

## Prospective Seroepidemiologic Study of Human Papillomavirus and Other Risk Factors in Cervical Cancer

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### Abstract

**Background:** Several sexually transmitted infections (STI) have been reported to interact with human papillomavirus (HPV) in the etiology of cervical cancer. A large cohort study is required to obtain a both unbiased and stable estimate of their effects.

**Methods:** Four major biobanks in the Nordic Countries containing samples from about 1,000,000 subjects were linked with nation-wide cancer registries. Serum samples from 604 women with invasive cervical cancer (ICC) diagnosed on average 10 years after sampling and 2,980 matched control women were retrieved and analyzed with serology for key STI.

**Results:** Exposure to HPV16 was the strongest risk factor for cervical cancer [OR = 2.4; 95% confidence interval (CI), 2.0–3.0], particularly for squamous cell carcinoma (OR = 2.9; 95% CI, 2.2–3.7). HPV18 was strongly associated with increased risk for adenocarcinoma (OR = 2.3; 95% CI, 1.3–4.1). Baseline seropositivity for HPV16 did not confer any increased risk for HPV18 DNA-positive cancer and conversely HPV18 seropositivity had no association with HPV16 DNA-positive cancers. HPV6 had no effect on its own (OR = 1.1; 95% CI, 0.9–1.3), but had an antagonistic effect on the risk conferred by HPV16 ( $P < 0.01$ ). Herpes simplex virus 2 had little or no association (OR = 1.1; 95% CI, 0.8–1.4). Previous exposure to *Chlamydia trachomatis*, as indicated by serum antibodies, had a strongly increased risk for cervical cancer (OR = 1.9; 95% CI, 1.5–2.3).

**Conclusions:** A large prospective study has assessed the role of different STIs in cervical cancer.

**Impact:** Prospective evidence supports cofactor role of some STI in cervical cancer. *Cancer Epidemiol Biomarkers Prev*; 20(12); 2541–50. ©2011 AACR.

### Introduction

Infection with human papillomavirus (HPV) is established as a necessary cause of cervical cancer, and HPV types 16 and 18 are the types most frequently detected in cervical cancer worldwide (1). A large body of evidence has

suggested that different sexually transmitted infections (STI) may act as cofactors to HPV16 and HPV18 in cervical carcinogenesis. Infection with multiple high-risk HPV types has been suggested to increase the risk for cervical cancer compared with single infections (2, 3), although there is also evidence that coinfections may act independently of each other in the progression to cervical cancer (4). A protective effect on cervical cancer by history of condyloma acuminata has been reported (5) and seroepidemiologic studies have found an antagonistic effect of the condyloma-related HPV type 6 on the cervical cancer risk mediated by HPV16 (6, 7). Women who were only seropositive for HPV16 were at higher risk of cervical cancer than women who were seropositive for both HPV6 and HPV16 at the same time (6, 7).

History of *Chlamydia trachomatis* infection has also been associated with cervical cancer (8–10) and appears to increase the probability that infections with high-risk HPV types will become persistent (11, 12). Some studies have also reported herpes simplex virus type 2 (HSV-2) as a co-factor (13), but this has not been confirmed in longitudinal studies (14). In addition to infectious co-factors, smoking has been indicated as a risk factor both for high-grade cervical intraepithelial neoplasia (CIN; ref. 15) and for cervical cancer (16, 17).

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The serum antibody response to HPV is stable over time and is therefore used as an indicator of past or present HPV infection (18, 19). The use of type-specific HPV serology has previously provided prospective epidemiologic links between HPV and cervical cancer (20, 21). Prediagnostic seropositivity has been found to correlate well with the type of HPV DNA found in subsequent cancers, for several HPV-associated cancer sites (22, 23), but the issue of whether different HPV types interact with each other in cervical carcinogenesis is not yet resolved. Although prospective studies minimize the risk for biases in study design, it has been difficult to obtain sufficiently large numbers of prospectively occurring cases of invasive cancers to have sufficient statistical power to be able to disentangle the role of the interrelated possible cofactors in cervical cancer.

In the present report, we assembled a large study base from several large biobanks with samples from almost 1,000,000 subjects that we followed up for up to 25 years to enable delineation of how the different infectious risk factors, co-risk factors, and effect modifiers interact in the etiology of cervical cancer.

## Materials and Methods

### Study base

The study base comprised 4 population-based biobanks to which a total of 974,000 female residents of Finland, Norway, Iceland, and Sweden, have donated blood samples, presented in more detail in Pukkala and colleagues (24).

The Finnish Maternity cohort contains blood samples from almost all (~ 98%) pregnant women in Finland. The samples were retrieved from women during early pregnancy to screen for congenital infections. In 2003, the bank contained about 1.4 million samples, stored at  $-25^{\circ}\text{C}$ , from 681,000 female donors. Enrolment began in 1983 and is still ongoing.

The Janus Biobank project in Norway was commenced in 1973 to search premorbid sera for changes that might indicate cancer development at early stages, or is indicative for increased risk of cancer. In 2003, the bank contained approximately 493,000 samples from 332,000 donors of whom 158,000 were females. A majority of the women, 144,000, took part in population-based health check-ups, and 14,000 women were enrolled at a blood donor center in Oslo.

The Northern Sweden Maternity cohort has since 1975 stored samples from the population-based screening of pregnant women for rubella immunity in Northern Sweden. Samples from 118,000 pregnancies (approximately 86,000 unique women) are stored at  $-20^{\circ}\text{C}$ .

