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HUMAN PAPILLOMAVIRUS TEST AND VACCINATION – IMPACT ON CERVICAL CANCER SCREENING AND PREVENTION

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To friends and family

My aspirations wrapped up in a book

ABSTRACT

Human papillomavirus (HPV) is the world's most common sexually transmitted infection and its consequence cervical cancer is one of the world's most common cancer forms. Revolutionary advances in HPV testing and HPV vaccination have the potential to radically change women's health. However, several challenges remain before effective cervical cancer control can be reached.

This thesis has exploited the excellent Swedish register and biobank infrastructure, for studies aiming to inform HPV-based prevention of cervical cancer. To this end, we have combined epidemiological, virological and biostatistical investigation of the use of HPV-tests in organized cervical screening. We also investigated awareness of HPV and acceptability of HPV vaccination in the population.

Study I was a molecular epidemiological case-control study, nested within the Swedish cervical screening program, including 515 women with cancer in situ (CIS), 315 women with invasive squamous cervical cancer (SCC), and matched control women. 2772 archival cervical smears were gathered and subjected to full HPV-typing. The median follow-up was 5-7 years. We provide prospective evidence that infection with non-16/18 high-risk HPV types, and persistent infection with HPV16, both confer increased risks for future invasive cervical cancer.

In Study II, we extended this case-control study to include 621 women with CIS and 457 women with SCC; a uniquely large sample. Here, 5665 archival smears were tested for HPV, and HPV16 positive smears further analyzed for HPV16 viral load through realtime-PCR. The median follow-up was 6-8 years. We show that HPV16 viral load predicts risk for both CIS and SCC, but also that the risk functions differ per diagnosis and over time. Thus, HPV16 viral load appears highly complex which may limit its use in HPV-based cervical screening. We further show unexpectedly low viral loads early in invasive disease, which may carry implications for the weighing of sensitivity against specificity in HPV testing.

In Study III, we report results from a cross-sectional population-based survey examining awareness and knowledge of HPV in 24,513 adult Swedish respondents. We show that awareness of condyloma and cervical cancer was high, but that awareness and understanding of their causal factor HPV was poor. The knowledge that men could contract HPV needs to be improved. Education campaigns on HPV should particularly target young men, and those of low education.

Finally, Study IV was also a cross-sectional survey, examining acceptability of HPV vaccination among 10,567 young adults age 18-30 in Sweden. We show that willingness to vaccinate was quite high, but that information on the benefits of vaccinating before sexual debut is important. Few adults stated their health-care related behavior would change after vaccination, but a number were uncertain, suggesting an educational need when vaccinating this group. A perceived risk of side effects was the largest potential barrier to vaccination.

Our findings should assist risk stratification in HPV-based screening, and design of HPV vaccination campaigns. Future research should include investigations of cervical screening attendance, HPV vaccine uptake and acceptability of HPV-based screening.

LIST OF PUBLICATIONS IN THIS THESIS

I. Prospective study of human papillomavirus (HPV) types, HPV persistence, and risk of squamous cell carcinoma of the cervix.
Sundström K, Eloranta S, Sparén P, Arnheim Dahlström L, Gunnell A, Lindgren A, Palmgren J, Ploner A, Sanjeevi CB, Melbye M, Dillner J, Adami HO, Ylitalo N.
Cancer Epidemiol Biomarkers Prev. 2010 Oct;19(10):2469-78.

II. Prospective study of HPV16 viral load and risk of in situ and invasive squamous cervical cancer.
Sundström K*, Ploner A*, Arnheim Dahlström L, Palmgren J, Dillner J, Adami HO, Ylitalo N, Sparén P. * *These authors have contributed equally.*
Cancer Epidemiol Biomarkers Prev. 2012 Nov 15. [Epub ahead of print]

III. Awareness and knowledge of human papillomavirus in the Swedish adult population. Dahlström LA*, Sundström K*, Young C, Lundholm C, Sparén P, Tran TN. * *These authors have contributed equally.*
J Adolesc Health. 2012 Feb;50(2):204-6. Epub 2011 Jun 25.

IV. Acceptability of HPV vaccination among young adults aged 18-30 years--a population based survey in Sweden.
Sundström K, Tran TN, Lundholm C, Young C, Sparén P, Dahlström LA.
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ADDITIONAL PUBLICATIONS

(NOT INCLUDED IN THESIS)

Assessing perceived risk and STI prevention behavior:
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Submitted.

CONTENTS

LIST OF ABBREVIATIONS

AC	Adenocarcinoma
AIS	Adenocarcinoma in situ
CIN1-3	Cervical intraepithelial neoplasia grade 1-3
CIS	Cancer in situ
HPV	Human papillomavirus
HPV+	HPV-positive
HPV-	HPV-negative
HSIL	High-grade squamous intraepithelial lesion
LSIL	Low-grade squamous intraepithelial lesion
OR	Odds ratio
Pap	Papanicolaou (smear/test)
PCR	Polymerase chain reaction
RR	Relative risk
SCC	Squamous cervical cancer/squamous cell carcinoma
STI	Sexually transmitted infection
VL	Viral load

1 INTRODUCTION

Human papillomavirus (HPV) is the world's most common sexually transmitted infection (STI) and causes significant morbidity and mortality. Yet, it is probably the STI of which, until recently, the least had been spoken. One of its chief consequences is invasive cervical cancer: one of the most common cancers in the world and which, if unscreened for, frequently strikes women of a young age.

Since the introduction of a national organized cervical screening program, incidence and mortality in cervical cancer have been considerably reduced in Sweden. The evidence-based rationale for such screening is therefore very strong. However, the incidence of squamous cell carcinoma is no longer decreasing and adenocarcinoma of the cervix is increasing.

Fortunately, and perhaps at the turn of the tide, promising new HPV-based prevention methods have emerged. Ensuring the proper evaluation of, and equal access to, these new prevention methods should be an important ethical priority for public health research. If the full potential of these methods is realized, it might be achievable to reach the final, visionary goal: the eradication of cervical cancer.

It has been the goal of this thesis to exploit the excellent Swedish register and biobank infrastructure for studies aiming to advance the prevention of cervical cancer. To this end, it has combined epidemiological, virological and biostatistical investigation of
i) the use of HPV tests and HPV viral load in organized cervical screening and
ii) awareness of HPV and acceptability of vaccination against HPV. The work presented here therefore encompasses several aspects of cervical cancer causation and prevention; from the significance of viral etiology and molecular markers to societal and health behavioral research.

2 BACKGROUND

2.1 HUMAN PAPILLOMAVIRUS INFECTION

Papillomaviruses are a diverse family of viruses found in most mammals and birds, capable of causing epithelial tumors in humans and some related species. The name derives from the Latin word *Pailla* - a nipple-like projection and the Greek word *oma* - a swelling or tumor. The types found in humans are termed human papillomavirus (HPV) and constitute a large family in the human host; over 100 HPV types have been identified, of which 40 infect the genital tract. Many of these types have been shown to be ubiquitously distributed around the globe (1), and some appear to have become more common in the population (2, 3).

HPV transmission occurs primarily during sexual activity, and as such HPV infection is the world's most common sexually transmitted infection (STI). The prevalence in women is most common before age 30, but there is a second incidence peak later on in life. In men, however, the prevalence is more stable across all ages (4). Most infections are asymptomatic with most women (75-80%) infected with HPV at some time during their life, but longitudinal studies show that 90% of infections are cleared within one-two years (5). It is unclear whether HPV is sufficiently infectious to be transmissible during the entire duration of the infection(6). Oral HPV is mainly transmitted through oral sex or open-mouth kissing (7), whereas genital HPV is transmitted through skin-to-skin contact during sexual activity, and not the exchange of body fluids like in other (bacterial) STI's such as Chlamydia and gonorrhea. This means that protection conferred by condoms exists, but is not complete (8). Other transmission routes known for HPV include vertical transmission from mother to infant, a risk which is greater after vaginal than caesarean birth (9).

HPV has a particular affinity to so-called squamocolumnar junctions, where there is a transition between squamous and glandular cells in an epithelium. Such junctions are found in the cervix, anus and tonsils and HPV is also capable of residing on skin tissue, e.g. on the penis (5). In addition to this, HPV infections appear capable of site-to-site transmission (or auto-inoculation), meaning that a woman who is cervicovaginally positive may well become anally positive for the same type within a certain time interval (4), and indeed, anal HPV infections are common in young women (10).

HPV types are traditionally divided into low-oncogenic-risk types and high-oncogenic-risk types – which will below be referred to as low-risk (LR-HPV), and high-risk (HR-HPV), respectively. This since the different types exhibit different disease-causing characteristics. The most noted low-risk types, where cancer risk is negligible (11), are HPV6 and HPV11, responsible for 90 percent of genital warts, and causative of the rare disease recurrent respiratory papillomatosis (RRP) (12). Around 13-15 HR-HPV types have been identified, the most noted of which are HPV16 and HPV18, together held accountable for 70% of cervical cancer tumor cases and are as such the primary targets for globally intended vaccines (see further below). HPV16, which has been described as "the major player" (13), occupies a particularly important place in the HPV-related disease pantheon due to its strong carcinogenicity.

Apart from viral genotype, it is now understood that persistence of infection is a main determinant of cancer risk in HPV-infected women, although only 10-30% of infections will persist beyond 2 years (5). Persistence of HPV infection is defined as infection detected a number of years (such as 4-12 years) after incident infection, and it appears that long-term persistence of HR-HPV without development of cervical disease is less common than previously thought (4). The potential for latency, re-activation and/or re-infections with HPV is still however not fully understood (14), although repeated infections appear common in young women (15).

HPVs are small round particles that consist of around 8 kilo-basepairs' worth of circular double-stranded DNA wrapped in a protein shell composed of two capsid proteins (L1 and L2). Copies of these shell particles are used in vaccines to induce immunity to HPV (see below). Three oncogenes, E5, E6 and E7, modulate the virus transformation process and two regulatory proteins, E1 and E2, regulate transcription and replication. Together, these replicate the viral DNA and assemble newly produced viral particles within infected cells (11). The E1, E2 , L1 and L2 genes are particularly well-conserved and HPVs are genetically very stable; sequence changes by mutations or recombination are very rare events (1). HPV infections are exclusively intra-epithelial, and in the cervix infect basal cells, probably through abrasions in the epithelium. HPV then uses the natural differentiation of the epithelium to procreate, ultimately shedding complete virions while back up at the cervical surface (11).

Normally, this process and the activities of E6 and E7 are tightly controlled, but in persistent infections the probability of molecular accidents deregulating the oncogenic expression in mitotically active cells can increase (13). Briefly, the neoplastic potential in E6 and E7 oncogenes is due to their ability to interact with human key cell cycle control regulators p53 and Rb. Constant E6 and E7 activity therefore confers an increasing genomic instability, loss of cell-growth control, and ultimately cancer. During progression, the viral genome frequently integrates into the host genome, thereby aggravating the de-regulation of oncogenes since the regulatory E2 gene is often lost (11).

2.2 CERVICAL SCREENING AND CERVICAL CANCER

Infection with HPV was known, but regarded as trivial until the 1970's, when it was gradually discovered that mild dysplasia of the cervix exhibited the cytological and histological features of papillomavirus infection. In connection with this, two novel HPV DNA types were cloned from genital warts and classified as HPV6 and 11 (13). HPV11 DNA was then used by the zur Hausen group to the revolutionary discovery of HPV16 and 18 in cervical cancer biopsies, which gradually led to the dawn of a new understanding that HPV was responsible for a considerable disease burden.

With an estimated half a million new cases and more than a quarter of a million deaths each year, cervical cancer is the third most common cancer among women globally (16). The median age of diagnosis is 57 years of age, compared to 72 years for other cancers in the U.S. (17) and cervical cancer, more than other major cancers, affect women under 45 years (18).

Globally, the incidence varies between <3 to >50 cases/100 000 women for low- and high-burden countries, respectively (18). The annual incidence in Sweden is 9/100 000 women, translating to around 460 cases, and 150 deaths each year due to the disease (19), despite the presence of an organized screening program with cytological (Pap-) smears. Following screening, the overall decrease in cervical cancer incidence has been ~70% over a 40-year period (20) but the incidence is no longer decreasing (19).

As stated above, persistent HPV infections can lead to malignant transformation of the cervix uteri, of varying severity. These lesions are termed cervical intraepithelial neoplasia grade 1-3 (CIN1-3) and may, similarly to HPV infections, either resolve spontaneously or progress to invasive cancer. This is a process usually spanning over decades but examples of more rapid progression are also known. Exfoliated cells from the different precancerous CIN stages can be detected through microscopic examination of a cytological smear using the Papanicolaou stain to dye cells – hence the vernacular term "Pap test". The detection and subsequent treatment of CIN abnormalities forms the basis of the cervical screening programs that have substantially decreased the incidence of cervical cancer in industrialized countries (21).

The focus in the cervical program is early detection of these precancerous changes, not cases of actual invasive cancer (although this is a desirable second priority) (22), hence programs will here mainly be termed "cervical screening" rather than "cervical cancer screening". Therefore, young women are invited already at the age of 23, and screening continues until age 55-60. Participation in Sweden is around 80%, although slightly lower in those age 23-25 (65-70%) and 26-30 (~75%) (23). An individual smear can have a relatively low sensitivity, but this increases with repeated testing and therefore, three-year intervals in the program are recommended (24). In the smear; cells are judged according to their appearance as either normal, or abnormal of a varying degree. Abnormalities can be detected either in cells of squamous epithelial origin, or glandular origin. A combination of squamous and glandular cervical cytological abnormalities is possible. A cytological diagnosis which has gathered more notoriety in the past few years is atypical cells of undetermined significance (ASCUS), not the least since a seminal trial showed that the underlying prevalence of CIN3 in this group was 5% (25). Diagnosis of a cytological abnormality will typically lead to examination with colposcopy (macroscopic examination of the cervix via a gynecological exam), and concurrent biopsy (tissue sample) from the cervix. Every year, around 20 000 Swedish women receive an abnormal screening result which requires some sort of further investigation, and around 3300 women get a diagnosis of cervical cancer in situ (19).

The ultimate potential consequence of an abnormality of squamous origin is a diagnosis of squamous cervical cancer *in situ* (CIS; where *in situ* refers to the cancer on histological examination not having broken through the basal epithelium) and invasive squamous cervical cancer (SCC). The ultimate potential consequence of a glandular abnormality is cervical adenocarcinoma *in situ* (AIS) and invasive cervical adenocarcinoma (AC). Thus, cervical cancer as a diagnosis is constituted by both SCC and AC, and although SCC is significantly more common, AC is increasing in the population (26).

This could be because AC is more difficult to detect on a traditional cytological smear since such cells derive from above the squamocolumnar junction, or transformation zone, in the cervix. This zone retracts further up the cervix as a woman ages, and thus becomes more difficult to reach with representative cytological sampling (27). However, no difference in cure proportions between the two diagnoses has been observed (22). The extent to which SCC and AC is caused by HPV has been the focus of numerous studies (26, 28). Attributable fraction in cervical cancer is traditionally calculated as the proportion of incident tumors which are HPV-positive, and for which HPV type (29). Using the traditional method, the attributable fraction appeared to be lower for AC than for SCC (27), although this could be due to artifactual explanations and more recent studies have detected similar prevalences of HPV in AC as in SCC (30, 31).

Treatment of colposcopically and histopathologically verified CIN means removal of the lesion by preferably excision (since this yields a histopathological verification of radicality and nature of the lesion), but in some cases destructive techniques such as laser vaporization can be acceptable (32). Of note, women who have been successfully treated for CIN3/CIS still have a 25-year long increased risk for invasive cancer. Therefore, recommendations are to pay extra heed to these women (33). Treatment options for invasive SCC include the fertility-preserving operation trachelechtomy, which involves removal of the cervix and part of the vagina, but leaves the rest of the uterus intact. In more advanced cases, total hysterectomy and/or radiation and/or chemotherapy are needed (34).

2.3 CANCERS IN SITES OTHER THAN THE CERVIX UTERI

With its affinity for multiple anatomical sites, HPV has been implicated in several other cancers than cervical: chiefly anal, oropharyngeal, penile, vaginal and vulvar cancer. Around 90% of anal cancer and a smaller subset (<50%) of these other cancers are attributed to HPV. If this is correct, HPV would be accountable for more than 5% of the entire global cancer burden – the highest of all infectious agents (5). In several of these other cancers, it does however appear that there may be several parallel etiological tracks to disease. In oropharyngeal and vulvar cancer, for example, a substantial proportion of tumors are HPV-negative. HPV-negative disease in these cases is associated with factors such as a higher degree of smoking, alcohol drinking and higher age, whereas HPV-positive disease is associated more with sexual habits. Interestingly, the prognosis for non-HPV related disease appears worse in oropharyngeal cancers, for reasons that are not entirely understood (35). Therefore, some consider HPV-positive and HPV-negative tumors in these sites as different cancers (36, 37).

Although these diseases are often substantially less common than cervical cancer, and not exclusively HPV-related, several of them afflict both women and men, and are thus potentially responsible for significant morbidity. Several of these cancer forms are also increasing in the population, such as anal cancer where a steep increase in incidence has been observed in both genders (38). In addition, for these other cancer forms there are no functioning screening programs, meaning that a viral etiology would be particularly important to identify, since such findings could lend support to mass vaccination.

2.4 OTHER HPV-RELATED DISEASE

As stated above, low-risk HPV types (LR-HPV) have negligible carcinogenic potential, but nevertheless are common and capable of causing a large morbidity burden in humans. A relatively common consequence of LR-HPV is condylomata acuminata (also called condyloma or genital warts), which has an incidence peak similar to other STI's, i.e. in those age 15-24 years (39). Incidence rates in Sweden in 2007 were estimated at around 400 cases/100 000 for both men and women age 10-44 years (40). In a population-based study from the Nordic countries, 1 in 10 women had experienced condyloma before the age of 45, with an increasing occurrence in younger age cohorts (41). Condyloma is a self-limiting disease and will frequently not cause too much trouble, yet can lead to painful symptoms as well as stigma and shame in the patient. Some women and men have severe problems and may require pharmacological and/or surgical treatment of the warts. Therefore, although benign, the condition is associated with significant costs to society (41).

Condyloma has been linked to subsequent risks for HPV-related cancers in several studies (42, 43). Obviously, this risk association could be confounded by several different risk factors, above all concurrent infection with HR-HPV types, the status of which is unknown in register-based studies (42). In fact, in molecular epidemiological studies that have had access to data on both LR- and HR-HPV status, an inverse association has been seen. Recently, HPV6 was associated with an antagonistic effect on the risk for cervical cancer conferred by HPV16 (44) in a seroepidemiological study. Whether this is a true biological effect, or caused by detection bias (due to women with condyloma being genitally screened more often and potentially discovered more frequently in the pre-cancerous stage) remains to be investigated.

2.5 RISK FACTORS FOR CERVICAL CANCER

Since most women will be exposed to HPV at some point in their life, yet only few develop persistence of infection and subsequent serious cervical disease, other factors are likely to play a role in the pathogenesis. Risk factors for (squamous) cervical cancer, apart from HPV infection, traditionally list early age at sexual debut; a large life-time number of sexual partners; use of hormonal contraceptives; high parity, smoking and other STI:s (44, 45).

