CHLAMYDIA TRACHOMATIS AND INVASIVE CERVICAL CANCER: A POOLED ANALYSIS OF THE IARC MULTICENTRIC CASE-CONTROL STUDY

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To determine whether Chlamydia trachomatis infection is consistently associated with an increased risk of invasive cervical carcinoma (ICC) after accounting for the strong effect of human papillomavirus (HPV) infection, a case-control study of 1,238 cases of ICC and 1,100 control women from 7 countries was carried out (hospital-based studies in Thailand, the Philippines, Morocco, Peru, Brazil and population-based studies in Colombia and Spain, all coordinated by the International Agency for Research on Cancer, Lyon, France). Ch. trachomatis serum antibody detection was made by means of a microfluorescence assay. Among HPV DNA-positive cases and controls, the risk of squamous cell ICC was elevated in Ch. trachomatis seropositive women (OR = 1.8; 95% CI = 1.2–2.7) after adjustment for age, center, oral contraceptive use, history of Pap smears, number of full-term pregnancies and herpes simplex virus 2 seropositivity. The effect of Ch. trachomatis seropositivity on squamous cell ICC risk increased with increasing Ch. trachomatis antibody titer and was higher in women under 55 years of age. Ch. trachomatis antibodies were not associated with adenoc- or adenosquamous cell carcinoma (OR = 1.0; 95% CI = 0.53–1.9) in HPV DNA-positive women. An association of Ch. trachomatis with squamous cell ICC was found among all cases and control women with or without adjustment for HPV.

Key words: Chlamydia trachomatis; cervical cancer; human papillomavirus

Human papillomavirus (HPV) is the main, and likely necessary, cause of invasive cervical carcinoma (ICC). Recent studies of the etiology of ICC aim to identify factors that may influence susceptibility to or progression of HPV infection to cervical neoplasia or ICC. Identification of cofactors acting in conjunction with HPV, such as exogenous hormones, multiparity, smoking and other sexually transmitted infections (STIs), is important because these factors may be amenable to prevention.

Among STIs other than HPV, Chlamydia trachomatis may be an important HPV cofactor for cervical carcinogenesis. C. trachomatis are obligate intracellular bacteria that infect genital and ocular tissue. Genital C. trachomatis infections may induce chronic inflammation, epithelial tissue damage and pelvic inflammatory disease in some cases and have been clinically associated with cytologic cervical atypia and the induction of cervical metaplasia, which in turn may increase a woman’s risk of cervical neoplasia.

Positive associations between C. trachomatis microimmunofluorescence (MIF) seropositivity and ICC have been found in a case-control study from England (OR = 2.2) and a pooled analysis of cohort studies from Finland, Norway and Sweden (OR = 2.5) that adjusted for HPV virus-like particle seropositivity. However, residual confounding due to HPV cannot be ruled out in these studies because the serologic methods used to measure HPV infection were of limited sensitivity and narrow spectrum of HPV types. A Swedish cohort study indicated that C. trachomatis DNA was highly predictive of ICC development (OR = 17.1), but the yield of HPV and chlamydial DNA was low from archived Pap smear slides.

We have previously reported findings on seropositivity for C. trachomatis antibodies and ICC from 2 studies in Brazil and the Philippines and have shown an OR of 2.1 (95% CI = 1.1–4.0). To confirm and better quantify the association between prior C. trachomatis infection and ICC in different geographical settings, we expanded our pooled analyses and included 5 additional case-control studies of ICC coordinated by the International Agency for Research on Cancer (IARC).

MATERIAL AND METHODS

Contributing studies and data collection

The 7 countries in this pooled analysis include high-HPV-infection populations in Morocco, Brazil, Peru and Colombia.

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bacteria; intermediate-ICC-incidence populations in Thailand and the Philippines; and a low-ICC-incidence population in Spain. Each study used a similar protocol and questionnaire for recruitment and data collection.

