Chlamydia trachomatis and Anti-MUC1 Serology and Subsequent Risk of High-Grade Serous Ovarian Cancer: A Population-Based Case–Control Study in Northern Sweden

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Abstract

BACKGROUND: Chlamydia trachomatis salpingitis causes inflammatory damage to the fallopian tube and could potentially cause initiation and progression of high-grade serous ovarian cancer (HGSC). Furthermore, *C. trachomatis* infection may stimulate mucin 1 (MUC1) protein production, possibly affecting anti-MUC1 antibody levels. The aim of this study was to examine if serology indicating past infection with *C. trachomatis* as well as anti-MUC1 production was associated with subsequent risk of HGSC. *MATERIALS AND METHODS:* In a prospective nested case–control study within the Northern Sweden Health and Disease Study and the Northern Sweden Maternity Cohort, the prevalence of chlamydial and anti-MUC1 antibodies was analyzed in blood samples drawn more than one year before diagnosis from 92 women with HGSC and 359 matched controls. Matching factors were age, date at blood draw, and sampling cohort. Plasma *C. trachomatis* IgG was analyzed using commercial micro-immunofluorescence test; chlamydial Heat Shock Protein 60 IgG (cHSP60) and anti-MUC1 IgG were analyzed with ELISA technique. *RESULTS:* The prevalence of *C. trachomatis* IgG and cHSP60 IgG antibodies, as well as the level of anti-MUC1 IgG was similar in women with HGSC and controls (16.3% vs. 17.0%, $P = 0.87$; 27.2% vs. 28.5%, $P = 0.80$; median 0.24 vs. 0.25, $P = 0.70$). Anti-MUC1 IgG and cHSP60 IgG levels were correlated ($r = 0.169$; $P < 0.001$). *CONCLUSIONS:* The findings of this prospective nested case–control study did not support an association between *C. trachomatis* infection, as measured by chlamydial serology, or anti-MUC1 IgG antibodies, and subsequent risk of HGSC.

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Introduction

Epithelial ovarian cancer (EOC) can be divided into five subgroups; high-grade serous (HGSC), low-grade serous, endometrioid, clear-

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cell, and mucinous cancer [1]. HGSC is the most common and lethal subtype of EOC. Traditionally, the ovarian surface epithelium has been viewed as the site of tumor origin. However, accumulating evidence now suggests that the distal part of the fallopian tube, many times the junction between the ciliated epithelium of the endosalpinx and the peritoneal mesothelium, is the primary origin of HGSC [2,3]. Carcinogenesis and potential risk factors for HGSC is not fully elucidated [4].

Chlamydia trachomatis is a sexually transmitted, Gram-negative intracellular bacterium causing cervicitis and if not resolved can ascend to the upper female genital tract, causing acute and chronic inflammation of the fallopian tubes [5,6]. In experimental animal models, *C. trachomatis* bacteria have been shown to infect the

secretory cells of the distal fallopian tube [7,8], the same cell type where serous tubal intraepithelial carcinoma, the suggested precursor of HGSC, is found. There are several mechanisms described explaining *C. trachomatis* potential carcinogenic properties. First, *C. trachomatis* is suggested to induce DNA damage in the host cell, inhibit DNA repair, and resist apoptotic stimuli [9,10]. Second, if left untreated, *C. trachomatis* can enter a viable nonreplicative chronic state [11−13] and the association of chronic inflammation with cancer is well documented [14,15]. Third, during chronic inflammation *C. trachomatis* produces a 60-kDa protein called chlamydial heat shock protein 60 (chsp60) [11]. The protein chsp60 is suggested to stimulate tissue damage by triggering the immune response as well as inducing resistance to apoptotic stimuli [6,12]. The ability to induce chronic inflammation generating an environment favorable for malignant transformation in combination with the ability to induce DNA damage and avoid apoptosis increases the risk for cancer initiation.

