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JOINT EFFECTS OF INFECTIONS WITH DIFFERENT TYPES OF HUMAN PAPILLOMAVIRUS ON RISK OF CERVICAL NEOPLASIA

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ACADEMIC DISSERTATION

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ABSTRACT

Cancer of the cervix uteri is the second most common cancer in women in the less developed countries and the eleventh in the more developed countries. Human papillomavirus (HPV) types 16 and 18 are the most prevalent and important risk factors for cervical cancer among the 12 oncogenic, high-risk (hr) HPV types, deemed to be carcinogenic in the cervix (IARC 2012a). Cervical infection with more than one hrHPV type is not uncommon, complicating assignment of (causal) carcinogenicity (Clifford *et al.* 2011). There are also non-oncogenic, low-risk (lr) HPV types, such as types 6 and 11, which cause benign lesions. Only a small proportion of women with cervical HPV infection(s) develop cervical cancer. Therefore, joint effects between different HPV types and putative co-factors in cervical carcinogenesis are of interest. For example, infection with *Chlamydia trachomatis* and tobacco smoking have been associated with an increased risk of cervical cancer also among hrHPV-positive women. Very little is, however, known about the interplay between these factors during cervical carcinogenesis.

The primary objectives of this work were 1) to identify joint effects of past infections with the hr and lrHPV types, 2) to identify joint effects of past infections with hrHPV types and *C. trachomatis* in the etiology of cervical cancer and 3) to reveal the chain(s) of events between the different HPV infections and the co-factors by investigating the order of solitary and joint effects of persistent and incident HPV and *C. trachomatis* infections on the risk of developing cervical cancer.

For the first two primary objectives, two case-control studies were nested within a joint cohort of three and four population-based Nordic biobanks to which 626 900 and 974 000 women donated serum or blood samples by the end of 1994 and 2002, respectively. In the first study with a shorter follow-up, (Study I), linkage to cancer registers identified 182 cases of invasive cervical carcinoma (ICC) with a donated serum sample before diagnosis. In Study II, the number of cases after re-examination of Study I histologies was 178; of these 148 were squamous cell carcinomas (SCCs). The material of the Study III with a longer follow-up comprised 604 ICC cases. Study III did *not* share any cases with Studies I and II. Three and five controls per case were randomly selected in Studies I and III, respectively. Material was restricted to women and matching was individual for age at serum sampling (±2 years), storage time of the first serum sample (±2 months), region and donor subgroup (participant in health examinations, blood donor).

For the third primary objective, a case-control study in a serial setting was nested within a cohort of women from the county of Västerbotten, who participated in a population-based, invitational cervical cancer screening programme in 1969–1995. In Study IV, 118 invasive cervical cancer cases were identified, and one control with two normal smears was age, and sampling-time matched to each case. In addition, a case-cohort study in the Finnish Maternity Cohort was based on women with a minimum of two pregnancies within 5 years (Study V). At the midpoint of the pregnancies, in 1995–2003, women were under 29 years of age, and were followed

on average for 4.8 years, from the second pregnancy sample until the end of 2004. Follow-up of 490 women ended at diagnosis of cervical high-grade precancer. For comparison, a subcohort of 2796 women was randomly selected by sampling from strata defined by age and calendar time.

IgG antibodies to capsids of HPV types 6, 11, 16, 18, 31, 33 and 45, *C. trachomatis* and herpes simplex virus type 2 were determined by ELISA methods. Serum cotinine, a marker for recent smoking, was measured by radioimmunoassay (Studies I and II) and by semiquantitative enzyme immunoassay (Study III). HPV DNA in the biopsy specimen of the cases was examined by polymerase chain reaction (PCR) in Studies II and III. HPV DNA-positive specimens were typed with E6 and E7 type-specific primers (Study II) and by enzyme immunoassay and reverse dot blot hybridization or multiplex fluorescent bead-based assay (Study III). All smears and biopsies for the cytological serial sample study (Study IV) were examined for HPV and *C. trachomatis* DNA by PCR.

Rate ratios and 95% confidence intervals were estimated by conditional logistic regression. In Study V, rate ratios and 95% confidence intervals were estimated fitting pseudolikelihood by Cox model with separate strata-specific baseline hazards. Misclassification of HPV serology was corrected for by back-calculus or by using as a gold standard the rate ratio for SCC related to HPV type 16 DNA positivity before diagnosis.

In the earlier seroepidemiological nested case-control study, we found no excess risk of cervical carcinoma among women seropositive for both HPV16 and non-oncogenic HPV types. In the later study, there was 2.4-fold excess risk, but the joint effect was again significantly smaller than the expected additive and multiplicative joint effects. Most importantly, we showed that if infection with major lrHPV type 6 preceded infection with hrHPV type 31, there was little excess risk of *in situ* cervical carcinoma; and in HPV18 DNA-positive ICCs, HPV16 seropositivity was associated with no excess risk. The antagonistic interaction, *i.e.* a joint effect smaller than expected, between different HPV types was probably due to a cell-mediated immune reaction (surveillance), of which the serum antibodies were a surrogate. The risk of ICC was highly increased not only among women whose first smear was HPV DNA-positive but also among *C. trachomatis* DNA-positive women. The risk was even higher among those, who were DNA positive (HPV or *C. trachomatis*) both at the start and end of follow-up. The risk of *in situ* cervical carcinoma was highly increased among women whose HPV18/45 and *C. trachomatis* infections were incident if not concomitant within a narrow time window. The risk of SCC was increased related to *C. trachomatis*, after adjusting for HPV, both in the total material and in the strata of HPV16 and HPV18 seronegatives and -positives.

C. trachomatis should not be ignored in the preventive efforts against cervical cancer. These studies support early HPV vaccination in cervical cancer prevention.

TIIVISTELMÄ

Kohdunkaulan syöpä on kehitysmaiden naisten toiseksi yleisin, ja kehittyneiden maiden naisten yhdenneksitoista yleisin syöpä. Kahdestatoista, syöpävaarallisesta, suuren riskin (hr) ihmisen papilloomaviruksen (HPV) tyypistä tyypit 16 ja 18 ovat tärkeimmät kohdunkaulan syövän riskitekijät. Kohdunkaulassa niitä pidetään karsinogeenisinä (IARC 2012a). Kohdunkaulan infektoituminen useammalla kuin yhdellä hrHPV-tyypillä ei ole harvinaista, ja voi tehdä tyyppikohtaisen kausaalisen karsinogeenisuuden määrittämisen vaikeaksi (Clifford *et al.* 2011). Tunnetaan myös pienen riskin (lr) HPV-tyyppejä, kuten HPV6 ja HPV11, jotka aiheuttavat hyvänlaatuisia kohdunkaulan limakalvovaurioita. Vain pienelle osalle naisista, joilla on kohdunkaulan HPV-infektio/infektioita, tulee kohdunkaulan syöpä. Tämän vuoksi yhteisvaikutukset eri HPV-tyyppien ja mahdollisten kohdunkaulan syövän lisäriskitekijöiden välillä ovat mielenkiintoisia. Esimerkiksi *Chlamydia trachomatis* -infektio ja tupakointi on liitetty lisääntyneeseen kohdunkaulan syövän riskiin, myös hrHPV-positiivisilla naisilla. Näiden tekijöiden yhteisvaikutuksista tiedetään kuitenkin hyvin vähän.

Tämän työn päätavoitteet olivat: 1) identifioida aiempien hrHPV- ja lrHPV-infektioiden yhteisvaikutukset, ja 2) identifioida aempien hrHPV- ja *C. trachomatis* -infektioiden yhteisvaikutukset kohdunkaulan syövän synnyssä sekä 3) selvittää eri HPV-infektioille ja lisäriskitekijöille altistumisen järjestyksen merkitystä kohdunkaulan syövän kehittymisessä tutkimalla persistoiviin ja insidentteihin HPV- ja *C. trachomatis* -infektioihin liittyviä, näiden tekijöiden omia ja yhteisvaikutuksena syntyviä riskejä.

Kahta ensimmäistä päätavoitetta varten käytettiin kahta tapaus-verrokkiaineistoa. Ne olivat peräisin kolmesta ja neljästä väestöpohjaisesta, pohjoismaisesta biopankista, joihin 626 900 ja 974 000 naista oli antanut seerumi- tai verinäytteen vuosien 1992 ja 2002 loppuun mennessä. Edellisessä tutkimuksessa, osajulkaisussa I, jossa oli lyhyempi seuranta-aika, syöpärekistereihin yhdistämisissä löydettiin 182 invasiivista kohdunkaulan syöpätapausta (ICC), jotka olivat antaneet näytteen ennen diagnoosia. Osajulkaisussa II tapausten määrä oli histologisen uudelleenluokittelun jälkeen 178, joista 148 oli kohdunkaulan levyepiteelisyöpiä (SCC). Jälkimmäinen tutkimus, osajulkaisu III, sisälsi 604 ICC-tapausta, jotka eivät kuuluneet edelliseen tutkimukseen. Tapausta kohti valittiin satunnaisesti kolme ja jälkimmäisessä tutkimuksessa viisi verrokkia, jotka kaltaistettiin tapauksen sukupuolen, näytteenottohetken iän (+/-2 vuotta), näytteen säilytysajan (+/- 2 kuukautta), alueen ja osapopulaation (verenluovuttajat, terveystutkimukseen osallistuneet) suhteen.

Kolmatta päätavoitetta varten oli käytettävissä Västerbottenin läänin kohorttiin upotettu tapaus-verrokkiaineisto, jossa oli käytössä perättäisiä näytteitä kohdunkaulan syövän seulontaan vuosina 1969–1995 osallistuneista naisista. Osajulkaisussa IV oli 118 invasiivista kohdunkaulan syöpätapausta, joista jokaiselle oli käytettävissä näytteidenottoikien ja näytteidenottoajankohtien suhteen kaltaistettu verrokki. Lisäksi osajulkaisun V tapaus-kohorttitutkimus perustui Äitiysneuvola-

seerumipankkiin (FMC) näytteen antaneisiin naisiin, joilla oli ollut kaksi raskautta alle 29-vuotiaina, ja joita oli seurattu keskimäärin 4,8 vuotta toisen raskausnäytteenoton jälkeen aina vuoteen 2004 asti. Seuranta päättyi 490 naisella kohdunkaulan syövän pisimmälle kehittyneen esiasteen diagnoosiin. Mainitut ehdot täyttäneistä naisista poimittiin satunnaisesti 2 796 naisen alikohortti, joka oli ositettu iän ja näytteenoton kalenteriajankohdan suhteen.

IgG-vasta-aineet HPV-tyypeille 6, 11, 16, 18, 31, 33 ja 45, sekä klamydialle että herpes simplex -virus tyyppi 2:lle määritettiin ELISA-menetelmillä. Äskettäiseen tupakointiin viittaava merkkiaine, seerumin kotiniini, määritettiin radioimmunoanalyysilla osajulkaisuissa I ja II ja semikvantitatiivisella entsyymi-immunoanalyysilla osajulkaisussa III. HPV DNA osoitettiin biopsianäytteistä polymeraasiketjureaktiolla (PCR) osajulkaisuissa II ja III. HPV DNA -positiiviset löydökset tyypitettiin tyyppispesifisillä E6- ja E7-alukkeilla osajulkaisussa II, ja multiplex-menetelmällä osajulkaisussa III. Kaikki osajulkaisun IV sytologiset ja biopsianäytteet tutkittiin HPV ja *C. trachomatis* -DNA:n suhteen PCR-menetelmällä.

Tiheyssuhteet ja niiden 95 %:n luottamusvälit estimoitiin ehdollisella logistisella regressiolla. Osajulkaisussa V tiheyssuhteet ja niiden 95 %:n luottamusvälit estimoitiin Coxin regressiomallilla käyttämällä ositekohtaisia riskitiheyksiä. HPV-serologian virheluokitusta korjattiin käyttämällä kultastandardina SCC-diagnoosia edeltävän HPV16 DNA -positiivisuuden tiheyssuhdearviota.

Aikaisemmassa seroepidemiologisessa, upotetussa tapaus-verrokkitutkimuksessa ei havaittu kohdunkaulan syövän lisäriskiä naisilla, joilla oli vasta-aineita sekä HPV16:lle että ei-syöpävaarallisille HPV-tyypeille. Myöhemmässä tutkimuksessa riski oli 2,4-kertainen, mutta yhteisvaikutus oli edelleen merkitsevästi pienempi kuin mitä oli odotettavissa additiivisen tai multiplikatiivisen yhteisvaikutuksen perusteella. Suuren riskin HPV31-infektioon liittyvä kohdunkaulan syövän pisimmälle kehittyneen esiasteen riski lähes katosi, jos nainen oli aikaisemmin sairastanut HPV6-infektion. Myöskään HPV18 DNA -positiivisilla ICC-tapauksilla (aikaisempi) HPV16-seropositiivisuuteen ei liittynyt lisäriskiä. Antagonistiset, odotettua pienemmät eri HPV-tyyppien yhteisvaikutukset luultavasti selittyvät soluvälitteisellä immuniteetilla, jonka surrogaattina seerumin vasta-aineet toimivat.

Naisilla, joilla oli HPV DNA -positiivinen tai *C. trachomatis* DNA -positiivinen sytologinen löydös, oli suuresti kohonnut ICC:n riski. Riski oli vielä suurempi naisilla, jotka olivat joko HPV tai *C. trachomatis* DNA -positiivisia sekä seurannan alussa että lopussa. Kohdunkaulan syövän pisimmälle kehittyneen esiasteen riski oli merkittävästi koholla naisilla, joilla oli joko yhtäaikaiset tai lähellä toisiaan tapahtuneet HPV18/45- ja *C. trachomatis* -infektiot. *C. trachomatis* -infektioon liittyi lisääntynyt levyepiteelisyövän riski HPV-vakioinnista huolimatta niin HPV16/18-seronegatiivisten kuin -seropositiivisten ositteissa.

Klamydiaa ei saisi unohtaa kohdunkaulan syövän ehkäisyssä. Tutkimustulokset tukevat HPV-rokotuksia nuorella iällä.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals.

I Luostarinen T, af Geijersstam V, Bjørge T, Eklund C, Hakama M, Hakulinen T, Jellum E, Koskela P, Paavonen J, Pukkala E, Schiller JT, Thoresen S, Youngman LD, Dillner J, Lehtinen M. No excess risk of cervical carcinoma among women seropositive for both HPV16 and HPV6/11. Int J Cancer 1999;80:818–22. Cit[*]:28, IF[#]:5.007.

II Luostarinen T, Lehtinen M, Bjørge T, Abeler V, Hakama M, Hallmans G, Jellum E, Koskela P, Lenner P, Lie AK, Paavonen J, Pukkala E, Saikku P, Sigstad E, Youngman LD, Dillner J, Hakulinen T. Joint effects of different HPV and *Chlamydia trachomatis* infections on risk of squamous cell carcinoma of the cervix uteri. Eur J Cancer 2004;40:1058–65. Cit[*]:12, IF[#]:4.819.

III Arnheim Dahlström L, Andersson K, Luostarinen T, Thoresen S, Ögmundsdottír H, Tryggvadottír L, Wiklund F, Skare GB, Eklund C, Sjölin K, Jellum E, Koskela P, Wadell G, Lehtinen M, Dillner J. Prospective seroepidemiologic study of human papillomavirus and other risk factors in cervical cancer. Cancer Epidemiol Biomarkers Prev. 2011;20:2541–50. Cit[*]:9, IF[#]: 4.324.

IV Wallin KL, Wiklund F, Luostarinen T, Ångström T, Anttila T, Bergman F, Hallmans G, Ikäheimo I, Koskela P, Lehtinen M, Stendahl U, Paavonen J, Dillner J. A population-based prospective study of *Chlamydia trachomatis* infection and cervical carcinoma. Int J Cancer 2002;101:371–4. Cit[*]:106, IF[#]: 5.007.

V Luostarinen T, Namujju PB, Merikukka M, Dillner J, Hakulinen T, Koskela P, Paavonen J, Surcel HM, Lehtinen M. Order of HPV/*Chlamydia* infections and cervical high-grade precancer risk: a case-cohort study. Int J Cancer 2013;133:1756–9. Cit[*]:3, IF[#]:5.007.

[*]No. of citations (self-citations of all authors excluded) by November 5, 2014. Source: SCOPUS.

[#]Journal impact factor in 2013. Source: ISI Web of Knowledge[SM], Journal Citation Reports®.

Publication I was published in the thesis by Veronika af Geijersstam, the Karolinska Institute in 2000. Manuscripts with preliminary material for Publication III were published in the theses by Lisen Arnheim, Karolinska Institute, in 2005 and Kristin Andersson, Lund University, in 2010.

ABBREVIATIONS

C. trachomatis	*Chlamydia trachomatis*
CD4+ level	CD4+ T cell count/mm^3 of blood or T helper cell count/mm^3 of blood
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIN 2	Cervical intraepithelial neoplasia grade 2: moderate dysplasia
CIN 3	Cervical intraepithelial neoplasia grade 3: severe dysplasia or carcinoma *in situ*
CIS	Carcinoma *in situ*
ELISA	Enzyme-linked immunosorbent assay
GP5+/6+	General primer 5+/6(+)-mediated polymerase chain reaction assay
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hrHPV	High-risk human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
HSV-2	Herpes simplex virus type 2
IARC	International Agency for Research on Cancer
ICC	Invasive cervical carcinoma
ICD	International Classification of Diseases
ICD-O	International Classification of Diseases for Oncology
IgG	Immunoglobulin G
lrHPV	Low-risk human papillomavirus
MONICA	Multinational Monitoring of Trends and Determinants in Cardiovascular Disease
NBSBCCC	Nordic Biological Specimen Banks working group on Cancer Causes and Control
OC	Oral contraceptive
OR	Odds ratio
PCR	Polymerase chain reaction
RR	Rate ratio
SCC	Squamous cell carcinoma
SIR	Standardized incidence ratio
STI	Sexually transmitted infection
VIP	Västerbotten Intervention Programme
VLP	Virus-like particle

1 INTRODUCTION

Cancer of the uterine cervix is the second most common cancer in the less developed countries and the eleventh in the more developed countries (Ferlay *et al.* 2013). The aetiology of invasive cervical carcinoma is better known than that of cancers in most of the other anatomical sites. Sufficient evidence is reported to exist for the carcinogenicity of 12 human papillomavirus (HPV) types, most notably types 16 and 18, in the cervix uteri (IARC 2012a). These high-risk (hr) HPV types are sexually transmitted. Persistence of at least one hrHPV type in the cervix is considered to be a prerequisite for the progression of transformed cells to precancer, and finally, invasion.

Low-risk (lr) HPV types, 6 and 11, cause benign genital lesions. Oncogenic HPV infections are more likely to persist in women with a previous *C. trachomatis* infection (Samoff *et al.* 2005, Silins *et al.* 2005), which is the most common sexually transmitted bacterial infection and apparently is independently associated with cervical neoplasia (Lehtinen *et al.* 2011). Smoking has been associated with an increased risk of cervical squamous cell carcinoma, the most common morphological type of invasive cervical carcinoma, among hrHPV-positive women (International Collaboration of Epidemiological Studies of Cervical Cancer 2006a) and hrHPV seronegative and -positive women (Kapeu *et al.* 2009).

Cervical co-infection with at least two HPV types and subsequent acquisition of a new HPV type is quite common among HPV-infected women (Thomas *et al.* 2000, Clifford *et al.* 2005, Mendéz *et al.* 2005). However, the joint effects of HPV types, interactions between high- and low-risk HPV types and co-factors, such as *Chlamydia trachomatis* and smoking, on the risk of invasive cervical carcinoma are not well known.

2 REVIEW OF THE LITERATURE

2.1 DESCRIPTIVE EPIDEMIOLOGY OF CERVICAL NEOPLASIA

2.1.1 WORLDWIDE

Cervical cancer is the most common cancer of women in Eastern and Middle Africa (Ferlay *et al.* 2013, 2014). An estimated 445 000 new cases of cervical cancer occurred in 2012 in the less developed countries (*i.e.*, world excluding Europe, Northern America, Japan, Australia and New Zealand). Cervical cancer accounts for 12% of all cancers (non-melanoma skin cancer excluded) in women in the less developed countries. Only cancer of the breast is more common.

The burden of cervical cancer is higher in less developed than more developed countries. Of the new cervical cancer cases worldwide, 84% were from the less developed countries in 2012, whereas 57% of all new female cancer cases were from the less developed countries. In the more developed countries, cervical cancer accounts for 2.9% of all the female cancers, and cervix uteri is the eleventh among the primary sites of cancer.

