

To investigate these matters, oral and cervical samples were analyzed for the presence of HPV DNA, a questionnaire was used to investigate the sexual experiences of youth, and HPV DNA and p16 expression was analyzed in relation to survival in samples of hypopharyngeal cancer.

In Paper I, we could show that oral HPV prevalence was significantly less common in high school students from a middle sized municipality in Sweden (1.8%) than what was observed in 2009-2011 in the Stockholm youth clinic (9.3%).

In Paper II, we could show that there were no differences between HPV vaccinated and non-vaccinated women regarding condom use and self-reported STI history, however, vaccinated women were more likely to have had vaginal intercourse and one-night stands (p=0.005, and p=0.046, respectively).

In Paper III, we found a low oral HPV prevalence also at the Stockholm youth clinic (1.4%) in 2013-2014 which was lower than what was previously observed at the same clinic (p=0.00001). Cervical HPV 16, 31 and 70 prevalence was now less common in vaccinated than in non-vaccinated individuals (p=0.0002, p=0.019, and p=0.006, respectively).

In Paper IV, we expanded the cohort from paper 3 to also include samples from the fall of 2014 and the spring of 2015. Oral HPV prevalence remained low (1.5%) and cervical HPV 16, 31 and 6 were less common in vaccinated than in non-vaccinated women (p=0.0006, p=0.038 and p=0.009, respectively).

In Paper V, we could show that the proportion of HPV positive cases of hypopharyngeal cancer have not increased in Stockholm and that p16 is a poor surrogate marker of active HPV infection in this cancer type.
LIST OF SCIENTIFIC PAPERS


LIST OF RELATED PUBLICATIONS


# CONTENTS

1 Introduction .................................................................................................................. 1

1.1 What is cancer? ........................................................................................................ 1

1.2 The Emergence Tumor Virology ........................................................................... 1

1.3 History of the human papillomavirus .................................................................... 1

1.4 HPV structure and proteins ................................................................................. 2

  1.4.1 Early proteins ..................................................................................................... 2

  1.4.2 Late proteins .................................................................................................... 5

1.5 HPV life cycle ........................................................................................................ 6

1.6 Persistence and clearance of HPV infections ....................................................... 6

1.7 HPV in disease ....................................................................................................... 7

  1.7.1 HPV-associated cancers .................................................................................... 7

1.8 HPV in other conditions ....................................................................................... 12

1.9 HPV epidemiology .................................................................................................. 13

1.10 HPV detection methods ...................................................................................... 14

  1.10.1 Sampling methods and sample types ............................................................... 14

  1.10.2 Direct and indirect detection of presence of HPV .......................................... 14

1.11 HPV vaccination .................................................................................................... 15

  1.11.1 Prophylactic HPV vaccination ....................................................................... 15

  1.11.2 Therapeutic HPV vaccination ....................................................................... 16

  1.11.3 Vaccine controversy ...................................................................................... 17

  1.11.4 Vaccine safety and efficacy ......................................................................... 17

  1.11.5 Upscaling of vaccination programs .............................................................. 18

1.12 Sexual habits in youth .......................................................................................... 19

1.13 Sexually transmitted infections and reproductive health in youth ................. 20

2 Aims .......................................................................................................................... 21

3 Material and Methods .............................................................................................. 22
3.1.1 Paper I ................................................................. 22
3.1.2 Paper II ................................................................. 22
3.1.3 Paper III ................................................................. 22
3.1.4 Paper IV ................................................................. 22
3.1.5 Paper V ................................................................. 23
3.2 DNA extraction ......................................................... 23
3.3 HPV detection .......................................................... 23
3.4 Immunohistochemistry .............................................. 23
3.5 Questionnaire .......................................................... 24
3.6 Statistical analysis ...................................................... 24
4 Results and Discussion .................................................. 25
  4.1.1 Paper I ................................................................. 25
  4.1.2 Paper II ................................................................. 26
  4.1.3 Paper III ............................................................... 26
  4.1.4 Paper IV ............................................................... 27
  4.1.5 Paper V ............................................................... 30
5 Summary and Conclusions ............................................. 31
6 Acknowledgements ...................................................... 32
7 References .................................................................. 35
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APM</td>
<td>Antigen Processing Machinery</td>
</tr>
<tr>
<td>ASC-US</td>
<td>Atypical Squamous Cells of Undetermined Significance</td>
</tr>
<tr>
<td>BOTSCC</td>
<td>Base of Tongue Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>BPV</td>
<td>Bovine Papillomavirus</td>
</tr>
<tr>
<td>CC</td>
<td>Cervical Cancer</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>CKC</td>
<td>Cold Knife Conization</td>
</tr>
<tr>
<td>E6AP</td>
<td>E6-Associated Protein</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EV</td>
<td>Epidermodysplasia Verruciformis</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FEH</td>
<td>Focal Epithelial Hyperdysplasia</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin Fixed Paraffin Embedded</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HR</td>
<td>High-Risk</td>
</tr>
<tr>
<td>HSIL</td>
<td>High-grade Squamous Intraepithelial Lesions</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
</tbody>
</table>
ISH  
**In Situ** Hybridization

LCR  
Long Control Region

LR  
Low-Risk

LSIL  
Low-grade Squamous Intraepithelial Lesions

MHC  
Major Histocompatibility Complex

MSM  
Men who have Sex with Men

OC  
Oral Contraceptive

OPSCC  
Oropharyngeal Cancer

ORF  
Open Reading Frame

Pap  
Papanicolaou

POTS  
Postural Orthostatic Tachycardia Syndrome

RCT  
Randomized Control Trial

rpm  
Rotations per Minute

RRP  
Recurrent Respiratory Papillomatosis

RSV  
Rous Sarcoma Virus

STI  
Sexually Transmitted Infection

TSCC  
Tonsillar Squamous Cell Carcinoma

US  
United States of America

VIA  
Visual Inspection with Acetic Acid

VLP  
Virus-Like Particles
1 INTRODUCTION

1.1 WHAT IS CANCER?

Today, cancer is one of the most common causes of death worldwide and was estimated to have caused approximately 8.2 million human deaths in 2012 and, over a lifetime; more than one in three will be diagnosed with cancer\textsuperscript{1,2}. As a consequence, the search for a cure for cancer has been a priority for the scientific community for decades, and in 1971, US President Richard Nixon signed the National Cancer Act; officially declaring a war on cancer\textsuperscript{3}. Yet, in 2016 we remain far from finding the final solution to the cancer issue.

Why is it then that we have not come further? One critical point to be made is that cancer is not one disease; rather, it’s a number of diseases that share the characteristics of uncontrolled cellular division and the ability of the cells to expand beyond the boundaries of their original compartment. Most cell types in the body can indeed give rise to cancer, resulting in a vast diversity in clinical presentation, as well as in prognosis for the person affected. As such, the causes and potential treatment options for cancer diseases are widely disperse and thus, a large amount of branches have emerged within the field of cancer research. One such field is the field of tumor virology.

1.2 THE EMERGENCE TUMOR VIROLOGY

While the germ theory of disease was first proposed in the mid-16\textsuperscript{th} century, the idea that infections might also be a potential cause of cancer was foreign to most researchers. Following the famous experiments by Francis Peyton Rous, showing that filtered extracts from chicken tumors were indeed transferrable, this perception slowly began to change\textsuperscript{4}. The subsequent discovery of what became known as the Rous sarcoma virus (RSV) was to be the first of many tumor causing viruses discovered in the following century.

The identification of the first virus linked to human cancer was made in the 1960’s and can be seen as a direct result of the fortunate meeting between the pathologist Michael Anthony Epstein and the surgeon Denis Parsons Burkitt\textsuperscript{5}. The latter had recently described an endemic form of lymphoma in children (Burkitt’s lymphoma) predominantly found in equatorial Africa and it was soon established that the tumors contained large quantities of viral particles. The virus was named Epstein-Barr virus (EBV) after the researchers who discovered it; Michael Anthony Epstein and Yvonne Barr\textsuperscript{6}. The transforming capabilities of the virus were later confirmed experimentally by, among others, Gertrude Henle and Harald zur Hausen. Since then several viruses such as human papillomavirus (HPV) hepatitis B, hepatitis C, human T-cell leukemia virus 1 and Merkel Cell polyomavirus have been demonstrated to be involved in human cancer, and the focus of this thesis is on the first of these; the HPV family\textsuperscript{7-10}.

1.3 HISTORY OF THE HUMAN PAPILLOMAVIRUS

The essential work on Epstein-Barr virus was far from the last contribution Harald zur Hausen would make to the field of tumor virology. His major field of interest, however, afterwards changed to another virus family, HPV.
Already back in 1949, Maurice J Strauss and colleagues had discovered the presence of virus-like particles (VLPs) in skin papillomas\textsuperscript{11}. At that time, the importance of these particles was to remain a mystery yet for some decades.

By the mid 1980’s, zur Hausen and colleagues had discovered that Human Papillomavirus (HPV) types 16 and 18 are prevalent in cervical cancer\textsuperscript{12–14}. Although this was not the first finding of an oncogenic virus in humans, the discovery was still ground breaking in its own right. As it turns out, HPV 16 and 18 together cause approximately 75\% of all cervical cancer (CC), the fourth most common type of cancer in women\textsuperscript{1,15}.

1.4 HPV STRUCTURE AND PROTEINS

While previously classified as a part of the family \textit{Papovaviridae} together with the polyomaviruses and SV40, the papillomaviruses are today recognized as constituting their own viral family; \textit{Papillomaviridae}\textsuperscript{16}. Within the \textit{Papillomaviridae} family there are a number of genera, indicated by Greek letters, which are then further subdivided into species and, finally, types. To date, there are 202 confirmed HPV types and new ones are being described at an increasing rate since the development of metagenomic sequencing\textsuperscript{17,18}. While all HPVs are epitheliotropic, some preferentially infect cutaneous epithelia, and others are mainly found in mucosal epithelia. Depending on their oncogenic capabilities, the HPV types are classified as either low-risk (LR) or high-risk (HR), with some types being assigned as putative, or potential, HR due to a lack of conclusive evidence of their oncogenicity.

