Tubal factor infertility is associated with antibodies against *Chlamydia trachomatis* heat shock protein 60 (HSP60) but not human HSP60

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**BACKGROUND:** Serum antibodies against major outer membrane protein (MOMP) and heat shock protein 60 (HSP60) from *Chlamydia trachomatis* are correlated with sequelae following infection. Since bacterial and human HSP60 share considerable sequence homology, cross-reactivity to human HSP60 is suggested as being involved in tubal factor infertility (TFI). The aim was to investigate whether antibodies to human HSP60 are associated with TFI, and to evaluate antibody testing in TFI diagnosis.

**METHODS:** Serum levels of antibodies against chlamydial MOMP and HSP60 from *C. trachomatis*, *Salmonella enterica* Enteritidis, *Campylobacter jejuni* and human HSP60 were analysed by enzyme-linked immunosorbent assay in three groups of infertile women: women with TFI (*n* = 70), controls with normal fallopian tubes (control group 1, *n* = 92) and a subgroup of women with normal fallopian tubes and sero-positive for either chlamydial MOMP or chlamydial HSP60 (control group 2, *n* = 28).

**RESULTS:** Serum levels of immunoglobulin (Ig)G1 and IgG3 antibodies against MOMP and HSP60 from *C. trachomatis* were elevated in patients with TFI compared with non-TFI individuals (group 1; *P* < 0.001), while levels of IgG3 against MOMP and IgG1 against HSP60 were higher in the TFI group compared with control group 2 (*P* = 0.04 and *P* = 0.03, respectively). Levels of antibodies against human HSP60 did not differ between groups.

**CONCLUSIONS:** Our findings confirm an association between TFI and antibodies to MOMP and HSP60 from *C. trachomatis*, suggesting antibody testing as a supplement in TFI diagnosis. No connection was observed between TFI and antibodies to human HSP60, pointing to an infectious rather than an autoimmune inflammation as the cause of TFI.

**Key words:** *Chlamydia trachomatis* / infertility / heat shock protein 60 / autoimmunity / immunoglobulin gG subclasses

**Introduction**

Infection with *Chlamydia trachomatis* is a major cause of sexually transmitted disease worldwide with extensive consequences for fertility resulting from damage of the fallopian tube (Gerbase *et al.*, 1998).

*Chlamydia trachomatis* is an obligate intracellular bacterium, infecting cervical epithelial cells in women. Generally, the infection resolves without sequelae, but occasionally it spreads from the lower to the upper genital tract and pelvic inflammatory disease may develop (Paavonen *et al.*, 1985; Morre *et al.*, 2002). This may lead to scarring of the fallopian tube, causing occlusion, possibly resulting in ectopic pregnancy or tubal factor infertility (TFI). Prolonged and recurrent infection increases the risk of sequelae (Beatty *et al.*, 1994a,b). In accordance with this, prevalence and high serum titre of immunoglobulin (Ig)G antibodies against *C. trachomatis* have been correlated to TFI (Machado *et al.*, 2007; Malik *et al.*, 2009).

The pathogenic processes causing the sequelae are thought to be partly immunological (Beatty *et al.*, 1994a,b). However, the
mechanisms behind this immunopathology and the development of TFI have not been fully elucidated.

Heat shock proteins (HSPs) are a group of functionally related chaperones primarily involved in correct folding of intracellular proteins. They are found in both eukaryotic and prokaryotic organisms and are highly conserved throughout evolution. HSPs are stress-proteins, and the expression is significantly up-regulated when cells are exposed to stressors, such as increased temperature, oxidative stress or inflammation (Zugel and Kaufmann, 1999). Furthermore, bacterial HSPs are very immunogenic (Rajaiah and Moudgil, 2009). During C. trachomatis infection, epitopes from the bacterial HSP are proven to be strong T and B cell antigens, resulting in a powerful cellular and humoral immune response (Zugel and Kaufmann, 1999; Kinnunen et al., 2002), and an association between C. trachomatis heat shock protein 60 (HSP60) antibodies and sequelae following infection has been observed (Dieterle and Wollenhaupt, 1996; Dutta et al., 2008; Jakus et al., 2008). Several T cell epitopes in C. trachomatis HSP60 and human HSP60 are homologous (Yi et al., 1993). Therefore, autoimmunity to human HSP60, resulting from cross-reactivity, has been suggested to be involved in the pathogenesis of TFI following C. trachomatis infection (Yi et al., 1993; Domeika et al., 1998; Witkin et al., 1998; Witkin, 2002).