The Icelandic Maternity Cohort has since 1980 stored samples from the population-based screening of pregnant women for rubella immunity in Iceland. Samples from 91,000 pregnancies (approximately 49,000 unique women) are stored at  $-20^{\circ}\text{C}$ .

To identify cases, data files from the biobanks were linked with the corresponding cancer registries. The linkage was done on basis of personal identification numbers. To ensure that only incident cases of invasive cervical cancer (ICC) were included, a woman had to have at least 1 serum sample taken more than a month before diagnosis. If there were several serum samples available for each case, the first (oldest) sample was chosen. The cancer registries used are population based. Notifications have been received from hospitals, pathology and hematology labs, and doctors since the 1950s. Coding of primary site is based on ICD-7. Reporting of the cancer cases is compulsory in Finland, Norway, and Sweden, and was voluntary in Iceland until 2007. In Sweden, private practitioners reported on voluntary basis until 1984.

Five controls were individually matched for sex, age at serum sampling (within 2 years), storage time (within 2 months), and for region (Norway, Finland, Iceland, or Sweden and in Norway also for county and blood donor status). If 5 controls could not be found within the matching criteria on age at serum sampling and storage time, these time spans were successively widened until a control could be found. The age at serum sampling differed more than 731 days between 11 cases and 13 controls. Mean age at enrollment for cases was 31.4 (range 15.5–60.4) and 31.5 (range 14.8–59.7) for controls. The storage time differed more than 62 days between 7 cases and 20 controls.

The linkage identified 653 cases diagnosed during the period 1975 to 2002. Thirty-four cases were lost because the samples could not be located or the sample volume was too low. Fifteen cases were benign or without reported histology and were not included. In total, 604 cases and 2,980 controls were included (Norway: 212 cases and 1,053 controls; Finland: 174 cases and 854 controls; Iceland: 103 cases and 500 controls; and Sweden: 115 cases and 573 controls). The majority of women were classified as having squamous cell carcinoma (SCC;  $n = 470$ ), 111 women were diagnosed with adenocarcinoma (AC) and 21 with adenosquamous carcinoma (ASC). There were also 2 undifferentiated carcinomas. Time since serum sampling until diagnosis ranged from 3 months to 25.5 years and was on average 9.6 years. The number of women-years of follow-up for each age group and for each biobank cohort is shown in Table 1. Mean age at diagnosis for cases was 41.0 years (range 21.5–70.5). Cases included in previous seroepidemiologic studies of STIs and cervical cancer (7, 8, 14, 22, 25) were not included in the present study. Whereas the previous study published the effect of the different exposures in a series of separate publication (7, 8, 14, 22, 25), we have in this second, larger, and independent data set decided to combine the effect of all the different STIs in the same paper, for completeness. However, the effect of serum cotinine has been published separately (16).

Paraffin-embedded cancer tissue and when possible histopathologic slides were collected from the pathology departments at the hospitals where the cases were

**Table 1.** Women-years of follow-up from enrolment until end of study (31st December, 2002)

| Age, y | Finnish maternity cohort | Icelandic maternity cohort | Janus biobank, blood donors | Janus biobank, health check-up | Northern Sweden | Total      |
|--------|--------------------------|----------------------------|-----------------------------|--------------------------------|-----------------|------------|
| 10–14  | 81                       | 38                         | 0                           | 0                              | 50              | 169        |
| 15–19  | 57,936                   | 10,982                     | 1,111                       | 1,009                          | 7,909           | 78,947     |
| 20–24  | 535,052                  | 69,871                     | 20,017                      | 13,317                         | 87,226          | 725,483    |
| 25–29  | 1,394,745                | 126,489                    | 51,548                      | 38,651                         | 215,630         | 1,827,063  |
| 30–34  | 1,919,558                | 140,060                    | 79,457                      | 66,632                         | 272,808         | 2,478,515  |
| 35–39  | 1,755,218                | 122,674                    | 96,955                      | 132,222                        | 245,534         | 2,352,603  |
| 40–44  | 1,189,354                | 83,887                     | 104,598                     | 929,495                        | 174,356         | 2,481,690  |
| 45–49  | 582,727                  | 43,442                     | 96,449                      | 1,323,104                      | 99,774          | 2,145,496  |
| 50–54  | 192,371                  | 16,374                     | 78,056                      | 1,184,581                      | 45,051          | 1,516,433  |
| 55–59  | 32,397                   | 3,875                      | 55,360                      | 546,560                        | 13,480          | 651,672    |
| 60–64  | 2,429                    | 405                        | 36,482                      | 302,406                        | 2,478           | 344,200    |
| 65–69  | 86                       | 10                         | 23,092                      | 237,220                        | 317             | 260,725    |
| 70–74  | 27                       | 0                          | 13,602                      | 142,069                        | 47              | 155,745    |
| 75–79  | 0                        | 0                          | 6,688                       | 44,124                         | 14              | 50,826     |
| 80–84  | 0                        | 0                          | 2,434                       | 879                            | 1               | 3,314      |
| 85+    | 0                        | 0                          | 637                         | 264                            | 0               | 901        |
| Total  | 7,661,981                | 618,107                    | 666,486                     | 4,962,533                      | 1,164,675       | 15,073,782 |

diagnosed and treated. Cancer samples collected from Iceland because of legislation on export of biobank samples. A senior pathologist reexamined newly sectioned hematoxylin and eosin-stained slides and when available also the original diagnostic histopathologic slides for confirmation of diagnosis of cervical cancer. Samples negative for  $\beta$ -globin and samples where re-review found that cancer cells were no longer present in the block were excluded.