Based on the abovementioned, *C. trachomatis* has been implicated in ovarian cancer development [16−18]. Antibodies to *C. trachomatis* and chsp60 is associated with pelvic inflammatory disease (PID) [19,20]. Recent studies have shown an increased risk of HGSC after PID [21,22]. During an inflammatory process, the glycoprotein mucin 1 (MUC1), normally expressed by epithelial cells, are overexpressed to provide a barrier between the epithelium and the extra cellular milieu against infections [23]. This results in a humoral immune response and anti-MUC1 antibody production. Not only inflammation but also epithelial adenocarcinomas including EOC induces overexpression of MUC1 protein [24] and anti-MUC1 antibody production [25]. On the contrary, higher anti-MUC1 antibody levels in prospective blood samples have been associated with events known to reduce the risk for ovarian cancer (e.g. using oral contraceptives, parity, tubal ligation, hysterectomy, and salpingectomy) [25−28], suggesting that natural immunity against MUC1 might have a long-term protective effect [26]. Low-grade chronic events such as increasing number of ovulatory cycles and use of talc have been shown to reduce the antibody level suggestively because of immune tolerance [27,28]. Accordingly, infection of the female genital tract with *C. trachomatis* could stimulate an immune response to MUC1 protein and potentially both increase or decrease anti-MUC1 antibody levels depending on the chronicity of the infection.

Based on experimental and epidemiologic data, we hypothesized that *C. trachomatis* may play a role in the development of HGSC, and *C. trachomatis* antibodies to be associated with increased risk of HGSC. The aim of this study was to assess the association of *C. trachomatis* and anti-MUC1 antibodies with HGSC, in a prospective population-based case−control study.

Materials and Methods

Study Population

This is a nested case−control study within the Northern Sweden Health and Disease Study (NSHDS) and the Northern Sweden Maternity Cohort (NSMC) to compare the prevalence of *C. trachomatis* and the levels of anti-MUC1 antibodies in prospective blood samples from women with HGSC and matched controls. Blood samples, drawn more than one year before ovarian cancer diagnosis were identified in NSHDS and NSMC, both previously reported in detail [29−31]. In brief, the NSHDS cohort contains

three subcohorts. Plasma samples used in the present study were collected from participants in the Västerbotten Intervention Program (VIP) and the Mammography Screening Project (MSP). VIP invites all residents of Västerbotten County to a general health examination at 10-year intervals at ages 40, 50, and 60 years. The MSP collects blood samples at mammography screening visits and the cohort consists of women aged 18−82 years at blood draw, of whom 95% are between 48 and 70 years. The NSMC collects serum samples for research purpose from pregnant women attending the maternity health care during the end of the first trimester of pregnancy. All participants provided informed consent before donating their blood samples for research purposes.

Ethical approval was given from The Human Ethics Committee of the Medical Faculty, Umeå University (Dnr 06−053), Sweden.

Nested Case−Control Study Participant Selection

Women diagnosed with HGSC between 1993 through 2011 with at least one prospective blood sample in NSHDS or NSMC were included in the study. Cases were identified through the national Cancer Register having one of the following malignant neoplasms; peritoneum, ovary, fallopian tube, abdomen, or pelvis (International Classification of Diseases 9th revision codes 158 and 183; 10th revision code C48.1−2, C56.9, C57.0−8, C76.2−3). A pathology report review was conducted. Only serous histotypes were included. Serous adenocarcinomas Silverberg grade III [32] were defined as HGSC. All cases with serous histotype Silverberg grade II or unknown differentiation were reevaluated blindly by a senior consultant in gynecologic pathology to confirm or reject HGSC diagnosis as defined by the World Health Organization classification (2014).

Women with HGSC were matched to four controls each with respect to age (±1 year), date at blood draw (±3 months), and sampling cohort (VIP, MSP, NSMC). Except for nonmelanoma skin cancer, cases and controls had no history of cancer before blood collection. Women having had bilateral oophorectomy before diagnosis of the index case were excluded.

Plasma Antibody Analysis

C. trachomatis IgG. *C. trachomatis* IgG antibodies, as well as *Chlamydia pneumoniae* IgG antibodies, were determined by the serovar D−K specific micro-immunofluorescence (MIF) test (Focus Diagnostics, USA). *C. pneumoniae* IgG antibodies were included in the analysis to determine possible covariance/cross-reaction with *C. trachomatis*. All procedures were performed according to the manufacturer's protocols. In short, serum dilutions 1/16 were used. Serum was added to the well, incubated for 30 min, in 37 °C. Plates were washed in PBS to remove unbound serum antibodies. Fluorescein-labeled antibody IgG was added and samples were incubated for 30 min at 37 °C. Then again washed, dried, and mounted. All samples were examined by the same observer, blinded to case−control status, using fluorescence microscopy. Positive and negative controls from the kit were included.

cHSP60 IgG. Analysis of the levels of chlamydial Heat Shock Protein 60 (cHSP60) IgG antibodies was performed using a commercial ELISA technique (Medac, Germany) and optical density (OD) values were measured (at 450 nm; reference wavelength at 620 nm). Protocol and validation criteria of the assay were followed according to the manufacturer's instructions. The OD values were analyzed continuously and dichotomized; cutoff was defined as the

mean OD value of the negative control plus 0.350 and results are presented as positive (+) or negative (−). Plasma presenting OD values ± 10% of the cutoff value were interpreted as negative to exclude false positive results. Levels of cHSP60 IgG were also analyzed in quartiles based on the distribution in the control population; the highest and lowest quartiles were compared (Q4 vs. Q1). Human HSP60 (hHSP60) IgG was included in the analysis to determine possible covariance/cross-reaction with cHSP60 IgG. Analysis of hHSP60 IgG was performed using the same method as for cHSP60 IgG antibodies.

