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# Risk Factors for Cervical Cancer Development

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Institutet**

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Cover figure: Image of woman and Human Papillomavirus adapted from:  
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*I dedicate this work to my parents, John Beaudry Gunnell and  
Noelene Jean Gunnell.*

*And to my wonderful wife and daughter, Kristjana  
Einarsdóttir and Lísá Sóley Gunnell.*

## ABSTRACT

Cervical cancer remains one of the leading causes of cancer mortality globally, predominantly in less developed countries. It is widely accepted that certain ‘oncogenic’ types of HPV (Human papillomavirus) are necessary causes of cervical cancer development and a number of co-factors (including smoking) have also been implicated. The introduction of Papanicolaou testing has achieved a large reduction in cervical cancer incidence over the last 4 decades however its use is not without problems, particularly with regards to its relatively low specificity. Recently HPV vaccines targeting HPV-16 and HPV-18 have been developed, which hold the promise of reducing cervical cancer incidence further. In the four studies included in this thesis, we investigate several risk factors related to cervical cancer formation, test the efficiency of Pap smear screening in reducing the incidence of the two main cervical cancer types (cervical squamous cell carcinoma and adenocarcinoma), investigate possible maternal transmission of cervical cancer *in situ*, and estimate the effects that vaccines against HPV-16/18 might have had on the Swedish population over the last 30 years had they been implemented.

Using a population-based case-control study, we examined the individual risks for cervical cancer *in situ* (CIS) associated with HPV-16 presence, HPV-16 load, and smoking. After analysis of first cervical smears for 375 case and 363 control women, we found higher increased risks associated with HPV-16 presence in smokers (adjusted OR=14.4; 95% CI 5.6-36.8) than non-smokers (adjusted OR=5.6; 95% CI 2.7-11.2), compared with HPV-16 negative women. Risk for CIS was even higher in smokers with high HPV-16 load (adjusted OR=27.0; 95% CI 6.5-114.2) compared to smokers who were HPV-16 negative. In contrast, non-smokers with high HPV-16 load had a lower risk (adjusted OR=5.9; 95% CI 2.4-14.6). Neither HPV-16 presence nor load were found to significantly interact with smoking status, although significant interaction was found between current smoking status (at first smear) and duration of smoking ( $p=0.03$ ).

Analysis of Swedish nation-wide data covering 1968-2002 provided evidence for the beneficial nature of Pap smear screening in reducing cervical squamous cell carcinoma (SCC), but not cervical adenocarcinoma (AC). We also found that higher reported CIS incidence rates in certain counties did not lead to future reductions in SCC incidence. This was also the case for adenocarcinoma *in situ* (AIS) reported, and future AC incidence. On the whole, our results suggest there is over-treatment of CIS. Also it appears likely that inefficiencies exist in the use of Pap smear screening for AC prevention.

In a cohort of mother-daughter pairs, we found a 24% excess risk for CIS in daughters whose mothers exhibited CIS within 10 years after their daughter’s conception compared to daughters whose mothers had CIS more than 10 years after their conception. This may conceivably be the result of maternal transmission of HPV during pregnancy or childhood.

Using a population-based cohort of Swedish women, we estimated that a 61.2% reduction in SCC incidence could have been achieved through the removal of HPV-16/18 from the population during 1969-2002. In comparison, removal of all measured high risk HPV types may have reduced SCC incidence by 83.9%. Removal or reduction of HPV-16 and -18 from the Swedish population will give considerable benefit in reducing cervical cancer. However, the risks associated with other non-HPV-16/18 oncogenic types are worthy of consideration in future monitoring and prevention strategies.

## LIST OF PUBLICATIONS

This thesis is based on the following papers:

- I. Gunnell AS, Tran TN, Torrång A, Dickman PW, Sparén P, Palmgren J, Ylitalo N.  
**Synergy between Cigarette Smoking and Human Papillomavirus type 16 in Cervical Cancer *in situ* Development.**  
*Cancer Epidemiology Biomarkers and Prevention* 2006; 15(11): 2141-7.
- II. Gunnell AS, Ylitalo N, Sandin S, Sparén P, Adami H-O, Ripatti S.  
**A longitudinal Swedish study on screening for squamous cell carcinoma and adenocarcinoma; Evidence of effectiveness and over-treatment.**  
*Cancer Epidemiology Biomarkers and Prevention* (in press).
- III. Gunnell AS, Ripatti S, Sandin S, Sparén P, Ylitalo N.  
**Cervical Cancer *in situ* in Mothers and Subsequent Risk for Daughters: Maternal Transmission of HPV?**  
*Submitted 2007.*
- IV. Gunnell AS, Sparén P, Ripatti S, Adami H-O, Dillner J, Ylitalo N.  
**Risk for severe dysplasia/cancer *in-situ* and invasive squamous cell carcinoma conferred by common oncogenic HPV types in Sweden.**  
*Submitted 2007.*

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## ABBREVIATIONS

AC	Cervical Adenocarcinoma
AIS	Cervical Adenocarcinoma <i>in situ</i>
CIS	Severe Dysplasia / Cervical Cancer <i>in situ</i>
CIN	Cervical Intraepithelial Neoplasia
DNA	Deoxyribonucleic Acid
GEE	Generalized Estimating Equation
HPV	Human Papillomavirus
HRHPV	High-Risk HPV
HSIL	High-grade Squamous Intraepithelial Lesion
LCR	Long Control Region
LLETZ	Large Loop Excision of the Transformation Zone
LRHPV	Low-Risk HPV
OR	Odds Ratio
Pap	Papanicolaou
PAR	Population Attributable Risk
PCR	Polymerase Chain Reaction
RDBH	Reverse Dot Blot Hybridisation
RR	Relative Risk
SCC	Cervical Squamous Cell Carcinoma
SIR	Standardised Incidence Rate



## INTRODUCTION

We live in an exciting period regarding the evolution of cervical cancer management. During the last 5 decades, cervical cancer deaths have been considerably reduced through the implementation of cytological Papanicolaou (Pap) testing. It has long since been accepted that cervical cancer generally progresses through a series of stages prior to its development. Careful monitoring of at-risk women, through cytological screening of cervical samples, allows clinicians to identify and remove dysplastic cells before they progress to cervical cancer.

Removal of dysplastic lesions has been immensely successful but is far from perfect. Apart from the physical discomfort bestowed upon patients during their regular visits and the slightly elevated risks for birth-related complications, logistic problems exist. Women must be vigilant in their compliance to testing and even in countries with highly organized screening programs, lack of attendance remains a major cause of cervical cancer incidence. Ironically, another major problem related to this form of prevention is over-treatment. Some studies have shown that whilst Pap screening is effective, far too many dysplastic lesions are removed without any obvious benefit; meaning that many of the precursor lesions would not have progressed into cervical cancer. This of course relates to our lack of understanding as to how cervical cancer actually develops.

Most researchers would agree that cervical cancer is fundamentally a symptom of viral infection. The virus in question – *Human Papillomavirus* (HPV) – resides in the cervical epithelia and usually causes nothing more innocuous than occasional benign lesions. HPV is one of the most common sexually transmitted infections and yet, even though up to 80% of women will contract this infection over their lifetime, very few women will develop cervical cancer. Clearly HPV is not a sufficient cause but it does appear to be a necessary cause. So what characteristics of this usually harmless virus allow it to become deadly?

From a simplified point of view, the main determinants of HPV's ability to cause cervical cancer appear to be HPV type, HPV persistence, HPV load, physical state of HPV (episomal or integrated), and possibly the existence of multiple HPV infections. Other co-factors have also been implicated, such as smoking, HLA type, parity, and sexual behaviour, although many of these (and other) co-factors are likely to work in combination with one or more of the 'main' HPV-related determinants.

This knowledge has led researchers to the inevitable conclusion; if we can control HPV, we can control cervical cancer. Based on this concept, pharmaceutical companies have joined the fray and developed vaccines against two of the main HPV types thus far associated with cervical cancer (HPV-16 and HPV-18). Countries who embrace these vaccines are now set to enter a new stage of cervical cancer management. Will this mark an end to cervical cancer?

Widespread introduction of HPV vaccination for women will undoubtedly have a considerable impact on cervical cancer development and related mortality. In their present forms, the two current vaccines will not eradicate cervical cancer. Recent randomized

clinical studies involving the two vaccines have shown almost complete protection against HPV-16/18 and resulting cervical intraepithelial neoplasia 2+ (CIN2+), which is considered to be a precursor to cervical cancer. It remains to be seen how this will translate into practice when distributed to a population however, which includes different age groups and women with prior HPV infections. Also, since the studies have used CIN2+ as an endpoint (instead of cervical cancer), there is still a question of how many cervical cancers will indeed be removed from the population as a result of HPV-16 and HPV-18 removal.

The studies included in my thesis aim to address some key questions related to the risk factors involved in the development of cervical cancer and subsequent management of cervical cancer.

The first study addresses the risk associated with one co-factor (smoking) in the development of CIS. In this study, we take a step beyond the generally accepted fact that smoking is involved in cervical cancer formation, to see whether its role as a risk factor may be related to an interaction with HPV presence and even the viral burden (HPV load).

My second study explores the efficiency of cervical cancer management over the last 3 decades in Sweden. With regards to organized Pap smear screening for cervical cancer prevention, Sweden possesses one of the most rigorous programs in the world. Using extensive centralized registry records, we were able to retrieve information on ‘county by county’ incidence rates of cervical cancer and its precursor lesion CIS. Armed with this data, we delved into the differences in CIS and cervical cancer incidence between counties. Specifically, we were investigating whether the variations in CIS incidence between counties corresponded to similar variations in cervical cancer incidence, which one might expect.

My third study stemmed from an interest in HPV vaccines and the idea that they may not show as great an efficacy in already HPV-exposed women as they do in women without prior HPV exposure. For population immunization programs, the age of vaccination is chosen to allow the greatest effectiveness of the HPV vaccine – prior to sexual debut. But what if there are alternative means of transmission? In previous studies, a surprisingly large number of children have been reportedly HPV positive. One possible explanation for this phenomenon could of course be the transfer of HPV from mother to child, and some evidence exists to support this. Since we did not possess HPV information on mothers at their children’s birth, we used CIS as a surrogate marker for HPV presence in mothers (since HPV should also be present). We then looked at the likelihood that daughters of women with CIS near delivery were themselves at higher risk for CIS in later life.

The last study also investigated an aspect related to the introduction of HPV vaccines. Namely, we were interested in the likely contribution of HPV-16 and HPV-18 to cervical cancer development in Sweden and the possible remaining risks attributable to other HPV types than HPV-16/18. We collected women’s archival smears from 6 counties of Sweden for women with CIS or cervical cancer, along with matched controls. HPV typing was performed and we were able to estimate relative risks and population attributable risks associated with HPV-16/18 and non-16/18 high risk HPV types.

Progress towards improved prevention for one of the most preventable cancers is well underway. The next decade in HPV and cervical cancer research should prove to be dynamic and exciting. The next stage of cervical cancer prevention has begun.

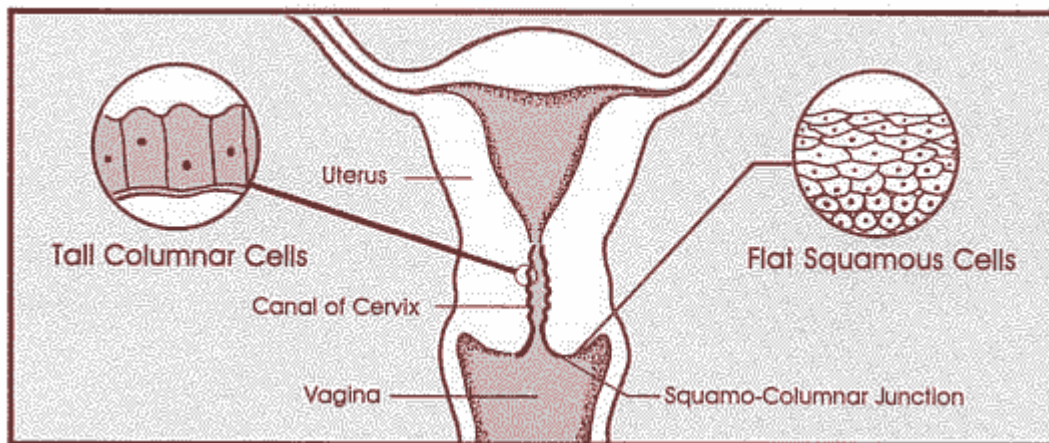
## BACKGROUND

### The Cervix

The cervix is a narrow portion of the uterus that extends to the top of the vagina (Figure 1) and correspondingly derives its name from the Latin word for 'neck', referring to the neck of the womb. The main role of the cervix appears to revolve around the maintenance of normal pregnancies in women. Ironically, its activities in assisting with the creation of life are often overshadowed by its infamous relationship with cancer-related death.

The predisposition of the cervix towards cancer formation relates to the high cell turnover that occurs in an area referred to as the transformation zone. In this area of constant epithelial shedding, differentiation of basal and parabasal cells must occur in order to replenish the epithelial layer. It is within these totipotent cells that the path towards cervical cancer begins.

**Figure 1: The Cervix.**

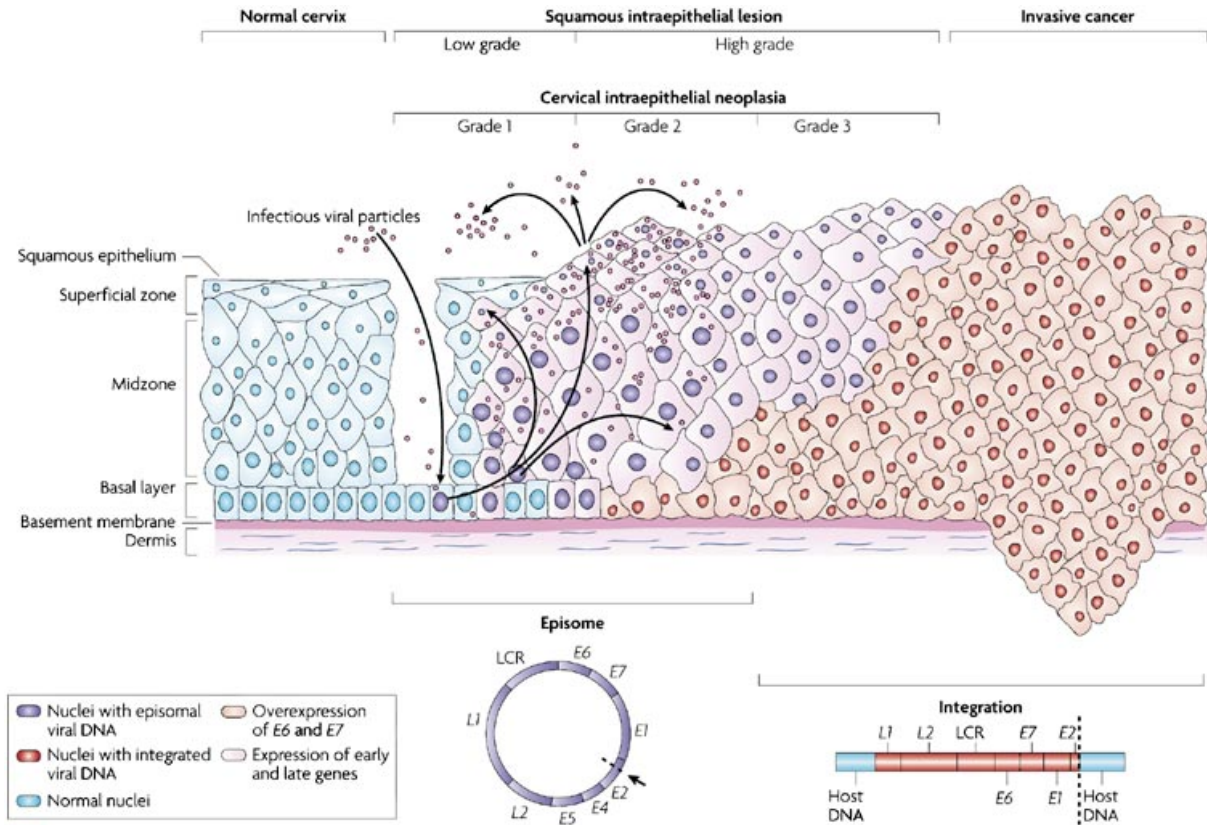


## **Cervical Intraepithelial Neoplasia**

Cervical squamous cell carcinoma exhibits a well-defined pre-malignant phase which, incidentally, allows for successful management using Pap smear testing. This pre-malignant phase is characterized by various stages through which cells proceed prior to the onset of invasive disease. These stages are accompanied by numerous measures of cancer-related changes in the proliferating primitive cells, such as nuclear abnormalities; including nuclear size, number of mitoses, and presence of abnormal mitotic forms (1).

Basal and parabasal cells form on the basement membrane of the dermis and develop, through a process of differentiation, into flat squamous cells which comprise the squamous epithelium. It is during this process that the carcinogenic process occurs and subsequently is the basis for the cervical intraepithelial neoplasia (CIN) staging system (Figure 2). The first stage of CIN, namely CIN 1, applies when the proliferation of parabasal-like cells is confined to the lower one-third of the epithelium. If the proliferating cells extend into the middle third of the epithelium, they are marked as CIN 2. Once these cells involve the upper third of the epithelium, they are considered to be severe dysplastic lesions (CIN 3). If they replace the entire thickness of the epithelium, they are termed carcinoma *in situ* (1). Cervical squamous cell carcinoma occurs when the proliferating cells gain the ability to invade the stroma.