#### Laboratory methods

All analyses were carried out with the analyzing laboratory blinded to the identity of the samples.

**HPV serology.** The method used was the standard VLP ELISA (serum dilution 1:30; ref. 26) for detecting IgG antibodies specific for HPV6, HPV16, and HPV18, using baculovirus-expressed capsids containing the L1 protein. As a background control antigen, bovine papillomavirus capsids (disrupted by treatment with 0.1 mol/L carbonate buffer pH 9.6) was used (27). As positive control, a reference serum from patients with CIN was used at dilutions 1:10, 1:30 (in duplicates), and 1:100.

The cutoff levels for determining seropositivity for the HPV types analyzed has been established in previous studies (6). The OD values of the reference serum in the present study were normalized to correspond to the OD values in the previous studies.

**HSV-2 serology used two different methods.** First, infected cell-derived antigen blocked for HSV-1 (28) used HSV-2 antigen, generated from HSV-2 infected cells, coated onto microtiter plates. As negative control, HSV-2-negative cells were used. A positive control reference serum from patients positive for HSV-2 as well as the

patient sera was used in 3 dilutions (1:31, 1:100, and 1:316). The seropositivity of a sample was based on the relationship between the optical density (OD) of the sample and the OD of the reference serum. Optical density values were transformed into antibody levels using the PLL (parallel line) method (29). Cutoff values for antibody levels were preassigned. A sample was considered positive when the antibody level was more than 0.15 units and the correlation between the 3 dilutions was more than 0.9. Second, immunoglobulin G antibodies to HSV-2 were also determined by a commercially available HSV-2 glycoprotein gG-2-based ELISA (Biokit), according to the manufacturer's recommendations (30).

**Chlamydia trachomatis. IgG EIA** Serum IgG antibodies to *C. trachomatis* were measured by a major outer protein (MOMP) peptide ELISA using a commercial kit from Ani LabSystems. The antigen consists of 4 different peptides from variable domains of MOMP proteins. These peptides represent B-, C-, and intermediated serotype groups.

**Cotinine serology.** Cotinine was measured using a qualitative immunoassay method (OraSure Technologies) that was carried out as a quantitative assay based on the competition between free cotinine in the sample and cotinine bound to horseradish peroxidase-labeled cotinine. Concentration was quantified by measuring the light absorbance at 450 nm and 630 nm and by comparing the cotinine concentration of each sample to the standard curve. Regression dilution bias was assessed by measuring paired samples repeatedly with the same batch from the Assay Kit.

We categorized the level of measured cotinine into 3 groups: nonsmokers and persons passively exposed to

tobacco smoke less than 20 ng/mL, light smokers 20 to 100 or less ng/mL, and heavy smokers 100 or more ng/mL.

**DNA testing of formalin-fixed paraffin-embedded cancer tissue.** To avoid and check for cross-contamination between the blocks, a blank block containing only paraffin was cut between the case blocks and included in the analysis. In addition, the microtome was cleaned thoroughly with DNAZap (Ambion) between every case. From each case block, six 5 µm thick sections were cut. The first and last sections were put on glass slides and stained with hematoxylin-eosin for histopathologic review. Samples that did not contain CIN3 or worse were excluded from the DNA analysis as there was then no evidence of cancer tissue in the intervening sections that had been used for the HPV DNA testing. The 4 sections in the middle were put in a test tube and used for DNA extraction.

Before digesting the formalin-fixed tissue, the paraffin was removed using 2 × 1 mL xylene for a minimum of 30 minutes and the tissue was washed twice with 500 µL ethanol. After removal of paraffin, the tissue was treated with proteinase K (200 µg/mL in 50 mmol/L Tris HCl buffer with 1 mmol/L EDTA and 0.5% Tween 20, pH 8.5). The samples were tested for amplifiability in a PCR targeting the beta-globin gene and detection of the amplicon in an enzyme immunoassay (ELISA; ref. 31). Negative samples were re-tested in dilution 1:10 and if still negative the sample was tested with real-time PCR for the beta-globin gene. Samples that were not amplifiable were excluded.

The extracted samples were tested for the presence of HPV DNA by PCR using the GP5<sup>+</sup>/6<sup>+</sup> primer system (32) and the types were detected by EIA and reverse dot blot hybridization (RDBH; ref. 31) or a multiplex fluorescent bead-based assay (33). If the samples were negative in both tests, the samples were retested in the same PCR system diluted 1:10 and if still negative the samples were amplified by multiple displacement amplification (MDA) using the TempliPhi 100 Kit (GE Healthcare) according to the manufacturer's instructions prior to a new round of both beta-globin and HPV PCR analysis, where the amplified material was tested in 3 dilutions (1:10, 1:100, and 1:1,000).

### Statistical analysis

Data from biobanks, cancer registries, and analyzing laboratories were submitted for joint statistical analysis at the Finnish Cancer Registry. ORs were estimated with conditional logistic regression with SAS 9.1 software (SAS Institute Inc; ref.34). Multivariate models were adjusted for the infectious agents previously found to be risk factors for ICC [HPV16, HPV18, and *C. trachomatis* (25)] and cotinine. When the analyses were restricted to the high-risk HPV seropositives, the ORs were estimated by unconditional logistic regression adjusting for cotinine and matching variables (age in years, calendar year, country and in Norway also for sub-cohort [population-based health check-up or blood donor]). The 95% confi-

dence limits for parameters of the conditional analyses were on the basis of Wald-type statistic and those of unconditional analyses on likelihood ratio.

