

## Chlamydia trachomatis, Chlamydial Heat Shock Protein 60 and Anti-Chlamydial Antibodies in Women with Epithelial Ovarian Tumors<sup>1,2</sup>



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### Abstract

**OBJECTIVE:** *Chlamydia trachomatis* (*C. trachomatis*) infection has been suggested to promote epithelial ovarian cancer (EOC) development. This study sought to explore the presence of *C. trachomatis* DNA and chlamydial heat shock protein 60 (chsp60) in ovarian tissue, as well as anti-chlamydial IgG antibodies in plasma, in relation to subtypes of EOC. **METHODS:** This cross-sectional cohort consisted of 69 women who underwent surgery due to suspected ovarian pathology. Ovarian tissue and corresponding blood samples were collected at the time of diagnosis. In ovarian tumor tissue, p53, p16, Ki67 and chsp60 were analyzed immunohistochemically, and PCR was used to detect *C. trachomatis* DNA. Plasma *C. trachomatis* IgG and cHSP60 IgG were analyzed with a commercial MIF-test and ELISA, respectively. **RESULTS:** Eight out of 69 women had *C. trachomatis* DNA in their ovarian tissue, all were invasive ovarian cancer cases (16.7% of invasive EOC). The prevalence of the chsp60 protein, *C. trachomatis* IgG and cHSP60 IgG in HGSC, compared to other ovarian tumors, was 56.0% vs. 37.2%  $P = .13$ , 15.4% vs. 9.3%  $P = .46$  and 63.6% vs. 45.5%  $P = .33$  respectively. None of the markers of *C. trachomatis* infection were associated with p53, p16 or Ki67. **CONCLUSIONS:** *C. trachomatis* was detected in invasive ovarian cancer, supporting a possible role in carcinogenesis of EOC. However, there were no statistically significant associations of chsp60 in ovarian tissue, or plasma anti-chlamydial IgG antibodies, with any of the subtypes of ovarian tumors.

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## Introduction

### Ovarian Tumors

Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer in the developed parts of the world [1]. It is a heterogeneous group of tumors, and based on histopathology, immunohistochemistry and molecular genetic analyzes, at least five main subtypes of epithelial ovarian carcinomas with different clinical characteristics are identified: endometrioid, clear-cell, mucinous, low-grade serous and high-grade serous carcinomas (HGSC) [2]. HGSC's are fast-growing

and highly aggressive tumors characterized by p53 and p16 mutations combined with very high Ki67 proliferation [3–8]. A recent meta-analysis by Wentzensen et al., illustrates that risk factors, once accepted for all ovarian cancer subtypes, are strongly associated only to non-serous and low-grade serous carcinomas [9]. Risk factors for the most common and fatal subtype, HGSC, are sparsely described. Systematic examination of the fallopian tubes and ovaries from salpingo-oophorectomies suggest that the secretory cells of the fallopian tube is the site of HGSC origin [10–13]. It is unclear what triggers the neoplastic transformation of these cells, however in

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experimental animal models *Chlamydia trachomatis* (*C. trachomatis*) bacteria have been found to infect the same type of secretory cells of the fallopian tubes [14,15]. Shanmughapriya et al. identified *C. trachomatis* in tissue of 70% of ovarian tumors and none in benign or normal ovaries, suggesting that *C. trachomatis* could be associated with ovarian carcinogenesis [16].