Methods and findings from each study have been described previously. In summary, eligible cases were patients with incident, histologically confirmed, squamous cell, and adenocarcinoma constituent cell ICC. Cases had had no previous treatment for cervical carcinoma. Skilled pathologists reviewed the histologic slides of carcinoma cases and confirmed diagnosis. Clinical cancer stage was defined according to the International Federation of Gynecology and Obstetrics classification system.

Controls were population-based in Spain and Colombia and hospital-based in the other countries and were frequency-matched to cases by quinquennium of age. For the hospital-based studies, women with conditions related to risk factors for ICC (e.g., reproductive tract neoplasias or tobacco-related diseases) were not eligible to participate.

Personal interviews were conducted by trained interviewers using a standardized questionnaire including information on sociodemographic factors, smoking, sexual and reproductive history, history of Pap smears and selected STIs. Participants were asked to provide 10 ml of blood for the detection of C. trachomatis and herpes simplex virus type 2 (HSV-2) antibodies. Blood samples were processed by centrifugation at the site of collection. The separated serum was placed into vials, frozen at −20°C and shipped to IARC for storage. All protocols were cleared by the IARC and local ethical research committees in accordance with the Helsinki Declaration of 1983.

Chlamydia trachomatis antibody detection
C. trachomatis IgG antibodies were determined by an MIF assay, which is considered to be the reference standard for chlamydial serology. MIF assays were conducted without knowledge of case or control status. The antigen panel consisted of purified elementary bodies of C. trachomatis (serovar A and 3 pooled serovar groups of BDE, CJHI and FGK) and C. pneumoniae, which was included to monitor cross-reactive genus-specific antibody responses against all chlamydial species. Sera were screened for C. trachomatis at 1:8 dilution and titered to endpoint (1:8, 1:32, 1:128, ≥ 1:128). An IgG titer of ≥ 1:8 against any C. trachomatis serovar group was considered evidence of past C. trachomatis infection. An IgG titer of ≥ 1:16 against C. pneumoniae was considered evidence for past C. pneumoniae respiratory infections. Sera with identical titers for all C. trachomatis and C. pneumoniae species were also tested against C. psittaci (avian-strain 6BC) to determine the presence of genus-specific antibody responses. Sera from 6 cases and 11 controls with identical antibody titers for all chlamydial species were screened for HSV-2 IgG antibodies with the Gull HSV-2 ELISA, and all sera with positive, equivocal or borderline negative results were retested with the WB to obtain type-specific results.

Statistical analysis
Only cases and controls with available and valid C. trachomatis serology and HPV DNA laboratory results were included in the present analysis. The number of cases and controls thus slightly differ from those in the previous publications.

Unconditional logistic regression models were fitted to individual data. For squamous cell ICC, separate analyses for each study center were performed, then data from all centers were pooled together adjusted for center. Only pooled results adjusted for center are presented for adenocarcinoma and adenosquamous cell ICC due to the limited number of cases. Summary ORs and 95% CIs were computed from the above models, including terms for age, study center, history of Pap smears, oral contraceptive (OC) use, number of full-term pregnancies and HSV-2 seropositivity, plus number of lifetime sexual partners and age at first sexual intercourse when indicated. Women who reported hormonal contraceptive use were considered as oral contraceptive users because the use of injectable contraceptives was rare in study areas.

Tests for trend were based on the likelihood ratio test between the models with and without a linear term for the variable of interest. To test for heterogeneity among the study centers, we compared the difference between the log likelihood of the model that estimated a common OR and the model that estimated an OR for each center to the chi-square distribution with degrees of freedom equal to the number of centers minus one.

Graphs are presented, displaying for each center a black square, whose center corresponds to the estimated OR and whose size is inversely proportional to the variance of the OR. The corresponding 95% CIs are drawn as a line. Diamonds plot the summary ORs for all squamous cell ICC or for all adenocarcinoma and adenosquamous cell ICC data together. The centers of the diamonds represent the OR and the extremes show the 95% CIs.