Testing for multiplicative versus nonmultiplicative interaction of exposures was done with a likelihood ratio test to compare 2 nested models, one for the solitary effects only and the other for the joint effects. Relative excess risk due to interaction (RERI) and the confidence interval (CI) for RERI were estimated according to Hosmer and Lemeshow (35).

The study was approved by the Karolinska Institutet Ethical Review Board.

### Results

The risk of HPV16 seropositive women to develop ICC was more than twice as high as the risk for seronegative women (OR = 2.4; 95% CI, 2.0–3.0). There was also a weakly increased risk of acquiring ICC when seropositive for HPV18 (OR = 1.5; 95% CI, 1.2–3.0). An increased risk was also observed for women who were seropositive for *C. trachomatis* (OR = 1.9; 95% CI, 1.5–2.3; Table 2). Among the women seropositive for HPV16 and/or HPV18, the OR for ICC related to *C. trachomatis* was 1.4 (95% CI, 1.1–2.0; data not shown). The corresponding point estimates for SCC was 1.5 (95% CI, 1.1–2.1) and for AC 1.4 (95% CI, 0.6–3.2). Women who were HPV6 seropositive had a small increased risk of developing ICC in crude analysis, but the risk disappeared in the multivariate model (Table 2). Similarly, the small excess risk associated with HSV-2 was materially eliminated in the multivariate model (Table 2). The 2 different HSV-2 antibody tests that were used showed very similar results (Table 2).

HPV16 was associated with the highest risk of developing SCC (OR = 2.9; 95% CI, 2.2–3.7), whereas HPV18 was associated with the highest risk for AC (OR = 2.3; 95% CI, 1.3–4.1). *C. trachomatis* was clearly associated with SCC and had an association of borderline significance with AC and ASC (Table 2). HPV6 and HSV-2 seropositives had no associations with any specific ICC histologic type (Table 2).

When stratifying the *C. trachomatis* and HSV-associated risks of ICC and SCC by seropositivity for HPV16 or HPV18, the risk associated with *C. trachomatis* was found to be similarly increased among both HPV18 seropositives and seronegatives (Table 3). The *C. trachomatis*-associated risk was significantly increased among HPV16 seronegatives, but not among seropositives (Table 3). Stratification by HPV16 or 18 seropositivity had little effect on HSV-associated risk estimates (Table 3).

The risk estimate for developing ICC was highest in HPV16 seropositive women, who were adolescent at serum sampling (OR = 5.5; 95% CI, 1.8–17). The relative risk for ICC leveled down by age, to increase again among women 40 years or older (Table 4, age at serum sampling). Neither lag time between serum sampling nor cancer diagnosis (Table 4, lag) or calendar period of sampling time (also reflecting storage time) had any detectable effect (Table 4, period of serum sampling).

**Table 2.** Number of positives, ORs and 95% CIs of cervical cancer associated with seropositivity for HPV16, HPV18, and *C. trachomatis*, Herpes Simplex virus 2 (comparing 2 ELISA methods)