**Figure 2: Cervical Intraepithelial Neoplasia.**



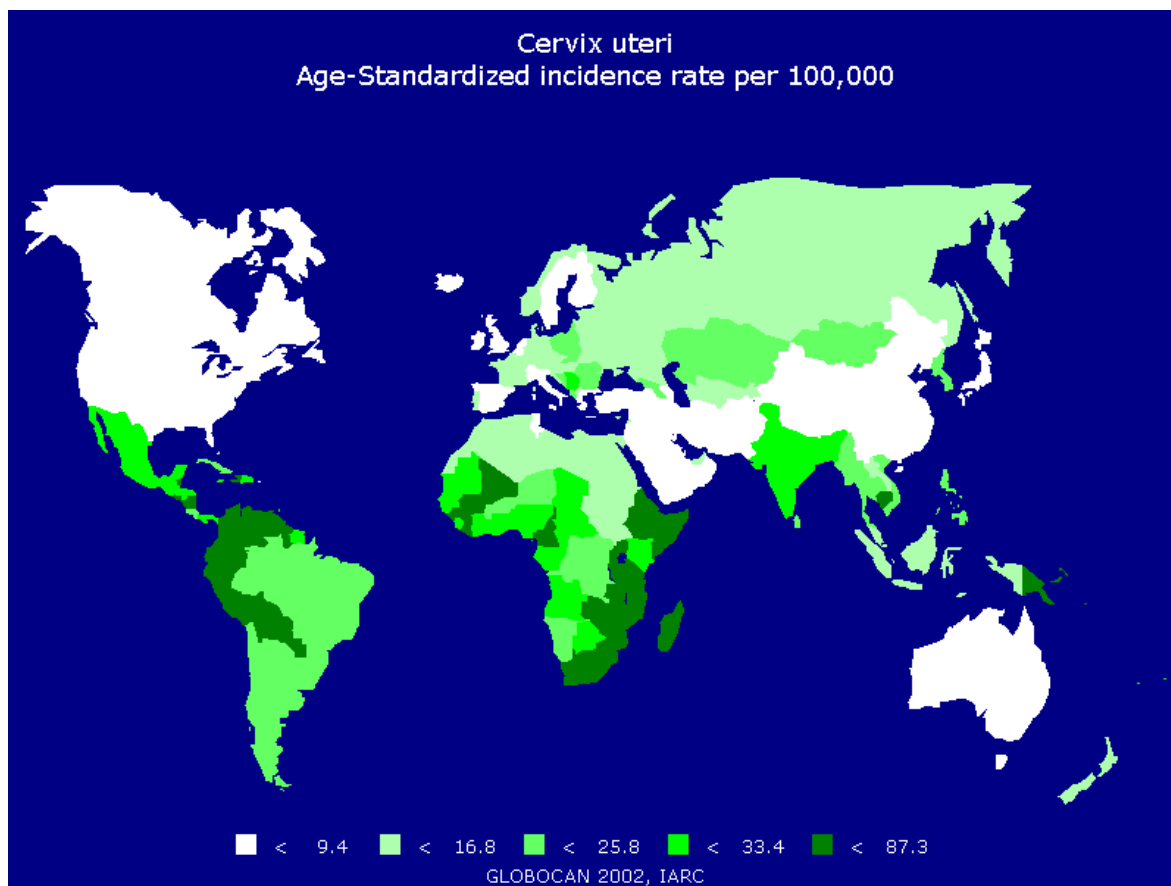
Nature Reviews | Cancer

Woodman, et al; 2007 (2)

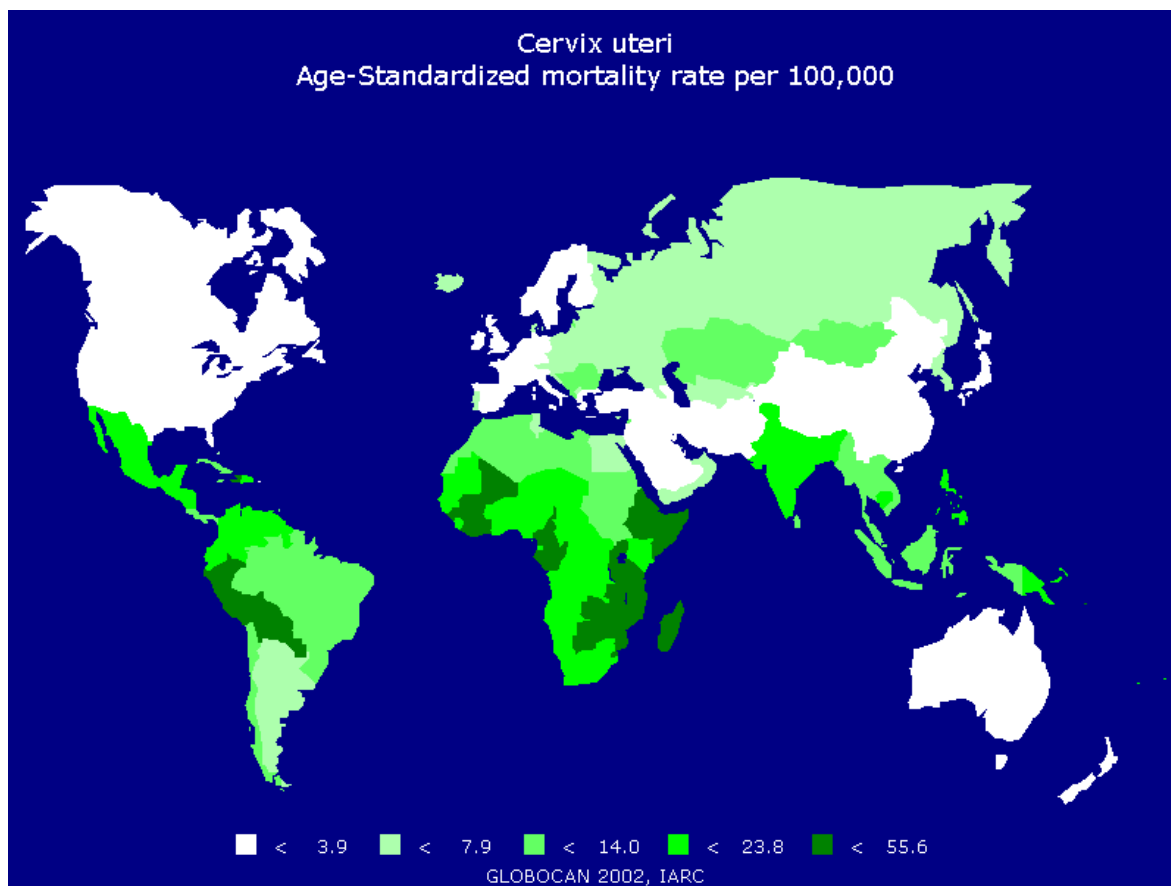
## Cervical Cancer Globally

Cervical cancer is a disease whose greatest toll is borne by developing countries (Figures 3 and 4). In 2002, an estimated 493,000 women worldwide were diagnosed with cervical cancer, with roughly 83% of these cases occurring in developing countries (3). This makes cervical cancer the second most common cancer amongst women globally. Age-standardised incidence rates (ASR) per 100,000 women, range from 5.8 in Western Asia, up to 42.7 in Eastern Africa. These correspond to observed mortality rates (age-standardised) of 2.9 per 100,000 in Western Asia and 34.6 per 100,000 in Eastern Africa. In Northern Europe the age standardised incidence is relatively low (9.0 per 100,000), as is the mortality rate (3.6 per 100,000) (3).

**Figure 3: World-Wide Cervical Cancer Incidence Rates.**



**Figure 4: World-Wide Cervical Cancer Mortality Rates.**





## **The Effectiveness of Papanicolaou Testing**

The large disparity between cervical cancer incidence in 'Developed' and 'Developing' countries is generally attributable to the lack of effective Pap smear screening strategies and adequate access to medical care. Indeed, when women migrate from regions of high cervical cancer incidence (with well organized cytological screening) to those with low incidence rates, they may acquire rates which more closely resemble those of the new host country (4). This highlights the effectiveness of Pap smear screening. Despite the unequivocal benefits associated with the use of Pap smear screening, there are however many controversial issues regarding its specific implementation.

Probably foremost in the debate on Pap smear screening are the benefits of organized versus opportunistic screening, and frequency of testing. It is generally held true that organized screening reduces the incidence and mortality of cervical cancer, although this has been disputed (5). The frequency of testing required to maintain effective prevention of cervical cancer is a little less obvious. In a review on this topic (6), it was noted that many European countries conducting organized screening at 3-yearly intervals possessed similar cervical cancer related mortality to those countries employing 5-yearly screening. In some countries (Austria and Germany), who performed annual testing, the mortality rate was even higher than in some neighbouring countries performing 3- or 5-yearly testing (Belgium, Italy, Netherlands and France). Obviously it is difficult to directly compare mortality from different countries in this way since they potentially have underlying differences in risk for this disease. Nevertheless, it suggests that the use of 5-yearly screening does not in itself appear to decrease the effectiveness of screening.

A second issue of importance in the use of Pap smear screening to prevent cervical cancer is its lack of specificity. The percentage of severe dysplasia/cervical cancer *in situ* lesions that are thought to progress towards cervical cancer has been estimated at more than 12% (7, 8). The corollary of this is that the majority of lesions ordinarily removed as a matter course in cervical cancer prevention, would not have progressed towards an invasive endpoint. Whilst the benefits attributable to Pap smear testing have been considered to outweigh its distinct lack of specificity, identification of an intermediate (and more specific) predictor of progression would be immensely useful. HPV-related factors such as HPV persistence, HPV load, physical state of HPV, and multiple HPV infections, may prove to be useful in this regard, although specifically which one (and how they can be utilised) continues to be a matter for discussion.

## **Human Papillomavirus – A Necessary Cause of Cervical Cancer**

The role of Human Papillomavirus (HPV) in cervical cancer development has been studied in depth, culminating in the conclusion that the presence of certain HPV types is necessary for the development of cervical cancer (9-13).

HPV is not considered to be a sufficient cause of cervical cancer. During their lifetime, more than 50% of women worldwide will acquire a genital HPV infection (14). Clearly,

this far exceeds the likelihood for women developing cervical cancer which suggests that HPV in itself is not a sufficient cause. A possible reason for this is that one or more co-factors are also necessary to trigger cervical cancer formation. This may be through alteration of the host environment (eg, immune system), direct carcinogenic potential (eg, creation of genotoxic adducts), or interaction with HPV in another way. It could also be that the use of blunt measures for HPV exposure may be inadequate for identifying a sufficient cause. For example, different HPV types exhibit differing oncogenic potentials. The use of an oncogenic HPV exposure would be deemed more sufficient than a non-oncogenic type. Likewise, perhaps a particular strain of an oncogenic HPV type might later be found to exhibit properties which make it a sufficient cause.

More recently, researchers have attempted to refine their knowledge by trying to determine exactly what conditions are necessary for HPV to cause cervical cancer. Most studies now focus upon particular characteristics of specific HPV infections that predispose individuals towards cervical cancer development.

## **Human Papillomavirus Type**

HPV typing was one of the first discriminating tools used to gain a more accurate portrayal of HPV's involvement as a causal factor in cervical cancer development. This was an important step since, of the 100 or more HPV types identified thus far, 40 different HPV types are known to infect the genital tract (15). A large number of studies have investigated the prevalence of different HPV types in various countries (16-20). Others have quantified the risks they impose upon cervical cancer incidence (12, 13, 21-24). These (and other) studies have been vital in assigning carcinogenic potential to different HPV types. Of the 40 HPV types inhabiting the genital tract, at least 14 are considered to be significantly associated with the progression to cervical cancer (25) and are referred to generally as high risk, or oncogenic, HPV types.

One of the key findings gained from studies on individual HPV types was the considerable role of HPV-16 and HPV-18 in cervical cancer development. These types are generally considered to confer the greatest risk for cervical cancer development, responsible for an estimated 70% of cervical cancer (18, 26). This knowledge paved the way for development of vaccines against HPV-16/18 (27-30) to combat cervical cancer.

## **Human Papillomavirus Load**

As a further step towards the isolation of characteristics of HPV infections that might predict carcinogenic potential more accurately, a number of researchers have investigated the potential role of HPV load (31-35). One landmark study in this field utilized a population-based case-control study in Sweden to assess the association between HPV-16 load and CIS (31). These researchers found that high HPV load, present in cervical smears taken within a year prior to diagnosis of CIS, placed women at a 43-fold increased risk for CIS compared to HPV negative women. This was in distinct contrast to the 3.1-fold

increased risk (compared to HPV negative women) seen in women whose last smear within a year prior to diagnosis contained low HPV-16 load.

## **Human Papillomavirus Persistence**

Most HPV infections are considered transient and will be cleared or suppressed by cell-mediated immune mechanisms within one or two years following initial exposure (36). As one might expect, there is a positive relationship between the lack of clearance (persistence) of an HPV infection and subsequent development of cervical lesions (12, 13, 37). Persistence of HPV infections has gained great acceptance among many in the HPV research community as a marker for high cervical cancer risk. As with HPV load, persistence of HPV infections appears to signify a much greater risk for cervical cancer development than HPV presence alone.

## **Human Papillomavirus Integration**

One particularly interesting marker for increased cervical cancer risk is the physical state of HPV in the host. HPV DNA may exist in episomal (extra-chromosomal) or integrated (within the host cell genome) forms. During a normal life-cycle, high risk HPV genomes replicate as episomal molecules (38). However, integration of HPV viral DNA into the host cell genome is frequently observed in cervical carcinomas and in a subset of CIN3 lesions (39-42). This is not entirely unexpected since the current understanding of HPV's carcinogenic potential revolves around the disruption of a regulatory HPV gene (E2), thought to occur mainly as a result of integration into the host cell genome (43).

## **HPV Pathogenesis**

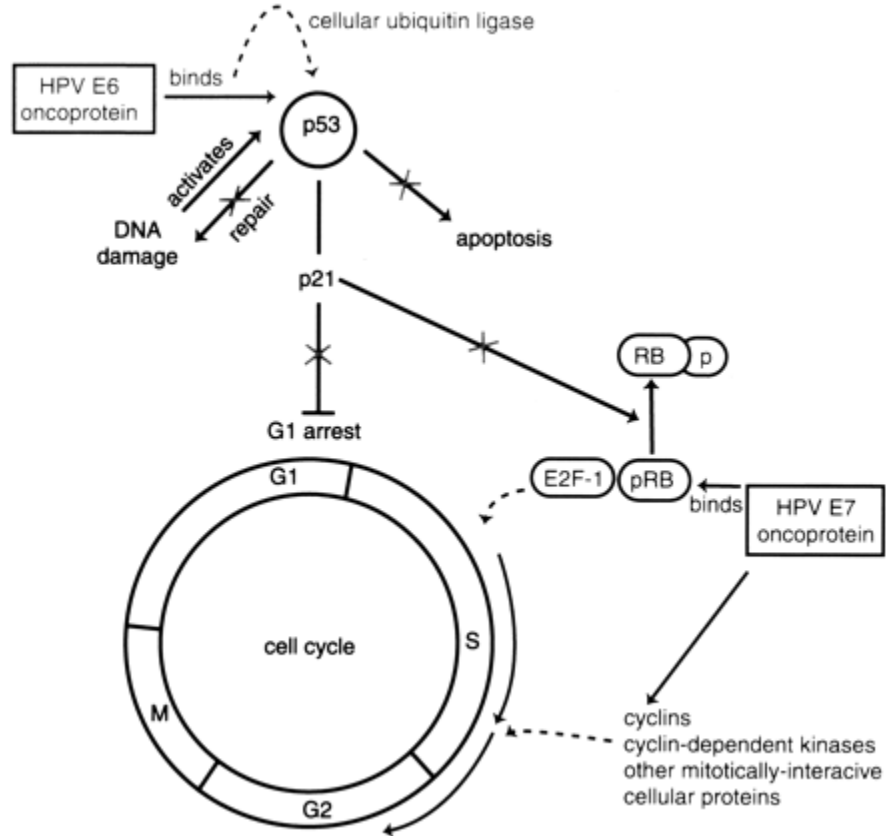
The natural life-cycle of HPV involves infection of basal or para-basal cells (presumably accessed through micro-abrasions of the epidermis), replication of its own DNA during basal cell differentiation and subsequent amplification of its DNA to high copy numbers, along with capsid protein synthesis and viral assembly, in the differentiated keratinocytes (43).

The HPV life-cycle is controlled by relatively few viral proteins and the virus must utilize host cell factors to regulate viral transcription and replication. Of particular interest are the HPV E6, E7 and E2 proteins. The E6 and E7 proteins are coded for in a region referred to as the long control region (LCR). These two proteins are produced early in the viral cycle and are used to deregulate the host cell growth cycle. They do this by binding with certain tumour suppressor proteins, cell cyclins, and cyclin-dependant kinases, effectively inactivating them (43) (Figure 5). Two of these inactivated proteins – the tumour suppressor protein p53 and the retinoblastoma gene product pRB – are major players in cell growth. HPV E6 proteins bind to p53 and ensure its degradation through ubiquitination, whereas HPV E7 proteins bind to hypophosphorylated pRB forms and abrogates their ability to form a complex with the cellular transcription factor E2F-1. Once liberated, E2F-

1 initiates transcription of genes required for the cell to enter S-phase of the cell cycle (43, 44). The outcome of these (and other) activities by E6 and E7 proteins is continuous cell proliferation and stimulated DNA synthesis. Subsequently, after a period where E5 proteins encourage continued proliferation and delayed differentiation of the host cells, DNA binding proteins (E2) are translated, which block transcription of E6 and E7 proteins. At this point, E1 proteins are able to bind to the viral origin of replication, which enables extrachromosomal replication. Furthermore, due to E2's antagonism of E6 and E7, regulatory elements of the host's cell cycle (such as p53 and pRB) return to normal and allow the differentiation process to continue. Viral particles are then assembled in the host's nucleus and mature HPV virions are later released when the cells are shed.