The standard procedure for diagnosing TFI is based on laparoscopy and hysterosalpingography (HSG). Although HSG is less costly and less invasive than laparoscopy, it still carries the risk of complications and unexplained infertility or male factor infertility in case of normal result of HSG (control group 1, n = 92). Furthermore, within control group 1, a third group of patients, who were sero-positive either against MOMP or chlamydial HSP60 but had normal Fallopian tubes, was defined (control group 2, n = 28).

The patients were recruited to the project when they began their first IVF treatment. The serum samples were taken in connection with the routine pregnancy test, which was performed 2 weeks after embryo transfer.

**Materials and Methods**

**Patient sera**

Sera were obtained from 162 Danish women undergoing IVF treatment. The study was approved by the local Scientific Ethical Committee (journal no. 1998/4219) and written informed consent was obtained from each patient.

All women were examined by HSG and unless normal passage through two normal, calibrated tubes was found, laparoscopy with insufflations of methylene blue was also performed.

On the basis of these procedures, the women were classified into two groups according to cause of infertility: TFI related to lack of passage through one or both tubes, verified by laparoscopy (TFI group, n = 70) and unexplained infertility or male factor infertility (control group 1, n = 92). Furthermore, within control group 1, a third group of patients, who were sero-positive either against MOMP or chlamydial HSP60 but had normal Fallopian tubes, was defined (control group 2, n = 28).

The aim of the present study was to confirm the association between TFI and the presence of antibodies to C. trachomatis major outer membrane protein (MOMP) or recombinant HSP60 from C. trachomatis, and thereby validate antibody detection as a diagnostic test for TFI. Instead of measuring total IgG, as in earlier studies, we measured the IgG subclasses in an attempt to optimize the diagnostic sensitivity of the test. Furthermore, we wanted to investigate whether antibodies to chlamydial HSP60 are likely to cross-react with HSP60 from other bacteria and with human HSP60, in order to evaluate a possible connection between autoantibodies to human HSP60 and TFI.

Three populations of women were studied: women with TFI, controls with normal Fallopian tubes (control group 1) and women sero-positive for either chlamydial MOMP or HSP60 from C. trachomatis but with normal fallopian tubes (control group 2).

**Statistical analysis**

The data were analysed by GraphPad Prism version 5.0a for Mac OS X (Graphpad Software Inc., La Jolla, CA, USA), using individual samples as experimental unit.

Mann–Whitney U-test was used to analyse the differences between antibody levels in the three groups (TFI, control group 1 and control group 2). Spearman non-parametric correlation was used to analyse the correlation between antibody levels obtained using different antigens.

In addition, the χ² test was used to determine the difference in number of sero-positive individuals between the groups. Probabilities < 0.05 were considered as significant.

**Enzyme-linked immunosorbent assay**

Prevalence of antibodies was determined by enzyme-linked immunosorbent assay (ELISA) using IgG subclass-specific secondary antibodies. *Chlamydia trachomatis-IgG-ELISA-plus* plates (medac, Hamburg, Germany) (Jones et al., 2003) were used for the MOMP peptide-based analysis and *chHSP60-IgG-ELISA* plates (medac) (Bax et al., 2004) were used for C. trachomatis HSP60.

The ELISA for Campylobacter jejuni, Salmonella enterica Enteritidis and human HSP60 was performed as described by Drasbek et al. (2004). ELISA plates were coated with 4 µg/ml human HSP60, C. jejuni HSP60 or S. enterica Enteritidis HSP60.

Full length human HSP60 was obtained from Loke Diagnostics (Risskov, Denmark). The functionality of this antigen is established from analyses made on serum from patients with spondyloarthritis (unpublished data). These patients have been proven sero-positive against human HSP60 (Handley et al., 1996).