The highest cervical cancer risk areas in terms of age-adjusted (world standard population) incidence rate are Eastern, Southern and Middle Africa and Melanesia. The age-adjusted incidence rate exceeds 30 per 100 000 woman-years in these areas, whereas in the low-incidence areas, Western and Eastern Asia, Australia, New Zealand, Northern America, Northern Africa, Northern, Southern and Western Europe and Micronesia, it is below 10 per 100 000 woman-years. The extremes on the country level are even more outstanding, 2.0 per 100 000 woman-years in the State of Palestine and 76 in Malawi. The cumulative risk of developing cervical cancer before age 75 is 1.4% worldwide, 1.6% in the less developed countries and 0.9% in the more developed countries. On the country level, the cumulative risk ranges from 0.2% to 7.4%.

Cervical cancer is the second most common cancer of women in the age group of 15–44 years, in both the less and more developed countries. The age-specific incidence rate is lower among women aged 15–39 years in the less developed countries, 7.9 per 100 000 woman-years, than in the more developed countries, 9.7, but higher in the older age groups. In the less developed countries, the incidence rate estimate is almost constant, 44–46 per 100 000 woman-years, among 50- to 69-year-old women, declining thereafter. In the more developed countries, the incidence rate is almost constant, 21–22 per 100 000 woman-years, among 40- to 59-year-old women, declining thereafter. Before screening in the more developed countries, the incidence rates increased steeply until middle age, typically attaining a peak of at least 50 per 100 000 woman-years and decreasing with age (Gustafsson *et al.* 1997).

The incidence rates of cervical cancer decreased during 1973–1987 in most of these countries for which cancer incidence data for that period are available (Coleman *et al.* 1993). Cervix uteri ceded its place as the leading cancer site of women in the less developed countries to the breast between the years 1985 and 1990 (Parkin *et al.* 1999). The estimate for age-adjusted incidence rate decreased in the less developed countries from 18.2 per 100 000 woman-years in 1990 to 15.7 per 100 000 woman-years in 2012, and in the more developed countries from 11.2 per 100 000 woman-years in 1990 to 9.0 in 2008, but increased to 9.9 in 2012 (Parkin *et al.* 1999, Ferlay *et al.* 2010, 2013). The trend in age-adjusted incidence rate during the two most recent five-year periods was decreasing in several countries, except in the Netherlands, many Eastern European countries, Thailand and Uganda (Vaccarella *et al.* 2013a). In North Europe, the recent trend was less favourable than that over several decades.

The number of new cervical cancer cases in the less developed countries is predicted to reach 479 000 in 2015 and 533 000 in 2020 (Ferlay *et al.* 2013). The burden of cervical cancer is predicted to increase also in the more developed countries, 85 000 in 2015 and 86 000 in 2020, although the number of new cases among women younger than 65 years is expected to decrease between the years 2015 and 2020.

Squamous cell carcinoma is the predominant histological type of cervical cancer, representing about 75% of the new cases (Vizcaino *et al.* 2000). The incidence rates of squamous cell carcinoma were, with few exceptions, decreasing in the more developed countries and, to some extent, also in a few less developed countries with time series data during 1973–1991. In several European countries, increasing cohort trends have been observed in generations born after 1930 (Bray *et al.* 2005a). Adenocarcinoma and adenosquamous cell carcinoma account for 10–15% of cervical cancer cases (Vizcaino *et al.* 1998). There was an upward trend in the incidence of adeno- and adenosquamous cell carcinoma among 25- to 49-year-old women during 1973–1991 in several countries. A significant decrease in incidence was recorded only in three countries among 25- to 74-year-old women. In 12 out of 13 European countries, the trend in incidence of cervical adenocarcinoma for the period 1983–1997 was estimated to have been on the rise (Bray *et al.* 2005b). The increases in risk of adenocarcinoma were typically seen in generations born since the 1940s. The upward trends may partly be explained by parallel declines in the incidence rates of unspecified cervical cancer/carcinoma, but also by changes in distribution and prevalence of aetiological factors as well as the inability of cytological cervical cancer screening to reduce the incidence of adenocarcinoma (Bray *et al.* 2005b).

Cervical cancer is the third most common cause of cancer death among women in the less developed countries, comprising a tenth of cancer deaths (Ferlay *et al.* 2013). In the more developed countries, cervical cancer is the ninth most common cause of cancer death, causing 2.8% of cancer deaths among women. Cervical cancer was the main contributor to the overall burden of age-adjusted years of life lost because of cancer among women in 54 countries, including India, Sub-Saharan Africa, Oceania (excluding Australia and New Zealand) and many Latin American countries, in 2008 (Soerjomataram *et al.* 2012). Cervical cancer made the largest

contribution mostly in the same countries to disability-adjusted life-years from cancer, sum of years of life lost because of premature cancer mortality and years lived with disability due to cancer.

2.1.2 IN FINLAND, ICELAND, NORWAY AND SWEDEN

The respective incidences of immediate precursors of invasive cervical cancer, cervical intraepithelial neoplasia (CIN) grade 3 and adenocarcinoma *in situ* (AIS), were highest, 135 and 5 per 100 000 woman-years, among 30- to 34-year-old Finnish women during 2004–2008 (Salo *et al.* 2013). The corresponding age-adjusted incidence rates were 1.3 and 39.1.

The incidence of invasive cervical cancer has decreased markedly in Finland from the late 1960s until the early 1990s mainly due to the successfully organized mass screening programme (Anttila *et al.* 1999, Nieminen *et al.* 1999, Engholm *et al.* 2013). According to the Finnish Cancer Registry files, only 129 new cervical cancer cases were diagnosed in 1991, corresponding to an age-adjusted incidence of 2.8 per 100 000 woman-years. This incidence increased in two years to 4 and has since remained at this level. The mean annual number of new cervical cancer cases was 151 during 2007–2011, accounting for 1.2% of all (except skin, non-melanoma) female cancer cases (Engholm *et al.* 2010, 2013). The cumulative risk of developing cervical cancer before age 85 was 0.5%, based on incidence rates of 2007–2011. The age-specific rates among women <70 were less than 10 during 1998–2002, even if corrected for cervix-at-risk using the coefficients derived from 1995–1999 by Luoto *et al.* (2004).

The age pattern of cervical cancer incidence was most beneficial for women born in the forties (Hakulinen 2004). In Finland, the cohort effects on the relative risk of ICC were estimated to be the smallest among women born in that decade (Vaccarella *et al.* 2013a). For women born since the late 1940s, the incidence has increased more steeply with age and year of birth (Anttila *et al.* 1999, Hakulinen 2004, Laukkanen *et al.* 2012). During the last decade, among women in the age range of 30–34 years, which is within the targeted age range (30–60 years) of the screening programme, the cervical cancer incidence has been at the same level as in the same age range prior to the onset of the screening programme in 1963. Furthermore, during 2007–2011, the age-specific incidence rates were higher than 10 per 100 000 woman-years among 35- to 44-year-old women. The adverse changes in incidence have been suggested to be attributable to changes in sexual behaviour, screening attendance and quality of screening (Anttila *et al.* 1999, Nieminen *et al.* 2002). Laukkanen *et al.* (2003) showed that an increase in HPV16 incidence without increase in HPV6/11 incidence among women in their twenties during 1983–1997 preceded the increase of ICC incidence in Finland.

The proportion of squamous cell carcinomas was 76% prior to screening in 1958–1962 and 62% in 2000–2009 (Lönnberg *et al.* 2012a). From 1955 to 1999, the incidence of cervical squamous cell carcinoma declined by 4.7% per year, on average (Bray *et al.* 2005a). In the nineties, the incidence increased by 8% per year.

The proportion of adenocarcinomas was only 6% prior to screening and 29% in 2000–2009. The incidence rate of cervical adenocarcinomas decreased 2% per year among women aged 50–74 years during 1973–1991 (Vizcaino *et al.* 1998). Overall, the age-adjusted incidence of cervical adenocarcinoma has been fairly stable, and increasing steeply only in the nineties (Anttila *et al.* 1999). The annual increase in incidence of cervical adenocarcinoma was 2.6% during 1983–1997 among women aged less than 75 years (Bray *et al.* 2005b). The upward trend was seen among women born since 1945.

In Iceland, a screening programme for cervical cancer was launched in 1964 and became nationwide in 1969 (Sigurdsson and Sigvaldason 2006). The age-adjusted incidence decreased steeply until the late seventies, reaching 9 per 100 000 woman-years, climbed to a new peak in the early 1980s, returned to 9 per 100 000 woman-years during the latter half of the eighties and has since plateaued (Engholm *et al.* 2013). The mean annual number of new cases was 16 during 2007–2011, accounting for 2.5% of female cancer cases. The cumulative risk of developing cervical cancer before age 85 was 0.9% based on incidence rates of 2007–2011 (Engholm *et al.* 2013). While the incidence rate of squamous cell carcinoma in 1964–1969 was 3.5 times higher than in 1992–2002, that of adenocarcinoma was 2.4 times lower (Sigurdsson and Sigvaldason 2006).

In Sweden, the age-adjusted incidence of cervical cancer was fairly stable at a level exceeding 15 per 100 000 woman-years until many counties started screening in the late 1960s, which was followed by a downward trend to reach a constant incidence of 7 since 1998 (Engeland *et al.* 1993, Dillner 2000a, Anttila *et al.* 2004, Engholm *et al.* 2013). In Finland, the age-adjusted incidence has been below 7 since the late 1970s.

The age-specific incidence rates were less than 20 per 100 000 woman-years for all age groups during 2007–2011 (Engholm *et al.* 2013). The mean annual number of new cervical cancers, 446, diagnosed during 2007–2011, accounted for 2.0% of female cancer cases. The cumulative risk of developing cervical cancer before age 85 was 0.8% based on incidence rates for 2007–2011.

The age-adjusted incidence of squamous cell carcinoma was stable during 1958–1967 and decreased by 3.7% per year during 1968–1995 (Bergström *et al.* 1999). The decline was modest, 0.8% per year, in the nineties (Bray *et al.* 2005a). Adenocarcinomas accounted for 5% of the cervical cancers in 1958 and for 19% in 1995 (Bergström *et al.* 1999). The age-adjusted incidence of adenocarcinoma increased annually by 1.8% from 1958 to 1995. The annual increase in incidence was only 0.4% during 1983–1997 among women aged less than 75 years (Bray *et al.* 2005b).

In Norway, the age-adjusted incidence rate peaked in the middle of the seventies and decreased, excluding the early nineties, until the early 2000s (Engholm *et al.* 2013). The age-adjusted incidence rate has been since 1999 at 9 or 10, and in Sweden below this figure since 1983. The cervical cancer screening programme was introduced in Norway in 1995 (Nygård *et al.* 2002).

The age-specific incidence rates were higher than 15 in most age groups during 2007–2011 (Engholm *et al.* 2013). The mean annual number of new cervical

cancers, 299, diagnosed during 2007–2011, accounted for 2.5% of female cancer cases. The cumulative risk of developing cervical cancer before age 85 was 1.0% based on incidence rates for the period 2007–2011.

Squamous cell carcinomas accounted for 90% of cervical cancer during 1971–1975 and for 81% during 1986–1990 (Bjørge *et al.* 1993). The incidence rate decreased 1.1% annually from 1953 to 1997 and 1.2% from 1988 to 1997 (Bray *et al.* 2005a). Adenocarcinomas accounted for 5% of the cervical cancers in 1971–1975 and for 12% in 1986–1990 (Bjørge *et al.* 1993). The age-adjusted (European standard) incidence rate of cervical adenocarcinoma decreased from the peak of the early sixties until the end of the seventies and reached a second higher peak during the eighties (Bjørge *et al.* 1993). The annual increase in incidence was 1.1% during 1983–1997 among women aged less than 75 (Bray *et al.* 2005b). The upward trend was seen among women born from 1930 onwards.

Brustugun *et al.* (2014) estimated that the average number of years of life lost due to cervical cancer was 23.7 years in Norway in 2012. The loss estimate was smaller for all other cancer types.

2.2 AETIOLOGY OF CERVICAL CANCER

2.2.1 HUMAN PAPILLOMAVIRUS (HPV) INFECTION

Human papillomavirus (HPV) is a small (8000 base pairs), unenveloped virus with double-stranded DNA. Each HPV type has less than 90% sequence similarity with other HPV types (Ylitalo *et al.* 2008). More than 50 distinct HPV types are known to infect the genital mucosa (Schmitt *et al.* 2013). Most genital HPV infections are transient, self-limited or cleared completely by the cell-mediated immune system (Lowy and Howley 2001). Persistent infections are of long duration. Wide variation exists in the definitions of persistence. According to the first summary of studies on persistent genital HPV infection among female populations of average risk, the median duration for HPV infections with types 16, 31 and 33 was longer than a year and with the rest of the HPV types less than a year (Rositch *et al.* 2013).

The high-risk genital HPV types, *e.g.* 16 and 18, are sexually transmitted and highly transmissible (Dillner *et al.* 1996, Barnabas *et al.* 2006, Burchell *et al.* 2006). As a group, genital HPVs represent the most common sexually transmitted infections (STIs) (Schiffman and Kjær 2003). The prevalence of HPV in cytologically normal women has ranged from 1.5% (before first intercourse) to 44% (sexually active young) (Winer and Koutsky 2004). In a worldwide study, age-standardized HPV prevalence varied greatly, from 1.4% in Spain to 26% in Nigeria (Clifford *et al.* 2005). There was heterogeneity in HPV type distribution between continents and within Asia between countries, but HPV16 was usually the most common hrHPV type. While HPV prevalence peaks at a younger age, in some populations a second peak is observed in older age groups, possibly explained by greater HPV persistence

(Castle *et al.* 2005). In Eastern Africa, up to one-half of women aged 25–34 years were HPV-positive (Bruni *et al.* 2010). New sexual partnership within the previous two years, life-time number of partners and husband's extramarital relationships were associated with increased risk of being HPV-positive (Deacon *et al.* 2000, Vaccarella *et al.* 2006).

In a longitudinal study, cumulative risk of acquiring any HPV infection was 44% at 3 years and 60% at 5 years among initially HPV-negative, sexually active young women (Woodman *et al.* 2001). Winer *et al.* (2003) reported comparable cumulative incidences. It has been estimated that the median time from first intercourse to first detection of HPV is three months (Collins *et al.* 2002).

Low-risk HPV types, *e.g.* 6 and 11, can cause not only anogenital warts but also lesions up to CIN grade 2 (moderate dysplasia, CIN 2). About 90% of the anogenital warts are attributable to types 6 and 11. High-risk HPV types can cause cervical precancer, *i.e.* CIN 3 (severe dysplasia or carcinoma *in situ*) and invasive cervical carcinoma (ICC). Most HPV infections clear and lesions regress spontaneously. Molano *et al.* (2003) reported that HPV16 and its phylogenetic relatives, alphapapillomavirus types of clade 9, showed lower clearance rates than lrHPV types. The rate of regression decreases by severity of lesion (Myers *et al.* 2000). In a study on sexually active young women, 7% of those with incident HPV16 or HPV18 infection were diagnosed with CIN 3 during a follow-up of three years (Winer *et al.* 2005). The median incubation period between HPV16 infection and diagnosis of cervical carcinoma *in situ* (CIS) is estimated to be 7–12 years (Ylitalo *et al.* 2000a). Progression from untreated CIN 3 to ICC is highly probable. One-fifth and almost one-third of women with minimally disturbed CIN 3 lesions were diagnosed with cancer of the cervix or vaginal vault within 10 and 30 years, respectively, after CIN 3 diagnosis (McCredie *et al.* 2008). The cumulative incidences were even higher, 31% and 50%, if CIN 3 was known to persist.

Unlike other virus families, papillomavirus infection requires availability of basal layer cells, mucosal or epidermal, able to proliferate (zur Hausen 2002). Three early genes of the hrHPV genome, E5, E6 and E7, have a proliferation-stimulating activity. E5 stimulates cell growth in the early course of infection, complexing with cellular growth receptors, and prevents apoptosis following DNA damage. Expression of E5 is usually terminated by integration of the viral genome into the host chromosome. Once the hrHPV genome is integrated, the viral E2 gene controlling for transcription of E6 and E7 is also usually disrupted or lost (Ylitalo *et al.* 2008), which may contribute to the cells not dying from productive infection (directed by an intact E2 gene). E6 and E7 are crucial for cervical carcinogenesis and are always present in HPV-associated cervical tumour samples (Boccardo *et al.* 2010, Franco *et al.* 2004). E7 protein is able to immortalize human keratinocytes, but the combination of E6 and E7 is highly efficient at immortalizing human cells (Moody and Laimins 2010). E6 protein binds to p53 protein, which has an important role in cell-cycle control. The degradation of p53 leads to loss of DNA repair function, and the host cell is prevented from undergoing apoptosis. E6 also activates telomerase expression and activity and can bind many other proteins independently of p53 inactivation. The main transforming protein, E7, induces uncontrolled cell

proliferation by binding to the retinoblastoma protein, which participates in cell-cycle regulation. E7 also promotes a calpain-driven cleavage of the retinoblastoma protein (Yugawa and Kiyono 2009). E7 binds to several other cell-cycle regulators. E6 and E7 enable the infected cell to re-enter the replicative S phase of the cell cycle. The biological properties of the HPV types agree well with their epidemiological classification into high- and low-risk types (Muñoz *et al.* 2003). The low-risk HPV types are not able to immortalize cells (Lowy and Howley 2001). E6 and E7 of lrHPV types have no or very low transforming activity *in vitro* (Villa 2006a).

No single gold standard HPV test exists (Gravitt and Viscidi 2004). The modern assays for measurement of HPV DNA, such as polymerase chain reaction (PCR), are of high sensitivity and specificity. Contamination is a more probable reason for false positivity than the assay itself (Eklund *et al.* 2012a). The quantity of HPV DNA (viral load) can be determined with real-time PCR assays (Josefsson *et al.* 2000); the currently used assays measure HPV types 11, 16 and 18 at the smallest sample volume (Eklund *et al.* 2012a). A commonly used PCR assay, general primer-mediated 5+/6+ (GP5+/6+) (de Roda Husman *et al.* 1995), has poor sensitivity for detection of HPV52 (Chan *et al.* 2006). Another common PCR assay, MY09/11 (Manos *et al.* 1989), substantially underdetects some genotypes (Gravitt and Viscidi 2004). Furthermore, both of these assays are limited in their capacity to genotype HPV types 18, 31, 51, 52 and 58 if the specimen contains HPV type 16 (Mori *et al.* 2011). Incorrect detection and typing of multiple HPV types are continuing problems that introduce a systematic detection bias. Formalin-fixed paraffin-embedded tissues are suitable for amplification by PCR (Brink *et al.* 2007).

Another method for HPV detection is to amplify the chemiluminescent signal instead of the target DNA. Hybrid capture 2 (Qiagen, Gaithersburg, MD) is the most widely used commercially available signal amplification assay for clinical purposes (Clavel *et al.* 1998). It was approved by the U.S. Food and Drug Administration in 2003 and validated clinically. It detects 13 hrHPV types, but cannot determine multiple infections (Gravitt and Viscidi 2004). It has lower analytical sensitivity than PCR assays (Snijders *et al.* 2003). FDA approval does not include use on formalin-fixed paraffin-embedded tissues. As the assay detects hrHPVs only, it has been used in screening for HPV. In Finland, the assay was used in a randomized trial within an organized cervical cancer screening programme (Leinonen *et al.* 2013).

Serology is a useful tool for defining past infection with HPV. Zhou *et al.* (1991) introduced HPV virus-like particles (VLPs), which are essential for HPV serology and development of cervical cancer vaccines (Reynolds and Tansey 2009). The HPV16 seroprevalence has ranged from 2% to 43% in a series of cancer-free control women (Winer and Koutsky 2004). In an HPV serology proficiency study, 6/10 laboratories met the criteria of at least 50% sensitivity and 100% specificity relative to a standardized, HPV16 VLP-based enzyme-linked immunosorbent assay (ELISA) (Eklund *et al.* 2012b). The sensitivity to detect an incident HPV16 infection by serological HPV16 VLP enzyme immunoassays is approximately 65% (Kjellberg *et al.* 1999, Carter *et al.* 2000, Gravitt and Viscidi 2004). The VLP assays of other HPV types probably have a sensitivity comparable with the HPV16 VLP assay,

except for HPV18, with a reported sensitivity within the range of 35–55%, although Kjellberg *et al.* (1999) have reported a similar sensitivity to detect HPV16 and HPV18 antibodies by the standard VLP assay. In unvaccinated populations, reactivity to multiple HPV VLP types is, however, more likely due to cumulative exposure to multiple HPV types than to serological cross-reactivity between types. Availability of high-quality VLPs has been a problem for laboratories worldwide (Eklund *et al.* 2012b). Neutralization assays using HPV pseudovirions are considered the gold standard for HPV serology (Eklund *et al.* 2012b). While HPV virions are type-specific in inducing antibodies in natural infection, artificially made VLPs most likely are not (Dubin *et al.* 2005). Especially, in large-scale studies ELISA is preferred to complex neutralization assays. Interlaboratory agreement is not good with sera from vaccinees (Ferguson *et al.* 2006). Moreover, the correlation between ELISA serology and GP5+/6+ PCR is not very good at an individual level, but is high at a population level (Vaccarella *et al.* 2010a).