| Seropositivity        | Cases +/all | Positive% | Controls +/all | Positive% | Crude OR (95% CI) | Adjusted <sup>a</sup> OR (95% CI) |
|-----------------------|-------------|-----------|----------------|-----------|-------------------|-----------------------------------|
| <b>ICC</b>            |             |           |                |           |                   |                                   |
| HPV16                 | 197/604     | 32.6      | 425/2,979      | 14.3      | 2.9 (2.4–3.5)     | 2.4 (2.0–3.0)                     |
| HPV18                 | 122/604     | 20.2      | 305/2,977      | 10.2      | 2.2 (1.7–2.8)     | 1.5 (1.2–2.0)                     |
| <i>C. trachomatis</i> | 277/588     | 47.1      | 818/2,846      | 28.7      | 2.3 (1.9–2.7)     | 1.9 (1.5–2.3)                     |
| HPV6                  | 220/603     | 36.5      | 856/2,974      | 28.8      | 1.4 (1.2–1.7)     | 1.1 (0.9–1.3)                     |
| HSV-2 (gG-2)          | 99/588      | 16.8      | 324/2,846      | 11.4      | 1.6 (1.2–2.1)     | 1.1 (0.8–1.4)                     |
| HSV-2 (standard)      | 123/603     | 20.4      | 417/2,968      | 14.0      | 1.6 (1.3–2.0)     | 1.1 (0.9–1.5)                     |
| <b>SCC</b>            |             |           |                |           |                   |                                   |
| HPV16                 | 167/470     | 35.5      | 332/2,316      | 14.3      | 3.3 (2.7–4.2)     | 2.9 (2.2–3.7)                     |
| HPV18                 | 92/470      | 19.6      | 236/2,315      | 10.2      | 2.1 (1.6–2.8)     | 1.4 (1.1–1.9)                     |
| <i>C. trachomatis</i> | 224/457     | 49.0      | 632/2,207      | 28.6      | 2.5 (2.0–3.0)     | 1.9 (1.6–2.4)                     |
| HPV6                  | 169/469     | 36.0      | 662/2,311      | 28.6      | 1.4 (1.1–1.8)     | 1.1 (0.8–1.3)                     |
| HSV-2 (gG-2)          | 78/457      | 17.1      | 249/2,207      | 11.3      | 1.6 (1.2–2.2)     | 1.1 (0.8–1.5)                     |
| HSV-2 (standard)      | 96/470      | 20.4      | 321/2,311      | 13.9      | 1.6 (1.2–2.1)     | 1.1 (0.8–1.5)                     |
| <b>AC</b>             |             |           |                |           |                   |                                   |
| HPV16                 | 24/111      | 21.6      | 80/549         | 14.6      | 1.6 (0.96–2.6)    | 1.1 (0.6–2.0)                     |
| HPV18                 | 26/111      | 23.4      | 57/548         | 10.4      | 2.6 (1.6–4.4)     | 2.3 (1.3–4.1)                     |
| <i>C. trachomatis</i> | 41/108      | 38.0      | 149/526        | 28.3      | 1.6 (1.02–2.4)    | 1.4 (0.9–2.2)                     |
| HPV6                  | 41/111      | 37.0      | 152/549        | 27.7      | 1.6 (1.02–2.5)    | 1.3 (0.8–2.1)                     |
| HSV-2 (gG-2)          | 18/108      | 16.7      | 66/526         | 12.5      | 1.4 (0.8–2.5)     | 1.1 (0.6–2.0)                     |
| HSV-2 (standard)      | 23/110      | 20.9      | 83/543         | 15.3      | 1.5 (0.9–2.5)     | 1.2 (0.7–2.2)                     |
| <b>ASC</b>            |             |           |                |           |                   |                                   |
| HPV16                 | 5/21        | 23.8      | 13/104         | 12.5      | 2.2 (0.7–7.2)     | 2.0 (0.6–7.1)                     |
| HPV18                 | 4/21        | 19.0      | 11/104         | 10.6      | 1.9 (0.6–6.2)     | 1.6 (0.4–5.6)                     |
| <i>C. trachomatis</i> | 11/21       | 52.4      | 34/104         | 32.7      | 2.4 (0.9–6.4)     | 2.4 (0.9–7.0)                     |
| HPV6                  | 9/21        | 42.9      | 42/104         | 40.4      | 1.1 (0.4–3.0)     | 0.9 (0.3–2.5)                     |
| HSV-2 (gG-2)          | 3/21        | 14.3      | 9/104          | 8.7       | 1.7 (0.4–6.5)     | 2.0 (0.4–8.8)                     |
| HSV-2 (standard)      | 3/21        | 14.3      | 12/104         | 11.5      | 1.3 (0.3–4.7)     | 1.2 (0.3–5.0)                     |

<sup>a</sup>Adjusted for HPV16, HPV18, *C. trachomatis*, and cotinine.

The relative risk of SCC for women seropositive for both HPV16 and HPV6 (OR = 2.4; 95% CI, 1.7–3.4) was significantly decreased ( $P < 0.01$  in both additive and multiplicative interaction models) compared with the risk expected on the basis of solitary effects of HPV16 and HPV6 (Table 5). The antagonistic interaction was also seen in ASC, but not in AC (data not shown). To evaluate whether the interaction was specific for HPV6 or a more general phenomenon seen for all STIs, similar interaction analyses were carried out between HPV16 and HPV18 or *C. trachomatis* (Tables 6 and 7). HPV18 and *C. trachomatis* showed a tendencies for interaction with HPV16 that were not significant in the additive model and had lower point estimate of the RERI [0.957, 95% CI (–2.6 to 0.69) and –0.645, 95% CI (–2.5 to 1.2)] than the interaction between HPV6 and 16 [RERI –2.12, 95% CI (–3.6 to –0.67)]. In the multiplicative model, *C. trachomatis* and HPV6, but not HPV18, had significant interactions with HPV16 (Table 5–Table 7).

To evaluate whether the interactions seen may be attributable to confounding with other HPV types than

HPV16, that may also cause cervical cancer, we restricted the analysis to cervical cancer cases containing HPV16 DNA only (as persistent presence of HPV DNA is established as essential in cervical carcinogenesis). The magnitude of the interaction between HPV6 and HPV16 was about the same (Table 8), suggesting that the phenomenon is relevant for HPV16-associated cancers.

HPV16 seropositivity conferred an increased risk for HPV16 DNA-positive cervical cancer (3.2; 95% CI, 2.1–5.0) but there was no excess risk of cervical cancer with detectable HPV18 DNA. Similarly, HPV18 seropositivity conferred an increased risk for HPV18 DNA-positive cervical cancer (3.2; 95% CI, 1.3–7.7) but no excess risk of cervical cancer with detectable HPV16 DNA (Table 9).

## Discussion

We report the by far largest prospective study of HPV and other STIs with ICC as endpoint. Several interesting findings emerged. Notably, *C. trachomatis* seropositivity