### *C. trachomatis*, inflammation and cancer

*C. trachomatis* is a Gram-negative obligate intracellular bacterium and the most common sexually transmitted bacterial disease worldwide. Since the infection is usually asymptomatic the exact incidence is not clear. Chlamydia bacteria have evolved successful mechanisms to avoid the host immune system. It has been suggested that without treatment up to 50% of infected women continue to be infected more than one year [17]. The sequelae of these infections, causing a chronic inflammatory state of the female upper genital tract, include for example tubal factor infertility and chronic pelvic pain [18]. Several mechanisms have been described explaining how *C. trachomatis* could be carcinogenic. Firstly, *C. trachomatis* has the potential to become chronic, and the link between chronic infection/inflammation and cancer is well documented [19,20]. Secondly, *C. trachomatis* bacteria can enter a viable, non-replicative persistent state and evade the host cell immune response [21–23]. In this persistent form the chlamydia bacteria up-regulate the synthesis of a conserved anti-apoptotic 60-kDa protein called chlamydial heat shock protein 60 (chsp60) [21,23], and down-regulate the synthesis of structural and membrane proteins. Thirdly, *C. trachomatis* blocks the release of mitochondrial cytochrome C and caspase 3, which allows the infected cell to escape intrinsic apoptosis [24]. The ability to avoid apoptosis prolongs the life of the infected host cell and facilitates potentially DNA damaged cells to survive, thereby increasing the risk for cancer initiation. Consequently, *C. trachomatis* bacteria could tentatively be carcinogenic.

The aim of this cross-sectional study was to explore the prevalence of markers of *C. trachomatis* infection in ovarian tissue and plasma from women with ovarian tumors of different subtypes, furthermore to study if these markers were more prevalent in women with HGSC compared to women with other ovarian tumors.

### Materials and methods

This cross-sectional study was undertaken to analyze ovarian tissue and plasma samples of women with suspected ovarian pathology. Ethical approval was given from The Human Ethics Committee of the Medical Faculty, Umeå University (Dnr 06-053), Sweden.

### Cohort

Ovarian tissue and corresponding blood samples were obtained from women undergoing laparotomies due to suspected ovarian pathology between 1993 and 2008, at the Department of Obstetrics and Gynecology, University Hospital of Northern Sweden, Umeå, Sweden. Women were included in the study after oral and written informed consent. The women were mainly from the Västerbotten County in northern Sweden. Tumors were classified according to World Health Organization and FIGO criteria [25]. The pathology reports were reviewed. All cases were reevaluated blindly by a senior consultant subspecialized in gynecologic pathology.

### Immunohistochemistry

Representative samples of the ovarian tumors were primarily fixed in 10% buffered formalin and embedded in paraffin according to standard procedures. Serial four micrometer thick tissue sections were cut, to

perform immunohistochemical staining with antibodies to chsp60 (Enzo, A57-B9; dilution 1:150) and hhsp60 (Enzo, LK-1; dilution 1:100), as well as tumor and proliferation markers for HGSC; p53 (Novocastra, D0-7; dilution 1:100), p16 (Santa Cruz, JC8; dilution 1:200) and Ki67 (DAKO, MIB-1; dilution 1:50). The immunohistochemical staining was performed according to the manufacturer's protocols using Benchmark Ultra® (Ventana) automated staining machine, after optimization of the methods. The immunohistochemical stainings were evaluated twice by the same observer using light microscopy. Discordant cases were reviewed a third time by a senior pathologist, followed by a conclusive judgement.

*p53*. Immunoreactivities of p53 were quantified with a weighted score [3]. First, the proportion of positive cells in each section was scored with a 5-point scale: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (>75%). After that, the intensity of immunoreactivity for positive cases was evaluated with a 3-point scale: 1 (weak), 2 (moderate) and 3 (intense). The weighted score for each section was obtained by multiplying the proportion score by the intensity score, giving the range of score 0–12. Yemelyanova et al. compared nucleotide sequencing and immunohistochemistry, and showed that if the two patterns of immunohistochemical labeling were combined (60–100% of tumor cells strongly positive, indicating p53 mutation, and tumors completely negative for p53 staining, indicating p53 null mutation) it correlated with p53 mutations determined with nucleotide sequencing in 94% of cases [4]. Thus, in accordance with the results of Yemelyanova et al., score 0 and 12 were regarded as p53 mutations.

*p16*. Positive p16 required nuclear staining, and was evaluated as positive if moderate or intense diffuse staining in more than 25% of cells was encountered [5]. Staining of cytoplasm or cell membrane alone was regarded as negative.

*Ki67*. Percentage of stained Ki67 tumor cells was quantified in hot spots using 400× magnification. High Ki67 proliferation was defined as >25% stained cells. This cut-off level is based on biological evidence of HGSC having higher proliferation compared to borderline tumors and low-grade serous cancers [3,7,8].

