Article

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Abstract: *Chlamydia trachomatis* (CT) infection affects female fertility. The purpose of our study was to assess the association between serological and follicular fluid markers of CT infection and *in vitro* fertilization (IVF) success. This prospective multicentre cohort study included female patients undergoing IVF procedure in Serbia. IVF procedure was performed according to the standard protocol. Serum and follicular fluid samples were collected during IVF, and IgG and IgA anti-MOMP antibodies titers were determined by ELISA test. Significantly higher embryos implantation rate was detected among patients with negative antibodies titers in follicular fluid (OR [95% CI]: 5.254 [1.055; 26.152]). There was a trend toward increased risk of IVF failure in patients positive for either IgG or IgA in follicular fluid, or positive for IgG in serum. Older age was associated with lower odds for successful implantation (OR [95% CI]: 0.888 [0.820; 0.962]), biochemical pregnancy (OR [95% CI]: 0.890 [0.817; 0.969]) and live birth (OR [95% CI]: 0.906 [0.833; 0.985]). Our results suggest that the presence of chlamydial IgG and IgA anti-MOMP antibodies in serum and follicular fluid of infertile women could be indicative of lower IVF success rate, and that the advanced maternal age is associated with higher risk of IVF failure.

Keywords: *Chlamydia trachomatis*; IgG; IgA; age; IVF

1. Introduction

Infertility has been defined as a disease of the reproductive system, presented by failure to establish clinical pregnancy after one year of regular trying [1]. Affecting approximately 17.5% of the adult world population [2], it represents a significant medical, social and psychological burden at an individual level, and a major ongoing challenge for public health globally. Infertility affects both sexes, yet in women the problem is usually more complex, and often further aggravated by treatment

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invasiveness and its health, economic and career-related complications, as well as by frequent stigmatization and victimization in parenthood-oriented societies [3,4].

There are many causes of female infertility, such as older age, ovulatory disorders, uterine and tubal abnormalities [5–10], but the main preventable ones belong to sexually transmitted infections [3,11,12]. Among more than 30 different pathogens that are sexually transmissible [11], the most common bacteria worldwide is *Chlamydia trachomatis* (CT) [13]. This infection usually displays asymptomatic clinical course [14] that often hinders timely diagnosis, causing detrimental treatment delay: up to 45% of women with untreated CT infection develop complications such as pelvic inflammatory disease (PID) or tubal factor infertility (TFI), which dramatically increase the risk of both infertility and ectopic pregnancy [15–19]. To combat female infertility, different strategies have been developed, including lifestyle changes, controlled ovarian hyperstimulation with or without intrauterine insemination, operative hysteroscopy, but *in vitro* fertilization (IVF) has been associated with the highest live birth rates as compared to other methods [5,20].

Due to significant association of CT infection with female infertility [17,21], corresponding cervical screening has been strongly recommended as a prenatal test to be performed prior to initiating IVF, thus becoming a national standard of care in many countries [22]. However, the usefulness of serological and follicular fluid evidences of CT infection in predicting the outcome of IVF procedure has not been established yet. Therefore, the main objective of our study was to assess the potential association between serological and follicular fluid markers of CT infection and pregnancy or birth rates as indicators of IVF success.

2. Results

There were 121 patients with a median age of 35 (IQR: 32 – 39; range: 23 - 48) included in the study. After IVF procedure, at least one embryo was successfully implanted and biochemical pregnancy achieved in 55 (45.5%), with 51 of them (42.1% of the study population) giving birth to a living child or children. Women that achieved biochemical pregnancy had significantly higher number of oocytes retrieved and the number of embryos obtained ($U=1030.0$, $p<0.001$, and $U=704.5$, $p<0.001$, respectively), and the same was observed in those with successful delivery ($U=1083.0$, $p<0.001$, and $U=742.5$, $p<0.001$, respectively) (Table 1). Up to 3 embryos have been transferred per patient during IVF, with no significant difference in their number between those with successful and unsuccessful outcome, as assessed by both biochemical ($\chi2(3)=3.498$, $p=0.321$) and clinical indicators ($\chi2(3)=3.963$, $p=0.266$). The frequency of patients positive for IgG or IgA antibodies in serum or follicular fluid, as classified according to major IVF outcomes, is presented in Table 1.