The challenge is to determine to what degree these risk factors possess an inherent capacity to actually induce cervical cancer, or if they are actually advanced proxy measures for the risk for present and/or past HR HPV infection. For example, the risk associated with hormonal contraceptives could be explained by a hormonal influence on the cervical mucosa (thus rendering it more susceptible to persistent/progressing infection). It could also be related to a higher risk for HR-HPV due to a concurrent tendency to A) use fewer condoms since already on contraception otherwise and B) being more likely to have an active sexual life than those not employing contraception. Therefore, it is important to adjust for information on these factors as well as possible, and in large meta-analyses, an association to contraceptive use has still been demonstrated (46).

The association between early age at sexual debut (typically before the age of 14) has likewise been an area of debate. Option A) could be that early age at sexual debut is correlated with other risk-taking behavior later on in life, for example a tendency to practice less safe sex (47). Option B) could be that women who start having sex early have more sexual partners, thus increasing the risk of contracting high-risk HPV (48). Again, a combination of A) and B) may exist. Yet another, very interesting, option C) could be that early age at sexual debut leads to early-life HR-HPV infections, that have the potential to last so long as to cause carcinogenesis to a larger extent than infections acquired later on in life. In fact, if this theory holds; it leads to convenient, if perhaps controversial, implications for HPV vaccination (45), which will be discussed further below.

Smoking has been established as a risk factor for cervical cancer in several studies, including large meta-analyses adjusting for the other factors mentioned here (49), and a synergism between smoking and HPV16 status has been shown (50). Proposed mechanisms include local genotoxic effects in the cervical epithelium, stimulation of carcinogenesis, or localized immunosuppression. Furthermore, several studies have noted a correlation between *Chlamydia trachomatis* and persistent HPV or risk of cervical cancer (44, 51). Finally, high parity has been shown to be a risk factor in developing countries, however this risk is most apparent if comparing >7 births to 1-2 (52), meaning that few women in developed countries will be affected.

So far, knowledge of these risk factors has not led to any major policy implications since several are difficult to modify, and confer only modestly increased risks compared to the risk associated with HPV. In the future, in the context of universal vaccination, they will likely diminish further in importance, unless vaccination fails to reach high-risk groups.

2.5.1 Is heredity a risk factor for cervical cancer?

Apart from viral facors, several host genetic factors, such as HLA-haplotype, might play a role in the cervical disease process (53). However, despite cervical cancer being so common, few reports exist on familial clustering of the disease. In the few reports there are, the observed association may be due to shared exogenic risk factors and lifestyles, shared genetic risk factors, or a combination of these. Several studies have therefore utilized family-based designs, and did find increased relative risks for first degree relatives of affected women (54). A Swedish study found a significantly increased risk of CIN3 and cervical cancer to biological, but not non-biological, first degree relatives of women with cervical tumors (55). These findings have however not resulted in any larger health policy changes so far.

2.6 AWARENESS OF HPV

HPV infection is nearly ubiquitous, yet paradoxically the symptoms are often few and most women will never know they had it. Despite the scientific community's understanding of its role in cancer, until relatively recently HPV was not very actively spoken of with women, since there was no means for treating the infection except screen for its consequence cancer.

There may have been a reluctance to discuss the sexually transmitted nature of the cancer, to avoid stigmatizing women and causing feelings of self-blame. In qualitative studies, the mention of the relationship between an STI (HPV) and cervical cancer has indeed been shown as associated to shock among study participants (56, 57). This since the information seems inconsistent with the view of cancer striking an unfortunate victim “out of the blue” (58).

In 2007, only 2.5% of a British population-based sample of women could identify HPV as the cause of cervical cancer without being prompted, a figure that had not improved much from 2002 (when it was virtually non-existent at 0.9%) (59). In 2008, the Nobel Prize in Physiology or Medicine was given to professor Harald zur Hausen for his pioneering work in the field and the publicity generated from likely helped illuminate the public (at least in Sweden). Further discussion has been generated by the introduction of school-based vaccination, where parental consent is needed. However, obtained study rates of awareness are highly sensitive to the phrasing of questions and selection of populations. Depending on study question and selection of the sample, a wildly varying range of HPV awareness of between 13-93% was shown in a review in 2008 (60). Understanding of the HPV-condyloma and HPV-cervical cancer links was likewise inconsistent (0.6-68%, and 5-83%, respectively). More updated reviews still show awareness rates of between 5-66%, which means firm conclusions on the state of HPV awareness are continuously difficult to draw (61). Although it appears likely that overall awareness should have increased, there still appears to be a definite lag of public understanding behind rapidly advancing science (62). More population-based studies on these factors in both genders would be valuable.

Clearly, along with the development of HPV-based prevention of cervical cancer, new information horizons have dawned and need to be actively and correctly communicated to women and men. This would be important not the least in the context of HPVtesting, where care needs to be taken to give correct information, while at the same time ensuring that it does not raise unnecessary anxiety (58).

2.7 HPV TESTS

Ever since the etiological role of HPV in cervical cancer was established, the potential to screen women for cervical HPV (the agent) instead of cervical cytological abnormalities (the symptom) has been recognized. HPV testing has long been proposed to replace the cytological smear as a primary screening tool – one reason is that identification of HPV-negative women allows reduced screening for this low risk group. HPV molecular testing therefore determines the need for screening, allowing a personalized medicine approach in programs which hitherto have been relatively agnostic of a woman’s past history.

HPV-negative women may safely extend their screening interval to six years, instead of the current 3-5 years that apply for everyone regardless of HPV status (63). Recent data indicate that the protection conferred is valid for up to 14 years (64). Also, HPV tests have repeatedly been shown to possess sensitivity rates superior to those of cytology (65-67).

However, it is important to use HPV-based screening in appropriate age groups since transient HPV infection is so common among young sexually active women, and the test specificity for underlying lesions must be acceptable. The current evidence from randomized trials favors cytological screening until approximately age 30-35, and then a switch to HPV-based screening (68, 69). However, the trial results have not yet been transferred to clinical practice and at present HPV-tests are mainly used for triage (risk-grading) of cytologically abnormal women (70, 71). The fact that most of Sweden has switched to performing cytology using liquid-based cytology (LBC), where the same sample can be used both for HPV typing and cytology, will greatly facilitate a randomized implementation of HPV screening and allow real-life studies of policy effects (71, 72).

With the advent of HPV tests used as a triage tool in screening, indeed in the future as a primary screening method; improved tools for risk stratification of HPV-positive women are crucial. The infections are very common with approximately 10% of cytologically normal women being positive for HPV at any point in time (73). It has been estimated that almost 300 million women worldwide are carriers of HPV DNA (73). Clearly, separating the few HPV-positive women at high risk for cervical cancer from the many with smaller risk will become a critical issue. Studies with long-term follow-up addressing particularly the issue of persistence of infection have been called for (74, 75). Also, in order to properly assess primary prevention strategies in a country such as Sweden, nation-specific data on the epidemiological variation of HPV are needed (76). Such data can provide vital clues to inform both HPV vaccination and HPV-based screening in Sweden.

2.7.1 Adjuncts to HPV testing

Currently, the Hybrid Capture II (HCII), GP5+/6+, Cobas 4800 and Abbott hrHPV PCR can be considered clinically validated assays for use in primary screening, but the drawback of lower specificity in HPV testing still needs to be addressed. Reflex testing with cytology is an effective triage method, as cytology in HR-HPV positive women clearly stratifies the risk of CIN3+ and cancer (67). Another established risk stratifier is genotyping for HPV16/18, which has a good evidence-base in terms of predicting 10-year cumulative risk for CIN3+ (17% for those HPV16-positive, 14% for those 18-positive, and 3% for those positive for other high-risk types than 16/18) (77). Other candidate markers are testing for viral RNA, p16INK4a immunohistochemistry or p16INK4a i67 staining (78). However, these are not yet established and should first show the same level of evidence as demonstrated by original HPV DNA screening tests (67).

2.7.2 Viral load

A special potential adjunct to HPV testing is viral load, which is the amount of viral DNA copies that can be detected in a cervical sample, typically normalized to the human DNA content in the sample. Applying a quantitative measure of HPV infection could present an attractive option in screening, if a reliable and consistent risk association with severe dysplasia could be shown. Indeed, in the year 2000, a seminal Swedish study was published using a nested case-control design capable of showing such an association for an assay measuring type-specific HPV16 viral load (79).

Initially rebutted by concerns that the relation was confounded by differing classifications of abnormal cytology (80), nevertheless multiple studies went on to show similar results in varying study designs and settings (81). A high viral load of HPV16 has since been established as a prognostic factor for cervical lesions (82-84). It is best measured through quantitative real-time PCR assays (85) although semi-quantitative methods have also been used via HCII. It is conceivable to use both high viral load as a disease marker for women at increased risk for cervical disease, but also low viral load as a distinguishing sign of HPV infection at low risk of progressing (86).

The clinical use of viral load is complex but if those limitations could be overcome, the technology could offer the benefits of increased specificity and objective, quantitative input to screening and management (87). However, there is a paucity of risk prediction data for invasive cancer. Some studies have shown conflicting results; viral loads in invasive cancers have either been quite low (88) or very high (89). The studies were also very small; only 11 and 22 cases, respectively. Another study showed no additive value of HPV16 viral load but only analyzed 77 cases of CIN3/invasive cancer combined (90). Larger studies are needed to inform this issue.

2.8 HPV VACCINATION

Along with the realization that HPV could serve as a screening tool, it was also proposed that prophylactic HPV vaccination potentially could reduce significant amounts of HPV-related disease. After much method-development, today this proposition has become a reality. Two HPV vaccines are approved for clinical use: the bivalent vaccine Cervarix (GlaxoSmithKline) and the quadrivalent vaccine Gardasil (Merck and Co., Inc.). The vaccines are based on the virus-like particle (VLP) technology, where viral genes encoding surface proteins are used to produce empty virus shells, capable of inducing effective immune responses without any infectious or malignant potential. Both the vaccines are aimed at HPV16/18 for their predominant role in cancer. Both have shown virtually complete protection against HPV16 and 18-related precancerous lesions in HPV-negative individuals participating in randomized clinical trials with a follow-up time of up to 5 years (91, 92). Gardasil also protects against HPV6 and 11, which cause 80-90% of condyloma (91).

Even when including previously exposed subjects (who were seropositive indicating past infection, but negative for cervical HPV DNA indicating no ongoing cervical infection), vaccination has shown protective effects in those up to age 25-26 (91, 92), and to some degree even up until age 45 (93). The bivalent vaccine has also shown significant cross-protective efficacy against HPV31, 33, 45, and 51, and against anal HPV infection (94, 95). In the subset of women with signs of ongoing infection at time of vaccination (i.e. who are both seropositive and DNA-positive), no protective benefit on high-grade cervical lesions is derived (96). However, recent data indicate that the quadrivalent vaccine may confer protection against recurrence of HPV-related disease in women who had surgical treatment for such, but the mechanism behind this is not understood (97).

It should here be noted that no effect on invasive cervical cancer has as yet been shown, and likely will take some time to achieve due to the long incubation of the disease.

Meanwhile, the shorter incubation disease CIN3 is the best surrogate endpoint we have. Although it is not perfect (4), CIN3 is a true precursor to invasive cancer and has an innate considerable propensity to progress to invasive disease (98). As such, CIN3 or worse (CIN3+) has been accepted by regulation agencies as an acceptable study outcome.

Also note that the long-term duration of protection with HPV vaccines, and need for boosters, is quite unknown, and there is a lack of validated immunological measures of immunity. On a speculative note, however, if only early-life infections are relevant in cancer risk, a lifelong protection against HPV might not actually be necessary (45).

The Swedish National Board of Health and Welfare decided in 2008 that HPV vaccination should be included in the national vaccination program, and administered to girls age 10-12 (99). The public tender was initially won by Cervarix, but after a complicated tender process, a new tender was won by Gardasil which will be used for the next few years in Sweden. Opportunistic vaccination has been available since 2006 and was subsidized for girls aged 13-17 2007-2011. A free catch-up vaccination program is now available for 13-18 year olds. Approximately 130 000 Swedish females have been vaccinated with Gardasil, and only very few with Cervarix (100). Most of them have been within the 13-17 year interval but several thousand older women have also elected to get vaccinated. Recently, subsidized vaccination was made available for women until age 26, and Stockholm County Council will offer it for free until this age.

HPV-vaccinated young adult women will have a continued need for screening, as sexually active women may have been exposed before the vaccine was given and effectiveness is reduced (96). Screening programs specifically for HPV vaccinated women will therefore need to be designed, and women's continued participation needs to be monitored. Also, about 30% of all cervical cancer is caused by HPV types other than HPV16 and 18, and although cross-protection appears to exist, complete protection is not guaranteed. Second generation HPV vaccines that contain numerous HPV types may offer a more complete cervical cancer protection, but data from such trials are not yet available.

In men, vaccine efficacy has been studied for the quadrivalent vaccine, and high efficacy against external genital lesions has been shown, although most of the prevented lesions were condyloma, rather than penile lesions (101, 102). In a subgroup study among men who have sex with men (MSM), efficacy was also high against anal intraepithelial neoplasia. However, penile and anal cancers are rare and will require long follow-up studies to determine vaccine protection (102). Modeling studies have predicted that the greatest benefit of HPV vaccination will be achieved through vaccination of girls alone, but these models are sensitive to assumptions such as whether only cervical cancer incidence, or other HPV-related disease, is also considered (102). It has been estimated that introducing a male vaccination program of similar coverage to that in women (71-78%) would be expected to decrease new HPV infections with an additional 24% by 2050, with an estimated additional long-term reduction in male HPV-associated cancers of 6-8% (103). However, where female uptake is low, it has been shown that protection against cervical cancer might be improved by concentrating on improving uptake in females rather than males (104).

A special case is MSM populations, where anal cancer is more prevalent than in heterosexual men, and where herd immunity derived from female vaccination is less likely to have a large impact. In MSM, HPV vaccination might be more cost-effective under certain scenarios (105).

2.8.1 Efficacy versus effectiveness studies

Randomized trials can be capable of demonstrating technical vaccine efficacy required for the introduction of new interventions, but they employ several selection criteria, and are often not fully reflective of real-life situations in which the intervention will actually be implemented. Studies of real-life vaccine effectiveness, and safety, is therefore a key component of the evaluation of HPV vaccination post-randomized trials. A spectrum of intermediate outcomes is available for monitoring of the early HPV vaccine impact, where condyloma forms a significant part, as the shortest incubation-disease available (106). Four years after HPV vaccination implementation, ecological studies have shown dramatic declines in both HPV prevalence and incidence of condyloma in Australia (107, 108).

A recent Swedish study using a more advanced study design showed high effectiveness against condyloma in vaccinated women, but only if vaccinated at younger than 22 years (100). Declines in heterosexual men have also been shown in Australia, but no declines among adults >30 or homosexual men (109). The partial protection in non-vaccinated heterosexual men, probably conferred from having sex with vaccinated women and thus hindering spread, is commonly referred to as “herd immunity” in the HPV field, although it can be noted that this term in other contexts signifies the *total* protection from disease in non-vaccinated, implying having achieved a threshold of immunes above which no infection can persist in the population (110).

2.8.2 Cross-protection or type replacement?

A commonly mentioned concern in discussions on vaccination is whether the large-scale removal of HPV16 and 18 will leave an empty ecological niche, ripe for the take-over of other HR-HPV types now free to cause cervical cancer instead. For this to occur; two factors should be fulfilled. Firstly, there should be evidence of competition between HPV types, whereas in reality, multiple infections are common and no clear signs of type competition are apparent (5). Secondly, there should be no cross-protection of vaccination, whereas on the contrary, quite extensive such has been observed (94). Thus, in combination with the knowledge that HPV has a very slow evolutionary process, the risk for type replacement in cervical cancer appears low, although the best way of proving this will be monitoring of cervical cancer incidence, with full HPV typing of incident tumors.

2.8.3 Acceptability of HPV vaccination in different groups

Regardless of technical advances in vaccine development, unless the target population is accepting of the intervention, no success will be reached in vaccination programs. Therefore, studies into the acceptability of HPV vaccination are called for.
As in studies on awareness, the degree of acceptability is sensitive to setting, and thus, population-based studies should be a priority to improve generalizability of the results.

In a population-based study on Swedish parents to girls and boys age 12-15, we found that although awareness of HPV was low, the willingness to vaccinate one's children against it was high at around 75% if the vaccine was for free.
Vaccine safety was the most important concern (111). In line with this, a review found that most parents were positive towards HPV vaccination, if effectiveness was perceived as high, a physician recommended it, and HPV infection was perceived as likely (112). This review further found lower education to be associated with higher hypothetical acceptability among parents (a finding which concurs with ours) but also identified some potential controversies in HPV vaccination (112).

Even though vaccination of girls before sexual debut is acknowledged as the first priority for national vaccination schemes, young adult women and men could also benefit from vaccination, as described above. Reviews in young women have found that acceptability overall is high (at around 50-96%), if adequate information is given, HPV infection is perceived as likely, and the cost is affordable (112, 113), but that more data from developing countries are needed (114). In men, despite low awareness of the virus, some or most have been willing to vaccinate, with acceptability between 33%-78% depending on the selection of the sample. Recent data on English school boys reported 41% were interested in receiving the vaccine (115). Acceptability has been reported to be higher in MSM than in heterosexual populations (116, 117), at around 75% whether HIV-positive or not (118). A large majority of these studies in adults are however based on selected convenience samples: in a recent review, less than 25% were population-based (119). Therefore, caution should be exercised when reviewing the results, and more population-based studies appear called for.

2.9 ANTICIPATED EFFECTS OF HPV VACCINATION

In summary, organized HPV vaccination programs are today believed to result in reductions in the following infections and diseases around the globe:

- Incident infection with HPV16/18
- Incident infection with HPV31/33/45/51
- Cervical intraepithelial lesions (CIN) and cervical cancer
- Anal intraepithelial lesions (AIN) and anal cancer
- Vulvar intraepithelial lesions (VIN) and vulvar cancer
- Vaginal intraepithelial lesions (VaIN) and vaginal cancer
- Penile intraepithelial lesions (PIN) and penile cancer
- Condyloma

For recurrent respiratory papillomatosis and some head and neck cancers, particularly tonsillar cancer, it is as yet unknown whether HPV vaccination will lead to decreases in oral HPV infection and subsequent development of these diseases, but some observations suggest that a protective effect might be plausible (12). The potential for virtually complete protection against cervical carcinoma in situ/CIN3 in unexposed individuals means that, under optimal conditions, in the future such a large proportion of both HPV, and cervical disease, could be removed from the population that it might be possible to speak of *eradication*. However, this currently remains a visionary goal, and we do recognize the great length of the road that still lies ahead.

3 AIMS OF THE THESIS

The aim of this thesis has been to inform cervical screening practices in the era of HPV testing, to assist in estimating the effects of HPV vaccination, and aid in the development of HPV vaccination campaigns. To this end, it has involved the following studies:

Study I: A molecular epidemiological case-control study on the risk profile of HPV infections, and the relative contribution of different subtypes of HPV to *in situ* and invasive squamous cervical cancer.
Study II: A molecular epidemiological case-control study on the significance of high viral load of HPV16 as a risk factor in the development of *in situ* and invasive squamous cervical cancer.
Study III: A cross-sectional survey examining awareness and knowledge of HPV in the adult Swedish population.
Study IV: A cross-sectional survey examining acceptability of HPV vaccination among young adults in Sweden.

Limitations

Some limitations to this thesis work should be acknowledged when reviewing these studies.