It would appear that HPV has no need (or desire) to transform cells. On the contrary, evidence suggests that HPV generally has a vested interest in ensuring the host cells resume their normal course. For HPV, it is important that the host cells continue to differentiate since some of its late-stage development can only occur in differentiating cells (44). So what happens? It would appear that the problem lies in disruption of the E2 protein. Integration of HPV DNA into the host's DNA is often accompanied by disruption of the E1 or E2 genes (43, 45). Loss of expression of the E2 gene leads to a loss of E2 protein function which corresponds to an increased expression of the E6 and E7 proteins. Effectively, this results in increased proliferation of the host cell and genomic instability. Consequently, the host cell accumulates a greater amount of damaged DNA and has a reduced ability to repair it. This process may finally result in the accumulation of mutations which allow fully transformed cancer cells to form (43).

**Figure 5: Mechanism for HPV Pathogenesis.**



Burd, et al; 2003 (43).

## HPV Transmission

It is common knowledge that the main route for transmission of HPV is through sexual intercourse. However, reports of unusually high incidence rates of HPV infection in children raises the issue of alternative methods for transfer of the virus. Numerous studies have reported the presence of HPV in oral cavities and genitalia of children and infants, as described recently in a review by Cason and Mant (46). The likely mode of HPV transmission in these cases is cause for debate however. Certainly, a proportion of these cases could be attributable to sexual abuse (47, 48), although presumably this is less likely in infants than older children (48). Another possibility is the acquisition of HPV in utero, intrapartum or postpartum (46). Evidence has accumulated in favour of this 'vertical' form of transmission (49-52) although some studies have been unable to confirm this (53-55).

The route of transmission raises important issues which may influence the effectiveness of HPV vaccination programs. The currently held dogma is that vaccination should be introduced to females prior to sexual debut since efficacy is likely to be compromised if an HPV infection is current. Furthermore, it is not known how *previous* infections may influence vaccine effectiveness.

## Smoking

Presence of HPV infection cannot be considered a sufficient causative agent due to the numbers of HPV infected women who do not develop cervical cancer. The roles of other potential risk factors in cervical carcinogenesis therefore need to be considered. An increased risk of cervical cancer associated with tobacco smoking has been established on the basis of a number of epidemiological studies since the 1980's (56, 57). Whether this link is related to genotoxic DNA adducts of smoking in the cervix epithelium (58-60), its effect on malignant transformation of HPV infected cells (61), or its influence on HPV infections via localised immunosuppression (62), has been discussed. It has also been debated whether an association of smoking and cervical cancer is merely an artefact of confounding by HPV, through smoking's association with sexual activity and the subsequent risk of acquiring an HPV infection (63, 64). A number of studies, restricted to HPV positive women, have shown an increased risk for cervical cancer in smokers compared with non-smokers (65-71) but to our knowledge only three studies have formally tested for interaction between HPV and smoking in cervical cancer development (67, 72, 73), two of which appear to have small numbers or a less appropriate study design for detecting interaction (72, 73). In addition, to our knowledge there appears to be a lack of information concerning the combined effects of HPV load and smoking in cervical cancer development.

# AIMS

## General Aims

This collection of projects aimed to investigate specific attributes related to the main risk factors involved in cervical cancer development. Ultimately, risk factors are studied with a view to their usefulness in preventing, managing, or treating a disease. We therefore also assessed the current management strategy (and its associated inadequacies) for cervical cancer management in Sweden and delved into certain factors (HPV transmission and residual risks from non-HPV16/18 types) that might influence efficacy of the recently developed HPV vaccines.

## Specific Aims

### Paper I:

1. To examine potential individual and combined effects of cigarette smoking and HPV-16 on risk for cervical cancer *in situ*.

### Paper II:

1. To assess the efficiency of cervical cancer screening (in Sweden) in reducing cervical cancer incidence over the last 3 decades.

### Paper III:

1. To investigate the risk for cervical cancer *in situ* in daughters whose mothers had cervical cancer *in situ* previously, with particular emphasis on the time between the daughter's birth and cervical cancer *in situ* detection in mothers.

### Paper IV:

1. To assess the likely impact that HPV-16/18 targeted vaccines will have upon cervical cancer incidence in Sweden. More specifically, to measure the residual risk for cervical cancer remaining in the population (after removal of HPV-16 and HPV-18) that is attributable to other non-16/18 high risk HPV types.

## SUBJECTS AND METHODS

### Subjects

#### Swedish Population Base

Each of the four studies was conducted using Swedish population-based data. The source population of women from whom the *in situ* or invasive cervical cancer cases arose was numbered at 3,991,867 women in 1968 and grew to 4,507,708 women in 2002. There are 21 municipalities currently existing in Sweden and healthcare gives equal access to medical care for all citizens.

#### Cancer Registration

The Swedish National Cancer Registry was established in 1958. Reporting of all cancers and *in situ* lesions has been ongoing and, in more recent years, is considered to include virtually 100% of incident cancer cases in Sweden (74). This is achieved through the contribution of the 21 counties' pathologists and clinicians who separately report their results to six regional oncologic centres who in turn report to the National Swedish Cancer Registry.

The end result is a registry containing cancer information for all Swedish residents, stretching back to 1958, where an individual's county of residence at time of diagnosis can be traced. Diagnoses for virtually all cervical cancer reports contained within the cancer registry are based on biopsy (rather than cytological) interpretation.

#### Multi-generation Registry

The Multi-generation register (MGR) contains people who have been registered in Sweden at any time since 1961, or who were born in Sweden in 1932 or later. These individuals are referred to as index persons. Connections between these index persons and their biological parents comprise the MGR which itself is a part of the registry system called the Total Population Register, which contains information from the National Tax Board in Sweden. Using these registries it is possible to retrieve data on such variables as immigration/emigration dates, birth-dates, parent-offspring relationships, cancer diagnosis dates and type, and date of death. We accessed information between the years 1961-2001 for use in our study. The recent version utilized in our third study contained data between the years 1958-2001, which incorporated 13,605,121 individuals.

#### Cervical Cancer Screening and Management

Papanicolaou smear (Pap smear) screening began in Sweden during the early 1960's as opportunistic screening. Organised screening was introduced from 1967 onwards, with some counties refraining until the early 1970's. From early 1970's, women aged 30-49



years were invited to attend Pap smear testing once every 4 years. From the mid-1970s this increased to once every 3 years. Currently, women aged 23-50 years are invited for testing every 3 years, or 5-yearly for those who are 51-60 years of age. Test results for cytology testing are stored in a National Cytology Register.

In Sweden, the typical collection method for cervical samples prior to the 1980s was the spatula. Post-1980s the cytobrush was incorporated in combination with the use of spatula collection. Liquid-based cytology has not been used routinely in Sweden. Before the 1980's, high-grade cervical dysplasia was treated using cold knife conization. During the 1980's however, more conservative mode of treatment such as cauterization, cryotherapy, and laser vaporization or conization were more readily employed. In 1990, Large Loop Excision of the Transformation Zone (LLETZ) was introduced and remains the procedure of choice for management of high-grade cervical lesions.

## **Study Participants**

### *Paper I:*

The source population for this study comprised all Swedish women (N=146,104) who participated in cytological screening in Uppsala county at any time during the years 1969 to 1995. All smears were stored at the Uppsala University Hospital and, given the publicly funded nature of Swedish health care, equal access for women to health facilities could be assumed.

Within this source population, a cohort of 105,760 women was identified who possessed the following characteristics: 1) Their first registered smear during the study period was cytologically normal (PAP=1), 2) They were born in Sweden, and 3) They were less than 50 years old at entry to the cohort (date of the first smear). Utilising the Swedish cancer registry, all cervical cancer *in situ* (CIS) cases within the cohort were identified. For each cancer *in situ* case, one control, individually matched on date of entry into cohort (+/- 3 months) and age, was identified from the cohort. These controls were free of malignant disease of the cervix and had not undergone a hysterectomy prior to the time (and age) of CIS diagnosis for their matched case. 499 eligible cases and their 499 matched controls remained after review of the cases histological slides by an experienced pathologist to confirm the CIS diagnosis. Each case woman and her matched control were considered a risk set.

### *Paper II:*

This study utilized the entire Swedish cohort of women registered in the Total Population registry between the years 1968 and 2002. We observed 125,543 cases of CIS and 17,399 cases of SCC. AIS and AC lesions were reported in 1,260 and 3,602 women respectively during the same period. The source population of women from whom these cases arose was numbered at 3,991,867 women in 1968 and grew to 4,507,708 women in 2002. There were

a total number of 145,252,306 person-years observed for Swedish women during follow-up, estimated using mid-populations for each calendar year.

### Paper III:

This population-based cohort study comprised all Swedish-born women residing in Sweden between the years of 1961 and 2001. Of 6,713,558 women originally identified from the multi-generation registry, we observed 3,928,774 biological mother/daughter pairs of whom 3,498,565 contained daughters born in Sweden. Data from sisters may be correlated. To avoid problems in the analyses related to this, we chose to consider the 1<sup>st</sup> born sisters only. This resulted in 2,372,394 biological mother/daughter pairs being available for analysis.

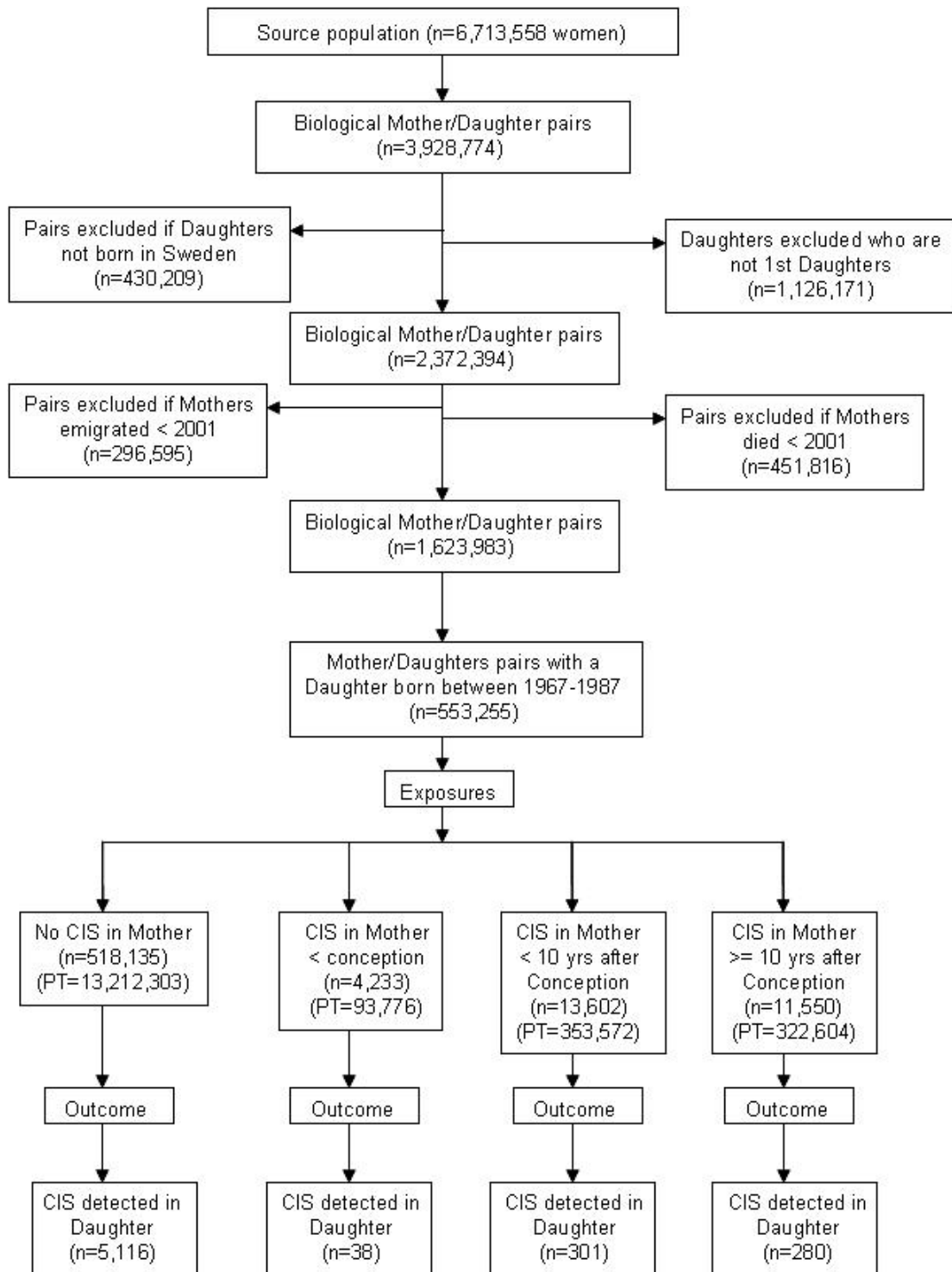
Since complete exposure information was not available for the entire follow-up period if mothers emigrated or died prior to the year 2001, we removed these mother/daughter pairs from the analysis (n=296,595 and n=451,816 pairs respectively). Following these exclusions, we identified 553,255 mother/daughter pairs where the daughters were born between the years 1967 and 1987 to be used in our study population (Figure 6). We chose daughters whose age exceeded 15 years based on the minimum age for detection of CIS lesions in our study sample. Our exclusion of daughters born before 1967 was related to the completeness of reporting for CIS in their mothers prior to or around the time of pregnancy. From 1967 onwards, we considered CIS reporting in the Swedish cancer registry to be reliable.

CIS was identified as a first cervical lesion in 29,385 mothers, out of a population of 553,255 mothers. During the follow-up, our outcome of cervical cancer *in situ* (CIS) was documented as a first CIS in 5,735 daughters out of 553,255 daughters at risk.

### Paper IV:

Using the Cytology Register, we identified 624,599 women from the source population (where the source population included 801,795 women from Helsingborg, Karlskrona, Kristianstad, Lund, Gothenburg and Vasteras municipalities) whose first registered smear during the study period was cytologically normal (PAP=1). The Swedish cancer registry was then used to identify women who tested positive for either severe dysplasia/cancer *in situ* (CIS) or squamous cervical cancer (SCC). A diagnosis of severe dysplasia or cervical cancer *in situ* in the Swedish cancer registry translates to a diagnosis of cervical intraepithelial neoplasia, grade 3 (CIN 3). All SCC cases (n=534) and a random selection of CIS cases (n=635) were chosen. Using the nation-wide Swedish cytology register, one control woman - matched on county, date of entry into cohort (+/- 3 months) and age - was randomly selected to act as an individually matched control for each CIS and SCC case. Of the 2,338 women initially identified (1169 cases and 1169 controls), cervical smears from 908 women were retrieved.

**Figure 6: Study Selection profile for Paper 3.**



## **Methods**

### *Paper I:*

#### ***DNA Preparation and HPV Analyses***

All available cervical smears were retrieved from archival storage. Samples from cases and controls within risk sets were re-coded and mixed to ensure blinding during analysis. DNA extraction from cervical slides was performed using a previously described protocol (75). PCR based detection of HPV-16 and quantification of HPV-16 load was performed on DNA from all cervical slides for each woman using real-time detection of accumulated fluorescence (Taqman) as previously described (31). Women with only B-actin negative smears during follow-up (12 cases and 20 controls) were excluded from our analyses. Of the 3771 smears tested, 2970 (78.8%) tested positive for B-actin. To omit smears taken as part of the diagnostic workup we also disregarded all smears taken within one year prior to the CIS diagnosis date for cases or their matched controls in the subsequent statistical analyses. Consequently, 35 case and 32 control women who possessed only a single smear taken within 1 year prior to diagnosis were removed from analyses.

#### ***Interview Data***

Detailed information regarding smoking habits (age at starting, duration, intensity) and other covariates (oral contraceptive use, parity, age at sexual debut, number of sexual partners, socioeconomic factors) were collected from cases and controls via telephone interviews. The interviewers were blinded as to case-control status. The methodology for collection of this data has been described previously for this study (76). Thirty cases and their matched controls from the eligible 499 risk sets were excluded because they did not have access to a telephone. Of the 469 cases and 469 controls approached by telephone, 422 (90%) case women and 422 (90%) control women agreed to participate.

#### ***Combined Biological and Interview Data***

Of the 844 women (422 cases and 422 controls) from whom we had interview data, 738 (375 cases and 363 controls) were included in all statistical analyses apart from the analyses utilising our surrogate persistence marker. These women had an HPV-16 result in at least one B-actin positive smear collected more than one year prior to diagnosis of a CIS lesion. Women were included in the persistence surrogate analysis (256 cases and 224 controls) if they had two or more smears in addition to having satisfied the aforementioned criteria. The first eligible smear for each woman was used in our analyses in order to study the joint effect of HPV-16 and smoking at an early stage during carcinogenesis.