All HPVs have a \textasciitilde{}8kb double stranded DNA genome with a low degree of genomic diversity within species and a low mutation rate. The current classification system of HPV is based on sequence relatedness of the L1 gene\textsuperscript{16}.

The HPV genome is frequently described as consisting of three major regions; the non-coding long control region (LCR), the early region, and the late region, encoding the early and late proteins respectively\textsuperscript{19,20}.

All of the early proteins possess more than one function during infection and HPV induced transformation, and to describe all functions exerted by these proteins would make a book in its own right. Below, some of the main functions of the HPV early proteins have been summarized.

1.4.1 Early proteins

The early region of HPVs has eight open reading frames (ORFs) that, often, produce the proteins E1, E2 and E4-E7. While the E3 gene, is not known to code for a protein, the E8 gene forms a fusion protein with E2 that seems to be repressing viral transcription\textsuperscript{21}. Here follows a summary of the functions of the major HPV early gene transcripts.
1.4.1.1 E1 and E2

The HPV E1 and E2 proteins are expressed early in the viral lifecycle and are necessary for the virus to integrate into the host genome. After viral integration, one or both of the genes may be lost. E2 loss in particular has been associated with high-grade cervical lesions, making it a commonly used marker of viral integration. It has also been shown that loss of E2 is related to a worse clinical outcome in several types of cancer. This has been shown to be the case for both cervical cancer, and more recently, tonsillar and base of tongue cancer, although evidence for the latter remain inconclusive.

1.4.1.2 E4

E4 is a protein that is highly prevalent during latter stages of HPV infection and coincides with vegetative viral genome amplification and similarities in E4 intracellular localization suggest an evolitional functional retention. The protein is expressed as an E1^E4 fusion protein that is involved in the sequestration of viral protein, the transcript may also be involved in the regulation of E2 expression.

1.4.1.3 E5, E6 and E7

The proteins E5, E6 and E7 are the HPV products with the most well-documented oncogenic activity in HR HPV types. These proteins often exhibit different function or binding capabilities in LR HPVs, as compared to HR HPVs.

1.4.1.4 E5

E5 is the smallest of the oncoproteins and several different subtypes exist among HPVs of different oncogenic capacity. In fact, not all genera of HPV express E5 at all. The evolutionary retention of E5 in many HR HPVs suggests that, while not necessary for cellular transformation, E5 gives an added benefit to the virus. Several types of E5 proteins have also been shown to have independent transforming activity in murine keratinocytes and fibroblasts.

1.4.1.5 E6

The E6 protein of HR HPV types has a well-documented ability to immortalize cells of various origins in vitro and, in some instances, even induce transformation independent of other viral proteins. In HR HPVs E6 is best known for its ability to interfere with p53, "the guardian of the genome". E6 binds to the E6-associated protein (E6AP), the E6/E6AP complex targets p53, causing it to become ubiquitinated and degraded in the proteasome. While p53 degradation...
takes place in most HPV 16 associated cancers, there are documented cases of HPV-positive cervical cancer where p53 expression remains seemingly unaffected. It is likely that in these tumors, an alternative mechanism of p53 independent postmitotic checkpoint alteration may be in play. While the exact details of this proposed mechanism remain unknown, Cdk1 is implicated to play a crucial role as a mediator.

Another important function of HR HPV E6 is its ability to interact with so called PDZ domains. Such domains are found in a number of other cellular proteins, several of which are also targets of E6 binding including: hDly, hScrib, MUPP1, 14-3-3ζ, Na+/H exchange regulatory factor 1, PATJ, PDZRN3/LNX3 as well as MAGI and TIP family proteins. Interestingly, while HR HPV E6s have a class 1 PDZ binding motif at the C-terminus, no such motif is present in LR HPVs and this could be a contributing factor to why different HPVs have varying oncogenic capacity. Furthermore, while the E6 PDZ binding domain has been found to be facilitating efficient growth in human foreskin keratinocytes, it does not appear essential for HPV induced immortalization.

Another interesting feature of E6 activity is its interaction with the WNT pathway. In association with E6AP, E6 stabilizes β-catenin and works to augment WNT signaling. Additionally, E6 has been shown to cause translocation of β-catenin, possibly in an EGFR related manner.

1.4.1.6 E7

E7 is an oncoprotein that, in some contexts, has an even more potent transforming activity than E6. In HR HPVs E7 de-regulates cell cycle control, primarily by competitively binding to pRb, and thus releasing bound E2F1. E2F1 will then transactivate cell cycle related genes such as cyclin A and E, subsequently stimulating cell cycle transition by dysregulating the G1/S checkpoint. Also the E7 protein of LR HPV has the ability to bind pRb but does so to a lesser extent.

In addition to their transforming activities, both E5 and E7 have been described to interfere with the cellular antigen processing machinery (APM) and downregulate the expression of the major histocompatibility complex (MHC). This association has not been verified in oropharyngeal squamous cell carcinomas.

E7 has also been used as a target in the development of both prophylactic and therapeutic vaccines. The usage of E7 in therapeutic vaccines is particularly exciting as there are currently no therapeutic vaccines for HPV related disease on the market. A 2014 study showed that oral vaccination with *Lactobacillus Casei* expressing modified E7 was able to downgrade CIN3 lesions to CIN2 in 9 weeks.
1.4.2 Late proteins

The transcripts of the two late proteins L1 and L2 were identified following the discovery of the early gene transcripts. As postulated, the L1 and L2 proteins are structural proteins that together make up the viral capsid\textsuperscript{56}.

1.4.2.1 L1

L1 is the major capsid protein of HPV. In total, the capsid consist of 72 L1 pentamers, each of which consist of an outwards facing star like structure and a trunk through which runs a channel to the capsid interior. The protein has the ability to self-assemble into virus-like particles (VLP) both with and without L2 in a fashion similar to what had previously been observed for the VP1 protein of the closely related polyomaviruses\textsuperscript{57–60}.

The HPV vaccines currently on the market consist of empty L1 capsids of different HPV types. Already in 1987 it was shown that antisera produced using purified HPV particles was reactive against L1\textsuperscript{56}. At a relatively early stage it was also shown that the human antibody-reactive epitope of L1 proteins were specific for different HPVs with a certain degree of cross-reactivity, a fact that has become critical in the development of HPV vaccines\textsuperscript{61,62}.

1.4.2.2 L2

For each pentamer present in the viral capsid, there is up to one molecule of the minor capsid protein L2, although the average virion seems to contain somewhat fewer molecules randomly distributed between the binding sites\textsuperscript{63,64}.

\textit{Figure 3. Oncogenic mechanisms of the HPV E6 and E7 proteins}
It would seem that the L2 protein is involved in encapsidating DNA in the virions, since only L2, and not L1 has the capability to bind HPV DNA\textsuperscript{65}. Furthermore, it has been found that capsids with mutated L2 have a 10-fold decrease in the ability to encapsidate viral DNA. Interestingly, this reduced ability to take up viral DNA did not seem to fully explain the observed decrease in infectiousness of the mutant particles, highlighting the fact that the protein has multiple roles in the viral life cycle\textsuperscript{66}. Examples of such functions are facilitating capsid uptake into the target cells and mediation of viral entry into the ER\textsuperscript{67,68}.

L2 is more evolutionarily conserved than L1, and it has therefore been suggested as a potential tool in vaccine development; however, antibody titers produced against L2 have been weak in comparison to responses elicited by L1 vaccines\textsuperscript{69}. No L2 based vaccines are currently in clinical use.

### 1.5 HPV LIFE CYCLE

As previously mentioned, HPV infect epithelial cells. More specifically, HPV infects epithelial cells of the basal lamina of stratified epithelium through micro-wounds by interacting with cell surface heparin proteoglycans\textsuperscript{70,71}. Over time, these infected cells divide and the daughter cells spread towards the epithelial surface. In an HPV driven lesion, different cellular layers are in different phases of the cell cycle and produce different viral proteins. Somewhat simplified, the lower layers produce the oncoproteins E6 and E7, thus pushing the cells to divide. Further up in the lesion, E4 is produced and the genome is amplified. In the cells closest to the surface, the capsid proteins L1 and L2 are produced and the viral genomes are packed in capsids (figure 3).

### 1.6 PERSISTANCE AND CLEARANCE OF HPV INFECTIONS

It is likely that there is a genetic component to HPV infections persisting in an individual. For example, it appears as if cervical HPV infection is more common in young women of African American ethnicity than in women of European American ethnicity although incidence rates were the same\textsuperscript{72}. Furthermore, it seems that pregnancy is protective against persistent cervical HPV infection\textsuperscript{73}. Genetic variations in the virus can also be associated with a less efficient viral clearance; for example, it has been shown that variations within HPV 16 E6 and E2 increase the risk of persistent infection\textsuperscript{74}. 
1.7 HPV IN DISEASE

As earlier mentioned, HPV types can be divided into HR and LR types depending on their oncogenic potential. According to the 2007 IARC monograph on papillomaviruses, approximately 15 HPV mucosal types should be considered as high-risk. These types are HPV 16, 18, 39, 45, 59, 68, 26, 31, 33, 35, 51, 52, 55, 56, and 58\textsuperscript{75}. Low-risk mucosal types are HPV 6, 11, 40, 42, 51, 53, 54, 57, 66, 73, 82, 83, and 84\textsuperscript{75}. There are varying degrees of evidence on the oncogenicity of the different HPV types in different cancer sites; however, in the majority of HPV related malignancies, HPV 16 is the dominating type\textsuperscript{75}.