Infection with multiple HPV types as well as sequential infection with new HPV types seem to be common (Winer and Koutsky 2004). In a worldwide study, 9% of cytologically normal women were HPV-positive, 27% of whom had at least two HPV types (Clifford *et al.* 2005). In a meta-analysis of one million cytologically normal women, the crude and regionally adjusted HPV prevalences were 7.2% and 11.7%, respectively (Bruni *et al.* 2010). One-fifth of HPV-positive women had infections with multiple HPV types.

In a study on pregnant Finnish women, baseline HPV16-seropositive women were at threefold higher risk of later infections with HPV18 than baseline HPV11/16/31-seronegative women (Kaasila *et al.* 2009). *Vice versa*, baseline HPV18-seropositive women were at increased risk of later infection with HPV16. The youngest women, ≤20 years of age at first pregnancy, who were HPV18-seropositive at baseline had a highly increased risk of later HPV11 infection. Rousseau *et al.* (2003) reported that the cumulative probability of acquisition of multiple (PCR-detected) HPV infections was higher for younger women (18–24 years) than for women in older age groups. Concurrent acquisition of multiple HPV types or clustering of incident infections with multiple HPV types, of both low- and high-risk types, occurred more often than would be expected by chance (Thomas *et al.* 2000, Mendéz *et al.* 2005). The odds ratios (ORs) of incident, concurrent infections ranged from 3.3 to 25 (Mendéz *et al.* 2005). Vaccarella *et al.* (2010b) suggested that the excess of multiple infections was an artifact of enzyme immunoassay genotyping. The excess of multiple infections was not evident when reverse line blot was used as a genotyping method. Carozzi *et al.* (2012) reported that the observed-to-expected ratio for infections with multiple HPV types was 1.21 (95% credible interval, 1.13–1.30), but no evidence emerged for specific HPV types occurring in co-infections more or less often than expected at a significance level 0.01. In a study of Swedish subjects, the HPV type combination 18 and 6 was found more often than expected (Vaccarella *et al.* 2013b).

In a study by Thomas *et al.* (2000), the risk of acquiring HPV6 infection was increased when it was subsequent to infection with HPV45, and *vice versa*. The risk of HPV18 infection was increased when it was subsequent to HPV6 or HPV11

according to study by Mendéz *et al.* (2005). Non-significant relations comprised HPV18 prior to HPV33 and HPV45, and HPV31 prior to HPV6. Thomas *et al.* (2000) reported that the risk of acquiring a new HPV type was not decreased among women with a prior HPV infection. Liaw *et al.* (2001) reported that the risk for acquisition of HPV types 6/11, 18 and 45 was increased among those who were HPV16 DNA-positive at enrolment. Rousseau *et al.* (2003) reported that HPV16 and HPV18 co-occurred with other oncogenic HPV types and with HPV6/11 less frequently than expected. Chaturvedi *et al.* (2005) found that HPV16 and its phylogenetic relatives, alphapapillomavirus types of clade 9, were less likely involved in multiple HPV infections than alphapapillomavirus types of other clades. Mejlhede *et al.* (2010) reported that all odds ratios for co-infection with HPV16 and each of 24 HPV types were ≤1. HPV16 did not affect the persistence of concomitant infections (Liaw *et al.* 2001). Persistence of HPV was independent of co-infection with other HPV types (Rousseau *et al.* 2001). By contrast, Ho *et al.* (1998) and Samoff *et al.* (2005) reported that infection with multiple HPV types was associated with increased risk of HPV persistence. Clearance of HPV infection has been suggested to be independent of co-infection with other HPV types (Liaw *et al.* 2001, Molano *et al.* 2003).

Merikukka *et al.* (2011) reported a possible competitive advantage for HPV33 over the genital HPV types in an unvaccinated population. A virtual longitudinal survey showed that cross-immunity among HPV types is consistent with current epidemiological data, and removal of targeted HPV types would increase the prevalence of non-targeted types considerably (Durham *et al.* 2012). On the other hand, type replacement is considered unlikely, and the replacing types probably entail a lower risk of cancer (Tota *et al.* 2013). According to the mathematical model of three or more epidemiologically interacting bacterial serotypes by Lipsitch (1997), it is possible that the prevalence of a non-targeted, competing serotype may increase more than the prevalence of a single targeted serotype decreases. It has been observed that replacement of pneumococcal serotypes is a combination of expansion of pre-existing serotypes and an introduction or identification of new serotypes important in invasive pneumococcal disease and/or carriage in a population (Scott *et al.* 2012).

2.2.2 HPV AND CERVICAL NEOPLASIA

Human papillomavirus (HPV) type 16 and 18 DNAs were cloned from cervical carcinoma biopsies in 1983 and 1984, respectively (Dürst *et al.* 1983, Boshart *et al.* 1984). Walboomers *et al.* (1999) suggested that HPV is a necessary cause of invasive cervical carcinoma worldwide, as only two adequate cervical carcinoma specimens (0.3%) in their material from 22 countries were HPV-negative. There are genital HPV types, most notably types 16 and 18, considered to be associated with significantly increased occurrence of cervical carcinoma (Muñoz *et al.* 2003). An IARC working group suggested that twelve HPV types can lead to cervical cancer (IARC 2012a).

The association between HPV and ICC has been evaluated under all proposed standard sets of causality criteria (Bosch *et al.* 2002). The association is very strong and universally consistent. The requirement for a biological gradient has been met for cervical precancer, including CIS, but not for ICC (Josefsson *et al.* 2000, Ylitalo *et al.* 2000b, Moberg *et al.* 2004, Spence *et al.* 2005). Experimental evidence from HPV vaccination studies shows that CIN 2 associated with HPV16/18 can be prevented by vaccination (Harper *et al.* 2004, Villa 2006b). However, not until ICC can be prevented by HPV vaccination can the role of HPV be considered causal for the development of ICC. To prevent ICC, it would be sufficient to remove infection with the hrHPVs if a hrHPV was a necessary cause of ICC. ICC is the first human cancer with a proposed necessary cause (Franco *et al.* 2004).

The major steps in the natural history of cervical cancer are acquisition of hrHPV, infection persistence with at least one hrHPV type, progression to precancer and invasion (Schiffman and Kjær 2003). Most cervical cancers and their precursors develop at the transformation zone of the uterine cervix. Thus, hrHPVs must infect the transformation zone, where columnar cells with the potential to differentiate along squamous or glandular lines are located (Stoler 2004). The transformation zone is located at the exocervix in 94% of women younger than 25 years and moves to the endocervical canal with age (Autier *et al.* 1996).

According to a worldwide meta-analysis of 11 600 CIN 3 and 40 600 ICC cases, 93% of women with CIN 3 and 89% of those with ICC were HPV DNA-positive (Guan *et al.* 2012). Among CIN 3 patients, HPV16 was the most prevalent, 59%, and the order of types 31, 52, 33 and 58 depended on the source of DNA. The most common types among ICC patients were 16 (65%), 18 (17%), 58, 33 and 45. The relative contribution of HPV types 16 and 18 in ICC and squamous cell carcinoma (SCC) of cervix uteri remained stable in a material of 11 countries from Central-South America, Europe and Asia over seven decades until 2007 (Alemany *et al.* 2014).

In SCC, the most prevalent hrHPV types worldwide were 16, 18, 45, 31 and 33 (Clifford *et al.* 2003). In the International Agency for Research on Cancer (IARC) multicentre study, these five HPV types accounted for 83% of the HPV DNA-positive SCCs and 75% of all SCCs (Muñoz *et al.* 2003). There were small differences between the regions with regard to the other hrHPVs. In a more recent study, the same five HPV types predominated (de Sanjosé *et al.* 2010). The proportion of HPV DNA-positives was 87%.

Alemany *et al.* (2014) reported that in 11 countries, the relative contribution of HPV16 in adenocarcinoma increased and that of HPV18 decreased over seven decades, with HPV16 becoming more prevalent than HPV18 after the 1960s. Clifford *et al.* (2003) reported in their meta-analysis that in adeno- and adenosquamous carcinoma of the uterine cervix, the predominant HPV type, 18, was followed by 16 and 45 in every region in the world. In an IARC multicentre study, the order of HPV types 16 and 18 was reversed, and the five most prevalent HPV types, 16, 18, 45, 59 and 35, accounted for 96% of the HPV DNA-positive adenocarcinomas and 89% of all adenocarcinomas (Castellsagué *et al.* 2006). In the more recent study, 16, 18 and 45 were the most common HPV types among both

adeno- and adenosquamous carcinomas, and the proportions of HPV DNA positivity were 62% and 81%, respectively (de Sanjosé *et al.* 2010). The most common HPV types in a precursor to adenocarcinoma, AIS, in Europe and North America were 18, 16 and 45 (Guan *et al.* 2013).

If HPV is a necessary cause of ICC, then all cases of ICC are attributable to HPV infection. The estimated number of ICC cases worldwide in 2008 was 530 000, corresponding to 93% of cancer cases attributable to HPV, 48% of cancer cases attributable to any infectious agent and 8.8% of all cancer cases in women (de Martel *et al.* 2012). The pooled OR for ICC associated with the presence of HPV was 158 (95% confidence interval (CI), 113–221), and the hrHPV type-specific pooled ORs were not smaller than 45 in the IARC multicentre study (Muñoz *et al.* 2003). The ORs for the strongest associations, HPV16 and squamous cell carcinoma and HPV18 and adenocarcinoma, led to attributable risks greater than 95% (Bosch *et al.* 2002). However, as the IARC multicentre study is based on pooled cross-sectional studies, it does not contribute to the temporality criterion of causality. According to a meta-analysis of longitudinal studies, the estimate for HPV-associated relative risk of cervical carcinoma, both invasive cervical carcinoma and carcinoma *in situ*, was 17 (95% CI, 8.2–33) and the estimate for the proportion of cervical carcinoma cases attributable to HPV16 was 27–44% (Lehtinen *et al.* 2001). In the fertile-aged Finnish female population, 61% (95% CI, 18–85%) of SCC cases and 6% (95% CI, -19–35%) of CIN 3 cases were estimated to be attributable to HPV16 after adjustment for several variables, among them invitations to organized cervical cancer screening (Laukkanen *et al.* 2010).

2.2.3 CO-FACTORS

Human papillomavirus may be a necessary but *not* a sufficient cause of cervical cancer. Only a small proportion of women with HPV infection develops cervical cancer. Therefore the role of co-factors in cervical carcinogenesis, *e.g.* how they modify the cancer risk among HPV-infected women, is an important issue. A classification of candidate co-factors into three groups has been suggested: 1) environmental co-factors, such as use of oral contraceptives, tobacco smoking, parity and other STIs; 2) host co-factors, such as endogeneous hormones and factors related to the host's immune response; and 3) HPV co-factors, including virus variants, viral load, and viral integration (Castellsagué and Muñoz 2003). The last class concerns HPV *per se* and might be called HPV factors instead of co-factors. When assessing the contribution of co-factors in cervical carcinogenesis, it has become a standard procedure to restrict the analyses to hrHPV-positive subjects. This is not necessarily a safe procedure because, for example, smoking and other STIs are surrogates for risk-taking behaviour that increases the exposure to hrHPVs (Castle and Giuliano 2003).

Chlamydia trachomatis

Chlamydia trachomatis is an obligate intracellular bacterium that replicates in cervical epithelial cells (Paavonen *et al.* 2003). *C. trachomatis* is among the most common STI agents, with an estimated 46 million new infections in women worldwide in 1995 (Gerbase *et al.* 1998). About three-quarters of women infected with *C. trachomatis* are symptom-free, and some *C. trachomatis* infections persist (Golden *et al.* 2000). *C. trachomatis* is a common cause of urethritis and cervicitis and may induce pelvic inflammatory disease (Paavonen *et al.* 2003). Co-infection of HPV and *C. trachomatis* may result in a more profound inflammatory state than HPV or *C. trachomatis* infections alone (Ylä-Outinen *et al.* 1990, Castle and Giuliano 2003). Oncogenic HPV infections may be more likely to persist among women with a previous *C. trachomatis* infection (Samoff *et al.* 2005, Silins *et al.* 2005).

Antibodies to *C. trachomatis* were shown to be associated with an increased risk of cervical precancer and cancer some time ago, but the role of HPV was not controlled (Schachter *et al.* 1982, Hakama *et al.* 1993). *C. trachomatis* seropositivity was associated with an increased risk of cervical squamous cell carcinoma adjusted for HPV or among HPV DNA-positives (Koskela *et al.* 2000, Smith *et al.* 2004, Castellsagué *et al.* 2006). There was no excess risk of adenocarcinoma among women seropositive for *C. trachomatis* in these studies. Nauclér *et al.* (2007) reported that *C. trachomatis* was associated with an increased risk of incident CIS+. Safaeian *et al.* (2010) reported without showing results that they failed to find any association between *C. trachomatis* IgG or DNA status and risk of CIN 3+ or ICC among HPV-infected women. The risk of CIN 3/AIS associated with *C. trachomatis* positivity was only slightly increased among hrHPV-positive women participating in the placebo arms of two multinational HPV vaccination trials (Lehtinen *et al.* 2011). Castellsagué *et al.* (2014) reported that mucosal-HPV L1-serology-adjusted *C. trachomatis* was associated with a non-significantly increased risk of CIN 3/CIS, but the risk of ICC was higher and statistically significant, with respective ORs of 1.3 and 2.3. In two longitudinal studies, the ORs for squamous cell carcinoma were 3.0 and 6.6 related to *C. trachomatis* serotypes G, F, K or G (Lehtinen *et al.* 1996, Anttila *et al.* 2001). In a cross-sectional IARC multicentre study, the OR for *C. trachomatis* seropositivity among HPV DNA-positives was 1.8 (95% CI, 1.2–2.7) (Smith *et al.* 2004). The risk of squamous cell carcinoma increased by *C. trachomatis* antibody titres. The proportion of cervical neoplasia attributable to *C. trachomatis* varied between 15% and 20% depending on the background prevalence in a meta-analysis by Lehtinen *et al.* (2010).

Herpes simplex virus type 2

Since the late 1960s until the 1980s, herpes simplex virus type 2 (HSV-2) was considered the major cause of ICC (Rawls *et al.* 1968, Lehtinen *et al.* 2002). This hypothesis was abandoned due to no difference in HSV-2 antibody prevalence

between cases and controls in a longitudinal study (Vonka *et al.* 1984), and consistent identification of HPV DNA in cervical carcinoma (Dürst *et al.* 1983). As HSV-2 DNA could not be identified consistently in cervical carcinoma, Galloway and McDougall (1983) proposed a "hit and run" mechanism, suggesting that HSV-2 is an initiator in cervical carcinogenesis. In a meta-analysis of longitudinal studies, not a single study reported a significantly increased relative risk for cervical carcinoma related to HSV-2 and the weighted mean of relative risks was 0.9 (95% CI, 0.6–1.3) (Lehtinen *et al.* 2002). Recent longitudinal studies have confirmed this finding (Castellsagué *et al.* 2014).

In the cross-sectional IARC multicentre cervical cancer study, among HPV DNA-positive women, HSV-2 was associated with an increased risk of squamous cell carcinoma and adenocarcinoma, with respective ORs of 2.2 and 3.4 (Smith *et al.* 2002). A longitudinal Nordic study of invasive cervical carcinoma reported an increased risk of the same level during the year prior to diagnosis (Lehtinen *et al.* 2002). Probably HSV-2 preferably infects cancerous tissue, and due to the fact that HSV-2 seropositivity is a strong marker of sexual activity (Cowan *et al.* 1994), increased risk estimates are seen in cross-sectional studies.

Human immunodeficiency virus

On the basis of a meta-analytic pooling of cross-sectional studies, HPV and human immunodeficiency virus (HIV) seem to interact synergistically to increase the risk of CIN (Mandelblatt *et al.* 1999). Infection with HIV causes suppression of cell-mediated immunity, and lower CD4+ level is the marker for immunosuppression among HIV-positive individuals (Palefsky and Holly 2003). Among HIV-positive women, lower CD4+ level is related to high HPV DNA levels and a large number of HPV types in cervical specimens (Palefsky and Holly 2003). HIV-positive individuals tend to have a higher prevalence and a longer duration of genital HPV DNA than HIV-negative individuals (Ho *et al.* 1994, Winer and Koutsky 2004). Immunosuppression is strongly associated with the early stages of dysplasia (Palefsky and Holly 2003).

Smoking

Regardless of body fluid, blood, urine or saliva, cotinine, the major primary metabolite of nicotine, is highly specific, 99–100%, and sensitive, 96–97%, and currently is the most widely used biomarker of tobacco smoke uptake (Jarvis *et al.* 1987). Cotinine reflects exposure to tobacco smoke over the past 2–3 days. Individual differences are present in the amount of nicotine converted to cotinine. However, the cotinine levels are clearly higher in smokers than in non-smokers and directly correlated with the number of cigarettes smoked per day. Both nicotine and cotinine have also been found in cervical mucus (Sasson *et al.* 1985). The mucus concentrations correlate with reported cigarette consumption (Schiffman *et al.* 1987).

Cotinine is the best biomarker for exposure to secondhand smoke uptake (IARC 2004).

In a pooled analysis restricted to the HPV-positive women of the cross-sectional IARC multicentre case-control study, the OR estimate for SCC associated with current smoking was 2.3 (95% CI, 1.3–4.0) and with smoking in past 1.8 (95% CI, 0.95–3.4) (Plummer *et al.* 2003). In a collaborative re-analysis of studies, including also the above-mentioned ones, the respective OR estimates were 2.0 (95% CI, 1.5–2.5) and 1.6 (95% CI, 1.0–2.6) (International Collaboration of Epidemiological Studies of Cervical Cancer 2006a). The respective OR estimates for adenocarcinoma (including adenosquamous carcinoma) were 1.1 (95% CI, 0.4–2.7) and 1.7 (95% CI, 0.3–8.5). Heavy smoking, with high levels of serum cotinine, was associated with an increased risk of SCC among both HPV16/18-seronegative and -seropositive women (OR, 2.7; 95% CI, 1.7–4.3) (Kapeu *et al.* 2009). Among HPV L1-seropositive women, current smokers had twofold increased risks of CIN 3/CIS and ICC associated with smoking ≥10 years and ≥30 years, respectively, as compared with never-smokers (Roura *et al.* 2014). The HPV L1-seropositive former smokers had non-significantly decreased risks of CIN 3/CIS and ICC in all time categories since quitting smoking, relative to HPV L1-seropositive current smokers. Furthermore, the effect of smoking on the risk of cervical SCC did not diminish with the adjustment for hrHPV infection (HPV DNA detection), or in the analysis restricted to hrHPV DNA-positives (IARC 2004).

Unlike SCC, cervical adenocarcinoma appears to show no clear association with smoking (IARC 2012b). This is a major difference between cervical SCC and adenocarcinoma (Berrington de González *et al.* 2004, International Collaboration of Epidemiological Studies of Cervical Cancer 2007a). An estimated 12% of HPV-positive cervical cancer cases were attributable to ever-smoking in the IARC multicentre study material (Castellsagué and Muñoz 2003). Passive smoking has been investigated in couples where the woman has been monogamous (Louie *et al.* 2011). Among them, compared with non-smoking couples, the OR was 1.6 if the woman was an ever-smoker and the man a non-smoker, whereas the OR was 2.3 if they were both ever-smokers.

