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ORIGINAL ARTICLE

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Predicting tubal factor infertility by using markers of humoral and cell-mediated immune response against *Chlamydia trachomatis*

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Problem: The accuracy of *Chlamydia trachomatis* antibody test in predicting tubal factor infertility (TFI) is limited, and more accurate methods are needed. Cell-mediated immune response (CMI) is crucial in the resolution of pathogen, but it may play an important role in the pathogenesis of *C trachomatis*-associated tubal damage. We studied whether combining the markers of *C trachomatis*-induced CMI to humoral immune response improves the accuracy of serology in TFI prediction.

Method of study: Our prospective study consists of 258 subfertile women, of whom 22 (8.5%) had TFI. Women with other causes for subfertility served as a reference group. Serum *C trachomatis* major outer membrane protein (MOMP) and chlamydial heat-shock protein 60 (cHSP60) IgG antibodies were measured by ELISA. CMI was studied by lymphocyte proliferation assay in vitro.

Results: Serological markers were more prevalent in women with TFI than in other subfertile women (40.9% vs 12.3% for MOMP IgG and 27.3% vs 10.2% for cHSP60 IgG). The best test combination for TFI was *C. trachomatis* MOMP and cHSP60 antibody with an accuracy of 90.3%, sensitivity of 22.7% and specificity of 96.6%. Positive post-test probability of this combination was 54.2%, and negative post-test probability was 12.4%. Adding of the markers of CMI did not significantly improve the accuracy of serology in TFI prediction.

Conclusion: The accuracy of TFI prediction increases when the combination of *C trachomatis* MOMP and cHSP60 antibody tests is used. *C trachomatis*-induced CMI was common in our study population, but the markers of CMI did not predict TFI.

KEYWORDS
cell-mediated immune response, *Chlamydia trachomatis*, diagnostic test, serology, tubal evaluation, tubal factor infertility

1 | INTRODUCTION

Chlamydia trachomatis infection is the most prevalent bacterial sexually transmitted disease with over 130 million cases detected annually worldwide.[1] Up to 80% of infections are asymptomatic and may therefore remain undiagnosed. If the infection is not resolved, *C trachomatis* can ascend to the upper genital tract and cause pelvic inflammatory disease (PID) which increases the risk of tubal factor infertility (TFI).[2]

Laparoscopy has remained the gold standard for the diagnosis of TFI. However, it is costly, invasive and can potentially cause complications. Hysterosalpingography (HSG) and hysterosalpingosonography

(HSSG) are less risky procedures for TFI diagnosis, but false-positive results are common.[3,4] HSG and HSSG are also highly observer-dependent, and peritubal adhesions are not visualized by these procedures. In addition, these methods do not reveal impaired tubal function. In a recent meta-analysis, the sensitivity and specificity were 95% (78%-99%) and 93% (89%-96%) for HSSG, and 94% (74%-99%) and 92% (87%-95%) for HSG compared to laparoscopy.[3]

Numerous studies have shown that positive *C. trachomatis* serology is associated with tubal damage.[5,6] Therefore, *C. trachomatis* IgG antibody testing (CAT) has been used in infertility work-up to screen subfertile women with the highest risk of tubal pathology.[7,8] However, the accuracy of these tests is limited. The main limitations are the high rate of false-positive results and low positive predictive value (PPV).[9,10] More accurate, non-invasive methods are needed for TFI diagnostics.

In chronic *C. trachomatis* infection, the expression of chlamydial heat-shock protein 60 (cHSP60) is highly upregulated. Several studies have shown that immune responses to cHSP60 are associated with PID, tubal occlusion and ectopic pregnancy (EP).[11,12] Furthermore, the presence of serum antibodies against cHSP60 may also predict TFI.[13,14]

The clearance of *C. trachomatis* is known to depend on cell-mediated immune response (CMI).[15] However, in addition to the protective function, CMI is also associated with inflammatory pathological processes resulting in tubal damage.[16-18] The role of CMI in predicting TFI has been examined in our earlier studies, in which in vitro lymphocyte response against *C trachomatis* was more often detected in women with TFI than in healthy controls.[14,18] As a result, we hypothesized that TFI prediction model could be further improved by adding markers of CMI to serology.