### *Exposures and Covariates*

A 'current smoker' was defined as someone who smoked within 2 years prior to the date that a given smear was taken. A 2 year period was chosen to try and capture relatively short term effects of smoking. The corresponding 'non-smoking' group was a combination of women who either had never smoked or who had not smoked within 2 years prior to a given smear being taken. 'Duration of smoking' was categorized based on years of smoking prior to first smear (0, <5, >=5). Smoking intensity was measured as pack-years at time of first smear (0, <2.5, >=2.5).

Covariates included in the multivariable model were age, number of years between first smear and diagnosis, current oral contraceptive use (never or more than 2 years prior to smear, within 2 years prior to smear), parity (nulliparous, 1, 2, 3) and number of sexual partners up to the date of the first smear. Other potential confounders were not included as covariates in the model as they were considered surrogate measures for the number of sexual partners (marital status and sexual debut) and/or inclusion/exclusion from the multivariable analysis did not appreciably alter the risk estimates for the main exposure variable in the model.

We derived a variable to be used as a surrogate marker for persistence of HPV-16 infection based on the proportion of total smears collected for each woman that were HPV-16 positive, also taking into account the number of smears per woman. The number of HPV-16 positive smears per woman was divided by the square root of the total number of smears per woman:

HPV-16 Proportion = #HPV-16 positive smears per woman /  $\sqrt{\text{total \# smears per woman}}$ .

Eg: Two HPV-16 positive smears out of a total of 2 smears will be ranked lower than a woman with 5 HPV-16 positive smears out of a total of 5. Their respective values in this example would be 1.4 and 2.2. A woman with 2 HPV-16 positive smears out of a total of 5 smears would have a lower value than the previous examples (0.9). We used the square root of the total number of smears in the denominator, to account for the differences in number of smears per woman. Clearly, a woman with 5 smears that are all positive for HPV-16 is more likely to have a truly persistent infection than an individual possessing only 2 smears, both of which were positive. Likewise, it seems more plausible that a woman with 2/2 smears (HPV-16 positive smears / total number of smears) possesses a persistent infection than a woman possessing 2/5 smears that are HPV-16 positive. Women were considered to have a low proportion (persistence marker) of HPV-16 infections if their calculated value was lower than 0.5, where 0.5 was the median value observed in HPV-16 positive non-smokers. Women possessing an HPV-16 proportion (persistence marker) value greater than or equal to 0.5 were considered to have a high proportion of HPV-16 infections.

## ***Outcome***

Severe dysplasia/CIS was the outcome of interest. As mentioned previously, cytological diagnosis of severe dysplasia/CIS was confirmed through blinded histological review by a single experienced pathologist. Controls had no history of CIS or cervical cancer prior to the date of diagnosis for their corresponding matched case.

## ***Statistical Analyses***

To improve the power to detect interaction between smoking and HPV-16 in CIS development (as well as main effects), we used unconditional logistic regression in our analyses to produce odds ratios (OR's). We interpreted these OR's as estimates of relative risk. Since matching was performed on several variables, we treated the data as group matched on age and number of years between first eligible (B-actin positive and collected >1 year prior to diagnosis of CIS) smear and diagnosis. Comparisons were made with analyses performed using conditional logistic regression, taking into account the matching criteria. As expected, these risk estimates did not differ appreciably to those generated using unconditional logistic regression. We therefore report results from unconditional analyses since this enabled us to utilise incomplete risksets in our analyses to gain precision.

Tests for interaction were based on departure from multiplicativity of the odds ratios associated with smoking and HPV-16. A multiplicative logistic regression model was utilised, where the odds ratio for HPV-16 or HPV-16 load was allowed to vary for different levels of smoking status, duration or intensity. Effectively this meant that an interaction effect was added to the main effect.

Tests for trend were performed using the Wald test. This was used to test whether a linear trend existed with respect to the log odds for a given exposure. These and the aforementioned analyses were performed using SAS version 9.1.3.

## ***Paper II:***

### ***Exposures and Outcomes***

For this study, the exposure variables were CIS (severe dysplasia/CIS) or AIS (adenocarcinoma *in situ*). CIS is a well established precursor to cervical squamous cell carcinoma (SCC) and AIS is considered to be a precursor to cervical adenocarcinoma (AC). The outcomes of interest were SCC and AC.

Typically, initial diagnoses of CIS, AIS, SCC and AC were based on two separate diagnosis notifications to the cancer registry.

### *Statistical Analyses*

We calculated age-standardized incidence rates for each county and time period using the Swedish census population in 1970 as a reference. Incidence rates were reported as the number of case women per 100,000 women in the population.

For each county, Spearman correlations between CIS and SCC rates, in addition to AIS and AC rates, were estimated. To take into account the expected lag period between CIS development and SCC detection, we compared current age-standardized incidence rates for SCC to those for CIS in the same county and cohort 5, 10 or 15 years before. The same analyses were performed for AIS and AC.

In order to more effectively compare CIS/SCC correlations between counties, we used a generalized estimating equation (GEE) model where we allowed the variances to differ for different counties and time periods. We then added the CIS incidence (current, -5 years or -10 years) as a continuous covariate, estimating the association between SCC and lagged CIS. The 15 year lag period was omitted from these analyses because of power constraints.

### *Paper III:*

#### *Exposures, Covariates and Outcome*

The main exposure of interest in this study was CIS in mothers. Based on previous observations, the presence of CIS should correspond to the presence of oncogenic HPV infection (77-81).

We divided CIS status of mothers into 4 categories, depending on the incidence and/or proximity of the CIS diagnosis to their daughter's conception. Mothers who did not develop CIS at any stage up to December 2001 were classified as CIS negative. The remaining 3 exposure categories (with respect to mothers CIS status) were 'CIS prior to conception', 'CIS < 10yrs after conception' and 'CIS  $\geq$  10 yrs after conception'.

Date of conception was defined as being 280 days prior to the recorded date of birth. The 10 year category was chosen to include the likely induction period between HPV infection and CIS detection (82-84). Thus, the majority of women who developed CIS within 10 years following conception should have harbored an oncogenic HPV infection during, or relatively soon after, pregnancy. Relative to this group, mothers presenting with CIS more than 10 years after conception are *less likely* to have contracted an oncogenic HPV infection until well after pregnancy.

Information regarding the occupation of mothers was also gleaned from the multi-generation registry. This was used as a loose measure of social behaviour which was adjusted for in the analyses in an attempt to lessen residual confounding by maternally-acquired social behaviour. Mother's occupation was classified as blue collar workers, lower level white collar workers, higher level white collar workers, or self employed.

The outcome for this project was severe dysplasia/CIS in daughters.

### ***Statistical Analyses***

Kaplan-Meier estimates of the survival curves were plotted, comparing the three exposure groups and mothers with no CIS diagnosed during follow-up. The CIS incidence between the three exposure groups was compared by the fitting of three different Poisson regression models. In the first model, crude rates were calculated. Confounding by age was adjusted for by including daughters attained age (15-20, 20-25, 25-30, 30+) in the second model, and also mother's age at her daughter's conception as a continuous variable in the final model.

Residual confounding by maternally-acquired social behaviour was tested by adjusting for a categorical variable representing mother's occupation. Unfortunately we did not possess information on some other previously identified covariates such as smoking, oral contraceptives, and parity, and were thus unable to adjust for them in the analyses.

For inference purposes, rate ratios and the associated two-sided 95% confidence intervals were calculated.

A supplementary model evaluating the functional form between CIS incidence and time from conception to CIS diagnosis in mothers (without any apriori assumptions of time categories) was evaluated using fitting of splines.

All analyses were performed using the SAS Genmod procedure (version 9.3) or R software (version 2.4.0).

### ***Paper IV:***

#### ***DNA Preparation and HPV Analyses***

All available cervical smears were retrieved from various counties' archival storage. During extraction of DNA from archival cervical smears, using a previously established protocol (75), each sample was relabelled with a barcode to ensure blinding during analysis. Samples were batched together in risksets (where a riskset contained a matched case and control) during DNA extraction and HPV analysis.

Each smear per woman was tested for the presence of 23 HPV types (HPV-6, -7, -11, -16, -18, -31, -33, -35, -39, -42, -43, -45, -51, -52, -56, -58, -59, -66, -68, -70, -73, -82 and -90) using a reverse dot blot hybridisation (RDBH) assay. This involved amplification of a consensus region using GP5+/6+ primers, followed by hybridisation with HPV type-specific oligonucleotide probes representing the aforementioned HPV types. The presence of sufficient DNA for PCR amplification was determined by the amplification of the housekeeping gene Beta-globin.



Of the 2,182 smears analysed, 35 smears were found to be B-globin negative and subsequently excluded from the study. Of the remaining 2,147 smears (n=902 women), only those who contributed towards a complete riskset were included (n=808 women). We chose to include only one smear per woman in our study because a large number of women contributed only a single sample (330 women) during follow-up, thereby limiting our power to perform analyses on women with multiple smears. For women with multiple smears, the last smear before diagnosis was chosen because the median number of years before diagnosis in last smears was similar to that for smears obtained from women with a single smear.

### ***Exposures, Covariates and Outcome***

Pooled low-risk HPV types (LRHPV) and high-risk HPV types (HRHPV) were used as exposure variables, rather than analysis of each HPV type separately, since we did not possess sufficient power to obtain reliable results for individual HRHPV types in association with CIS or SCC risk.

Specifically, exposures were classified in the following manner:

1. LRHPV - where the last smear was positive for one or more low risk HPV types (HPV-6, -7, -11, -42, -43, -70 or -90);
2. HPV-16/18 – where the last smear was positive for HPV-16 and/or HPV-18; or
3. Non-16/18 HRHPV - where the last smear was positive for one or more high risk HPV types other than HPV-16 or HPV-18 (HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68, -73 or -82).

The covariate ‘riskset’ was included in adjusted analyses, which encompassed the matching criteria (county, age and date of entry into the cohort).

### ***Statistical Analyses***

In light of the fact that we used a matched case-control study design with relatively few individuals in certain strata, exact conditional logistic regression was employed to estimate odds ratios (ORs). Analyses were conditioned on the ‘riskset’ variable.

Cervical cancer being a rare disease, we interpreted OR estimates as relative risk (RR) estimates, namely the risk of an outcome (CIS or SCC) in exposed women, relative to unexposed women. For each HPV risk group, crude and adjusted (for other measured HPV risk groups) OR’s were estimated using SAS version 9.1.3.

Population attributable risks were calculated as:

$$\text{PAR}\% = [P_e (\text{RR} - 1) / (P_e (\text{RR} - 1) + 1)] \times 100$$

$P_e$  is the proportion of women in the Swedish population with the exposure. Prevalences of the exposures in our control group were assumed to be representative of the population during the period 1980-2002 and were used to estimate  $P_e$ . Adjusted RR’s were used for

calculation of PAR's for HPV-16/18 and non-16/18 HRHPV variables, whereas crude RR's were used for calculation of the 'All HRHPV type' PAR. When calculating upper and lower confidence intervals, the HPV prevalence among controls was assumed to be fixed.

## RESULTS

### Paper I:

#### ***Characteristics of Participants***

For both cases and controls, the median number of smears per woman was three (range = 2-16 in cases and 2-14 in controls). The mean numbers of smears per woman were 3.98 and 3.57 for cases and controls respectively. The median age at first smear was 25.5 years (range ~ 15-49 years) and the median time between first smear and diagnosis was almost 9 years (range ~ 1-25 years). The risk of CIS increased in relation to current OC use ( $p=0.01$ ), parity of 1-2 children ( $p<0.001$ ) and increasing number of sexual partners ( $p<0.001$ ).

#### ***HPV and Smoking as Risk Factors for Cervical Cancer***

Multivariable analyses (Table 1) showed that women positive for HPV-16 in their first smear had an 8-fold increased risk of CIS (adjusted OR=8.4; 95% CI 4.8-14.7) compared to women negative for HPV-16. Having low or high HPV-16 load in the first smear corresponded to an increased adjusted risk of 5.5 (95% CI 2.4-12.6) or 11.0 (95% CI 5.3-22.6) respectively ( $P_{\text{trend}}<0.0001$ ) compared to being HPV-16 negative. Likewise, having a high proportion of HPV-16 positive smears per total number of smears increased a woman's relative risk for CIS, when compared to HPV-16 negative women (adjusted OR=11.8; 95% CI 7.2-19.5). Those with a low proportion of HPV-16 positive smears showed no increased risk (adjusted OR=0.8; 95% CI 0.3-2.0) compared with HPV-16 negative women.

Current smoking at time of first smear conferred a 70% increase in risk for development of CIS (adjusted OR=1.7; 95% CI 1.2-2.4) compared to non-smokers (Table 2). The adjusted odds ratios for smoking debut, intensity and duration were similar to those seen for current smoking.

**Table 1: OR and 95% CI of Cervical Cancer *In situ* in relation to HPV-16 and smoking habits.**

Variable	Cases/Controls	Crude OR (95%CI)	Adjusted OR* (95%CI)
<u>HPV-16 status</u>			
Negative	268 / 346	Ref	Ref
Positive	107 / 17	8.4 (4.9-14.5)	8.4 (4.8-14.7)
<u>HPV-16 Load</u>			
Negative	268 / 346	Ref	Ref
Low	31 / 8	5.1 (2.3-11.3)	5.5 (2.4-12.6)
High	76 / 9	11.4 (5.6-23.4)	11.0 (5.3-22.6)
P for trend (Wald)			p<0.0001
<u>HPV-16 proportion/persistence<sup>§</sup></u>			
Negative	94 / 181	Ref	Ref
Low	8 / 18	0.8 (0.3-2.0)	0.8 (0.3-2.0)
High	154 / 25	12.2 (7.4-19.9)	11.8 (7.2-19.5)
<u>Smoking Status<sup>¶</sup></u>			
Non-smokers	131 / 181	Ref	Ref
Current smokers	244 / 182	1.9 (1.4-2.6)	1.7 (1.2-2.4)
<u>Smoking duration (years)</u>			
0	119 / 171	Ref	Ref
< 5	61 / 56	1.5 (1.0-2.4)	1.5 (0.9-2.4)
>= 5	195 / 136	2.1 (1.5-2.9)	1.7 (1.2-2.4)
<u>Smoking Intensity (pack years)</u>			
0	119 / 171	Ref	Ref
< 2.5	116 / 88	1.9 (1.3-2.8)	1.7 (1.2-2.6)
>= 2.5	140 / 104	1.9 (1.4-2.7)	1.6 (1.1-2.3)

\*Odds ratios (ORs) are adjusted for age at first smear, #years between date of smear and diagnosis, current oral contraceptive use and parity as categorised in Table 1. Smoking associated variables were adjusted for HPV-16 status (negative, positive) and HPV-16 associated variables were adjusted for current smoking status (non, current). Number of sex partners prior to first smear were included as a continuous variable.

<sup>§</sup>Categories for HPV-16 proportion: (0, <0.5, >=0.5).

<sup>¶</sup>Current smokers = subject smoked within 2 years prior to the smear date. Non-smokers = subject never smoked or smoked more than 2 years prior to the smear date.

### ***Joint Effects of HPV and Smoking in Cervical Cancer *In situ* Development***

When stratified by current smoking status (Table 2), non-smoking women who were positive for HPV-16 had a 5-fold increased risk (adjusted OR=5.6; 95% CI 2.7-11.5) of CIS compared to non-smoking HPV-16 negative women. However, being a smoker and HPV-16 positive was related to a 14-fold increased risk (adjusted OR=14.4; 95% CI 5.6-36.8) compared to HPV-16 negative non-smokers. Non-smoking women with a high viral

load had an approximately 6-fold increased risk (adjusted OR=5.9; 95% CI 2.4-14.6) compared to non-smoking HPV-16 negative women. In comparison, current smokers with a high HPV-16 viral load had an increased risk of 27.0 (95% CI 6.5-114.2) compared to current smokers without HPV-16 infection. Tests for interaction on a multiplicative scale between HPV-16 infection and current smoking status (p=0.12), or HPV-16 load and current smoking status (p=0.21) were not significant.

**Table 2: Multivariable analysis of Cervical Cancer *In situ* in relation to HPV-16 status at first smear, stratified by smoking status.**

Variable	Non-smokers <sup>†</sup> Cases/Controls	Non-smokers <sup>†</sup> OR* (95%CI)	Current smokers <sup>‡</sup> Cases/Controls	Current smokers <sup>‡</sup> OR* (95%CI)	p value interaction
<u>HPV-16 status</u>					
Negative	96 / 169	Ref	172 / 177	Ref	0.12
Positive	35 / 12	5.6 (2.7-11.5)	72 / 5	14.4 (5.6-36.8)	
<u>HPV-16 load</u>					
Negative	96 / 169	Ref	172 / 177	Ref	0.21
Low	12 / 5	5.1 (1.7-15.4)	19 / 3	6.0 (1.7-20.8)	
High	23 / 7	5.9 (2.4-14.6)	53 / 2	27.0 (6.5-114.2)	
P for trend		p<0.0001		p<0.0001	

\*Odds ratios (ORs) adjusted for age at first smear, # years between date of smear and diagnosis, current oral contraceptive use and parity. Number of sex partners prior to first smear was included as a continuous variable.