RESULTS
A total of 1,139 squamous cell ICC, 99 adenocarcinoma and adenosquamous cell ICC cases and 1,100 controls were included (Table 1). Most squamous cell ICC (94.6%) and adenocarcinoma or adenosquamous cell ICC cases (90.9%) were HPV DNA-positive, in contrast to
14.9% of controls. Overall, C. trachomatis antibodies were more frequently in squamous cell ICC cases (53.2%) or adeno- or adenosquamous cell ICC cases (39.4%) than in control participants (30.8%). In contrast, C. pneumoniae seropositivity was similar in the 3 groups. C. trachomatis seropositivity varied significantly by country among control participants, ranging from 19.4% in Brazil to 50% in Colombia.

In control participants, C. trachomatis seropositivity was the highest in women under 35 years of age (Table II) and was significantly associated with indicators of sexual behavior. Independent risk factors were having no education, being single, separated, divorced or widowed, having a first sexual intercourse before 17 years of age, having 2 or more lifetime sexual partners and HSV-2 seropositivity. In contrast, C. pneumoniae seropositivity was not significantly associated with the indicators of sexual behavior available (data not shown), but was modestly associated with C. trachomatis seropositivity (OR = 1.4; 95% CI = 1.0 – 1.9; data not shown).

Figure 1 shows the ORs for ICC in HPV DNA-positive cases and controls according to C. trachomatis seropositivity by center and overall. The pooled OR was 1.80 (95% CI = 1.22–2.66) for overall.
squamous cell ICC and 1.03 (95% CI = 0.53–2.01) for adeno- or adenosquamous cell ICC. Especially strong associations emerged in Brazil (OR = 2.42) and Colombia (OR = 5.97), whereas the OR was 1.05 in Spain. No significant heterogeneity between centers for either squamous cell or adeno- or adenosquamous cell ICC (Fig. 1). If the data from Colombia and Spain (i.e., those centers where earlier, less sensitive tests for HPV detection were used) were eliminated, the association between C. trachomatis seropositivity and squamous cell ICC would persist (OR = 1.77; 95% CI = 1.16–2.70).

If only women positive for 14 high-risk HPV types were included, the OR for C. trachomatis seropositivity would have been 1.89 (95% CI = 1.18–3.02) for squamous cell ICC (based on 990 cases and 104 control women) and 0.95 (95% CI = 0.44–2.07) for adeno- or adenosquamous cell ICC (based on 85 cases and 85 control women). Among 1,031 HPV-positive squamous cell ICC where the clinical stage of ICC was known, the effect of C. trachomatis seropositivity was similar for stage I or II cancers (OR = 1.58; 95% CI = 1.19–2.90; data not shown).

Table III shows that elevated C. trachomatis antibody titers were particularly associated with an increased risk of squamous cell ICC in HPV DNA-positive women (p for trend < 0.001), with the highest risk in women with antibody titers ≥ 128 (OR = 3.58). The effect of C. trachomatis was similar for the different C. trachomatis serovar groups (OR = 2.17 for A, 2.21 for BED, 1.65 for CJHI and 1.87 for FGK). The presence of 2 or more serovar groups was associated with a nonsignificantly elevated OR of 1.60 (95% CI = 0.84–3.03) compared to the presence of a single serovar group of BED, CJHI or FGK (data not shown). C. pneumoniae seropositivity was not associated with an increased risk of squamous cell ICC (OR = 1.03).

When the association between C. trachomatis infection and squamous cell ICC was analyzed by categories of age at diagnosis (Fig. 2), the effect of C. trachomatis appeared to be attenuated in older-age women, with no increased risk observed over 55 years of age (OR = 0.95).

Analyses were conducted to examine the association of C. trachomatis seropositivity and squamous cell ICC in strata of selected indicators of socioeconomic status (data not shown). The effect of C. trachomatis tended to be nonsignificantly lower (p = 0.43) in women with less education (no education: OR = 1.07, 95% CI = 0.47–2.39; primary education: OR = 1.74, 95% CI = 0.95–3.19; secondary or more education: OR = 2.55, 95% CI = 1.15–5.67). Results were consistent in women with rural (OR = 1.24; 95% CI = 0.54–2.84) and urban (OR = 1.84; 95% CI = 1.08–3.07) residence.

