UNIVERSITY OF MEDICINE AND FARMACY
„GR.T.POPA” IAŞI
FACULTY OF MEDICINE

PhD THESIS

EFFECT ON ENDOMETRIUM QUALITY IN INFERTILITY UTERINE INFECTIOUS ETIOLOGY. CORRELATIONS BETWEEN INFLAMMATORY FACTORS AND MICROBIAL AGENTS (*Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*).

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IAŞI, 2013
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The PhD thesis contains:

- 120 pages;
- 12 tables;
- 43 figures,
- 190 references;
- Notice of the Research ethics committees;
- List of project indicators;
- 3 original articles in extenso (1 B+ and 2 ISI).

This summary is kept the numbering of the thesis for the content, tables and figures.

**Keywords:** female infertility, *Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, infectious endometritis, cytokines.

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INTRODUCTION

In the last decade, infertility became an increasing pathology, among both women and men, and unfortunately, age at which it is diagnosed is continuously decreasing. One in four women is affected at some time, and approximately 20% of couples consult the general practitioner because of difficulty in conception of a child; among them, 10% requires specialized consultation.

10-15 years ago, women addressing to gynecological services for difficulty in conception of a child, were usually between the ages of 35-40 years, currently, the average of age decreased greatly, and the pathology due to sexually transmitted infections acquired a fairly large proportion. The chosen research topic responds to this problem pointing the importance of endometrial pathology by infectious origin as a causes of infertility.

Sexually transmitted diseases have also acquired a special scale, thanks to the modern concepts regarding sexual freedom and the absence of national screening programs. The incidence of genital infections with *Chlamydia trachomatis* and/or mycoplasmas reached an alarming rate among women of reproductive age, and patients presented late to consult a specialist, when the complications of pelvic inflammatory disease are already installed. Complication with the greatest impact on the women is the infertility and it has important consequences on family life, social integration and quality of life in general.

Is well known the connection between women’s genital infections and its effect on reproductive function. The most
studied genital infection was with *Chlamydia trachomatis*, especially the presence of high titers of antibodies in the serum of patients, correlated with tubal damage.

For a long time, mycoplasmas have been considered commensal of the urogenital tract, with no particular importance, but, multiple studies demonstrated that their presence at this level can cause significant disturbances in the vaginal flora, with the possibility of ascension and the generation of sequelae, among which the most frequently is infertility.

With the development and improvement of human assisted reproduction techniques, more and more specialists, given increasing importance to the quality of the endometrium, which is essential for embryo implantation and maintenance of pregnancy.

The normal reactions of the cell-mediated immunity plays an essential role in the preparation of the endometrium throughout the menstrual cycle for embryo implantation and maintenance of pregnancy. Cytokine secretion is strictly controlled in numerous interrelated reactions and any disturbance, even minor, results in the failure to obtaining pregnancy. Currently there are many studies that have shown that genital infections cause disturbance reactions of cell-mediated immunity through exacerbation or cytokine secretion deficiency, which disrupts their normal balance to the endometrium, and the result is infertility.

Currently there are various protocols for the investigation of infertility, but all of it includes investigation of tubal patency and uterine cavity. Uterine and tubal assessment can be performed using histerosalpingography, histerosonography and hysteroscopy. Hysteroscopy is considered the „gold standard” method for the diagnosis / treatment of enduterine pathology, because it allows direct visualization of the uterine cavity and
tubal ostiums, and it is a minimally invasive procedure that can be performed in ambulatory, even with the local anesthesia. Endometrial biopsy performed during hysteroscopy is extremely valuable as it allows investigation of the uterine lining both morphologically and functionally.

Because of increasingly high proportion of infertility cases reported, both in our country and worldwide, it is necessary the reassessment of the investigation protocols of infertile couple and implementation of national screening programs for sexually transmitted diseases.