Table 1. Age, IVF parameters, and CT antibody titers in subjects classified according to major indicators of IVF success.

		Biochemical pregnancy		Live birth	
		Yes	**No**	**Yes**	**No**
Age[1]		34.0 (30.0 – 37.3)	36.0 (33.0 – 40.0)	34.0 (30.0 – 36.3)	36.0 (33.0 – 40.0)
Number of oocytes[1]	**Retrieved**	9 (7 – 12)	6 (3 – 9)	9 (7 - 12)	7 (3 – 9)
	Obtained	6 (4 - 8)	3 (2 – 4)	6 (4 - 8)	3 (2 – 4)
	Transferred	2 (1 – 2)	2 (2 – 2)	2 (1 – 2)	2 (2 – 2)
	Implanted	1 (1 – 1)	0 (0 – 0)	1 (1 – 1)	0 (0 – 0)
IgG titer[2]	**Serum**	3 (5.7%)	11 (17.5%)	3 (6.1%)	11 (16.4%)
	Follicular fluid	2 (3.7%)	8 (12.3%)	2 (4.0%)	8 (11.6%)
IgA titer[2]	**Serum**	4 (7.4%)	8 (12.3%)	4 (7.8%)	8 (11.8%)
	Follicular fluid	1 (2.0%)	3 (4.8%)	1 (2.1%)	3 (4.5%)

[1] presented as median (IQR); [2] presented as number (frequency) of subjects.

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Comparison between patients in terms of IVF success rate revealed significant association between older age and lower odds of achieving both biochemical pregnancy (Table 2) and live birth (Table 3). As expected, older patients were also less likely to have successful implantation per embryos transferred (Table 4). Regarding antibodies titers in serum or follicular fluid, significant association with IVF outcome was not observed. Yet, there was a trend toward increased risk of IVF failure in patients positive for IgG in serum, or positive for either IgG or IgA in follicular fluid, which further bootstrapping analyses indicated as statistically significant (Tables 2 and 3).

Table 2. Summary of variable estimates using univariable logistic regression analysis related to biochemical pregnancy after IVF.

		Univariable logistic regression analysis						Bootstrapping analysis			
Variables		B	SE	Wald $\chi 2$	p	OR	95% CI[1]	B	SE	p	95% CI[2]
Age		-0.117	0.043	7.284	**0.007**	0.890	**0.817; 0.969**	-0.117	0.042	**0.005**	**-0.210; -0.043**
IgG	**Serum**	-1.260	0.681	3.426	0.064	0.284	0.075; 1.077	-1.260	4.188	**0.037**	-21.202; 0.021
	Follicular fluid	-1.294	0.813	2.532	0.112	0.274	0.056; 1.350	-1.294	6.688	0.062	-21.293; 0.092
IgA	**Serum**	-0.562	0.642	0.766	0.381	0.570	0.162; 2.007	-0.562	2.807	0.385	-2.416; 0.765
	Follicular fluid	-0.933	1.171	0.635	0.425	0.393	0.040; 3.901	-0.933	11.505	0.175	-21.329; 21.336
IgG or IgA	**Serum**	-0.874	0.564	2.403	0.121	0.417	0.138; 1.260	-0.874	1.563	0.114	-2.628; 0.160
	Follicular fluid	-1.550	0.800	3.756	0.053	0.212	0.044; 1.018	-1.550	6.442	**0.038**	**-21.263; -0.215**

B - the regression coefficient or estimate; SE - standard error; Wald $\chi 2$ - Wald test statistics for the degree of freedom of 1 (df = 1); p – probability; OR - odds ratio, calculated as exponent of B; 95%CI - 95% confidence interval; [1] - 95% CI for the estimated OR; [2] - percentile 95% CI for the estimated B; negative antibody titers were used as a reference category.