- The first two studies are restricted to squamous cervical cancer; adenocarcinoma was excluded as the two histological types exhibit different characteristics.
- All studies were based in Sweden, a highly industrialized country with an established organized screening program that has national coverage and is actively offered to all women of screening ages. Therefore, the results have been found in a relatively privileged society where screening functions well and cervical cancer is uncommon, which may impact for example acceptability of HPV vaccination.
- Furthermore, Study I and II use the screening program as a sampling frame, thus by design excluding women that do not participate. Since non-adherence to screening recommendations is a major risk factor for cervical cancer morbidity and mortality in Sweden, strictly speaking this strategy might mean excluding a risk group. However, in the study of screening tests such sampling is necessary and does not constitute selection bias.

Note on funding:
Study I and II were financed by grants from the National Institutes of Health, and the Swedish Cancer Society. The data collection in Study III and IV was funded by Sanofi Pasteur MSD, Sweden. All research took place independent from the study sponsors.

4 METHODS

4.1 EPIDEMIOLOGICAL STUDY DESIGN AND ANALYSIS

The goal of medical epidemiological research is frequently to make a statement about cause and effect in a given scenario; we aim to identify a cause of illness, to enable us to prevent or treat disease. Obviously, this requires a degree of causal inference so that we are reasonably sure of which clinical decisions on prevention or treatment to make. The best method to establish causality is often considered to be the randomized study, where subjects are randomly assigned to an exposure or not (typically a randomized clinical trial – RCT - exposing participants to a medical treatment or a control intervention). The incidence of outcome (typically disease or mortality) can then be studied among exposed and non-exposed, respectively, and if there is a difference, causality can be attributed to the exposure given all other things equal.

However, in many scenarios, randomized studies are unfeasible, and/or unethical. The exposure of interest may not be a pharmaceutical, but instead viruses, environmental factors, diet, physical activity etc. We can naturally not randomize women to either be infected with different types of HPV, or not, and then determine who gets cervical cancer. In such scenarios, we frequently rely on observational (i.e. non-experimental) epidemiological studies instead, although they cannot prove causality (120). As this thesis utilizes observational epidemiological designs, some basic characteristics of these, and how they are analyzed, are discussed here.

4.1.1 Cohort, case-control and nested case-control studies

A *cohort study* describes the experience of a group of individuals followed over time, where both exposure/-s and outcome/-s are recorded continuously. The incidence of disease can thereby be studied over time, enabling calculation of the incidence rate (IR) at different time points. The incidence rates can then be compared in exposed and non-exposed groups, respectively, and an **incidence rate ratio** (**IRR**) can be calculated. Typically, this will be interpreted as a **relative risk** (**RR**) – the risk for the outcome in those exposed, compared to the risk in those non-exposed. The advantage of a cohort study is that several different measures of outcome can be calculated, and that the study numbers can enable the study of rare exposures. However, if the outcome is rare, even large cohorts will struggle to assess that outcome effectively due to a low number of cases accrued.

For rare outcomes, the *case-control design* can often be more efficient (121). As the name implies, a number of cases are identified through, for example, medical records at a clinic, an approach which ensures that a certain number of cases are met. A number of controls (i.e. subjects without the disease) are selected as a comparison group, although defining controls representative of the entire population at risk can be challenging and may succeed to varying degree. Then, exposure status is assessed, through either objective sources such as health registers, or subjective sources such as the cases and controls themselves (through questionnaires, interviews, etc).

To increase precision, several controls per case may be selected, where four is usually considered optimal (122). However, since the proportion of cases in the study is pre-determined by the investigator, and the time to event is not known, the IRR cannot be estimated. Instead, the **odds ratio** (**OR**) is estimated, where the odds of being a case if exposed are compared to the odds of being a case if non-exposed. The possibility of this may appear counterintuitive, since the probability of being a case in the study is what is known at the outset (as the ratio of cases to controls is determined by the investigator), whereas the exposure status is not. However, based on Bayes theorem, conveniently the OR of having disease if exposed is the mathematical identity of the OR of being exposed if having disease, an important foundation for the case-control study. A further advantageous feature of the OR is that, if the outcome is rare, the OR will approach the RR estimate (123), thereby under the "rare disease assumption" enabling the interpretation of the OR as a relative risk, which is often easier to communicate.

The *nested case-control study* was proposed as lying in the intersection between these two study designs. All cases and controls, after all, originate in a population of some sort, and it was gradually realized that case-control studies can be viewed as nested within a larger population or cohort. Frequently, this cohort is ill-defined, but in instances where it can be more closely detailed, it is possible to construct a case-control study which is clearly nested in, and preserves the validity of, the underlying cohort. Briefly, this requires defining a cohort of subjects who are closely followed until development of disease, with accurate registration of follow-up time. When disease occurs, that subject becomes a case, and *at that time,* from all the remaining subjects still at risk for the disease (i.e. the *risk-set,* those not dead, emigrated or censored for other reason), controls are randomly selected. Since the controls are randomly sampled from the remaining population, their experience of exposure (or non-exposure) is considered representative of the remaining risk-set.

Controls are therefore selected from the person-time at risk, and matched on time to the cases (121), meaning that the OR will be a good approximation of the IRR from the corresponding cohort study, although with lower precision inherently due to the sampling procedure of controls (124). This precision deficit can be improved if sampling a greater ratio of controls to cases than 1:1. This way of sampling controls – incidence density based – further entails that the rare disease assumption is not required to interpret the OR as a IRR (124) although there are some limitations to this statement (125). With accurate knowledge of the sampling fractions, it is also possible to estimate the **absolute risk** (**AR**) for the outcome from a nested case-control study, a measure otherwise reserved for cohort studies (126).

The nested case-control study is particularly appealing for studies on *molecular epidemiology*, which combines traditional register-based variables with biological measurements (127). This was a main motivation for using the nested design in our Studies I and II. The study base required to yield the desired number of cases exceeded one million women: performing a cohort study with full HPV typing in this population would obviously be impossible. The nested design instead allows a methodologically sound and cost-effective approach while also maximizing the number of cases available (124).

In all types of studies mentioned above, the collection of data on exposure can be either prospective or retrospective. Prospective refers to data collection which took place before the outcome was known. We have therefore termed Study I and II "prospective", since they measure HPV prevalence in archival smears taken and stored in the years before diagnosis of disease. Thus, we were able to objectively assess HPV status before the outcome was known. "Retrospective" refers to data collection performed when it is already known who is a case and who is a control in the dataset; the classical example perhaps being a questionnaire assessing previous exposure to risk factors for a disease which the study subjects answer themselves. Such a retrospective data collection may be more prone to biases such as recall bias (see below) than a prospective such, but it should be noted that retrospective does not necessarily mean worse quality of the data.

4.1.2 Cross-sectional studies

Cohort and nested case-control studies are longitudinal in nature: exposure and outcome can be separated in time. Depending on the research question, study designs without a time dimension can also be informative. For example, many survey studies are cross-sectional in nature: the outcome of interest can be current opinion ("outcome") on some matter, and what factors ("exposures") may be related to that opinion. The design aims to provide a "snapshot" of the population at that time, not to calculate incidence of outcome/disease over time (124). The measure calculated in a cross-sectional study is typically an odds ratio, sometimes called prevalence (rather than an incidence) odds ratio to emphasize the cross-sectional aspect. For Study III and IV in this thesis, the question of interest was to which degree respondents were currently aware of HPV, and willing to vaccinate against it. For this purpose, a cross-sectional study design was deemed appropriate. However, this design also places restrictions on how associations can be interpreted (see below), and for that reason we termed the independent variables "correlates" rather than "exposures".

4.2 ERRORS IN EPIDEMIOLOGICAL RESEARCH

4.2.1 Systematic error

The overriding issue in observational epidemiology is that there is no randomization procedure to confer protection against systematic error in the estimation of the effect of exposure on the risk of outcome. In reality, subjects are not randomized to exposure or non-exposure and all other factors are not equal: many may vary systematically between exposed and non-exposed, and in those with the outcome and without. Thus, exposure and outcome may share (other) common causes, which will induce a spurious association between the two, even though there is no causal relationship between them. Such a spurious association is termed *confounding* (120). Common confounding factors are gender and age but in general, confounding is unique to each situation and deciding on which confounders may be relevant requires detailed subject matter knowledge.

Briefly, some methods for handling confounding are implemented on the design stage, such as restriction and matching (others are implemented at the analysis stage, see further below). *Restriction* entails restricting the study population to, for example, one gender.

Thus, gender is constant across exposure and outcome and cannot confound the estimates of the effect. *Matching* is another way of holding certain factors constant between cases and controls: for each case, controls are selected not entirely randomly, but so that they match the case in terms of e.g. age and living area. Done judiciously, matching can remove confounding and increase efficiency in estimating an effect of exposure and outcome, but this also means that an effect for the actual matching variable/-s cannot be calculated – which is why care should be taken not to match on a variable which may later be of interest. Also, the analysis must always take the matching factors into account, or the estimate will be biased (128). Further, it should be emphasized that matching to a degree will force cases and controls to be more alike in the study than in real life, which means that matching on too many variables may induce bias rather than prevent it. Study I and II in this thesis were matched designs, the reasons for which are discussed below.

Another systematic error of note is *selection bias*, where the effect of exposure on outcome is different among those who participate in the study, than those who do not. The association in those who do not participate is usually unknown, and presence of selection bias is usually therefore inferred, rather than observed (124). In Study I and II below, we discuss whether there is such selection bias affecting whether our results are generalizable to the wider population or not. In Study IV below, we attempt to formally investigate non-participation to the study, and whether it can be adjusted for via a separate analysis.

Thirdly, *misclassification bias* concerns the crucial part of an epidemiological study of classifying subjects regarding exposure and outcome status. This classification is the "alpha and omega" of any research study, yet there is frequently the risk of getting it wrong. Misclassification of exposure can be *non-differential* with respect to outcome status, i.e. equally present whether the subject is a case or not. This will tend to bias obtained estimates towards the null, i.e. an attenuate the effect. If the misclassification is unequally distributed with respect to outcome status (*differential* misclassification), this will either exaggerate or underestimate an effect and the direction may sometimes be difficult to predict (120). A classic case of differential misclassification is *recall bias*, where individuals with a disease recall exposure in a systematically different way from those that never suffered from disease. Additionally, there can be non-differential or differential misclassification of outcome in relation to exposure status. In our Study I and II, outcome status (cervical cancer) was registered in the Swedish Cancer Register with no knowledge of exposure (HPV) status. Exposure (HPV) status was determined by laboratory technicians who were blinded to outcome (case/control) status.

4.2.2 Random error

The goal of most epidemiological studies is to reflect the real life ("true") estimate that would have been obtained if exposure-outcome relation could have been studied in the whole population, rather than just a sample of it. After all, if another study had been conducted on another sample from the same underlying population, it may have reached a different result. Thus, we must account for how likely it is that our observed result arose merely as a result of sampling variability.

For this reason, we do not only report point estimates of our effect measure (such as OR=2), but we also consider the risk for *random error* in our study, chiefly through the use of *confidence intervals* (CIs). The CI is constructed as the sample (point) estimate plus or minus its standard error (SE) multiplied by an appropriate percentage point (based on a normal distribution unless the sample size is small). Typically, the SE is multiplied by 1.96 to yield a 95% CI. The CI gives a range of values within which we are reasonably confident that the true population difference lies (123). For example, the OR for an exposure related to an outcome could be reported as 2.0 (95% CI 1.8-2.4). CI:s are used regardless of the type of outcome measure used (OR; RR; IRR, etc.). The SE also determines the test-statistic, which is used to derive the *p-value* for the association. The larger the test statistic is, the smaller the p-value becomes. The p-value is usually interpreted as the probability of the observed result, even though the null hypothesis of no difference between exposure groups is true. Hence, the smaller the p-value, the lower the probability that the results arose by chance (123).

The SE is inversely related to the sample size and will thus be smaller as the study increases in size, which means that with increasing size, the CI becomes tighter and the p-value smaller. With infinite size, the study's random error would be removed as the sample estimate would be the same as the population estimate (124). Indeed, this has been the motivation for some to debate the use of CI:s when considering whole population data such as national cancer registers. However, even national registers can be considered samples from the population, and a CI is then usually an appropriate caveat (129).

The CI and p-values are naturally related, as they are both derived from the SE. Together, they constitute measures of *precision* in the study. All the studies in this thesis report CI:s, Study III and IV also report p-values for some or all associations. Study III and IV state the use of a convention of considering p-values <0.05 as significant (the equivalent of a 95% CI not containing the null value). However, the CI:s were naturally also taken into account.

4.2.3 Logistic regression modeling

In epidemiology, we frequently wish to conduct analyses considering several exposures at once, rather than just one exposure and outcome. A flexible analysis method for estimating effects, and controlling confounding due to several variables, in case-control and cross-sectional studies is the *logistic regression* model (123). It is similar to linear regression, but considers a binary outcome (e.g. cervical cancer yes/no, accurate knowledge of HPV yes/no) instead of a linear one. It estimates the probability of outcome Y given exposure x: $P(Y=1 \mid x)$, also expressed as π. This probability is transformed to a logit function (hence the name logistic regression):

$$\text{Logit}[\pi] = \log(\pi/(1-\pi)) = \log(\text{odds}) = \beta_0 + \beta_1 x_1$$

Where β_0 is the baseline log odds for the outcome in the unexposed and x_1 equals 1 for those in the exposed group and 0 in the unexposed group (130). The transformed logit[π] has many of the desirable properties of a linear regression model; it can be continuous and range from $-\infty$ to $+\infty$. (Note: "log" here refers to natural log, not $\log_{10}$.)

The interpretation of the formula above is that when exposure x_1 increases by one unit, the log odds of the outcome will increase by β_1. This assumes linearity of the logit, an assumption which should be investigated. If needed, this assumption may be relaxed by categorization of a continuous exposure variable or by the use of methods such as splines (131), which were used to model HPV16 viral load in Study II.

The logistic regression model conveniently extends to accommodate exposures with more than one level, and to consider more than one exposure (terms $\beta_2 x_2 \ldots \beta_k x_k$ are added, leading to a *multivariate* analysis). When the parameter β_1 is back-transformed, it yields the desired odds ratio for exposure x_1 (in a multivariate analysis adjusted for the other variables x_k included), along with a CI and p-value for the effect. The logistic regression model with a binary outcome can also be extended to consider instead an outcome with three or more levels. The model then estimates the effect of one or more exposures on the probability that the outcome is in a certain category (123). This is termed *multinomial* logistic regression, used in Study III to determine willingness to vaccinate a) not at all, b) only if for free or c) even if at a cost.

If a case-control is individually matched on certain factors, these factors should be taken into account in the analysis in order not to bias the effect estimates. The standard approach is to use *conditional* logistic regression, a variant of logistic regression where the pairing of cases and controls is preserved in the analysis (123).

$$\text{Unconditional OR} = \frac{\text{odds of outcome in exposed group}}{\text{odds of outcome in unexposed group}}$$

$$\text{Conditional OR} = \text{ratio of discordant pairs} = \frac{\text{no. of pairs in which case exposed, control not exposed}}{\text{no. of pairs in which control exposed, case not exposed}}$$

Concordant pairs on the exposure contribute no information; only pairs discordant for the exposure do. If data on a case or a control is missing, this means that information from the pair is lost, and if only few discordant pairs are available in the analysis, precision will be low. It is possible to break the matching and manually adjust for the matching factors, although care must be taken to compare the estimates from the matched and non-matched analysis to ensure that bias is not introduced (128). We have however chosen to consistently employ the standard approach of conditional logistic regression in Study I and II which were matched, and accept that this may lead to a loss in precision.

Another advantage of multivariate logistic regression modeling is that it allows the assessment of interaction effects in the data. Interaction, or effect modification, is not a confounding effect (i.e. inducing a spurious association between exposure and outcome), but a true effect that may be of interest to examine (122). Effect modification means that the OR for the outcome in the exposed differs according to a third variable, such as gender. If no interaction terms are included in the logistic regression model, it is assumed that no such interaction exists. We actively investigated interaction effects with multiple HPV type infections in Study I, and interaction effects with gender in Study III and IV.

4.3 INTERNAL AND EXTERNAL VALIDITY

Finally, some brief words on the concepts of study validity, which are frequently discussed in epidemiological contexts. As reviewed by Steckler and McLeroy (132), different types of validity of a study have been defined; such as

1. internal validity (whether the study correctly estimates what it aims to estimate)
2. statistical conclusion validity (whether statistical conclusions made in the study are justified)
3. construct validity (whether operational variables adequately represent theoretical constructs)
4. external validity (whether (causal) relationships can be generalized to other populations and times)

Statistical conclusion validity and construct validity can be considered parts of internal validity, a key feature of which is the quality of design and measurements in the study. Our ability to generalize the findings, i.e. the degree of external validity in the study, may be more dependent on judgment (132). A high internal validity is usually considered a prerequisite for high external validity, but is not a guarantee for it – as demonstrated by the difference between the results in even very high-quality RCT:s once applied in less selected populations (133, 134). Therefore, RCT:s and observational studies complement one another. Perhaps a suitable axiom in this case would be the following, as paraphrased from Nallamothu et al (134):

"All types of evidence for new therapies [or exposure-outcome associations, author's note] rely primarily on the rigor with which individual studies were conducted, regardless of the methodological approach, and the care with which they are interpreted."

4.4 REGISTERS USED IN THIS THESIS

- The National Cervical Screening Register (NCSR)

Initially established ten years ago at MEB, KI, the NCSR is a highly reliable quality register with 100% coverage of new smears. Encompassing screening information back to the late 1960's, it contains records of over 15 million cervical smears and 2.5 million histological samples, from around 3.3 million Swedish women. In 2011, the register was transferred to the auspices of Stockholm County Council (23), and now forms part of the National Quality Register for Cervical Cancer Prevention. This register is led by a national steering group with complementary expertise in epidemiology, gynecology, pathology, virology and oncology. Evaluation of data delivery, coverage and multiple quality indicators is carried out annually, enabling bilateral feedback.

- The Swedish Cancer Register (SCR)

The SCR was founded in 1958 and is maintained by the National Board of Health and Welfare. It has national coverage and reporting of malignant and certain benign tumors is compulsory to the register from the health care provider, via six regional cancer registries around the nation. A cancer report has to be sent for every cancer diagnosed at clinical, morphological and other laboratory examinations, and those diagnosed at autopsy. In a validation study, the completeness of the Register was evaluated formally for the year 1998, through comparing all cases in the Register to those in the National Hospital Discharge Register. It was found that the overall completeness of the SCR is high and although there is some underreporting, this rate was very low for female genital cancers (135).

- The Swedish Total Population Register (TPR)

The TPR is maintained at Statistics Sweden, the national Swedish statistical agency. It contains information on every resident registered in Sweden at the end of each year since 1968. Each individual is recorded through a unique personal identification number (PIN). Data on births, deaths, immigration, emigration etc. are reported from local taxation authorities to the register continuously, and after 30 days the coverage in TPR is nearly complete, although slightly lower for emigration statistics (136).

- The Longitudinal Integration Database for Health Insurance and Labor Market Studies (LISA)

This is a longitudinal database at Statistics Sweden which integrates data from the labor market, educational and social sectors and which is updated each year. Abbreviated LISA in Swedish, it includes among others, information on employment, disposable income, country of birth, country of parental birth, education, and residence (137).

Register linkage procedure
These databases were all linked through the individually unique Swedish personal ID-numbers, which allow for linkage through virtually 100% of the Swedish health care system (138).

4.5 METHODS IN STUDY I AND II

In these first two studies, the following study questions regarding risk for cervical cancer in situ (CIS) and invasive squamous cervical cancer (SCC) were posed:

- What is the category-specific risk associated with different HPV type infections?
- What is the effect of persistent infection with HPV16?
- What are the predicted effects of the current and future HPV vaccines?
- What is the risk associated with HPV16 viral load?