**Objectives**

The main objective of the research in this thesis, is to identify a causal relationship between genital infections with *Chlamydia trachomatis* and/or mycoplasmas (*Mycoplasma hominis, Ureaplasma urealyticum*) and impaired quality of endometrium for patients with primary or secondary infertility.

The secondary objective, but no less important, is the way in which these genital infections affecting cell-mediated immunity. The research focused on identifying of local inflammatory factors (cytokines) which are capable of supporting endometrial inflammation and thus interfere with it’s normal function, with difficulties in obtaining and maintaining a pregnancy. Achieving this objective would allow early diagnosis of pelvic inflammatory disease, with the rapid establishment of the proper treatment and prevention of sequelae, especially those generating infertility.
The ultimate goal of the research is to develop a protocol for diagnosis and treatment of these genital infections, which prevent the most redoubttable complications of pelvic inflammatory disease - infertility.

The general part of the thesis is structured in six chapters:

Chapter I summarizes some notions about embryology, morphology and physiology of the endometrium and endometrial pathology found in women of reproductive age (endometrial polyp, endometrial hyperplasia, uterine synechiae and infectious endometritis).

Chapter II presents some notions about female infertility and here are exposed the newest investigative protocols and regimens based on identified pathology.

Chapter III and IV are dedicated to genital infections with Chlamydia trachomatis and mycoplasmas (Mycoplasma hominis and Ureaplasm a urealyticum). Here were described etio-pathogenic mechanisms of pelvic inflammatory disease caused by genital infection with these microorganisms, disease progression and the adverse effects on the reproductive potential of women. Were also exposed the new ways of diagnosis and treatment of genital infections and pelvic inflammatory disease.

Chapter V of the general part, systematize recent data from the literature about the relationship between inflammatory markers (cytokines), genital infections with Chlamydia trachomatis, Mycoplasma hominis, Ureaplasm a urealyticum and infertility.
In the sixth chapter is presented hysteroscopy as a minimally invasive method of diagnosis and treatment of endouterine pathology, and practical applicability, especially in the field of infertility. Also, the technique described endometrial biopsy per-hysteroscopy and the enormous benefits of such an investigation.

The experimental part of the thesis includes four studies that aimed at achieving the objectives in doctoral research:

The first two studies relate the prevalence of genital infections with *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis* for patients with infertility. Identification of genital microorganisms was performed using Real Time PCR method, that consists of detecting of bacterial DNA by direct amplification of specific regions of the genome. Detection and quantification of the resulting product is achieved in real time. This provides high specificity (98%) and sensitivity (95%) method and the results are ready in a very short time (2 hours). Our results are comparable with the literature and the detection of antibodies for *Chlamydia trachomatis* by ELISA method, has proved to be very helpful for staging pelvic inflammatory disease and guide the clinician to further investigations.

Materials and methods

Investigated patients (n = 176) are aged between 21 and 40 years (mean 30.5 years) and 79 patients were diagnosed with primary infertility (44.88%) and 97 patients with secondary infertility (55.12%). Most patients (84%) came from urban
areas. Infertility time is between 1 year and 9 years (median 4 years). From all the patients were collected biological samples for identification of microbial agents (endocervical samples for detecting *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis*).

We used the following kits:

- **Chlamydia trachomatis** Quant Real-TM (Sacace Biotechnologies, Italia).
- **Ureaplasma species** Quant Real-TM (Sacace Biotechnologies, Italia);
- **Mycoplasma hominis** Quant Real-TM (Sacace Biotechnologies, Italia).

The samples were processed with *Applied Byosystem 7300 RT PCR* (Applera, USA).

The detection of antibodies for *C. trachomatis* was performed using ELISA method (Enzyme Linked Immuno Assay) and the kit- *Nova Lisa Novatec* (Immunodiagnostic GmbH, Germany).

After the identification of the strains of mycoplasma in the positive samples, was performed sensitivity spectrum on 9 antibiotic for each strain separately using microplate *Mycoplasma IST2* (bioMérieux, France).