In 2009, before introduction of public HPV vaccination in Sweden, the estimated cost of HPV related cervical cancer and genital warts alone was approximately €106.6 million\textsuperscript{76}.

1.7.1 HPV-associated cancers

1.7.1.1 HPV in anogenital cancers

Around 1970, there was a suspicion that papillomaviruses, which had been found in different types of human warts, could in fact also be causing cancer, however, evidence at this time was scarce and Herpes simplex virus (HSV) remained the major suspect in the hunt for a virus causing anogenital cancer\textsuperscript{77–79}. In 1971, there was a case report showing what appeared to be papillomavirus particles in an anal carcinoma in situ, however, the first cancer where enough evidence could be gathered to support a causal role for HPV was cancer of the cervix uteri\textsuperscript{13,80}. Since then HPV, has also been found in a considerable portion of vulvar cancer, vaginal cancer, penile cancer and anal\textsuperscript{81–85}. Below, cancer of the cervix uteri will be described followed by a more superficial discussion on HPV in other anogenital sites.
1.7.1.2 Cervical cancer

With 528,000 newly diagnosed cases worldwide in 2012, carcinoma of the uterine cervix is the fourth most common cancer in women and a major cause of cancer related death, accounting for 7.5% of cancer related deaths in women. The geographical discrepancy in the prevalence and mortality of cervical cancer is pronounced; more than 85% of cervical cancer deaths occur in less developed areas of the world with the highest mortality being reported for Eastern and Middle Africa, and Melanesia.

There are two main types of cervical cancer; squamous cell carcinoma and adenocarcinoma. These two share many of the same risk factors and both have been associated with HPV in prospective epidemiological studies. Of the two types, squamous cell carcinoma accounts for the majority of all cases and approximately 80% of these cancers are caused by either HPV 16 or 18, close to all of the rest being caused by other HR HPVs.

Since long it has been known that invasive cervical cancer is foregone by precancerous lesions, which can be defined in different ways.

One common way of classifying these lesions is by subdividing them into grades of cancer intraepithelial neoplasia (CIN), where CIN 1 represents mild abnormal cell growth encompassing a maximum of 1/3 of the basal epithelium, CIN 2 represents abnormal cell growth encompassing 2/3 of the basal epithelium, and CIN 3 spans more than 2/3 of the epithelium and can effectively be classified as carcinoma in situ.

The histological grading of CIN is corresponded by cytological grading in the Bethesda system where atypical squamous cells of undetermined significance (ASC-US) is a common result of a Papanicolao (Pap) test, which is most often not indicative of cervical carcinoma, but should be investigated further, preferably by HPV testing.

The denomination Low-grade Squamous Intraepithelial Lesion (LSIL) roughly corresponds to the presence of a CIN 1, while High-grade Squamous Intraepithelial Lesion corresponds roughly to a CIN 2 or 3. The system also includes codes for glandular abnormalities.

Notably, CIN lesions can be removed through ablative or excisional methods and have a low recurrence rate, especially in women with negative posttreatment Pap tests. A fraction of CIN lesions will ultimately progress into invasive carcinoma if left untreated, this risk has been shown to be low in women who have HPV L1 specific antibodies.

Tests for screening for atypical cells in the uterine cervix were first invented in the 1920s, independently by Georgios Papanicolaou and Aurel Babeş. Of the two tests, the one that won international recognition and was put into routine clinical practice in large parts of the world was the Papanicolao (Pap) test. The test, referred to as a conventional pap test, is performed either using a spatula, smearing the cells directly onto a glass slide or, more recently by liquid based cytology, using a brush, which is suspended in a preservative liquid until analysis.
Since the introduction of the Pap test, the proportion of cervical cancers diagnosed at an advanced stage has decreased in Sweden. Despite its obvious merits; the performance of the Pap test is less than optimal. A Pap test is able to distinguish between high grade lesions and other conditions with a high specificity of 0.96, but with a rather low sensitivity of only 0.63. Conversely, the test can detect any abnormality regardless of grade, with a low specificity of 0.53 but with a high sensitivity of 0.91\(^9\).

The suboptimal performance of the Pap test, together with the emerging possibilities to more easily assay for presence of HPV have led to the development of new screening protocols, which may now also include screening for HPV.

When using liquid based cytology, but not conventional cytology, it is possible to use the same sample for HPV testing\(^9\). In Sweden, The National Board of Health and Welfare (Socialstyrelsen) now recommends that all women of 30–49 years of age should be offered HPV testing every third year, for women of 50–64 years of age testing should be performed every seventh year. In case of a positive sample, the HPV test should be followed up by cytology. In women younger than 30, Pap testing is still recommended as a primary screening method, partly due to the fact that a large proportion of young women are carriers of HR HPV\(^9\). Another test that is used in some settings is visual inspection with acetic acid (VIA) that is cheap and easy to perform, since it in essence only requires some basic tools, a table and acetic acid. The performance of this test is similar to that of the Pap test; the test is therefore often the screening method of choice in low resource settings\(^9\).

An alternative to classical gynecological visits is HPV testing by self-sampling. This method gives reliable results as compared to testing at a gynecologist and also allows for women to be screened without having to undergo a pelvic exam which is often described as embarrassing \(^96,97\). Importantly, self-sampling may have potential to reach women who for cultural or other reasons choose not to attend gynecological visits. It may also be an option in low resource settings where access to health care facilities is limited and cervical cancer incidence is high. The main concern expressed by women who have tried cervical self-sampling is the fear that the procedure will not be carried out correctly and that the personal contact with the gynecologist will be lost. There also seems to be a generational difference in the acceptance of an at-home test with younger women favoring the classical gynecological visits to a higher extent than older women\(^97\). It would also seem that educational level is important for women’s likelihood in accepting the self-sampling procedure\(^98\).

In cervical cancer, overexpression of p16 has often been used as a prognostic marker and a marker of active HPV infection. In this setting, p16 as a marker seems to function rather well, having a clear correlation to patient \(^99,100\). The marker also seems to facilitate diagnosis of CIN2+ lesions as compared to using hematoxylin and eosin staining alone for morphological evaluation\(^101\).

Cervical conization is a common treatment option for CIN lesions and is often carried out using either cold knife conization (CKC) or what is known as loop electrosurgical excision procedure
(LEEP), however, a subset of the patients are left with residual or recurrent disease. The risk of persistent or recurrent disease is increased in patients who remain HPV positive 6 months after treatment, and who have conization specimens positive for TPX2 and PD-L1. For cervical cancer, treatment can be either fertility sparing or radical. The type of treatment received is critical for the recovery of the patients, where those undergoing fertility sparing treatment have fewer lost workdays as compared to patients undergoing more extensive treatment.

1.7.1.3 Vulvar, penile, anal cancer

Vulvar, vaginal, penile and anal cancer are not as common as cervical cancer in unscreened populations. There are no regular screening methods at present to detect these tumors, however antibodies against HPV 16 E6 can be found in 29% of individuals who will later develop anal cancer already 10 years before diagnosis. Notably, to present HPV 16 E6 antibodies at this time point is rare in other anogenital cancers but common in OPSCC.

Tumors from other subsites in the vicinity are rarely HPV positive. In fact, despite its anatomical proximity to the uterine cervix, endometrial cancer seems to stem mainly from other causes rather than HPV infection, neither does it seem to play a role in urothelial bladder cancer.

1.7.1.4 HPV in head-neck cancers

Since long, papillomaviruses have been known to be associated with laryngeal papilloma, and while the virus was suspected to have a role also in laryngeal carcinoma, no definitive correlation could be established.

1.7.1.5 Oropharyngeal cancer, with emphasis on tonsillar and base of tongue cancer

It was later shown that HPV16 could indeed be found in a number of head neck cancers, with the highest prevalence being in oropharyngeal cancers (OPSCC). These and other reports lead to the International Agency for Cancer Research (IARC) recognizing HPV as a causative agent for oropharyngeal cancer in 2007 alongside the traditional risk factors smoking, and alcohol. Subsequent studies have shown that HPV is primarily found in tonsillar and base of tongue cancers (TSCC and BOTSCC), which together constitute a majority of OPSCC. A meta-analysis by Abogunrin et al. showed that between 2002 and 2012, the prevalence of HPV in head-cancers in Europe was 40%, with the highest prevalence being for tonsillar cancer at 66.4%. A recent study by our research group on tumors from the County of Stockholm has demonstrated an even higher incidence, at 80% and 64% for tonsillar and base of tongue cancer respectively. This difference may in part be attributable to differences in smoking habits between countries.

Determining whether a tumor of the head and neck is caused by HPV has been a matter of debate. To determine if a cancer is HPV driven, using only the presence of HPV DNA in the tumor is not conclusive. To detect an active infection, there are a few major approaches. What has sometimes been considered the most reliable measure is the detection of HPV E6 and E7 mRNA. This is optimally done in fresh frozen material, which is not always available at all times and especially not for retrospective studies.
P16 overexpression has sometimes been used as a pseudomarker of HPV infection and, while using p16 alone has now been shown to have a non-satisfactory correlation to the presence of HPV DNA in the tumor, using the two markers in combination has shown greater promise\textsuperscript{114}. Using HPV DNA and p16 in combination has been shown to be almost as sensitive and specific as detection of HPV E6 and E7 mRNA in OPSCC. Approximately 15% of HPV negative head-neck cancers are p16 positive\textsuperscript{115}.

HPV positive OPSCC is prone to metastasize. Common sites of metastasis include locoregional metastases in the head-neck region, as well as distant metastasis in bone and lung. As the metastases generally retain their HPV positivity, HPV positive metastases of cancer of unknown primary of the head and neck region can be strongly suspected to be of oropharyngeal origin and warrant tonsillectomy and/or resection of the tongue base\textsuperscript{116–118}.