**Table 3.** Adjusted ORs for risk of cervical cancer associated with seropositivity for *C. trachomatis* and Herpes Simplex 2 in HPV type 16 and 18 seropositive and seronegative women

|                              | ICC<br>OR (95% CI)                     | SCC<br>OR (95% CI) | ICC<br>OR (95% CI)                     | SCC<br>OR (95% CI) |
|------------------------------|--|--------------------|--|--------------------|
| <b><i>C. trachomatis</i></b> | HPV type 16 seropositives <sup>a</sup> |                    | HPV type 18 seropositives <sup>b</sup> |                    |
|                              | 188 cases                              | 159 cases          | 119 cases                              | 89 cases           |
|                              | 410 controls                           | 319 controls       | 297 controls                           | 229 controls       |
| Seronegative                 | 1                                      | 1                  | 1                                      | 1                  |
| Seropositive                 | 1.2 (0.9–1.8)                          | 1.2 (0.8–1.8)      | 1.9 (1.2–3.0)                          | 2.1 (1.2–3.7)      |
|                              | 391 cases                              | 290 cases          | 460 cases                              | 360 cases          |
|                              | 2,471 controls                         | 1,920 controls     | 2,584 controls                         | 2,010 controls     |
| Seronegative                 | 1                                      | 1                  | 1                                      | 1                  |
| Seropositive                 | 2.2 (1.7–2.7)                          | 2.4 (1.8–3.1)      | 1.9 (1.5–2.3)                          | 1.9 (1.5–2.5)      |
| HSV-2                        | HPV type 16 seropositives <sup>c</sup> |                    | HPV type 18 seropositives <sup>d</sup> |                    |
|                              | 188 cases                              | 159 cases          | 119 cases                              | 89 cases           |
|                              | 410 controls                           | 319 controls       | 297 controls                           | 229 controls       |
| Seronegative                 | 1                                      | 1                  | 1                                      | 1                  |
| Seropositive                 | 0.8 (0.5–1.2)                          | 0.8 (0.5–1.2)      | 0.8 (0.5–1.4)                          | 0.8 (0.4–1.4)      |
|                              | 391 cases                              | 290 cases          | 460 cases                              | 360 cases          |
|                              | 2,471 controls                         | 1,920 controls     | 2,584 controls                         | 2,010 controls     |
| Seronegative                 | 1                                      | 1                  | 1                                      | 1                  |
| Seropositive                 | 1.3 (0.96–1.7)                         | 1.2 (0.9–1.7)      | 1.2 (0.9–1.6)                          | 1.1 (0.8–1.6)      |

<sup>a</sup>OR adjusted for age at serum sampling in years, calendar year, country, blood donor status, cotinine, and HPV18.

<sup>b</sup>OR adjusted for age at serum sampling in years, calendar year, country, blood donor status, cotinine, and HPV16.

<sup>c</sup>OR adjusted for age at serum sampling in years, calendar year, country, blood donor status, cotinine, HPV18, and *C. trachomatis*.

<sup>d</sup>OR adjusted for age at serum sampling in years, calendar year, country, blood donor status, cotinine, HPV16, and *C. trachomatis*.

**Table 4.** OR and 95% CI of ICC and SCC among women seropositive for HPV16 by (a) age at serum sampling (b) lag, and (c) period of serum sampling.

|                                    | ICC                               |            |               | SCC                  |            |               |
|------------------------------------|-----------------------------------|------------|---------------|----------------------|------------|---------------|
|                                    | Adjusted OR <sup>a</sup> (95% CI) | Case +/all | Control +/all | Adjusted OR (95% CI) | Case +/all | Control +/all |
| A. Case's age at serum sampling, y |                                   |            |               |                      |            |               |
| 15–19                              | 5.5 (1.8–17)                      | 12/38      | 10/187        | 9.0 (2.1–39)         | 10/30      | 7/148         |
| 20–24                              | 2.7 (1.6–4.4)                     | 48/125     | 88/613        | 3.7 (2.1–6.5)        | 42/102     | 66/500        |
| 25–29                              | 2.4 (1.6–3.8)                     | 47/147     | 112/722       | 2.8 (1.7–4.5)        | 38/112     | 83/550        |
| 30–34                              | 2.0 (1.0–3.7)                     | 22/80      | 51/396        | 2.4 (1.1–5.0)        | 18/56      | 38/276        |
| 35–39                              | 1.7 (0.9–3.2)                     | 20/72      | 56/355        | 1.6 (0.7–3.3)        | 16/53      | 42/260        |
| 40–44                              | 2.4 (1.5–4.0)                     | 36/117     | 82/582        | 2.5 (1.5–4.3)        | 31/96      | 74/478        |
| 45–60                              | 3.1 (1.1–8.6)                     | 12/25      | 26/124        | 5.2 (1.6–17)         | 12/21      | 22/104        |
| B. Lag, mo                         |                                   |            |               |                      |            |               |
| 2–11                               | 3.7 (0.95–15)                     | 7/15       | 13/75         | 3.4 (0.7–17)         | 5/12       | 8/60          |
| 12–59                              | 2.3 (1.4–3.6)                     | 36/117     | 86/577        | 2.8 (1.7–4.7)        | 35/102     | 73/502        |
| 60–119                             | 2.7 (1.8–3.9)                     | 78/216     | 151/1,064     | 2.9 (1.9–4.5)        | 65/169     | 124/829       |
| 120–305                            | 2.3 (1.6–3.2)                     | 76/256     | 175/1,263     | 2.9 (1.9–4.3)        | 62/187     | 127/925       |
| C. period of serum sampling        |                                   |            |               |                      |            |               |
| 1973–1979                          | 2.7 (1.6–4.4)                     | 34/113     | 70/560        | 3.4 (1.9–5.9)        | 29/94      | 55/467        |
| 1980–1989                          | 2.6 (1.9–3.4)                     | 130/396    | 277/1,952     | 2.9 (2.1–4.0)        | 109/300    | 218/1,477     |
| 1990–2001                          | 2.0 (1.2–3.4)                     | 33/95      | 78/467        | 2.6 (1.4–4.8)        | 29/76      | 59/372        |

<sup>a</sup>Adjusted for HPV18, *C. trachomatis*, and cotinine.