**Results and Discussion**

From the 176 samples tested, *C. trachomatis* - Ag was detected only in three patients (1.7%); 124 samples (70.4%) were negative for all three kinds of *C. Trachomatis* antibodies (IgA, IgM și IgG); the remaining 52 samples (29.6%) were positive for one or two types of antibodies (Fig.II.3.6.). There was no sample with all three types of antibodies.
Following serological determinations, we found that the highest prevalence have Ig G (43 cases - 24.43%), with the predominance in the patients of older age and urban area of origin, between these dates there is a significant correlation (p <0.001) (Table.II.3.II.).

Table II.3.II. The risk factors correlated with infertility

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>OR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT IgG</td>
<td>0.151</td>
<td>0.015</td>
</tr>
<tr>
<td>Age</td>
<td>0.183</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Living area</td>
<td>5.437</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Fig. II.3.6. The distribution of Ab and Ag for *Chlamydia trachomatis* in the studied group
Identification of the genital infection with the Mycoplasma hominis and Ureaplasma urealyticum:

From 176 cases examined, 104 samples (59%) were negative, and 72 samples (41%) were positive. The distribution of both microorganisms of positive samples was as follows (Fig.II.4.3.)

- only *Mycoplasma hominis* – 2 samples (1,13%);
- only *Ureaplasma urealyticum* – 57 samples (32,38%);
- *Mycoplasma hominis* + *Ureaplasma urealyticum* – 13 samples (7,38%).

*Fig. II.4.3. The percentage distribution of M.hominis and U.urealyticum in the study group (Real Time PCR)*

In the group of infertile patients the features of the infection with the mycoplasma are:
- average of age 32.4 years for *Mycoplasma hominis* and 31.9 years for *Ureaplasma urealyticum*;
- much larger frequency of the strains of *Ureaplasma urealyticum* opposed the *Mycoplasma hominis* (39.8% versus 7.4%);
- infection with the mycoplasma were more common in the age group > 25 years;
- the greater frequency in the patients from rural areas (Table.II.4.I.).

Table II.4.I. Distribution of micoplasmas infection

<table>
<thead>
<tr>
<th></th>
<th><em>M. hominis</em> negative</th>
<th><em>M. hominis</em> positive</th>
<th><em>U. urealyticum</em> negative</th>
<th><em>U. urealyticum</em> positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>The average age (years)</td>
<td>31,3</td>
<td>32,4</td>
<td>31</td>
<td>31,9</td>
</tr>
<tr>
<td>Age &lt;25 years</td>
<td>18</td>
<td>1</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>94,7%</td>
<td>5,2%</td>
<td>68,4%</td>
<td>31,6%</td>
</tr>
<tr>
<td>Age &gt;25 years</td>
<td>145</td>
<td>12</td>
<td>93</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>92,4%</td>
<td>7,6%</td>
<td>59,2%</td>
<td>40,7%</td>
</tr>
<tr>
<td>Urban</td>
<td>140</td>
<td>8</td>
<td>91</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>94,6%</td>
<td>5,4%</td>
<td>61,5%</td>
<td>38,5%</td>
</tr>
<tr>
<td>Rural</td>
<td>23</td>
<td>5</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>82,1%</td>
<td>17,9%</td>
<td>53,6%</td>
<td>46,4%</td>
</tr>
<tr>
<td>Totally</td>
<td>163</td>
<td>13</td>
<td>106</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>92,6%</td>
<td>7,4%</td>
<td>60,2%</td>
<td>39,8%</td>
</tr>
</tbody>
</table>

Regarding the spectrum of antibiotic sensitivity for mycoplasma’s strains identified, our results are in accordance with the literature [Uuskula, Kohl, 2002] for the first 7
antibiotics (Pristinamycin, Josamycin, Clarithromycin, Azithromycin, Tetracycline and Doxycycline), instead the discrepancy is greater for fluoroquinolones (Ciprofloxacin and Ofloxacin). The resistance rate is considerably higher than the other comparative studies (CIP: 51% for *U.urealyticum* and 73% for *M.hominis*; OFL: 17% for *U.urealyticum* and 27% for *M.hominis*), probably due to uncontrolled administration of antibiotics, insufficient duration of treatment (less than 5 days), but also due to preferential use of fluoroquinolones (mainly Ciprofloxacin) without documentation of infection.