Anti-MUC1 IgG. Anti-MUC1 IgG was measured by ELISA as previously published [33]. Immulon 4 (Thermo Fisher Scientific) microtiter plates were coated overnight at 4 °C with 1 μg of synthetic MUC1 100-mer peptide (vaccine antigen) dissolved in 0.9% Dulbecco's PBS. Corresponding control plates received PBS but no antigen. The plates were washed three times with PBS and 1-hour incubation in room temperature with 2.5% bovine serum albumin (BSA) in PBS (PBS-BSA) to fully coat the microtiter plate wells with protein and block nonspecific binding. PBS-BSA was removed and plasma diluted in PBS-BSA was added to the wells. After 1-hour incubation at room temperature, the plates were washed 5 times with PBS with 0.1% Tween-20 (Sigma−Aldrich), and alkaline phosphatase−conjugated anti-human IgG, IgM, or IgA secondary antibody (Sigma−Aldrich) in PBS-BSA was added. After a 1-hour incubation, the plates were washed 5 times and the substrate, p-nitrophenyl phosphate (Sigma−Aldrich), was added to each well. The reaction was terminated after 1-hour by adding 0.5 mol/L NaOH. The results were read at OD 405 nm on a spectro-photometer. The OD values from the control wells containing no antigen were subtracted from the OD values in test wells coated with peptide. Every sample was assayed multiple times at multiple dilutions, in at least triplicate wells. The OD values were analyzed continuously and dichotomized. Based on a previous study, a cutoff for high anti-MUC1 IgG level was set at an OD value ≥ 1.0 [26].

Statistical Analysis

Statistical analysis was performed using the SPSS software (version 25.0). The differences in proportions of the categorical variables were evaluated by chi-square or Fisher's exact test, whichever appropriate. Nonparametric Mann−Whitney U test was applied to analyze continuous data not normally distributed. Spearman rank test was used analyzing correlations. Association of antibodies with HGSC was also evaluated by lag time between blood draw and diagnosis, using the median time as a cutoff (1−7.3 years or > 7.3 years) as well by age group using mean age at diagnosis as cutoff. A two-sided P-value less than 0.05 was considered significant.

Results

Cohort

Ninety-two women diagnosed with HGSC were included. Most cases were low-differentiated serous adenocarcinomas ($n = 75$, 81.5%) followed by serous surface papillary carcinoma ($n = 12$, 13.0%) and serous cystadenocarcinoma "not otherwise specified" ($n = 5$, 5.4%). Cases were diagnosed at a mean age of 62 years (range 38−83 years) and matched to four controls each. However, for nine cases, only three controls fulfilling the criteria were identified. Forty-two cases (46%) had more than one prospective blood sample. Most blood samples were collected within the NSHDS cohort ($n = 317$, 70.3% whereof MSP: $n = 188$, 59.3%; VIP: $n = 129$, 40.7%) and the rest from NSMC ($n = 134$, 29.7%). The median time between blood collection and diagnosis was 7.3 years (range 1.1−34.0 years).

Chlamydial Antibodies and HGSC

Women having *C. trachomatis* IgG antibodies were younger at blood collection than women who did not have *C. trachomatis* IgG antibodies (median 47.1 vs. 58.7 years, respectively). There was no correlation between age and the level of *C. pneumoniae* IgG or cHSP60 IgG (data not shown). The prevalence of *C. trachomatis* IgG and cHSP60 IgG was similar in the different sample cohorts among cases as well as controls (data not shown). The prevalence of *C. trachomatis* IgG was similar in women with HGSC and controls (16.3% vs. 17.0%, $P = 0.87$) (Table 1). There was no difference in cHSP60 IgG antibodies between cases and controls neither as continuous parameter ($P = 0.85$) (Table 2) nor dichotomized (27.2% vs. 28.5%, $P = 0.80$) (Table 1). There was no difference in the proportion of cHSP60 IgG antibodies in Q4 vs. Q1 (quartile cutoff based on control population) between cases and controls ($P = 0.79$). Analysis on lag time between blood draw and diagnosis (1−7.3 years or > 7.3 years) did not result in any significant differences in