As described above, a nested case-control study using archival cervical smears was deemed appropriate for answering these questions, since ever after the development of a validated laboratory method for such smears (139); this observational study design has been used in Sweden with great success (79, 140).

4.5.1 Classification of outcome

Study I and II: Sampling frame and study participants

For Study I, our initial source population comprised all women who participated in Swedish cervical screening sometime during 1969-2002, n=757,690, in a number of county laboratories. For Study II, this source population was expanded to include 1,459,258 women. For each study, we then identified a cohort of women whose first registered smear (defining cohort entry) was classified as cytologically normal (Study I: 739,072 women, Study II, 1,431,724 women).

For each study, the cohort was subsequently linked to information from the Swedish Cancer Register (SCR) to identify all women with a first histologically confirmed diagnosis of cancer in situ (CIS) or invasive squamous cell carcinoma (SCC) after cohort entry. A diagnosis of CIS in the SCR translates internationally to a diagnosis of CIN grade 3 (CIN3). Since the incidence of CIS is higher than the incidence of SCC (19, 141, 142), we included only a random sample of CIS to achieve case-groups of more similar size.

To be eligible as a control, a woman could not have had a histologically confirmed diagnosis of CIS/SCC at the time of diagnosis of the case, i.e. she must still be at risk. Among all eligible control women (i.e. the entire at-risk-set), one control woman – matched on county laboratory, date of cohort entry (+/- 3 months), and age at first normal smear (+/- 1 year) – was randomly selected for each CIS and SCC case. (Nota bene: since she is still at risk, a feature of the nested case-control study is that a control may later become a case, however this only occurred in one control woman from the CIS group, and none in the SCC group.) Since both the HPV exposure and risk of a CIS/SCC outcome may vary systematically over age and time period, this matching was done to minimize confounding from these factors. The matching on county was implicit from a logistical perspective as biobanks agreed to the study on a county (laboratory) level.

All available smears from the case patient and the control woman taken prior to the date of diagnosis of the case were retrieved from biobank archives included in the studies.

4.5.2 Ascertainment of outcome

Study I and II: Histopathological re-review

To verify the original histological diagnosis, all available histological specimens from the case women were re-reviewed by one senior pathologist. Due to the multiplicity of pathology laboratories involved (in total 38 such), the overall proportion of missing histologies in this study was 18% for SCC and 21% for CIS. Given our nation-wide approach and our study time spanning several decades, we hold this to be acceptable given that our previous study had a missing rate of 11%, in just one county laboratory (79). In several instances, we contracted laboratories to re-section new cases from old paraffin-embedded tissue since the original slides had been disposed of. On several occasions, due to sparsity of material to re-section, our pathologist was not able to review all original tissue blocks representatively. It is therefore not certain whether all the cases that were re-classified as lower-grade (than originally) truly are of lower grade. It could also be that our pathologist was not able to review them to the same precision as the original diagnostician.

Usually, it is the epidemiological tradition to exclude all cases that were not confirmed. However, all cases in the study were originally histo-pathologically verified. The reduced power due to missing histological re-review, and partially unclear validity of the reclassification, means that we therefore hold sensitivity analyses, comparing results in the full and the re-classified data, to instead be the most informative.

4.5.3 Classification of exposure

Study I: Qualitative infection with HPV

Each archival cytological smear was re-coded and re-labeled to ensure blinding of case-control status to the analyzing staff; samples belonging to the same matched case-control pair were included in the same analysis batch at the same calendar time. DNA extraction was performed by validated methods (139). All smears were analyzed for the presence of seven low-risk HPV types (HPV 6, 7, 11, 42, 43, 70, and 90), and 16 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82). The polymerase chain reaction (PCR) amplification of a consensus region using GP5+/6+ primers (143) was followed by HPV type detection through a PCR-EIA (enzyme immunosorbent assay) and reverse dot blot hybridization procedure (RDBH) (144) or detection of biotinylated HPV amplicons by a multiplex fluorescent bead-based assay (145). Positive controls (HPV16 DNA) and multiple negative controls (Sigma water) were included in all runs to ensure absence of contamination. Presence of amplifiable DNA in samples was determined by PCR-EIA or real-time PCR for the housekeeping β-globin gene.

HPV analyses were performed in the WHO HPV LabNet Global Reference Laboratory, Malmö, Sweden.

Study II: Quantitative infection with HPV16

Study II focuses on risk for CIS and SCC according to infection with HPV16 measured quantitatively (i.e. amount of infection, not just infection present yes/no). In HPV16-positive samples, the viral load was measured as an absolute number of viral copies of the E7 gene per microliter, using the Taqman real-time quantitative PCR method (146). The GeneAmp 5700 Sequence Detection System continuously measures the PCR product accumulation.

4.5.4 Statistical methods

Study I: Data structuring and model construction

Study I focuses on type-specific risks for CIS and SCC according to category of HPV infection measured qualitatively (infection present yes/no). In all studies concerning HPV type-specific risks, some decision must be made as to how to handle multiple infections, which are usually present to at least some degree. This decision will depend on the research question involved. It is possible to handle multiple infections either at the exposure classification stage (147), or the modeling stage (66).

For the purpose of our study, we defined exposure categories as follows: (a) HPV16, the first/last smear being positive for HPV16; (b) HPV18, the first/last smear being positive for HPV18; (c) HPV16/18, the first/last smear being positive for HPV16 and/or 18; (d) non-16/18 HRHPV, the first/last smear being positive for one or more HRHPV types but not HPV16 or 18; and (e) LRHPV, the first/last smear being positive for one or more LRHPV types. We thus chose to treat multiple infections first at the classification stage, which meant that many of the multiple infections were handled by grouping. However, some women were still positive for two categories in the same smear and thus, further refinement was required.

For estimating odds ratios associated with each category, taking the effect of multiple categories into consideration, we constructed a conditional logistic regression model including terms for the main effect for each category, and terms for possible two-way interactions between them (it was not possible to analyze three-way interactions due to a lack of power). The following model was fitted:

$$Logit\,(Y=1 \mid 16/18\ HRHPV,\ non16/18\ HR\ HPV,\ LRHPV) = \beta_0 + \beta_1 HPV16/18 + \beta_2 non16/18HR\ HPV + \beta_3 LRHPV + \beta_4 HPV16/18 * non16/18HR\ HPV + \beta_5 HPV16/18 * LRHPV + \beta_6 non16/18HR\ HPV * LRHPV$$

In this analysis, the main effects (reported in the paper) represent the risk association, adjusting for multiple category infections. This estimation requires using HPV-negative women as a reference level, since only they are free of all possible combinations of HPV-positivity. When studying a single HPV type, use of an HPV-negative reference level – instead of women negative for only that HPV type – will inflate odds ratios compared to a mixed reference level (which is why we also present results for a mixed reference level, “all other women”).

In our case, however, this use was called for in order to obtain etiological fractions for non16/18-HR HPV free of contamination from concurrent 16/18-infections.

We also examined HPV16 persistence as an exposure in this study. Persistence of HPV16 infection in the first two consecutive smears was defined as: (a) negative (both smears negative for HPV16); (b) transient (first positive, second negative); (c) acquired (first negative, second positive); or (d) persistent (both smears positive for HPV16). All risk associations with the exposure were analyzed separately in the first and in the last smear prior to the diagnosis of the case, to describe changes in risk over time to diagnosis. As discussed above, the ORs obtained in this study (and Study II) are interpretable both as the IRR and the RR. We do not explicitly mention the IRR in the articles since it was judged that a relative risk interpretation was the most communicable in either case.

Attributable risk proportions (ARPs) and 95% confidence intervals (95% CI) were calculated based on ORs obtained from the conditional logistic regression models (148). The formula used was the following:

$$ARP = pd((RR-1)/RR)$$

where *pd* is the proportion of cases exposed to risk factor, and *RR* is the adjusted OR for the main effect in each category of HPV-positivity, retrieved from the conditional logistic regression models (for a discussion of different formulas for ARP, see also (149)).

We performed sensitivity analyses using histologically re-confirmed cases only. In these analyses, the results remained robust.

Study II: Data examination and model construction

For our study II, we focused on HPV16 viral load and began with evaluating multiple time scales for their relevance for the dynamics of HPV16 viral load. Because control women had few HPV16-positive smears, we modeled viral loads among case women only. We fitted linear models for the logarithmized (base 10) viral loads with time before diagnosis as primary time scale; and then systematically added individual and combined age, period and cohort (APC) effects to the basic model.

These effects were evaluated both for their statistical significance and size. These preliminary models indicated weak or no effect of birth cohort and age at smear on viral load in either case group. However, we did find a significant effect of calendar period of smear for CIS, where viral loads decreased systematically over time: the highest levels were found during the 1980s, with levels at the end of the 1990s almost an order of magnitude lower. Sensitivity analysis indicated that this period effect affected risk estimates for CIS, but not for SCC; consequently, only viral loads for CIS were adjusted for period of smear.

Independently of these adjustments, we also found that viral loads for cases and controls varied systematically within the period of HPV analyses, with later measurements tending to be lower during the analysis period (2005-2010). Consequently, all viral loads were adjusted for calendar date of analysis.

Risk estimation through viral load imputation

We used conditional logistic regression (as defined above) to study the association between HPV16 viral load and risk of CIS and SCC at 1-10 years prior to diagnosis, using HPV16-negative women as reference. A practical consideration when modeling the risk associated with viral load cross-sectionally over time was the irregular pattern in which smears were available due to our study being nested in a screening program with variable participant compliance. This meant that few matched case-control pairs originally had measurements in the same intervals. As a consequence, only few matched pairs would contribute to the estimation of each cross-sectional odds ratio, leading to wide confidence intervals and problems with separation. To address this issue, we implemented a within-person imputation scheme for each individual, as done previously by our group (79). For each subject and each year, we imputed HPV16 measurements from all observations within a fixed window (±5 years), where observations closer in time received larger weights.

Formally, this entailed that for any individual with observed HPV16 status $Y_1 , \ldots Y_k$ and corresponding viral loads $L_1 , \ldots L_k$ at time points $t_1 , \ldots t_k$, we estimated the probability of being HPV16 positive at any time point t as

$$p(t) = \frac{\sum_{d_i \leq w} Y_i / d_i}{\sum_{d_i \leq w} 1/d_i}$$

where $d_i = |t - t_i|$ is the absolute distance from the target time point to the observational time points, and w is the width of the time window. For an empty window, the corresponding probability and measurements were set to missing. For a single imputation run, we used $p(t)$ to impute $Y(t)$ at yearly intervals up to 15 years prior to diagnosis or up to first smear. For an imputed HPV16-negative smear, the corresponding viral load $L(t)$ was set to zero; for an imputed positive smear, the corresponding viral load was selected randomly from the observed loads L_j within the current time window, with selection probability proportional to distance in time:

$$p_j(t) \sim 1/d_j$$

with a large finite value substituted for $d_j = 0$.

For the final results, we performed 100 imputation runs for all individuals, resulting in 100 imputed data sets. The final estimate of the odds ratio at each time point t was calculated as the average of the estimates from the imputed data sets.
The corresponding variance was calculated as the sum of the average of the within-imputation variance and the between-imputation variance, according to Rubin's formula (150). Alternative values of four and six years for the imputation time window w had little effect on the results.

We used a marginal mean approach to modeling longitudinal effects in the viral loads of case women and the risk of cervical cancer associated with HPV16 viral load. Individual trajectories of viral load in women with multiple smears can vary substantially, which is why we opted for the per-subject imputation scheme outlined above for estimating risk. Our results are consequently valid and interpretable on an average population level.

We performed sensitivity analyses using histologically re-confirmed cases only, and analyses restricted to the pre-1995 period. The results remained robust in these analyses.

4.6 METHODS IN STUDY III AND IV

The main research questions for these studies were

- What degree of awareness of HPV was there in the Swedish population pre-large scale HPV vaccination campaigns?
- What degree of correct knowledge did people possess regarding HPV?
- How many are willing to vaccinate against HPV?
- What are the concerns regarding HPV vaccination?
- Might some health behaviors change after HPV vaccination?

For these questions, a cross-sectional study assessing current opinion was deemed appropriate, building on previous experience from similar survey studies on condyloma (41, 151).

4.6.1 Sampling frame and study participants

Study III and IV

Study III and IV are based on a nationwide population-based cross-sectional survey in two demographic groups: parents to children aged 12-15, and young adults aged 18-30. The rationale for this was that parents will make decision regarding school vaccination for their children, but young adults will participate in, or make their own decisions regarding, catch-up vaccination. The first group consisted of 20 000 parents of children aged 12-15 years (16 000 parents to girls and 4000 parents to boys) and the second consisted of 20 000 young adults aged 18-30 years (16 000 women and 4 000 men). Parents to girls and young women were purposefully over sampled since they constitute the main target of HPV vaccination and to enable adequate power in a planned follow-up study. Both groups were randomly selected from the Swedish Population Register from which home address and telephone number was retrieved, which were used to contact the study sample.

In Study III, both the parents and young adults were included. Study IV focuses on young adults only, as acceptability among parents had already been assessed separately (111).

4.6.2 Survey data collection

Study III and IV

The survey was conducted during January to May, 2007. A multi-modal method for data collection was used to improve response rates to the study. First, an invitation letter was sent out which included a log-in to a web-based form of the questionnaire. If no response was made, participants were reminded by post two more times (three weeks apart) and given opportunity to also respond by a paper-based questionnaire in the first reminder and by a telephone interview in the second reminder. Participation was voluntary and by answering the questionnaire, subject consented to take part in the study.

In order to examine survey non-response; additional socio demographic data on the entire initial sample including non-respondents was obtained from the LISA database at Statistics Sweden, after de-identification of the data.

In the parents' group, the questionnaire recorded information on demographic factors (section 1), awareness and knowledge of HPV and HPV-related diseases (section 2), attitudes toward vaccines in general (section 3) and acceptability and perceptions of HPV vaccine (section 4). They were also asked at the end of the questionnaire whether HPV vaccination might alter children's future health-care related behavior (section 5). In the young adult group, the questionnaire recorded information on demographic factors (section 1), sexual habits (section 2), awareness and knowledge of HPV and HPV-related diseases (section 3), attitudes toward vaccines in general (section 4) and acceptability and perceptions of HPV vaccine (section 5). Female respondents were also asked questions on awareness of and participation in cervical screening, and intention towards screening if they were to be vaccinated against HPV (section 6).

4.6.3 Classification of exposure

Study III and IV

As this was a cross-sectional survey, it should be noted that there are no absolute boundaries between what is considered exposure (cause) and what is considered outcome (effect) in the study. Technically speaking, that would require causal inference which is not possible without exposure and outcome being separated in time. In both Study III and IV, many different variables were considered regarding potential relation to the outcome (knowledge of HPV in Study III and acceptability of HPV vaccination in Study IV), but since the study was cross-sectional in nature, we have consistently opted to term these variables "correlates" to the outcome, rather than "exposures". Many potential correlates were assessed in both studies, and these can be grouped into a) socio-demographic variables, b) variables describing sexual habits (only in young adults) and c) awareness of HPV and HPV-related diseases. The socio-demographic variables mainly utilized the data from Statistics Sweden, but were in some cases also complemented by questionnaire data. Variables were selected into the model based on prior knowledge and potential association with the outcome.

4.6.4 Classification of outcome

Study III: Awareness and knowledge of HPV

HPV awareness was assessed in section 2 (parents) or 3 (young adults) by an item asking *"Had you heard of a virus called human papillomavirus (HPV) prior to your participation in this study?"*. Correct knowledge was assessed by asking respondents who had heard about of HPV if they thought that 1) HPV can cause cervical cancer, 2) HPV can cause other cancers, 3) HPV can cause condyloma (a.k.a. genital warts), 4) HPV is sexually transmitted, 5) men can be infected with HPV and 6) women can be infected with HPV. All questions could be answered with "Yes"/"No"/"Don't know". An HPV knowledge score was then constructed, where the respondent was given one point for each correctly answered question (the correct answer to all questions was "Yes"). The range was therefore 0-6 points.

Persons who had not heard of HPV were given 0 points in the analysis. Please note that, whereas some tend to use the term "awareness" and "knowledge" interchangeably, strictly speaking: "awareness" refers to having heard of something (as in being aware of something) whereas "knowledge" signifies correct understanding of an issue (as in ability to answer specific questions on the topic).

Study IV: Acceptability of HPV vaccination

In the beginning of section 4 or 5, respondents were given written information phrased as follows: *"We would like to inform you that HPV is a sexually transmitted virus which can cause condyloma in men and women, and cervical cancer in women. An effective HPV vaccine has been developed and is ready for use"*. In case of the telephone interviews, the same information was read to the respondent over the phone. Respondents to the paper questionnaire were in connection with this information asked not to go back and alter any previous answers in the survey. For respondents to the web or telephone questionnaire, no such going back was possible. After the information, two questions on acceptability were posed; the first one asking "*Would you want to be vaccinated against HPV if the vaccine is for free?"*, and the second asking "*Would you want to be vaccinated against HPV if the vaccine costs?*". (The cost of the vaccine was not pre-specified, as this was unknown when the questionnaire was designed.)

Responses on acceptability were grouped into three different levels: 1) those unwilling/unsure if willing to vaccinate even if the vaccine was for free; used as reference category, 2) those willing to vaccinate if the vaccine was for free but unwilling/unsure if willing to vaccinate if the vaccine cost and 3) those willing to vaccinate and willing to pay for the vaccine if there was a cost. Note that the theoretical construct that we wished to investigate in Study IV is usually termed "acceptability" (or "acceptance"), whereas our actual study question measuring this construct used (the Swedish term for) "wanting to" or "willingness". Therefore, the article refers to both terms, depending on context.

4.6.5 Statistical analysis

Study III

Logistic regression model

A binomial logistic regression model was used to examine correlates of high levels of HPV knowledge. Respondents were grouped in two groups; those with below median or median results on the HPV knowledge score (i.e. ≤4 points) – termed low knowledge – and those with above median score (i.e.>4 points) – termed high knowledge. The regression model was built through examining all potential correlates in a first full model. A backward stepwise approach was then taken, removing variables with non-significant categorical effects whose removal did not affect the confidence intervals of other variables by more than 10%. This approach was repeated until only significant variables remained.

The prevalent odds ratios (OR) with 95% confidence intervals from the regression analyses are presented together with p-values for each categorical effect in the model, based on the Wald Chi-square statistic. A p-value of <0.05 was considered significant.

Study IV

Non-response analysis

Since the overall response rate among young adults was only around 55%, it was apparent that some form of non-response analysis was appropriate. We opted to construct so-called response homogeneity groups (RHG), by a method previously described (152). This method tries, as accurately as possible, to describe the unknown response mechanism of the sample. Since data from Statistics Sweden were gathered for the entire study sample, it was possible to calculate a survey response rate according to a number of demographic characteristics. To determine the main factors predicting survey non-response, these socio-demographic variables were entered simultaneously into a logistic regression model using non-response to the survey as the outcome. In this analysis; the main factors correlated to non-response was gender, education level, birth country of the respondent's mother, and presence of social welfare grant in the family. These factors were used to construct the RHG:s through the following approach.