**Fig. II.4.4. Antibiotic sensitivity of *Ureaplasma urealyticum* strains**

We noticed that the spectrum of antibiotic sensitivity for *Ureaplasma urealyticum* and *Mycoplasma hominis* strains varies greatly from one country to another, and probably due to several local factors, such as circulating serovars in the population, the most commonly used antibiotics for various conditions, the addressability of the population to medical services, and not the least, the release regime of antibiotics in pharmacies.
In the second study we tried to establish a correlation between the presence of genital infection with *Chlamydia trachomatis* and/or mycoplasmas and the presence of chronic endometritis.

**Materials and methods**

Initial group of patients (n = 176) were subdivided into two groups: group 1 - patients with genital infections with *Chlamydia trachomatis* and/or mycoplasmas (n1 = 101) and group 2 - patients with all negative microbiological determinations (n2 = 75). For all patients was performed diagnostic hysteroscopy and uterine cavity, tubal ostiums and the uterine lining were evaluated. Device being used is Karl Storz Endoskope (Germany, 2008), hysteroscopy were performed with rigid hysteroscope with dual flow working channel, telescope diameter of 2.7 mm, with 6.5 mm sheath and
30° visual angle. During the procedure was taken a sample of endometrial tissue that was sent for anatomo-pathological examination. The diagnosis of chronic endometritis was established accordance with the conventional criteria, namely, identifying the endometrial stroma on at least 5 PMN / field (at 400x magnification) and on at least one plasma cells / field (at 100x magnification) [Wiesenfeld, et al, 2002].

Results and Discussion

In case of endometritis, the detected aspect of diagnostic hysteroscopy can take many forms: sometimes we observe numerous diffuse or localized hemorrhagic focal points (Fig.II.5.4.), other times, the diagnosis is suggested by indirect signs (friable uterine lining, blocked tubal orifices, small membranous synechiae).

Fig. II.5.4. Chronic endometritis - hysteroscopy aspect (our clinic archive)

According to the anatomo-pathological results was demonstrated that infectious endometritis is present in a much
higher proportion in the group of patients with genital infections (28% vs 5%).

Table II.5.I. Distribution endouterine pathology at the 2 groups

<table>
<thead>
<tr>
<th>Microbiology</th>
<th>Normally</th>
<th>chronic endometritis</th>
<th>Other pathologies (polyps, synechiae, fibrom)</th>
<th>Totally</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>41</td>
<td>28 (27,7%)</td>
<td>32 (31,68%)</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>(40,59%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>42 (56%)</td>
<td>4 (5,33%)</td>
<td>29 (38,67%)</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>totally</td>
<td>83</td>
<td>32</td>
<td>61</td>
<td>176</td>
</tr>
</tbody>
</table>

More frequently, chronic endometritis was diagnosed in the patients with genital infections with *Chlamydia trachomatis* and / or mycoplasmas, compared with patients with no genital infection; between the two groups there is a significant difference (*p* = 0,0012). Much greater frequency of endometritis at infertile patients with genital infections with *Chlamydia trachomatis* and / or mycoplasmas suggests a possible causal relationship between the presence of these organisms in the female genital tract and the damage of uterine lining in pelvic inflammatory disease.
The third study is the new element and originality of this thesis, because it brings a new technology for analysis - Biochip Array Technology - BAT.