Several biological mechanisms have been suggested for the association of smoking with cervical neoplasia (Szarewski and Cuzick 1998). Smoking may weaken the immune response to hrHPV, allowing the virus to persist longer among smokers than non-smokers (Barton *et al.* 1988). Minor-grade cervical lesions regress more slowly among current than past smokers (Szarewski *et al.* 1996). On the other hand, smoking was found to be protective against hrHPV persistence in two prospective studies (Hildesheim *et al.* 1994, Ho *et al.* 1998). According to a mechanism of direct effects, cigarette smoke metabolites found in the cervical tissue may transform cells infected by hrHPVs (Szarewski and Cuzick 1998). A prospective study demonstrated results supporting a promoter role of smoking in the early phase of cervical cancer development (Giuliano *et al.* 2002). Cigarette smoke by-products may affect the early evolution of HPV-related lesions in the transformation zone of the cervix uteri, possibly by increasing the rate of cell turnover (Cotran *et al.* 1989, Harris *et al.* 2004).

Parity

Multiparity has for decades been suspected of being associated with an increased risk of cervical cancer (Muñoz *et al.* 2002). Parous compared with nulliparous women more likely have the transformation zone located on the exocervix (Autier *et al.* 1996). Autier *et al.* (1996) suggested that with an increasing number of livebirths the transformation zone is for longer periods directly exposed to external agents capable of inducing premalignant lesions. Seroreactivity against HPV16 is higher in non-pregnant women than in pregnant women, suggesting a low humoral immune response against HPV infections during pregnancy (Sethi *et al.* 1998).

In a pooled co-factor analysis restricted to the HPV DNA-positive women of the cross-sectional IARC multicentre study, the OR estimate for invasive and *in situ* SCC associated with seven or more full-term pregnancies compared with nulliparity was 3.8 (95% CI, 2.7–5.5) (Muñoz *et al.* 2002). In a collaborative re-analysis of studies including the above-mentioned ones, the corresponding OR estimate was 5.0 (95% CI, 3.5–7.1) (International Collaboration of Epidemiological Studies of Cervical Cancer 2006b). The risk of SCC increased with an increasing number of full-term pregnancies in most major studies on HPV-positive (restrictively) women (Castellsagué and Muñoz 2003). The effect of this restriction was considerable in the IARC study, as the OR among all women was 1.6 (95% CI, 1.2–2.2) (Muñoz *et al.* 2002). The association of parity with adenocarcinoma risk was weaker than that with SCC risk, with the OR estimates no higher than 2.4 among HPV-positive women and 1.5 among all women (Berrington de González *et al.* 2004, Castellsagué *et al.* 2006, International Collaboration of Epidemiological Studies of Cervical Cancer 2006b). In a Finnish study of multiparous women the SCC incidence exceeded the national average slightly (standardized incidence ratio (SIR), 1.2; 95% CI, 1.05–1.4), while that of adenocarcinoma did not essentially differ from the average (Hinkula *et al.* 2004). The age at first full-term pregnancy was associated inversely with the risk of ICC, and this association remained within strata defined by age at sexual debut in the collaborative re-analysis of studies of cervical cancer (International Collaboration of Epidemiological Studies of Cervical Cancer 2006b).

Oral contraceptives

In vitro and *in vivo* experiments have revealed an effect of 16α-hydroxysterone, an oestrogen metabolite, in enhancing hrHPV gene activity (de Villiers 2003). This is in line with epidemiological studies showing an increased risk of cervical cancer related to long-term oral contraceptive (OC) use in HPV-infected women. In the pooled, cross-sectional IARC multicentre case-control study restricted to the HPV-positive women, the OR estimate for invasive SCC associated with at least five-year OC use was 4.0 (95% CI, 2.0–8.0) (Moreno *et al.* 2002). The OR estimates did not vary by time since first or last use. A systematic review of 28 studies reported that the OR estimates in HPV-positive women associated with long-term OC use were broadly similar for CIS and ICC and for SCC and adenocarcinoma (Smith *et al.*

2003). A collaborative re-analysis study of individual data reported that the combined use of oral contraceptives for at least five years was associated with increased RR, 1.5, of ICC, and past use with increased RR, 1.4, of both ICC and CIN 3/CIS, among HPV-positive women (International Collaboration of Epidemiological Studies of Cervical Cancer *et al.* 2007b). In a Finnish register-based study, postmenopausal oestradiol-progestagen therapy was not associated with risk of CIN 3 or AIS lesions, but at least six-month use and at least five-year use were associated with decreased risk of SCC and increased risk of AC without adjustment for HPV status, respectively (Jaakkola *et al.* 2012). An estimated 4% of the HPV-positive cervical cancer cases were attributable to being ever-users of OCs, but 16% were attributable to at least a five-year use *versus* shorter or no use (Castellsagué and Muñoz 2003).

Diet and nutrition

The available evidence for an association between diet and nutritional status and cervical carcinogenesis taking HPV infection into account is not yet convincing according to a review by García-Closas *et al.* (2005). They suggested that folate, homocysteine, retinol and vitamin E are probably associated with cervical neoplasia and cancer. Whereas homocysteine may increase the risk of cervical neoplasia, the other three micronutrients may be protective.

A prospective population-based serological study did not show any protective effect of serum retinol, but suggested larger than multiplicative joint effect of low levels of retinol and HPV (types 16, 18 and 33) seropositivity on the occurrence of ICC (Lehtinen *et al.* 1999). However, as a whole, decreased risk of ICC was associated with low levels of serum retinol and the lowest levels of α-tocopherol. In another study, low levels of serum retinol were associated with an increased risk of ICC, and the combination of high levels of serum retinol and HPV16 with a decreased risk of ICC (Lehtinen *et al.* 1994). Shannon *et al.* (2002) reported an increased risk of ICC to be associated with high dietary E levels (Shannon *et al.* 2002). Older studies on this association have been less consistent than those on vitamins A and C and risk of ICC (Potischman and Brinton 1996). A serological case-control study suggested that β-carotene might have a protective role in the aetiology of cervical cancer (Potischman *et al.* 1991). In dietary studies, the OR estimates of ICC association with high *versus* low carotenoid levels have ranged from 0.5 to 1.0 (Potischman and Brinton 1996, Shannon *et al.* 2002). The same applies to studies on dietary vitamin C. In a recent, prospective questionnaire-based study, the only finding that remained significant after correction for measurement error was an inverse association between ICC and daily intake of fruit (González *et al.* 2011). Of the nutrients, vitamin C and retinol had closest to significant inverse associations with the risk of ICC. The risk of CIS was not associated with any dietary factors.

Socio-economic status

The incidence of ICC in the highest social class (based on occupational classification) was below the national average among Finnish women aged 45–64 years in 1971–1995 (SIR, 0.6; 95% CI, 0.5–0.7) (Pukkala and Weiderpass 1999). The SIR for ICC in the lowest social class was 1.3 (95% CI, 1.2–1.4). The authors concluded that the observed socio-economic differences were possibly due to differences in lifestyles or life conditions such as viral infections, smoking, reproductive patterns and diet. Waiters, drivers, beverage and tobacco workers were among the occupations with the highest SIRs for ICC, 1.5–2.0, in Nordic countries in 1961–2005 (Pukkala *et al.* 2009). Exposure to hrHPV types and tobacco smoking as well as participation in cervical cancer screening programmes may vary by occupation. A meta-analysis of ten studies on income status and the risk of ICC reported an OR estimate of 2.7 (95% CI, 2.3–3.1) associated with low compared with high income (Parikh *et al.* 2003). A meta-analysis of 27 studies on education level and risk of ICC reported an OR estimate of 2.0 (95% CI, 1.8–2.2) associated with low compared with high social class (Parikh *et al.* 2003). The authors concluded that the socio-economic differences in Western Europe were primarily due to differences in lifestyles such as history of sexual (risk-taking) behaviour and elsewhere equally importantly due to differences in access to cervical cancer prevention programmes. It is important to note that both the acquisition of HPV infection and many of the co-factors are determined by risk-taking behaviour. A combination of two IARC multicentre studies found that the excess of ICC in women with five years of education or less was not explained by HPV prevalence, but largely by the ages at first sexual intercourse and first pregnancy (Franceschi *et al.* 2009).

Male circumcision

According to an IARC multicentre cervical cancer study, male circumcision was associated with a moderate, non-significant decrease in the risk of ICC in the men's female partners (OR, 0.7) (Castellsagué *et al.* 2002). The decrease was significant if the women were monogamous and their circumcised male partner had a history of at least six sexual partners (OR, 0.4; 95% CI, 0.2–0.8). The ICC risk among monogamous women was non-significantly increased, if the circumcised male partner had less than six life-time sexual partners, (OR, 1.4). Male circumcision may protect against ICC by preventing HPV transmission. Circumcised men were less likely than uncircumcised men to have penile HPV infection (OR, 0.4; 95% CI, 0.2–0.9) (Castellsagué *et al.* 2002). Women with circumcised partners compared with those with uncircumcised partners also had a decreased risk of testing seropositive for *C. trachomatis* (OR, 0.2; 95% CI, 0.1–0.6) (Castellsagué *et al.* 2005).

Endogeneous hormones

The first prospective study on levels of circulating steroidal sex hormones and ICC risk found a significant positive association with free testosterone in premenopausal women, and with testosterone in postmenopausal women (Rinaldi *et al.* 2011). Sex hormone-binding globulin was inversely associated with ICC risk in premenopausal women. Oestradiol showed a non-significant positive association among postmenopausal women. No associations were detected between any hormone and risk of CIN 3.

Genetics and immune response genes

Transplant recipients tend to have higher HPV DNA prevalence or HPV antibody levels prior to diagnosis of skin cancer than controls (Bouwes Bavinck *et al.* 1993). High HPV DNA levels in immunosuppressed individuals support the vital role of cell-mediated immunity in the body's ability to clear HPV infection (Ho *et al.* 1994, Winer and Koutsky 2004). A study utilizing the Swedish Family Cancer Database reported that, compared with daughters of mothers without ICC, the familial relative risk estimate for ICC in daughters of mothers with ICC was 2.1 (95% CI, 1.7–2.4) (Hemminki *et al.* 1999). The familial relative risk estimate for ICC in mothers of daughters with ICC as compared with mothers of daughters without ICC was higher, 3.9 (95% CI, 3.0–4.9). On the contrary, tobacco-, immunosuppression- and HPV-related cancers were aggregated in the cervical cancer families. Heritability of liability to ICC was estimated to be between 0.22 and 0.34. According to a meta-analysis by Castro *et al.* (2007), human leukocyte antigens (HLA) A11, B7 and DR2 were identified as susceptibility genes and DR6 and B15 as resistance genes. In Caucasian populations, also DR10 had a significant association with susceptibility to cervical cancer. Castro *et al.* (2009) found out that variations in genes/alleles IL-6, LTA and DRB1*1301 were associated with a decreased risk, and genes/alleles CCND1, DRB1*0401 and DRB1*1501 with an increased risk of CIN 3/cervical cancer. The first genome-wide association study of CIS/cervical cancer confirmed the previously observed increased risk of CIS/cervical cancer associated with alleles DRB1*1501, DQB1*0602 and B*0702, and the decreased risk associated with alleles DRB1*1301, DQA1*0103 and DQB1*0603 (Chen *et al.* 2013). They found also three novel loci in the major histocompatibility complex region associated with CIS/cervical cancer. The above-mentioned genes and alleles are involved in the cell cycle and immune control.

2.3 DESIGN AND ANALYSIS OF CASE-CONTROL STUDIES

The rationale for case-control studies is the comparison of exposure histories between a group of diseased cases and a group of randomly selected, non-diseased controls in order to find differences of importance. The first modern case-control studies were conducted in the 1920s, but the methodology gained popularity only after publication of four case-control studies successful in linking tobacco smoking and lung cancer in 1950 (Paneth *et al.* 2002, Breslow 2014). The study by Doll and Hill (1950, 1952) is still a model for case-control studies. Doll and Peto (pp. 1259–60, 1981) foresaw the pertinent role of case-control design in large biobank studies on lifestyle and environmental aspects and risk of cancer death.

In seroepidemiological studies, the volume of the serum sample decreases each time a droplet is needed for a new study. At most serum banks, the whole sample must be frozen and thawed, which can affect many biomarkers. Furthermore, the effort required to analyse the samples of all cohort members may be beyond the capacity of the laboratory. Valuable samples should not be wasted in full cohort analysis. The number of studies a serum bank can serve should be maximized by implementing more efficient study designs.

Nested case-control design and *case-cohort design* are efficient sampling methods to measure exposure-disease associations in a cohort and are appropriate especially when a full cohort design is not feasible. The *case group* comprises preferably all cases in the study population who contracted the disease of interest during follow-up. The *control group* is a random sample of all subjects at risk in the study population selected at design-dependent time-points and meeting possible matching criteria.

2.3.1 NESTED CASE-CONTROL DESIGN

In epidemiology, the nested case-control design refers, in practice, to sampling from a well-defined cohort (Läärä 2011, Kass 2014). In biostatistics, this design originally suggested by Duncan Thomas (1977) refers to a *time-matched sampling* of controls from the risk set (Läärä 2011). *Time-matching* means here that the controls are selected at the time of diagnosis of each new case. Matching fixes the time-scale of the study. The subjects in the *risk set* are at the time of the case's diagnosis *at risk*, *i.e.* alive, without the outcome disease, and under follow-up, and fulfilling possible further matching criteria. Time-matched sampling is a special case of (incidence) *density sampling*. The controls are randomly sampled *without* replacement from the risk set to a case-control set. Sampling is independent across risk sets, and the probability of being selected from the risk set is the same for all control candidates. Therefore, a control can be a member of more than one case-control set. As a case is at risk until diagnosis, she can serve as a control for one or more cases with an earlier date of diagnosis (Greenland and Thomas 1982). There is no need to follow

up the exposure history of controls beyond the case's diagnosis in nested case-control studies. As the controls are made similar to cases, they do *not* form a representative sample of the full cohort.

Usually, matching is not solely limited to time of diagnosis. It is possible to reduce biases caused by storage and laboratory analyses by matching for storage time, number of freeze-thaw cycles and analytic batch (Rundle *et al.* 2005). Age and sex are commonly used in matching. *Matching* makes the distributions of matching variables in controls similar to those in cases. Matching on a strong confounder related to exposure increases efficiency and precision, but overmatching on a non-confounding variable reduces efficiency. Matching makes a case-control study vulnerable to missing information due to, for instance, excessively low serum volume if there is only one case and/or control per case-control set. The nested case-control design becomes less flexible when the number of matching variables increases. *Counter-matching* is an efficient approach in nested case-control design to assess interaction between a risk factor measured in the full cohort and another risk factor measured only in the case-control sample (Langholz and Borgan 1995).

Exposure odds ratio between cases and controls is the measure of exposure-disease association in nested case-control design. It is a valid and efficient estimate of *incidence rate ratio* between those exposed and not exposed in the cohort (Breslow 2014) and *hazard rate ratio* (Läärä 2011). Also absolute risks of diseases can be estimated from nested case-control studies. For the assessment of co-factors, it is necessary that their effects on outcome are estimated among those having exposure that is considered causal, *e.g.* among human papillomavirus positives in cervical cancer aetiology. This leads to a missing data problem, unless the exposure is a matching variable. One approach is to break matching, to model with unconditional logistic regression, and to reduce bias due to confounding, adjusting for the matching variables. Another is to analyse with conditional logistic regression the case-control sets with at least one case and one control. A third approach, a *missing-indicator method*, a combination of unmatched and matched analysis, has been suggested (Huberman and Langholz 1999). Unfortunately, none of these approaches is satisfactory under all conditions (Li *et al.* 2004).

The controls can be reused with restrictions as controls for new outcomes of interest by applying the likelihood approach or the probability weighting approach (Saarela *et al.* 2008, Støer *et al.* 2014). A drawback of reuse is that the effects of analytic batch, storage time and freeze-thaw cycles will be present.

For a valid study, not only the study design but also the methods must be valid. Matched case-control studies are analysed with conditional logistic regression, maximizing conditional likelihood, or proportional hazards regression, maximizing partial likelihood.

2.3.2 CASE-COHORT DESIGN

This design was originally suggested by Ross Prentice (1986). The control group, a *subcohort*, is sampled at the beginning of the follow-up. Sampling is *not* time-

matched, which is the key difference between the case-cohort and the nested case-control design. The subcohort, a simple random sample from the cohort, is used as a comparison group for all cases in the cohort. The subcohort is selected without regard to disease status. The subcohort provides information about the person-time experience in the full cohort.

Stratification is a means to increase the efficiency in case-cohort studies. The subcohort is selected by applying stratified random sampling. There are two types of stratified case-cohort studies, confounder-stratified to deal with confounding and exposure-stratified to increase efficiency (Cologne *et al.* 2012). In *confounder-stratified* studies, sampling and modelling strata correspond with each other, and in *exposure-stratified* studies, sampling is stratified, but the model is not (Langholz and Jiao 2007). In exposure-stratified studies, exposure is known for the entire cohort, and interactions with exposure are of interest.

Also in case-cohort design, incidence rate ratios and hazard rate ratios are estimable (Läärä 2011, Breslow 2014). Furthermore, absolute risks and cumulative risk ratios can be estimated (Langholz and Jiao 2007, Breslow 2014). The subcohort of the case-cohort study can be used for several diseases and for extended follow-up.

Case-cohort studies are analysed with proportional hazards regression based on weighted exact (or approximate) pseudolikelihood. Analysis of a confounder-stratified study is a stratified version of an unstratified study. For analysis of an exposure-stratified study, a swapper method is recommended (Cologne *et al.* 2012). Case-cohort design is flexibly adapted to fit other kinds of survival models (Petersen *et al.* 2010, Li *et al.* 2012).

Control for batch and storage effects and freeze-thaw cycles is more difficult in case-cohort design than in nested case-control design. Batch effect will cause bias when subsequent case series are investigated in a case-cohort design (Rundle *et al.* 2005).

As an example of the sophisticated use of case-control design, Gilbert and Hudgens (2008) developed an approach for measuring principal surrogate predictiveness based on case-cohort sampling from a large clinical trial. They illustrated the approach assessing HIV-specific immune response to a vaccine as a surrogate endpoint for the HIV infection.

2.3.3 COMPARISON

The case-cohort design has clear advantages over nested case-control design: multiple outcomes, less sensitive to missing data, freedom to choose a time-scale, more flexible choice of models, sampling of the subcohort and data collection can start immediately, subcohort serves also new cases, *etc*. But, biomarkers tend to suffer from lengthy storage, batch effects and freeze-thaw cycles. The nested case-control design provides simple tools for dealing with such issues and is therefore often a more appropriate design for seroepidemiological studies, especially those investigating a single outcome. Nested case-control is a low-cost design for which standard software is easily available. Choice of design depends on the parameter of

interest, aim of the study, data already available (exposure levels for a subcohort or cohort), costs, *etc*. For example, for an aim, risk prediction for a marker, either case-cohort design or nested case-control design will do, but the latter only if matching is not too stringent (Ganna *et al.* 2012). Finally, it should be kept in mind that the principle of random sampling of controls is not necessary in more complex case-control designs. Of more importance is that 'the method of control selection must be incorporated into the analysis' (Langholz and Goldstein 2001).

3 OBJECTIVES OF THE STUDY

Longitudinal study designs are needed

1) to distinguish causes of multiaetiological cervical cancer from confounders or mere correlates.
2) to disclose interactions between different causes or confounders.
3) to disclose temporal relationships between different causes or confounders.

Study-specific objectives were as follows.

Studies I and III

To evaluate the joint effects of past infections with the oncogenic and non-oncogenic HPV types on the risk of subsequent development of cervical cancer by applying a nested case-control design on independent materials.

Studies II and III

To assess the joint effects of past HPV and *C. trachomatis* infections on the risk of squamous cell carcinoma of cervix uteri by applying a nested case-control design on independent materials.

Study IV

To investigate the solitary and joint effects of persistent and incident HPV and *C. trachomatis* infections on the risk of developing cervical cancer by applying a nested case-control design in a serial setting.

Study V

To evaluate the order and joint effects of incident and persistent HPV and *C. trachomatis* infections on the risk of developing high-grade cervical precancer by applying a stratified case-cohort design in a serial setting.

4 MATERIALS AND METHODS

Efficient and valid study designs, nested case-control and case-cohort design, were applied, and analyses were conducted with valid methods.

4.1 STUDY BASES

Studies I, II and III were the fruit of the collaboration between Finnish, Icelandic, Norwegian and Swedish biobanks, cancer registries and laboratories, together forming the Nordic Biological Specimen Banks working group on Cancer Causes and Control (NBSBCCC). Twelve papers with NBSBCCC study numbers 6, **10**, 11, 13, 14, 16, 20, 21, 22, 23, **26** and 30 were based on the first Cervical Cancer Study of the working group (Pukkala 2011).