Table 3. Summary of variable estimates using univariable logistic regression analysis related to live birth after IVF.

		Univariable logistic regression analysis						Bootstrapping analysis			
Variables		B	SE	Wald $\chi 2$	p	OR	95% CI[1]	B	SE	p	95% CI[2]
Age		-0.099	0.043	5.327	**0.021**	0.906	**0.833; 0.985**	-0.099	0.042	**0.010**	**-0.193; -0.2029**
IgG	**Serum**	-1.103	0.681	2.621	0.105	0.332	0.087; 1.261	-1.103	4.081	0.057	-20.958; 0.048
	Follicular fluid	-1.147	0.814	1.985	0.159	0.318	0.064; 1.566	-1.147	6.810	0.095	-21.079; 0.314
IgA	**Serum**	-0.449	0.643	0.488	0.485	0.638	0.181; 2.249	-0.449	2.806	0.491	-2.219; 0.838
	Follicular fluid	-0.784	1.171	0.448	0.503	0.457	0.046; 4.530	-0.784	10.930	0.239	-21.131; 21.389
IgG or IgA	**Serum**	-0.751	0.564	1.771	0.183	0.472	0.156; 1.426	-0.751	2.003	0.159	-2.459; 0.229
	Follicular fluid	-1.391	0.800	3.022	0.082	0.249	0.052; 1.194	-1.391	6.866	**0.049**	**-21.146; -0.025**

B - the regression coefficient or estimate; SE - standard error; Wald $\chi 2$ - Wald test statistics for the degree of freedom of 1 (df = 1); p – probability; OR - odds ratio, calculated as exponent of B; 95%CI - 95% confidence interval; [1] - 95% CI for the estimated OR; [2] - percentile 95% CI for the estimated B; negative antibody titers were used as a reference category.

On the other hand, significantly higher embryos implantation rate was detected among patients with negative antibodies titers in follicular fluid. Subsequent bootstrap resampling confirmed the observed association, and additionally suggested positive IgG test in either serum or follicular fluid as predictive of lower implantation rate per embryos transferred (Table 4).

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Table 4. Summary of variable estimates using ordinal regression analysis related to EI/ET ratio after IVF.

		Univariable logistic regression analysis						Bootstrapping analysis		
Variables		B	SE	Wald $\chi 2$	p	OR	95% CI[1]	B	SE	95% CI[2]
Age		-0.118	0.041	8.495	**0.004**	0.888	**0.820; 0.962**	-0.118	0.040	**-0.211. -0.055**
IgG	Serum	1.308	0.681	3.688	0.055	3.698	0.973; 14.053	1.308	3.625	**0.239; 18.053**
	Follicular fluid	1.431	0.836	9.926	0.087	4.181	0.812; 21.535	1.431	5.426	**0.327; 18.073**
IgA	Serum	0.688	0.640	1.157	0.282	1.990	0.568; 6.977	0.688	2.520	-0.326; 2.525
	Follicular fluid	1.186	1.237	0.920	0.337	3.275	0.290; 36.991	1.186	7.558	-0.274; 17.107
IgG or IgA	Serum	0.909	0.557	2.663	0.103	2.481	0.833; 7.391	0.909	1.148	-0.055; 2.594
	Follicular fluid	1.659	0.819	4.104	**0.043**	5.254	**1.055; 26.152**	1.659	5.312	**0.522; 18.124**

EI/ET ratio - the implantation rate per embryos transferred; B - the regression coefficient or estimate; SE - standard error; Wald $\chi 2$ - Wald test statistics for the degree of freedom of 1 (df = 1); p – probability; OR - odds ratio, calculated as exponent of B; 95%CI - 95% confidence interval; [1] - 95% CI for the estimated OR; [2] - percentile 95% CI for the estimated B; positive antibody titers were used as a reference category.