Campylobacter jejuni and S. enterica Enteritidis HSP60 genes were cloned in pET30ek-LIC vector (Invitrogen, Carlsbad, CA, USA). The C. jejuni HSP60 gene was amplified with the forward primer 5′GACGACGACAAGTGGCAAAAGAATTTTTTTTGAGTAAAGC3′ and reverse primer 5′G-CGGAGAGACCCGGGTTACATCTCCATCCCGAGCC3′. For S. enterica Enteritidis HSP60 gene, the primers 5′GACGAGGACAAGATGGCGACGA-GGAAAGTTTTATTTCAGTAAAGC3′ and 5′GAGGAGAAGACCCGGTATTACA-TCAATGGCGCCGCC3′ were used. The PCR products were cloned into pET30ek-LIC by ligase-independent cloning, according to the manufacturer’s instructions. The proteins were expressed in Escherichia coli BL21 (DE3) using 1 mM IPTG for 2 h. The recombinant HSP60 proteins were purified by Ni²⁺ affinity chromatography (Franken et al., 2000).

The human sera were diluted 1:50 in Bac-dil (medac) before use. The secondary anti-human IgG antibodies used were horse-radish peroxidase conjugated, sheep-anti-human IgG1, IgG2, IgG3 and IgG4 (Binding site, Birmingham, UK), diluted 1:10 000 in Bac-dil.

For quantification of IgG subclasses, NUNC Maxisorp plates were coated with dilution series of native IgG1, IgG2, IgG3 and IgG4 from human myeloma plasma (EMD Biosciences, San Diego, CA, USA) in CCB-buffer (50 mM NaHCO₃, pH9.6). The respective secondary antibodies were added to the standards.

**Materials and Methods**

**Statistical analysis**

The data were analysed by GraphPad Prism version 5.0a for Mac OS X (Graphpad Software Inc., La Jolla, CA, USA), using individual samples as experimental unit.

Mann–Whitney U-test was used to analyse the differences between antibody levels in the three groups (TFI, control group 1 and control group 2). Spearman non-parametric correlation was used to analyse the correlation between antibody levels obtained using different antigens.

In addition, the χ² test was used to determine the difference in number of sero-positive individuals between the groups. Probabilities < 0.05 were considered as significant.
A receiver operating characteristic (ROC) curve, plotting the true-positive rate (sensitivity) on the vertical axis against the false-positive rate (1 – specificity) on the horizontal axis, was used to quantify the overall ability of the particular test to discriminate between individuals with and without the disease. For the overall measure of the test’s accuracy, the area under the curve (AUC) was used. A value of 0.5 represents no better than chance and a value of 1 equals perfect sensitivity and specificity.

The cut-off values were calculated as the SD of the blanks (wells incubated without sample) times two [cut-off = SD(Blanks) × 2]. Furthermore, clinical diagnostic cut-off values were determined based on the ROC curves by assessing the serum concentration where the curve breaks (the point closest to 100% sensitivity and specificity). Individuals with a serum antibody concentration above the cut-off values were considered sero-positive.

**Results**

**Antibodies against MOMP**

Serum levels of IgG1 and IgG3 antibodies were elevated in patients with TFI compared with control group 1 (Fig. 1 A and B, \( P < 0.001 \)). Serum levels of IgG3 antibodies were also higher in the TFI group compared with control group 2 (Fig. 1 B, \( P = 0.04 \)). No IgG2 or IgG4 antibodies against MOMP were detected.

The ROC curve, used to quantify the overall ability of antibody detection in predicting TFI, demonstrated that prevalence of IgG1, and especially IgG3, antibodies to the MOMP peptide was associated with TFI (Fig. 2 A and B, IgG1, AUC = 0.77; IgG3, AUC = 0.82).

