

Chlamydia trachomatis and risk of cervical intraepithelial neoplasia grade 3 or worse in women with persistent human papillomavirus infection: a cohort study

Kirsten E Jensen,¹ Louise T Thomsen,¹ Sven Schmiedel,² Kirsten Frederiksen,² Bodil Norrild,³ Adriaan van den Brule,⁴ Thomas Iftner,⁵ Susanne K Kjær^{1,6}

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¹Unit of Virus, Lifestyle, and Genes, Danish Cancer Society Research Center, Copenhagen, Denmark

²Unit of Statistics, Bioinformatics and Registries, Danish Cancer Society Research Center, Copenhagen, Denmark

³Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark

⁴Laboratory for Molecular Diagnostics, Department of Medical Microbiology and Pathology, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands

⁵Medical Virology, Section of Experimental Virology, University Hospital of Tübingen, Tübingen, Germany

⁶Gynecologic Clinic, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

Correspondence to

Professor Susanne K Kjær, Unit of Virus, Lifestyle, and Genes, Danish Cancer Society Research Center, Strandboulevarden 49, Copenhagen DK-2100, Denmark; susanne@cancer.dk

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ABSTRACT

Objectives Some studies suggest that *Chlamydia trachomatis* (CT) enhances cervical carcinogenesis; however, a possible confounding effect of persistent human papillomavirus (HPV) infection was not addressed. We examined the potential role of CT infection in the development of subsequent cervical intraepithelial neoplasia grade 3 or worse (CIN3+) in women with prevalent HPV infection and in a subgroup of women with persistent HPV infection.

Methods Participants in this population-based cohort study underwent a structured interview, including history of CT infection, and subsequently cervical exfoliated cells were obtained for HPV DNA and CT DNA testing. Women with high-risk HPV DNA infection and no prevalent cervical disease constituted the overall study population (n=1390). A subgroup of women with persistent HPV infection (n=320) was also identified. All women were passively followed for development of cervical lesions in the national Pathology Data Bank. HRs and 95% CIs for CIN3+ during follow-up (up to 19 years) were estimated in an accelerated failure time model.

Results Women who reported more than one CT infection had a statistically significantly increased risk of CIN3+ (high-risk HPV-positive, HR=2.51, 95% CI 1.44 to 4.37) (persistent HPV infection, HR=3.65, 95% CI 1.53 to 8.70). We found no association between CT DNA and subsequent risk of CIN3+ among women who were HPV-positive or had a persistent HPV infection at baseline.

Conclusions Repeated CT infections increased the risk of CIN3+ among women with prevalent as well as persistent high-risk HPV infection.

INTRODUCTION

Cervical cancer is a rare event following infection with high-risk human papillomavirus (HPV), and persistent HPV infection is necessary in cervical carcinogenesis.^{1–2} It has been debated whether *Chlamydia trachomatis* (CT) also increases the risk of developing cervical cancer and its precursors.^{3–4} Some longitudinal studies have observed CT infection to increase the risk of HPV acquisition^{4–5} or HPV persistence.⁶ If these associations between CT and HPV are true, this could lead to an indirect association between CT infection and cervical cancer.

In the assessment of whether CT more directly influences cervical carcinogenesis in addition to

HPV, only few studies of HPV-positive women have been conducted with equivocal results.^{4–7–11} Some of these studies reported no association between CT DNA or CT antibodies and cervical intraepithelial neoplasia grade 3 or worse (CIN3+) among HPV-positive women,^{4–10–11} whereas others observed an association between CT antibodies and cervical cancer,^{7–9} and one reported an association with CIN2.¹¹ A new approach to clarify the influence of CT on CIN3+ is to follow women with persistent HPV infection in relation to CT infection. Such a design more stringently complies with the natural history of HPV as essential in cervical carcinogenesis.^{1–2} A growing body of literature suggests possible molecular mechanisms for a role of CT in cervical carcinogenesis such as inhibition of host cell apoptosis,¹² induction of chromosome instability¹³ and release of reactive nitric oxide and oxidative species,^{14–15} which may result in DNA damage and thus promote carcinogenesis.^{15–16}

Our aim was to further examine whether CT increases the risk of progression from high-risk HPV infection to CIN3+. We analysed data from a prospective, population-based cohort study with up to 19 years of follow-up and various measures of CT infection (DNA, self-report). The analysis was restricted to women who were high-risk HPV DNA-positive at baseline; in a subanalysis, we examined the risk of progression in relation to CT infection among women with persistent high-risk HPV infection.