<sup>†</sup>Non-smokers = subject never smoked or smoked more than 2 years prior to the smear date.

<sup>‡</sup>Current smokers = subject smoked within 2 years prior to the smear date.

The potential interaction between HPV-16 and smoking was further explored (Table 3), using other indicators of smoking status (duration and intensity). In stratified analyses, the strongest risk was observed among high duration smokers ( $\geq 5$  years) who were positive for HPV-16 infection at time of first smear, compared to HPV-16 negative, high duration smokers (adjusted OR=35.9; 95% CI 8.6-150.2). The corresponding risk among non-smokers who were HPV-16 positive was 4.8 (95% CI 2.2-10.3). Presence of a multiplicative interaction between duration of smoking and HPV-16 status was found (p=0.03). Smoking intensity followed a similar risk pattern but did not show significance in a formal test for interaction.

**Table 3: Multivariable analysis of Cervical Cancer *In situ* in relation to HPV-16 status in first smear, stratified by smoking attributes at first smear.**

Variable	HPV-16 status	No. of Cases/Controls	Adjusted OR* (95%CI)	p value interaction
<u>Smoking duration (years)</u>				
0	Negative	91 / 160	Ref	
	Positive	28 / 11	4.8 (2.2-10.3)	
< 5	Negative	49 / 52	Ref	
	Positive	12 / 4	3.3 (1.0-11.2)	
≥ 5	Negative	128 / 134	Ref	0.03
	Positive	67 / 2	35.9 (8.6-150.2)	
<u>Smoking intensity (packyears)</u>				
0	Negative	91 / 160	Ref	
	Positive	28 / 11	4.8 (2.2-10.2)	
< 2.5	Negative	86 / 84	Ref	
	Positive	30 / 4	7.9 (2.6-23.7)	
≥ 2.5	Negative	91 / 102	Ref	0.12
	Positive	49 / 2	26.9 (6.3-114.6)	

\*Odds ratios (ORs) are adjusted for age at first smear, # years between date of smear and diagnosis, current oral contraceptive use and parity. Number of sex partners prior to first smear was included as a continuous variable.

## Paper II:

### ***Descriptive Data***

During the years 1968 to 2002, the reported incidence (standardised) of CIS and SCC in Sweden generally declined (Table 4, Figure 7), whereas the incidence of AIS and AC increased dramatically (Table 4, Figure 8).

There appeared to be no shift in the age at which women acquired CIS, SCC, AIS or AC between the 3 decades. There were however differences in the age distributions of the 4 histological types. Both SCC and AC incidence were, on average, reported at later ages than their respective pre-cursors CIS and AIS.

### ***Age Standardised Incidence Rates***

Standardised incidence rates (SIR) throughout Sweden for the 4 histological types were calculated (Figures 7 and 8). County-specific SIR's were also calculated (data not shown). These county-specific rates showed that a variation existed between counties with respect to the reported incidence of various lesions. This variation was particularly evident for CIS, AIS, and AC, and most likely represented poor standardisation of histopathologic criteria used for classification of CIS and/or random variation due to low numbers (for AIS and AC).

### ***Correlations between In situ and Invasive Lesions***

Spearman correlation analysis of the standardized incidence rates for SCC and CIS (5 or 10 years prior to SCC) showed weakly (but statistically non-significant) positive correlations between the incidence of SCC and that of CIS, 5 years ( $r=0.19$ ;  $p=0.051$ ) or 10 years ( $r=0.16$ ;  $p=0.095$ ) prior (Table 5). Similar analyses for AC and AIS (5 or 10 years prior to AC) showed slightly stronger positive correlations of  $r=0.31$  ( $p=0.001$ ) for the 5 year and  $r=0.28$  ( $p=0.003$ ) for the 10 year lag periods (Table 5). In addition, we performed similar analyses using a lag period of 15 years. There were no apparent correlations between incidence of SCC and CIS (-15 years), or between AC and AIS (-15 years).

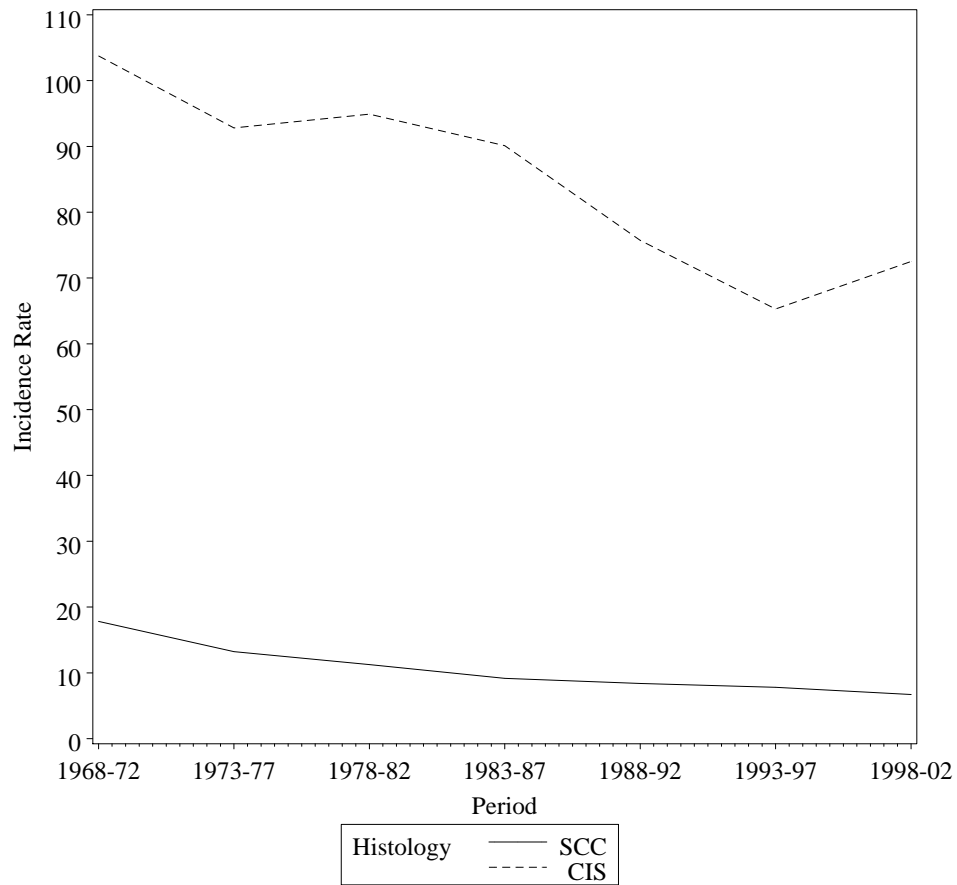
These findings were verified utilizing GEE models (Table 5). The relative change in SCC for an increase of 100 CIS cases per 100,000 women years was positive rather than inverse and estimated to 1.05 (CI: 0.99 - 1.10) for the 5 year and 1.02 (CI: 0.96 - 1.08) for the 10 year lag periods. For the AC and AIS relationship there were fewer cases and as a consequence lower statistical power. The relative change in AC for an increase of 10 AIS cases per 100,000 women was estimated to 1.17 (CI: 0.90 - 1.52) and 1.08 (CI: 0.81 - 1.43) for -5 and -10 years lag respectively. The lack of an inverse correlation suggests that increased reported incidence of CIS in certain counties does not forecast a reduction in SCC for those counties. Thus, while the GEE statistical model reduces some of the variability present in the Spearman correlations, there was still no support for an association between SCC and -5 or -10 year CIS rate or between AC and -5 or -10 year AIS rate.

**Table 4: Number of incident cases of Squamous Cell Carcinoma *In situ* (CIS), Squamous Cell Carcinoma (SCC), Adenocarcinoma *In situ* (AIS) and Cervical Adenocarcinoma (AC) between 1968 and 2002, by age of Diagnosis.**

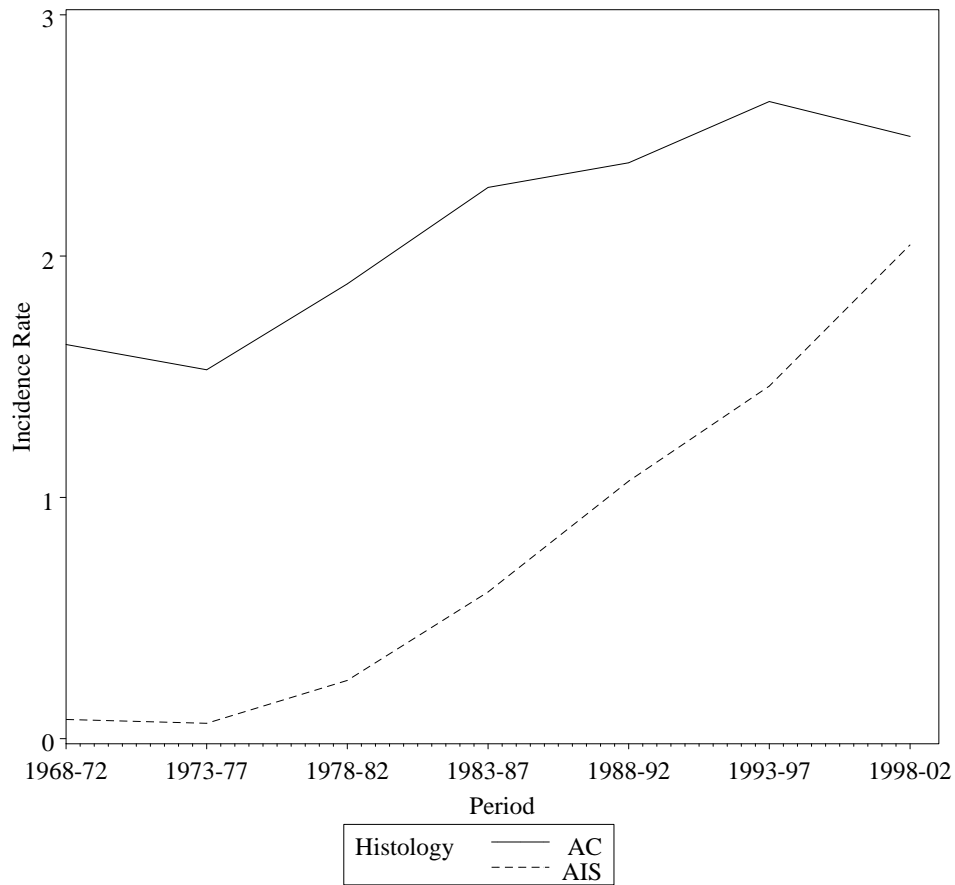
Age at Diagnosis	CIS			SCC			AIS			AC		
	1968- 1979	1980- 1991	1992- 2002	1968- 1979	1980- 1991	1992- 2002	1968- 1979	1980- 1991	1992- 2002	1968- 1979	1980- 1991	1992- 2002
<20	658	583	190	7	2	0	0	0	0	1	1	1
20-29	16,513	15,698	10,978	413	355	235	12	49	179	46	67	71
30-39	17,738	17,737	12,150	1,119	1,104	828	15	119	318	147	261	303
40-49	8,598	7,535	6,148	1,621	896	873	8	115	219	179	262	356
50-59	2,643	1,946	2,613	1,799	718	605	8	45	97	172	221	230
60-69	878	804	824	1,585	1,056	586	0	15	35	148	191	195
70-79	265	386	440	860	942	706	0	3	10	111	181	187
80+	39	78	101	284	377	428	1	5	7	46	110	115
All ages	47,332	44,767	33,444	7,688	5,450	4,261	44	351	865	850	1,294	1,458



**Figure 7: Squamous Cell Carcinoma and Cervical Cancer *In situ* Standardised Incidence Rates (per 100,000) for Sweden.**



**Figure 8: Adenocarcinoma and Adenocarcinoma *In situ* Standardised Incidence Rates (per 100,000) for Sweden.**



**Table 5: Correlations between *In situ* and Invasive Cervical Cancer.**

	SCC rate Vs CIS-5yrs	SCC rate Vs CIS-10yrs	AC rate Vs AIS-5yrs	AC rate Vs AIS-10yrs
<b>Correlation</b> <sup>†</sup>	0.19	0.16	0.31	0.28
P value	0.051	0.095	0.001	0.003
<b>GEE model</b>				
Relative Risk Estimate*	1.05	1.02	1.17	1.08
Confidence Interval	(0.99 - 1.10)	(0.96 - 1.08)	(0.90 - 1.52)	(0.81 - 1.43)

<sup>†</sup> Spearman's rank correlation coefficient.

\* The relative change in SCC for an increase of 100 CIS cases per 100,000 women years.

### Paper III:

#### ***Descriptive Data***

Characteristics of the 553,255 participants, by mothers CIS status relative to conception, are presented in Table 6. The observed median age of mothers who had CIS lesions prior to their daughters' conception was higher than in mothers with CIS after conception. The opposite trend was observed in relation to age of CIS diagnosis in mothers, where the median age of diagnosis was highest in mothers who developed CIS more than 10 years after their daughter's conception. No differences were observed in the daughters' median ages at CIS diagnosis in relation to if/when their mothers developed CIS. Comparable numbers of daughters were present in the exposure groups whose mothers developed CIS at, or following, conception. Notably fewer numbers of daughters were available in the group whose mothers developed CIS prior to conception.

#### ***Survival Analyses***

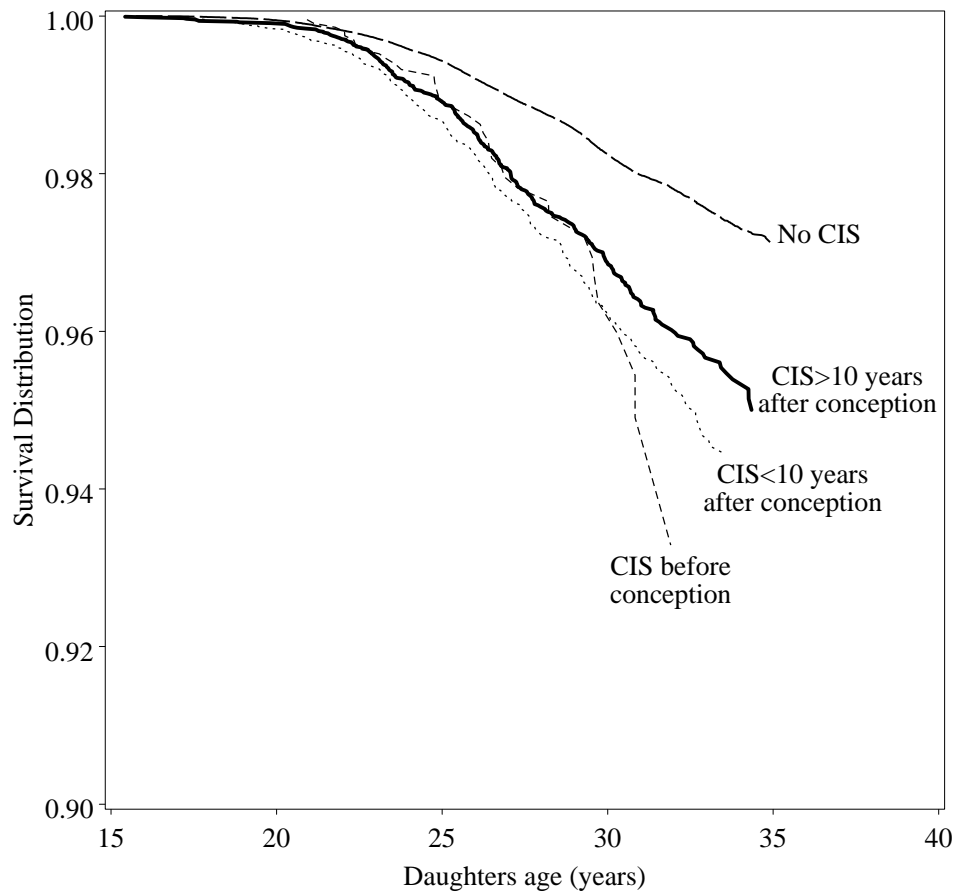
Kaplan-Meier estimates (unadjusted) for survival functions of CIS in daughters, in relation to mothers CIS status prior to and after conception, were plotted (Figure 9). The probability of CIS in daughters was clearly lowest among women whose mothers were CIS negative during follow-up. There appeared to be slight differences in the likelihood of daughters developing CIS, relative to whether their mothers reported CIS within, or more than, 10 years after conception (relative to conception). However, the 3 exposure groups - CIS prior to conception, CIS < 10 years after conception, and CIS > 10 years after conception - were compared using a log-rank test and showed no significant differences ( $p=0.1009$ ).

#### ***Relative Risk for CIS in Daughters***

We identified two likely confounding variables (attained age of daughters, and mother's age at daughter's conception) and adjusted for them in the analyses. After adjustment for mother's age at daughter's conception, we observed an almost twofold (adjusted RR=1.92; 95% CI 1.76-2.09) increased risk for developing CIS in women whose mothers were diagnosed with CIS during follow-up, compared to women whose mothers did not present with CIS in the same period (Table 7).

We then divided the analyses using our exposure of interest, namely CIS status in mothers at different time points relative to conception (Table 7). The largest difference between the crude and adjusted models was observed within the 'CIS in mothers prior to conception' group. For this exposure group, the crude relative risk in daughters shifted from a protective effect (RR=0.66; 95% CI 0.47-0.93) to a non-significant increased risk (RR=1.24; 95% CI 0.88-1.74), compared to the 'CIS in mothers  $\geq$  10 years' group.