Table 1. Prevalence of *Chlamydial trachomatis* IgG, cHSP60 IgG, and Anti-MUC1 IgG in Prospective Blood Samples in Women With High-Grade Serous Ovarian Cancer and Matched Controls

Antibodies	Cases			Controls			P^*
	n	$n+$	(%+)	n	$n+$	(%+)	
C. trachomatis IgG	92	15	(16.3%)	358[†]	61	(17.0%)	0.87
1−7.3 years	46[‡]	8	(17.4%)	176[‡]	24	(13.6%)	0.52
>7.3 years	69[‡]	14	(20.3%)	270[‡]	45	(16.7%)	0.48
cHSP60 IgG[§]	92	25	(27.2%)	358[†]	102	(28.5%)	0.80
1−7.3 years	46[‡]	16	(34.8%)	176[‡]	54	(30.7%)	0.59
>7.3 years	69[‡]	18	(26.1%)	270[‡]	67	(24.8%)	0.83
Anti-MUC1 IgG[¶]	92	11	(12.0%)	359	24	(6.7%)	0.09
1−7.3 years	46[‡]	2	(4.3%)	177[‡]	13	(7.3%)	0.74[#]
>7.3 years	69[‡]	9	(13.0%)	270[‡]	17	(6.3%)	0.06

Abbreviations: *C. trachomatis* IgG = *Chlamydia trachomatis* IgG; cHSP60 IgG = chlamydial Heat Shock Protein 60 IgG; MUC1 = mucin 1.
* Chi-square test.
† One control was not evaluated because of technical problems.
‡ The same individual might have samples in both the 1−7.3 years or >7.3 years subgroups.
§ Cutoff for cHSP60 IgG was defined as the mean optical density value of the negative control plus 0.350 and results are presented as positive (+) or negative (−).
¶ Cutoff for high anti-MUC1 IgG level was considered as an optical density of ≥1.0.
# Fisher's exact test.

Table 2. Optical Density Values of cHSP60 and Anti-MUC1 Antibodies in Prospective Blood Samples in Women With High-Grade Serous Ovarian Cancer and Matched Controls

Antibodies	Cases			Controls			P^*
	n	Median	(min–max)	n	Median	(min–max)	
cHSP60 IgG	92	0.53	(0.15–7.80)	358[†]	0.58	(0.01–8.59)	0.85
1–7.3 years	46[‡]	0.58	(0.15–7.80)	176[‡]	0.62	(0.01–8.59)	0.59
>7.3 years	69[‡]	0.53	(0.15–6.06)	270[‡]	0.50	(0.03–8.09)	0.51
Anti-MUC1 IgG	92	0.24	(0.00–2.45)	359	0.25	(0.01–3.67)	0.70
1–7.3 years	46[‡]	0.23	(0.00–2.05)	177[‡]	0.24	(0.02–3.67)	0.51
>7.3 years	69[‡]	0.24	(0.05–3.29)	270[‡]	0.26	(0.03–3.63)	0.51

Abbreviations: cHSP60 IgG = chlamydial Heat Shock Protein 60 IgG; MUC1 = mucin 1.
* Mann-Whitney U test.
† One control was not evaluated because of technical problems.
‡ The same individual might have samples in both the 1–7.3 years and >7.3 years subgroups.

prevalence of *C. trachomatis* IgG nor cHSP60 IgG between cases and controls (Table 1). There were no significant differences in the results in analysis by age group (< 62 or ≥ 62 years at diagnosis) (data not shown).

Anti-MUC1 Antibodies and HGSC

There was a negative correlation between age and the level of anti-MUC1 antibodies (r = −0,162, *P < 0.001*). The prevalence of anti-MUC1 IgG was similar in the different sample cohorts among cases as well as controls (data not shown). The median OD values between the cases and controls were equal (0.24 vs. 0.25, *P = 0.70*) (Table 2). The prevalence of higher anti-MUC1 IgG antibody levels was similar between cases and controls, based on anti-MUC1 IgG cutoff with OD ≥ 1.0 (12.0% vs. 6.7%, *P = 0.09*) (Table 1). Women with HGSC had a trend of higher anti-MUC1 IgG levels compared with controls, when analyzing blood samples collected more than 7.3 years before diagnosis and using anti-MUC1 IgG cutoff OD ≥ 1.0 (13.0% vs. 6.3%, *P = 0.06*) (Table 1). There were no significant differences in the results in analysis by age group (< 62 or ≥ 62 years at diagnosis) (data not shown). Sensitivity analysis excluding the cohort of pregnant women did not change the results in any of the analyses aforementioned (data not shown).