MATERIALS AND METHODS

Data collection

Our study population was women participating in a population-based cohort study initiated in Denmark in 1991.¹ In total, 17 949 women aged 20–29 years were randomly sampled from the general female population of Copenhagen through the computerised national Central Population Register, in which all inhabitants of Denmark are recorded with a unique personal identification number. Women who had moved out of the study area before our contact were ineligible for the study (n=1604). The remaining 16 345 women were invited to participate. Between May 1991 and January 1993, 11 088 women (response rate, 68%) participated in the first study visit. Approximately 2 years later (October 1993–January 1995), the participants were reinvited in the same order as their original enrolment. In total, 8656 women

(response rate, 78%) participated in the second visit. All participants gave written informed consent before entering the study. The study protocol was approved by the national Scientific Ethics Committee and the national Data Protection Board. At both visits, the women underwent a gynecological examination, at which a Pap smear was taken and endo-ectocervical exfoliated cells were obtained for later HPV DNA and CT DNA detection. The women also participated in a structured interview to obtain information on sociodemography, lifestyle, contraceptive use, sexual behaviour, reproductive history and history of sexually transmitted infections, including questions concerning self-reported CT infections, number of CT infections and age at first CT infection.

HPV DNA testing

HPV DNA in cervical samples was detected by Hybrid Capture 2 (HC2; Qiagen-Hilden, Germany).¹ The cut-off of 1.0 pg/mL recommended by the US Food and Drug Administration was used, with only the high-risk probe that detects at least 13 oncogenic types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68).¹⁷ The HC2 was used to define high-risk HPV positivity in the overall study population. Among the women high-risk HPV-positive by HC2, genotyping was performed by a PCR-based method, LiPA2 (Innogenetics, Inc., Ghent, Belgium).¹⁸ This assay identifies 24 HPV types; we were interested in the high-risk types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The LiPA2 results were used to define persistent high-risk HPV infection.

CT DNA testing

CT DNA was detected in cervical samples by PCR, as described previously.^{19, 20} PCR products were subsequently analysed by a non-radioactive enzyme immunoassay.²¹ As positive controls, dilutions of CT serovar L2 DNA were used. Cut-off values used in PCR enzyme immunoassay were OD values corresponding to 0.01–0.1 inclusion forming unit L2 DNA.¹⁹

Study population

Baseline was defined as the date of the second study visit. Among the 8656 women who participated in both visits, 1435 tested positive for high-risk HPV at baseline. We excluded 29 women with an inadequate baseline smear or a prevalent abnormal cervical diagnosis (moderate dysplasia or worse) at baseline, within 1 year before baseline or up to 60 days after baseline. Furthermore, 16 women did not undergo a cervical examination during follow-up and were excluded. Thus, our final study population consisted of 1390 women with high-risk HPV infection (HC2) at baseline. Furthermore, we identified a subgroup of women positive for the same high-risk HPV type at baseline and the examination 2 years before baseline. This subgroup was defined as having persistent high-risk type-specific HPV infection ($n=320$) (LiPA2).

Follow-up

The cohort was followed passively through the national Pathology Data Bank (PDB). We have previously reported on parity and smoking as HPV cofactors with follow-up until 2007.^{22, 23} For the present analysis, we extended follow-up to 31 December 2012. The PDB contains information on all cervical cytology and histology performed in Denmark. Pathology departments communicate with the PDB through an online real-time data reporting system. By linking the unique personal identification number of each participant to the PDB, we collected individual information about incident abnormal cervical

cytology and histology. In the PDB, abnormal cervical diagnoses are usually reported as atypia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ or cancer. For our analysis, the histological diagnoses were translated into the CIN nomenclature and severe dysplasia, carcinoma in situ and cancer were categorised as CIN3+ and chosen as outcome.