**Figure 9: Kaplan-Meier estimates (unadjusted) for survival functions of CIS in daughters in relation to mothers CIS status prior to and after conception.**



**Table 6: Characteristics of 553,255 biological mother/daughter pairs used in analyses.**

	CIS negative	<u>Mother's CIS status relative to conception</u>		
		CIS prior to conception	CIS<10yrs after conception	CIS>=10yrs after conception
<b>Mothers median age at daughter's conception (quartiles)</b>	24.8 (21.8-28.2)	28.6 (25.7-32.1)	24.0 (21.1-27.1)	22.2 (19.6-25.4)
<b>Mothers age at diagnosis (median)</b>	-	25.4 (22.7-28.3)	29.2 (26.3-32.6)	38.5 (34.4-43.5)
<b>Daughters age at diagnosis (median)</b>	26.1 (23.6-29.0)	26.0 (23.3-28.2)	25.9 (23.3-28.6)	26.2 (23.5-29.0)
<b>Daughters with CIS (N)</b>	5,116	38	301	280
<b>Daughters at risk (N)</b>	518,135	4,233	13,602	11,550
<b>Daughters at risk (Person-Years)</b>	13,212,303	93,776	353,572	322,604

After adjustment for the two confounders (attained age of daughters and mother's age at daughter's conception), we found a significantly increased risk in daughters whose mother presented with CIS within 10 years following conception (adjusted RR=1.24; 95% CI 1.05-1.46), compared with daughters whose mothers developed CIS ten or more years following conception (Table 7). A similar estimate for risk was observed in daughters whose mothers developed CIS prior to their daughter's conception, however this result was not significant. Daughters of CIS negative mothers had a decreased risk for CIS themselves, compared to daughters whose mothers developed CIS more than 10 years following their daughters' conception (adjusted RR=0.58; 95% CI 0.52-0.66).

To address the possibility of residual confounding by maternally-inherited social behaviour, we adjusted for the occupation of the mother in our analyses. We found no significant change to our estimates of risk for daughters whose mother presented with CIS within 10 years following conception (adjusted RR=1.23; 95% CI 1.05-1.45), compared with daughters whose mothers developed CIS ten or more years following conception.

In order to check for possible bias related to removal of mother/daughter pairs where mothers had died or emigrated prior to end of follow-up, we performed analyses including these pairs. No considerable differences in results were noted.

**Table 7: Analyses of Cervical Cancer *In situ* risk in daughters as a consequence of their mother’s Cervical Cancer *In situ* status relative to time since daughter’s conception.**

	<u>Case Daughters (N)</u>	<u>Daughters at risk (Person-Years)</u>	<u>Crude RR (95% CI)</u>	<u>Adjusted RR* (95% CI)</u>	<u>Adjusted RR† (95% CI)</u>
<b>Mother’s CIS status</b>					
Negative	5,116	13,212,303	Ref	Ref	Ref
Positive	619	769,952	2.03 (1.87-2.20)	2.00 (1.84-2.17)	1.92 (1.76-2.09)
<b>CIS in mothers relative to conception</b>					
Never	5116	13,212,303	0.49 (0.44-0.56)	0.54 (0.48-0.61)	0.58 (0.52-0.66)
Prior	38	93,776	0.66 (0.47-0.93)	1.04 (0.74-1.46)	1.24 (0.88-1.74)
<10 yrs after	301	353,572	1.08 (0.91-1.27)	1.19 (1.01-1.40)	1.24 (1.05-1.46)
>=10 yrs after	280	322,604	Ref	Ref	Ref

\* Adjusted for attained age of daughter.

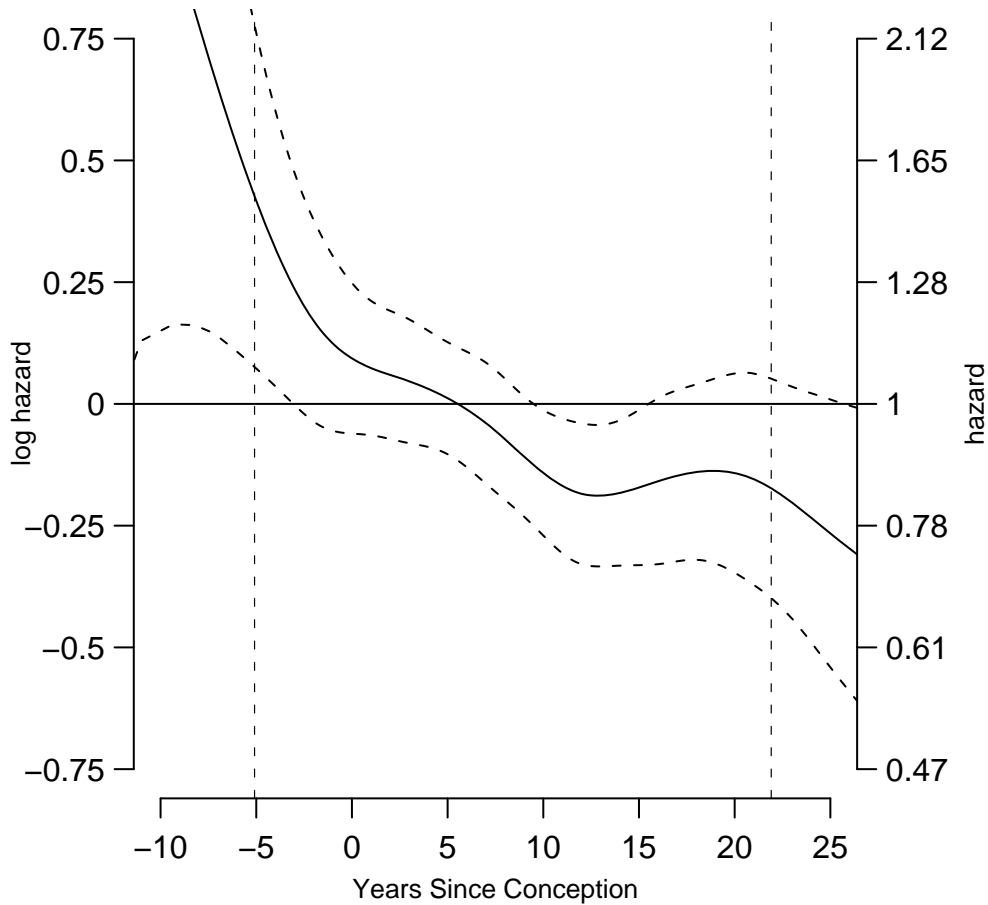
† Adjusted for attained age of daughter and mother’s age at daughter’s conception.

To ensure that our lower age restriction in daughters did not bias our results, we repeated our analyses using a higher minimum age restriction of 20 years of age for daughters (instead of 15 years of age) and found our results changed only marginally (data not shown). We therefore used the 15 year of age restriction in order to gain as many case daughters as possible.

We did not consider it appropriate to combine mothers with invasive cervical cancer and mothers who developed CIS, since the induction period between HPV and CIS should be different to the induction period between HPV and cervical cancer. We did however test whether the addition of cervical cancer case daughters might influence our results and found no considerable differences in our results (data not shown).

Since the CIS categorization in mothers (in relation to time since pregnancy) was critical in our study, we investigated the plausibility of our categorizations by plotting hazard ratios (adjusted for mother’s age at conception) for CIS detection in daughters against years since conception that CIS occurred in mothers estimated using splines (Figure 10). We observed an apparent plateau effect shortly after the 10 year point, supporting our choice of using a 10 year cutoff.

**Figure 10: Estimates of Log Hazard Vs. Time for CIS diagnosis in mothers (years) since conception in CIS positive mothers.**



Paper IV:

**Descriptive Data**

Our final cohort for this study consisted of 808 women, including 225 CIS cases, 179 SCC cases, and 404 individually matched controls. The median number of years between the last smear and the case's diagnosis within a given riskset, was 0.6 years (min=0 years, max=15.9 years) for cases and 2.8 years (min=0 years, max=15.1 years) for controls. When stratified by outcome, the median number of years prior to diagnosis was 0.3 years (0-14.2 years) for CIS cases and 2.4 years (0-14.3 years) for their matched controls. For SCC cases, the median number of years prior to diagnosis was 2.4 years (0-15.9 years) and 3.4 years (0-15.1 years) for their matched controls.

### ***HPV Prevalences***

The presence of one or more HPV infections in the last smear per woman was detected in 81.8% of CIS cases, 72.1% of SCC cases, and 11.9% of controls (Table 8). A very similar pattern of HPV-16/18 infection was observed for CIS and SCC cases; 54.7% of women with CIS, and 54.2% of women with SCC, possessed an HPV-16 and/or HPV-18 infection in their last smear. This was in contrast to the observed prevalence of 4.7% for HPV-16/18 in control women. The prevalence of non-16/18 high-risk (oncogenic) HPV infections (non-16/18-HRHPV) was expectedly higher in both CIS (51.6%) and SCC cases (29.6%) compared to control women (9.2%) (Table 8).

We found that 184 CIS cases possessed an HPV infection, 55 (29.9%) of whom had multiple HPV infections in the same cervical smear. In SCC cases however, multiple HPV infections were only seen in 15.5% (20/129) of women with a positive HPV test. In control women with HPV, 22.9% (11/48 women) - had multiple HPV infections (Table 8).

### ***HPV-related Risk for In situ or Invasive Squamous Cell Carcinoma***

The risks for developing CIS or SCC in women exposed to HPV types other than HPV-16 and HPV-18 were estimated (Table 9).

Crude risks associated with the presence of non-16/18 HRHPV infections (compared to HPV negative, LRHPV positive, or HPV-16/18 positive women) were 17-fold (RR=16.8; 95% CI, 6.8-41.4) for CIS, 6-fold (RR=6.1; 95% CI, 2.7-16.2) for SCC, and 11-fold (RR=10.6; 95% CI, 5.8-21.0) for pooled CIS+SCC cases.

After adjustment for HPV-16/18 and LRHPV infections however, the risk associated with the presence of a non-16/18 HRHPV infection in the last smear dramatically increased (due to exclusion of HPV-16/18, and LRHPV infections from the reference group). We found that women with a non-16/18 HRHPV infection were at a 25-fold increased risk (adjusted RR=25.3; 95% CI, 8.1-108.5) for developing CIS, compared to women without an HPV infection. Similar analyses for the SCC outcome revealed an increased risk of 10 (adjusted RR=9.9; 95% CI, 3.3-39.8), for women with a non-HPV-16/18 HRHPV infection compared to HPV negative women. The presence of HPV-16/18 in the last smear (Table 9) corresponded to a 33-fold increased risk for CIS development (adjusted RR=33.4; 95% CI, 11.9-125.5) and a 34-fold increased risk of developing SCC (adjusted RR=34.5; 95% CI, 10.9-177.3), relative to HPV negative women.

### ***Population Attributable Risks (PAR) Associated with HPV Exposures***

To look into the likely benefit to be gained through population-wide introduction of vaccines targeting HPV-16 and HPV-18 (Figure 1), we estimated the population attributable risk for HPV-16/18. For CIS, 60.4% of cases would not have occurred during the follow-up period in the absence of HPV-16/18 (under an assumption of 100% efficacy



of both vaccines and complete population coverage/compliance). Furthermore, a reduction of 61.2% of SCC cases was estimated in the absence of HPV-16/18 in the population.

**Table 8: Prevalence of HPV types in the last recorded Cervical smear for Swedish women with and without Cervical Cancer *In situ* (CIS) and Squamous Cell Cervical Cancer (SCC).**

HPV Type	Low Risk (LR) or High Risk (HR)	CIS		SCC		Controls* n=404	% HPV* positive
		Cases* n=225	% HPV* positive	Cases* n=179	% HPV* positive		
HPV-6	LR	1	0.4	0	0	2	0.5
HPV-7	LR	2	0.9	0	0	1	0.2
HPV-11	LR	0	0	0	0	1	0.2
HPV-16	HR	108	48.0	85	47.5	13	3.2
HPV-18	HR	22	9.8	14	7.8	7	1.7
HPV-31	HR	20	8.9	9	5.0	8	2.0
HPV-33	HR	23	10.2	8	4.5	4	1.0
HPV-35	HR	8	3.6	1	0.6	2	0.5
HPV-39	HR	1	0.4	6	3.4	4	1.0
HPV-42	LR	11	4.9	2	1.1	7	1.7
HPV-43	LR	0	0	1	0.6	1	0.2
HPV-45	HR	12	5.3	16	8.9	3	0.7
HPV-51	HR	12	5.3	1	0.6	2	0.5
HPV-52	HR	8	3.6	1	0.6	3	0.7
HPV-56	HR	11	4.9	6	3.4	4	1.0
HPV-58	HR	5	2.2	1	0.6	2	0.5
HPV-59	HR	7	3.1	3	1.7	2	0.5
HPV-66	HR	5	2.2	1	0.6	3	0.7
HPV-68	HR	2	0.9	0	0	0	0
HPV-70	LR	2	0.9	0	0	1	0.2
HPV-73	HR	0	0	0	0	0	0
HPV-82	HR	2	0.9	0	0	0	0
HPV-90	LR	0	0	0	0	1	0.2
Any LRHPV	LR	16	7.1	3	1.7	14	3.5
HPV-16/18	HR	123	54.7	97	54.2	19	4.7
Non-16/18 HRHPV	HR	116	51.6	53	29.6	37	9.2
Any HRHPV	HR	180	80.0	128	71.5	39	9.6
Any HPV	HR or LR	184	81.8	129	72.1	48	11.9
Multiple HPV infected women	HR or LR	55	24.4	20	11.2	11	2.7

\* Addition of individual HPV types exceeds the number of women due to multiple HPV infections in some women. Therefore, the sum of percentages of positive HPV infections may exceed 100%.

**Table 9: RR and 95% CI of CIS, SCC and CIS/SCC in relation to the presence of HPV types in the last recorded smear prior to diagnosis for cases compared to individually matched controls.**

	<b>CIS (n=225)</b>		<b>Adjusted<sup>‡Ψ</sup></b>	
	<b>Crude<sup>Ψ</sup></b>	<b>95% CI</b>	<b>RR</b>	<b>95% CI</b>
<b>Reference</b>	All other women		HPV negative women	
<b>LRHPV<sup>†</sup></b>	2.8	1.0 – 9.9	6.5	1.0 – 54.3
<b>HPV-16/18<sup>‡</sup></b>	23.2	9.7 – 72.8	33.4	11.9 – 125.5
<b>Non-16/18 HRHPV<sup>*</sup></b>	16.8	6.8 – 41.4	25.3	8.1 – 108.5
	<b>SCC (n=179)</b>		<b>Adjusted<sup>‡Ψ</sup></b>	
	<b>Crude<sup>Ψ</sup></b>	<b>95% CI</b>	<b>RR</b>	<b>95% CI</b>
<b>Reference</b>	All other women		HPV negative women	
<b>LRHPV<sup>†</sup></b>	0.5	0.1 – 2.3	0.4	0.0 – 6.7
<b>HPV-16/18<sup>‡</sup></b>	31.0	10.3 – 153.0	34.5	10.9 – 177.3
<b>Non-16/18 HRHPV<sup>*</sup></b>	6.1	2.7 – 16.2	9.9	3.3 – 39.8
	<b>CIS+SCC (n=404)</b>		<b>Adjusted<sup>‡Ψ</sup></b>	
	<b>Crude<sup>Ψ</sup></b>	<b>95% CI</b>	<b>RR</b>	<b>95% CI</b>
<b>Reference</b>	All other women		HPV negative women	
<b>LRHPV<sup>†</sup></b>	1.5	0.7 – 3.6	2.4	0.6 – 10.5
<b>HPV-16/18<sup>‡</sup></b>	26.1	13.0 – 61.3	35.2	16.0 – 91.1
<b>Non-16/18 HRHPV<sup>*</sup></b>	10.6	5.8 – 21.0	15.7	7.2 – 38.8

<sup>Ψ</sup>All estimates were controlled for matching criteria (county, date of entry into cohort, and age).

<sup>‡</sup>RR's further adjusted for all other HPV variables.

<sup>\*</sup>Non-16/18 HRHPV = any high risk HPV infection apart from HPV-16 or HPV-18.