Table IV shows the combined effect of C. trachomatis and selected indicators of sexual behavior and oral contraceptive use on the risk of squamous cell ICC in HPV-positive women. The association between C. trachomatis infection and squamous cell ICC (Fig. 1) was slightly attenuated, but remained significant, after further adjustment for number of sexual partners and age at first intercourse (OR = 1.69; 95% CI = 1.16–2.51). After additional adjustment of C. trachomatis in the fully adjusted model, a woman’s reported number of sexual partners was not significantly associated with squamous cell ICC risk (OR = 1.29; 95% CI = 0.83–2.0), whereas young age at first intercourse (OR = 2.44 for < 17 vs. ≥ 21 years) and ≥ 5 years oral contraceptive use (OR = 2.69) remained statistically significant risk factors.
TABLE III - ODDS RATIOS OF SQUAMOUS CELL INVASCIVE CARCINOMA OF THE CERVIX AND CORRESPONDING 95% CONFIDENCE INTERVALS AMONG HPV-POSITIVE WOMEN ACCORDING TO CHLAMYDIA TRACHOMATIS AND CHLAMYDIA PNEUMONIAE SEROPOSITIVITY OVERALL AND IN STRATA BY AGE GROUP AT DIAGNOSIS

| CHLAMYDIA TRACHOMATIS | HPV+ cases (%) | HPV+ controls (%) | OR (95% CI) |
|-----------------------|---------------|------------------|------------|
| Titers                |               |                  |            |
| Seronegative          | 500 (46.4)    | 104 (64.4)       | 1          |
| Seropositive          | 413 (39.5)    | 123 (75.0)       | 1          |
| 8                     | 176 (16.3)    | 25 (15.2)        | 1.28 (0.76–2.14) |
| 32                    | 239 (22.2)    | 24 (14.6)        | 1.71 (1.01–2.87) |
| ≥ 128                 | 163 (15.1)    | 11 (6.7)         | 3.58 (1.73–7.39) |

Chi-square trend (p-value)

Serovars

A

Seronegative

Seropositive

BED

Seronegative

Seropositive

CHI

Seronegative

Seropositive

FGK

Seronegative

Seropositive

Chlamydia pneumoniae

Seronegative

Seropositive

756 (70.1)

500 (46.4)

104 (64.4)

413 (39.5)

123 (75.0)

1.27 (1.14–1.43)

1.83 (1.49–2.25)

1.33–2.52

1.35 (0.63–2.94)

3.57 for titers of 8, OR 1.19 for titers of 32, OR 2.03 for titers of ≥ 128.

DISCUSSION

This study, based on data from 1,238 case and 1,100 control participants in 7 countries worldwide, shows that C. trachomatis serum antibodies were associated with a 1.8-fold increased risk of squamous cell ICC. An increased squamous cell ICC risk was consistently found in all countries considered, except for Spain, although C. trachomatis seropositivity in controls varied greatly by country. This risk was higher in women with elevated C. trachomatis antibody titers, and in women under 55 years of age. C. trachomatis and C. pneumoniae species-specific serum antibodies were differentiated using MIF assay, and an increased risk of squamous cell ICC was found in women with C. trachomatis but not with C. pneumoniae antibodies. Our study thus supports the possibility that C. trachomatis increases squamous cell ICC risk, after accounting for cervical HPV infection using highly sensitive PCR-based methods, and other risk factors for ICC. Our ability to restrict analyses to HPV DNA-positive case and control participants, albeit justified by the causal link between HPV infection and ICC, did not alter the relationship between C. trachomatis and squamous cell ICC risk. The effect of C. trachomatis was similar for HPV DNA-positive women and for all cases and controls after adjustment for HPV DNA status or HPV DNA type. After adjustment for major confounding factors, but not for HPV DNA status, the OR for squamous cell ICC was 2.2 (i.e., slightly more elevated than after stratification or adjustment for HPV).