The aim of our study was to evaluate if combining markers of cell-mediated and humoral immune response against *C. trachomatis* improves the accuracy of serology in the prediction of TFI in clinical practice.

2 | MATERIALS AND METHODS

2.1 | Study population

Our study population consisted of 258 subfertile women referred for infertility investigations to Helsinki University Hospital during July 2007 - December 2010. Infertility work-up was performed according to our routine protocol after at least one year of unprotected intercourse. The first visit in the fertility clinic included gynaecological examination and ultrasonography of uterus and adnexa. Ovulatory menstrual cycle was monitored by the growth of the follicle in ultrasound and by measuring serum mid-luteal progesterone. Hormonal disorders were diagnosed by determination of serum gonadotropins and progesterone and by analysing serum prolactin and thyroid function tests. First-void urine or a cervical swab specimen was collected for the diagnosis of *C. trachomatis* by the nucleic acid amplification test (NAAT). Semen samples of the male partners were analysed according to the criteria of the World Health Organization (WHO).

Tubal patency was evaluated either by HSSG or by diagnostic laparoscopic tubal evaluation. TFI was defined as a visible occlusion of at least one tube. HSSG was performed by a fertility specialist by instilling 0.9% saline and air into uterus and following the path of air bubbles through the fallopian tubes. Tubal status in HSSG was classified as no occlusion, unilateral occlusion or bilateral occlusion. Bilateral tubal occlusion or unclear result in HSSG was confirmed by laparoscopy. Laparoscopy was performed also in a suspicion of deep rectovaginal endometriosis, in the presence of large endometrioma requiring operational treatment or if severe symptoms consistent with endometriosis were present. Laparoscopic tubal evaluation was performed by chromopertubation, where methylene blue was injected through a catheter into the uterine cavity and the passage of dye was followed through the fallopian tubes. Laparoscopic criterium for endometriosis was a direct visualization of ectopic endometrial lesions, usually accompanied by pelvic adhesions. Cases with EP before or during infertility evaluation (n = 11) were included in the unilateral TFI group. Subfertile women with another aetiology for subfertility, such as ovulatory disorder (n = 69), endometriosis (n = 37), male factor infertility (n = 25), unexplained infertility (n = 96) or other sporadic cause (hormonal disorder, structural anomaly or pelvic adhesions due to previous pelvic surgery; n = 9), served as the reference group.

Participants were followed to the first delivery or until June 2014. Clinical data on the results of infertility examinations, treatment and subsequent pregnancy were collected from the patient register of Helsinki University Hospital.

2.2 | Serological analysis and lymphocyte proliferation assay

Blood samples for immunological analysis were collected at the first visit and analysed at the National Institute for Health and Welfare in Oulu, Finland. Both humoral and cell-mediated immune responses to *C. trachomatis* were studied. *C. trachomatis* MOMP-specific and cHSP60-specific IgG antibodies were analysed by commercially available ELISA kits (Medac Diagnostika, Hamburg, Germany) according to manufacturer's instructions. This peptide-based *C. trachomatis* MOMP ELISA is considered species-specific with minimal cross-reactivity to other *Chlamydia* species.[9] Results were obtained as a mean absorbance of duplicated samples at 450 nm. Less than 10% variation was seen in doublets (OD > 0.2). Cut-off for a positive antibody level (mean OD value of the negative control +0.350) was OD > 0.4.

Chlamydia trachomatis-specific CMI was analysed in vitro by lymphocyte proliferation test. Viable cells for the analysis were available from 234 patients. *C. trachomatis* elementary body (EB; serovar E and F, total protein concentration 3 μg/mL) and recombinant CHSP60 (2.5 μg/mL) were used as lymphocyte-stimulating antigens.[15] The results were expressed as stimulation indices (SI, or mean count per minute in the presence of antigen, divided by mean count per minute in its absence) for triplicate cultures. SI > 5 was

considered a positive response to chlamydial EB antigen, and SI > 2.5 was considered a positive response to cHSP60 antigen.

2.3 | Statistical analysis

Chi-squared test was used for the analysis of categorical data. Continuous variables were compared by Student's *t* test or Mann–Whitney *U*-test as appropriate. To evaluate the prognostic value of immunological tests for tubal pathology, we calculated sensitivity, specificity, PPV, negative predictive value (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR–). Post-test probability was calculated as (pre-test odds*likelihood ratio)/(1 + pre-test odds*likelihood ratio), where pre-test odds = pre-test probability/(1–pre-test probability). Estimated TFI prevalence of 15% was used when calculating post-test probability. The diagnostic performance of the tests was analysed by receiver operating characteristic (ROC) curves. Statistical analyses were performed with IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA).