*chsp60*. Anti-chlamydial hsp60 antibodies recognize hsp60 epitopes specific for Chlamydia [26]. Antibodies against hhsp60 have unique specificity for mammalian hsp60 and do not cross react with bacterial counterparts [27]. The intensity of chsp60 immunoreactivity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (intense). Formalin embedded tissue from children, below 10 years of age, were used as negative controls. chsp60 and hhsp60 stained weakly and moderately in all of the control tissue samples, but not intensely in any of them. Thus score 3 was considered positive, and scores 0, 1, and 2 as negative. Human heat shock protein 60 (hhsp60) (Enzo, LK-1) was included in the immunohistochemical analysis in order to determine potential covariance between chlamydial and human hsp60. Assessment of hhsp60 immunoreactivity was carried out with the same method as chsp60. Human hsp60 was present in all cells, cut-off for overexpression was set at score 3 (intense) whereas scores 0–2 were considered as normal expression, in accordance with the results mentioned above.

### PCR

Paraffin-embedded ovarian tissue blocks were sampled twice with 10µm thick sections and transferred into sterile Eppendorf tubes. Tissue was solubilized and DNA extracted with Kapa Express Extraction kit (KAPA Biosystems, Cape Town, South Africa), total

volume 100 µl/sample, according to the manufacturer's instructions. To detect *C. trachomatis* DNA in the samples a PCR reaction was directed against a 109bp long region within ORF2 of the chlamydial plasmid [28]. The reaction mix consisted of primers and TaqMan probe as specified before [28], Kapa Force Probe qPCR master mix (KAPA Biosystems, Cape Town, South Africa) and 1 µl extracted DNA was added to a total reaction volume of 20 µl. Primers and probes were ordered from EurofinsGenomics, Ebersberg, Germany. Amplification reactions included an initial 15-minute denaturation step at 95°C, followed by 45 cycles of 15s at 95°C, and 30s at 60°C. The reactions were performed on an ABI QuantStudio5 instrument, and results were analyzed with the QuantStudio Design & Analysis software set to automatic baseline and threshold (Automatic C<sub>T</sub>).

### Plasma antibody analyzes

Plasma drawn at the time of diagnosis, at the day of (or a few days prior to) surgery, was analyzed. Plasma *C. trachomatis* IgG and cHSP60 IgG antibodies were used as markers of previous chronic *C. trachomatis* infections since women rarely are aware of a previous infection due to its often asymptomatic nature.

**C. trachomatis IgG.** *C. trachomatis* IgG antibodies serovar D–K specific, as well as *Chlamydia pneumoniae* (*C. pneumoniae*) IgG antibodies, were determined by the micro-immunofluorescence (MIF) test (Focus Diagnostics, USA). *C. pneumoniae* IgG antibodies were included in the analysis in order to determine possible covariance 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 using fluorescence microscopy. Positive and negative controls from the kit were included.

**cHSP60 IgG.** Analyzes of the concentrations of chlamydial HSP60 IgG antibodies were performed using a commercial ELISA technique (Medac, Germany) and optical density values were measured (at 450 nm, reference wavelength at 620 nm). The values of cHSP60 IgG were categorized in quartiles. Association of cHSP60 IgG with tumor subtypes was evaluated by comparing cases in the highest vs. the lowest quartiles. Human HSP60 antibodies were included in the analysis in order to determine possible covariance with chlamydial HSP60 IgG. Analyzes of hHSP60 IgG were performed with the same method as analyzes of cHSP60 IgG antibodies.

### Statistical analyzes

Statistical analyzes were carried out using the SPSS version 23.0 software package. The differences in proportions of categorical variables were evaluated by  $\chi^2$  or Fisher's Exact test, whichever was appropriate. Correlation was calculated by the Pearson correlation coefficient, and t-test was used comparing means. All *P*-values were two-sided and *P* < .05 was considered statistically significant.

## Results

### Cohort

Ovarian tissue and corresponding plasma samples were collected from 69 women (mean age 57 years, range 36 to 81 years) between 1993 and 2008. Histopathological evaluation identified 26 HGSC, the distribution of tumors is described in Table 1.