Materials and methods

Biochip is a solid substrate, chemical functional of 9x9 mm, which facilitates the spatial disposing of the catch agents in distinct test areas. Analytes in the sample are bound of complementary polyclonal antibody to the biochip surface; the technology is based on traditional principles of ELISA method. For the interpretation of the results was used Evidence Investigator device.

![Scheme of working method for the quantification of cytokines](image)

**Fig. II.6.4.** Scheme of working method for the quantification of cytokines
Quantitative determinations of cytokines have been performed on the analyzer Evidence Investigator (Randox Laboratories, UK), which is able to determine 12 cytokines simultaneously from the same sample. The system uses the basic immune principles: competitive sandwich method is based on the capture of monoclonal antibody (on the principle of ELISA). The detection method is enzymatically induced chemiluminescence (with peroxidase) using a CCD camera (Charge Coupled Device) with high sensitivity. The great advantage of Biochip method is that it not requires further processing of the samples, avoiding degradation through manipulation and the results are obtained simultaneously from the same sample in a short time.

This is a comparative study; the study group is represented by the 28 patients diagnosed with chronic endometritis and positive microbiological determinations. The control group is formed from the other 28 infertile patients with normal results at hysteroscopy examination and all microbiological determinations negative. The samples were represented by endocervical cells, that were initially properly processed, then have been made simultaneous determinations for 4 inflammatory markers: IL-1β, IL-10, TNF-α and IFN-γ.

Results and Discussion

In the study group (patients with infectious endometritis) was observed higher values of inflammatory markers, with statistically significant differences compared to the control group (p < 0.001).
### Table II.6.III. The average values of cytokines for the two groups

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Control group</td>
<td>28</td>
<td>2,245000</td>
<td>1,1067252</td>
</tr>
<tr>
<td></td>
<td>Study group</td>
<td>28</td>
<td>116,183571</td>
<td>128,8787912</td>
</tr>
<tr>
<td>IL-10</td>
<td>Control group</td>
<td>28</td>
<td>4,758571</td>
<td>2,6101209</td>
</tr>
<tr>
<td></td>
<td>Study group</td>
<td>28</td>
<td>387,996786</td>
<td>304,6235459</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Control group</td>
<td>28</td>
<td>15,070357</td>
<td>7,3413136</td>
</tr>
<tr>
<td></td>
<td>Study group</td>
<td>28</td>
<td>466,442143</td>
<td>469,1413002</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Control group</td>
<td>28</td>
<td>3,387143</td>
<td>2,0983801</td>
</tr>
<tr>
<td></td>
<td>Study group</td>
<td>28</td>
<td>95,240714</td>
<td>109,5715921</td>
</tr>
</tbody>
</table>

Finally, we analyzed the distribution of the four cytokines for each type of genital infection, at the patients from the group with chronic endometritis, and we obtained the following results:

► for patients with *Chlamydia trachomatis* infection, the highest values were obtained for IFN-γ and IL-10, TNF-α registered a moderate increase and IL-1β had normal values (Fig.II.6.16).

► for patients with genital infection with mycoplasmas (*Mycoplasma hominis* and / or *Ureaplasma urealyticum*), the highest values were for IL-1β then IL-10 and TNF-α with similar levels and the lowest values were recorded for IFN -γ (Fig.II.6.17).

► for patients with mixed infections (*C.trachomatis* + mycoplasmas) were detected high levels of all cytokines, but IFN-γ and IL-10 showed higher growth. IL-1β and TNF-α registered a moderate growth (Fig.II.6.18).
Fig. II.6.16. Distribution of IL-1β, IL-10, IFN-γ and TNF-α for patients with *Chlamydia trachomatis* infection

![Bar chart showing distribution](image1.png)

Fig. II.6.17. Distribution of IL-1β, IL-10, IFN-γ and TNF-α for patients with mycoplasmas infection