3 | RESULTS

3.1 | TFI in the study population

TFI was observed in 22 (8.5%) of the 258 women. The patency of fallopian tubes was evaluated by laparoscopy in 51 (19.8%) and by HSSG in 160 (62.0%) women. Both procedures were performed for eight (3.1%) women. Tubal patency was not evaluated in women who conceived spontaneously before tubal evaluation (n = 24) or were admitted directly to IVF (n = 23). Of all the 22 TFI cases, bilateral tubal occlusion was found in five cases, and 17 women had unilateral tubal occlusion. Altogether, 11 women included in the TFI group had EP before or during infertility work-up. Of the 11 women with EP, five had tubal occlusion and five had patent tubes. One of the 11 women with EP conceived spontaneously before tubal evaluation, but the pregnancy resulted in EP and was treated by salpingectomy. The flow chart of the study population is presented in Figure 1.

The baseline characteristics of the study population are shown in Table 1. Women with TFI were older than women with other causes of subfertility (mean 33.1 vs 31.2 years, $P = 0.04$) and were more likely to have secondary infertility (54.5% vs 27.1%, $P = 0.007$). Smoking was associated with TFI (38.1% vs 16.0%, $P = 0.03$). Altogether, 37 (14.3%) of the 258 women reported prior history of chlamydial infection and six (2.3%) reported recurrent (two or more episodes) infection. Three women (1.2%) reported a history of PID. None of the participants had a positive *C. trachomatis* NAAT from urogenital sample at the time of infertility evaluation. During the follow-up, the live birth rate was significantly lower in the TFI group than in the non-TFI group (58.8% vs 89.8%, $P < 0.001$).

3.2 | Immune response to *C. trachomatis*

The prevalence of *C. trachomatis*-induced humoral and cell-mediated immunological markers in the study population is shown in Table 2. All serological markers were more often positive in TFI cases than in cases with other causes for subfertility. Table 3 shows the performance of immunological tests and test combinations. *C. trachomatis* MOMP antibody test was the best single test (area under ROC curve 0.73; 95% CI 0.61-0.85) in the detection of TFI, with an accuracy of 83.7%, sensitivity of 40.9% and specificity of 87.7%. PPV of the MOMP antibody test (in our population with 8.5% TFI rate) was 23.7% and NPV was 94.1% (Figure 2,

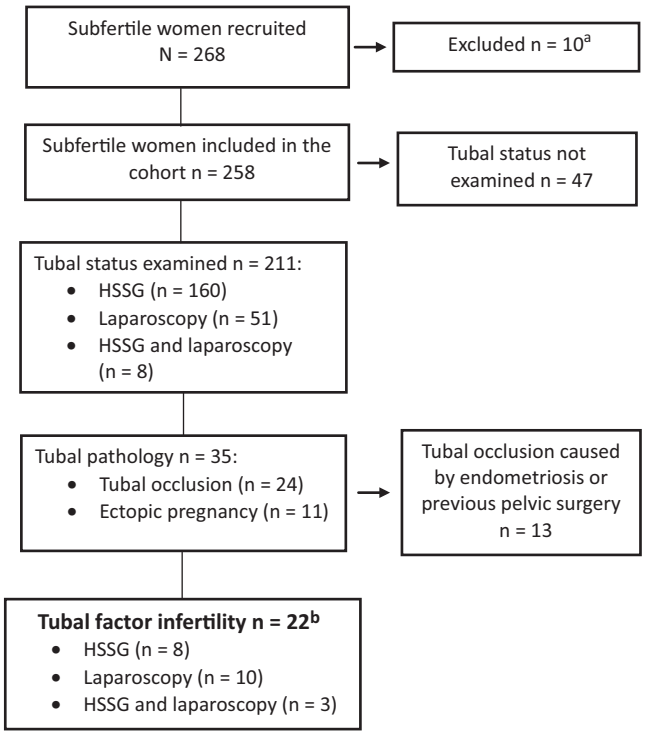

FIGURE 1 Flow chart of the study population. [a]Ten women were excluded for the following reasons: not meeting the criteria for infertility investigation (n = 5), not willing to have infertility investigation (n = 2) or referred directly to IVF from another clinic (n = 3). [b]One of the 11 women with ectopic pregnancy (EP) conceived spontaneously before tubal evaluation, but the pregnancy resulted in EP and was treated by salpingectomy