As mentioned above, HPV DNA positivity and p16 immunostaining show a relatively good but not absolute correlation, however, this correlation is not as good at other cancer sites. A previous report from the Tina Dalianis research group showed that for hypopharyngeal cancer, p16 and HPV DNA did not seem to correlate to any larger extent\textsuperscript{119}. Further data has since been presented by e.g. Sgaramella et al., strengthening the notion that p16 may not be a suitable pseudomarker of active infection in head neck cancers outside of the oropharynx\textsuperscript{120}.

The causative role of HPV in OPSCC and the mere fact that the disease is caused by an infectious agent is of course interesting from an epidemiological perspective, especially as the
incidence of HPV positive OPSCC is increasing in many parts of the world. As a matter of fact, in Sweden, the incidence of HPV TSCC and BOTCC has increased\textsuperscript{121}. Moreover, HPV driven TSCC and BOTSCC have shown to have a different mutational profile as well as a markedly better prognosis than the corresponding HPV negative cancers when given a standard treatment regime. For example, in a 2007 study from our research group, patients with HPV positive tonsillar cancer had a disease specific survival of 81\% while the corresponding figure was 36\% for patients with HPV negative tonsillar cancer\textsuperscript{122}. This suggests that HPV positive and negative TSCC and BOTSCC should be considered as different disease entities in research and clinical care.

1.7.1.6 HPV in hypopharyngeal cancer

For hypopharyngeal cancer, estimates of the HPV attributable fraction have varied widely, from 0-82\% in different studies\textsuperscript{119,123–126}. To what degree this difference is due to methodological differences, and to what degree it reflects a true biological difference is unknown.

Risk factors for hypopharyngeal cancer are the classical risk factors for head neck cancer; smoking and alcohol. The disease is often diagnosed at late stages, since early symptoms are rare, which contributes to the poor prognosis associated with these cancers, where only between 15 and 30\% survive beyond five years. Due to the anatomical proximity of the hypopharynx to the oropharynx, it is reasonable to assume that a proportion of hypopharyngeal cancers are HPV related. Furthermore, the increase in the proportion of HPV positive cases observed in OPSCC brings up the question if a similar development is taking place in hypopharyngeal cancer. The role of HPV in hypopharyngeal cancer and its relation to p16 expression is explored in paper 5 in this thesis.

1.8 HPV IN OTHER CONDITIONS

Anogenital warts, or condylomata acuminate, are fairly common in the general population with an estimated incidence of about 200/100,000, peaking in young adulthood\textsuperscript{127}. The condition is caused by HPVs and numerous HPV types including both HR and LR types have been found in these lesions, where HPV 6 and 11 are the most common types\textsuperscript{128}. Almost all cases of condylomata acuminate have been found to contain HPV when investigated by deep sequencing\textsuperscript{128}.

Another, rather rare condition associated with HPV is recurrent respiratory papillomatosis (RRP), which just as condylomata acuminate, is mainly associated with HPV 6 and 11\textsuperscript{129,130}. The condition can have either a juvenile onset or an adult onset and the incidence was 0.54/100,000 in adults and 0.17/100,000 in children in a Norwegian study\textsuperscript{131}. In a study from northern Sweden, the median age of diagnosis was at 32 years of age\textsuperscript{132}. So far, treatment for RRP has been considered symptomatic rather than curative although there have been some recent progress using combination therapies\textsuperscript{133}. Eventually, a small subset of RRP patients will develop malignancies of the respiratory tract\textsuperscript{130}.

Sinonasal papilloma is another ailment affecting the respiratory tract, and a substantial fraction of these have also been found to contain HPV\textsuperscript{134}. 

12
Two other rare conditions associated with HPV are epidermodysplasia verruciformis (EV) and focal epithelial hyperdysplasia (FEH), also known as Heck’s disease. EV is a condition in which the patient’s immune system is incapable of handling certain HPV infections caused by HPV types such as 5 and 8, and patients develop skin lesions in the form of scaly macules and papules on the skin\textsuperscript{135,136}. Patients with EV is also at a higher risk of developing skin cancer due to malignant transformation of the lesions\textsuperscript{137,138}. FEH on the other hand, is a benign condition mainly associated with HPV 13, and 32 in which numerous papules develop in the oral cavity\textsuperscript{139–141}. Both of these conditions also have a genetic component which is made evident by the existence of families with a very high incidence of disease\textsuperscript{142–146}. Especially for FEH, there is also a pattern where certain ethnic populations have an increased incidence of the condition\textsuperscript{147}.

### 1.9 HPV EPIDEMIOLOGY

A large meta-analysis, which included one million women with normal cytological findings concluded that the estimated global cervical HPV prevalence was 11.7\%\textsuperscript{148}. The data also showed a peak in HPV prevalence in adolescents and young adults followed by a second peak in the middle aged\textsuperscript{148}. The most commonly found HPV types in these women were HPV 16, 18, 52, 31, 58, 39, 51, and 56, with 22.5\% of all infections being caused by HPV 16\textsuperscript{148}. In a study performed by our research group at a Stockholm youth clinic before the introduction of public HPV vaccination, the highest prevalence was seen in women of 21 years of age where 73\% were positive for HR HPV\textsuperscript{149}. The age distribution pattern seen in these studies is not mirrored in the prevalence of anal HPV in men who have sex with men (MSM). In a study from the US, 57\% of MSM were shown to be positive for anal HPV, with little variation over age groups\textsuperscript{150}.

A proportion of healthy subjects of different ages have been shown to have HPV DNA present in the oral cavity. The reported prevalence of oral HPV varies; however, the trend seems to be towards a low prevalence in pre-adolescents and adults of the general population and towards higher figures in sexually active youth\textsuperscript{151,152}. In our previous study from our research group, oral HPV prevalence in non-vaccinated young adults at a Stockholm youth clinic was high at 9.3\%\textsuperscript{153}. Similar figures have since been reported for certain high risk groups including MSM attending a sexual health clinic in London\textsuperscript{154}. Even higher numbers were reported for at-risk women in Ho Chi Minh City where a total of 24.6\% were positive for at least one HPV type by oral rinse and for women below 20 years of age in Yucatan, where 24.5\% where positive for at least one HPV type by buccal swab\textsuperscript{155,156}. Other studies, e.g. a study from the National Health and Nutrition Examination Survey in the US found a lower oral HPV prevalence of 3.8\% in the oral cavity of civilian, noninstitutionalized women\textsuperscript{157}.

Some studies have reported that also new-born infants can harbor oral HPV, suggesting a route of vertical transmission from mother to child\textsuperscript{158}.
1.10 HPV DETECTION METHODS

1.10.1 Sampling methods and sample types

To detect HPV in tumor samples, the type of sample obtained and the methods used for sample preservation play an important role.

Tumor samples that are available are often formalin-fixed paraffin embedded (FFPE) samples or more seldom fresh frozen tumor samples. DNA and RNA can be extracted both from FFPE samples or fresh frozen samples\(^{159}\). However, if the FFPE has been stored for many decades its quality may be affected and the ability to detect longer fragments of nucleic acids can be abrogated\(^{160,161}\).

To detect HPV in oral samples, from healthy individuals or cancer patients, swabs or oral rinses can be used, yielding comparable results with regard to HPV prevalence\(^{162}\). The liquid in which the sample is collected may be of importance as certain brands of mouthwash liquid have indicated to have a better performance with regard to i.e. DNA quality\(^{163}\). Other considerations in oral sampling include salivary production and whether the person who is being sampled has ingested any food or beverage close to the time of sample collection.

Swab samples are also frequently being used for detection of HPV in cervical specimens and to a certain extent for obtaining genital samples from men\(^{164,165}\). While swab samples from women are generally considered a reliable method of HPV detection, genital HPV sampling from men is more difficult and often requires combinatory approach of exfoliation of cells from various genital subsites and collection of urine\(^{166,167}\).

1.10.2 Direct and indirect detection of presence of HPV

In clinical settings, p16 immunohistochemistry (IHC) is still commonly used as a surrogate marker for HPV in cancer diagnostics. As earlier mentioned and also pointed out by many, this practice is suboptimal as p16 status and HPV status, as defined by more direct methods of detection are often discordant\(^{119,168,169}\). Today, p16 is mostly considered as a useful marker when used in combination with other methods detecting viral DNA or RNA\(^{170}\).

One of the most frequently utilized ways of detecting HPV DNA is by PCR. The regions commonly amplified using this method are L1, E6 and E7. PCR amplification of the relatively well conserved L1 region enables for detection of a wide range of HPVs using either primers with degenerative nucleotides or consensus primers which can bind to a large variety of HPVs when using a relatively low annealing temperature\(^{171}\). The different HPV types detected by these methods can then be identified using various detection methods such as sequencing, or bead-based methods like Luminex MagPix. Some studies have suggested that a lower false negative rate can be achieved by using E6 or E7 primers, since these regions are more likely to be retained over the course of disease progression and/or chromosomal integration, however, since these genomic sequences differ more across HPV types, they may be more suited to detect specific HPV types than for use in larger screenings\(^{172}\).
Another method, which has received more attention in recent years, is the detection of E6/E7 mRNA, this method is often considered the preferred method of detecting an active HPV infection\(^{173}\). As mentioned above, detection of the oncogenes E6 and E7 should be possible in a large proportion of HPV associated lesions. By detecting E6 and E7 rather than L1, you gain the added benefit of being able to identify HPV that is transcriptionally active and thus has a greater chance of being clinically relevant. Detection of HPV mRNA can be done either by PCR or by mRNA in situ hybridization (ISH). However, for detection of mRNA the samples should ideally be rapidly fresh frozen, or possibly formalin fixed\(^{159}\).