![Bar chart showing distribution](image2.png)
General conclusions

Pelvic inflammatory disease (PID) is one of the most common infections diagnosed in women of reproductive age and still remains one of the most important public health problems in developed countries. Chlamydia trachomatis is the main etiologic agent identified in approximately 40% of cases of pelvic inflammatory disease. Following the microbiological investigations performed in the protocol for infertility, we noticed a strong association between genital infection with Chlamydia trachomatis and the presence of pelvic inflammatory disease. All patients diagnosed with pelvic inflammatory disease...
have been presented for a specialist consult because of difficulties in obtaining a pregnancy and not for the specific symptoms of the disease. This demonstrates once again the silent evolution infections and it’s immense destructive potential. Most cases of infertility (about 89%) are situated in between the age 25-40 years, 84% from urban origin. Both patients <25 years and > 25 years predominated chronic infection with *Chlamydia trachomatis* without statistically significant differences between the two categories. In all cases, patients with positive samples in urban areas, are about 5 times higher than in rural areas. *Chlamydia trachomatis* antigen was identified in 1.7% of patients (3/176).

We identified antibodies IgA, IgM and IgG with the ELISA method in the serum of patients. The highest prevalence have type G immunoglobulins (43 cases - 24.43%), with predominance in the patients of older age and urban origin between these dates there is a statistically significant correlation ($p<0.001$).

Detection of *Chlamydia trachomatis* antigen in cervical secretion is not intended to be used as the sole method of diagnosis for the presence of pelvic infection because isn’t gives us any indications of the type of infection and its age. Performing serology for *Chlamydia trachomatis*, the identification of three types of antibodies, provides diagnostic value, allowing the clinician to stage the disease and guide the following investigations based on it. IgG antibodies are the marker previous exposure to microbial agent having quite high predictive value for the presence of pelvic inflammatory disease.

We found that the rate of genital mycoplasma infection in infertile patients is quite high (41%), with predominance for *Ureaplasma urealyticum* (32.28%) and the combination of the two microorganisms was detected in 7.38% of patients. Most of
the patients with positive microbiology for mycoplasmas were older than 25 years (average of 32 years), they are from rural areas and have a history of significantly fewer pregnancies, more abortions, births than patients with negative microbiology.

Regard the antibiotic susceptibility, Mycopasma hominis strains present resistance of 18% for OFL and 51% for CIP and t Ureaplasma urealyticum strains 27% for OFL and over 60% for CIP. The resistance rate is considerably higher than the other comparative studies, probably due to uncontrolled administration of antibiotics, insufficient duration of treatment (less than 5 days) and due to preferential use of fluoroquinolones (mainly ciprofloxacin) without documentation of infection and antibiogram.

Chronic endometritis was diagnosed more frequently in the patients with genital infections with Chlamydia trachomatis and / or mycoplasmas against the patients who these microorganisms were not identified, between the two groups there is a statistically significant difference (p = 0.0012). Share of total cases of chronic endometritis at infertile patients is approximately 16%, a value that aligns with those obtained in other studies, but at lower limit.

The values of all 4 cytokines (IL-1β, IL-10, TNF-α and IFN-γ) are higher at the patients with chronic endometritis and with positive microbiological samples, compared to controls (p < 0.001).

IL-10 has been identified constantly with high values in all patients with genital infections and chronic endometritis compared with the controls, so it seems that it is the most reliable inflammatory marker that correlated with chronic inflammation at the infertile patients.

At Women of reproductive age, quality, integrity and normal functioning of the endometrium is essential for
implantation, growth and development of the embryo, and to maintain pregnancy. Also, in cases where it is necessary to obtain a pregnancy through assisted human reproduction technique, the endometrium is extremely important for the success of the procedure. In these situations it may be useful endocervical dosage of cytokine in order to detect early any change and increase the chances of success of the procedure.

Considering that in our country there is no screening protocols for genital infections with these organisms, this research can be very helpful for develops strategies for early diagnosis and treatment of genital infections to prevent pelvic inflammatory disease and its sequelae installation, especially infertility.
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