Initially, a matrix was set up where all subjects in the sample were classified according to their different combinations of the identified main non-response factors (for example: gender=male, education level=high school, birth country of mother=Sweden, social welfare grant =no). This classified all subjects (whether respondents or not) into 16 possible combinations (here called groups) of these factors. Subsequently, the proportions of acceptability were retrieved for the *respondents* in each group and entered into the matrix, to represent the acceptability for *all* members in that group (i.e. also the non-respondents). A total proportion of willingness to vaccinate was then weighted together from all the groups, according to which relative representation that particular group had in the total *original* study sample (e.g. if group 1 had 12% representation in the original study sample, it would now be allotted 12% weight in the overall summation).

In effect, this simulates what the overall acceptability proportions could have been, if all subjects in each group had answered to the questionnaire (instead of just the respondents). Thus, it corrects the underrepresentation in response of certain socio-demographic groups in the actual study sample obtained, compared to the original sample. In so doing, one critical assumption is made: that people within the same (socio-demographical) group answer more similarly to each other than to members of another group, so that one can use the given answers among respondents in one group as proxies for answers among non-respondents in that group. Effectively, it is assumed that data is missing at random within the sample subgroups. However, such an assumption is not necessarily unreasonable, and usually presents a significant improvement over more naïve models that assume data are missing at random throughout the entire study population (152).

Note that the only item in the article adjusted for non-response is these proportions of acceptability presented in Table 2, all other frequencies and proportions are observed.

Logistic regression model

We used multivariate multinomial logistic regression to examine factors that may be associated with acceptability of HPV vaccine, because this outcome had three levels. The model included variables obtained from the questionnaire, and in certain cases, from the socio-demographic data obtained from Statistics Sweden. To test whether being female or male influenced the effect of the explanatory variables on acceptability of HPV vaccination, models with interaction terms between gender and each of the other explanatory variables were fitted. Since only few interaction effects were detected, the correlates are presented with the genders combined, with the interacting variables reported also separately.

In order to examine potential correlates concerning sexual habits that applied to only sexually active respondents, a subgroup analysis among everyone excluding those who stated they had never had sex was conducted.

To be able to present results regardless of univariate significance, the potential correlates were simultaneously entered into the models without further selection criteria. A p-value of <0.05 was considered statistically significant (although most of the p-values obtained in the analysis were much smaller). All prevalent OR:s in the acceptability analysis were mutually adjusted for all other variables in the table.

5 RESULTS

5.1 STUDY I

5.1.1 Characteristics of the study participants

515 women with cancer in situ (CIS) and 315 women with squamous cervical cancer (SCC) were included, along with their matched control women. The median age at diagnosis was 33 years in the CIS group and 40 years in the SCC group. The median study time was 5 years for the CIS group and 7 years for the SCC group, with the last smear on average 0.6 years before diagnosis of CIS and around 2 years before diagnosis of SCC. Although the absolute number of smears varied significantly between women, the median number of smears was 2 per woman in all study groups.

5.1.2 HPV prevalence

HPV positivity in the first smear was three times as common in CIS and SCC cases compared to control women. Most of the HPV infections detected in the pre-diagnostic smears from cases and controls were due to HPV16/18 (35% in CIS cases and 44% in SCC cases). The prevalence increased from the first to last smear in cases but not in controls. Being positive for multiple HPV type categories (e.g. both HR and LR or 16/18 and non-16/18) was quite rare in the study – around 11-16% of CIS cases, 6-9% of SCC cases and a few percentage units among the control women.

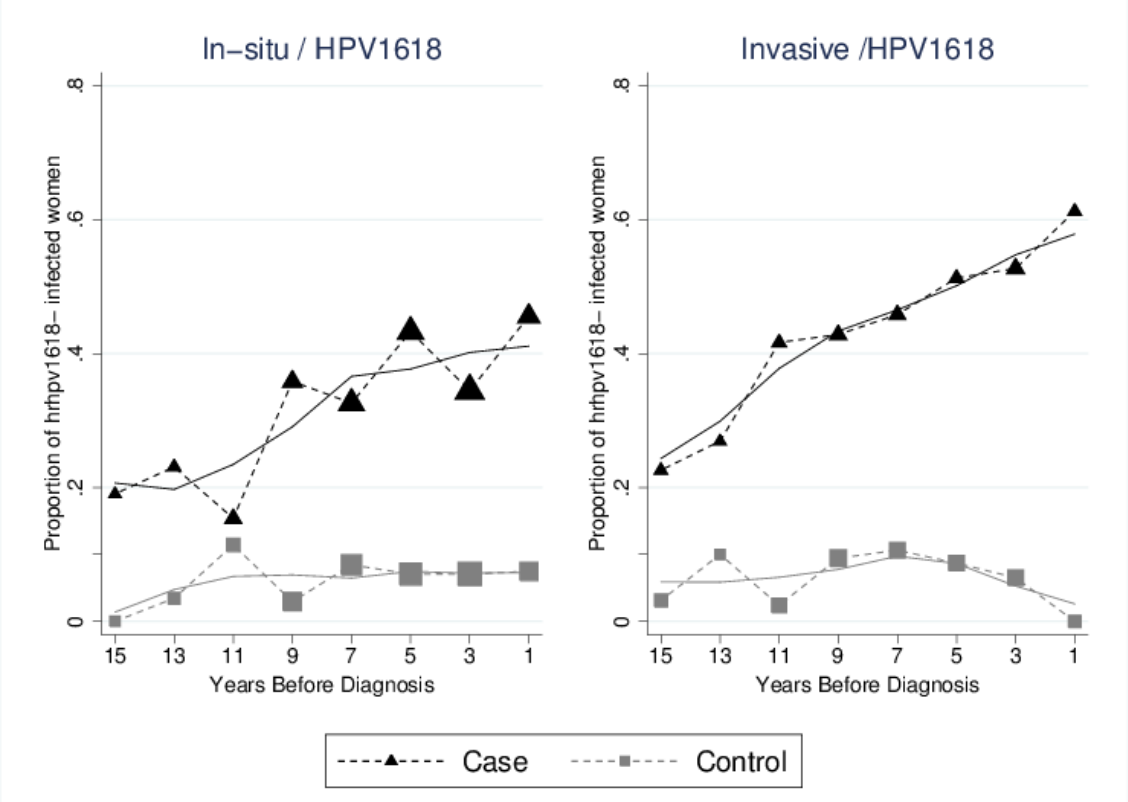


Figure 1. HPV16/18 prevalence in cases and controls in CIS (left) and SCC (right).

In an alternate comparison, complimenting the perspective of HPV-positivity in first/last smear; we investigated HPV-status in a continuum of smears taken from ten years before diagnosis and up until point of diagnosis. The probability of HPV16/18-positivity, and non-16/18-HR-HPV positivity was increased throughout ten years before diagnosis in cases compared to controls. However, the probability of being positive for LR-HPV was very similar in cases and controls over time before diagnosis.

5.1.3 Risk associated with different HPV types

The largest relative risk associations were seen for the HPV16/18-category, whether comparing to all other women, or just HPV-negative women. Further, the relative risks for CIS and SCC, respectively, were greater if HPV16/18 was found in the last smear than in the first. The same was seen for the non-16/18-HR HPV types, although the relative risks were not of the same magnitude as for 16/18.

Due to a lack of power, we could not obtain single-type-specific risks for HPV16 and HPV18 separately when assessing CIS and SCC as separate outcomes. Therefore, in an exploratory analysis these outcomes were combined (effectively replicating a CIN3+ outcome).

In the first smear, both HPV16 and HPV18-positivity was associated with a relative risk of around 11-12 compared to HPV-negative women. However, HPV16 showed a strong risk increase over time to a RR of 41 (95%CI 23-76), whereas the risk associated with HVP18 positivity increased only moderately over time to reach a RR of 16 (95%CI 6-43).

5.1.4 Risk associated with HPV16 persistence

Just over a third of both CIS and SCC case women were persistent for the same HPV type in the first and second consecutive smears, compared to a mere 4% of the controls. The main persistent type was HPV16, followed by HPV18, 31, 33 and 52. Power was only sufficient for HPV16 in a separate regression analysis.

This risk analysis showed a clear gradient of risk associated with HPV16, even though the low precision meant wide confidence intervals. Transience of HPV16-infection was not associated with any clear risk increase for either CIS or SCC, whereas having acquired an infection in the second smear was associated with an increased risk for particularly CIS, but also SCC.

Persistence in the first two smears was associated with almost 20-fold increased risks for both CIS and SCC. The exposure status for cases and controls in this analysis is given in the table below. Many cases, but few controls were exposed to HPV persistence, yielding the high OR for +/+. Furthermore, since the analysis was conditional, only discordant case-control pairs were used which limited precision somewhat.

Number of cases and controls in the HPV16 persistence analysis

CIS	Controls (n)	Cases (n)	DP*
-/-	218	138	14
+/-	11	11	9
-/+	8	32	30
+/+	4	60	60
Total	241	241	

SCC	Controls (n)	Cases (n)	DP*
-/-	138	89	11
+/-	5	10	10
-/+	8	18	17
+/+	4	38	36
Total	155	155	

** DP is the number of case-control pairs discordant on the exposure, used in analysis.*

Attributable risk proportions

The following attributable risk proportions were calculated for the different HRHPV categories, according to the main effects model described above.

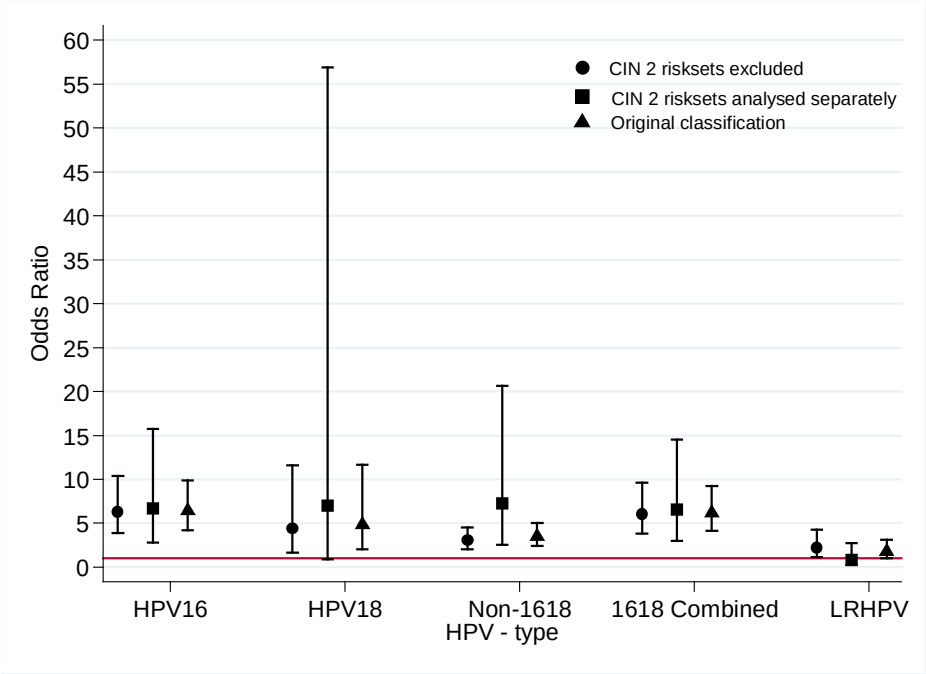
CIS	HPV16/18	Non-16/18 HRHPV
First smear	29% (24-34%)	20% (15-25%)
Last smear	49% (44-53%)	34% (29-39%)

SCC	HPV16/18	Non-16/18 HRHPV
First smear	41% (35-47%)	10% (4-16%)
Last smear	47% (41-53%)	19% (13-25%)

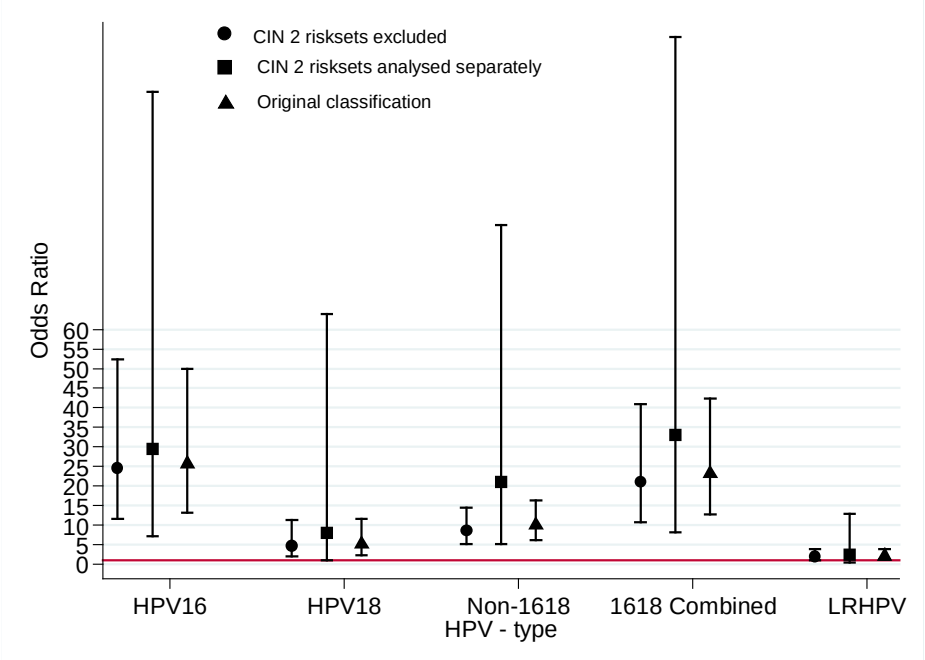
Additional data on sensitivity analyses according to histological re-review

Graphs illustrating how the estimated OR's are affected by the inclusion, or exclusion, of cases where the in-situ cases where downgraded from CIS (CIN3) to CIN2 in the histological re-review. As can be seen, the results in different classifications of disease (original versus revised) remained unchanged, although precision was low for assessing CIN2 risk-sets separately (also shown) conferring some variation in the point estimates.

Analysis in first smear

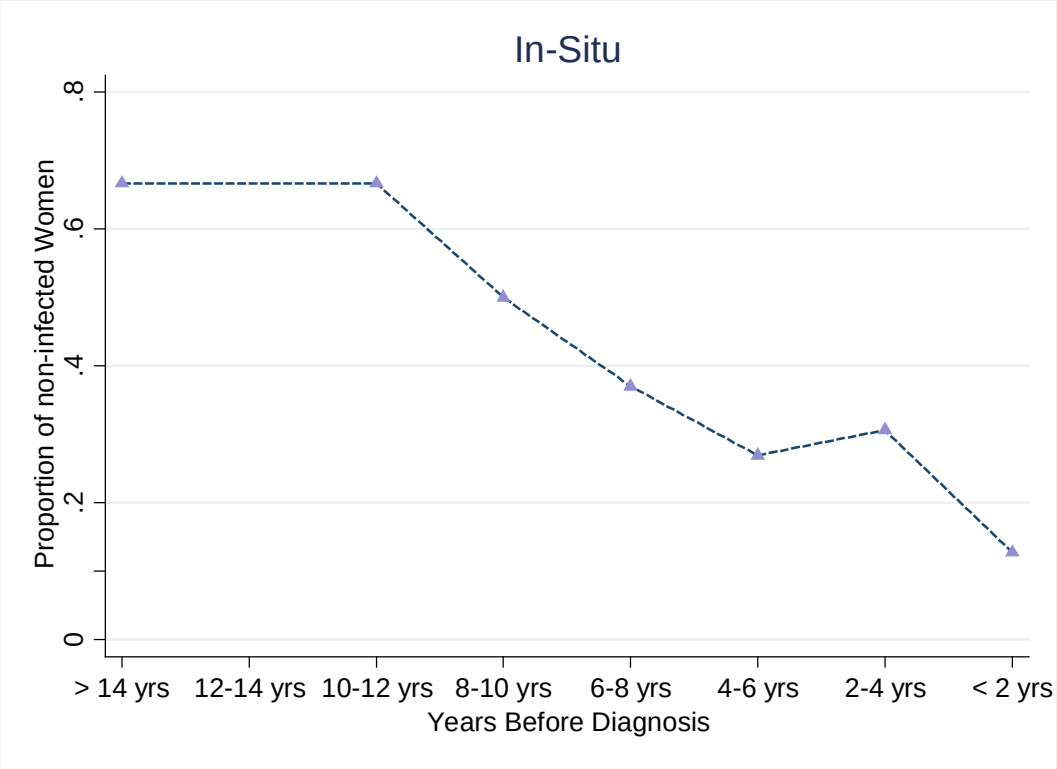


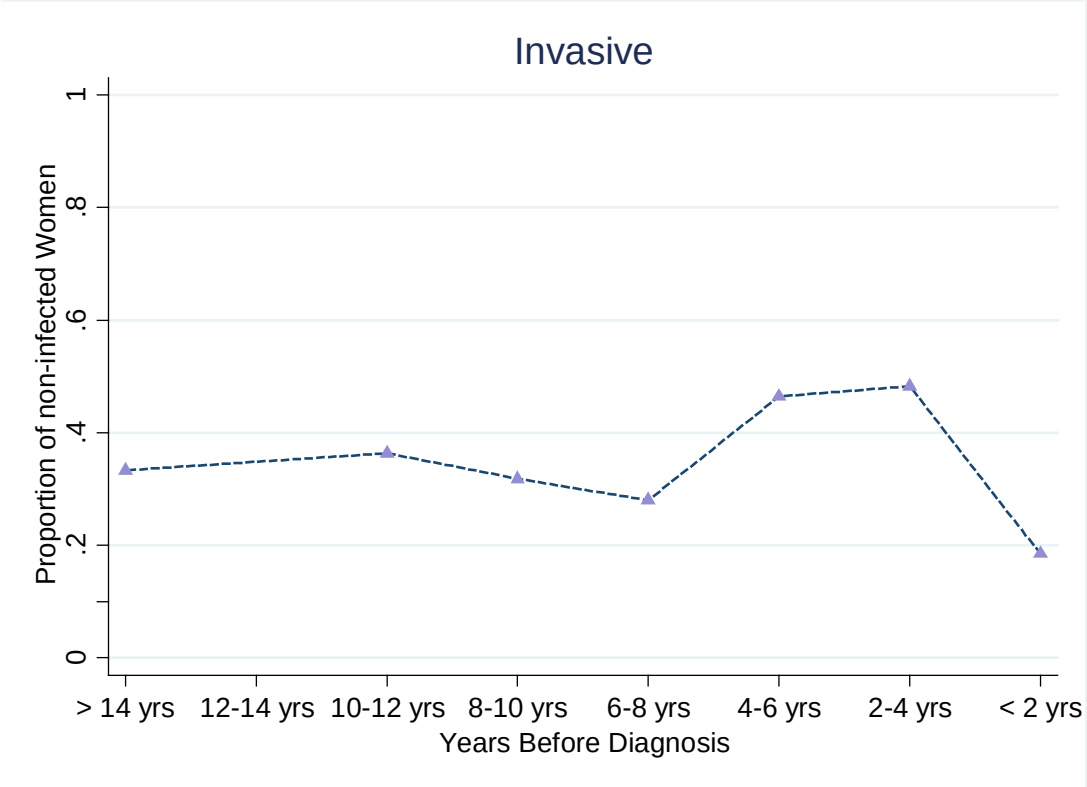
Analysis in last smear



Additional data on proportion of HPV-negative cases

Proportion of HPV-negative smears from case women by latency between last smear and diagnosis, in CIS ("In-situ") and SCC ("Invasive"). This proportion steadily decreases as time to CIS diagnosis draws near, although in SCC, the proportion was more stable. In case women whose last smear was less than 2 years before diagnosis, less than 20% were HPV-negative in that last smear.