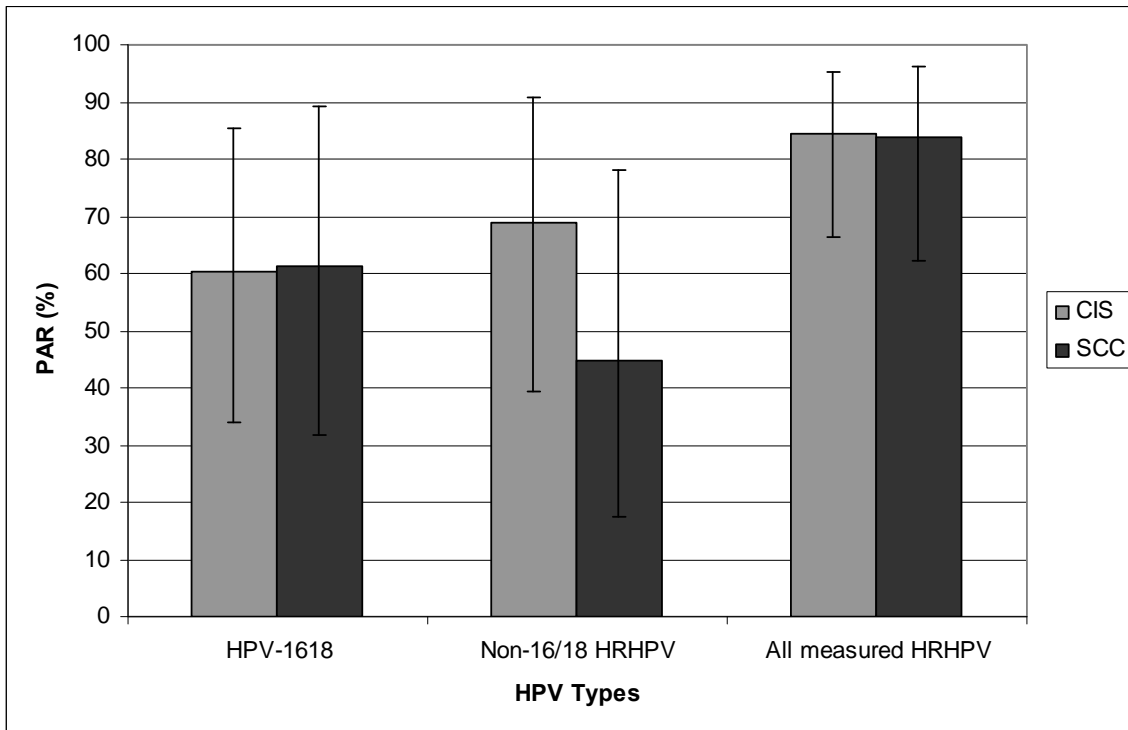
<sup>†</sup>LRHPV = any low risk HPV infection.

<sup>‡</sup>HPV-16/18 = any HPV-16 or HPV-18 infection.

Removal of Non-16/18 HRHPV types from the population would have reduced the CIS burden by 69% and SCC incidence by 44.9%. In our cohort, we also estimated the PAR of all HPV types measured in our study (Figure 11). Prevention of all 23 types we measured would have conferred an 84.8% reduction of CIS cases and an 83.9% reduction of SCC cases.

While interpreting the PAR figures, it should be kept in mind that the addition of PAR's for HPV-16/18 and non-HPV-16/18 infections will exceed 100%. This is partly due to the fact that HPV-16/18 infected women may also carry other HPV infections, and also lack of precision (as seen in the large confidence limits).

**Figure 11: Potential reduction in Incidence of CIS and SCC achievable through elimination of HPV-16/18, Non-16/18 High Risk HPV (HRHPV), or ‘All measured’ HRHPV types.**



## DISCUSSION

### Methodological Considerations

#### Study Design

All of the studies conducted for this thesis were population-based studies. This is an important consideration because epidemiological studies aim to extend findings to that of the general population. For the 1<sup>st</sup> and 4<sup>th</sup> papers, this is particularly pertinent due to their case-control structure.

Case-control studies are an extremely efficient tool in etiological research, particularly for rare diseases, whereby you can specifically target subjects possessing a disease in the population for collection. These individuals may then be assessed for previous exposure to various agents. By comparing the likelihood for a case (with disease) subject having a particular exposure to the likelihood of a control (without disease) subject having the same exposure, one can estimate the risk for exposed individuals developing the disease relative to the risk for unexposed individuals. This is the relative risk (RR).

A key factor in being able to conduct case-control studies is the ability to select control subjects that are representative of the population. That is, they should be chosen in such a way that they represent the source population from which the cases arose. For our studies in cervical cancer, this means that controls should be selected independently of the various exposures such as HPV, smoking, socio economic status, sexual habits, etc. Also it is important that the amount of time the controls contribute toward the studies should be comparable to that of cases. Generally speaking, controls should be given every opportunity to develop disease as the cases have.

For papers 1 and 4, all cases (CIS or SCC) reported during a specified period were initially identified from the relevant counties. For study 1 all available CIS cases were included, whereas in study 4 a selection of CIS cases was randomly chosen for inclusion. In addition, all available SCC cases were included in study 4. We employed individual matching when choosing controls. For example, in the 1<sup>st</sup> study each control woman was randomly selected if she shared certain characteristics with a case woman. Specifically, each control woman had to possess a similar age and date of entry into the cohort, to that of the matched case. This ensured that adequate numbers of women with a similar age and follow-up time were available for analysis.

For the 2<sup>nd</sup> and 3<sup>rd</sup> papers we did not need to conduct case-control studies since we had the ability to access all population information from the various population registries. This is of course preferable to case-control studies because it is possible to include all individuals, rather than a subset of individuals. The challenge with studies incorporating ‘all’ individuals is that the reporting of disease (in this case) should be virtually complete. For the calendar periods we studied - from 1968 to 2002 for paper 2, and from 1967 to 2001 for paper 3 - the Swedish cancer registry is considered to be virtually 100% complete (74).

## Validity

The validity of a study basically refers to the reliability of the results. In order for a study to be considered valid, it must be shown that the results are ‘true’ and do not merely reflect the presence of bias, confounding, or random error. Case-control studies are particularly prone to bias and problems related to temporality, and must be assessed accordingly before a result is considered to be valid.

## Chance

Statistical analysis of data allows us to make certain conclusions. One fundamental truth of statistics however is that there is no truth, only probability. Even if a particular result is repeated 10 times, there is no guarantee that the same result will occur on the 11<sup>th</sup> repetition. The same can be said for a result that is generated 100 times in a row. There is always the possibility that any given event has occurred randomly. This relates to variance within a probability distribution. However, the more often a test is repeated, the more confident you can be that the result is stable (assuming the test is performed correctly!). For example, a coin tossed twice is less likely to give an accurate picture of the likelihood that a ‘tail’ will occur than if the coin was tossed 1000 times.

This concept is extremely important and influences study design, analysis, and interpretation of results. Steps must be taken to decrease the possibility that *chance* is responsible for a result. Primarily, the study’s sample size should be large enough to provide confidence in the estimates. Often we will use p-values and confidence limits to aid us in determining how likely it is that a result is true. Whilst we endeavored to use sufficient numbers of women to allow confidence in our estimates for risk, it is still possible that some results were generated by chance.

In the first study, the main effects measured (smoking and HPV-16 presence, load, and persistence) were found to be statistically significant ( $p < 0.05$ ) and had confidence limits which excluded the null value. This strongly suggests that the results are not due to chance. Results related to the interaction between smoking and HPV are less clear since tests for multiplicative interaction require larger sample sizes in order to have sufficient power for detecting an association (if present). For the interaction tests between smoking and HPV presence (or HPV load), we did not achieve a significant result. However the results are arguably suggestive of an interaction being present. These results were further strengthened by the significant association we observed between smoking duration and HPV presence.

The second study utilized very large numbers of women, ensuring our ability to detect an association if present and also that our results are unlikely to be due to chance.

The third study made use of fewer women and thus we were unable to confirm the association or lack thereof for all exposure categories. Nevertheless, in our main exposure of interest (CIS detected in mothers within 10 years following their daughter’s conception),

the tight confidence limits for the adjusted RR estimate exclude the null. This suggests a significant result.

In the fourth study we realized the lack of data points necessary to address the individual associations for each HPV type with CIS and SCC outcomes. We therefore pooled the non-HPV16/18 high risk types, as well as combining HPV-16 and HPV-18 into a single exposure variable. This enabled us to gain more reliable RR and PAR estimates.

### *Bias*

A lack of study validity may also occur as a result of incorrect measurements of the exposure or outcome. Care should be taken to ensure the methods of testing an exposure or diagnosing an outcome do not vary considerably.

### *Selection Bias*

#### ***Control Selection Bias***

Control selection bias refers to a difference in the criteria used to select cases and controls, which may result in the selection of controls who do not accurately represent the population base from which the cases have arisen.

In studies 1 and 4 where a case-control structure was used, great care was taken to ensure controls were representative of the entire population of women who attended cervical cancer screening in Swedish counties from which the cases arose. The combination of using a population-based case-control approach, rather than a hospital-based case-control study approach for example, and random selection should ensure our control selection did not introduce any considerable bias.

For studies 2 and 3, control selection bias is highly unlikely due to the fact that we used all women who were not diagnosed with cervical cancer or cervical cancer *in situ* from the entire population.

#### ***Self Selection Bias***

This form of bias may occur if non-response or non-participation of individuals is different between cases and controls, and this difference is related to the exposure.

In study 1 there were an equivalent and extremely high percentage (90%) of responders to the questionnaire for cases and controls. For those who did not respond, it may be possible that their lack of response was related to the exposure, for example, if smoking resulted in poorer health and subsequent inability or difficulty to respond. This may in turn cause a degree of differential bias if there are more non-responding cases who smoke than non-responding controls. Given our high response rate however, we feel the effects on our estimates of risk would be minimal.

A low number of cases (n=12) and controls (n=20) were also excluded on the basis of insufficient DNA presence as measured by B-actin presence. We have no reason to believe this would be related to either smoking or HPV related exposures.

For studies 2 and 3, participation was dependant upon women's participation in Sweden's organized cervical cancer screening program. Given that all women are given equal access to cervical cancer screening in Sweden, we feel that self-selection bias is unlikely. Had we been using data obtained via opportunistic screening this may have been a concern, but we excluded the years prior to organized screening to reduce this likelihood.

As with study 1, participation in study 4 was reliant on women's participation in the Swedish cervical cancer screening program, however interview information was not sought in this study.

### ***Differential Diagnosis***

In the event that diagnosis is related to the exposure, cases *may* be selected on the basis of the exposure. In study 1 for example, this could have occurred if the diagnosis of CIS depended on the presence of HPV or smoking. This may be likely if opportunistic screening was the predominant form of cervical cancer management. However, since organized screening was in place for these studies it is unlikely we would see a strong effect related to differential diagnosis. The same principle applies to the other 3 studies.

### ***Observation Bias***

#### ***Recall Bias***

The possibility of recall bias applies to studies where past exposure information is required. Specifically it refers to any differences in recalling or reporting previous exposures between cases and controls.

Interview data is particularly prone to this form of bias, thus great care was taken in the 1<sup>st</sup> study to reduce such bias. Interviewers were not informed of the study hypothesis, nor did they have information on case/control status. Therefore we do not believe the interviewers were able to differentially influence subjects' recall. Furthermore, since the outcome for study 1 was CIS, women were generally healthy at the time of the interview and had presented with CIS lesions many years prior to the interview. Given that the detection of CIS generally does not have a huge impact on women's health many years after detection; it seems unlikely that the case-control status should affect their memory of previous risk factors considerably. However, the length of time between 'exposure to risk factors' and 'participation in interviews' would affect a subject's ability to accurately remember details of exposures like smoking history, sexual partners, etc. We therefore expect a certain amount of non-differential recall bias which may have resulted in slightly underestimated risk estimates.



Recall bias is not a concern for papers 2-4 since the exposures were ascertained either from registers (and reported by pathologists), or were determined through analysis of cervical smears (paper 4).

### ***Misclassification***

Misclassification may occur in the forms of non-differential or differential misclassification of either the outcome or exposures. The less severe non-differential type occurs when an exposure (for example) is misclassified, but the incorrect classification does not occur more frequently in cases or controls (the outcome). Comparably, if the classification of an exposure differs according to the outcome then a differential misclassification is observed. Differential misclassification may distort estimates away from, or towards the null whereas non-differential misclassification generally causes bias towards the null.

### ***Misclassification of Exposure***

For the first and fourth studies, every effort was made to ensure that there were no differential measurement errors in determination of HPV-16 presence by blinding the laboratory personnel to the case/control status of samples. Also, samples from cases and their matched controls were batched together during DNA extraction and HPV analyses to lessen the chance that any batch-related differences in laboratory analysis would induce a differential bias.

In an attempt to reduce non-differential misclassification of HPV related exposures, the presence of house-keeping genes was assessed in order to ensure that an HPV negative result was not generated as a result of insufficient DNA presence. We cannot assume a total absence of non-differential measurement errors in laboratory testing however, although any such errors would serve only to decrease our power to detect an association rather than inducing a differentially biased result.

Papers 2 and 3 relied on registry data for information on CIS status in women. Misclassification of CIS lesions is likely, although there is no reason to believe this bias would be differential since determination of CIS status by pathologists is unlikely to be related to either outcome from these two studies.

One issue related to study 3 is that we used CIS in mothers primarily as a surrogate marker of HPV status. Whilst it is generally assumed that one or more oncogenic HPV infections are present in women presenting with CIS, our stratification of mothers based on CIS detection relative to their daughter's conception is prone to problems with misclassification. Our belief however, is that a greater average number of mothers diagnosed with CIS within 10 years following conception would have an HPV infection around the time of conception/pregnancy compared to those mothers diagnosed with CIS more than 10 years following conception.

### ***Misclassification of Outcome***

For all four studies, the diagnoses of either CIS or SCC were reported by pathologists and mostly based on results from histology. There are unlikely to be many misclassifications of SCC diagnosis due to the relative ease of their identification. However, CIS reporting is likely to vary between pathologists. Indeed, this fact was utilized in the 2<sup>nd</sup> study to measure the effectiveness of SCC management. Nevertheless, we do not expect any differential misclassification of CIS to be present for papers 1 or 4 since a pathologist's classification of CIS is unlikely to be related HPV or smoking.

Study 3 is less clear however. Because the exposure is 'CIS in mothers' (relative to their daughter's conception) and the outcome is 'CIS in daughters', a pathologist's characterization of a lesion *may* be influenced if he is aware of the mother's history. This probably rarely happens however, so we doubt this would greatly influence our results.

### ***Loss to Follow-up***

Given the comprehensiveness of registration for women in Sweden (regarding cervical cancer reporting), we are confident that movements within Sweden did not greatly influence our ability to gain complete follow-up for our studies. One exception to this is migration to and from Sweden (for studies 2 and 3). We possessed migration data for study 3 and limited the follow-up in emigrants to the date at which they emigrated since their outcome status after emigration is unknown. For studies 1, 2 and 4, we did not possess such information however we do not foresee any problems related to differential bias since migration is unlikely to be associated with either the outcome or the exposure.

### **Confounding**

Confounding is often thought of as being a mixing of effects. It describes a situation where the true association between two factors is confused by one or more other factors, and is one of the central tenets of epidemiological theory. In order for confounding to occur, a few criteria must be met. Firstly, there must be an association between the potential confounder (PC) and the exposure. Secondly, the PC must be associated with the outcome. Lastly, the PC should not be an intermediate in the causal pathway between the exposure and outcome.

### ***Confounding - Paper I***

In the first study, we possessed information on many risk factors and identified a number of PC's based on previous studies, biological theory, and statistical association within our own study population. We then adjusted for these PC's in order to more accurately estimate risk associated with the risk factors of interest (smoking and HPV). One limitation in this study was our inability to adjust for the potential effects of other HPV types in the analysis. It is possible that some residual confounding may exist in relation to this. This is of particular concern in our estimates for smoking-related variables. However,

we believe the effects from this lack of adjustment for HPV types would be limited. This is based on our observation that adjustment for HPV-16 status gave little difference between crude and adjusted risk estimates for smoking.

### ***Confounding - Paper II***

It is difficult to envisage any confounding within this study that might significantly affect our results. One possibility might be migration between counties, however there is no reason to believe county-migration would be associated with cervical cancer (SCC or AC) incidence. These histological types are easily identified by pathologists (compared to CIS or AIS) so it is doubtful that a woman moving to another county is more or less likely to have a 'reported' cervical cancer than in her previous county. Furthermore, we do not believe differences between counties in background risk factors for cervical cancer are so great that they would have a large effect on our results.

One other issue relates to migration of women into Sweden. Sweden accepts many migrants, particularly from Europe. In the event that immigrants from certain countries possessed a higher risk for cervical cancer *and* that they were present in great enough numbers, it is possible that they might influence figures for incidence of cervical cancer. Ideally, if we had possessed information on country of birth in our dataset, we would stratify by country of birth in this case. However, it is worth noting that migrants from high cervical cancer risk countries may acquire a reduced risk in their new country of residence (4).

### ***Confounding - Paper III***

This study was unfortunately prone to several issues of confounding. Probably the greatest difficulty was in separating the effects attributable to HPV transmission from those related to genetic predisposition or maternally-acquired behavioral traits. Genetic predisposition is thought to give an almost 2-fold increased risk for cervical cancer (85). Other risk factors, such as sexual behavior or smoking (86), are likely to show a certain degree of maternal acquisition. Both genetics and certain maternally-acquired behavior (MAB) are also thought to be associated with HPV incidence.

To combat this problem, we defined a reference group of women who would represent a 'familial baseline' from which we could estimate the residual risk associated with the transfer of HPV from mother to daughter. This 'familial' exposure reference group contained *mothers who developed CIS more than 10 years following their daughter's conception*. A ten year period was chosen to reflect the estimated induction period between HPV incidence and CIS detection. The main exposure group of interest then, was *mothers who possessed CIS within 10 years following their daughter's conception*. Thus, a greater number of mothers in the exposed group should have possessed HPV infections during pregnancy compared to mothers in the reference familial group. Given this, it would seem reasonable for there to be an increased risk for CIS in daughters of exposed women compared to familial references *if* HPV transmission occurred during, or relatively soon after, conception. Whilst this is an imperfect method for controlling for confounding,

it should give an idea as to the residual risks (to daughters) associated with CIS in mothers, that is not related to genetics or maternally-acquired behavior (MAB). To further reduce confounding by MAB however, we adjusted analyses for socioeconomic status (SES) which is likely to be related to such factors as smoking and sexual behavior. Our significant risk estimates remained, even after adjustment for SES.