1.11 HPV VACCINATION

1.11.1 Prophylactic HPV vaccination

Since ancient times, attempts have been made at inoculating individuals with infectious agents to give rise immunity and prevent serious disease. Since then, the art of vaccine development has grown increasingly sophisticated and today, some main categories of vaccines can be identified; inactivated, live attenuated, toxoid, conjugate vaccines and subunit vaccines, where the prophylactic HPV vaccines in current use belong to the latter category. In the 1980’s, it was possible to effectively vaccinate cattle against papillomaviruses using L1 and L2 based vaccines and in the 1990’s, highly immunogenic VLPs were produced against human papillomaviruses\(^{174,175}\).

In 2006, the first prophylactic vaccine against HPV, Gardasil became available on the Swedish market. In 2007, the second vaccine Cervarix became available. Both of the vaccines are protective against HPV 16 and 18, which are the types most commonly found in cervical cancer. Gardasil is also protective against an additional two types, namely, types 6 and 11, which are commonly found in condylomata acuminate. Gardasil is indicated for use in both girls and boys from 9 years of age to protect against anogenital cancers, precancerous lesions of cervical, vulvar, vaginal, and anal cancers, and condylomata acuminate, while Cervarix is recommended for use in girls from 9 years of age against precancerous lesions of cervical cancer\(^{176,177}\).

As mentioned above, both Gardasil and Cervarix are subunit vaccines whose active component consists of VLPs of specific HPV types. The VLPs of Gardasil are produced in yeast, whereas the VLPs of Cervarix are produced in insect cells. Both vaccines are adjuvanted with aluminum based adjuvants\(^{177,178}\).

Since the introduction of the two first HPV vaccines, vaccine development has continued. Gardasil 9 is the first FDA approved nona-valent HPV vaccine. In addition to protecting against HPV 6, 11, 16 and 18 as the quadrivalent Gardasil, the nona-valent Gardasil also protects against HPV 31, 33, 45, 52, and 58 and is indicated for use in both girls and boys from 9 years of age to protect against anogenital cancers, precancerous lesions, and condylomata acuminate\(^{176}\).

When implementing large scale vaccination programs, there is a great chance that some individuals will not be vaccinated, either due to medical reasons or to personal beliefs. To a certain extent, these individuals will obtain protection from the disease as well, given that the proportion of vaccinated individuals is sufficiently high. This phenomenon is named “heard immunity”.

15
The dynamics of sexually transmitted infections are unique in the sense that vaccinating one sex will grant protection for the other sex as well - at least for the individuals who have exclusively heterosexual relationships\textsuperscript{179}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{herd_immunity.png}
\caption{Herd Immunity. In scenario A, infection spreads throughout the population. In scenario B, the vaccinated individuals protect those at risk and the infection is contained.}
\end{figure}

\subsection{Therapeutic HPV vaccination}

Attempts at curing HPV related disease using vaccination have been made even before the virus was identified. Since long, extracts of papillomas have been used in veterinary practice with some success\textsuperscript{180}. In the 1960ies, trials on autogenous laryngeal papilloma vaccines were performed in humans. Although the vaccines were somewhat effective in limiting the spread of papillomas and even causing them to regress, far from every patient benefitted from the treatment and some patients were forced to discontinue the treatment due to adverse events\textsuperscript{181}. Over time, the approaches towards therapeutic papillomavirus vaccination grew more sophisticated. After the invention of recombinant technology, it became possible to elicit immune responses using recombinant viral proteins, a principle that was tried out in cattle in the early 1990ies\textsuperscript{182}. At this time, Campo et al. showed that it was possible to vaccinate calves both prophylactically and therapeutically against bovine papillomavirus (BPV) 4, a mucosal BPV type causing papillomas of the bovine alimentary canal\textsuperscript{182}. Major targets in the development of therapeutic vac-
cines for use in humans have been the E6 and E7 proteins of HR HPV types. Examples of therapeutic vaccines that have been developed include a Venezuelan equine encephalitis based vaccine using a fused HR HPV E6 and E7, and the previously mentioned example of *Lactobacillus Casei* expressing modified E7, however, to date, no therapeutic HPV vaccine is available on the market\textsuperscript{55,183}.

### 1.11.3 Vaccine controversy

While vaccination arguably can be considered as one of humanities greatest innovations and triumphs, the implementation of vaccination programs is still received with some suspicion by the general public. Concerns that are being raised include the fear of adverse effects due to autoimmunity or toxicity caused by vaccine components such as adjuvants, corruption within the pharmaceutical industry as well as government directed efforts for population control\textsuperscript{184,185}.

Although it is easy to dismiss these concerns as ignorance and conspiracy theories, it is important to remember that the history of medical science is indeed littered with some true low water marks. The infamous Nazi experiments on human physiology, as well as the Tuskegee syphilis study, and the Vipeholm experiments on dental decay constitute examples of what is today to be deemed severe scientific misconduct\textsuperscript{186–188}.

Also, despite the best intentions, not every new vaccine or therapeutic agent have the desired safety and efficacy when taken into human trials or clinical use. One such example is the unexpected side effects that surfaced after the large scale implementation of the H1N1, or swine flu, vaccine\textsuperscript{189,190}.

Not surprisingly, also the HPV vaccines are subject to some controversy. Regarding the HPV vaccines, some main causes of concern have been the fear of post-vaccination infertility due to ovarian failure as well as onset of postural tachycardia syndrome (POTS). Clinical data on these syndromes in relation to vaccination remain scarce; regarding primary ovarian failure, only a handful of cases have been reported and also concerning POTS, the evidence is limited and non-conclusive due to small sample size and poor study design\textsuperscript{191,192}. This does not mean that the studies should be dismissed. Post-vaccination monitoring of adverse events remains of immense importance to identify potential risk-groups and in the development of new vaccines.

In the case of the reported incidences of POTS, the European Medicines Agency (EMA) undertook an investigation which showed no increase in the overall incidence of the condition in the relevant age group. The EMA concluded that there was no evidence supporting the notion that HPV vaccination would be causing POTS\textsuperscript{193}.

### 1.11.4 Vaccine safety and efficacy

Despite frequent claims of the contrary, the HPV vaccines currently on the market were extensively studied prior to licensure and have been deemed to have acceptable risk-benefit ratios. In August 2015, there were 84 national programs and 38 pilot programs ongoing which is resulting in a very high number of people vaccinated worldwide, facilitating large scale epidemiological studies\textsuperscript{194}.
So far, no large scale studies of HPV vaccine safety have shown the vaccines currently in use to be associated with any serious adverse effects. The side effects that have been described are often located to the injection site and include pain, swelling, erythema, and pruritus, as well as systemic side effects such as nausea, syncope, headache and pyrexia\textsuperscript{195,196}. Also in pregnant vaccine-recipients, no concerning pattern of adverse events have been observed\textsuperscript{196}.

Since cancer development takes many years, it is still not possible to detect changes in the incidence of HPV related cancers; however, the reduction seen in precancerous lesions is highly encouraging and provides reliable evidence of vaccine efficacy\textsuperscript{197,198}.

1.11.5 Upscaling of vaccination programs

It has been demonstrated that HPV vaccination is primarily beneficial before first exposure to the virus. Because of this, vaccination programs have primarily been aimed at young girls that are statistically unlikely to be sexually active, and, have often been performed in a school based setting. This approach may well lead to a good vaccine uptake in the target population, however, since only selected age groups are targeted this leads to a suboptimal protection in other cohorts. To provide a better coverage on a population basis, catch-up vaccination of older girls has been used in some regions.

Although HPV vaccination has been suggested to be less efficient in older women than in young girls, catch-up vaccination seems to be both beneficial and safe. A systematic review by Couto \textit{et al.} reporting on 13 randomized control trials (RCTs) of women until the age of 45, including close to 40,000 individuals, showed a reduced risk for all VIN2+ and VaIN2+, HPV related CIN2 and condylomata acuminate. Serious adverse events were not more common in the catch-up vaccinated group as compared to vaccination in young girls\textsuperscript{199}.

Increased vaccination coverage in adolescent girls has been estimated to be the most cost effective mean of upscaling a vaccination program targeting only 11-year old girls when considering cervical prevalence of HPV16 and 18\textsuperscript{200}.

Another option in increasing vaccination coverage is to also include young boys. This effort alone seems to be less effective than vaccinating older girls; however, these estimates usually do not include other outcomes than cervical disease and is also dependent on what percentage of girls that are vaccinated. Another obstacle that has emerged in areas that already offer gender neutral vaccination programs is the fact that boys and their parents simply do not know that vaccination is beneficial and readily available\textsuperscript{184}. In 2013, the vaccination coverage of boys in the United States was 34.6\% and dose series completion was 13.9\%\textsuperscript{201}.

Whether upscaling of vaccination is cost effective in absolute figures, as measured by e.g. cost/quality-adjusted life year gained, is dependent on a number of factors including predicted vaccine efficacy, base line HPV prevalence, vaccine coverage, number of birth cohorts included and vaccine cost\textsuperscript{202}. One attractive option in addressing the latter is to oversee the number of doses needed to give adequate protection. Studies have shown that two doses, and maybe even one dose, can elicit antibody responses comparable to that of a three dose regimen\textsuperscript{203,204}. If the re-
sponse of such a regimen would prove stable over time, this might well be a feasible option to decrease the cost of the vaccination program and achieve better vaccination coverage.

1.12 SEXUAL HABITS IN YOUTH

During adolescence and young adulthood, most individuals become sexually active. This is often referred to as the sexual debut, and the age at which a person experiences this event is known as the sexarche. Sexarche is on average said to occur sometime in the late teens and, about one year earlier in men than in women. The mean age of sexarche varies, both over time and over geographical regions. Other aspects such as gender, sexual orientation and sociodemographic aspects also play a role in when a person becomes sexually active. Factors associated with an earlier sexual debut include poor impulse control, childhood behavioral problems, and for some behaviors, high BMI. Conversely, a Swedish study showed a later sexual debut to be correlated to a number of factors including having caring or overprotective fathers, and parents born outside of Europe, however, the most important factor was a lower sexual desire.