Data analysis

We examined the risk of CIN3+ according to CT status as determined by DNA or self-report. A woman was considered CT DNA-positive if the CT DNA test results from at least one of the two examinations were positive. CT DNA-positive women were further categorised according to the number of CT DNA-positive tests (0/1/2). Because of small numbers, this categorisation was done only for the overall study population. A woman was considered to have had a self-reported CT infection if she reported a history of CT infection at either of the study

Table 1 Distribution of demographic, lifestyle, reproductive and sexual factors among *Chlamydia trachomatis* (CT) DNA-positive women, women with a self-reported previous CT infection and in the overall study population of high-risk HPV-positive women

	CT DNA-positive ($n=131$) (%)	Self-reported CT ($n=355$) (%)	Overall ($n=1390$) (%)
Age (years)			
22–24	39.7	30.9	29.9
25–27	35.9	34.0	33.2
28–32	24.4	35.1	36.8
Length of schooling (years)			
≤9	7.6	5.6	5.8
10–11	29.8	25.8	25.5
≥12	62.6	68.5	68.5
Smoking			
Never	38.2	31.7	36.0
Previous	22.9	23.9	20.2
Current	38.9	44.4	43.7
Childbirth			
Never	90.8	83.2	83.4
Ever	9.2	16.8	16.4
Use of oral contraceptives			
Never	14.5	12.4	13.7
Previous	42.0	48.0	47.6
Current	43.5	39.6	38.6
Use of intrauterine devices			
Never	80.2	71.9	77.1
Previous	12.2	19.1	15.2
Current	7.6	9.0	7.7
Age first intercourse (years)			
≤15	26.7	35.4	31.9
16–17	42.0	41.0	38.6
≥18	31.3	23.6	29.4
Lifetime number of partners			
≤4	4.6	5.3	13.7
5–9	35.1	23.0	27.6
10–14	22.9	27.3	24.2
≥15	37.4	44.4	35.5
Pelvic inflammatory disease			
Never	83.2	72.5	79.4
Ever	16.8	27.5	20.6

visits. We additionally examined whether the number of self-reported CT infections (0/1/≥2) or age at first CT infection (continuous and categorical (≤19 years/20–24 years/≥25 years)) was associated with the risk of CIN3+.

The associations between CT infection and risk of CIN3+ were estimated in an accelerated failure time model, taking into account that the exact date of development of CIN3+ was unknown (interval-censored response variable). A Weibull distribution was used for failure time. HRs with corresponding 95% CIs were estimated for the overall study population of high-risk HPV-positive women and for women with persistent HPV infection. Women with missing information concerning CT DNA or self-reported CT infection were excluded from the particular model and included in the other models.

To determine the influence of number of cervical examinations during follow-up, we assessed the average number of cervical smears per year between baseline and end of study in relation to CT status among women without cervical lesions during follow-up (n=888) using F-test. Follow-up was calculated as the difference between the individual baseline date and end of study.

The HRs were adjusted for age, childbirth (ever/never), length of schooling (≥12 years/10–11 years/≤9 years) and smoking status (never/former/current) at baseline. When initially adjusting for self-reported age at first intercourse, lifetime number of partners and pelvic inflammatory disease, it did not markedly change the results, and therefore these variables were not included in the final model.

RESULTS

Baseline characteristics of CT DNA-positive women, women with a self-reported CT infection and in the overall study population of HPV-positive women are presented in table 1. CT DNA-positive women were younger and less likely to be smokers or to have given birth than women with a self-reported CT infection.

The overall follow-up was 18.1–19.2 years, and CIN3+ was diagnosed in 200 of the HPV-positive women. Table 2 presents

mean follow-up, proportion of CIN3+, prevalence of CT DNA and self-reported CT infection among high-risk HPV-positive women. The mean follow-up was similar between CT categories and varied between 18.6 and 18.8 years. There was some overlap between CT DNA-positive women and women with self-reported CT infection: 21.7% of women who reported two or more CT infections, 14.2% of women with one previous CT infection and 7.5% of women with no self-reported CT infection were CT DNA-positive. Table 2 also shows that the average number of yearly cervical smears did not differ significantly by self-reported number of CT infections (p=0.2) or by self-reported age at first CT infection (p=0.2). Women with one or two CT DNA-positive tests had a lower average number of cervical smears (0.26 or 0.24 smears/year) than women with no CT DNA-positive tests (0.29 smears/year) (p=0.01).

Table 3 presents the HRs for CIN3+ according to CT status in high-risk HPV-positive women. We observed no association between CT DNA and subsequent CIN3+ in the age-adjusted model or after adjustment for age, childbirth, length of schooling and smoking (HR=0.88, 95% CI 0.53 to 1.47). Furthermore, no association was observed according to the number of CT DNA-positive tests. When we subdivided the women with one CT DNA infection into those with incident CT (CT-negative at visit 1, CT-positive at visit 2, n=24) and those with transient CT (CT-positive at visit 1, CT-negative at visit 2, n=86), we also found no association between the pattern of CT DNA detection and CIN3+ (data not shown). No association was seen between the overall measure of self-reported CT infection and CIN3+, whereas a statistically significantly increased risk was observed for women who reported ≥2 CT infections (HR=2.51, 95% CI 1.44 to 4.37). For self-reported age at first CT infection, there was no specific pattern (table 3).