Another concern was related to possible confounding by age. Firstly, the attained age of daughters will clearly influence their likelihood of developing CIS so we adjusted for this variable accordingly. Secondly, in case any genetic components for CIS risk *might* result in a heightened risk for daughters whose mothers developed CIS at a younger age, we adjusted for mother's attained age at daughter's conception.

We were unable to adjust for other potential confounders such as smoking, oral contraceptive use and sexual behavior factors. Whilst these factors may confound our association between 'mother's CIS status' and 'daughter's CIS status', we do not believe they would be problematic in our associations between 'CIS in mothers relative to conception' and 'CIS in daughters'. In order to confound the latter associations, the risk factors should be associated with both the exposure and the outcome. Particularly with respect to our main finding, it is not obvious that characteristics of the mother such as socioeconomic status (SES) should be related to when a mother develops CIS relative to conception and how this might affect the future CIS status in her daughter. Nevertheless, since SES is deemed important in relation to the maternally-acquired social behaviour of the daughter, we adjusted for the occupation of the mother in our analyses and indeed found no difference in our observed RR. Had we possessed information regarding the other aforementioned risk factors (smoking, OC use, etc), we believe their effect on our results would also be negligible.

### ***Confounding - Paper IV***

For this study, we did not collect information on many of the common risk factors other than HPV and consequently were unable to adjust for them in the analyses. This may have resulted in slight over-estimation of risk associated with various HPV types. Since our main purpose was to look at residual risks associated with non-16/18 high risk HPV types however, we do not feel that these other risk factors would considerably alter our findings. Of course this is still a possibility if in fact different HPV types are differentially affected by certain risk factors such as smoking.

One particularly strong feature of this study however, was our ability to identify all of the common oncogenic HPV types. This allowed us to observe risks associated with pooled HPV type variables after adjustment for other HPV types. As can be seen in Table 9 of this thesis, the difference between crude estimates and those adjusted for other HPV types can be considerable.

## Generalisability

After ensuring that a given study design is internally valid – after addressing previously mentioned issues related to bias, confounding, etc – one must consider the external validity of any findings, or generalisability. This involves consideration of the populations for which the results apply. Namely, are our results generalisable to other populations outside of our study cohort?

All four studies that contributed towards this thesis were population-based studies. This means that the cases and controls were selected from either whole municipalities (Papers 1 and 4), or the whole of Sweden (Papers 2 and 3).

Clearly, for studies 2 and 3, our results should be representative of the Swedish population (assuming internal validity). Caution must be taken in extending these results to other countries citizens however. It is known that the prevalence of various HPV types differs between countries. Also, management for cervical cancer differs considerably between countries. There are numerous other differences that exist between nations in relation to genetics and lifestyle factors. Therefore, whilst the general tenets of our results for these papers are likely to apply to other countries, we might expect slight differences in magnitude of the effects. For example, in study 2 we appear to see something of a saturation of benefit related to management of SCC by CIS detection and removal in Sweden. In many countries this would not be the case if they have previously employed a less aggressive strategy for removing CIS lesions.

Studies 1 and 4 were performed on counties in Sweden, rather than the whole Swedish population. We are confident that results from these studies would apply to other Swedish counties due to the relative conformity in behaviour and health-care between counties. It is doubtful that there are major differences in prevalences of specific HPV types between municipalities. Of course the prevalence of different HPV types will differ between countries but again, the general idea should be generalisable even though the actual RR's or PAR's might differ between nations.

## **Interpretation of findings**

### Paper I – Smoking and HPV-16

Many studies have shown both smoking (56, 57, 65-70) and HPV-16 (12, 13, 21-24, 84) to be independently associated with an increased risk for cervical cancer. Few studies have examined the possible synergism between these two factors however (67, 72, 73), and to our knowledge, none have examined potential interactions between HPV load and smoking status.

Results from study 1 suggested an early synergistic effect exists between smoking and HPV-16 in CIS development. Furthermore, current smokers with high HPV-16 viral load at time of first smear were at a particularly increased risk for CIS (27-fold), compared to

current smokers without HPV-infection. Within non-smokers however, high HPV-16 load contributed only a 6-fold increased risk compared to HPV-16 negative non-smokers at time of first smear. Interaction on a multiplicative scale was observed ( $p=0.03$ ) between duration of smoking and HPV-16 presence at time of first smear in CIS development.

Studies have found that, as part of HPV's ability to evade immune recognition, it can inhibit various components of the innate immune system (87-91), which may in turn promote a Th2-biased immune response. Such a shift in immunity would favour viral persistence rather than viral clearance and may aid tumour progression by subverting immune surveillance mechanisms (92). It has also been reported that smoking may result in localised immune suppression (62, 93-95) which may lead to heightened risk for cervical cancer. The fact that some cigarette constituents have the ability to manipulate cytokine expression in a similar manner to HPV would allow for the possibility that smoking may enhance the ability of HPV to avoid the immune system and, at the same time, increase the chances of neoplastic progression via further imbalance of the Th1 and Th2 cytokine profile. This could be one explanation for the apparent interaction between smoking and HPV-16 that we observed.

### *Paper II – Efficiency of Cervical Cancer Screening in Sweden*

Pap smear screening has been the corner-stone for management of cervical cancer over the last several decades. It is undoubtedly one of the greatest success stories with respect to cancer prevention (96, 97). However, recent studies have raised concerns about the non-specific nature of Pap smear screening and the possibility that over-treatment might occur in some countries (6, 98, 99). One of these studies (98) made use of existing variability (between Swedish counties) in reported incidence of pre-cursor lesions (of cervical cancer) to estimate the effectiveness of Pap smear screening. The hypothesis was that counties having high reported incidence of CIS should have a lower reported incidence of SCC, compared with those counties reporting low incidence of CIS.

We chose to examine whether the lack of association observed in the previous study (98) on CIS and SCC continued over the last decade. We were also interested in using a similar methodology to investigate the effectiveness of Pap smear screening also on AC incidence.

Firstly, we found the differences in CIS incidence per county did not appear to influence SCC incidence 5, 10, or 15 years later. Since severe dysplastic/CIS lesions are typically removed, this suggests that treatment of a higher number of CIS lesions does little to reduce the incidence of SCC. This assumes that the levels of background risk factors are similar across counties, which may not hold true for all counties. The implication of this finding is that there appears to be over-treatment of CIS lesions in Sweden. Whilst no one would suggest that Pap smear screening be removed, certain aspects of the screening procedure should be revisited. Particularly, the period between visits, compliance of individuals, and perhaps a relaxation of criteria for pre-cursor lesion removal should be assessed.

Secondly, we found that the incidence of adenocarcinoma (AC) and its supposed precursor (AIS) has been increasing over the last 30 years. This may be related to an increase in background risk factors or could be a result of a greater awareness of AC, resulting in a higher reported incidence. We investigated the correlations between AIS incidence and future AC incidence in the same way as described for CIS/SCC, and found no association between them. In other words, the treatment of AIS lesions appeared to have little effect on future AC incidence. One possible explanation for this is the lower efficiency in finding, and therefore treating, AIS lesions. However the fact that both AIS and AC are increasing may also indicate that the number of AIS lesions that progress to AC are less than expected or that AIS (or a proportion of AIS) are not in fact precursors to AC. Indeed, whilst a great many people assume AIS is a precursor to AC, the evidence for this remains somewhat sparse (100-105).

### *Paper III – Maternal Transmission of HPV?*

Upon review of literature on childhood incidence of HPV, it becomes clear that HPV is not only an infection confined to sexually active people (46, 106, 107). Some reports of childhood HPV infection are alarmingly high and it is difficult to imagine that sexual abuse can explain the numbers. Certainly a proportion of these infections could also occur through normal post-partum contact with parents. Equally likely however, is the possibility that HPV is contracted intra-partum or in utero (107). Research into the possible reasons for this high HPV incidence in children is mottled with contradictory findings, probably due to differences in study designs, cohorts, limitations in sample sizes, and the likelihood of multiple modes of transmission. Studies investigating the possible consequences of HPV transmission from parents to offspring are not in abundance however some studies have found that the presence of maternal genital warts is a risk factor for juvenile-onset recurrent respiratory papillomatosis (108, 109).

We wanted to take this a step further by investigating the possibility that HPV contact during birth or early childhood might increase the risk for cervical cancer.

Initially we tested the association between CIS in mothers and CIS in daughters. The two-fold increased risk of CIS in daughters of mother with CIS that we observed however, could be attributable to genetics, maternally acquired behavior, or vertical transmission. Literature suggests there is an almost two-fold increased risk for cervical cancer associated with genetic factors so our next step was to try and tease the familial risks from those that might be related to direct HPV transmission from mother to daughter.

The most challenging aspect of the study was to construct a sub-group of mothers who could serve as a ‘genetic and social inheritance exposure’ group. This would allow us to observe the residual risks exerted by a mother with CIS (and HPV) after taking into account the other familial risk factors. Our firmly hypothesis-driven categorization, the appropriateness for which we subsequently tested using splines, should dispel a certain amount of criticism related to the stratification of CIS in mothers.

Our finding of a 24% increased risk of CIS in daughters whose mothers had CIS within 10 years following conception (of the daughters) is compared to an exposure group made up of mothers who also had CIS. Thus, the daughters of mothers in the exposure 'reference' group should also be at a higher risk for CIS due to familial factors. Understanding that various other confounding factors could also explain the result we observed, we had adjusted for the most likely problematic factors.

The implication of our results is that a higher risk for CIS does appear to exist in women whose mothers had CIS, which may be in addition to those explained by genetic and other familial factors such as acquired social behavior. In my view, the most likely explanation for this is the direct transmission from mothers to daughters. From our study, it is impossible to surmise whether this occurs during pregnancy, labor, or early childhood. Regardless, it is important to determine whether or not direct transmission is occurring between mothers and children. If so, further studies can address the mechanism in more detail.

#### *Paper IV – HPV type-specific risks for CIS and SCC in Sweden*

With the advent of vaccines targeting HPV-16 and HPV-18, people within and outside of the HPV research community have been hypothesizing on the likely benefit. It has been estimated that HPV-16/18 contributes towards roughly 67% of SCC cases and 52% of HSIL (high-grade squamous intraepithelial lesion) cases (19). It remains to be seen however, what role the other oncogenic HPV types will play in cervical cancer incidence after effective removal of HPV-16/18 from the community.

Using a population-based cohort of Swedish women, our investigation of the relative and population attributable risks associated with CIS and SCC incidence provided a hint of what the future might hold.

Initial results confirmed the high RR's associated with the presence of HPV-16/18 for both CIS and SCC. They also highlighted the considerable RR's associated with other oncogenic HPV types. Even more pertinent to current concerns however were our estimates for PAR. Our estimates of an approximately 60% reduction in CIS and SCC through removal of HPV-16/18 certainly appears to be in line with what one might expect. Interestingly however, our observed 45% PAR for non-16/18 HRHPV types suggests the benefits achievable through HPV-16/18 removal could be less than expected.

An important observation in our study was the considerable number of women with multiple HPV infections. Their presence partly explains the non-additive nature of our observed PAR's (the other explanation being the imprecision of our estimates as seen by our wide confidence intervals) and also draws attention to one reason for a possible lessening of benefit from HPV vaccines. Many previous studies have not fully investigated co-infection of HPV types. In fact, many researchers have not even looked for other HPV types than their type(s) of interest (84, 110-113). Without fully investigating the risks associated with HPV types other than HPV-16 and HPV-18, one cannot truly isolate the



attributable risks associated with HPV-16 and -18. Our study suggests that these other HPV types have yet to show their true capacity for cervical cancer causation.

Despite the apparent role of non-16/18 HRHPV types, our results provide evidence that vaccines against HPV-16 and HPV-18 will significantly enhance our ability to prevent cervical cancer. Also, the possibility that vaccines may confer a certain level of cross-protection to other HPV types (27, 30) could enhance their effectiveness further. It will be interesting to see how far, and across how many types, this cross-protection will extend.

## Implications and Future Research

Every new piece of evidence uncovered brings us one step closer to the ultimate goal of eradicating cervical cancer. Significant changes, such as the implementation of Pap smear screening and HPV vaccine development, have been made possible through the connection of numerous pieces of existing information that have been reported by thousands of researchers. The papers outlined in this thesis are examples of such studies.

The importance of smoking and its interaction with HPV in causing cervical cancer has an immediate application in preventing cervical cancer. It is unlikely that a majority of women who smoke will reduce or stop smoking based on the results from this study (and others), although perhaps some will. In itself, this is a beneficial consequence. However, another benefit is the observation that smoking and HPV interact. Experimental and epidemiological evidence compliment each other in aiding future research. It may be useful to further investigate the mechanism by which smoking exacerbates the carcinogenic potential of HPV, both to increase our understanding of HPV pathogenesis and to understand how smoking might be involved in pathogenesis of other diseases.

Cervical cancer screening has proved to exhibit extraordinary cervical cancer reducing abilities. However, results from our study (and others) highlight potential insufficiencies in its use. Further studies into duration between testing in women and compliance to testing are warranted. Furthermore, the possibilities of introducing triaging of CIS cases based on HPV-related measures may decrease some of the non-specificity related to Pap smear testing. Before taking such a step however, careful consideration must be given to the costs and benefits associated with such a move. Also, I feel that there is still insufficient information available to make a sound decision on how this should be achieved. Should one triage based on HPV load, persistence, or physical state of HPV (integrated or not)? Currently, HPV load is generally measured using semi-quantitative methods at best, the definition for persistence seems not to have reached a consensus in the academic community, and more research is necessary to really understand the role of integration in cervical cancer development. Still, I believe that ultimately triage of severe dysplasia/CIS cases based on an HPV-related marker would benefit cervical cancer management.

Considering the number of researchers globally who study HPV and cervical cancer, it is surprising how little is known about the possibility of maternal transmission of HPV. A number of studies have reported high incidence of HPV in children. Further research is necessary to definitively confirm the mechanism by which young children are acquiring HPV infections. Also, the ramifications of this with respect to HPV vaccine efficacy should be investigated. Finally, the future health consequences for female children who acquire HPV during early ages should be examined.

HPV vaccination has the potential to strongly influence future cervical cancer incidence. The remaining questions related to population-wide distribution of such vaccines are numerous however. Not least among these issues is the potential efficacy of vaccines in the real world. Thus far, the impressive results from clinical trials have generally been based on relatively narrow study cohorts; women aged 15-25 years, mostly white and from

developed nations, and seronegative for HPV-16/18 at enrolment (114). It remains to be seen whether these efficacies will remain, once vaccines are given to more representative populations of women containing greater heterogeneity. Also, compliance (to taking the full 3 vaccine doses) may be lower in the general population. Finally, the use of a surrogate marker for cervical cancer occurrence (precancerous cervical disease), rather than cervical cancer, leaves a degree of uncertainty as to the actual efficacy for cervical cancer.

The 4<sup>th</sup> study in my thesis investigates one aspect of HPV vaccine introduction which will surely compromise its ability to reduce cervical cancer incidence, namely the presence of other high risk HPV types. No doubt there will be many future studies able to prospectively view the residual risks for cervical cancer attributable to these other HPV types after vaccine deployment. It will be very interesting to see how much cross-protection is achievable using these vaccines. It is conceivable that certain HPV types other than HPV-16/18 will be prevented by the current vaccines and in fact, evidence already suggests this may well be the case. Numerous studies will also be necessary to observe efficacy of the vaccines in preventing both HPV-16/18 and cervical cancer in the general population, in consideration of their heterogeneous nature.

## CONCLUSIONS

### ***HPV-16 and Cigarette Smoking***

- As independent risk factors, HPV-16 presence, HPV-16 load, and smoking significantly increase the risk for cervical cancer *in situ*.
- Women who both smoke and possess HPV-16 infections appear to show a higher risk for CIS than would be expected from the sum of their independent risks. This suggests that smoking and HPV interact to heighten the risk for CIS.

### ***The Realities of Pap Smear Screening in Sweden***

- Pap smear screening in Sweden has greatly reduced the incidence of CIS and SCC in Sweden over the last 4 decades however over-treatment of CIS lesions seems to provide little added benefit to furthering SCC reduction. Consideration should be given to widening the time intervals between testing, tightening criteria for inclusion of treatable lesions, and/or investigation of women's compliance to screening.
- Incidences of AC and AIS are increasing, which may relate to increases in background risk factors. Prevention of AC through Pap smear testing appears ineffective, potentially due to problems in detection or subsequent removal of AIS lesions. Conceivably, this lack of effectiveness could put into question the current hypothesis that AIS is a precursor to AC, rather than a separate disease entity.

### ***Maternal Transmission of CIS risk***

- We found an excess risk for CIS in daughters whose mothers exhibited CIS within 10 years after their daughter's conception compared to daughters whose mothers had CIS more than 10 years after their conception. This may conceivably be the result of maternal transmission of HPV during pregnancy or childhood, although further studies are necessary to substantiate this.

### ***HPV Vaccination in Sweden***

- Based on the apparent causal HPV types prevalent in Sweden over the last 30 year period, the introduction of vaccines against HPV-16 and HPV-18 is likely to greatly impact the incidence of cervical cancer in Sweden. However, the risks associated with other high-risk HPV types should not be discounted. Their inclusion in any future multi-valent vaccines will ensure further improvements in cervical cancer reduction.

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