5.2 STUDY II

5.2.1 Characteristics of the study participants

In total, Study II included 621 women with CIS, 457 women with SCC; and their matched controls. The median number of smears was similar, likewise the total range, although Table 1 in this study specifies inter-quartile range. Ages at diagnosis were similar to Study 1, as was study time although it was a little longer in both CIS and SCC. We here also describe the density of smears over 20 years before diagnosis, where it is apparent that cases and controls had the same amount of smears except for the last year before diagnosis.

5.2.2 HPV prevalence

The overall HPV positivity was 65% in smears from CIS cases, and in 55% of SCC cases, the majority of which were due to HPV16. 12-15% of control women's smears were HPV-positive, of which 3-4% was due to HPV16. (Please note that these proportions are not stratified on first/last smear, as was done in Study I.)

5.2.3 HPV16 viral load dynamics

From Figure 1, it is apparent that average HPV16 viral loads increased continuously in HPV16-positive smears taken from 10 years before, and up until time of diagnosis of CIS. The strongest surge was seen in the last year before diagnosis. In SCC, there was more of a plateau phase in contrast, but here also a surge was seen especially during the last two years before diagnosis.

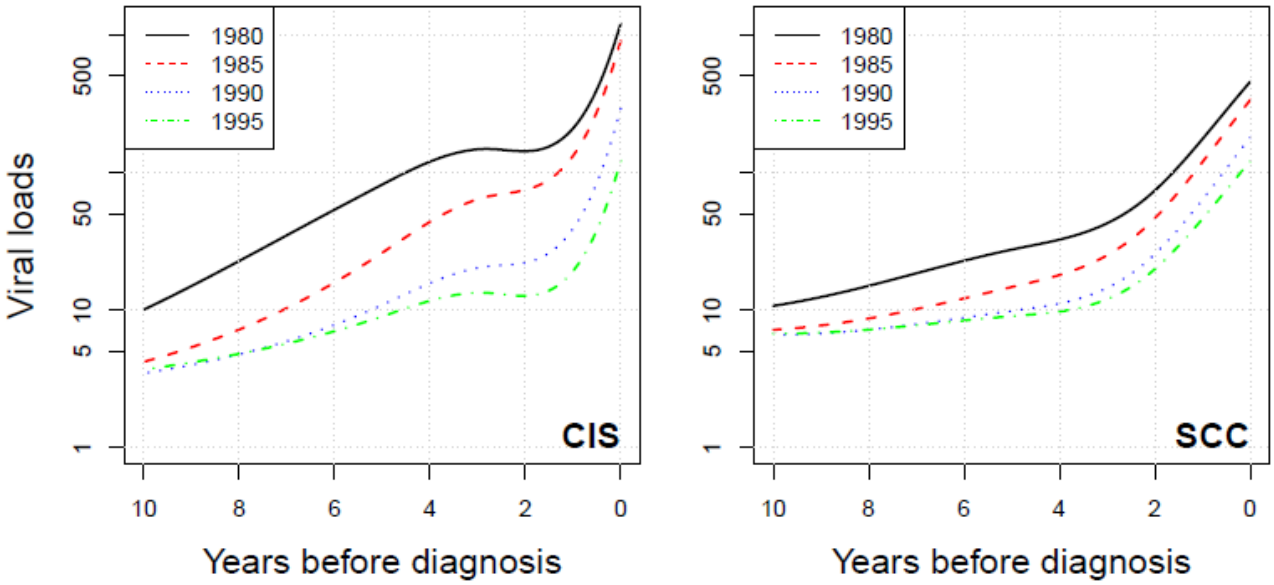


Figure 2. HPV16 viral load in copies/microliter over time to diagnosis, in cases of CIS (left) and SCC (right). Case-case comparison.

Since a strong period effect was found for viral load in CIS (see Methods), no raw viral load data (i.e. without taking period into account) are presented, as such results would be difficult to interpret. Therefore, Figure 1 shows viral load levels stratified for calendar period in case women with CIS, or SCC, respectively. For CIS cases, women who were followed for ten years in the earliest period had the highest loads at diagnosis, whereas women who were followed for ten years in the last periods had the lowest. A similar trend was visible for SCC cases but this was not statistically significant.

We further observed that women later diagnosed with CIS or SCC initially had unexpectedly low viral loads, especially in the later calendar periods. In SCC, this was quite pronounced from ten years to about four years before diagnosis. The late surge, however, resulted in high average viral loads at diagnosis in both groups.

5.2.4 Risk associated with HPV16 viral load

Initially, imputation models were constructed using tertiles of HPV16 viral load levels as cut-offs for categorization of exposure, as described above.

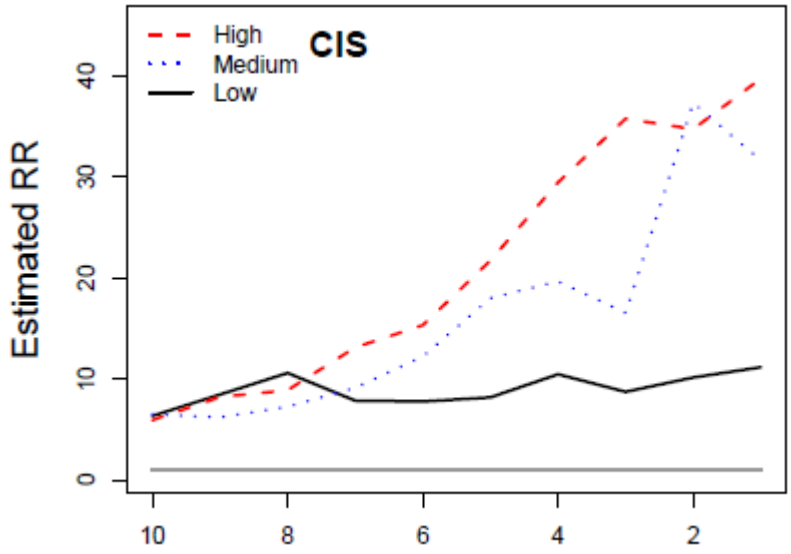


Figure 3. Relative risk (RR) for individual tertiles of HPV16 viral load in CIS, over time before diagnosis in years. The horizontal line at RR=1 is the reference level of HPV16-negative.

In the imputation model for CIS, the medium and highest tertiles aligned in risk throughout ten years before diagnosis. To increase precision, these were therefore collapsed into one. (See Supplementary Data for further information on this.) In the final CIS model; being HPV16-positive of the lowest viral load tertile was associated with a constant 10-fold increased risk throughout ten years before diagnosis, compared to HPV16-negative women. Initially, the risk associated with medium/high HPV16 viral load was similar to this.

However, at around seven years before diagnosis, there was a risk separation between the lowest and the medium/high category, so that women with a medium/high HPV16 viral load eventually had a relative risk of around 40 during the last year before diagnosis. This risk separation, although low in precision, was robust in sensitivity analyses.

Overall HPV16-positivity conferred a risk profile similar to that of the low viral load category, until the last 2-3 years before diagnosis where a more intermediate position between the two was assumed. High-risk HPV positivity was similar to HPV16-positivity over this time.

In the SCC initial model, the low and medium tertiles were similar and thus were collapsed into a low/medium viral load category.

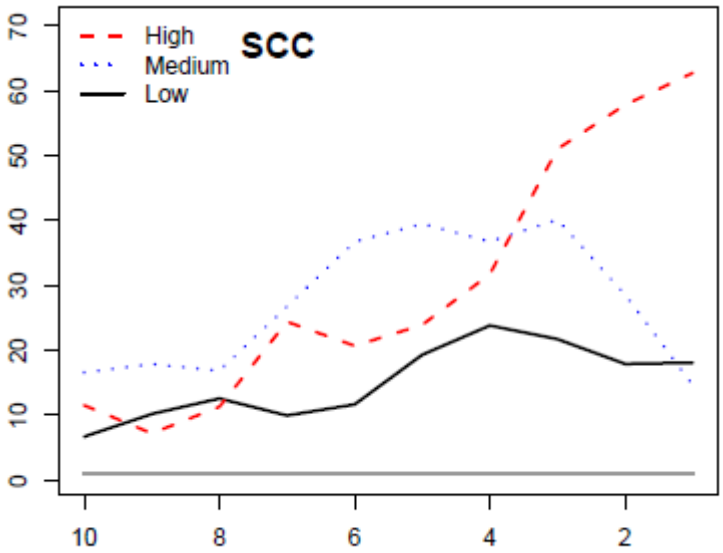


Figure 4. Relative risk (RR) for individual tertiles of HPV16 viral load in SCC, over time before diagnosis in years. The horizontal line at RR=1 is the reference level of HPV16-negative.

Between ten to three years before diagnosis, both low/medium and high viral load was similar in terms of risk, but then a risk separation similar to that in CIS occurred as well. So, although for the majority of the study time the risk for SCC appeared mainly associated with HPV16-positivity of any viral load level, from around 3-4 years before diagnosis the high category did appear to confer a significantly increased risk. Ultimately, this risk increase ended at around 70-fold, in contrast to the 20-fold risk associated with the low/medium category, compared to HPV16-negative women. Again, this risk separation was robust in sensitivity analyses, although somewhat less dramatic when considering histologically re-confirmed cases only.

HPV16-positivity and HR-HPV-positivity conferred much the same risk profile as the low/medium category of HPV16 viral load.

5.3 STUDY III

5.3.1 Characteristics of the study samples

- Parents

The median age of parents was 44 years. Almost equal numbers answered through the web questionnaire (42%) and paper version (39%). Most were born in Sweden (87%) and the majority lived in rural or small town areas. 76% were either married or had a partner, and the mean number of children per household was 2.2. 38% had higher education than high school and 87% were gainfully employed.

- Young adults

The median age was 24 for women and 23 for men. Most chose the web-based questionnaire as response method. More men than women responded via telephone interview. Most respondents were born in Sweden (89-90%) and were non-married (90-96%). Two thirds of the respondents lived outside of Sweden's largest cities. The degree of highest education in the sample varied, as expected given the age demographic of the group.

5.3.2 Response rates

- Parents

The response rate was 70% among mothers and 69% among fathers. Mothers accounted for 58% of the respondents.

- Young adults

The response rate among women was 55% and among men 43%. Women accounted for 84% of the respondents.

5.3.3 Awareness and knowledge of HPV

Since awareness of cervical cancer and condyloma was high, but awareness of HPV was low; understanding of the causal link between the two was naturally limited. Even among those that had heard of HPV, how many were aware of the link between HPV and cervical cancer varied strongly within the respondent groups. Slightly more concurrent between groups, but even lower absolutely speaking, was the knowledge that HPV caused condyloma. Female parents generally were the most knowledgeable, and young men the least. Interestingly, there was one case where this did not apply; namely the question on whether HPV can cause other cancers. This question clearly was the most difficult for respondents to answer, with around 64-69% of respondents in all groups being uncertain.

There was a contrast between the item on whether HPV is sexually transmitted, which a large majority of respondents stated "Yes" to, and the item on whether men can become infected with HPV, to which 10 percentage units fewer respondents said "Yes".

In combination with the fact that almost all respondents stated that women could become infected with HPV, this may point to some of the complexities involved in conceptualizing HPV as an STI when it is still mainly associated with cervical cancer in women.

5.3.4 Correlates to correct knowledge of HPV

It should be said that many potential correlates were assessed to establish whether there was a relation with the outcome HPV knowledge (for the young adults all variables listed in Table 3 in Study IV). However, (surprisingly) few correlates came up stable. In young adults, no questions on sexual habits showed significant effect in the model build. Classic demographic factors were the main correlates for high knowledge; gender, income, education and country of origin. In young adults, a (paradoxical) correlation between low income and high knowledge was seen, but this may be due to residual confounding, from not having entirely updated information on educational degree in this mobile young cohort.

A note should be made to the significance of the response method, where those that responded through the internet showed higher knowledge. As discussed further in article IV (see the Discussion section in that paper), respondents were not randomized to different response methods, but self-selected whether to answer online or not.

5.3.5 Sensitivity analysis

Although not included in the paper due to the small changes in results, and the brief format, the same non-response analysis carried out in Study IV (see Methods) was carried out also for this study for the young adults group. The proportions of awareness for cervical cancer and condyloma shifted somewhat downwards when under-represented respondent groups were brought in. However, HPV awareness remained very stable.

		Young women		Young men	
		Non-adjusted (%)	Adjusted (%)	Non-adjusted (%)	Adjusted (%)
Heard of HPV	Yes	19,9	19,2	12,6	12,2
	No	80,1	80,8	87,4	87,8
Heard of cervical cancer	Yes	91,4	90,4	69,7	68,4
	No	8,6	9,6	30,3	31,6
Heard of condyloma	Yes	91,4	89,3	84,7	82,5
	No	8,6	10,7	15,3	17,5

Table. Adjusted and non-adjusted proportions of awareness of HPV, condyloma and cervical cancer.

5.4 STUDY IV

5.4.1 Characteristics of the study sample

As the same sample of young adults was used, please see above under Study III.

5.4.2 Response rates

As stated above, the response rate in the young adults was significantly lower than in the parents' group, 55% overall versus 75%. Therefore, in this study a more formal non-response analysis was performed. The lowest response rates were seen among men in general, and – among both women and men – those respondents born outside the Nordic area, where birth country was unknown, and among those on social welfare grants.

5.4.3 Acceptability of HPV vaccination

The overall response to the main study question is given in the table below.

	Unwilling/unsure if willing even if for free	Willing to vaccinate but only if for free	Willing to vaccinate even if it cost
Women	25%	34%	41%
Men	32%	37%	31%

Men who reported only homosexual contacts had very similar levels of acceptability compared to men who reported only heterosexual contacts. Men who reported bisexual contacts appeared to have higher interest than other men to vaccinate whether if for free or not, but this did not reach statistical significance (p=0.3).

When adjusting these proportions for the main correlates of non-response, they remained virtually unchanged. Thus, the factors predicting non-response did not appear to predict acceptability to the same degree, meaning that when under-represented response groups were allotted more weight in the analysis, our results remained largely unchanged. (Indeed, as seen in Table 3, the factors correlated to non-response were not the most correlated to acceptability.)

5.4.4 Correlates to acceptability

In our analysis of correlates to willingness to vaccinate, we found several significant correlates, although most estimates were moderate, with OR:s of around 1.5-1.7 at the highest (indicating a 50-70% increased willingness relative to the reference level, i.e. those not willing to vaccinate even if for free). Some of the largest estimates were seen for bisexual respondents, and for those who perceived themselves to be at a fairly large or large risk for contracting an STI. The latter was also the strongest correlate to willingness to vaccinate even if it cost; with an OR of 2.00 (95% CI 1.6-2.6).

The chief correlates to lowest interest to vaccinate whether if for free or at a cost were, perhaps logically, related to non-belief in the efficacy of vaccination to prevent disease (OR for willingness to vaccinate even if it cost 0.3, 95% CI 0.2-0.4), and to a non-belief that vaccines are safe. Less expected was the finding that not having made one's sexual debut was correlated to a decreased willingness of approximately the same size as non-belief in the safety and efficacy of vaccines (OR for willingness to vaccinate only if for free 0.4, 95% CI 0.3-0.5 and OR for willingness to vaccinate even if it cost 0.4, 95% CI 0.3-0.4).

In the subgroup analysis among those that had made their sexual debut, several factors were correlated with an increased willingness to vaccinate: young age at sexual debut, having tried anal and/or oral sex, and having had an above median number of partners in the last year. For women, awareness of cervical screening, or participation in cervical screening, were not strong correlates to willingness to vaccinate.

5.4.5 Perceptions of HPV vaccine and health behaviors

Among both men and women, only around 13-15% believed they would be fully protected against condyloma after HPV vaccination. A large proportion (around 43%) was uncertain. Few women (6%) stated they might use fewer condoms after vaccination, and 80% stated a definite "No". However, around 12% of men stated that could be the case and men were also more uncertain of whether they might have more unsafe sex or not.

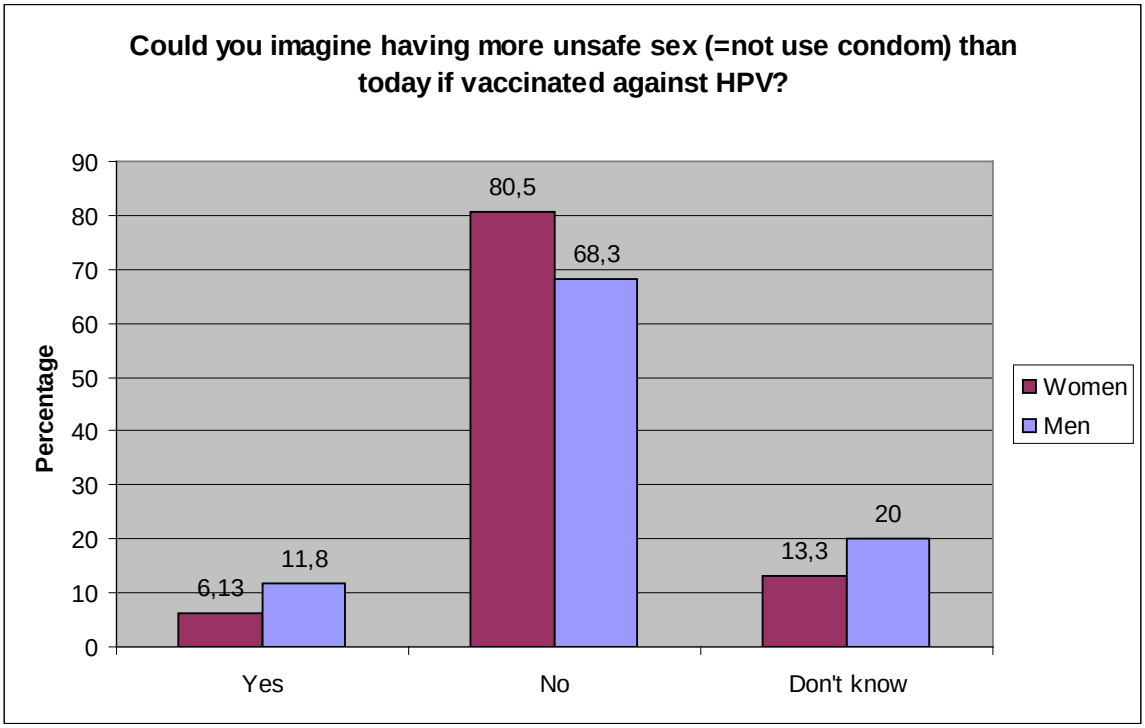


Figure 5. Response to item on potential change in sexual habits, stratified on gender.

Although only few women (around 6%) believed they would be fully protected against cervical cancer after HPV vaccination, around the same amount of women believed they would not attend screening as often after vaccination. Among women who had not participated or heard of cervical screening before the study (i.e. the young women, see graph below), the uncertainty was widespread in comparison to the older women.