The study base for Studies I and II was a joint cohort of approximately 627 000 women who had donated serum or blood samples to three Nordic population-based biobanks in 1974–1994 (Table 1). Most Study I cases and controls were also Study II cases and controls, and *vice versa*. Therefore these studies are referred to as Studies I/II in the materials section.

Study III was the Cervical Cancer Study II of the working group. The study base was a joint cohort of four Nordic population-based serum banks to which more than 900 000 women had donated serum samples during 1973–2002 and contributed to follow-up of approximately 15 000 000 woman-years by the end of the year 2002.

The study base for Study IV consisted of women aged 25–59 years who had participated in the population-based cytological screening programme for cancer of cervix uteri in Västerbotten county (Sweden) from 1969 to 1995. Women eligible for the study had an unoperated cervix and had had at least one cytologically normal smear and at least one additional Pap smear.

The study base for Study V consisted of 94 349 women who had donated to the Finnish Maternity Cohort serum samples of two consecutive pregnancies within five years and were younger than 32 years in 1992–2004. The women were eligible for the study until diagnosis with CIN 3 or ICC, hysterectomy, emigration, death or closing date of the study, *i.e.* 31 December 2004. The women had contributed to follow-up of approximately 450 000 woman-years.

4.2 SERUM BANKS (I/II, III, V)

4.2.1 JANUS SERUM BANK (I/II, III)

The Janus Serum Bank was established in Norway in 1973 to store sera collected from originally healthy individuals (Jellum *et al.* 1995, Langseth *et al.* 2009). The Norwegian Cancer Society owned the Janus Serum Bank up to 1 May 2005, after which the bank has been overseen by the Cancer Registry of Norway (Gislefoss and Jellum 2006). By the end of the year 1992, approximately 144 000 women had donated serum samples to the serum bank during regular health examinations, particularly in connection with evaluation of risk factors for chronic diseases (Table 1). The number of female donors was virtually the same in 2003.

During phase I, covering the period 1974–1978, the study area comprised one county from western, central and northern Norway, Sogn og Fjordane, Oppland and Finnmark, respectively. During phase II, in 1981–1992, the study area included the whole country, except the two southern counties of Buskerud and Hordaland. Most of the phase II subjects donated a serum sample in the context of a cardiovascular health examination of 40- to 42-year-old Norwegians. The Janus health examination donors had a lower cancer incidence than the comparable general Norwegian population (Table 1) (Pukkala *et al.* 2011), but the Janus Serum Bank is considered to be representative of the Norwegian population (Langseth *et al.* 2009).

For Study III, also another subcohort of the Janus Serum Bank, 14 000 female Red Cross blood donors from Oslo, was included. The women were enrolled in 1973–1991 and 1997–2000. The subcohort is not considered to be representative of the Norwegian population (Langseth *et al.* 2009).

4.2.2 FINNISH MATERNITY COHORT (I/II, III, V)

The Finnish Maternity Cohort (FMC) serum bank has collected serum samples during early pregnancy (first trimester) in order to screen for congenital infections in Finland since 1983 (Bardy *et al.* 1993). The serum samples are drawn at the maternity clinics, and almost all (more than 98%) pregnant women have donated serum samples to the bank. At the end of 1994, the bank contained serum samples from approximately 465 000 women. By 2003, a total of 681 000 women had donated about 1.4 million samples to the FMC. The National Institute for Health and Welfare (until 2008, the National Public Health Institute of Finland) owns the Finnish Maternity Cohort serum bank. An informed consent for the research use of samples has been obtained from donors since 2001, and samples donated before 2001 can be used for medical research according to Finnish law (Kaasila *et al.* 2009).

Table 1. Characteristics of the biobanks utilized in Studies I–III and V.

Biobank	Material	Storage temperature	Geographical location	Period of serum/ blood sampling [1]	Participation (%)	No. of female donors		SIR (cancer, all sites) [2]	95% CI [2]
						1994	2003		
Janus Serum Bank	Serum	–25°C	Three counties, Norway	1974 - 1978	85	29 000	29 000	0.91[3]	0.90–0.92
			Several counties, Norway	1981 - 1992	75	115 000	115 000		
			Oslo, Norway[4]	1973 - 1991, 1997 - 2000			14 000	0.95[3]	0.91–0.99
Finnish Maternity Cohort	Serum	–25°C	Finland	1983+	>98	465 000	681 000	0.95	0.93–0.96
Northern Sweden Health and Disease Study VIP[5]	Blood	–80°C	Västerbotten County, Sweden	1985+	65	16 500		0.92[3]	0.88–0.96
MONICA [6]			Counties of Västerbotten and Norrbotten, Sweden	1986/-90/-94/-99/2004	77	1700		0.89[3]	0.79–1.00
Northern Sweden Maternity Cohort	Serum	–20°C	Four northernmost counties, Sweden	1975+	>95		86 000	0.85	0.81–0.89
Icelandic Maternity Cohort	Serum	–20°C	Iceland	1980+	>99		49 000	0.99	0.94–1.04

[1]Female donors only; [2]Amended from Pukkala et al. (2007, 2011), national reference rates, closing year for standardized incidence ratio (SIR) estimation: Norway 2001, Finland and Iceland 2005, Sweden 2003; [3]Males included; [4]Red Cross blood donors; [5]Västerbotten Intervention Programme; [6]Multinational Monitoring of Trends and Determinants in Cardiovascular Disease

Sample pair cohort and subcohort of the FMC (V)

Previously, a cohort of all FMC sample pairs of consecutive pregnancies within five years of women, who were aged under 29 years in 1995–2003, at the midpoint of the sample withdrawals, had been identified for studies on HPV co-infections and population-level competition of HPV types (Kaasila *et al.* 2009, Merikukka *et al.* 2011). The cohort comprised 123 773 sample pairs donated by 97 124 women. It was divided to 12 strata by age, <20, 20–22, 23–25 and 26–28 years, and calendar time, 1995–1997, 1998–2000 and 2001–2003, at the midpoint of the pregnancies. From each stratum, 200 or 400 samples were randomly selected for a subcohort of 3600 sample pairs, which were donated by 3569 women. Serology results for HPV types 6, 11, 16, 18, 31, 33 and 45 and *C. trachomatis* were available for the sample pairs of the subcohort (Merikukka *et al.* 2011).

4.2.3 NORTHERN SWEDEN HEALTH AND DISEASE STUDY (I/II)

The Västerbotten Intervention Programme (VIP) and the Northern Sweden MONICA (abbreviation for the Multinational Monitoring of Trends and Determinants in Cardiovascular Disease) are population-based sub-banks of the Northern Sweden Health and Disease Study Cohort. In 1985, the VIP cohort was initiated in a northern Swedish county, Västerbotten (Dillner *et al.* 1994, Hallmans *et al.* 2003). Each year, all residents aged 40, 50 or 60 years are invited to participate in a health-promoting project, including the donation of biological samples for future medical research. The participation rate was about 65%. By the end of the year 1994, the VIP cohort had received blood samples from 16 500 women.

The Northern Sweden MONICA project contains material from screenings for risk factors of cardiovascular diseases. The first screenings were carried out in 1986, 1990 and 1994. The screenees come from the counties of Västerbotten and Norrbotten. The first screening round was not available for the Cervical Cancer Study. The MONICA project received blood samples donated by 1700 women in 1990 and 1994. Half of the women are also included in the VIP cohort (Pukkala *et al.* 2007). The blood samples of the Northern Sweden Health and Disease Study Cohort are stored at the Medical Biobank in Umeå University Hospital.

4.2.4 NORTHERN SWEDEN MATERNITY COHORT (III)

The Northern Sweden Maternity Cohort has since 1975 collected rubella screening samples of pregnant women from Västerbotten county and since the 1980s from a larger area, the four northernmost counties in Sweden. In 2003, the virus laboratory of Umeå University contained samples of 86 000 women.

4.2.5 ICELANDIC MATERNITY COHORT (III)

The Icelandic Maternity Cohort has collected rubella screening samples from Iceland since 1980. In 2003, samples of 49 000 women were contained within the Department of Medical Virology of Landspitali University Hospital. About 6% of the donors have emigrated after serum sampling (Pukkala *et al.* 2011). Their samples and data cannot be used in the studies, as the date of emigration is not available at the biobank.

4.3 ORGANIZED CERVICAL CANCER SCREENING PROGRAMME IN VÄSTERBOTTEN COUNTY (IV)

The population-based invitational cytological screening programme for cancer of the cervix uteri was started in Västerbotten county in 1969. The screening programme was targeted to women 25–59 years of age. Invitations were issued at four-year intervals. The attendance rate was higher than 80%. Until the early 1980s, smears were taken using a wooden Ayers spatula and a cotton tip applicator. The latter was replaced by a cytological brush in the mid-1980s (Cytobrush®, Medscand Medical AB, Malmö, Sweden). All smears of the screening programme, or obtained outside the programme and histological specimens were recorded and stored at one hospital, Umeå University Hospital, where all diagnoses based on cytological findings in Pap smears were made.

4.4 CANCER REGISTRIES

The Cancer Registry of Norway, the Finnish Cancer Registry, and the Icelandic Cancer Registry are nationwide, and started operating in 1952 in Norway and Finland and in 1953 in Iceland, respectively. The nationwide Swedish Cancer Registry started operating in 1958. In the late 1970s, cancer registration was decentralized in Sweden, and data since 1958 were transferred to six regional cancer registries (Association of the Nordic Cancer Registries 2000). The regional cancer registries send coded data on an annual basis to the central cancer registry at the National Board of Health and Welfare. The regional cancer registry at the Oncological Centre in Umeå covers the four northernmost counties in Sweden (Association of the Nordic Cancer Registries 2000). All of these registries are population-based. Reporting of new cancer cases is compulsory for hospitals, physicians and pathology laboratories. In Iceland, reporting of cancer cases was voluntary until 2007. The primary basis of registered cancer diagnosis is morphology.

The coverage of the cancer registries for solid tumours is excellent, close to 100% (Lund 1981, Mattsson and Wallgren 1984, Teppo *et al.* 1994). The Norwegian

cancer register was 98.8% and 99.97% complete with regard to invasive cervical cancer in 1985 and 2001–2005, respectively (Bilet *et al.* 2009, Larsen *et al.* 2009). The Norwegian cancer register data can be considered almost complete four years after the year of diagnosis (Larsen *et al.* 2007). The Finnish cancer register was 99% complete with regard to invasive cervical cancer in 1998–2007 (Lönnberg *et al.* 2012b). The Finnish cancer register data are considered a reliable source of information for follow-up of cancer incidence in large cohort studies (Korhonen *et al.* 2002). According to Hospital Discharge Registry record linkage, the invasive cervical cancer data of the Icelandic cancer register was 100% complete during 2005–2009 (Sigurðardóttir *et al.* 2012). In 1998, the Swedish cancer register was 96% complete according to a sample survey (Barlow *et al.* 2009). For further details of the cancer registries and registration, and other registers see Technical Appendix 1.

4.5 STUDY DESIGN

4.5.1 NESTED CASE-CONTROL DESIGN, ONE SERUM SAMPLE (I/II, III)

Case-control design nested in the joint cohort of serum banks was adopted. The design was longitudinal, as the biological markers of the putative risk factors for invasive cervical carcinoma were identified from prediagnostic sera. The follow-up time was maximized by studying the first serum samples donated by the subjects.

Identification of cases (I/II, III)

In Studies I/II, the patients eligible to be included in these studies (as cases) were diagnosed with invasive carcinoma of the uterine cervix (International Classification of Diseases 7th revision code 171) at least 15 days after serum sample donation. Cases were identified by linking the data files of the serum banks and the cancer registers using personal identification numbers.

Altogether 196 cases were identified by the linkages. Fourteen cases were excluded, four carcinomas *in situ*, one leiomyosarcoma, three without a reported histology, four whose serum sample was postdiagnostic or donated too close to the date of diagnosis and two whose serum sample could not be located. The remaining 182 cases were included in Study I. The follow-up time of the cases was five years on average, with a minimum of 15 days, and a maximum of 16 years. There were 178 ICCs and 148 SCCs in Study II after histological reclassification (for details, see Technical Appendix 2). At the time of linkage in 1994, about 120 000 serum samples of the Janus Serum Bank were incorrectly labelled. Cases among the donors of these samples could not be identified. More than 30 women, who were donors to the Health examinations sub-bank of the Janus Serum Bank and were registered as

patients with invasive cervical carcinoma before the linkage, could not be identified. Donors of 15 000 samples to the FMC could not be identified because of incorrect or missing personal identification number.

In Study III, linkage to national cancer registers identified 653 cases of invasive cervical carcinoma diagnosed during 1975–2002. The cases donated the first serum sample more than one month before diagnosis. Forty-nine cases were excluded from the study, as the sample could not be located or the sample volume was too low (34 cases), behaviour of the neoplasm was benign or morphology was not reported to the cancer register (15 cases). After re-examination of cancer tissues and histopathological slides (see Technical Appendix 2), morphologies of the final 604 cases were squamous cell carcinoma (470 cases), adenocarcinoma (111), adenosquamous carcinoma (21) and undifferentiated carcinoma (2). The cases were enrolled in the study on average at an age of 31.4 years (range, 15.5–60.4 years) and controls at 31.5 years (range, 14.8–59.7 years). The cases were followed for on average 9.6 years (range, 3 months–25.5 years) and were on average 41.0 years (range, 21.5–70.5 years) at diagnosis. This study does *not* have any cases in common with Studies I/II.

In comparison with the national reference rates, incidence of cervical cancer was significantly lower than expected among donors to the Janus Serum Bank (Table 2). Among donors to the maternity cohorts, cervical cancer incidence was close to the expected figure.

Table 2. Study-specific numbers of cases and controls and cases' mean age at serum sampling and diagnosis, and standardized incidence ratio (SIR) and 95% confidence interval (CI) for cervical cancer, by biobank and sub-bank.

Biobank		No. of cases/controls			Age[1] at serum sampling/diagnosis			SIR (cervival cancer)[2]	95% CI[2]
		Study I	Study II	Study III	Study I	Study II	Study III		
Janus Serum Bank	Three counties	80/240	79/237	36/178	40.7/46.7	40.6/46.9	38.6/56.6	0.82	0.75–0.90
	Several counties	48/144	47/141	130/647	44.7/47.3	44.9/47.5	40.7/47.6		
	Oslo[3]	–/–	–/–	46/228			34.2/45.0	0.75	0.56–0.99
Finnish Maternity Cohort		49/139	48/137	174/854	30.0/34.7	30.1/34.7	28.9/38.4	0.95	0.87–1.03
Northern Sweden Health and Disease Study	VIP[4]	4/12	3/9	–/–	49.2/51.1	46.6/47.9		0.72	0.47–1.06
	MONICA[5]	1/3	1/3	–/–	56.7/57.0	56.7/57.0		1.48	0.48–3.46
Northern Sweden Maternity Cohort		–/–	–/–	115/573			26.6/37.1	0.97	0.82–1.14
Icelandic Maternity Cohort		–/–	–/–	103/500			25.7/34.4	1.01	0.84–1.18

[1]Cases' mean age; [2]Amended from Pukkala et al. (2007, 2011), national reference rates, closing year for SIR estimation: Norway 2001, Finland and Iceland 2005, Sweden 2003; [3]Red Cross blood donors; [4]Västerbotten Intervention Programme; [5]Multinational Monitoring of Trends and Determinants in Cardiovascular Disease

Matching (I/II, III)

In Studies I/II, for each case, three cancer-free female controls were randomly selected, individually matched for age at first serum sampling (±2 years), storage time of the first serum sample (±2 months) and area of residence (Finland, Northern

Sweden and Norway), and in Norway for county. The alive status of the control candidates at the time of case diagnosis was probably not checked for in all centres. If three controls for a case could not be found, the matching criteria on age and storage time were widened. The age at serum sampling of six controls differed by more than four years from that of the case, and difference in storage time was never greater than six months. The serum samples of eight controls could not be located. Finally, there were 182 cases and 538 controls for Study I, and 178 cases and 527 controls for Study II (Table 2).

In Study III, five female controls were individually matched to a case. Age at serum sampling (within two years), storage time (within two months), area of residence (Finland, Iceland, Northern Sweden and Norway) and in Norway, county and blood donor status were individually matched for. The matching criteria on age and storage time were widened if the number of control candidates was insufficient. The final material comprised 604 cases and 2980 controls.

4.5.2 NESTED CASE-CONTROL DESIGN, TWO SMEARS (IV)

Identification of cases

Linkage between the cytology registry and the regional cancer registry at the Oncological Centre in Umeå identified 133 eligible women, whose smear taken before diagnosis with invasive cervical carcinoma was normal. Four of them had incorrect entry in the cancer registry and 11 had non-invasive cervical neoplasia. After theses exclusions, 118 cases with ICC remained.

Matching

Women eligible to be controls did not develop ICC before the time-point of diagnosis of the corresponding case. For each case, one control was matched for age (same calendar year of birth) and time-point of sampling of the baseline smear. The average age when the prediagnostic Pap smear was taken was 44.2 years (range, 19.1–74.1 years) among the cases and 44.1 years (range, 19.5–74.4 years) among the controls. The date of smear taking differed 1 month on average. The mean time between the sampling of the baseline smear and cancer diagnosis was 5.6 years (range, 0.5 months–26.2 years). The controls were required to have been followed up beyond the date of diagnosis of the corresponding case. The control's normal smear immediately after the date of diagnosis was chosen. The time-point of cancer biopsy and the second normal smear of the control differed on average by 9.6 months.

Frequency matching was applied to increase statistical efficiency. Matched pairs were pooled so that 118 matched pairs reduced to 74 strata. In each stratum, there were 1 to 5 cases and the same number of controls. Those strata were available for

analyses of baseline smears. One hundred and four cases and controls in 70 strata were available for analyses of baseline smears, second smear and cancer biopsy.

4.5.3 CASE-COHORT DESIGN, TWO SERUM SAMPLES (V)

Conversion of the sample pair cohort into the study cohort

A case-cohort design was adopted to investigate the order of infections and risk of high-grade cervical precancer. The sample pair cohort and the subcohort of the FMC were not as such eligible for a study with incidence of cervical precancer as the outcome. In this study, cohort members were women instead of sample pairs. Follow-up of a given woman for cervical precancer started at the beginning of the month directly after the date of the second serum withdrawal. To be at risk during follow-up, a woman must not have emigrated from Finland, needed to have a Finnish personal identification number and cervix uteri without a former diagnosis of cervical precancer or cancer. In other words, follow-up ended at diagnosis with cervical precancer or just before censoring due to hysterectomy, emigration, diagnosis with cervical cancer, death or the common closing date of 31 December 2004. For studies with incidence of cancer or precancer as outcome, time since the serum withdrawal until diagnosis is usually maximized, *i.e.* the first sample pair of a given woman was preferred.

A starting point for a cohort was the 97 124 women of the sample pair cohort and their records related to the first sample pair. A total of 2385 women were excluded because they had donated the second serum sample in December 2004 or later. According to the linkage to the cancer register, 347 women had to be excluded because they were diagnosed with cervical precancer or cancer before (or during the same month and year) donating the second serum sample. Linkage to the Population Information System (for details of the register, see the Technical Appendix 1) revealed that 42 women had emigrated before the second serum sampling, and thus, were excluded (they could not be followed for CIN 3/AIS during their stay abroad). One woman was excluded, as her samples were not withdrawn within five years. The final cohort size was 94 349 women. The subcohort size decreased mainly due to exclusion of the second and third serum sample pairs from 3569 women to 2796 women. The first and second available/eligible samples were not necessarily related to the woman's first and second pregnancy. A third of the cohort members reported at the maternal welfare clinic that the pregnancy is the third or later in connection with donating the second serum sample of the sample pair. About 13% of the women had given birth to at least two children before withdrawal of the second serum sample.

Identification of cases

Linkage of the Finnish Maternity Cohort and the Finnish Cancer Registry, permitted by the Ministry of Health, identified 516 cervical precancer or cancer cases diagnosed after the second serum sampling by the end of the year 2004. One case had emigrated before diagnosis, and was excluded before emigration. There were 490 cases, of whom 474 had CIN 3 and 16 AIS diagnosis. Twenty-three were members of the subcohort and were included in the analysis. Twenty-five women were diagnosed with invasive cervical carcinoma. Among the FMC donors, the SIR for CIN 3/AIS was 1.07 (1.04–1.09) (Pukkala 2011). Closing year of the estimation was 2005.