Overall, the mean age of sexual debut seems to be decreasing in many parts of the world. The reasons for this may include an increased cultural acceptance as well as greater opportunity to intermingle with individuals of similar age as the world becomes increasingly urbanized. Nevertheless, sexual habits remain a sensitive topic and behavioral research has to rely on self-reported information which is inherently prone to bias. This issue is made evident when comparing questionnaire administration modes. A systematic literature review by Langhau et al found that response rates as well as reported behaviors differed quite extensively between administration modes. In general, lower frequencies of different sensitive behaviors were found using self-administered questionnaires or face-to-face interviewing, as compared to when using audio computer-assisted survey instruments, highlighting the influence of social desirability norms in these types of studies. In Sweden, the mean age of sexual debut appears stable at somewhere between 16 and 17 years of age since the 1960’s while the mean age of sexual debut seems to be decreasing for both sexes in the US.

One change that has taken part over last decades in Sweden is an increase in the average number of lifetime partners. In 1967, the median number of sexual partners reported by a selection of Swedes of 18-74 years of age was 1.4 for women and 4.7 for men. In 1996 the corresponding figures were 4.6 and 7.1 respectively and similar trends have been shown in the US.

The greatest degree of sexual intermingling seems to take place in adolescence and early adulthood. This trend also reflects in the incidence of sexually transmitted infections (STIs). It has been estimated that 50% of the 19.7 million incident infections of STI in the US were among young men and women aged 15-24 and the majority of infections were caused by HPV. Similarly, the age-specific incidence of cervical infections prior to the introduction of public HPV vaccination shows a peak in the early twenties. As a side note, prevalence data from a number of countries display a second peak in the late forties which may be related to a high rate of divorce and children being likely to have left the home.
Research on trends and behaviors in youth remain crucial for understanding the spread of sexually transmitted infections (STIs) and their associated complications.

1.13 SEXUALLY TRANSMITTED INFECTIONS AND REPRODUCTIVE HEALTH IN YOUTH

Together with educational interventions and vaccination against some pathogens, youth clinics constitute an important aspect of primary STI prevention. In Sweden, youth clinics are available for individuals up to 23 years of age. These clinics are found in most larger communities and offer counselling, advice on contraception and family planning as well as testing for STIs.

Studies have shown Sweden to have the highest rate of abortions in the Nordic countries and a high incidence of chlamydia acuminate\textsuperscript{213,214}. One of the explanations for these observations may be inconsistent condom use and a relatively low age of sexarche in international comparison. A Swedish study also showed positive association between oral contraceptive (OC) use and, nationwide abortion and chlamydia infection rates\textsuperscript{214}. This association may be explained by a lower use of condoms in individuals taking OC as well as failure by individuals to follow the instructions for use of the drug\textsuperscript{214}. Similarly, it has been shown that cervical HPV infection is indeed very common in young Swedish women\textsuperscript{153}.

The practice of “safe sex” using barrier type contraceptives such as condoms and dental dams is inconsistent in adolescents and young adults in Sweden\textsuperscript{215}. Even though many know how to protect themselves against STIs and unwanted pregnancies, this does not translate into safe sexual practice\textsuperscript{215,216}. The reasons for not using condoms during sexual contact include convenience, the belief that it condom use would make sex less enjoyable and the fear that sexual partners may react negatively to suggested condom use\textsuperscript{216,217}. This issue is being addressed through informational campaigns and by handing out free condoms in different contexts.
2 AIMS

- To examine oral HPV prevalence, and its association with HPV vaccination status in youth at a youth clinic in Stockholm and in high schools in a middle-sized Swedish municipality (Papers I, III, and IV)

- To investigate how HPV vaccination status relate to sexual experiences and sexual risk-taking in a cohort of Swedish youth (Paper II)

- To examine type-specific cervical HPV prevalence, and its association with HPV vaccination status in youth at a youth clinic in Stockholm (Papers III, and IV)

- To evaluate how commonly HPV can be found in hypopharyngeal cancer and to what extent this correlates to p16 expression (Paper V)

- To determine if there has been an increase in the proportion of HPV positive hypopharyngeal cancer over time (Paper V)
3 MATERIAL AND METHODS

The studies included in this thesis were approved by a Regional Ethical Review Board and conducted in accordance with ethical permissions Regional Ethical Review Board of Uppsala No. 2010/369, Regional Ethical Review Board of Stockholm No. 2012/1756-31/2, No. 2008/870-31/4, No. 2009/1147-31/2, and No. 2009/1278-31/4.

Studies on sexual habits and HPV prevalence in healthy subjects were cross-sectional and did not individually consider temporality.

The patient material and samples from healthy subjects were collected from several source populations. Diagnosis codes used in the classification of hypopharyngeal cancers in Paper V was in accordance with the ICD-classification system ICD10. The study design and the respective cohorts of patients and healthy subjects are described in further detail below.

3.1.1 Paper I

In total, 335 third-year high school students aged 17–21 years (median age 18 y), from 13 schools in a municipality with a population of 140,000, were examined for oral HPV prevalence. Data were also collected on sex, and HPV vaccination status of the participants. In addition, the students answered a questionnaire, and data on sexual experiences could thus be accessed (for details of the questionnaire see paper 2). This study was cross-sectional in design.

3.1.2 Paper II

355 female third grade high school students aged 17–21 years (median age 18 y) were included in this study of which 338 answered the questionnaire in the classroom setting and 17 who participated by postal questionnaire. Questions on HPV vaccination were not included in the original study and this study should thus be considered cross-sectional as information is only gathered at one time point.

3.1.3 Paper III

In total, 211 women and 87 men aged 15-23 years, attending a Stockholm youth clinic, participated in the study. A total of 287 mouthwash samples were collected in 50% Listerine and water. The study is a follow-up on a previous study on HPV prevalence conducted at the same clinic.

3.1.4 Paper IV

In this study, HPV prevalence was investigated in cervical, and mouth wash samples collected between October 2014 and May 2015. Mouthwash samples were collected from 335 women and 112 men. Cervical samples were collected from 338 women. The study was an extension of the study presented in Paper III.
3.1.5 Paper V

In this follow up study, HPV prevalence and p16 overexpression was examined in formalin fixed paraffin embedded (FFPE) tumor biopsies of 82 patients diagnosed with hypopharyngeal cancer diagnosed in 2008-2013 at the Karolinska University Hospital. The survival analysis included 142 hypopharyngeal cancer patients diagnosed between the years of 2000 and 2013 at the Karolinska University Hospital, which were treated with curative intent and had a 3-year follow-up period.

3.2 DNA EXTRACTION

In order to detect the presence of HPV DNA in the samples, DNA was extracted using one of the following DNA extraction kits:

- High pure PCR template preparation kit (Roche®) (for cervical swabs)
- Gentra Puregene Buccal Cell Kit (Qiagen®) (for mouthwash samples)

The High pure PCR template preparation kit is a spin column based method which was used for cervical swab samples. The Gentra Puregene Buccal Cell Kit, which functions through a modified “salting-out” mechanism, was used for extraction of DNA from buccal cells in mouth wash samples. Both protocols have the potential to yield high quality DNA suitable for a range of downstream applications.

3.3 HPV DETECTION

Presence of 27 mucosal HPVs including all known HR HPVs was performed using a bead-based multiplex assay as follows:

The DNA was amplified using BSGP5+/6+ HPV L1 consensus primers, and specific primers for HPV 16 and HPV 33 E6, and β-globin along with the Qiagen Multiplex PCR kit (Qiagen®). Specific HPV types were the detected using a bead-based MagPix instrument (Luminex Inc. ®). The micro-beads were coupled to the HPV type specific probes on-site in the laboratory. The assay used in these studies contained specific beads for simultaneous detection of 27 mucosal HPV types, namely HPV6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, and 82. Bound DNA was detected using fluorescent r-phycoerythrin which was linked to the PCR products through a biotin-streptavidin complex. This method of HPV detection is semi-quantitative in nature and enables detection even of very few viral genomes. A dilution series of DNA from the HPV 16 positive cell line SiHa was used as positive control for HPV DNA. The detection of β-globin was used as a positive control for the amplification of cellular DNA.

3.4 IMMUNOHISTOCHEMISTRY

4μm sections of formalin fixed paraffin embedded (FFPE) tumor material was de-paraffinized in xylene of decreasing concentrations and rehydrated in ethanol. Antigen retrieval was done by heating of the samples in citrate buffer in a microwave oven. Endogenous peroxidase was blocked by incubation with 0.03% H2O2. Sections were stained
for p16 using the E6H4™ Mouse monoclonal antibody, CINtec® p16 histology (Ventana, Tucson, Arizona) followed by a secondary biotinylated horse anti-mouse antibody (Vector Laboratories, California) diluted 1:200. ABC-HRP (Vectastain, Vector laboratories) was then used for antigen detection and the slides were developed with chromogen 3’ dianinobenzidine (DAB). The slides were counterstained with hematoxylin and mounted using Pertex® mounting media (Histolab, Göteborg, Sweden) and evaluated by two researchers. Samples with strong staining in more than 70% of the tumor cells were considered p16 positive.

3.5 QUESTIONNAIRE

The questionnaire was originally used by some of the co-authors in a previous study on sexual habits in youth. For this study, the questionnaire was modified to also include questions on HPV vaccination.