3. Discussion

In the present study, detection of chlamydial IgG and IgA anti-MOMP antibodies in serum and follicular fluid of infertile women demonstrated potential to be indicative of lower IVF success rate, irrespective of maternal age. In the era of dramatic decline in fertility rates worldwide [23], when IVF represents the most efficient assisted reproductive technology (ART) method [5,20], and sexually transmitted infections list among the most recent WHO global research priorities [24], we trust our findings to at least partly contribute to this extremely important, yet still unresolved topic [25]. In addition, we confirmed advanced maternal age to be associated with higher risk of IVF failure, irrespective of CT infection. Witnessing a prominent societal shift toward delayed parenthood [26], with IVF featuring possibility of oocyte donation [27], we believe our modest contribution to further clarification of the importance of age for IVF outcome can help optimizing personalized fertility solutions.

In an attempt to conceive and deliver, women with CT infection face significant risk of failure, which calls for successful treatment as an important step in reaching the goal of child birth [28]. This could hold especially true in couples deemed infertile and preparing for IVF procedure, regardless of whether the infection represents the sole cause of infertility [29], or they also face other issues that hinder embryo implantation [5]. Unfortunately, not all forms of CT infections are easy to detect [30], so routine cervical screening often needs to be supplemented with other diagnostic methods to confirm or rule out the presence of this disease [31]. Among several options offered so far [32], CT antibody testing has been proposed as one of the most cost-effective screening methods for CT-associated genital tract pathology [33]. However, investigations of IVF outcome in relation to CT serological and follicular fluid markers yielded conflicting results [25,30].

The presence of CT-specific IgG and IgA antibodies in follicular fluid of infertile women undergoing IVF was first detected 35 years ago by Lunenfeld et al. [34]. Failing to demonstrate the link between CT antibodies at the site of fertilization and fertilization rate, they proposed transudation of immunoglobulins from blood serum into ovarian follicles, rather than the presence of CT itself within the oocyte or embryo, as the most probable explanation for their finding. Subsequent similar study by Neuer et al. [35] confirmed the lack of association between IgG in follicular fluid and IVF outcome, but observed increased risk of failure after embryo transfer in women positive for follicular fluid CT anti- MOMP IgA, indicative of more recent or persistent form of disease [30,34,36]. While adhering to initial hypothesis of antibodies entering by transduction from the circulation, they introduced another possibility of local production of IgA in response to viable CT present and/or replicating in follicular fluid macrophages [35]. Later investigation by Pacchiarotti et al. [30], involving women entering IVF after antibiotic treatment of CT infection that resulted in negative cervical swab, showed that almost half of the patients remained positive for IgA in both sera

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and follicular fluids in spite of being clinically cured. What is more, IgA-positive women, as compared to those tested negative, had significantly reduced number of mature oocytes, and half as high pregnancy and implantation rates. The authors hypothesized that the association between anti-CT antibodies and IVF outcome stems from persistent immune response against both bacterial and host antigens that results in low embryo quality, and suggests antibody detection rather than cervical swab test as a confirmation of CT treatment success [30]. On the other hand, in the study by Muller et al. [37] on patients diagnosed with TFI, despite of observed correlation between follicular fluid anti-CT IgG and IgA positivity and severity of pelvic adhesions, women positive for follicular fluid antibodies did not significantly differ from others in terms of IVF success rate. In our study, the presence of IgG or IgA in follicular fluid showed clear association with worse implantation rate per embryos transferred, as well as the tendency to link with lower rates of biochemical pregnancy and live birth after IVF. Bearing in mind our relatively small sample size and relying on an association detected through approximation to a wider population, we are prone to believe that the presence of anti-CT antibodies in follicular fluid can indicate higher risk of unfavourable IVF outcome, thus could be considered useful additional diagnostic test in patients undergoing laparoscopy due to mechanical infertility.