**Table 1.** Distribution of Histopathological Diagnoses Among 69 Women with Epithelial Ovarian Tumors

| Histopathologic Diagnosis             | <i>n</i> | %    |
|---------------------------------------|----------|------|
| High-grade serous carcinoma           | 26       | 37.7 |
| Endometrioid carcinoma <sup>a</sup>   | 4        | 5.8  |
| Clear cell carcinoma                  | 3        | 4.3  |
| Mucinous cystadenocarcinoma           | 2        | 2.9  |
| Low-grade serous carcinoma            | 5        | 7.2  |
| Carcinosarcoma                        | 1        | 1.4  |
| Mixed tumor <sup>b</sup>              | 4        | 5.8  |
| Non-differentiated carcinoma          | 3        | 4.3  |
| Mucinous cystadenoma                  | 1        | 1.4  |
| Borderline ovarian tumor <sup>c</sup> | 20       | 29.0 |

Abbreviations: EC = endometrioid carcinoma; CCC = clear cell carcinoma; MC = mucinous carcinoma.

<sup>a</sup> FIGO II, *n* = 2; FIGO III, *n* = 2.

<sup>b</sup> EC / CCC, *n* = 2; EC / MC, *n* = 2.

<sup>c</sup> serous subtype, *n* = 10; mucinous subtype, *n* = 10.

### Tumor and proliferation markers and high-grade serous carcinoma (HGSC)

Table 2 presents the tumor and proliferation markers in relation to HGSC and other ovarian tumors. As expected, p53 mutations are seen in greater extent in HGSC than in other ovarian tumors (92.3% vs. 23.8%, *P* < .001). There was also a significantly higher p16 expression in HGSC compared to other morphologic subtypes (100% vs. 60.5%, *P* < .001). A larger proportion of women with HGSC had a high Ki67 proliferation index compared to women with other ovarian tumors (73.1% vs. 37.2%, *P* < .005).

### C. trachomatis DNA and heat shock proteins in ovarian tumors

Eight out of 69 ovarian tumors were positive for *C. trachomatis* DNA (Table 3). *C. trachomatis* DNA was only found in invasive ovarian cancer (16.7% vs. 0%, *P* = .10). Four of the *C. trachomatis* DNA positive cases were HGSC's, three were low-grade serous carcinomas and one was a mucinous carcinoma. The age of the women with *C. trachomatis* DNA positive ovarian tumors was within the range 42-75 years. Age at time of diagnosis did not affect the risk of having a *C. trachomatis* DNA positive tumor (mean 58.0 vs. 57.1 years, *P* = .84). Chlamydial hsp60 protein was present in 56.0% of HGSC tissue compared to 37.2% in other ovarian tumors (*P* = .13). There were similar proportions of chsp60 in invasive compared to

**Table 2.** Tumor and Proliferation Markers in High-Grade Serous Carcinoma (HGSC) vs. Other Epithelial Ovarian Tumors<sup>a</sup>

| Tumor marker         | HGSC            |      | Other Tumors <sup>a</sup> |      | <i>P</i> |
|----------------------|-----------------|------|---------------------------|------|----------|
|                      | <i>(n</i> = 26) |      | <i>(n</i> = 43)           |      |          |
|                      | <i>n</i>        | %    | <i>n</i>                  | %    |          |
| Tumor marker         |                 |      |                           |      |          |
| p53 <sup>b</sup>     | 24              | 92.3 | 10 <sup>c</sup>           | 23.8 | <.001    |
| p16 <sup>d</sup>     | 26              | 100  | 26                        | 60.5 | <.001    |
| Proliferation marker |                 |      |                           |      |          |
| Ki67 <sup>e</sup>    | 19              | 73.1 | 16                        | 37.2 | <.005    |

<sup>a</sup> Other tumors = endometrioid carcinoma, clear cell carcinoma, mucinous carcinoma, low-grade serous carcinoma, carcinosarcoma, mixed tumor, non-differentiated carcinoma, mucinous cystadenoma and borderline ovarian tumor.