The instrument used in this study had a total of 48 multiple-choice questions, covering different aspects of sexual health and behaviors as well as questions on HPV vaccination. The questionnaires were placed in envelopes by the students and anonymized before analysis. All students present in the visited classrooms received a small gift bag with condoms, candy and a lottery ticket.

3.6 STATISTICAL ANALYSIS

Fischer’s exact test or Chi-squared test was used for categorical data. In paper 1, confidence intervals for prevalence data were calculated using a 1-sample proportion test with continuity correction. In paper 5, survival data were stratified on HPV 16 and p16 status or p16 alone and analyzed for overall and disease specific survival by nonparametric log-rank test. All statistical analyses were performed in R Statistics version 2.15.3 with the exception of paper 2 where IBM Statistical Packages of Social Sciences, SPSS 20 was used.
4 RESULTS AND DISCUSSION

4.1.1 Paper I

Oral and cervical HPV was shown to be common at a Stockholm youth clinic in youth who were not vaccinated against HPV in studies performed 2008–2010 and 2009–2011. In a previous study by our research group, oral HPV prevalence in young adults not vaccinated against HPV at a Stockholm youth clinic was 9.3\%\textsuperscript{153}. An oral prevalence of 9.3\% can be considered somewhat high by international standards, as a 2010 meta-analysis found an average HPV prevalence of 4.5\%\textsuperscript{218}. Similarly, cervical HPV was high by international standards at 70\%\textsuperscript{219,220}. The question was raised whether or not this high prevalence could be due to the fact that the youth clinic constituted a selected cohort of high risk of contracting an HPV infection. Consequently, we decided to examine whether the prevalence was equally high in another, less selected cohort of Swedish youth.

In conjunction with a study on lifestyle and sexual habits which was a collaboration between researchers from several Swedish universities, we here collected mouthwash samples and vaccination data from Swedish 3\textsuperscript{rd} year high school students from a middle sized municipality in Sweden. At the same time, a questionnaire study was carried out to shed some light on the sexual experiences of the young adults participating in this study- an aspect that was missing in the original youth clinic study (for details see Paper II). The results of these studies were interesting in that the HPV prevalence was much lower (1.8\%) than in the original youth clinic study. Methodologically, the studies were similar although three additional HPV types were screened for in this study.

Of the women participating in this study, 64\% had received at least one dose of HPV vaccine. Of all oral samples, 1.8\% were positive for the presence of HPV DNA, and there was no statistically significant difference between men and women. Four of the women tested positive for HPV 16, all of which were vaccinated, but none before their sexual debut. The fact that vaccination took place after the sexual debut means that the virus could have been acquired prior to vaccination. No statistically significant difference could be seen in the oral HPV prevalence between vaccinated and non-vaccinated women.

The prevalence of oral HPV was low in this study, as compared to the original youth clinic studies. One thing that should be noted is that MFI values in the MagPix analysis from mouthwash samples are usually many times lower than what is found in other sample types such as cervical samples, and tumor biopsies. Nevertheless, results from similar studies have given similar results\textsuperscript{221,222}. We therefore speculated that the lower prevalence could be due to demographical differences, effects of the HPV vaccination, or a combination of both. This study contributes to a more accurate picture of HPV prevalence healthy youth as the sample is classroom based rather than clinical.
4.1.2 Paper II

In the context of the introduction of the HPV vaccines, it was speculated as to whether or not getting vaccinated would influence sexual habits in youth. In conjunction with a follow-up study on sexual habits in youth, we collected data on HPV vaccination, lifestyle, and sexual habits from Swedish high school students one year after the vaccines became free of charge for young women.

Of the 65% of individuals who were vaccinated against HPV, 62% were vaccinated after their sexual debut. There were no significant differences between vaccinated and non-vaccinated individuals with regard to condom use, self-reported STIs, experiences of oral or anal sex, or having had a friends-with-benefits relationship. Having had sexual intercourse and “one-night stands” was more common in the vaccinated, than in the non-vaccinated women (p=0.005, and p=0.046) At the time of the sample collection, 64% of the women included in the study reported having received at least one dose of HPV vaccine, albeit on average one year after sexual debut, suggesting that the lower HPV prevalence could at least in part be related to protection from the vaccine.

This study showed that being HPV vaccinated did not have a major impact on sexual risk-taking which is in line with a previous study from the U.S. Since the risk of feeling a false sense of being protected against STIs in general is a common argument against HPV vaccination, this study adds information that may be of great benefit for public health. It should also be noted that due to the cross sectional nature of this study, no conclusions can be drawn regarding causality. This means that the observed differences between vaccinated and non-vaccinated women may be a result of women with higher risk taking choosing to get vaccinated to a larger extent.

It should also be noted that the administration mode of questionnaires can affect the results and that such effects can differ between different populations. Additionally, repeating this study in other populations would improve the generalizability of the results.

4.1.3 Paper III

In two previous studies, in 2008–2010 and 2009–2011, our group had studied oral and cervical HPV prevalence in youth aged 15–23 years and found a very high oral and cervical HPV prevalence. In 2013, in Paper I, we therefore studied oral HPV prevalence in a different cohort, i.e. in of Swedish high school students and here oral HPV prevalence was much lower than in our initial studies at the youth clinic. However, by this time 65% of the female high school students had been HPV vaccinated. These results urged us to return to the Stockholm youth clinic visited in the original study to do a follow-up study, examine whether oral and cervical HPV prevalence remained at a high level in that setting also after the introduction of public HPV vaccination.

In this study, we could see a lower oral HPV prevalence also in the youth clinic cohort of 1.4%, and that oral HPV-prevalence which was very similar to that among high school students. Furthermore, it was clear that the vaccination coverage of the females in both
the school-based study (Paper I in this thesis), and in the present study was quite similar (1.8% and 1.4% respectively), and it was reasonable to assume that the fact that we were now investigating largely vaccinated cohorts may account for the difference observed in prevalence.

Methodologically, this study was similar to the original youth clinic study with the addition of screening for three extra HPV types. In this study, a proportion of the mouth wash samples were collected in the Scope® Original Mint mouthwash rather than Listerine®. This did not seem to affect the proportion of HPV positive samples.

In this study, 73% of the women donating a cervical sample were vaccinated against HPV, although we did not have data on whether vaccination occurred before after their sexual debut. Oral HPV DNA was detected in 1.4% of the young men and women donating a mouthwash sample with no difference between vaccinated and non-vaccinated individuals, or between women and men. The prevalence in this population was significantly lower than what was seen in the previous youth clinic study on oral prevalence from 2009-2011 (p < 0.00001). Cervical HPV DNA was detected in 61% and 70% of the samples of HPV- vaccinated and non-vaccinated women respectively. HPV 16, 31 and 70 were significantly less common in vaccinated than in non-vaccinated individuals. While HPV 16 was expected to have decreased and there have been previous reports of cross protection against HPV 31, the observed protection against HPV 70 has not been previously reported 225,226. This finding may not be of major clinical relevance as there is little evidence implicating HPV 70 in disease. There was also a tendency for the vaccine types HPV18, 6 and 11 to be less common. However, that there were no significant differences observed in the other vaccine type HPVs can be explained by the fact that they simply were not very common to begin with and a very large sample size would be needed to see any statistically significant differences in the prevalence of these types.

Further, more drastic, changes in the HPV prevalence among youth can be assumed to occur over time when those vaccinated at a young age within the school based vaccination program initiated in 2012 will reach the same age as the cohorts included in this thesis.

4.1.4 Paper IV

In our study 2013-2014 (Paper III), at the youth clinic in Stockholm, there had been a major decrease in oral HPV prevalence, and in cervical samples, total HPV as well as HPV 16 had decreased as compared to previous studies 2008-2011 at the same clinic. To detect if these changes persisted over time, we extended the study to also include samples from the fall of 2014 and the spring of 2015.

During this period HPV-vaccination frequency in women remained stable at 71%. No data to whether it was performed before or after sexual debut were obtained. When combining data from 2015 with previous results from 2013-2014, oral HPV prevalence remained low at 1.5%. Cervical HPV prevalence was 64.6% and 74.5% in vaccinated and non-vaccinated women respectively (p = 0.096). HPV 16, HPV 31 and HPV 6 were less common in vac-
cinated than in non-vaccinated women ($p = 0.0006$, $p = 0.038$ and $p = 0.009$, respectively). HPV16 prevalence was also significantly less common in this cohort than in the pre-vaccination youth clinic study from 2008-2011.

Methodologically, this study was a continuation of the study presented in paper 3. Here, all mouthwash samples were collected in Scope Original Mint Mouthwash.

Indeed, in this fourth study of this thesis, we found that adding an additional 160 mouthwash samples to our previous data did little difference to the total oral HPV prevalence, which remained low (1.5%). It should be noted that MFI values for mouth washes from healthy individuals were generally much lower than in cervical samples or in mouthwashes from cancer patients. It is therefore possible that some proportion of the differences observed may be due to minute methodological differences between studies even though we could find no evidence of such differences in the laboratory protocols.

Over the studies performed on HPV and sexual habits in youth, we could see that after the introduction of public HPV vaccination, vaccination coverage increased from 10% in the original youth clinic studies to stabilize at a level of about 70% in girls. In the same time period, vaccinated boys were extremely rare in every study. The fact that cervical HPV 16 was less common in both vaccinated and non-vaccinated women as compared to what was observed in the original 2008-2012 youth clinic study, which included non-vaccinated women indicates a certain degree of heard immunity effect. This also seemed to be the case with regard to oral HPV prevalence, since both vaccinated young women and non-vaccinated young women and men had a lower oral HPV prevalence than what was observed in the original youth clinic studies. The decrease from 9.3% to 1.5% was indeed rather dramatic, even when considering potential heard immunity and cross protective effects. As no socio-demographic data were collected from the study subjects we cannot address whether any such changes had taken place in the population visiting the clinic, however, no major changes in the daily work had been reported by the midwives at the clinic.