TABLE 1 Baseline characteristics of the study population

	TFI (n = 22)	Non-TFI (n = 236)	*P*-value
Age in years (mean [SD])	33.1 (4.9)	31.2 (4.0)	0.04
Secondary infertility (n, %)	12 (54.5)	64 (27.1)	0.007
Past genital chlamydia infection[a] (n, %)	4 (18.2)	33 (14.2)	0.54
Smoking[b] (n, %)	8 (38.1)	37 (16.0)	0.03
Body mass index[c] (median [range])	22.5 (18-35)	23 (17-41)	0.65

[a]Self-reported infection, no data in 4 cases.
[b]No data in 6 cases.
[c]No data in 2 cases.

TABLE 2 The prevalence (%) of positive immunological tests against *Chlamydia trachomatis* and cHSP60 in TFI cases and in women with other causes for subfertility

	TFI (N = 22)	Non-TFI (N = 236)	*P-value*
Humoral immune markers (%, n)			
C. trachomatis MOMP IgG	40.9 (9/22)	12.3 (29/236)	0.002
cHSP60 IgG	27.3 (6/22)	10.2 (24/236)	0.03
Cell-mediated immune markers[a] (%, n)			
C. trachomatis EB LP response	85.7 (18/21)	67.1 (143/213)	0.08
cHSP60 LP response	23.8 (5/21)	28.6 (61/213)	0.64
Combination of immune markers[a] (%, n)			
C. trachomatis MOMP IgG and cHSP60 IgG	22.7 (5/22)	3.4 (8/236)	0.002
C. trachomatis MOMP IgG and EB LP response	38.1 (8/21)	9.1 (21/213)	<0.001
C. trachomatis MOMP IgG and cHSP60 IgG and EB LP response	19.0 (4/21)	2.8 (6/213)	0.007

EB, *C. trachomatis* elementary body; LP, lymphocyte proliferation.
[a]Data on CMI response missing in 24 cases.

Table 3). PPV increased to 38.5% when the combination of MOMP and cHSP60 antibody was used. When combining MOMP antibody and EB lymphocyte proliferation test, the PPV was 27.6% and NPV was 93.7%. When the combination of MOMP antibody, cHSP60 antibody and EB lymphocyte proliferation test was used, the PPV increased to 40.0% (Table 3). Positive and negative post-test probabilities are shown in Table 3. Positive post-test probability of the combination of MOMP and cHSP60 antibody was 54.2%, and 54.4% when EB proliferation test was combined with the serological combination. LR+and LR– of MOMP and cHSP60 antibody combination were 6.7 and 0.8, respectively. Adding cHSP60 lymphocyte proliferation test to serological tests did not increase the performance of the tests.

4 | DISCUSSION

The aim of our study was to evaluate whether *C. trachomatis* serology for TFI prediction can be improved by combining tests of *C. trachomatis*-specific humoral and cell-mediated immune response. We found that the accuracy of TFI prediction increases when the combination of *C. trachomatis* MOMP and cHSP60 antibody tests is used. Adding the markers of CMI to the antibody tests did not significantly improve the accuracy of TFI prediction.

Of the single tests, cHSP60 IgG had the highest specificity for predicting TFI in our study, but the sensitivity of the test was low indicating a high number of cHSP60-negative cases among women with TFI *C. trachomatis* MOMP IgG test yielded better sensitivity, but as shown in previous studies ,[6,10] it had relatively low specificity