Our analysis of C. trachomatis in 99 adeno- or adenosquamous cell ICC cases is the largest to date, given the relative rarity of cervical adenocarcinoma. Our findings are consistent with another study of those from 32 adenocarcinoma cases that found no effect due to C. trachomatis. Overall, these data support the notion that some HPV cofactors may differ according to the histologic type of ICC.
measured *C. trachomatis* serum antibodies. The specificity of the observed MIF antibody responses was validated by the observation that *C. trachomatis*, unlike *C. pneumoniae*, antibody responses were correlated with indicators of sexual behavior.

The dose-response relationship between *C. trachomatis* MIF antibody titers and squamous cell ICC risk provides further support for an etiologic role for *C. trachomatis* in cervical carcinogenesis. Elevated *C. trachomatis* titers (≥ 128) are likely to be maintained by continued antigenic stimulation, which may be due to either repeated or chronic chlamydial infections. Elevated *C. trachomatis* titers have also been associated with adverse reproductive sequelae such as tubal infertility, pelvic inflammatory disease and the presence of heat shock proteins (HSPs). The latter finding is of particular interest given that HSPs appear to be involved in the immunopathogenesis of chlamydial infections.

No specific *C. trachomatis* serovar group had a higher association with squamous cell ICC in our study. *C. trachomatis* serotypes fall into 2 major antigenic groups, the B complex (consisting of serovars of B, D and E) and the C complex (consisting of serovars of C, J, H and I), with serovars of F, G and K as bridging serovars between the 2 major antigenic complexes (*i.e.*, they share minor antigenic profiles with both major complexes in addition to unique antigenic characteristics). Serologic assays such as the MIF measure cumulative exposure of an individual to antigens of *C. trachomatis*. An individual who is infected with one serovar and then subsequently exposed to another serovar from a heterologous antigenic complex will exhibit broad antibody reactivity to multiple serovars. A nested case-control study from Finland, Norway and Sweden found that serovar G was most strongly associated with squamous cell ICC but the confidence intervals were broad, and it is unclear how an increased risk for squamous cell carcinoma may be attributable to a single serovar using serologic assays since complex antigenic relationships exist between serovars of *C. trachomatis*.

The association between squamous cell ICC and *C. trachomatis* seropositivity appeared to be stronger in younger women (< 55 years of age), suggesting that cumulative exposure to chlamydial infection may be less important, or less-well measured by seropositivity after middle age. The prevalence of *C. trachomatis* IgG antibodies declined with age among squamous cell ICC cases but not among control women. Few reliable data are available on the natural history of *C. trachomatis* antibody profiles during persistent or acute infections. *C. trachomatis* antibodies have been shown to persist for years; however, a loss of *C. trachomatis* antibodies or a decrease in IgG antibody titers over time may occur, particularly if there is no continued antigenic stimulation. A case-control study showed a stronger association between *C. trachomatis* seropositivity and squamous cell ICC when the serum sampling was closer in time to ICC diagnosis.

**C. trachomatis** infection may increase the risk of squamous cell ICC by increasing host susceptibility to HPV or enhancing the effects of HPV. Inflammation, resulting from a chronic *C. trachomatis* infection, may result in the production of reactive oxygen species that may cause DNA damage and increase the risk of HPV-associated carcinogenesis. *In vitro* data suggest that Chlamydia-infected cells are also less likely to undergo the normal process of programmed cell death. Other laboratory data indicate that *C. trachomatis* may disrupt the normal structure of cadherin-catenin junctions in cervical epithelial cells, resulting in increased susceptibility to HPV and other infections. Alternatively, *C. trachomatis* seropositive women may be more likely to elicit a humoral-mediated (Th2) rather than cell-mediated (Th1) immune response to particular antigens. In this case, women infected with