In the subgroup of women with persistent high-risk HPV infection, CIN3+ was diagnosed in 80 women during follow-up. The HRs for CIN3+ in relation to CT status in this subgroup are given in table 4. Similar to the overall study population, we observed no association between CT DNA positivity

Table 2 Mean follow-up, proportion of cervical intraepithelial neoplasia grade 3 or worse (CIN3+), prevalence of *Chlamydia trachomatis* (CT) infection (DNA, self-reported) in the overall study population of high-risk human papillomavirus (HPV)-positive women (n=1390) as well as mean number of yearly cervical cytology examinations during follow-up among women with no cervical lesions during follow-up (n=888)

	N	Mean follow-up (years)	CIN3+ (%)	CT DNA pos. (%)	Self-reported history of CT (%)	Mean no. of cervical smears* (per year)	F-test (p value)
No. of CT DNA-positive test							0.01
0	1249	18.7	14.7	–	24.0	0.29	
1	110	18.7	12.7	–	46.4	0.26	
2	21	18.8	9.5	–	14.3	0.24	
Missing	10	18.6	10.0	–	20.0		
Self-reported no. of CT infections							0.2
0	1033	18.7	13.5	7.5	–	0.29	
1	309	18.7	15.2	14.2	–	0.30	
≥2	46	18.7	30.4	21.7	–	0.30	
Missing	2	18.6	0.0	0.0	–		
Self-reported age at first CT infection							0.2
Never	1033	18.7	13.5	7.5	–	0.29	
≤19	85	18.7	15.2	9.4	–	0.30	
20–24	205	18.7	17.6	16.1	–	0.30	
≥25	66	18.7	17.7	19.7	–	0.31	
Missing	1	18.8	0.0	0.0	–		

*Number of cervical cytology examinations per year during follow-up among women who did not develop cervical lesions during follow-up.

Table 3 HRs and 95% CIs for a diagnosis of cervical intraepithelial neoplasia grade 3 or worse (CIN3+) according to *Chlamydia trachomatis* (CT) status among 1390 women infected with high-risk human papillomavirus (HPV) at baseline

	HR*	95% CI	HR†	95% CI
CT DNA				
Negative	1		1	–
Positive	0.84	0.50 to 1.40	0.88	0.53 to 1.47
Number of CT DNA-positive tests				
0	1		1	–
1	0.87	0.51 to 1.50	0.90	0.52 to 1.56
2	0.67	0.17 to 2.72	0.73	0.18 to 2.98
Trend		p=0.5		p=0.6
Self-reported CT infection				
Never	1		1	–
Ever	1.30	0.96 to 1.76	1.28	0.95 to 1.73
Self-reported number of CT infections				
0	1		1	–
1	1.14	0.82 to 1.59	1.12	0.81 to 1.56
≥2	2.55	1.47 to 4.43	2.51	1.44 to 4.37
Trend		p<0.01		p<0.01
Self-reported age at first CT infection (years)				
Never	1		1	–
≥25	1.18	0.61 to 2.27	1.29	0.66 to 2.50
20–24	1.33	0.92 to 1.19	1.28	0.89 to 1.85
≤19	1.34	0.78 to 2.31	1.27	0.73 to 2.19
Continuous (per year)‡	0.96	0.89 to 1.05	0.98	0.90 to 1.07

*Adjusted for age at baseline.

†Adjusted for the baseline characteristics age, ever given birth, length of schooling and smoking status.

‡Among women who reported CT infection.

and subsequent CIN3+. An increased risk of CIN3+ was observed with ≥2 self-reported CT infections (HR=3.65, 95% CI 1.53 to 8.70). The HR was also increased for self-reported

young age at first CT infection (≤19 years old), although the estimate did not reach statistical significance (HR=1.62, 95% CI 0.77 to 3.41). The risk decreased by 9% per increasing year of age at first CT infection (HR=0.91, 95% CI 0.81 to 1.03). Mutual adjustment for self-reported number of CT infections and age at first CT infection did not affect the corresponding estimated associations among all HPV-positive women, nor among persistently HPV-positive women (data not shown).