**Table 5.** OR and 95% CI of cases and controls with SCC who were positive for HPV6, HPV16, or both

| HPV16 | HPV6 | Adjusted OR <sup>a</sup> (95% CI) | Case | Control |
|-------|------|-----------------------------------|------|---------|
| –     | –    | 1.0                               | 192  | 1,384   |
| –     | +    | 1.4 (1.1–1.9)                     | 97   | 458     |
| +     | –    | 4.1 (3.0–5.7)                     | 92   | 153     |
| +     | +    | 2.4 (1.7–3.4)                     | 67   | 160     |

NOTE: Expected multiplicative interaction = 5.8.

*P* for multiplicative interaction = 0.0004.

RERI = –2.12, 95% CI (–3.6 to –0.67).

*P* = 0.0043.

<sup>a</sup>Adjusted for HPV 18, *C. trachomatis*, and cotinine.

was associated with a clearly increased risk for cervical cancer. *C. trachomatis* has been reported as a co-factor for CIN and ICC in several previous studies (8, 36). *C. trachomatis* DNA is not present in cervical ACs (37) and prospective studies of *C. trachomatis* DNA in archival smears and the risk of cervical cancer have only found an effect in smears taken more than 6 years before the cancer diagnosis (36). These data suggest that an effect of a *C. trachomatis* infection may occur early in the carcinogenesis. This is also supported by a study of HPV DNA-positive women that had HPV-persistence as the endpoint, where *C. trachomatis* history was the only positively associated risk factor for HPV persistence (11). Other studies of the possible role of *C. trachomatis* in the etiology of cervical cancer are consistent with the notion that a possible role is not in the progression and persistence of cervical neoplasia, but possibly in the persistence of HPV infection (38, 39). A recent case–control study found that *C. trachomatis* did not affect the risk for progression to high grade CIN among HPV-positive women, suggesting that *C. trachomatis* may rather act by increasing susceptibility to HPV (40). *C. trachomatis* appeared, in our study, to associate with risk of cervical cancer only among HPV16 seronegative women, suggesting that *C. trachomatis* may increase the risk to acquire HPV16 or for HPV16 infections to persist long enough to elicit

an antibody response. Interestingly, *C. trachomatis*-associated risk was not affected by serostatus for HPV18. *C. trachomatis* infection has several ways of interfering with and evading the immune response (41), which might be a possible mechanism for an interaction of *C. trachomatis* with HPV.

We found clear prospective evidence that HPV16 and 18 preferentially cause different histologic types of cervical cancer. The fact that HPV18 preferentially associates with AC has been known for many years and there is now strong prospective epidemiologic evidence to support that exposure to different types of HPV determines the histologic type of cervical cancer (42). This study also provides prospective evidence with follow-up time of more than 10 years confirming that HPV16 and HPV18 cause cervical cancer. The fact that cervical cancer risks were about the same for HPV16 positive subjects less than 1 year before diagnosis and more than 10 years before diagnosis is consistent with the fact that the incubation time between HPV16 infection and diagnosis of ICC is typically more than 10 years.

Seropositivity for HPV16 or 18 did not confer any increased risk for cervical cancer when the converse type of HPV DNA was detected in the tumor tissue. This suggests that the HPV-associated risk is only mediated via persistence of HPV DNA and that exposure to other

**Table 6.** OR and 95% CI of cases and controls with SCC who were positive for HPV18, HPV16, or both

| HPV16 | HPV18 | Adjusted OR <sup>a</sup> (95% CI) | Case | Control |
|-------|-------|-----------------------------------|------|---------|
| –     | –     | 1.0                               | 245  | 1,705   |
| –     | +     | 1.9 (1.3–2.8)                     | 45   | 142     |
| +     | –     | 3.3 (2.5–4.4)                     | 115  | 235     |
| +     | +     | 3.2 (2.1–4.9)                     | 44   | 78      |

NOTE: Expected multiplicative interaction = 6.2.

*P* for multiplicative interaction = 0.052.

RERI = –0.957 95% CI (–2.6 to 0.69).

*P* = 0.25.

<sup>a</sup>Adjusted for *C. trachomatis* and cotinine.

**Table 7.** OR and 95% CI of cases and controls with SCC who were positive for either *C. trachomatis*, HPV16, or both

| HPV16 | <i>C. trachomatis</i> | Adjusted OR <sup>a</sup> (95% CI) | Case | Control |
|-------|-----------------------|-----------------------------------|------|---------|
| –     | –                     | 1.0                               | 148  | 1,363   |
| –     | +                     | 2.4 (1.8–3.1)                     | 142  | 482     |
| +     | –                     | 4.0 (2.9–5.5)                     | 80   | 179     |
| +     | +                     | 4.7 (3.3–6.7)                     | 79   | 134     |

NOTE: Expected multiplicative interaction = 9.5.

*P* for multiplicative interaction = 0.0036.

RERI = –0.645 95% CI (–2.5 to 1.2).

*P* = 0.49.

<sup>a</sup>Adjusted for HPV18 and cotinine.

types of oncogenic HPV does not affect, neither positively nor negatively, the risk of an HPV16 or HPV18 infection to cause cervical cancer. This is in contradiction to some other studies that have suggested that multiple infections

with high risk types increase the risk for cervical cancer compared with a single infection (2, 43).