Yet, due to its invasiveness, the detection of anti-CT antibodies in follicular fluid has long been recognized as inappropriate for routine use [34], prompting search for other potential markers as a suitable alternative. As the earliest evidences of CT infection leading to tubal infertility were based on serological studies [38–40], the possibility that serum anti-CT antibodies could be linked to decreased fertilization rate has been extended to IVF research. The main focus was put on anti-MOMP [41] IgG and IgA, which have been described as indicative of past, and active or persistent CT infection, respectively [32,42,43]. However, the initial results were contradictory, ranging from complete lack of association between seropositivity and the outcome of IVF [39] to conclusions that the presence of IgG in serum decrease IVF success rate of IVF by half [42], or that high prevalence of both IgG and IgA correlates with low chances of biochemical pregnancies achieved by IVF being ended in “bringing home a baby” [43]. While some of the subsequent studies conformed with the latter [30], others failed to detect any significant association [35], and the proposed link between CT seropositivity and lower IVF success rate remained unconfirmed [25,44]. In the present study, we failed to observe the association of seropositivity for IgG or IgA with live birth rate after IVF. Yet, we noticed a tendency of women positive for serum IgG antibody to display lower implantation rate per embryos transferred and be less likely to achieve biochemical pregnancy, further supported by predictions drawn from larger dataset obtained by resampling from our population. Although our study lacks clear-cut conclusion in terms of IVF outcome, our results align with the most frequent hypothesis of CT infection affecting fertilization success by hindering embryo implantation, either due to endometrium being less receptive, or due to cross-reactivity between chlamydial and human antigens triggering autoimmune response [17,37].

Serological testing is far from ideal, mostly because of false positive and false negative results, low negative predictive value, as well as the lack of scientific consensus on which phase of CT infection course over time the positive test results actually reflect [45–49]. However, being simple and safe, it can complement patient selection and preparation for IVF by initial screening for indications for other more invasive diagnostic procedures [33,38]. In addition, previous reports of other CT-related antibodies associated with tubal pathology and/or affecting IVF pregnancy rate [46,49–51] introduce a possibility of several serological markers being integrated into a serological panel, which would be predictive of unfavourable IVF outcome with greater sensitivity and specificity [32,52].

On the other hand, the importance of maternal age for childbearing in general has always been considered a common knowledge [53]. Following introduction of ART, the available data supported apparent expectations, and the advanced age of a mother entering the procedure was deemed the most significant negative factor affecting IVF outcome [54]. However, when controlling for other relevant variables, it was revealed that maternal age associates with live birth rates and not with embryo implantation [55], suggesting that the age of the oocytes rather than the age of a bearer

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represent the true obstacle in achieving pregnancy in otherwise healthy mothers [56]. Delay of childbearing that became omnipresent during the past several decades [57] thus welcomed oocyte donation as a promising solution for women with "biological clock" ticking [58,59]. Unfortunately, even with the help of a donor, the risk of adverse pregnancy outcomes in women older than 45 remains controversial [60,61]. In the present study, where the oldest participant was 48 years old and all the oocytes used were autologous, advanced maternal age was associated with significantly lower implantation rate per embryos transferred, and lower odds of both achieving biochemical pregnancy and giving birth to a living child or children. The effect we observed was independent of CT infection and its consequences. Almost half a century after the first oocyte donation took place [62], we believe advanced maternal age in relation to IVF still deserves attention of researchers, and hope our observations, together with future studies, will help improving IVF success rate.

In conclusion, our results support potential role of serum and follicular fluid IgG and IgA antibodies, and confirm the importance of maternal age, in predicting the outcome of IVF. Considering several important limitations our study suffers from, including small sample size, analysing only anti-MOMP CT antibodies, and the lack of data on other significant factors that could (or have been shown to) affect IVF success rate, we encourage other researchers to challenge our results by additional investigations.