DISCUSSION

In this prospective study with up to 19 years of follow-up, more than one self-reported CT infection increased the risk of developing CIN3+ in addition to the risk associated with prevalent as well as persistent high-risk HPV infection. Furthermore, we observed a tendency towards an increased risk with self-reported young age at first CT infection among women with persistent HPV infection, whereas CT DNA positivity was not associated with CIN3+. As CT and HPV DNA testing took place several years after the study examinations, the women and their health professionals were unaware of the test results, and the results were not used for clinical management. We did not observe any large differences in the number of follow-up cervical smears among women with 0, 1 or 2 CT DNA-positive tests (0.29, 0.26 and 0.24 smears/year), respectively. Additionally, we observed no significant difference in the number of cervical smears in relation to self-reported CT infection. Our results concerning CT DNA are in accordance with two previous prospective studies,^{4, 11} which observed no association between CT DNA and subsequent CIN3+ among baseline HPV DNA-positive women, although Lehtinen *et al*¹¹ reported an association between CT DNA and CIN2. However, this study¹¹ had short follow-up (3.7 years), which could have underestimated the effect of CT infection.

The presence of CT DNA in the cervix indicates current CT infection, whereas self-reported CT infection could be interpreted as previous exposure. Most studies of HPV-positive

Table 4 HRs and 95% CIs for a diagnosis of cervical intraepithelial neoplasia grade 3 or worse (CIN3+) according to *Chlamydia trachomatis* (CT) status among 320 women with persistent human papillomavirus (HPV) infection at baseline

	n*	% CIN3+	HR†	95% CI	HR‡	95% CI
CT DNA						
Negative	296	25.7	1		1	–
Positive	23	13.0	0.51	0.16 to 1.63	0.53	0.17 to 1.70
Self-reported CT infection						
Never	218	22.9	1		1	–
Ever	102	29.4	1.39	0.88 to 2.18	1.38	0.87 to 2.18
Self-reported number of CT infections						
0	218	22.9	1		1	–
1	94	25.5	1.18	0.73 to 1.93	1.20	0.73 to 1.95
≥2	8	75.0	4.55	1.95 to 10.63	3.65	1.53 to 8.70
trend				p=0.02		p=0.02
Self-reported age at first CT infection (years)						
Never	218	22.9	1		1	–
≥25	18	22.2	0.95	0.33 to 2.74	1.10	0.38 to 3.21
20–24	59	28.8	1.37	0.79 to 2.38	1.36	0.78 to 2.36
≤19	25	36.0	1.82	0.87 to 3.79	1.62	0.77 to 3.41
Continuous (per year)§			0.89	0.79 to 1.00	0.91	0.81 to 1.03

*One woman had missing information on CT DNA status. No missing information concerning self-reported CT infection.

†Adjusted for age at baseline.

‡Adjusted for the baseline characteristics age, ever given birth, length of schooling and smoking status.

§Among women who reported CT infection.

women used CT antibodies as the marker of previous CT exposure, and the results have been equivocal.^{4 7–10} Safaiean *et al*⁴ observed no association between CT antibodies and CIN3+. Conversely, another prospective study reported that CT antibodies increased the risk of cervical cancer among HPV18-positive women but not among HPV16-positive women.⁷

Unlike our study, none of the previous studies with analyses restricted to HPV-positive women^{4 7–11} used CT measures of number of infections or age at first infection. The significantly increased risk of CIN3+ with ≥ 2 self-reported CT infections and the finding of a significant linear increasing trend with increasing number of self-reported CT infections ($p < 0.01$) indicate a dose-response relation. If the temporal order of CT and HPV infection is important in relation to the role of CT in cervical carcinogenesis, then an increased number of CT infections enhances the likelihood of obtaining the precise timing that could facilitate cervical cancer development. Another explanation could be that women with more CT infections are more vulnerable to infections in general and have poorer immune response, which is associated with cervical carcinogenesis.²⁴ Given that HPV is necessary for cervical cancer development,^{1 2} the mechanisms in studies reporting associations between CT and subsequent CIN without stratifying for HPV status could either be that CT infection increases the risk of HPV acquisition or that CT infection increases the risk of CIN among already HPV-positive women.^{3 4} A publication using the same cohort as in our study found no increased risk of HPV acquisition with neither CT DNA nor self-reported CT.²⁵ Furthermore, when we analysed the risk of CIN3+ among high-risk HPV-negative women, we found no association with CT DNA or self-reported CT (data not shown).