Previous reports of an antagonistic interaction between HPV16 and HPV6 in cervical carcinogenesis (6, 7) were

**Table 8.** Adjusted OR and 95% CI of cases with HPV16 DNA-positive ICC and controls, who were seropositive for HPV16, HPV6, or both

| HPV16 | HPV6 | Adjusted OR <sup>a</sup> (95% CI) | Case | Control |
|-------|------|-----------------------------------|------|---------|
| –     | –    | 1.0                               | 60   | 455     |
| –     | +    | 1.7 (0.98–2.9)                    | 26   | 115     |
| +     | –    | 5.5 (3.2–9.5)                     | 38   | 49      |
| +     | +    | 2.1 (1.1–4.0)                     | 16   | 56      |

NOTE: Expected multiplicative interaction = 9.4.

*P* for multiplicative interaction = 0.00099.

RERI = –4.2 95% CI (–7.4, –0.92).

*P* = 0.012.

*P* for multiplicative interaction = 0.0036

RERI = –0.645 95% CI (–2.5, –1.2).

*P* = 0.49

<sup>a</sup>Adjusted for HPV18, *C. trachomatis* and cotinine.

**Table 9.** Risk of invasive cancer in case of HPV16/18 seropositivity, stratified by type of HPV DNA found in the tumor

|                        | Cases +/all | Positive% | Controls +/all | Positive% | Crude OR (95% CI) | Adjusted OR <sup>a</sup> (95% CI) |
|------------------------|-------------|-----------|----------------|-----------|-------------------|-----------------------------------|
| <b>All ICC</b>         |             |           |                |           |                   |                                   |
| HPV16 seropositive     | 84/244      | 34.4      | 171/1,210      | 14.1      | 3.2 (2.3–4.4)     | 2.7 (1.9–3.9)                     |
| HPV18 seropositive     | 56/244      | 23.0      | 139/1,209      | 11.5      | 2.2 (1.6–3.1)     | 1.3 (0.9–2.0)                     |
| <b>HPV16 DNA + ICC</b> |             |           |                |           |                   |                                   |
| HPV16 seropositive     | 57/150      | 38.0      | 111/742        | 15.0      | 3.4 (2.3–4.9)     | 3.2 (2.1–5.0)                     |
| HPV18 seropositive     | 27/150      | 18.0      | 78/741         | 10.5      | 1.8 (1.1–2.9)     | 1.0 (0.6–1.8)                     |
| <b>HPV18 DNA + ICC</b> |             |           |                |           |                   |                                   |
| HPV16 seropositive     | 10/34       | 29.4      | 29/170         | 17.1      | 2.0 (0.9–4.5)     | 1.0 (0.4–2.6)                     |
| HPV18 seropositive     | 16/34       | 47.1      | 28/170         | 16.5      | 4.3 (2.0–9.6)     | 3.2 (1.3–7.7)                     |

<sup>a</sup>ORs for only ICC (all, HPV16 DNA<sup>+</sup>, and HPV18 DNA<sup>+</sup>) related to HPV16 were adjusted for HPV18, *C. trachomatis*, and cotinine. The ORs for ICC (all, HPV16 DNA<sup>+</sup>, and HPV18 DNA<sup>+</sup>) related to HPV18 were adjusted for HPV16, *C. trachomatis*, and cotinine.

confirmed in the present study on an independent material, although we found that the magnitude of the antagonism was not strong. The interaction between HPV16 and HPV6 was seen also when restricted to HPV16 DNA-containing cancers and was strongest for HPV6, although interaction tendencies were also seen for other STIs. The mechanism of this antagonism is not known. It is clear that the antagonism is not mediated by one HPV type preventing infection by another, as antagonism is seen when there is seropositivity (a sign of past or present infection) for both HPV types. Possibly an antecedent HPV6 infection could shorten the duration of persistence and/or interfere with the oncogenic action of HPV16.

The role of HSV-2 as a co-factor in cervical cancer has been inconsistently reported for decades. We found only weak and barely significant associations that could conceivably be explained by residual confounding. Molecular analyses have failed to find any evidence of HSV-2 DNA in CIN or in cervical cancer, a finding that also does not support a role of HSV-2 in cervical cancer (14, 44).

A limitation of our study is the fact that exposure was measured only by serology and in only one sample per subject. For all the STIs measured serology is not a completely sensitive marker of exposure and also measures past exposures (20). This may result in incomplete adjustment for confounding and also means that we are not able to study the order in which the infections occurred. HPV serology is known to have high specificity, but low sensitivity (45). Incomplete adjustment for confounding may result in that secondary associations that are not of etiologic significance may partially remain significantly associated with disease also after adjustment. Because of this possibility, we have presented both crude and adjusted ORs in our tables with results. In case of residual confounding, a decline (but not total disappearance) of effect is

expected. When all STIs are adjusted for each other, the point estimates declined rather similarly for most of the STIs. We note that the decline of the point estimate for *C. trachomatis* was not any more pronounced than the decline for HPV16, suggesting that the *C. trachomatis* effect is not because of residual confounding, at least not entirely.

We conclude that our large prospective study found that (i) *C. trachomatis* was a cofactor for cervical carcinoma, possibly via an effect on HPV16, (ii) double infection with HPV16 and HPV18 does not affect the risk for developing ICC compared with a single infection, (iii) HPV6 is a weak antagonistic cofactor to HPV16, (iv) HSV-2 did not affect the risk for cervical cancer, and (v) both HPV16 and HPV18 seropositivity do not increase the cancer risk if the converse type of HPV DNA is present, underlining the importance of type-specific HPV DNA persistence in cervical carcinogenesis,

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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