<sup>b</sup> Tumors were scored with respect to distribution (0-4) and intensity (1-3) of immunoreactivity. An immunohistochemical composite score was calculated by multiplying the distribution and intensity scores (0-12). Score 0 and 12 were considered as p53 mutation.

<sup>c</sup> One case was not evaluated due to technical problems with immunohistochemical staining.

<sup>d</sup> >25% Moderat/intense diffuse nuclear staining was considered as p16 mutation.

<sup>e</sup> High Ki67 proliferation (> 25% stained cells).



**Table 3.** Presence of *Chlamydia trachomatis* and Chlamydial Heat Shock Protein 60 in Ovarian Tumors

|                                  | <i>C. trachomatis</i> <sup>a</sup> |      | <i>P</i> | chsp60 <sup>b</sup>  |      | <i>P</i> |
|----------------------------------|------------------------------------|------|----------|----------------------|------|----------|
|                                  | <i>n</i> / <i>N</i>                | %    |          | <i>n</i> / <i>N</i>  | %    |          |
| Tumor characteristics            |                                    |      |          |                      |      |          |
| HGSC                             | 4 / 26                             | 15.4 | 0.46     | 14 / 25 <sup>c</sup> | 56.0 | 0.13     |
| Other tumors <sup>d</sup>        | 4 / 43                             | 9.3  |          | 16 / 43              | 37.2 |          |
| Invasive EOC                     | 8 / 48                             | 16.7 | 0.10     | 22 / 47 <sup>c</sup> | 46.8 | 0.50     |
| Non-invasive tumors <sup>e</sup> | 0 / 21                             | 0    |          | 8 / 21               | 38.1 |          |

Abbreviations: *n* / *N* = number of positive cases / total number of tumor subtype; HGSC = high-grade serous carcinoma.

<sup>a</sup> *C. trachomatis* DNA analyzed with PCR.

<sup>b</sup> Intense staining of nucleus was considered chsp60 positive.

<sup>c</sup> One case was not evaluated due to technical problems with immunohistochemical staining.

<sup>d</sup> Other tumors = endometrioid carcinoma, clear cell carcinoma, mucinous carcinoma, low-grade serous carcinoma, carcinosarcoma, mixed tumor, non-differentiated carcinoma, mucinous cystadenoma and borderline ovarian tumor.

<sup>e</sup> Borderline ovarian tumor, *n* = 20; mucinous cystadenoma, *n* = 1.

non-invasive ovarian tumors (46.8% vs. 38.1%, *P* = .50). Overexpression of hsp60 was significantly higher in invasive ovarian tissue compared to non-invasive tumors (58.3% vs. 28.6%, *P* = .02), while no difference was found between HGSC and other ovarian tumors (46.2% vs. 51.3%, *P* = .69).

**Chlamydial antibodies and association with tumor characteristics, *C. trachomatis* DNA and chsp60 in ovarian tissue**

The prevalence of *C. trachomatis* IgG antibodies was 15.4% in women with HGSC compared to 9.3% in women with other ovarian tumors (*P* = .46) (Table 4). *C. trachomatis* IgG antibodies were equally distributed between invasive and non-invasive ovarian tumor groups (12.5% vs. 9.5%, *P* = 1.00). Chlamydial HSP60 IgG antibody levels in the highest quartile were present in 63.6% of the women with HGSC compared to 45.5% in women with other ovarian tumors (*P* = .33). Women with invasive ovarian tumors had cHSP60 IgG levels in the highest quartile in 60.0% compared to 38.5% in the non-invasive tumor group (*P* = .23).

**Table 4.** Chlamydial Plasma Antibodies in Relation to Tumor Characteristics, *Chlamydia trachomatis* and Chlamydial Heat Shock Protein 60 in Ovarian Tissue