If our finding of an increased, but statistically non-significant, risk of CIN3+ with young age at first CT infection among women with persistent HPV infection is a true association, a partial explanation might be that the cervix is not fully matured in young women and higher levels of inflammatory cytokines are observed in the immature cervical epithelium.²⁶ CT infections at a young age might therefore be characterised by severe cervical inflammation with production of reactive oxygen species, which could facilitate DNA damage and HPV integration and thereby increase the risk of cervical carcinogenesis.¹⁵ However, it cannot be excluded that the results are affected by the shared sexually transmitted nature of HPV and CT infections and that those women with young age at first CT infection are more likely to have longer-term persistent HPV infection.

No other study has investigated the association between CT infection and subsequent CIN3+ among women with persistent HPV infection. Previous studies measured HPV status only once,^{4 7–11} reflecting both transient and persistent infections, whereas we tested for HPV twice to identify women with persistent HPV infection. If the observed risk of CIN3+ in relation to self-reported CT infection among women with persistent HPV infection is real and not merely a chance finding, this result is not likely solely to be explained by an association between HPV persistence and CT infection, even though a previous study did observe self-reported CT infection to be associated with subsequent HPV persistence.⁶ Our results may indicate that additional CT infections also influence the risk of progression from HPV persistence to CIN3+.

The strengths of this study include its prospective design, a long follow-up and virtually no loss to follow-up because of the existence of the unique personal identification number and a nationwide PDB with almost complete coverage. Furthermore,

we used several measures of CT exposure (DNA, self-reported), and we restricted the analysis to women with persistent HPV infection, which ensured that the population was truly at risk of CIN3+. We defined HPV persistence as concordance in the genotype at two consecutive measurements 2 years apart. This interval is longer than the average clearance time for high-risk HPV infections.²⁷ This definition could, however, in theory, also capture reinfections with the same genotype. Nevertheless, our definition of persistence has proved to be highly predictive of genotype-specific progression.¹ Lastly, we were able to adjust for potential confounding variables that may influence progression from HPV infection to CIN3+.^{22 23 28}

The potential limitations include the use of self-reported CT infections as a proxy for lifetime CT exposure. This is an inaccurate measure of previous CT exposure because CT infections are commonly asymptomatic²⁹ and therefore underreported. Self-reported CT infections are, however, likely to capture symptomatic infections, which might share some biological characteristics, which in addition to high-risk HPV infection may confer a risk of CIN3+. This might be a partial explanation for the difference in risk pattern between CT measurements (DNA, self-reported). Second, the group of women with persistent HPV infection was small, and only a small proportion of them were CT DNA-positive, resulting in wide CIs. Third, we could not determine whether the temporal relation between CT and HPV infection influenced the risk of CIN3+. As highlighted by Miller and Ko,³ a thorough evaluation of this question would require a population of sexually naive women followed by frequently spaced specimen collection with a long follow-up. Finally, we had no information about treatment for the CT infections that could potentially have influenced our results.

In conclusion, this is the first study to assess the role of CT infection among women with persistent high-risk HPV infection. We observed an increased risk of CIN3+ with two or more self-reported CT infections as well as some effect of young age at first self-reported CT infection. We found no association between CT DNA and subsequent CIN3+. Since the biological mechanisms probably do not differ for women included and excluded from our study, the results are most likely generalisable to women with similar ages. However, further prospective studies in larger cohorts and with frequent data collection are necessary to fully determine whether and how CT infection affects the risk of progression from HPV infection to CIN3+.

Key messages

- ▶ Previous studies showed equivocal results concerning the effect of *Chlamydia trachomatis* (CT) on the development of high-grade cervical lesions in addition to human papillomavirus (HPV).
- ▶ Self-reported history of more CT infections significantly increases the risk of high-grade cervical lesions in addition to prevalent as well as persistent high-risk HPV infection.
- ▶ Young age at first CT infection tended to increase the risk of high-grade cervical lesions among women with persistent high-risk HPV infection.

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the cervical cytology samples. KEJ, SS, KF and SKK were involved in the design and conducting of the study. KEJ, LTT, KF and SKK participated in the interpretation of the results. All authors read, revised and approved the manuscript before submission.

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