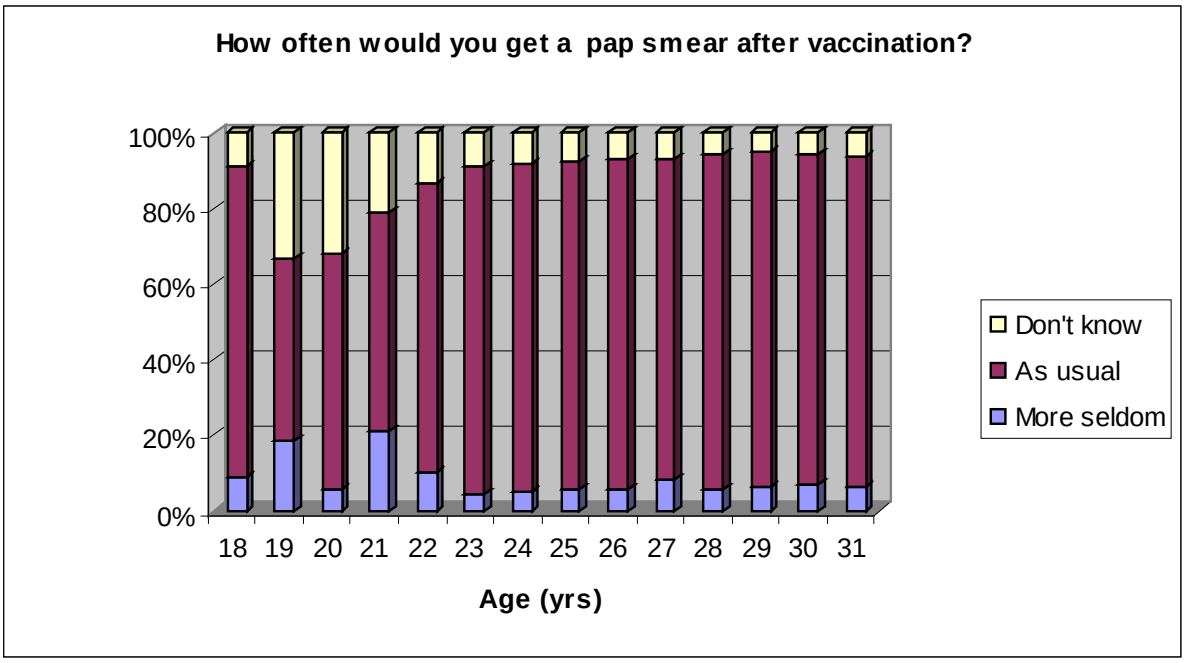


Figure 6. Proportions of response on cervical screening participation, stratified on age.

The main concern about HPV vaccination in both genders, and the main factor to abstaining from vaccination in both genders, was perceived risk for side effects of the vaccine.

6 DISCUSSION

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

T.S. Eliot.

6.1 STUDY I: HPV TYPES AND HPV PERSISTENCE

6.1.1 Main findings and interpretation

Study I provides prospective evidence of the strong risk profile of current HPV vaccine types HPV16/18 in both CIS and SCC, and also shows that non-16/18 high-risk HPV types increase the risk for invasive squamous cervical cancer. Therefore, it is estimated that a vaccine covering the non-16/18 HRHPV types could confer an additional 20% protection against SCC. It further confirms that persistent infection with HPV16 appears strongly linked to the risk for both CIS and SCC. These results support the use of persistent infection as an endpoint in HPV vaccine studies, a strategy which is not fully accepted.

6.1.2 Methodological considerations and validity

The strengths of Study I and II revolve around the quality of epidemiological, molecular and biostatistical expertise involved. Much time was spent on the design of the study and recruitment of county laboratories willing to participate, and further substantial efforts were dedicated to gathering smears for analysis and histological specimens for review. For a prospective study, the sample of invasive cancers is, to our knowledge, uniquely large. Subsequent large-scale efforts were devoted to DNA-extract and completely HPV type the smears using validated methods, and further to analyze and interpret the data. The laboratory analysis was accredited as an HPV reference laboratory, with very high standards for procedures, and was blinded to case-control status, and structures of risk-sets. This was done in order to disable the possibility of differential misclassification of exposure due to case-control status being known. Further, our pathologist was blinded as to whether the samples were originally coded as CIS or SCC. As such, the internal validity of our archival smear studies should be strong.

We did not reach 100% HPV-positivity in our case smears, which can be a ground for criticism. In smears taken close before diagnosis, we reached around 80% HPV positivity (see article I) which we conclude is the overall sensitivity of our archival smear method. We do not believe that 20% of tumors in our study were not caused by HPV, but instead, that the reduced HPV detection can be explained by several different factors. Firstly, the ubiquitously cited 99.7% attributable fraction to HPV was reached in tumor samples after subjecting them to three different PCR:s (153), whereas we only used one. Secondly, in a recent meta-analysis of invasive cancer cases, the overall HPV prevalence found was not 100%, but 89%, which is more similar to ours (154).

Moreover, we examined HPV prevalence in archival pre-diagnostic smears, rather than the actual diagnostic tissue biopsy. As such, the smear may be prone to sampling error, if not enough infected cells were picked up to trigger high-risk HPV detection. However, likely this reduced sensitivity could be non-differentially distributed between cases and controls, which would bias our results and attributable risk proportions towards the null, rather than inflate the risk associations (26).

In connection with this, ideally we should have HPV-typed the tumors belonging to the case women. This would have allowed us to study type concurrency in the smear and the tumors, and perform a closer investigation of the HPV-negative smears. However, to also type tumors was beyond the scientific scope of the study, as the original aim was to investigate screening tests over several years before diagnosis – i.e. where the future tumor type by necessity is unknown.

The sampling frame being the screening program in Sweden entailed that the results should be generalizable to a large part of the (Swedish) female population, and although it should be noted that cases and controls were matched on some basic criteria, these are unlikely to bias the findings much. Ultimately, we believe we have good generalizability in these studies as we have no reason to suppose that the overall risk profile of HPV infections, and relative contributions of HPV16 and other HR types, would be substantially different in non-screened groups. Further, although non-participation is a reason for cervical cancer in Sweden and needs to be improved, in the study of screening tests, sampling screening participants does not constitute selection bias. We did select women with a first normal smear, but this did not limit the study base much (see Methods), although we recognize that women with later disease may have been disproportionately present in that removed group. However, this strategy enabled us to study a more homogenous group which was deemed important in terms of internal validity, and enable the study of the progression from normal smear to cancer.

We did not have data on potential confounders such as oral contraceptive use, or smoking, as described in Introduction. However, compared to the large magnitude of the HPV effect size, these effect sizes have been shown to be modest, with ORs/RRs of around 2-3. Although of etiological interest, we therefore do not believe that they have heavily confounded our results (26). Another limitation to these studies include the large variation in the number of smears between women, and before these studies were launched, it was a point of discussion whether to also match on number of smears, to ensure comparable power of comparisons between case women and controls. Ultimately, it was felt that matching on number of smears would have run the risk of introducing bias into the study. This since the reasons for why case women and controls have different numbers of smears throughout life might correlate to HPV exposure status, and that forcing the smear density to coordinate could constitute over-matching (155). However, since the resulting median smear density was only around two smears per woman, it was not possible to evaluate more sophisticated measurements of persistence than HPV16-positivity in the first and second consecutive smears.

6.1.3 Comparison to other studies

The strong carcinogenic effect of HPV16 is now well-established, and some large studies have advocated the use of separate typing for HPV16/18 alone to risk stratify women in HPV-based screening (156). However, it has been shown that HPV31 and 33 are also associated with a high risk, and that other high-risk types are associated with differing risk for CIN2+ (157). We confirm this finding for our non16/18 HR HPV group, although their relative contribution was greater to CIS than SCC.

In terms of prospective risks for non16/18-HR HPV types in HPV-based screening, further follow-up from screening studies is needed to inform practice.

A recent study estimated the relative contributions (RC) of the nine types included in the coming nonavalent vaccine (HPV6, 11, 16, 18, 31, 33, 45, 52 and 58) based on data on HPV typing in invasive cancer tumors from 38 countries. By a proportional weighting method, and assuming 100% of cervical cancers were HPV-related, it was calculated that the RC of the nine vaccine types was close to 90% (158). Of these, the five non16/18 high risk types accounted for around 19%, which is in line with our attributable risk proportion of 19% for non16/18 HR HPV in the last smear before diagnosis.

6.1.4 Implications for continued research and practice

It appears crucial to continue the long-term follow-up of the risk associated with HPV types in cervical screening. Apart from long-term follow-up of the initial randomized HPV-based screening studies, also the typing of incident cervical CIN and manifest cervical tumors should be prioritized, to determine which HPV types are causing lesions and if any tumors might be HPV-negative. To establish a correct baseline containing both vaccination status and tumor status will serve as a valuable comparison for future studies of HPV vaccine effects on population-level, and inform decision making as well as detect potential vaccine failures. Such continued monitoring of potential cross-protection and/or type-replacement appears essential.

6.2 STUDY II – HPV16 VIRAL LOAD

6.2.1 Main findings and interpretation

In Study II, we demonstrate that the dynamics of HPV16 viral load appear to lend important clues to the understanding of HPV infectious natural history, but that these dynamics are also highly complex. The use of HPV16 viral load in HPV-based cervical screening may therefore be challenging and would require a diligent clinical evaluation. Furthermore, HPV16 viral loads were unexpectedly low in invasive cancer, which should be considered when weighing sensitivity against specificity in HPV-based screening.

6.2.2 Methodological considerations and validity

In terms of strengths and weaknesses of study design, the same principles as in Study I apply here also. In addition, the sample size was larger in this study, which was necessary to investigate the more complex outcome of quantitative infection. We used a type-specific realtime-PCR method for measuring viral load, which is methodologically more advanced than semi-quantitative measures (159).

Compared to Study I and qualitative infection, it was infinitely more challenging to comprehensively assess this viral load data. Upon close examination, it was evident that great care was needed in the statistical analysis. We therefore systematically assessed the data for potential error resulting from factors such as analysis person, date, or calendar period. When such tendencies were found, we adjusted for it as carefully as we could, resulting in several layers of adjustment. The relatively low density of smears limited the precision of Study II, since the more longitudinal smear information there is, the more the imputation model is informed. Taken together with the uncertainty added by the imputation procedure, the low density entailed that precision overall was low for the viral load risk analysis. As shown, the precision is significantly better when estimating risk according to qualitative infection with HPV16, or all high-risk HPV.

Even though our imputation is relatively imprecise, and is subject to additional layers of adjustment (i.e. for assay drift and calendar period effect) it still yields results for CIS comparable to other research, and we thus consider the validity for our SCC risk prediction to also be reliable.

The calendar period effect was the most dominating in our data, yet the most difficult to comprehensively explain. We assessed several different factors (time trends in HPV16 prevalence, histological re-review results, and systematic errors as described above) but could not derive any definite explanation from these. Also, whereas some multiple infections were present, these were not nearly common enough to explain the very strong calendar period effect, resulting in a viral load difference of one magnitude in the 1980's compared to the late 1990's and beginning of the 2000's. A potential explanation however, although not formally possible for us to evaluate, could be that cervical screening has improved and now detects smaller lesions, potentially of a lower viral load at diagnosis, than in the 1980's.

In terms of generalizability we believe our conclusion of the complexity of using HPV16 viral load should apply also in unscreened women, compared to the stability of using qualitative infection as a risk marker. However, as we discuss in the article, our decision to include only women with a first normal smear may have affected the viral load results in especially SCC. Thus, we acknowledge that viral loads in women screened with a first abnormal smear may exhibit different characteristics and a qualitatively different viral load kinetic curve over time before diagnosis.

6.2.3 Comparison to other studies

Some interesting complementary findings have been made recently in the viral load field. A case-cohort study has been carried out in Belgium, with quantitation of all single-type HPV infections in a large population-based material. In line with previous research, a strong risk association between viral load and risk of CIN3+ was demonstrated. Additional data on viral load kinetics showed that in HPV infections leading to CIN3+, viral load increased almost linearly each given day (160). This is well in line with our increasing curve in CIS/CIN3, although the number of cases of invasive cancer in the study was nigh on non-existent (n=7), precluding direct comparisons to invasive disease. In India, similar results were reported very recently, again confirming the large risk association between viral load and CIN3+ (161). As in the Belgian study, the small number of invasive cancer cases (n=16) precludes closer comparison, although it can be noted that 12 of these cases were found in the highest viral load category. Again, the minute number of invasive cancers accrued in these studies illustrates the power of our nested design.

The Indian study also reasons around the trade-off between sensitivity and specificity as related to viral load in their study. If their Hybrid Capture II cutoff had been increased to 2 relative light units (RLU) instead of 1, an additional 185 women with normal or CIN1 histology would have tested HPV-negative. However, one woman with CIN2 and one with cancer would have been missed. However, if they had reduced their RLU-limit to 0.5, it was calculated that only one additional CIN2 would have been detected, at the cost of 747 additional women who would have tested positive despite subsequent normal or CIN1 histologies (161).

6.2.4 Implications for continued research and practice

What does viral load actually stand for? The a priori hypothesis in this study very much was that HPV16 viral load drove progression to disease, whether CIS or SCC. As a seminal study on HPV16 viral load in CIS had previously demonstrated loads to be consistently high (79), we hypothesized that viral loads in invasive cancer would be even higher. A continuation of the upwards curve. However, as can be seen from Study II; this hypothesis was directly contradicted by our data. Viral loads in SCC exhibited a plateau phase, beginning at a very low level that was quite unexpected. Both disease groups showed a strong surge towards the end. This could represent a final disease-inducing effect of increasing viral load, or increasing viral load reflecting a larger, underlying expanding clone of HPV-infected malignant cells about to be diagnosed through cytology. It is difficult to determine which explanation is correct.

Whether viral load is the direct cause of HPV16-induced disease, or more of a risk marker, would be less central for screening inference so long as it stably served to single out women at highest risk (or those at lowest). Since the results for CIS were so strong, it was initially thought that, in the context of HPV-based screening, HPV16 viral load might indeed serve as a primary screening test, determining which women were at highest risk of cervical disease. Thus, it was foreseen that HPV16 viral load might dictate what follow-up could be appropriate for different women (155).

However, the picture from our Study II emerges more complicated than that. Due to the low precision of the study, it is difficult to draw detailed inference regarding exact effect sizes of the risk associated with viral load. However, it is still possible to interpret the overall implications as a result of the risk associations.

The differing thresholds for the risk separation in CIS and SCC means that, in a screening situation, it would be challenging to a) define what "high" viral load actually should be and b) risk stratify a woman with, e.g., a medium tertile HPV16 viral load. If it were close to time of diagnosis, she would be in the highest risk group for CIS, compared to HPV16-negative (and HPV-negative) women. However, she would not be in the highest risk group for SCC, whereas she would still be in a risk group for SCC compared to HPV16-negative women. Furthermore, the risk profile for CIS and SCC was dependent on time scale of disease development, since the risk separation occurred only late in SCC. Therefore, for a large part of CIS or SCC disease development, women are at similar risk regardless of viral load status.

Compared to instead triaging women according to the stable risk exhibited by being HPV16-positive, of any viral load level, at any point during ten years before diagnosis, the wide-spread use of HPV16 viral load in screening is not supported by this study.

6.3 STUDY III – AWARENESS AND KNOWLEDGE OF HPV

6.3.1 Main findings and interpretation

Pre-large scale HPV vaccination campaigns, awareness of HPV-related diseases was high but awareness of their causal factor HPV was low. Most respondents could correctly identify that women could be infected with HPV but whether men could be infected with HPV was not as clear to respondents, especially not among young men themselves. This should be corrected in vaccination campaigns, to promote better HPV knowledge. This would naturally be especially important if male vaccination were ever to be introduced.

The obvious limitation to this study is that it took place at such an early stage of HPV vaccination, and that the outcomes may be different today. It should therefore first be clarified that the intent was to serve as a baseline study. It was designed to investigate the degree of HPV awareness and knowledge before the introduction of large-scale vaccination campaigns, to help inform content educational messages and campaigns. Although the data are thus not totally current, we still believe the study holds several merits. These will be discussed here, along with an assessment of the relation of our results to today's situation.

6.3.2 Methodological considerations and validity

We note that the questionnaire used in both this study and study IV was not validated formally, a feature that is indeed frequently lacking in the field (62) although some examples are available (162). However, our questionnaire was constructed by researchers with significant experience from survey research, and processed through the Questionnaire Advisory Group at MEB, a group with long experience in survey design. The questionnaire was also pre-tested for question flow and logic, in a small sample of eligible adults (n=50) before being sent out, with a good result.

Regarding the survey design, the questionnaire may be critiqued for risking prompting response bias in the HPV knowledge section, since the correct answer to all questions was "yes". In a recent study which validated a measure of HPV knowledge, answers were instead varied between true and false (62). Nevertheless, in this study we did obtain significant variations in response patterns intra- and inter-respondent groups, and many chose the alternative "Don't know" instead of saying "Yes" to all questions.

In the questionnaire, it could furthermore have been good to have information on other factors of interest such as alcohol use, tobacco use, oral contraceptive use, and previous history of HPV-related disease, to correlate to knowledge and other outcomes. However, when designing the questionnaire it was felt that a previous study had charted several of these factors on a population level (41), and that the focus of the current survey should be more on awareness and knowledge of HPV (and acceptability).

Because few factors were strongly associated with having a high HPV knowledge score (as opposed to acceptability in Study IV), we opted for a backwards stepwise selection model to remove the many redundant variables.

We did however not formally assess model fit in Study III (or IV) and our discussion on this is perhaps a bit limited, given the significance of this factor (163). However, we still believe we reach an effective model for showing the main factors of relevance to the outcome.

Compared to many other surveys in selected samples such as university students or women attending dysplasia clinics, Study III and IV used the Swedish Total Population Register as a sampling frame. As such, the generalizability should be strong. However, whereas the response rate in parents was good (70%), the response rates in the young adults were lower, particularly among men (44%). Therefore, it should be acknowledged that we are not sure of the representativeness of the sample to the source population. However, in our non-response analysis the adjusted frequencies for awareness of HPV and related diseases did not change substantially, indicating that the bias from non-response may be limited.

6.3.3 Comparison to other studies

Although the literature on HPV awareness and knowledge has now increased exponentially, the wide-spread continued use of selected samples and diverging geographical settings means comparison is challenging, since recent large-scale population-based studies are still sparse. As a brief example, we show low awareness of HPV in 2007, with only 13-23% of Swedish young men and women, and 17-29% of fathers and mothers having heard of the virus, respectively. In a recent study among patients at a dermatological outpatient clinic in Germany – i.e. a quite selected material – still, only 40% of respondents had heard of HPV despite a median age of 44 in those attending. Of those HPV-aware, only around 66% stated that HPV causes genital warts, and only around 58% that it causes cervical cancer (164). Although the knowledge of condyloma was improved from our result of 40-56%, the understanding of cervical cancer was actually lower than our result of 56-82%, despite the selected nature of their sample, and several years' difference in time. This aspect is further emphasized by findings from a recent qualitative Swedish study among young HPV vaccinated women, which show a continued lack of understanding of the relation between sexual transmission, HPV and cervical cancer (165).

Similarly, in a recent population-based validation study, the item on whether HPV vaccine protects against condyloma (there termed genital warts) was still very difficult to answer correctly for respondents from three different countries (USA, the UK and Australia), along with items on whether it is true that HPV needs no treatment, and that no HPV means cancer risk is low (62). The correlation between self-rated HPV knowledge and objectively measured such was low, which emphasizes the need for objective assessment for inference regarding increased knowledge. The validated question set appears promising and could likely be valuable for the standardization of research across countries. However, this publication contained no actual data on awareness or knowledge proportions.