TABLE 3 Predictive value of the single and combination tests for predicting TFI

	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	LR+	LR–	Positive post-test probability (%)	Negative post-test probability (%)
Humoral immune response									
C. trachomatis MOMP IgG	40.9	87.7	83.7	23.7	94.1	3.3	0.7	37.0	10.6
cHSP60 IgG	27.3	89.8	84.5	20.0	93.0	2.7	0.8	32.1	12.5
Cell-mediated immune response									
C. trachomatis EB LP response	85.7	32.9	37.6	11.2	95.9	1.3	0.4	18.4	7.1
cHSP60 LP response	23.8	71.4	67.1	7.6	90.5	0.8	1.1	12.8	15.9
Combinations of immune markers									
C. trachomatis MOMP IgG and cHSP60 IgG	22.7	96.6	90.3	38.5	93.0	6.7	0.8	54.2	12.5
C. trachomatis MOMP IgG and EB LP response	38.1	90.1	85.5	27.6	93.7	3.9	0.7	40.5	10.8
C. trachomatis MOMP IgG and cHSP60 IgG and EB LP response	19.0	97.2	90.2	40.0	92.4	6.8	0.8	54.4	12.8

Post-test probability was calculated with pre-test probability value 15% for TFI.
EB, elementary body; LP, lymphocyte proliferation.

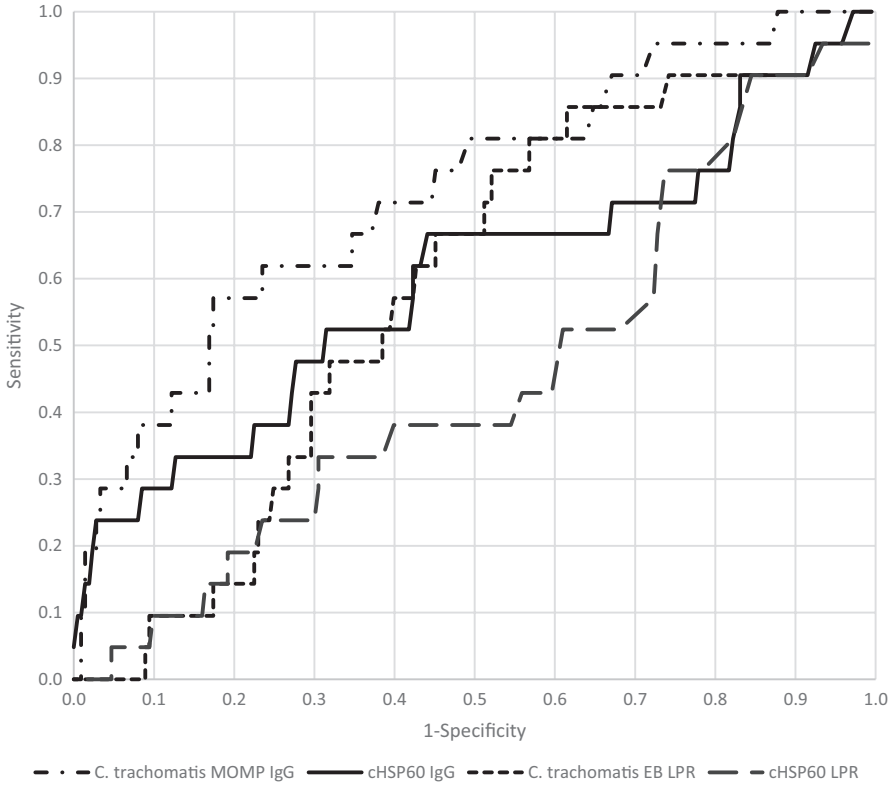

FIGURE 2 Areas under receiver operating characteristic -curves in the prediction of TFI by C. trachomatis-specific and cHSP60-specific immune markers. The best single test for TFI prediction was C. trachomatis MOMP IgG (area under ROC curve 0.73; 95% CI 0.61-0.85). EB, elementary body; LPR, lymphocyte proliferation response

in predicting TFI although this is a species-specific test for detecting serum *C trachomatis* antibodies.[9] This is likely to result from a high number of sero-positive cases among women with non-TFI subfertility.

We found that the specificity of *C trachomatis* serology can be improved by using the combination of MOMP and cHSP60 antibody. This combination had highest accuracy (90.3%) and PPV of 38.5% in our study population in which TFI prevalence was 8.5%. While PPV depends on the prevalence of the disease, the predictive value of the test combination would be higher in a population with higher TFI prevalence. With an estimated pre-test TFI prevalence of 15%, this antibody combination indicates a 54.2% risk of *C trachomatis*-induced TFI after a positive test result.