Follow-up of the study cohort

Finally, the study cohort was linked to the care register for hysterectomies (for details, see Technical Appendix 1). Follow-up of 490 women ended at diagnosis with CIN 3/AIS or due to censoring just before the closing date (93 387 women), hysterectomy (261 women), emigration (96 women), death (90 women) or diagnosis with invasive cervical carcinoma (25 women). The women were followed on average for 4.8 years, and the average time between sample withdrawals was 2.4 years. Thus, there was an average of six years since a possible seroconversion until the end of follow-up. Total follow-up time in the full cohort was approximately 450 000 woman-years.

Strata

The case-cohort design was confounder-stratified. In other words, stratification variables were confounders, and the sampling strata corresponded to the model strata. The strata were defined by age and calendar period at the midpoint of consecutive pregnancies, and in most of the material the pregnancies were the woman's first and second. The range of stratum-specific numbers of cohort members was 1746–18 936 and of subcohort members 140–366. The range of sampling fractions was 0.02–0.11. There were 1 to 140 cases per cohort stratum.

4.6 LABORATORY METHODS

4.6.1 SEROLOGY (I/II, III, V)

All serological analyses were performed on coded specimens.

HPV serology (I/II, III, V)

Immunoglobulin G (IgG) antibodies specific for oncogenic types (16 and 18, and in Studies I/II and V 33, and in Study V 31 and 45) and non-oncogenic types (6 and in Studies I/II 11) of HPV were determined by a standard ELISA using baculovirus-expressed HPV capsids (Kirnbauer *et al.* 1994). VLPs were kindly provided by Drs. B. Colau and F. Dessy (GlaxoSmithKline Biologicals, Rixensart, Belgium), Dr. J. Dillner (Karolinska Institute, Sweden), Dr. K. Jansen (Merck Research Laboratories, Philadelphia, PA) and Dr. R. Kirnbauer (University of Vienna, Austria). The cutoff levels for seropositivity had been validated in previous studies (Dillner *et al.* 1997, Silins *et al.* 1999, Laukkanen *et al.* 2003).

In Study V, the first and second subcohort samples were analysed for HPV16 at different times. Due to a technical error in the analyses, the HPV16 results of the first samples were excluded. The second serum samples of cases and subcohort members were analysed for HPV16 at the same time. The case and subcohort samples were analysed for the other HPV types at different times, and assay drift was imminent. The interval, (cut-off level − standard deviation, cut-off level + standard deviation) was considered a grey zone. The standard deviation was calculated for the absorbance values below cut-off level. Absorbance value below or above the grey zone was considered seronegative or seropositive, respectively. For further details of the grey zones, see Technical Appendix 3. Seroconversion was observed, if the first sample was seronegative and the second one seropositive. Two infections were considered overlapping and/or virtually concomitant if their seroconversions were observed between the first and second serum samplings.

C. trachomatis and HSV-2 serology (I/II, III, V)

In Studies I/II, IgG antibodies specific for *C. trachomatis* were determined by the microimmunofluorescence method (Wang and Grayston 1970). Elementary bodies of serovar pools B-group (B-E-D), C-group (C-H-I-J) and an intermediate group (G-F-K) were used as antigens (Washington Research Foundation, Seattle, WA). In Studies III and V, IgG antibodies were determined by a major outer membrane protein-derived peptide ELISA using a commercial kit (Labsystems, Helsinki, Finland) (Anttila *et al.* 2001). Titres of 1 to 16 or more were considered positive for *C. trachomatis*.

In Study II, a commercially available HSV-2 glycoprotein G-2 ELISA (Biokit SA, Barcelona, Spain) was used to determine IgG antibodies to HSV-2 according to the manufacturer's recommendations.

Cotinine serology (I/II, III)

Serum cotinine was measured by radioimmunoassay in Studies I/II (Parish *et al.* 1995). Cotinine measurements by radioimmunoassay and gas chromatography are in good agreement, although for samples containing high levels of cotinine radioimmunoassay tends to give higher results (Anderson *et al.* 1991). A serum cotinine level of 20 ng/mL and above was considered to indicate active smoking. In Study III, cotinine was measured using a competitive semiquantitative immunoassay method (OraSure Technologies, Bethlehem, PA). The correlation between the results of this and the gas chromatometric method was 95% across the range of 0–20 ng/mL (Boffetta *et al.* 2006). The cotinine levels were categorized into groups, less than 20 ng/mL for non-smokers or persons passively exposed to tobacco smoke, 20–100 ng/mL for light smokers and >100 ng/mL for heavy smokers (Kapeu *et al.* 2009).

4.6.2 DNA MEASUREMENT (II, III, IV)

HPV DNA measurement (II–IV)

In Study II, DNA was extracted from formalin-fixed, paraffin-embedded biopsy specimens containing cancer lesions from 133 cervical cancer cases (Sigstad *et al.* 2002). Six cases were excluded from the HPV-PCR because the quality of DNA extracts was not sufficiently good. In Study III, biopsies without CIN 3 or ICC and non-amplifiable, beta-globin gene-negative biopsies were excluded from the DNA analysis. In Study IV, DNA was extracted from Pap smears and biopsies using methods described by Chua and Hjerpe (1995, 1996) and Wallin *et al.* (1999). The diagnostic biopsy was eligible for PCR analysis in Study IV only if the paraffin block contained cancer lesions. An empty paraffin block was sectioned between each biopsy specimen to prevent cross-contamination. Knives were changed or the microtome cleaned thoroughly between each biopsy. In Study IV, PCR using human ribosomal gene S14 primers was used to test the quality of the DNA extracts.

The biopsies were tested for HPV DNA by PCR using the GP5+/6+ primer system (de Roda Husman *et al.* 1995, Jacobs *et al.* 1997). In Study II, specimens were typed with E6- and E7-derived, type-specific primers for HPV types 16, 18 and 33 (Lie *et al.* 1999). In Study III, 13 hrHPV types were measured by enzyme immunoassay and reverse dot blot hybridization (Söderlund-Strand *et al.* 2005) or a multiplex fluorescent bead–based assay (Schmitt *et al.* 2006). In Study IV, HPV types 16 and 18 were measured by type-specific PCR and types 16, 18, 31, 33 and 73 by DNA sequencing (Wallin *et al.* 1999).

Chlamydia trachomatis DNA measurement (IV)

PCR analysis for *Chlamydia trachomatis* was performed using a COBAS AMPLICOR™ CT test (Roche Molecular Diagnostics, Branchburg, NJ) with primers common to all serovars of *C. trachomatis*. Positive results were measured according to the manufacturer's instructions, including negative and positive controls. The test is highly sensitive and reproducible (Vincelette *et al.* 1999). PCR analyses were performed blinded to case-control status.

4.7 STATISTICAL ANALYSES

4.7.1 REGRESSION

Asymptotic logistic regression (I, II, III)

Exposure odds ratios were estimated by conditional logistic regression with EGRET software (Statistics and Epidemiology Research Corporation, Seattle, WA) in Study I, GLIM 4 software (Numerical Algorithms Group, Oxford, UK) in Study II and SAS version 9.1 software (SAS Institute Inc., Cary, NC) in Study III (Breslow and Day 1980). These are valid and efficient estimates of incidence rate ratios and hereafter will be referred to as *rate ratios* (RRs). The 95% confidence intervals (CIs) for the RRs were based on a Wald-type statistic in Study I and on profile likelihood in Study II (Nelder 1990). The RRs for HPV16 in strata containing incomplete case-control sets were estimated by unconditional logistic regression in Study II. In Study III, the 95% confidence limits for parameters of the conditional analyses were based on a Wald-type statistic and those of the unconditional analyses on profile likelihood.

The RRs were adjusted for other HPV types, smoking indicated by high levels of serum cotinine, and *C. trachomatis* in Study I and also for HSV-2 in Study II. In Study III, models were adjusted for HPV16, HPV18, *C. trachomatis* and cotinine. Analyses restricted to strata, HPV16 seronegatives, HPV16 seropositives, HPV18 seronegatives, HPV18 seropositives, were conducted by unconditional logistic regression adjusting the RR estimates for the other hrHPV, cotinine and matching variables, year of age, calendar year, country, county of Norway and Norwegian Red Cross blood donor status.

A model (1) with HPV16 and a model (2) with different parameters for the effect of HPV16 in the absence and the presence of a possible effect modifier, HPV 6 or 11 (6/11), were used to test for effect modification in Study I. No independent effect on risk was assumed for the effect modifier in model 2. The difference between the likelihood ratio statistics related to the hierarchical models 1 and 2 with one degree of freedom served as a test statistic for effect modification.

Exact logistic regression (IV)

Exposure odds ratios and 95% confidence intervals were estimated by exact conditional logistic regression with LogXact 4 software (Cytel, Cambridge, MA). As valid and efficient estimates of incidence rate ratios, they are referred to as *rate ratios*. If the conditional likelihood function could not be maximized in estimation of the point estimates, the less reliable median unbiased estimates were reported (Hirji *et al.* 1989). The confidence intervals are guaranteed to have at least 95% coverage for the point estimate.

Proportional hazards regression (V)

Rate ratios (RRs) and variances were estimated fitting pseudolikelihood by a Cox model with different strata-specific baseline hazards using SAS version 9.2 (SAS Institute Inc, Cary, NC) and macros written by Bryan Langholz (Langholz and Jiao 2007). Model strata corresponded to the sampling strata. Subcohort members, both cases and non-cases, enter at the beginning of the month immediately after second serum withdrawal and exit just before actual exit time as censored observations. Cases, both subcohort and non-subcohort, enter just before diagnosis and exit at diagnosis. Thus, subcohort cases contribute two rows to the analytic data set. Non-subcohort cases contribute to the study only at diagnosis. The pseudolikelihood method used exact pseudolikelihood estimator (Langholz and Jiao 2007). The estimator is score-unbiased, *i.e.* conditional expectation of the score from the estimator is zero. Simulation studies have shown that the estimator has optimal small-sample properties (Cologne *et al.* 2012). The estimator adjusts biased sampling due to over-sampling of cases. Both asymptotic variances based on the pseudo-score and robust 'sandwich'-type variances were estimated. The variance estimators are asymptotically equivalent (Langholz and Jiao 2007). Confidence (95%) intervals based on the asymptotic variance were reported, although those based on robust variance were also valid in this study. The confidence intervals based on the robust variance tended to be narrower. HPV16 seropositivity at the second serum sample was adjusted.

4.7.2 INTERACTION ANALYSES (I, II, III)

Statistical interaction of two risk factors requires departure from additivity in their effect on outcome (Berrington de González and Cox 2005). A specific type of interaction does *not* occur if the separate effects of the risk factors combine additively. The two most popular types of statistical interactions are additive interaction and multiplicative interaction. Studying multiplicative interaction is equivalent to studying additive interaction on the logarithmic risk scale. Statistical interaction is a non-additivity (Berrington de González and Cox 2007).

For simplicity, let us assume that two risk factors, *X* and *Z*, have two possible values, exposed (*x* and *z*) and not exposed ($\bar{x}$ and $\bar{z}$), respectively. In an additive model, the *excess rate ratio* for the additive joint effect is a sum of excess rate ratios for exposure to *X* in the absence of exposure to *Z* and for exposure to *Z* in the absence of exposure to *X*: $RR_{xz} - 1 = (RR_{x\bar{z}} - 1) + (RR_{\bar{x}z} - 1)$. Adding 1 to both sides gives the rate ratio for additive joint effect

(1) $$RR_{xz} = RR_{x\bar{z}} + RR_{\bar{x}z} - 1.$$

When studying the additive interaction, this is the expected RR for the additive joint effect to be compared with the observed RR for the joint effect. For brevity, it will be called *expected additive RR*.

In a multiplicative model, the rate ratio for the multiplicative joint effect is a product of rate ratios for exposure to *X* in the absence of exposure to *Z* and for exposure to *Z* in the absence of exposure to *X*, $RR_{xz} = RR_{x\bar{z}}\, RR_{\bar{x}z}$. When studying the multiplicative interaction, this is the expected RR for the multiplicative joint effect to be compared with the observed RR for the joint effect. For brevity, it will be called *expected multiplicative RR*. The rate ratios in the product above are solitary or separate effects.

A positive deviation from the expected joint effect indicates that exposures intensify each other's effects on the risk of disease, *i.e.* they *act synergistically* on the risk of disease. A negative deviation indicates that the exposures diminish each other's effects on the risk of disease, *i.e.* they *act antagonistically* on the risk of disease. When there is no deviation, the exposures act independently on the risk of disease.

The confidence limits for the expected additive RR were approximated by the delta method on the RR estimates. The confidence limits for the expected multiplicative RR were approximated by the delta method on the logarithms of the RR estimates. The P value for testing the null hypothesis, no additive interaction, against the alternative hypothesis, additive interaction, was obtained from standardized normal distribution using the difference between the observed and expected RRs for the joint effect divided by the standard error of the difference as a test statistic for a two-sided test. In Study III, additive interaction was detected by relative excess risk due to interaction and 95% CI, which were estimated according to Hosmer and Lemeshow (1992). Testing the null hypothesis, no multiplicative interaction of exposures, against the alternative hypothesis, multiplicative interaction of exposures, was carried out with likelihood ratio test to compare two nested models, one for the main effects only and the other including also an interaction term, by considering the difference between the model-specific scaled deviances.

In the model for estimation of the observed joint effect of two binary exposure variables *X* and *Z* on the risk of cervical neoplasia, there was always a variable with four categories, a reference category of no exposure to *X* and *Z*, a category of exposure to *X* and no exposure to *Z*, a category of no exposure to *X* and exposure to *Z* and a category of exposure to both *X* and *Z*. Variables to be adjusted were included in the model. The expected values for assessing additive and multiplicative

interaction were derived from the same model for estimating rate ratios for cervical neoplasia. The model required no *a priori* choice of the type of interaction to be studied.

Absence of a statistical interaction does not imply that there is no biological interaction. Statistical interaction refers to variation in a population measure of effect, whereas biological interaction refers to effects in individuals (Pearce and Greenland 2014).

4.7.3 SERIAL SAMPLE ANALYSES (IV, V)

In Study IV, the rate ratios for ICC related to baseline *C. trachomatis* DNA status were estimated by *lag time*, time from the case's prediagnostic smear to diagnostic biopsy, with lag up to three years as the reference category. The joint effects of transient (positive-negative), incident (negative-positive) and persistently positive combinations of HPV DNA and *C. trachomatis* DNA on risk of ICC were studied with persistently HPV DNA-negative and *C. trachomatis* DNA-negative women as reference category.

In Study V, the risk of CIN 3/AIS related to seroconversion of one or two infections was evaluated with dual seropositivity at first serum sampling as the reference class. Also seropositivity at first sampling of one infection and seroconversion related to another infection was compared with a situation in which the infections were in the reverse order. The magnitude of risk was illustrated with persistently double seronegative women as a reference class. HPV types 18 and 45 were combined, as observations in some single type categories were lacking and the type.wise results did not differ considerably.

The proportion of missing observations in each analysis was 14% in the subcohort and 12% in cases. All laboratory results were missing from 8% of the subcohort members and 3% of the cases.

4.7.4 IMPUTATION (II)

The serum cotinine level of four cases and three controls was missing. The missing values of smoking can be imputed (replaced) by 128 different combinations of values for smokers or non-smokers. One of the combinations is correct. Rate ratios for the joint effects of interest were estimated for the non-imputed data set and for the 128 differently imputed data sets. The RR estimate for the joint effect of HPV16 and HPV6/11 among non-imputed data did not lie within the range of the corresponding results from the imputed data sets. It was necessary to impute the missing values of smoking. For each participant, whose information about smoking was lacking, a subset of more than 20 controls was formed, with age at and year of serum sampling centred on those of the participant. A pseudo-random number in the range of 0–1 was generated. If the pseudo-random number was smaller than the

smoking prevalence in the control subset, the participant received a smoker status. Otherwise, the participant was given a non-smoker status.

4.7.5 CORRECTION FOR MISCLASSIFICATION (I, II)

Back-calculation (I)

Non-differential misclassification bias in the RR for the joint effect of HPV16 and HPV6/11 was assessed using frequencies of misclassification-corrected joint distributions of seropositivity for HPV16 and HPV6/11. Misclassification was adjusted by deriving the frequencies by back-calculation. Misclassification-corrected joint distributions were calculated using the observed joint distributions of these HPV types separately among SCC cases and their controls and assuming different levels of probabilities, sensitivity (S_{16} and $S_{6/11}$), specificity (W_{16} and $W_{6/11}$) and cross-reactivity ($P_{16|6/11}$ and $P_{6/11|16}$) for the HPV16 and HPV6/11 ELISA methods (T_{16} and $T_{6/11}$). The *cross-reactivity*, $P_{16|6/11}$, is here the probability that a case or control is classified as HPV16-seropositive by the T_{16} method because of antibodies induced by HPV6/11 infection. Cases and controls were assumed to have equal values of sensitivities, specificities, and cross-reactivities because non-differential misclassification bias was considered.

The following definitions and assumptions were made for the calculations: $T_{6/11}$ identifies true HPV6/11-seropositives with sensitivity, $S_{6/11}$. $T_{6/11}$ misclassifies true HPV6/11-seronegatives as HPV6/11-seropositives with complement probability of specificity, $1 - W_{6/11}$. $T_{6/11}$ classifies HPV6/11 seropositivity of HPV16 antibodies with cross-reactivity, $P_{6/11|16}$, equally likely whether or not the HPV6/11 antibodies are present. The probabilities of correct and misclassified determination of HPV6/11 seropositivity are:

$$\Pr(\text{observe HPV6/11+} \mid \text{true HPV6/11+ \& HPV16+}) = S_{6/11} + (1 - S_{6/11})P_{6/11|16},$$
$$\Pr(\text{observe HPV6/11+} \mid \text{true HPV6/11+ \& HPV16–}) = S_{6/11},$$
$$\Pr(\text{observe HPV6/11+} \mid \text{true HPV6/11– \& HPV16+}) = 1 - W_{6/11}(1 - P_{6/11|16}),$$
$$\Pr(\text{observe HPV6/11+} \mid \text{true HPV6/11– \& HPV16–}) = 1 - W_{6/11}.$$

The probabilities for observing HPV6/11 seronegativity given the true or misclassification-corrected event are complement probabilities of those given above. In the probability formulae for observing HPV16 seropositivity, 6/11 and 16 are interchanged in the subscripts. It was further assumed about the tests that $T_{6/11}$ succeeds or fails in identification of the HPV6/11 antibodies whether or not T_{16} succeeds or fails to identify HPV16 antibodies, and *vice versa*. It was assumed that $T_{6/11}$ and T_{16} have equal sensitivity and specificity and that the cross-reactivities $P_{6/11|16}$ and $P_{16|6/11}$ are equal, but these assumptions are not necessary for the calculations.

As we are evaluating the joint effect of two HPV types, 16 and 6/11, having two levels of seropositivity, seronegative and seropositive, there are four *misclassification-corrected frequencies* of interest: numbers of seronegatives for both types, seropositives for type 16 only, seropositives for type 6/11 only, and seropositives for both types. All of these frequencies have an effect on all four observed frequencies, numbers of seronegatives for both types, seropositives for type 16 only, seropositives for type 6/11 only, and seropositives for both types, unless S_{16}, $S_{6/11}$, $W_{6/11}$ or $W_{6/11}$ is equal to 0 or 1, or $P_{16|6/11}$ or $P_{6/11|16}$ is equal to 1. A set of four equations has to be solved. On the left-hand side of the equations there is the sum of unknown misclassification-corrected frequencies, each of which is multiplied by a coefficient in the range of 0–1. The coefficients of each misclassification-corrected frequency sum up to 1 in the set of equations. Observed frequencies are on the right-hand side of the equations. There is one solution because the number of equations is equal to the number of unknowns. The coefficients come from 4-by-4 observed-by-misclassification-corrected matrix. For example, the coefficient of misclassification-corrected frequency of those HPV16+ and HPV6/11– to calculate the observed frequency of HPV16+ and HPV6/11– is a product of conditional probabilities assumed to be independent.

Pr(observe HPV16+ & HPV6/11– | true HPV16+ & HPV6/11–) =
Pr(observe HPV16+ | true HPV16+ & HPV6/11–) •
Pr(observe HPV6/11– | true HPV16+ & HPV6/11–) = $S_{16}W_{6/11}(1 - P_{6/11|16})$.