6.3.4 Implications for continued research and practice

In this study, we assessed awareness and knowledge of HPV before large-scale implementation of HPV vaccination in Sweden. The vaccine had been introduced to the market in late 2006, and some advertising had taken place, above all for the subsidized group girls aged 13-17 years. In late 2008, the Nobel Prize was awarded to Harald zur Hausen for the discovery of HPV, which may have helped to raise awareness somewhat after the survey was conducted. However, it took two factors in Sweden to really increase awareness of HPV and HPV vaccination: the drawn-out, complicated vaccine tender process which included two appeals of the national purchase, and the introduction of school vaccination (166).

Both of these occurred after our data collection, and likely the results today, if a follow-up study were to be performed, would show higher awareness. Since a major discussion in the tender process was which value would be allotted to protection against condyloma (167), it is also conceivable that the past debate has helped understanding of the causal link between HPV and condyloma. Understanding of HPV's contribution to other cancer forms may also have started to trickle into the population as the subject of male vaccination has started to be discussed, but it is unclear to what extent.

However, as described above under 6.3.3, the actual extent to which media attention and vaccination campaigns have altered the degree of, and correlates to, degree of *accurate knowledge* that we demonstrated, is unclear. For example, despite all the attention surrounding the tenders, the current advertising campaign from Stockholm County Council for the catch-up vaccination with the quadrivalent vaccine does not prominently feature that the actual vaccine purchased protects against condyloma, although this information is mentioned in sub-pages of the campaign web site (168). It is further unclear how much (if at all) the relative difference between female and male parents, and female and male young adults, has improved. As such, follow-up studies of awareness and knowledge of HPV in the population would be of interest.

In order to ensure equal access to vaccination, follow-up studies should also be performed to assess whether non-uptake of vaccination is correlated to poor understanding of the nature of the viral mechanisms involved. The current advertising campaign for vaccination against cervical cancer is focused entirely on girls/women. But if boys and men are ever to be included in vaccination, campaigns ensuring correct understanding of HPV transmission and disease in males would naturally be needed.

6.4 STUDY IV – ACCEPTABILITY OF HPV VACCINATION

6.4.1 Main findings and interpretation

Study IV shows that theoretical acceptability of HPV vaccination was relatively high among young adults in Sweden pre-large scale HPV vaccination campaigns – although until 2010 only few had chosen to get vaccinated, likely due to the high cost. We also show that information about the benefit of vaccination before sexual debut should be improved. The main concern about HPV vaccination was whether the vaccine could have any side effects. Few respondents stated health behaviors could change, but this needs further follow-up.

6.4.2 Methodological considerations and validity

Regarding survey design, although the questionnaire section on acceptability had not been formally validated, it was developed with great care as described above under Study III. For future surveys, it can be noted that there have now been some validation studies on HPV vaccine acceptability which could be of use (169).

When we built our statistical model for acceptability, unlike in Study III; many factors actually correlated with the outcome. We wished to be as hypothesis-free as possible when building the model, since many other studies in the area select only few variables into the final model, without reporting all variables that were triaged. In the awareness/acceptability field, it is common to use forward selection procedures where the potential correlates are only assessed one by one in relation to the outcome, rather than with other potential correlates as well. We believe our method therefore holds advantages, although as in Study III, we could have more formally reasoned around, or assessed, model fit (163).

We did not enter the HPV knowledge score into the regression model since “mere” HPV awareness was a clear correlate of increased willingness to vaccinate, whether for free or not, and we believed this was of higher priority to report. It would have been informative to have a variable in the questionnaire on past history of HPV-related disease, to see how this was correlated with acceptability of vaccination. However, it is quite possible that our variable on STI risk perception captures some of the individual’s previous STI history, including that of e.g. condyloma, meaning that we assessed some of this factor in any case.

An individual who already had condyloma or a cervical lesion would likely have a reduced effectiveness against that HPV vaccine type and so, assessment of acceptance of prophylactic vaccination in that group might appear more of academic than policy-based interest. However, it would have been interesting to know something of about potential acceptability in those already HPV-afflicted. This could have been important information for observational studies of vaccine effectiveness as a function of age at vaccination. A recent Swedish study found no HPV vaccine effectiveness against condyloma if above 22 when vaccinating, which could be explained if older women with higher risk for, or even prevalent, condyloma self-selected vaccination (100). Information on acceptability in this specific group could thus have been useful.

Gender was clearly a determinant of acceptability. Although the questionnaire specified that men could also gain benefit from protection against condyloma, the described protection against cervical cancer likely led to the high acceptance in women. It has been shown that messages on cervical cancer for female partners do not resonate among adult males or parents, but that more data on HPV-related disease in general should be incorporated in studies on male acceptability (119). This was also confirmed in a recent study on acceptability among boys age 16-18 years (115). Had the information mentioned potential protection against other cancers in males, we acknowledge that this might have increased the acceptance in men. However, at the time of questionnaire construction, claiming protection against cancers in males and sites other than cervical, would have been difficult to defend from an evidence-based perspective.

Regarding the generalizability of findings, the potential for this is significantly strengthened due to the population-based nature of the survey. However, it is also limited due to the suboptimal response rates, again particularly among young men. Therefore, inference on generalizability should be carefully balanced. We believe we have strengthened the validity and generalization of findings since our non-response analysis, which used a standard method for examining survey non-response (152), did not identify any major bias in acceptability.

6.4.3 Comparison to other studies

Higher income was expectedly correlated to higher acceptability but we also found that a higher education was correlated to a decreased acceptability, a finding which mirrors that of our previous study in parents (111). Although the latter finding may appear counterintuitive, this was actually echoed in recent studies on HPV vaccine uptake in Canada and the USA, which have found lower uptake in children to parents with more education; girls attending private schools; and boys living in high-income households (170-172). Furthermore, an English study on non-uptake of HPV vaccination found that affluent parents were more likely to actively decline vaccination, whereas living in a deprived area was associated with passive non-response to offer of vaccination (173).

However, in the recent Swedish study on effectiveness of HPV vaccination against condyloma, it was found that opportunistic HPV vaccination of females was actually much more common if parents had high education (OR = 15) (100, 174).

Can we unite these findings? The tendency for higher education to correlate to negative attitudes might be more applicable in a school vaccination setting, where *actively* abstaining from the norm of vaccination might be easier for those with a high education (and, potentially, higher confidence in decision-making). Opportunistic, i.e. voluntary vaccination, although principally different, also requires *active* decision making, especially in settings where vaccination is not the norm (i.e. the low uptake in the Swedish catch-up program). Perhaps higher education confers more confidence to act, in either case? This remains speculative, and we acknowledge that this may be a sign that our acceptability results are not fully generalizable to actual population uptake.

Further studies on uptake are clearly called for, to investigate to what extent receipt of HPV vaccine is dictated by socioeconomic status, thereby creating social inequity. It would also be of great interest to, in detail, examine non-acceptants of vaccination, although recruiting to such a study might be challenging.

Along with licensing of HPV vaccination in the Western world, two concerns were raised around potential negative effects of vaccine receipt. In the US, it was speculated from conservative groups that HPV vaccination could lead to sexual disinhibition due to a perceived implicit approval to be sexually active, and these groups strongly opposed a mandated vaccination (175). This is a notion which may appear irrational and antiquated. Nevertheless, some media held up this concern and HPV vaccination was, among other things, described as "yet another green light on the road to promiscuity" (115). Therefore, although controversial, research is needed on this issue and an item on sexual behavior after vaccination was included in our survey, along with questions on sexual habits. We found that few respondents thought their safe sexual behavior could change, but that some did and more expressed uncertainty. We simply interpret this to signify that careful information on what protection HPV vaccination can, and cannot, confer should be a feature of vaccination campaigns.

Some studies have shown that girls think that they themselves would not change behavior, but that other girls might (176, 177). Perhaps it is easier in a survey to reason about the altered behavior of others, rather than state that oneself could pursue less safe sex, for example. However, reassuring data have now emerged on this issue. A recent study on managed care organizational data showed little difference in adverse outcomes of sexual activity (defined as pregnancy/STI-testing or diagnosis; or contraceptive counseling) between those who received HPV vaccine and those who did not (178). Furthermore, a recent study in Australia showed that HPV vaccinated university students held more positive attitudes toward safe sexual behavior than non-vaccinated peers (179), and an English study among school girls shows that neither being offered the HPV vaccine nor receiving it affected sexual behavior (180). Thus, data in uptake studies are positive, although if boys/men were to be vaccinated, similar uptake research would be called for.

The second concern on HPV vaccination is that vaccinated females might perceive themselves as fully protected against cervical cancer, and thus participate less in screening. In adult women, this would be highly risky, and even in school girls, continued screening participation will be key to proper evaluation of vaccine effects in the nearest future. In our study, we found that the youngest women were unsure of whether they needed as many pap smears as previously after vaccination. Again, researching this issue is called for and here, recent findings appear somewhat conflicting.

In Australia, one of the first countries to implement large-scale vaccination, it was found in a qualitative study that the majority of girls aged 12-16 believed they were indeed fully protected against cervical cancer after vaccination (181), and a quantitative study on women age 18-27 found that 19% viewed screening as being of low importance following vaccination (182).

A study on university students found that knowledge of cervical screening guidelines was generally poor but that intentions to undergo screening or uptake of screening did not differ between vaccinated and unvaccinated women (179). An Australian study on women age 18-28 found that among women aware of the vaccine, 96% knew that Pap tests were still needed after HPV vaccination, yet 20% thought that vaccination could prevent all cervical cancer – which raises the question whether there might be a disconnect between knowing the need to participate in screening, and truly understanding what screening is for (183). A similar lack of understanding of what cervical screening meant was recently shown among young HPV-vaccinated Swedish women (165).

In contrast to all this, a recent study from Canada showed that HPV-vaccinated women actually participated *more* in cervical screening than matched unvaccinated women (184). If they did, this would of course be advantageous compared to the alternative, that they would participate less. However, it would not be preferable from a health-economical perspective as it would mean that we doubly protect some women, whereas others are doubly cut-off from both screening and vaccination. This may be of growing concern in the field (185). Clearly, although cervical screening has been phenomenally successful in reducing cervical cancer burden in industrialized countries, perceptions and understanding of, as well as attendance to, these programs is key to safeguard. Regardless of vaccination status, all women will benefit from collective efforts to maintain high fidelity to screening programs.

6.4.4 Implications for continued research and practice

Returning to the Swedish situation, it appears that, despite high acceptability and media exposure, uptake of HPV vaccination in women above 18 has been very low, and virtually non-existent in males. This is likely due to a combination of reasons such as cost, practicalities and peer behavior. Even in the subsidized age groups, where the state has accepted a substantial part of the cost for the vaccine, so far only around 30% of eligible girls have vaccinated (100). School-based vaccination of girls and young women appears as the most realistic way forward for ensuring reasonably high to high coverage of HPV vaccination, especially in risk groups. Had the catch-up program been organized at a high-school level, instead of entirely opportunistic; potentially coverage could have been much increased. However HPV vaccination continues, as stated above, uptake research will be essential to monitor the degree of socioeconomic equality.

In contrast to the correlation between acceptability and self-perceived risk of STI, as described above, the acceptability of vaccination was low among respondents who had not yet made their sexual debut. The questionnaire information specified that HPV was an STI, but the benefit of vaccinating *before* sexual debut was clearly not obvious to all respondents. Perhaps virgin respondents felt that the vaccine was not intended for them, although it is prophylactic like all other vaccines and thus most effective if given before any exposure. In line with this, most respondents chose age 15-17 for age to commence HPV vaccination, which may indicate that understanding of general optimization of vaccination could be somewhat better, since in this age group, many will have made their sexual debut.

Now that school vaccination is a fact, much of the HPV vaccination benefit will be maximized in school children, and the Stockholm County Council advertising campaign website prominently mentions higher protection if vaccinated early on their portal page (168).

Should young adults really get vaccinated after sexual debut? This is clearly a complex issue. Efficacy trials indicate that young adults can have a substantial protective effect of vaccination up until age 26, yet some effectiveness studies do not (100). The limitation of the randomized trials is that participants had to meet certain criteria, which may limit generalizability of the findings. On the other hand, in real life an older group with high risk for, or even prevalent, condyloma may have vaccinated first, limiting the generalizability of those findings to those of the same age but lower exposure to HPV.

Age at vaccination is a proxy for risk of exposure to HPV: if a person has not had any HPV-related disease, and few sexual partners, protection should still be good even if she/he is around 25 years old. However, if a person of the same age has had many sexual partners, and perhaps even condyloma or cervical lesions, vaccine effectiveness will be reduced and the cost-effectiveness of vaccination both for the individual and society will be reduced likewise. It has now been decided that all women until age 26 will be offered subsidized vaccination, and in Stockholm, free such. Ultimately, if the now extended vaccine campaigns translate into high population-level coverage up until age 26, future studies will be able to assess real-life effectiveness even better, which may yield clearer answers than we have today.

The only main concern in this survey was that of vaccine safety. This was in 2007, long before an association between vaccination against pandemic H1N1-flu and narcolepsy was published (186). The association with narcolepsy garnered widespread attention in Swedish society (187), and now more than ever, it is of key significance to maintain public confidence in vaccination. In a recent report on national television, the fear of adverse effects such as narcolepsy was cited by young women as a contributing reason on why they and fewer than expected eligible girls had vaccinated against HPV. Reassuring safety data on HPV vaccination from Sweden and Denmark have already been produced (188) but clearly, to maintain public confidence in vaccination programs this is a key area of priority for future research and follow-up (189). Active continued information campaigns on the safety aspect of HPV vaccination are essential.

7 CONCLUSIONS

Study I and II:

- Persistent infection with HPV16 is strongly linked to the risk for both in situ and invasive squamous cell carcinoma.
- The cancer risk conferred by HPV16 increases over time, whereas the risk conferred by HPV18 is more stable over time.
- Non-16/18 high-risk HPV types increase the risk for both in situ and invasive squamous cell carcinoma, although their relative contribution is greater to carcinoma in situ.
- HPV16 viral load was initially unexpectedly low in invasive squamous cervical cancer. However, in both in situ and invasive squamous cell carcinoma, HPV16 viral load increases quickly in the last few years before diagnosis.
- HPV16 viral load is linked to future risk for both in situ and invasive squamous cell carcinoma, but the risk functions are complex and differ over time.
- Hence, HPV16 viral load may struggle to improve HPV-based screening but may carry important implications when weighing sensitivity against specificity in such screening.

Study III and IV:

- Pre-large scale vaccination campaigns; awareness of condyloma and cervical cancer was high in the young adult and adult Swedish population, but awareness of HPV and the causal relationship to these diseases was low.
- The understanding that men could contract HPV was deficient, and information campaigns for vaccination should correct this, especially if male vaccination is ever to become an avenue for infectious disease prevention.
- Hypothetical acceptability of HPV vaccination was quite high among young adults in Sweden pre-large scale vaccination campaigns, although until today, only few have gotten vaccinated. Information on the benefit of early vaccination is needed.
- Future research should concentrate on actual vaccine uptake, and vaccine safety.
- Few young adults stated their healthcare-related behavior would change after vaccination, which is reassuring. Correct information on the benefits and limitations of HPV vaccination should be given when vaccinating this group. Follow-up of young women's participation in cervical screening may be warranted.

8 SUGGESTIONS FOR FUTURE RESEARCH

The means to prevent cervical cancer have now been made available in the form of testing and vaccinating against HPV, and the scientific and clinical communities have an opportunity to remove such a large part of the disease that it might be possible to speak of eradication. Yet these tools are far from fully implemented. Thus, although revolutionary cancer vaccines have been developed, several public health challenges remain in order to achieve optimal cancer control. These are some general suggestions for future research and practice.

- Design of, and attendance to cervical screening

Of significance in cervical screening, the gap between science and practice should not be allowed to widen too far or too quickly. Several countries, even some highly developed European such, still today lack high-quality nation-wide organized cervical cytological screening programs, or only recently introduced such. In this context, HPV-based screening may present a fresh start, but can also require large-scale investment in countries which just recently managed to organize cytological screening.
Strategies to increase participation to screening would likely be successful in decreasing cervical cancer incidence in Sweden, whether this screening is cytological or HPV-based. Options to increase participation to screening might want to move beyond traditional reminders of attending. Similarly to mobile blood donations, a mobile cervical screening unit was introduced in parts of Sweden in 2010. Also, self-sampling for HPV in the home might be a future avenue for organized screening, although the women diagnosed with HR-HPV will still have to come in for further testing and the acceptability of self-sampling may differ between different groups.

In the future, the HPV-based screening scenario will present several specific logistical challenges. Since a negative HPV test confers longer protection test compared to a negative cytological smear, screening intervals will be lengthened and differ depending on HPV status. This will place high demands on screening organization and recall-systems. It might be a future challenge to bring women in to screening again after ten years, especially as some data indicate that women consistently underestimate the time since their last cervical screen.

- Acceptability and consequences of HPV-based screening

Whereas HPV-based screening conceptually speaking is logical, on the verge of elegant, in real life successful implementation relies on a number of factors. A new intervention is about to be presented into the female population, and research indicates that psychosocial effects could be seen. Of crucial importance, therefore, is to examine women's acceptability of HPV-based screening, and how well-equipped the clinical staff doing the follow-up is to handle women with positive results. For example, when HPV-based screening is introduced, some women might not be satisfied with being declared HPV+ but cytologically negative, and thus not in need of screening for another three years (according to proposed guidelines).

It will be of great interest to follow whether such women instead get more opportunistic cytological smears, or HPV testing, as a result.

- Uptake of HPV vaccination

As described, uptake in organized vaccination programs has varied so far. Programs in Australia and the UK have succeeded, while the Dutch program failed with to reach more than 56%. It is crucial to study those that do not accept vaccination for their children, although such research might likely be difficult. In Sweden, large social disparities in who participates in the catch-up program have been identified and need to be further examined.

Final reflections

Perhaps the greatest challenge in the coming years will be to clarify that cervical cancer prevention, at the end of the day, really is infectious disease prevention (190). At first, it was believed that HPV vaccination would be extremely expensive. The vaccine's preventive effect on cervical cancer, rather than the agent HPV, was therefore the natural focus of campaigns to introduce and subsidize the vaccine. With public tenders and two vaccine manufacturers in competition, however, the actual price obtained has been greatly reduced. This could mean a drastically changed cost-effectiveness ratio for, e.g., male vaccination. I would here like to state that it is my personal opinion that males should be vaccinated against HPV, since this would likely achieve the benefit of a quicker removal of HPV and related diseases from the population. However, the debate on whether to aim for eradication, or settle for herd immunity in men from female vaccination, is ongoing and likely needs to be informed by further trials before public health policy will be altered.

Further, the HPV vaccination field appears to be in a state of scientific flux: "truths" that previously were apparent become outdated in only a few years' time. Initially, it was believed that there would be limited, if any, cross-protection against non-vaccine types. An expanded vaccine was therefore actively called for (by among others, ourselves in Study I). Today these voices have somewhat diminished. The cross-protective effect in vaccine trials has surpassed expectations. Thus, the additional benefit might be less clear. Head-to-head trials on an expanded, versus the existing, vaccine should illuminate the issue, yet have not reported any results so far. Moreover, the effect on population level was thought to take some time to achieve, especially if only vaccinating girls, yet Australian data already show a drastic drop in HPV prevalence, and partial herd immunity. A chief future issue might not be the number of types included in the vaccine after all, but to reach a high population-level of coverage, and to reach the highest risk groups for cervical cancer, and other HPV-related cancer.

Although many obstacles remain, the potential to greatly reduce HPV-related morbidity and mortality appears within reach, and it will be the challenge for public health policy to see this potential realized. If successful, cervical cancer may come to serve as a model system for state-of-the-art disease prevention.

Karin Sundström, Stockholm, December 2012.

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