On the basis of the diagnostic cut-off value for IgG3 against MOMP obtained from the ROC curve (Fig. 2 B, 73% sensitivity and 80% specificity), we found that 52 of 70 individuals (74%) in the TFI group and 19 of 92 individuals (21%) in control group 1 were sero-positive (Table I, positive predictive value 73% and negative predictive value 80%). The number of sero-positive individuals differed between the TFI group and control group 1 (Table I, \( P < 0.001 \)). Eighteen of 28 patients (64%) were sero-positive in control group 2, which did not differ from the TFI group (Table I).

**Antibodies against C. trachomatis HSP60**

Higher serum levels of IgG1 and IgG3 against C. trachomatis HSP60 were observed in the TFI group compared with control group 1 (Fig. 1 C and D, \( P < 0.001 \)), whereas only serum levels of IgG1 were higher in the TFI group compared with control group 2 (\( P = 0.03 \)). IgG2 and IgG4 antibodies were not detectable.

Antibodies against C. trachomatis HSP60 were correlated with antibodies against MOMP (data not shown). The strongest correlation was found between IgG3 antibodies to the MOMP peptide and IgG1 antibodies to C. trachomatis HSP60 (\( r_s = 0.69, P < 0.001 \)).

From the ROC curves we observed IgG1 and IgG3 antibodies to chlamydial HSP60 to be associated with TFI (Fig. 2 C and D, IgG1, AUC = 0.81; IgG3, AUC = 0.74).

Using the diagnostic cut-off value for IgG1 antibodies against HSP60 (Fig. 2 C) 54 of 70 individuals in the TFI-group (77%) and 16 of 92 individuals in control group 1 (17%) were defined as sero-positive (Table I, positive predictive value 77% and negative predictive value 83%). In control group 2, 16 of 28 were sero-positive (Table I). The difference in number of sero-positive individuals between the TFI group compared with control group 1 and control group 2 was significant (Table I, \( P < 0.001 \) and \( P = 0.05 \), respectively).

A redefined sero-positivity criterion including both IgG3 antibodies against MOMP and IgG1 antibodies against C. trachomatis HSP60 revealed a positive predictive value of 89% and a negative predictive value of 80%, 69% sensitivity and 93% specificity (Table II).

**Antibodies to HSP60 from C. jejuni and S. enterica Enteritidis**

Serum levels of antibodies against HSP60 from C. trachomatis were correlated with antibodies to HSP60 from C. jejuni (IgG1: \( r_s = 0.43, P < 0.001 \)) and S. enterica Enteritidis (IgG1: \( r_s = 0.53, P < 0.001 \)). Despite this correlation, presence of antibodies against HSP60 from C. jejuni and S. enterica Enteritidis were not associated with TFI (Fig. 2 E and F).

The detected IgG antibodies against HSP60 from C. jejuni and S. enterica Enteritidis were IgG1 and IgG3 (data not shown). No IgG2 and IgG4 were detected.

**Antibodies to human HSP60**

Serum levels of IgG1 and IgG3 antibodies against human HSP60 were low in the TFI group as well as in the control groups (Fig. 1 E and F). No IgG2 and IgG4 were detected.

There was no correlation between antibodies to C. trachomatis HSP60 and human HSP60, and IgG antibodies to human HSP60 did not differ between groups (Fig. 2 G and H).

**Discussion**

Results from the present study confirmed the generally accepted hypothesis that severe or recurrent Chlamydia infection may lead to TFI, whereas a link of TFI to autoimmunity related to antibodies to human HSP60 was not substantiated. TFI was associated with the presence of antibodies against MOMP and HSP60 from C. trachomatis, and using appropriate cut-off values, measurements of serum IgG3 antibody levels against MOMP and IgG1 antibody levels against HSP60 proved to be powerful predictors of TFI, potentially operating as a diagnostic test.

The increased occurrence of sero-positive patients in the TFI group (Table I) as well as the increased levels of antibodies to MOMP and HSP60 from C. trachomatis in TFI patients compared with non-TFI controls (Fig. 1) support that TFI is associated with infection by C. trachomatis. This result is consistent with several previous studies demonstrating serum antibodies to MOMP and HSP60 from C. trachomatis in TFI patients (Jakus et al., 2008; Malik et al., 2009; Rodgers et al., 2010), however, we have expanded the analysis to include IgG subclass determinations. Furthermore, the present study demonstrated an increased antibody response in the TFI group compared with control group 2, consisting of sero-positive non-TFI patients (Fig. 1). This indicates that sequelae following Chlamydia infection is associated with a higher antibody level, consistent with the generally accepted hypothesis that TFI results from severe and recurrent infection (Beaty et al., 1994a,b).