Association and Correlation Between Antibodies

No association was found between *C. trachomatis* IgG and *C. pneumoniae* IgG ($\chi^2 = 0.012$; *P = 0.91*). There was a significant association between the presence of *C. trachomatis* IgG and cHSP60 IgG ($\chi^2 = 160.20$; *P < 0.001*). In nonparametric test, the level of cHSP60 IgG was similar in *C. trachomatis* IgG positive and negative women (*P = 0.81*). Spearman rank test showed no correlation between cHSP60 IgG and hHSP60 IgG (*P = 0.99*). Women with *C. trachomatis* IgG did not have higher or lower levels of anti-MUC1 IgG (median 0.26 vs. 0.23; *P = 0.49*), nor did women with *C. pneumoniae* IgG (0.23 vs. 0.23; *P = 0.75*). Spearman rank test showed a significant positive correlation between cHSP60 IgG and anti-MUC1 IgG (r = 0.169; *P < 0.001*). No correlation was found between hHSP60 IgG and anti-MUC1 IgG (r = 0.045; *P = 0.25*).

Discussion

We examined chlamydial and anti-MUC1 IgG antibodies in women with HGSC and matched controls using prospective blood samples collected one year or more before diagnosis. No significant association of *C. trachomatis* antibodies with subsequent risk for HGSC was detected. Neither were the levels of anti-MUC1 IgG antibodies significantly different between cases and controls. Women with

higher cHSP60 IgG antibodies had significantly higher anti-MUC1 antibody levels, suggesting that chronic chlamydia infection might stimulate MUC1 production thereby inducing a humoral immune response.

The results of previous studies are inconsistent regarding *C. trachomatis* serology and ovarian cancer. Our findings are in line with studies where no associations were found [34,35]. Recent studies show an association of *C. trachomatis* serology with subsequent risk for ovarian cancer [36,37]. However, their findings were specific to the *Chlamydia* Pgp3 antibody, while in this study, we used a commercial MIF-test which may explain the differences. Similar to our result, Trabert et al. found no association between cHSP60 IgG and ovarian cancer [36].

Our study did not support any association between anti-MUC1 IgG and subsequent risk for HGSC. This was in line with Cramer et al. [38]; they concluded that the anti-MUC1 antibody levels may be informative in the pathogenesis of EOC mucinous subtypes, but less useful for informing risk for all EOC. The focus of this study was HGSC and the mucinous subtype was not included.

We found that women with higher cHSP60 IgG antibody levels had significantly higher anti-MUC1 antibody levels, whereas *C. trachomatis* IgG and *C. pneumoniae* IgG were not associated with higher levels of anti-MUC1 antibodies. One explanation could be that the chronicity of *C. trachomatis* infection is important for induction of anti-MUC1 antibody production, but the result has to be interpreted with caution because the strength of the correlation was low. On the contrary, previous studies have shown that low-grade chronic inflammations are associated with lower levels of anti-MUC1 antibodies, suggestively because of immune tolerance [27,28]. Our results did not support lower anti-MUC1 antibody levels in women with serology indicating previous chronic *C. trachomatis* infection.

Strengths

This study consists of a well-defined cohort regarding the histopathological diagnosis. Most cases were successfully matched to four controls each with respect to age, date at blood donation, and sample cohort. All tests were performed with laboratory personnel unaware of case–control status, using validated methods.

Limitations

Only HGSC cases were included while results in more recent studies have shown associations with other histotypes that were not part of our aim. Reproductive variables were not collected in most cases and controls and therefore not adjusted for. It is shown that pregnancy influences the anti-MUC1 antibody level [25] and 30% of

the blood samples in our study were collected from pregnant women. However, sensitivity analysis of anti-MUC1 levels excluding the pregnant subcohort showed similar results.

Conclusions

In this prospective population-based case–control study, there was no significant association of *C. trachomatis*, cHSP60, or anti-MUC1 IgG antibodies with HGSC. Hence, the hypothesis that past infection with *C. trachomatis* would confer increased risk of HGSC was not supported. Given the divergent results in the current literature, the hypothesis needs to be further elucidated.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.09.007.

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