|                                    | <i>C. trachomatis</i> IgG |      | <i>P</i> | cHSP60 IgG                   |      | <i>P</i> |
|------------------------------------|---------------------------|------|----------|------------------------------|------|----------|
|                                    | <i>(n</i> = 69)           |      |          | <i>(n</i> = 33) <sup>a</sup> |      |          |
|                                    | <i>n</i> / <i>N</i>       | %    |          | <i>n</i> / <i>N</i>          | %    |          |
| Tumor characteristics              |                           |      |          |                              |      |          |
| HGSC                               | 4 / 26                    | 15.4 | 0.46     | 7 / 11                       | 63.6 | 0.33     |
| Other tumors <sup>b</sup>          | 4 / 43                    | 9.3  |          | 10 / 22                      | 45.5 |          |
| Invasive                           | 6 / 48                    | 12.5 | 1.00     | 12 / 20                      | 60.0 | 0.23     |
| Non-invasive <sup>c</sup>          | 2 / 21                    | 9.5  |          | 5 / 13                       | 38.5 |          |
| <i>C. trachomatis</i> <sup>d</sup> |                           |      |          |                              |      |          |
| Positive                           | 2 / 8                     | 25.0 | 0.23     | 2 / 3                        | 66.7 | 1.00     |
| Negative                           | 6 / 61                    | 9.8  |          | 15 / 30                      | 50.0 |          |
| chsp60 <sup>e</sup>                |                           |      |          |                              |      |          |
| Positive                           | 3 / 30 <sup>f</sup>       | 10.0 | 1.00     | 7 / 17                       | 41.2 | 0.22     |
| Negative                           | 4 / 38 <sup>f</sup>       | 10.5 |          | 10 / 16                      | 62.2 |          |

Abbreviations: cHSP60 IgG = chlamydial heat shock protein 60 IgG; HGSC = high-grade serous carcinoma.

<sup>a</sup> cHSP60 IgG optical density values using ELISA were categorized in quartiles. Highest and lowest quartiles were compared.

<sup>b</sup> Other tumors = endometrioid carcinoma, clear cell carcinoma, mucinous carcinoma, low-grade serous carcinoma, carcinosarcoma, mixed tumor, non-differentiated carcinoma, mucinous cystadenoma and borderline ovarian tumor.

<sup>c</sup> Borderline ovarian tumor, *n* = 20; Mucinous cystadenoma, *n* = 1.

<sup>d</sup> *C. trachomatis* DNA analyzed with PCR.

<sup>e</sup> Intense staining of nucleus was considered chsp60 positive.

<sup>f</sup> One case was not evaluated due to technical problems with immunohistochemical staining.

*C. trachomatis* DNA positive tumors were not significantly associated with *C. trachomatis* IgG or cHSP60 IgG (25.0% vs. 9.8%, *P* = .23 and 66.7% vs. 50.0%, *P* = 1.00) (Table 4). Furthermore, chsp60 in tumor tissue did not correlate to *C. trachomatis* IgG or high levels of cHSP60 IgG antibodies (10.0% vs. 10.5%, *P* = 1.00 and 41.2% vs. 62.2%, *P* = .22). Neither plasma antibodies nor *C. trachomatis* DNA or chsp60 in tissue were associated with any of the tumor and proliferation markers p53, p16 or Ki67 (data not shown).

**Covariance**

No covariance was found between *C. trachomatis* IgG and *C. pneumoniae* IgG antibodies (*P* = 1.00) nor between chsp60 and hsp60 in tissue (*P* = .83). Pearson correlation showed a significant, albeit weak, covariance between cHSP60 IgG and hHSP60 IgG (*r* = 0.32, *P* = .01).

**Discussion**

In our cohort of 69 women undergoing surgery due to suspected ovarian pathology *C. trachomatis* DNA was found in tumor tissue of eight women, all with invasive ovarian cancer. At the time of diagnosis, the prevalence of anti-*C. trachomatis* and anti-cHSP60 plasma antibodies, as well as chsp60 protein in ovarian tissue, was similar between HGSC and other ovarian tumors, although trends of higher prevalence were seen in women with HGSC. As expected, the tumor and proliferation markers p53, p16 and Ki67 were all significantly associated to HGSC [3–8], further verifying the histopathological diagnoses. Tissue and plasma markers of chlamydial infection were not associated with any of the tumor or proliferation markers.