This study revealed some correlation between C. trachomatis HSP60-specific antibodies and antibodies to HSP60 from C. jejuni and S. enterica Enteritidis, suggesting cross-reaction. However, no
Association between TFI and antibodies to HSP60 from S. enterica Enteritidis and C. jejuni was found (Fig. 2), indicating that regardless of the high degree of identity (Needleman and Wunsch, 1970), a specific antibody response is generated against C. trachomatis HSP60. Furthermore, no correlation between antibodies to chlamydial and human HSP60 was found, and no accumulation of human HSP60 sero-positive individuals was observed in the TFI group (Fig. 1). This indicates that even though HSP60 from C. trachomatis shares 48.5% sequence identity with human HSP60 (Needleman and Wunsch, 1970) and several epitopes of HSP60 from C. trachomatis are possibly involved in autoimmunity (Yi et al., 1993), no association between antibodies to human HSP60 and TFI exists (Fig. 2). This is contradicted by other studies, connecting previous C. trachomatis infection with antibodies to human HSP60 (Domeika et al., 1998; Witkin et al., 1998; Cortinas et al., 2004), however, to our knowledge, no association between TFI and human HSP60 has been reported.

Since no compelling evidence is available supporting the hypothesis of autoimmunity against human HSP60 in association with TFI, the inflammation and injury of the epithelial cells following infection are more likely caused by the immunological response against chlamydial
antigens, as proposed by Stephens (2003): he presents an alternative theory to the autoimmune hypothesis, arguing that the sequelae following chlamydial infection are primarily caused by the cellular responses induced by *Chlamydia*-infected cells. These cells secrete proinflammatory cytokines as well as cytokines, inducing tissue fibrosis (Stephens, 2003), which has been demonstrated to cause damage, also to uninfected epithelial cells (Hvid et al., 2007). This explains the inflammation and tissue fibrosis resulting in epithelial damage and the subsequent occlusion of the fallopian tube. Results from our study demonstrate a powerful antibody response against HSP60 from *C. trachomatis* indicating that HSP60 is a strong B cell antigen. Furthermore, chlamydial HSP60 is known to mediate proinflammatory responses through toll-like receptor 2 and 4 (Bulut et al., 2002; Da Costa et al., 2004). This suggests HSP60 as an important chlamydial antigen and inflammatory mediator in the generation of TFI.

Figure 2 ROC curves showing the true-positive rate (sensitivity) against the false-positive rate (1 – specificity) for the different possible cut-offs of the diagnostic analyses based on serum levels of IgG1 and IgG3 antibodies against chlamydial MOMP (A and B), HSP60 from *C. trachomatis* (C and D), *S. enterica* Enteritidis HSP60 (E), *C. jejuni* HSP60 (F) and human HSP60 (G and H) in patients with TFI and individuals with normal fallopian tubes (control group 1). The accuracy depends on the overall ability of the test to discriminate between individuals with and without the disease and is quantified by the AUC.
The strong association between TFI and serum antibodies demonstrated in the present study makes measurements of serum antibody levels against MOMP and HSP60 a potential predictor of TFI, if the cut-off values are set to reflect the enhanced antibody levels characteristic of sequelae following Chlamydia infection. On the basis of the present study, the most attractive candidates for a diagnostic test are IgG3 against MOMP and IgG1 against chlamydial HSP60, as these subclasses are predominant in the response (Fig. 1) and showed the greatest association to TFI (Fig. 2). By combining detection of IgG3 against MOMP and IgG1 against HSP60 from C. trachomatis, the best diagnostic value of the test was obtained, resulting in a sensitivity of 69% and a specificity of 93% (Table II).