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**TABLE IV - ODDS RATIOS OF SQUAMOUS CELL INVASIVE CARCINOMA OF THE CERVIX AND CORRESPONDING 95% CONFIDENCE INTERVALS AMONG HPV-POSITIVE WOMEN ACCORDING TO THE COMBINED EFFECT OF CHLAMYDIA TRACHOMATIS SEROPOSITIVITY AND SEXUAL BEHAVIOR**

| Category                          | Seronegative cases/controls | Seropositive cases/controls | OR^2 (95% CI) |
|----------------------------------|-----------------------------|----------------------------|---------------|
| Number of sexual partners        |                             |                            |               |
| 1                                | 344/78                      | 319/37                     | 1.00 (0.95–1.05) |
| ≥ 2                              | 156/26                      | 259/23                     | 1.00 (0.95–1.05) |
| Age at first intercourse (years) |                             |                            |               |
| ≥ 21                             | 149/48                      | 106/20                     | 1.00 (0.95–1.05) |
| 17–20                            | 211/31                      | 250/19                     | 1.00 (0.95–1.05) |
| < 17                             | 140/23                      | 222/20                     | 1.00 (0.95–1.05) |
| Oral contraceptive use           |                             |                            |               |
| Never                            | 347/69                      | 387/39                     | 1.00 (0.95–1.05) |
| ≤ 5 years                        | 81/28                       | 92/15                      | 1.00 (0.95–1.05) |
| ≥ 5 years                        | 71/6                        | 95/6                       | 1.00 (0.95–1.05) |
| OR (95% CI)                      | 1.69 (1.14–2.51)            |                            |               |

1Adjusted for age, study center, history of Pap smear, oral contraceptive use, number of full-term pregnancies, herpes simplex virus 2 seropositivity, number of sexual partners and age at first intercourse.–2Adjusted as above and for *C. trachomatis* seropositivity.–3Reference category.–4Chi-square for interaction = 0.00 (1 df), p = 0.95.–5Chi-square for interaction = 0.16 (2 df), p = 0.93.–6Chi-square for interaction = 0.05 (2 df), p = 0.70.

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C. trachomatis may have an impaired ability to clear an HPV infection or control HPV-induced cervical neoplasia. In a cohort study of Swedish women aged 32–38 years, self-reported history of C. trachomatis was associated with a 4-fold increased risk of HPV persistence after 12-month follow-up.39 We do not believe that our findings should be interpreted as C. trachomatis acting as a carcinogen of the cervix independently from HPV infection and note that no methods can distinguish transient from persistent HPV infection. The ascertainment of cervical HPV DNA is likely to have, for instance, a different meaning in case and control participants. HPV DNA in case women should indicate a persistent HPV infection, whereas some control women may be transiently infected or have been infected in the past and since cleared their infection. However, because the association between C. trachomatis and squamous cell ICC in HPV-positive women was not notably reduced after further adjustment or stratification for a woman’s number of sexual partners, age at first intercourse and HSV-2 seropositivity, the effect of C. trachomatis is not likely to represent simply exposure to HPV or other STIs.

Another limitation of our study is that the MIF assay used does not differentiate a woman’s exposure to genital or to ocular C. trachomatis infections. However, it is unlikely that our results are due to past exposure to ocular infections because endemic trachoma is rare or nonexistent in all study sites. Furthermore, the association between C. trachomatis and squamous cell ICC was consistent in strata of educational attainment and place of residence, although ocular infection should have been more frequent in low social classes and in rural areas. The use of hospital-based control subjects in 5 study sites may have also led to biased results if C. trachomatis seroprevalence in controls was not representative of the population source of the ICC patients. Hospital controls in our study, however, had a wide range of diagnoses and were ascertained in large public hospitals with reference populations similar to those where cases were identified.16–18,20,21

In conclusion, our results indicate that C. trachomatis infection may act in conjunction with HPV to increase the risk of squamous cell ICC, although this association is modest compared to the strong effect of HPV infection on ICC risk. Given that C. trachomatis is the most common bacterial STI worldwide, with over 12 million global cases estimated annually40 and 700,000 cases reported in the United States,41 additional data are needed to determine if screening and treatment of C. trachomatis infections may result in a lower incidence of low- and high-grade squamous intraepithelial lesions.

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APPENDIX

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