4. Materials and Methods

4.1. Study Design and Participants

This prospective, observational, multicentre cohort study included female patients undergoing IVF procedure at the Department of Medical Assistant Reproduction, University Clinical Centre Kragujevac, or at the Department of Artificial Reproductive Technology, The Obstetrics and Gynaecology Clinic "Narodni Front", Belgrade, Serbia. Study participants were recruited based on convenient sampling from hospitalized patients The main inclusion criteria aligned with the general eligibility requirements for IVF, as recommended by the European Society of Human Reproduction and Embryology (https://www.eshre.eu), and the Republic Fund of Health Insurance in Serbia (http://www.eng.rfzo.rs). In addition, study participants had to be free from active CT infection, as confirmed by negative polymerase chain reaction (PCR) analyses of endocervical and vaginal swabs. Patients younger than 18, those already participating in another study, and those that were not willing to give written informed consent for participation, were not included. The study conformed with the Declaration of Helsinki and the Good Clinical Practice, and was approved by the relevant Ethics Committees.

4.2. Data Collection and Analyses

Prior to entering the study, all study participants signed written informed consent form. IVF procedure at both study centres was performed according to the standard protocol. Serum and follicular fluid samples were collected during IVF procedure and stored at -20°C for subsequent analysis.

To determine the titers of IgG and IgA antibodies specific for CT major outer membrane protein (MOMP) antigen in serum and follicular fluid, EUROIMUN commercial ELISA test (Lubec, Germany) was used. According to the manufacturer's instructions, ELISA results for IgA were reported as negative for signal-to-cutoff (S/CO) ratio less than 0.8, borderline for values between 0.8 and 1.1, and positive for those higher than 1.1. Similarly, results of IgG test were considered negative, borderline, or positive if the obtained values of relative units (RU/ml) were less than 16, between 16 and 22, or above 22, respectively. In the present study, borderline cases of IgA and IgG were excluded from the analyses.

There were two primary outcomes in our study that served as major indicators of IVF success: a) biochemical pregnancy, defined as the serum β-HCG, measured 14 days after embryo transfer, higher than 25U/L, and b) live birth, i.e. giving birth to a living child or children after at least 28 weeks

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of pregnancy [63]. In addition, several other IVF parameters, including the number of oocytes retrieved, the number of embryos obtained, transferred, and implanted, were monitored, with the implantation rate per embryos transferred (EI/ET ratio) considered secondary outcome in the study also indicative of IVF success [64].

4.3. Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics, version 20 (IBM Inc, NY, US). Continuous data was tested for normality using Kolmogorov-Smirnov test, and presented as mean ± standard deviation (SD) or median with interquartile range (IQR), depending on the distribution. Categorical data were presented as count and percentage. The number of oocytes and embryos obtained and transferred in connection to IVF success was evaluated using Mann Whitney U or $\chi 2$ test. Potential association of antibody titers in serum and follicular fluid with indicators of IVF success, i.e. biochemical pregnancy, live birth rate and EI/ET ratio, was tested using univariable logistic or ordinal regression. Potential overestimation due to the relatively small sample size was corrected by internal validation of the model using bootstrapping analysis with 1000 bootstrap resamples. The strength of the observed association was presented as odds ratio (OR) with 95% confidence intervals, with the statistical significance level $p< 0.05$.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of University Clinical Center Kragujevac.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions, as they contain sensitive patient information.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ART	Assisted reproductive technology
B	The regression coefficient or estimate
CT	*Chlamydia trachomatis*
Df	Degree of freedom
IQR	Interquartile range
IVF	*In vitro* fertilization
MOMP	Major outer membrane protein
OR	Odds ratio
P	Probability
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
RU	Relative units
S/CO	Signal-to-cutoff
SD	Standard deviation
SE	Standard error

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TFI Tubal factor infertility
Wald $\chi 2$ Wald test statistics for df=1

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