On the basis of a meta-analysis assessing the accuracy of HSG, the resulting in a sensitivity of 69% and a specificity of 93% (Table II). In conclusion, the present study confirms the association between TFI and antibodies to MOMP and HSP60 from C. trachomatis, whereas no connection was observed between TFI and antibodies to human HSP60. This indicates that an infectious rather than an autoimmune inflammation is the cause of TFI. Our results suggest that antibodies against other C. trachomatis-specific proteins may have the potential to further improve an antibody-based diagnostic test.

Table I Number of sero-positive women in the group with TFI, control group 1 consisting of individuals with normal fallopian tubes and control group 2 which is a subgroup of group 1, with women who are sero-positive for either chlamydial MOMP or chlamydial HSP60 but with normal fallopian tubes.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>TFI group, n = 70 (%)</th>
<th>Control group 1, n = 92 (%)</th>
<th>Control group 2, n = 28 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydial MOMP (IgG1)</td>
<td>51 (73)</td>
<td>29 (32)***</td>
<td>19 (68)</td>
</tr>
<tr>
<td>Chlamydial MOMP (IgG3)</td>
<td>52 (74)</td>
<td>19 (21)***</td>
<td>18 (64)</td>
</tr>
<tr>
<td>Chlamydial HSP60 (IgG1)</td>
<td>54 (77)</td>
<td>16 (17)***</td>
<td>16 (57)*</td>
</tr>
<tr>
<td>Chlamydial HSP60 (IgG3)</td>
<td>43 (61)</td>
<td>16 (17)***</td>
<td>11 (39)*</td>
</tr>
<tr>
<td>Both chlamydial MOMP (IgG3) and chlamydial HSP60 (IgG1)</td>
<td>48 (69)</td>
<td>6 (7)***</td>
<td>6 (21)***</td>
</tr>
</tbody>
</table>

χ² test was used to compare the TFI group to control group 1 and control group 2, respectively. Ig, immunoglobulin; MOMP, major outer membrane protein; HSP60, heat shock protein 60.

***p < 0.001; **p < 0.01; *p < 0.05.

Table II Test performance: sensitivity, specificity and positive and negative predictive value for the combined TFI test based on sero-positivity for both IgG3 antibodies against chlamydial MOMP and IgG1 antibodies against C. trachomatis HSP60.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Patients with TF (n)</th>
<th>Control (n)</th>
<th>Positive predictive value</th>
<th>Negative predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both MOMP (IgG3) and HSP60 (IgG1)</td>
<td>48</td>
<td>6</td>
<td>89%</td>
<td>80%</td>
</tr>
<tr>
<td>Test-outcome (positive)</td>
<td>48</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test-outcome (negative)</td>
<td>22</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In total</td>
<td>70</td>
<td>92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity 69%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity 93%</td>
<td></td>
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</tbody>
</table>

In addition, antibodies against caseinolytic protease P from C. trachomatis have been correlated with TFI and, when included in an antibody-detection test, this increased the sensitivity (Rodgers et al., 2010). This suggests that antibodies against other C. trachomatis-specific proteins may have the potential to further improve an antibody-based diagnostic test.

In conclusion, the present study confirms the association between TFI and antibodies to MOMP and HSP60 from C. trachomatis, whereas no connection was observed between TFI and antibodies to human HSP60. This indicates that an infectious rather than an autoimmune inflammation is the cause of TFI. Our results suggest measurement of IgG3 against chlamydial MOMP and IgG1 against chlamydial HSP60 as a further diagnostic test for TFI, as a supplement to the methods which are used currently.

Authors’ roles

A.H.: contributions to conception and design, analysis and interpretation of data, drafting the article, final approval of the version to be published. G.C.: revising the article critically for important intellectual content, final approval of the version to be published. T.G.J.: contributions to revising the article critically for important intellectual content, final approval of the version to be published. H.J.I.: contributions to conception and design, acquisition of clinical data, final approval of the version to be published. S.B.: contributions to conception and design, revising the article critically for important intellectual content, final approval of the version to be published.
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Conflict of interest

S.B. and G.C. are shareholders in Loke Diagnostics (Århus, Denmark).

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