When evaluating the performance of a clinical test, LR is considered more useful measure than sensitivity and specificity.[19,20] LR summarizes how many times more (or less) likely patient with the disease is to have positive test result than patient without the disease. LR+ of the combination of *C trachomatis* MOMP IgG and cHSP60 IgG was 6.7, suggesting that this test combination is moderately useful in clinical practice to "rule in" women with TFI. However, the LR– of this combination was 0.8, which suggests a high number of false-negative cases who may have had tubal occlusion not related to *C trachomatis*, or who had not developed detectable immunological response to MOMP and cHSP60. Because false-negative test result may lead to ineffective treatment, we consider that high sensitivity and low LR– are more important than high specificity and high LR+ for TFI screening in clinical practice.

The sensitivity of cHSP60 antibody or cHSP60 lymphocyte proliferation test was low in our study, consistent with a high number of cHSP60-negative cases among women with TFI. The overall prevalence of cHSP60 antibodies and positive cHSP60 lymphocyte proliferation test was lower than expected. Immune response to cHSP60 has been linked to chronic or repeat chlamydial infection,[17] but our results suggest that cHSP60 test may be poorer marker of *C. trachomatis*-related sequelae than previously assumed.[12,21] Due to opportunistic *C. trachomatis* screening, chronic infections may have become less common. It is also possible that the virulence of *C. trachomatis* has changed over time, so that current serovars are less likely to ascend to the upper genital tract.[22] The relatively low prevalence of TFI (8.5%) in our study supports this hypothesis.

The course of a single *C. trachomatis* infection varies individually. The majority of women who have serological evidence of past exposure to *C. trachomatis* have cleared the infection without any reproductive consequences, whereas others have persistent infection increasing the risk of TFI. The individual factors influencing the pathogenic mechanisms of TFI are strongly related to the environment and host variables.[23] Furthermore, tubal damage can vary from visible intra-tubal adhesions to milder forms without tubal occlusion. In our study, tubal evaluation was mainly performed by HSSG which is known to have limited accuracy and low inter-observer reproducibility.[3] Moreover, peritubal adhesions or minor tubal damage (ie, destruction of functional tubal epithelium and loss of cilia) cannot be revealed by this procedure. These milder forms of TFI may lead to impaired function of fimbriae or fallopian tubes. In our study, women with EP were included in the TFI group, since EP suggests tubal disease.

Chlamydia trachomatis-associated proportion of all TFI is estimated to be between 29% to 45%, and repeat episodes of chlamydial

infection increase the risk of tubal damage.[24] However, the pathogenesis of TFI is multifactorial. Not all TFI is attributed to prior *C. trachomatis* infection which reduces the performance of *C. trachomatis*-specific immunological tests in the prediction of TFI.

Our finding of lower live birth rate in TFI patients than in patients with other causes of subfertility is consistent with previous studies.[25,26] It is well established that women with untreated hydrosalpinges are at higher risk of implantation failure and miscarriage.[27,28] In our study population, two women had untreated hydrosalpinges. It is also possible that women with post-infectious TFI have chronic endometrial inflammation resulting in impaired implantation.[29,30]

To the best of our knowledge, this is the first study evaluating the combinations of *C trachomatis*-induced humoral and cell-mediated immune responses in predicting TFI in clinical setting. *C trachomatis*-specific immune responses in the TFI cases were comparable to those in our earlier studies.[14,18] However, the study design and the study population were different. In the present study, immune responses against *C. trachomatis* were measured in an unselected subfertile population, whereas in previous studies immune responses were compared between TFI cases and healthy controls. Furthermore, the TFI cases in the previous studies had more severe forms of tubal damage, while in this study population only five women had bilateral tubal damage. In our study population, *C. trachomatis*-specific immune responses were common also in women with other causes for subfertility than TFI.

5 | CONCLUSIONS

In conclusion, the value of serology in evaluating *C. trachomatis*-associated TFI can be improved by combining serum *C. trachomatis* MOMP and cHSP60 IgG antibody tests, or combining EB lymphocyte proliferation test to *C. trachomatis* serology. However, *C. trachomatis* immune markers in the prediction of TFI are only of modest value due to the multifactorial nature of TFI development. Further studies are needed to elucidate the mechanisms of *C. trachomatis* ascension to the upper genital tract and its exact role in chronic inflammation leading to subfertility.

CONFLICT OF INTERESTS

The authors did not report any potential conflict of interests.

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