This study indicates that oral HPV prevalence have decreased after the introduction of the introduction of the HPV vaccines, which may have a substantial impact on the future burden of head neck cancers.
Figure 7. Type specific cervical HPV prevalence by HPV vaccination status and in comparison to previous data from the same clinic (Paper III).
4.1.5 Paper V

Based on the fact that the proportion of HPV positive tonsillar and base of tongue cancer has been increasing in the last few decades, we wanted to investigate if a similar increase had taken place in hypopharyngeal cancers- a cancer type with a particularly poor prognosis. During the period of 2000-2007 we had previously reported an HPV prevalence of 3.7% in hypopharyngeal cancer in Stockholm, and we anticipated that an increase in HPV prevalence may have occurred during the years 2008-2013. The goal of this investigation was to test this hypothesis.

Of the hypopharyngeal tumor biopsies in the present study, 3/82 (3.7%) were HPV16 DNA and p16 positive, while 12/82 (14.6%) were p16 positive, data similar to the study 2000-2007, thus there was no increase of HPV-prevalence over time. Combining the two studies, and including 142 hypopharyngeal patients diagnosed 2000-2011, the overall 3-year survival, was significantly better for those with HPV16 DNA and p16 positive tumors as compared to survival of the others (86% vs. 31%, p=0.0185). This is similar to what was previously shown for tonsillar and base of tongue cancer\textsuperscript{122}. We could find no evidence of an increase in the proportion of HPV positive hypopharyngeal cancer.

In similar international reports estimates for the HPV attributable fraction in hypopharyngeal cancer has varied between 0-82% and in a meta-analysis the average proportion of HPV positive hypopharyngeal cancer was 21%. Thus, our data showed a relatively low prevalence of HPV in hypopharyngeal cancer, despite the fact that the prevalence of HPV is high in oropharyngeal cancer in the Stockholm region.

P16 expression was found to be a poor surrogate marker for HPV in this sample since 12/82 (14.6%) of samples were p16 positive; all of which but three were found to be HPV DNA negative.

To conclude, HPV16 prevalence was 3.7% and there was no detectable increase in the HPV attributable fraction in this setting. p16 was not a suitable surrogate marker of active HPV infection in hypopharyngeal cancer since the majority of p16 positive tumors here were HPV DNA negative. One alternative way of detecting active infection in this sample type could be using HPV RNA ISH; however, the method has not been evaluated for this sample type and it has been suggested that the assay may not provide satisfactory discrimination between DNA and RNA\textsuperscript{173,227}. The fact that patients with HPV positive hypopharyngeal had a good clinical outcome is indeed interesting; however, the results should be taken with some caution due to the low sample number. Further studies are needed to confirm this finding.
5 SUMMARY AND CONCLUSIONS

- There was a relatively low prevalence of oral HPV (1.8%) in third year high school students in one Swedish municipality (Paper I)

- Although 65% of the women in the third year of high school in one municipality in Sweden were vaccinated against HPV, most were vaccinated after their sexual debut (Paper II)

- No differences were seen in condom use or STIs between vaccinated and non-vaccinated women in this cohort, however, experiences of sexual intercourse and one-night stands were more common in vaccinated, than in non-vaccinated women (Paper II)

- In 2013-2014, oral HPV prevalence was low at a Stockholm youth clinic as compared to previous data from the same clinic (1.4% as compared to 9.3% in 2009-2011) (Paper III)

- Cervical HPV 16 and 31 were significantly less common in vaccinated than in non-vaccinated women in both Paper III and Paper IV. HPV 70 was significantly less common only in the first, and HPV 6 was less common only in the second of the two studies (Paper III and Paper IV)

- HPV DNA was rarely detected in hypopharyngeal cancer from the Stockholm region and the prevalence remained stable over time (Paper V)

- Overexpression of p16 was not a reliable surrogate marker for HPV in these samples (Paper V)
6 ACKNOWLEDGEMENTS
Many thanks to my main supervisor Tina Dalianis for taking me into your lab and for truly caring about us students! Thank you, Torbjörn Ramqvist, for many good discussions and for all your help in the lab. Anders Näsm, thank you for introducing me to the lab and for helping me finish this thesis. As your first PhD-student, I hope I have made you proud!

Thank you Hanna Dahlstrand for being a wonderful mentor! I felt well looked after.

Many thanks to the rest of my “lab-family” for being there, brightening up every day. Some of you have moved on, some of you are still around, wherever you may end up, I sincerely hope that our paths will cross again.

Juan and Mircea, the first lab members I met- thank you for taking such good care of me when I was new and slightly lost (I’m also grateful that we never actually started work at five in the morning!). Thank you, Mathilda, for all your help in the lab, and for always having such a positive energy. Many thanks also to Lisa for supporting me when things were feeling difficult.

Thank you, Cinzia, for reading my thesis and giving me many helpful comments. You are very knowledgeable and I am very glad you decided to join us.

Thank you, Nikos, for being a friend and a source of inspiration; you make me want to be brave. See you at Zumba practice! Cecilia, I had a great time working with you. Thank you for always being there to share your knowledge in the lab and for all the good times in-between. Linnea, thank you for all the fun we had working together. Thank you also for all the dancing! When we are two people dancing in the corridor, it makes it less weird. Lars; a voice of reason. Thank you for always staying calm and collected, it helps when things are chaotic.

Joar, you make me feel somehow nostalgic about spinning down mouthwashes… Thank you for being a wonderful adoptive student.

Leila, I really admire your confidence and integrity. I also want to thank you for having such a wonderful work ethic; I knew I could always rely on you to get things done.

Andreas, your enthusiasm and creativity is truly inspirational. Thank you for all the fun!

Wilbert, Sandra, Jana, Stefan, Sara, Dairy, Georgia, Marlene, Jake and Susan, thank you for making the time in the lab a pleasant one. The lab wouldn’t be the same without the students.

Ruku and Michael, thank you for all the nice conversations, I’m happy I got to know you guys!
Eva, Andrea, Lalle, Linda and David, thank you for your dedicated work in the clinic and for strengthening the bridge between science and clinical care. Your work is very important.

My colleagues at other universities: Thank you, Tanja, Elisabet, Magdalena, Margareta, and Andreas, for a good collaboration.

Many thanks goes out to the wonderful staff at the Stockholm youth clinic. Kicki, Ingrid, Ingela, Lina and Sofie, without you this work wouldn’t have been possible.

I would also like to thank the rest of my coworkers at the department for making my time at CCK such a nice experience.

Thank you Adam, for a most valuable piece of career advice, and for always being kind and helpful!

Rolf, Yago, Mao, Maarten, Yuya, Tanja, Maria, Maria, Ulrika, Laura, Andreas, Veronika, Kristina, Dhifaf, Rosa, Caroline, Hannah, Jeroen, Charlotte, Tatjana, Majken, Emma, Pradeepa, Hákan, Mohammad, Ali, Amir, Barbro, Kia, Fariba, Lars, Ann, Elle, Elisabeth, Sofia, and the rest of the first floor: thank you for the fika and for making this the nicest floor in the building!

Erik and Matheus, many thanks for being great student representatives, I am happy to have had the opportunity to work with you both.

Many thanks also to my co-organizers at the various events I’ve been involved in at the department; Roger, Sophia, Emarndeena, Alessandro, Nikos, Sander, and Hanif, this place would be pretty boring without people like you!

Thank you, Inger Bodin, for being so helpful and knowledgeable, you make this building function!

Many thanks to the members of the members of the committee for equality; Anne, Yvonne, Pedram, Erika, Angelo and Daniel; keep up the good work!

I also want to thank some of my other sources of inspiration. Anita Bratt, and Olle Bergman, thank you for inspiring me to draw my own pictures! Many thanks also to Lars Flygar for making biology such fun!

Dagmar and Sandra, even if I ended up elsewhere, I am very grateful for your patients and for being wonderful teachers during my summer internships.
Thank you all former and present members of Kärleksakuten for your hard work and dedication. Without you, this thesis would never have been written. A special thanks to Sofie for putting up with me and helping me turn things around, you are a true fighter! Mahla, I will never forget you.

Klas, Anna, Anna, Leila, Marcus, Linnea, Erik, Susanne, Frasse, Anders, Andreas, Olof and the rest of the wonderful people joining us on game nights and other events. You keep me sane!

Bea and Hanna, we didn’t see each other as much in recent years, but the journey to this point didn’t start when I was registered as a PhD student. Who knows where I would have been without you.

Thank you, mamma, for listening to me when I have been stressed and upset. You are very strong and a true inspiration. Pappa, you helped me make sure that there was always food in the house, but more importantly, you have always been there to discuss all aspect of life, both big and small. Thank you both for always believing in me! Thank you, mormor, Nils, morfar and Ninni, you encouraged me to stay curious and creative, and you helped nurture my love for reading and learning.

Many thanks also to Henrik and the rest of the Hogland clan for the encouragement and all the cookies.

Nea, Robert, Weronica; my siblings. Thank you for always being there and for being a constant reminder of what matters most in life.

Thank you, Anton, for your patience and support over the years. Thank you for listening to my presentations even though it’s not even close to your field, thank you for joining me at work on Saturdays, and thank you for the compromises you have made. I’m looking forward to our next adventure!

El psy congroo.
7 REFERENCES


Alonso, I. *et al.* Does human papillomavirus infection imply a different prognosis in


137. Akoğlu, G. et al. Focal epithelial hyperplasia associated with human papillomavirus 13 and common human leukocyte antigen alleles in a Turkish family. Int. J. Dermatol. 54,


193. European Medecines Agency. HPV vaccines: EMA confirms evidence does not


208. Langhaug, L. F., Sherr, L. & Cowan, F. M. How to improve the validity of sexual