To calculate the misclassification-corrected rate ratio for the joint effect, the set of equations of both cases and controls has to be solved for given values of sensitivity, specificity and cross-reactivity. The solutions are not acceptable if there is a negative misclassification-corrected frequency. In such a case, the combination of sensitivity, specificity and cross-reactivity is not possible if the other assumptions made are correct.

An error in the numerical example in the Appendix of Study I has been corrected in the Errata.

Gold standard (II)

Misclassification of HPV serology was assessed by assuming a gold standard of OR for SCC related to HPV16 seropositivity in Study II, as described by Hakama *et al.* (2000). The gold standard, OR = 20, was based on comparable, systematically reviewed follow-up studies with PCR-based diagnosis of HPV16 infection (Lehtinen *et al.* 2001). As in those studies, ORs were valid and efficient estimates of incidence rate ratios, the gold standard of OR is referred to as the *gold standard of RR*. All acceptable specificity and sensitivity combinations consistent with the observed RR given the gold standard of RR were estimated. Cross-reactivity was not considered. Sensitivity analyses with RR from 12 to 50 as alternatives to gold standard of RR were also carried out.

The following set of equations was solved in search of a solution for misclassification-corrected frequencies:

$n_1 = S(n_{11} + n_{01}) + (1 - W)(n_{10} + n_{00})$
$n_{11} = S(n_{11} + n_{01})$
$n_1 = n_{10} + n_{11}$
$n_0 = n_{01} + n_{00}$.

Frequencies, n_1 and n_0, are observed numbers. In the subscript, 1 refers to HPV16 seropositives and 0 to HPV16 seronegatives. The frequencies with two digits in the subscript are unknown. The first digit refers to observed and the second to true HPV16 seropositivity status. Thus,

n_{11} = number of true HPV16 seropositives = n(observe HPV16+ & true HPV16+),
n_{10} = number of false HPV16 seropositives = n(observe HPV16+ & true HPV16–),
n_{01} = number of false HPV16 seronegatives = n(observe HPV16– & true HPV16+),
n_{00} = number of true HPV16 seronegatives = n(observe HPV16– & true HPV16–).

For a given value of sensitivity, *S*, a value of specificity, *W*, was numerically solved with a precision of up to 0.01%, or *vice versa*, which resulted in misclassification-corrected non-negative frequencies of cases and controls and misclassification-corrected RR of 20. For the analyses, all four observed cell frequencies of the case-control status-by-HPV16 seropositivity table, sensitivity and specificity were required.

The procedure was repeated in subgroups defined by the other risk factors being examined, *i.e.* in those who were *C. trachomatis*-seropositive or *C. trachomatis*-seronegative, in smokers or non-smokers or in HPV6/11-seropositive or HPV6/11-seronegative subjects. For graphical presentation, several sensitivity-specificity pairs or sensitivity-(1–specificity) pairs on an equipotential curve (for example, RR = 20) were calculated.

Statistical variability was taken into account by repeating the analyses with upper and lower 95% confidence interval limits of the estimated RRs and assuming fixed marginal frequencies of the 2-by-2 tables describing the association between HPV16 seropositivity and case-control status in the subgroups defined by measurements for *C. trachomatis* and smoking, or *C. trachomatis* and HPV6/11. For the analyses, four hypothetical cell frequencies of the case-control status-by-HPV16 seropositivity table, consistent with the confidence limit, sensitivity and specificity were required.

Inferences applied to the curves defined by the point estimates of RRs and the areas of admissible values defined by confidence intervals of RRs were similar. Any combination of specificity and sensitivity of the HPV16 antibody assay common to the subgroups, *C. trachomatis* seronegative and -positive or HPV6/11 seronegative and -positive, was taken as an indication of the possibility of non-differential misclassification bias to account for the observed interactions.

Differential misclassification of HPV16 serology due to *C. trachomatis* or HPV6/11 could have taken place if there were non-identical different subgroup-

specific combinations of sensitivity and specificity of HPV serology consistent with the difference between the estimated RR and the gold standard. A true interaction was regarded as a plausible explanation only if no combination of sensitivity and specificity of HPV serology could account for the difference between the observed RR and the gold standard, and if the assumed value of the gold standard is correct. However, it is possible that differential misclassification bias other than the type described above could account for the difference between the observed RR and the gold standard.

5 RESULTS

The main focus of this thesis is the *joint effects*. Three types of joint effects on risk of developing cervical neoplasia were studied. First, the joint effects of different human papillomaviruses, second, the joint effects of human papillomaviruses and *Chlamydia trachomatis*, and, third, the effects related to the order of these infections as compared with their reverse order, and other reference groups were investigated.

5.1 JOINT EFFECTS OF DIFFERENT HPVS ON RISK OF DEVELOPING CERVICAL NEOPLASIA

In Study I, the joint effects of prediagnostic type-specific HPV seropositivities on the risk of cervical neoplasia were smaller than expected on a multiplicative basis. The joint effect of seropositivity to HPV16 and HPV6/11 was antagonistic both for ICC and SCC. While the corresponding expected multiplicative RRs were 5.4 and 12, the observed RRs: for ICC 0.7 (95% CI, 0.2–2.5) and for SCC 1.0 (95% CI, 0.3–4.0), were significantly lower. All of the misclassification-corrected estimates (applying different levels of sensitivity, specificity and cross-reactivity for HPV serology) for SCC were more antagonistic. In addition to the hypothesis of no deviation from the multiplicative joint effect (no multiplicative interaction), another hypothesis, no effect modification by HPV6/11 was also rejected, and the antagonistic interaction was verified. The hypothesis, no multiplicative interaction, was also rejected in case of the antagonistic joint effects of HPV16 and HPV18 seropositivity on ICC and SCC risk and HPV16 and HPV33 seropositivity on SCC risk.

Also after re-examination of histology, in Study II, the joint effect of seropositivity to HPV16 and HPV6/11 on SCC risk was antagonistic. The RR of the observed effect of joint seropositivity was 1.1 (95% CI, 0.3–3.1), while the expected multiplicative RR was 8.7 and additive RR 5.3. Furthermore, although the joint effect of HPV16 and HPV6/11 on the risk of HPV16 DNA-positive SCC was slightly increased (RR, 1.9; 95% CI, 0.4–7.8), the hypothesis of no multiplicative interaction was again rejected, and the HPV16 and HPV6/11 antagonism verified.

In Study III, the RR for the joint effect of HPV16 and HPV6 seropositivity was increased, 2.4 (95% CI, 1.7–3.4), but it was significantly smaller than the expected multiplicative RR, 5.8. The estimate of relative excess risk due to interaction suggested a negative additive interaction, *i.e. antagonism*. There was neither increased nor decreased risk of HPV16 DNA-positive ICC among HPV18 seropositives. This was true also for HPV18 DNA-positive ICC among HPV16 seropositives.

5.2 JOINT EFFECTS OF HPVS AND *CHLAMYDIA TRACHOMATIS* ON RISK OF DEVELOPING CERVICAL NEOPLASIA

In Study II, the joint effect of HPV16 and *C. trachomatis* seropositivity on the risk of SCC was antagonistic. The hypotheses of no multiplicative interaction and no deviation from the additive joint effect (no additive interaction) between HPV16 and *C. trachomatis* were rejected in both the SCC and HPV16 DNA-positive SCC materials. Neither among *C. trachomatis* seropositives nor among HPV6/11 seropositives no combinations of sensitivity and specificity in the HPV16 antibody assay could account for the difference between the observed 0.6-fold HPV16-associated RRs and gold standard RR = 20. The *C. trachomatis*-associated RR was significantly increased among HPV16 seronegatives both for SCC and for HPV16 DNA-positive SCC.

In Study III, the *Chlamydia trachomatis*-associated risk of SCC was significantly increased among HPV18 seropositives (RR, 2.1; 95% CI, 1.2–3.7), but not among HPV16 seropositives (RR, 1.2; 95% CI, 0.8–1.8). The RR for joint effect of seropositivity to HPV16 and *Chlamydia trachomatis* on the risk of SCC was increased, 4.7 (95% CI, 3.3–6.7), but smaller than the expected multiplicative RR, 9.5. The joint effect of HPV18 and *C. trachomatis* on the risk of SCC was multiplicative, the observed and expected multiplicative RRs were equal to 2.8 (Table 3). Seropositivity for HPV18 was associated with significantly increased risk of SCC only if the women were also seropositive for *C. trachomatis*.

Table 3. Adjusted[1] rate ratios (RRs) and 95% confidence intervals (CIs) for squamous cell carcinoma of the cervix uteri in relation to seropositivity for HPV18 and/or *C. trachomatis* in a cohort of more than 900 000 women who donated serum samples to Nordic biobanks in 1973–2002.

Seropositivity for		Number of		RR	95% CI
HPV18	***C.trachomatis***	**Cases**	**Controls**		
Seronegative	Seronegative	197	1413	1	
Seronegative	Seropositive	163	527	1.9	1.5–2.5
Seropositive	Seronegative	31	129	1.4	0.9–2.2
Seropositive	Seropositive	58	91	2.8	1.9–4.1

[1]Adjusted for HPV16 and cotinine.

In Study IV, clearance of HPV DNA and/or *C. trachomatis* DNA positivity, as compared with persistent HPV DNA and *C. trachomatis* DNA negativity was non-significantly associated (RR, 15; 95% CI, 0.8–1500) with the risk of ICC (Table 4). Incident infection with either HPV or *C. trachomatis* by diagnosis was highly significantly associated with increased risk of ICC (RR, 110; 95% CI 17–4900). Persistent DNA positivity including subsequent/concomitant DNA positivity for HPV and/or *C. trachomatis* by diagnosis was extremely significantly associated with increased risk of ICC (RR, 220; 95% CI, 21–15 000). There were not enough *C. trachomatis* DNA-positives for a more detailed study of the joint effects.

Table 4. Rate ratios (RRs) and 95% confidence intervals (CIs) for invasive cervical carcinoma in relation to the presence of HPV DNA or *C. trachomatis* DNA in prediagnostic cytological smears or cervical biopsies donated by Swedish women from Västerbotten county in 1969–1995.

HPV DNA and *C. trachomatis* DNA positivity		Number of		RR[2]	95% CI
Prediagnostic phase smear	Diagnostic phase biopsy/smear[1]	Cases	Controls		
Both negative	Both negative	19	98	1	
One or both negative	Both negative	4	2	15	0.8–1500
Both negative	One or both negative	46	3	110	17–4900
One or both negative	One or both negative	35	1	220	21–15 000

[1]Case: diagnostic phase biopsy; Control: smear taken after the case's biopsy.

[2]Exact conditional logistic regression, frequency-matched.

In Study IV, the HPV DNA-adjusted RR, 17, for ICC associated with *C. trachomatis* DNA in prediagnostic smear was statistically significant, 95% CI (2.6–∞). Nine out of ten *C. trachomatis* DNA-positive cases at prediagnostic smear-taking were HPV DNA-negative. All of the controls were *C. trachomatis* DNA-negative. *C. trachomatis* DNA positivity was associated with long and HPV DNA positivity with short lag between the prediagnostic smear and cancer diagnosis. None of the cases were *C. trachomatis* DNA-positive in both smear and cancer biopsy.

5.3 ORDER OF DIFFERENT CARCINOGENS IN THE DEVELOPMENT OF CERVICAL NEOPLASIA

In Study V, paired prediagnostic (CIN 3) serum samples from a sizeable case-cohort material were analysed for HPV and *C. trachomatis* antibodies. The risk for CIN 3/AIS was very high (RR, 28; 95% CI, 4.3–190) among women who were infected with and seroconverted for *C. trachomatis* and HPV18/45 within approximately 2.4

years and on average 6 years before diagnosis, as compared with women seropositive for *C. trachomatis* and HPV18/45 already at first serum sampling. Their risk was even higher (RR, 96; 95% CI, 15–610) as compared with women seronegative for all three infections at both samplings.

The risk for CIN 3/AIS associated with persistent HPV6 seropositivity before HPV31 seroconversion was low (RR, 0.4; 95% CI, 0.0–4.4) compared with women seropositive for both HPV6 and HPV31 already at first serum sampling. By contrast, the risk associated with persistent HPV31 seropositivity before HPV6 seroconversion was high (RR, 10; 95% CI, 1.8–57). The RR related to HPV6 seropositivity before HPV31 seroconversion compared with HPV31 seropositivity before HPV6 seroconversion was very low, 0.04, and highly significant (95% CI, 0.003–0.7).

6 DISCUSSION

The objectives of this PhD thesis are in line with the original registered objectives for the doctoral thesis of 24 August 1998. The manuscript of Study I on the antagonistic joint effect of past infections with the oncogenic and non-oncogenic HPV types on risk of subsequent development of invasive cervical cancer was already available, as was the plan for a larger study (III). Study III was initiated to gain more power for stratified and interaction analyses. One of the objectives of Study III was to investigate whether the new finding, benign HPV infections protecting against invasive cervical carcinoma, would emerge again in an independent material.

The joint effect analyses of past HPV and *C. trachomatis* infections on the risk of invasive cervical cancer for Study II were ongoing at the time of the thesis registration. The original study plan covered two studies utilizing nested case-control design in a serial setting, one for evaluating the effects of incident and persistent HPV infections over time and the other the solitary and joint effects of incident infections with different HPV types on risk of developing CIN 3+. Studies IV and V concentrated not only on HPVs but on the joint effects of hrHPVs and *C. trachomatis* in a longitudinal/serial sample setting. The case-cohort design was introduced to Study V, as compared with nested case control design in the original plan, to better fulfill the original objectives.

The study bases represent Nordic women over four decades (1973–2004), but only the years 1992–1994 were common to all studies. Thus, the population-based nature of the utilized biobanks, except for the Red Cross blood donors from Oslo, a sub-bank of the Janus Serum Bank, was important. According to standardized incidence ratios, among the biobank donors the incidence of cancer of all sites combined was not higher than among a comparable group in the national population with the same age and gender distribution by calendar time. This means that the study bases represent a healthier portion of the target populations. Representativeness of the study base to the target population is, however, not necessary for studies with a scientific objective of understanding a phenomenon (Rothman *et al.* 2013, Ahrens *et al.* 2014). Three maternity cohorts contributed to the studies. Only a small proportion of pregnant women did not donate pregnancy-related serum samples to the maternity cohorts. Also the other biobanks stored serum/blood samples from projects in which participation was high. In each study, all or the majority of women were of reproductive age at the time of the first serum sampling or smear-taking. The population-based cytological screening programme of Västerbotten was optimal for Study IV, as all of the smears and histological specimens were recorded and stored in one place.

Most biobanks stored serum samples in –20˚C or –25˚C. Gislefoss *et al.* (2009) reported that immunoglobulin G is relatively stable in the Janus Serum Bank material and observed a non-significant difference in IgG concentrations with respect to storage time.

The Nordic cancer registries are nationwide and registration of invasive cervical carcinoma is complete or almost complete. The primary basis of diagnosis is morphology, and almost all invasive cervical carcinomas are morphologically verified. In the Finnish cancer register, CIN 3 registration is 80% complete. In Studies II–IV, histological slides and/or cancer tissues were re-examined for confirmation of cancer diagnosis. This made histology more comparable between the countries and over the long study periods. Unfortunately, Icelandic legislation forbade exporting the slides and tissues.

Linkages between cancer registers and biobanks and other registers were done utilizing unique personal identification numbers. The most remarkable obstacle to linkage for Studies I/II was that the cases among donors of 120 000 (28%) Janus Serum Bank samples could not be identified due to incorrect labelling. In Study III material, there are 34 SCC cases from the Janus health examinations sub-bank, with diagnoses made before the year 1992. We checked whether moving the matched sets of these cases to Study II material would cause major changes in the joint effect results of Studies II and III. Only minor changes in the estimates were observed.

During follow-up all women had to be at risk of developing invasive cervical carcinoma or, in Study V, CIN 3/AIS. As almost all cases were of working age at diagnosis, mortality among the controls before the case's diagnosis was rather low. In Studies I/II, it was not necessarily checked in all countries whether controls were alive or had emigrated before the case's diagnosis. Emigrated Icelandic women did not contribute to Study III, as the date of emigration was not available at the biobank. In Studies I/II and III, we did not check whether women had unoperated cervix uteri. In Study IV, women whose cervix uteri were operated did not contribute to the control series. This is reasonable, as the controls had to be at risk beyond the case's diagnosis. In Study V, women were at risk until the event which occurred first, diagnosis with CIN 3/AIS or ICC, death, emigration, hysterectomy or end of study at a common closing date. Studies I/II were based on the largest follow-up study incidence of invasive cervical carcinoma as outcome in the 1990s (Dillner *et al.* 1997). Study III was more than threefold larger than Studies I/II, with a twofold longer average follow-up time of almost ten years.

Age at serum sampling or baseline smear-taking, storage time or date of smear-taking, and country as well as alive status at the case's diagnosis were matching variables common to all the nested case-control studies, I–IV. In the seroepidemiological part of Studies I/II and III, the analytic batch was matched such that serum aliquots of a case and its controls were located on the same plate.

Matching is more detailed in the Janus Serum Bank material than in the other biobanks. County is matched in Norway because very different fractions of the county populations were sampled by the Janus Serum Bank. Red Cross blood donor status was matched in the Janus Serum Bank since the blood donors likely represent a healthier fraction of the population than other donors to the biobank (Langseth *et al.* 2010). Using blood donors as controls for population-based cases may result in an overestimation of risk, and using population-based controls for blood donor cases may result in an underestimation of risk. However, the SIR for cancer of all sites was higher among the Janus health examinations than among the blood donor

participants. The blood donors are Oslo residents, and a high proportion of health examinations donors are from the countryside. Urban inhabitants tend to have a higher cancer risk than rural inhabitants.

In Studies I/II, there was a wide variety of HPV seroprevalences among controls by region, which resulted in instability of the estimates. Therefore, the number of controls matched to a case was increased from three in Studies I/II to five in Study III.

The rate of HPV16 seroconversion is highest seven to eight months after detection of HPV16 DNA (Carter *et al.* 2000). The same applies to rate of HPV18 seroconversion after detection of HPV18 DNA. On average 7 to 18 months' time is needed to clear the HPV 18, 31, 33 and 52 infections (Bulkmans *et al.* 2007, Vänskä *et al.* 2013). Two to three weeks' time since the onset of symptoms is needed for *C. trachomatis* seroconversion (HM Surcel, personal communication, 9 December 2013). The mean duration of *C. trachomatis* infection is 1.4 years (Price *et al.* 2013). On the other hand, rapidly (within 3–6 years) increasing or decreasing over time trends affecting the Finnish HPV/*C. trachomatis* infection incidences/prevalences, respectively, during the study period have been documented (Laukkanen *et al.* 2003, Lyytikäinen *et al.* 2008). These ecological population biology phenomena due to changes in risk-taking sexual behaviour (Haavio-Mannila *et al.* 2001) set constraints for observational studies trying to understand interactions of the (most) important cervical cancer risk factors.

Possible confounding by age needs to be considered, especially in the context of Study V. Age at first sexual intercourse is a reasonable proxy for age at first exposure to HPV among young women (Plummer *et al.* 2012). Age at first sexual intercourse and age at first pregnancy are highly interrelated at least in the less developed countries (Louie *et al.* 2009). They are probably associated with early age at first exposure to hrHPV (Winer *et al.* 2003, Collins *et al.* 2005). HPV16 attack rates were highest among Finnish women with teenage pregnancies (Kibur *et al.* 2000) and were high in southwestern Finland among adolescents already in the 1980s (Lehtinen *et al.* 2006). In Study V, age at midpoint of consecutive first and second pregnancies was in the middle of the age interval of primary interest concerning *C. trachomatis* and type-specific HPV seropositivity and -conversion. Age at the midpoint of the first and second pregnancies, at least among the youngest women of Study V, was closely associated with early age at first exposure to HPV.

A co-factor role of long-term (≥10 years) oral contraceptive use has been suggested for CIN 3 by Luhn *et al.* (2013). In their study, a shorter term than 10 years' use of oral contraceptives was associated with non-significantly increased risk of CIN 3 as compared with women with a milder histological diagnosis than CIN 2. Almost half of the Finnish women aged under 25 used oral contraceptives in 2000 and had used these pills on average for 4 years (Kosunen *et al.* 2004). Age at first pregnancy is a surrogate for age at stopping oral contraceptive use (possibly also stopping smoking) before the first pregnancy, and thus is probably associated with CIN 3. Furthermore, during the last two decades the curve of age-specific CIN 3 incidence rates has been steeply increasing in Finnish women under 30 years of age (Salo *et al.* 2013). As the interval between the first and second pregnancies was on

average 2.4 years, age at the midpoint of these pregnancies was closely associated with age at stopping oral contraceptive use. Thus, confounding by age warranted control by stratification.