In the literature, there are contradictory findings regarding *C. trachomatis* involvement in ovarian cancer. One previous study has identified *C. trachomatis* in 70% of ovarian tumor tissue and none in benign ovaries [16]. Another study was not able to identify *C. trachomatis* in ovarian cancer tissue [29]. The methods used for detection, as well as technical quality of analyzed tissue, varies among studies, and this likely contributes to the reported discrepancy. Our detection targets DNA and chsp60, both markers of past or present chlamydial infection, but with potentially different dynamics. PCR detection of DNA benefits from a very high specificity, but might underestimate the prevalence because of DNA degradation [30]. To our knowledge, the prevalence of chsp60 protein in ovarian tumor tissue has never been described before. We interpret the high frequency found, that the protein is stable in the studied tissue and thus detectable even in cases negative for chlamydial DNA. Being selectively expressed during persistent infections, the ratio between protein and DNA might also be high.

There were no statistically significant associations between the presence of chlamydial plasma antibodies and HGSC at the time of diagnosis. Our results were consistent with two previous studies [31,32]. On the contrary, there are studies claiming association between chlamydial antibodies, more exactly cHSP60 IgG, and ovarian cancer [33,34]. We could not confirm this association. If *C. trachomatis* increases the risk of ovarian cancer development, it might have impacted the disease process several years or decades previously, and the infection could have been cleared at the time of diagnosis. Furthermore, the persistence of chlamydial antibodies is not completely elucidated. In one study, 58% of women with pelvic inflammatory disease caused by *C. trachomatis* had low or negative IgG titers after 3-6 years [35]. Therefore, assessment of *C. trachomatis*

plasma antibodies at diagnosis might have detected only a fraction of all previous infections in our study.

Furthermore, hHSP60 IgG and cHSP60 IgG levels were weakly correlated and a cross-reaction cannot be ruled out. Since hHSP60 IgG has been shown to not be associated with ovarian cancer [36], a possible cross-reaction might have diluted any association between cHSP60 IgG and HGSC. Another plausible explanation is that the secretion of both antibodies increase in parallel; *C. trachomatis* stimulates cHSP60 IgG secretion, while an inflammatory reaction stimulates an increase in hHSP60 antigen production followed by hHSP60 IgG secretion.

### Strengths

Strengths of this study are the well-defined cohort regarding histopathological diagnoses and tumor markers, as well as the corresponding high-quality blood samples. To ensure an optimal classification of the histopathological diagnoses a reevaluation of pathology reports and tumor sections was performed. Additionally, immunohistochemical staining of the tumors, including proliferation markers, strengthened the diagnostic evaluation. The methods applied, testing antibody prevalence in plasma, were commercially available validated standard methods, *C. trachomatis* IgG and cHSP60 IgG specific, minimizing the risk of misleading results. Covariance between chsp60 and hHSP60 protein in ovarian tissue has also been ruled out.

### Limitations

Limitations of this study include the small number of subjects which affects the generalizability of the results, and the extent to which statistical inferences can be made. A larger cohort with prospective plasma samples and a higher number of HGSC cases would have been preferable. The majority of the control group consisted of borderline tumors and other ovarian cancers, the results might thus have been different with a control group consisting of women without ovarian pathology. *C. trachomatis* infections could be involved in the carcinogenesis of ovarian tumors in general, and if so, it would have diluted any possible association to HGSC. In the future, with increasing opportunistic salpingectomy rates, controls with normal fallopian tube tissue might be a better and more available option. The evaluation of the immunohistochemical staining and MIF signal are subjective assessments. However, all samples were evaluated blindly in duplicate by the same observer, limiting inter-observer variation. In case of intra-observer discrepancy an experienced senior gynecologic oncology pathologist reassessed the sample and consensus was reached.

### Conclusion

In conclusion, *C. trachomatis* was identified in invasive ovarian cancer in women of all ages, giving some support to a possible role in EOC development. On the contrary, this was not supported by any statistically significant associations of chsp60 protein, plasma *C. trachomatis* IgG or cHSP60 IgG with invasive ovarian cancer or HGSC. Further research regarding the possible impact of *C. trachomatis* on the risk of EOC, and in specific HGSC, are needed in order to further elucidate the mechanisms underlying this deadly disease where few modifiable risk factors are well characterized.

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