Longitudinal designs, in contrast to cross-sectional designs, are necessary for studying temporal associations in carcinogenesis, for separating causes and effects. To enable representative sampling from biobanks, it is important that population and cancer registers are complete and population-based, and that the biobanks are fully enumerated. For reliable linkage between serum banks and registers, a unique personal identification code number is essential. In studies of cervical carcinogenesis, CIN 3/AIS is a feasible outcome, as more than half of the lesions may progress to ICC without treatment (McCredie *et al.* 2008). The final outcome, invasive cervical carcinoma, is the most important one since a large proportion of precancerous lesions may regress.

Studies on carcinogenicity usually require a long duration, from exposure until cancer diagnosis. Follow-up time was maximized by analysing the first pre-diagnostic serum sample or baseline smear. The HPV16 IgG antibody levels in serum wane very slowly, therefore revealing infections not only at the time of serum sampling but also several years before the serum sampling (af Geijersstam *et al.* 1998). HPV (capsid) IgG antibodies have been used as markers of cumulative HPV exposure (Dillner 2000b, IARC 2007).

As a subcohort with IgG antibodies for HPV types of interest and *C. trachomatis* at two time points per woman was available, a case-cohort design was ideal for meeting Study V objectives. In 6 out of 12 subcohort strata, there were more than eight (with a maximum of 174) subcohort members per case in the respective cohort stratum. A subcohort like this would not have been sampled for Study V if it had not been available from previous studies. Possibly a nested case-control design would have been chosen. In nested case-control design, the gain in efficiency is quite marginal, with more than four or five controls per case. Although the laboratory analyses are highly standardized, there may have been systematic differences between the laboratory results of cases and subcohort members due to longer storage time of cases' samples. Study V was the second study applying a case-cohort design with cervical neoplasia as the outcome in the Finnish Maternity Cohort, the first being that of Laukkanen *et al.* (2010) on the relative risks associated with hrHPVs and the population-attributable fraction of hrHPVs in SCC and CIN 3.

Factors may strengthen each other's effect on the risk of disease. When the combined or joint effect of two factors is greater than the expected joint effect under a statistical model, *e.g.* additive or multiplicative, it is said that the factors *act synergistically* or that there is *synergism*. The factors *act independently* if the joint effect is according to formula (1) under additive model or multiplicative if it is under multiplicative model. Finally, two factors may weaken each other's effect. When the joint effect of the factors is smaller than the expected joint effect, it is said that the factors *act antagonistically* or that there is *antagonism*.

One should avoid saying that there is no interaction instead of no additive interaction or no multiplicative interaction, as under another statistical model there probably is an interaction and a true biological interaction may exist. Statistical

interaction refers to effects at the population level, whereas biological interaction refers to effects at the individual level. Biological information to support one scale over another is usually not available. Study I focused on *multiplicative interaction*, but the statistical models would have been suitable for estimating also additive effects. In Studies II and III, no *a priori* specification was made for the type of statistical interaction.

An antagonistic interaction was repeatedly found between HPV16 and HPV6(/11) in cervical carcinogenesis in both additive and multiplicative models. The antagonism was so strong in Studies I/II that there was no excess risk of SCC or ICC. The antagonistic interaction was confirmed in the larger, independent material of Study III, where the joint effect was antagonistic also regarding the risk of HPV16 DNA-positive SCC. All joint effects were adjusted for at least cotinine and *C. trachomatis* and HPV18 antibodies. Correction for misclassification bias by applying back-calculus and a gold standard suggested that non-differential misclassification did not cause the antagonistic interaction. The antagonistic interaction between a hrHPV and HPV6/11 was specific to HPV16. In Study I, the joint effect of HPV18, which primarily affects columnar cells in the upper cervix, and HPV6/11 suggested antagonism, but the larger material of Study III did not support/confirm this observation. The joint effects of HPV16 and hrHPV types 18 and 33 were also antagonistic regarding the risk for SCC in Study I, but Study III again did not support/confirm this observation.

Silins *et al.* (1999) reported soon after Study I that in their cross-sectional seroepidemiological study the joint effect of HPV6 and HPV16 was antagonistic regarding the risk of ICC under both additive and multiplicative models. Chaturvedi *et al.* (2011) reported in their cross-sectional study that the risk of high-grade squamous intraepithelial lesion (HSIL) or worse was non-significantly decreased among women infected with HPV16 and at least one other HPV type, relative to HPV16 alone. In their large, cross-sectional HPV genotyping study, Wentzensen *et al.* (2014) did not find any evidence of synergy between carcinogenic HPV types for the risk of HSIL. The risk of HSIL was higher among those with multiple genotypes than among those with a single genotype, but the risk was lower than expected on the basis of additive effects.

In a longitudinal study by Trottier *et al.* (2006), the risk of HSIL associated with infections of four to six concurrent HPV types was synergistically increased on the additive scale of ORs. Two-year cumulative risks of CIN 3+ for different combinations of HPV16, and non-carcinogenic and carcinogenic HPV types at study entry were close to but not greater than that for DNA positive for HPV16 only (Wheeler *et al.* 2006). Nauclér *et al.* (2007) found an antagonistic joint effect between HPV6 seropositivity and HPV16 seropositivity on risk of ICC in a multiplicative model. Thomsen *et al.* (2014) reported that 8-year absolute risk of CIN 3+ was slightly lower among women who had both an hr- and an lrHPV infection at baseline than women who had only hrHPV.

The antagonistic interaction between different HPV types was probably due to a cell-mediated immune reaction (surveillance), the surrogate of which were the serum antibodies. A potential explanation for the antagonism between HPV6 and HPV16 in

cervical carcinogenesis is that prior infection with HPV6 prevents HPV16 infection from becoming persistent and causing cancer (Dillner 2000b).

Lehtinen and Dillner (2013) reviewed protective efficacies of bivalent HPV16/18 and quadrivalent HPV6/11/16/18 vaccines against a 6-month persistent cervical HPV infection among baseline HPV-seronegative women. The bivalent vaccine showed cross-protective efficacy of 35% against non-targeted lrHPV types 6/11 (Szarewski *et al.* 2012). The most notable cross-protective efficacies against non-vaccine hrHPV types observed for the bivalent vaccine were 65–77% against HPV31, 32–43% against HPV33, 73–79% against HPV45 and 19–20% against HPV52. The corresponding cross-protective efficacies of the quadrivalent vaccine were not higher against these types, 46%, 29%, 8%, and 18%. Against some non-vaccine types, also negative protective efficacies were observed, of up to -56%, but the negative estimates were non-significant. Cross-protective efficacy was primarily due to serum cross-neutralizing antibodies. The immune response after vaccination is qualitatively (more antigenic sites exposed/available) different than in natural infection, but it may interfere with the population biology of HPV types in the sexually active vaccinated population.

Unit risk of HPV18 DNA-positive ICC among HPV16-seropositive women, and conversely, unit risk of HPV16 DNA-positive ICC among HPV18-seropositive women suggest that the two hrHPV types do not affect each other's risk of cervical cancer. Observations of close to unit risks, ORs, 0.9 and 1.1, in corresponding order, were made already in the first study material (Sigstad *et al.* 2002).

In Study II, the joint effect of HPV16 and *C. trachomatis* was antagonistic regarding the risk for SCC, assuming both multiplicative and additive joint effects, but in Study III, the joint effect was close to additive. The RR of *C. trachomatis* was significantly increased among hrHPV seronegatives and HPV18 seropositives, and non-significantly among HPV16 seropositives. In Study IV, a highly increased risk of invasive cervical cancer was observed among women *C. trachomatis* DNA-positive in the prediagnostic smear. The rate ratio estimate was at the same level in the same women HPV DNA-positive in the prediagnostic smear (Wallin *et al.* 1999), suggesting that *C. trachomatis* facilitates (if not replaces) effect(s) of early hrHPV infection. It should, however, be noted that the median unbiased estimate for *C. trachomatis* DNA is less reliable than a maximum likelihood estimate. The risk of invasive cervical cancer was most increased among women who were positive for HPV or *C. trachomatis* in both the prediagnostic smear and cancer biopsy.

In Study V, the HPV16 adjusted risk of cervical precancer was highly increased among women who were HPV31 seropositive before becoming HPV6 seropositive as compared with HPV6 seropositivity before HPV31 seropositivity. This is in line with the longitudinal studies cited above. Within the time-windows/time-constraints, it also suggests that natural infection with benign HPV types acts as a natural (alive) vaccination against (consequences of) infections with an oncogenic HPV type. It sheds light on the success of prophylactic HPV vaccines and suggests that the mechanisms and consequences of (cross-)protection warrant further research.

The risk of CIN3 was similar whether HPV6 seropositivity was before or after HPV33 seropositivity. This is in line with the suggested competitive advantage of

HPV33 over other common low- and high-risk HPV types (Merikukka *et al.* 2011). The rate ratios associated with HPV18/45 seroconversion did not differ between HPV6 seroconverters and HPV6 seropositives at first sampling. The same applied to rate ratios associated with HPV18/45 seropositivity at first sampling.

The highest rate ratio estimate was observed for dual seroconversion of HPV18/45 and *C. trachomatis* between two consecutive pregnancies as compared with dual seronegativity to the infections at both samplings during the first trimesters. This indicates that virtually overlapping infections with HPV18/45 and *C. trachomatis* in the cervix <are especially carcinogenic. It is in line with an independent role for *C. trachomatis* in cervical carcinogenesis (Lehtinen *et al.* 2011) and suggests that *C. trachomatis* screening might have an effect on cervical neoplasia occurrence.

7 CONCLUSIONS

In the first longitudinal nested case-control study (I), excess risk of developing ICC among women seropositive for both the most carcinogenic HPV16 and the non-carcinogenic HPV6/11 was non-existent. This finding was supported by the second similar, albeit larger study (III) reporting a significantly smaller risk of developing ICC among women seropositive for both HPV16 and HPV6 than expected on the basis of multiplicative and additive joint effects. Other study groups have since reported similar results. Furthermore, the result of the most recent study (V), that the orders of major lrHPV type 6 and hrHPV type 31 have considerably different effects on risk of CIN 3/ICC, confirms the biological plausibility of the original finding of antagonistic interaction. The risk of CIN 3/AIS was 23 times higher if HPV31 preceded HPV6 than the reverse scenario. Earlier HPV6 infection and associated immune response may well prevent HPV16 and HPV31 from becoming persistent. The antagonistic interaction was probably due to cell-mediated immune response and associated immune surveillance. The finding that infection with a non-carcinogenic HPV type may act as a natural vaccination against high-risk HPV types supports HPV vaccination in cervical cancer prevention. The antagonistic additive interaction or even absence of an excess risk suggests that there is a public health interaction to be considered.

There was no excess risk of HPV18 DNA-positive ICC associated with HPV16 seropositivity, and also no excess risk of HPV16 DNA-positive ICC associated with HPV18 seropositivity. This is in line with type-specific HPV DNA persistence of these major oncogenic HPV types in cervical carcinogenesis.

The risk of SCC associated with *C. trachomatis* remained increased after adjusting for HPV, both in the total material and in HPV18-seropositive women. Virtually concomitant seroconversion for both *C. trachomatis* and HPV18/45, *i.e.* virtually concomitant infections, were associated with highly increased risk of CIN 3/AIS. Concomitant *C. trachomatis* and hrHPV infections should be considered in the preventive efforts against cervical cancer.

The public health implication of these studies is a support to the early HPV vaccinatioin in cervical cancer prevention.

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TECHNICAL APPENDIX 1 – THE NORDIC REGISTER DATA BASES UTILIZED IN THE STUDIES

Cancer registries and registration in Finland, Iceland, Norway and Sweden

(Referred to on page 46.)

The Finnish Cancer Registry is technically run by the Cancer Society of Finland, but is supervised by the National Institute for Health and Welfare, which owns the original patient data. The Icelandic Cancer Registry has been run by the Icelandic Cancer Society since 1954. The Directorate of Health has been responsible for the registration from 2007 onwards. The Cancer Registry of Norway is part of the South-Eastern Norway Regional Health Authority and an independent institution under Oslo University Hospital Trust. In Sweden, the local authorities of the 21 counties own and are responsible for the cancer data. The regional cancer registry at the Oncological Centre in Umeå has collected data since 1978.

The cancer registries are population-based. The populations include all residents in the areas who are entered in the official population statistics and have been given a personal identification number (Association of the Nordic Cancer Registries 2000).

The primary basis of diagnosis is morphology. Morphological confirmation of diagnosis was available for 92–100% of invasive cervical cancers at the national cancer register in Finland during 1983–1994 (Finnish Cancer Registry 1987–1996). All cases of invasive cervical cancer were morphologically verified in Iceland in 2005–2009 (Sigurðardóttir *et al.* 2012). In Norway, the proportion was 97.4% during 1989–1993 (Cancer Registry of Norway 1996) and 99.8% during 2001–2005 (Larsen *et al.* 2009).

The date of diagnosis is the earliest date on the received notification form (Association of the Nordic Cancer Registries 2000). The date of *in situ* carcinoma of cervix uteri diagnosis is the date of invasive cervical cancer diagnosis, if the new invasive tumour is diagnosed within 2 months of *in situ* diagnosis in Iceland, within 4 months in Norway and within 1 year in Finland and Sweden.

Up to and including 2006, Finnish Cancer Registry coded topography according to a modified version of International Classification of Diseases, 7th revision (ICD-7) (WHO 1957), and morphology according to the Manual of Tumor Nomenclature and Coding (MOTNAC) (American Cancer Society 1951). Since 2007, topography and morphology have been coded according to the International Classification of Diseases for Oncology, 3rd edition (ICD-O-3) (Fritz *et al.* 2000). The ICD-7 and MOTNAC codes have been translated into ICD-O-3.

The Cancer Registry of Norway coded topography according to ICD-7 in 1952–1982 and morphology according to a modified version of MOTNAC in 1968–1992

(American Cancer Society 1968). Since 1993, topography was coded according to ICD-O-2 (Percy *et al.* 1990) with the topography axis standardized with ICD-10, and morphology using ICD-O-2 with local modifications (Larsen *et al.* 2009). Topography was converted to ICD-7 codes.

Icelandic Cancer Registry coded topography according to ICD-9 since 1980 (WHO 1977), ICD-O-1 since 1983 (WHO 1976) and ICD-O-2 during 1991–2002 (Sigurðardóttir *et al.* 2012). Conversions to ICD-7 and ICD-9 are still done automatically. Morphology was coded according to the Systematized Nomenclature of Pathology until 1983 and since then according to the Systematized Nomenclature of Medicine (Wells 1965, Côté 1979).

In Sweden, morphology was coded according to C24 (WHO/HS/CANC/24.1) since 1958 and ICD-O-2 during 1993–2004. Topography was coded according to ICD-7 since 1958, ICD-9 since 1987 and ICDO-2/10 during 1993–2004. The codes were translated into ICD-7 and ICD-9.

High-grade cervical precancer at the Finnish Cancer Registy (V)

The Finnish Cancer Registry registers the following high-grade cervical precancers (ICD-O-3 codes for morphology/behaviour in parentheses), carcinoma *in situ*, not otherwise specified (M8010/2), squamous cell carcinoma *in situ* (M8070/2), dysplasia gravis (M6666/0), cervical intraepithelial neoplasia grade 3 (M8077/2) and adenocarcinoma *in situ* (M8140/2). Initially, carcinoma *in situ* was the only diagnostic term for high-grade cervical precancer. Registration of dysplasia gravis (severe dysplasia) and CIN 3 became official practice in the early 1990s (Hakama *et al.* 2004). All of the terms are still in use. In the following, all lesions except AIS will be called *CIN 3*. Registration of the CIN 3/AIS lesions was estimated to be 80% complete in 1998–2007 (Lönnberg *et al.* 2012).

Care Register for Health Care (V)

(Referred to on page 50.)

The Care Register for Health Care collects in Finland data on the activities of institutions providing inpatient care and on the clients treated in them as well as on home-nursing clients for the purposes of statistics, research and planning. The data are controlled by the National Institute for Welfare and Health. The Care Register for Health Care replaced the Hospital Discharge Register since 1994. The Hospital Discharge Register contained data only on patients discharged from inpatient care in hospitals, whereas the Care Register contains also outpatient data, *e.g.* on day surgeries. The register covers more than 95% of discharges, and positive predictive value for common diseases varies between 75% and 99% (Sund 2012).

Population Information System (V)

(Referred to on page 49.)

The Population Register Centre, founded in 1969, and the local register offices are controllers for the Finnish Population Information System. The Population Register Centre maintains and develops the Population Information System, its data and data quality. The Population Information System contains information on, for example, the time of death or the time of declaring a person dead, the municipality and the place of residence therein and the date of emigration. Statistics Finland is responsible for statistics on causes of death and maintains death certificates since 1936. The statistics are based on data in death certificates and data on deaths in the Population Information System. The cancer register receives data on emigrations from the Population Register Centre and data on deaths from Statistics Finland.

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TECHNICAL APPENDIX 2 – RE-EXAMINATION OF HISTOLOGY (II–IV)

The histological slides of 182 cases included in Study I and four cases excluded from that study (one leiomyosarcoma and three without reported histology) were retrieved for histological reclassification by two pathologists (E Sigstad and V Abeler). The reviewers were blinded to the registered diagnoses. The tumours were classified and graded according to an international histological classification of tumours blinded to registered diagnosis (Poulsen *et al.* 1975). The first biopsy or best-preserved specimen of 127 cases could be located, verified to contain ICC, and amplified by PCR (Sigstad *et al.* 2002). Paraffin rolls (5 cases) could not be tested for contamination and were not analysed by PCR. One hundred and seven of them were SCCs. Five of them were of other histology in Study I, and two were excluded from Study I due to no reported histology. The histology of eight SCCs in Study I changed in histological reclassification. There were 178 cases and 527 controls after histological reclassification.

Study II concentrated on SCC. The SCCs of Study I were not excluded from Study II if the histological specimen for the review was missing or non-representative. The SCCs reclassified as precancerous were not excluded if there was independent information of invasion. There were 148 SCCs and 438 matched controls.

For Study III, two senior pathologists (W Ryd and F Bergman) re-examined sections of paraffin-embedded cancer tissues and if available histopathological slides for confirmation of cervical cancer diagnosis. Icelandic legislation forbade export of cancer tissues and histopathological slides.

For Study IV, a senior pathologist (F Bergman) re-examined all histological slides for confirmation of cervical cancer diagnosis.

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(Referred to on pages 46 and 47.)

TECHNICAL APPENDIX 3 – THE GREY ZONES

For the cases not in the subcohort, grey zones of HPV type 6/16/18/31/33/45 antibodies were [0.2823,0.4837], [0.2674,0.4526], [1.1754,1.7906], [0.8758,1.3842], [0.8667,1.3533], [0.9278,1.4122] absorbance units (optical density at 405 nm), respectively. The grey zones for antibodies to HPV type 6/16/31/33/45 for the subcohort members were [0.3798,0.6202], [0.2674,0.4526], [0.3957,0.6243], [0.3834,0.5986], [0.3507,0.5433] absorbance units, respectively. The subcohort members had different grey zone for HPV18 antibodies at first sample ([0.3299,0.4911] absorbance units), and at second sample ([0.3149,0.4751] absorbance units).

(Referred to on page 51.)

ERRATA

In Publication I, on page 822 in the numerical example of the "Appendix", specificity of HPV16 and HPV6/11 antibody assays, Sx and Sy, should be 0.99 instead of 0.98.

In Publication III on page 2546, two rows of Table 3 are missing. In the table block of *C. trachomatis*, below row

" 2,471 controls 1,920 controls 2,584 controls 2,010 controls"

there should be row

" HPV type 16 seronegatives[a] HPV type 18 seronegatives[b]"

and in the table block of HSV-2 below row

" 2,471 controls 1,920 controls 2,584 controls 2,010 controls"

there should be row

" HPV type 16 seronegatives[c] HPV type 18 seronegatives[d]".

In Publication III on page 2548, there should not be in the footnote of Table 8 rows

"P for multiplicative interaction = 0.0036

RERI = -0.645 95% CI (-2.5, -1.2).

P = 0.49".

(Referred to on page 58.)