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# Role of TH-I based Endometrial Cytokines in Latent Genital Tuberculosis and Endometriosis Causing Infertility

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## Abstract

The aim of the present study was to find out a correlation of various inflammatory cytokines in infertile patients who either were diagnosed to have Latent Genital Tuberculosis (LGTB) or endometriosis or both. This can further facilitate the diagnosis and treatment of these patients. From January 2019 to January 2020, endometrial samples were collected from 150 patients between 20-35years of age, who had primary infertility due to LGTB or endometriosis or both as the causative factors. Patients were grouped into three categories: Category A (n-50): who were diagnosed to have LGTB only; Category B (n-50): who were diagnosed to have endometriosis and LGTB both and Category C (n-50): who had endometriosis only. DNA-PCR study and cytokine assay were carried out on these samples.

We had detected different levels of interleukins-IL-1$\beta$, IL-2, IL-6, IL-10, IL-13, IL-12, IFN-$\gamma$ and TNF-$\alpha$ in the samples. The mean value of all inflammatory cytokines were increased from normal range for category A. In case of category-B, only four inflammatory cytokines (IL-6, IL-12, IFN-$\gamma$, TNF-$\alpha$) were increased from detectable range. The mean value of IL-6, IL-12 and TNF-$\alpha$ were increased for category-C but IFN-$\gamma$ was in normal range. Moreover, TNF-$\alpha$ has no significant different average values for all three categories of patients. Both anti inflammatory cytokines IL-10 and IL-13 have almost same average value for all three categories of patients. Endometrial cytokine assay can be used as a screening method to identify the latent genital tuberculosis, endometriosis or both in infertile women.

**Keywords: Latent genital tuberculosis; Endometriosis; Infertility; Endometrial cytokine assay**

## Abbreviations

LGTB: Latent Genital Tuberculosis; IL: Interleukin; IFN: Interferon; TNF: Tumor Necrosis Factor; TST: Tuberculin Skin Test; MTB: *Mycobacterium tuberculosis*; IGRA: Interferon Gamma Release Assay; PCR: Polymerase Chain Reaction; ATD: Anti-Tubercular Drug

## Introduction

Endometriosis is an enigmatic disease. It might be asymptomatic in some whereas others present with severe pain during menstruation or infertility. Factors causing infertility in women with endometriosis may range from anatomical distortions due to adhesions and fibrosis to endocrine abnormalities and immunological disturbances. The overall prevalence of endometriosis in population-based studies varies from 0.8% to 6%; however, in case of associated infertility, the prevalence seems to be considerably higher, ranging from 20% to 50%, but with significant variation over time periods and the age of patients [1-5]. In a large cohort of women of reproductive age, the risk of infertility was increased two-fold in women <35 years with endometriosis compared with women without endometriosis [6]. Endometriosis is therefore a frequent cause of infertility, either by itself or in association with other fertility-reducing factors.

Latent Genital Tuberculosis (LGTB) is a major cause of infertility in women, and prevalence is generally underestimated because of the asymptomatic nature of the infection and diagnostic challenges. Almost 5-13% of infertile patients in the age group of 20-40 years, in our country have been diagnosed with LGTB [7]. Diagnosis is based on a positive Tuberculin Skin Test (TST), a delayed hypersensitivity reaction to the purified protein derivative of MTB or Interferon Gamma Release Assay (IGRA), a T-cell response to MTB-specific antigens and endometrial cytokines. It is believed that MTB remains viable in people with latent infections, not causing disease but maintaining its potential to do so [8]. Early diagnosis and correct treatment is vital to avoid complications and to

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restore fertility.

In the present study different interleukins, interferon and tumour necrosis factor were studied to find out a correlation of these inflammatory cytokines in infertile patients affected with LGTB or endometriosis or both. We had detected different levels of IL-1$\beta$, IL-2, IL-6, IL-10, IL-13, IL-12, IFN-$\gamma$ and TNF-$\alpha$ and statistically analysed. Hence endometrial cytokine assay can be used as a diagnostic screening method to identify the latent genital tuberculosis, endometriosis or both in infertile women affected by these conditions.

## Materials and Methods

A total of 260 infertile patients were referred to reproductive medicine unit of Calcutta fertility mission, over a period of January 2019 to January 2020. The data were collected from a total of 150 patients as cases between 20-40 years of age, who had primary infertility due to LGTB or endometriosis or both as the causative factors. Patients were grouped into three categories:

Category A (n-50): who were diagnosed to have LGTB only.

Category B (n-50): who were diagnosed to have, endometriosis and LGTB both.

Category C (n-50): who had endometriosis only.

### Inclusion criteria

Only infertile subjects within reproductive age group (20-40 years), had diagnosed lgtb by DNA-PCR and endometriosis by clinical symptom and history and both were considered.

### Exclusion criteria

Previous intake of ATD, previously corrected endometriosis or any such pains in pelvic region were excluded from this study. Other than any additional criteria have been taken.

### Consent and ethical clearance

Written informed consent of the study subject was also obtained prior to sample collection. The Ethical Committee of Institute of post graduate medical education and research ( I.P.G.M.E & R) and Calcutta fertility mission have given clearance for the retrospective study.

### Sample collection for DNA

Study subjects of three categories were also advised to attend the institute in the 2nd day of her menstrual cycle for menstrual blood collection for DNA-PCR. Those patients' endometrial aspirates were taken between 21st to 23rd day of the same cycle for DNA-PCR also. Two types of samples of each patient, were used for DNA-PCR to increase accuracy rate. After collecting the menstrual blood and endometrial aspirate, the contents were transferred to the lysis buffer as described previously in detail (Datta et.al., 2019) and stored it for DNA extraction. Then DNA was extracted from menstrual blood sample using Qiagen DNA mini kit (Qiagen, Hilden,Germany). Then eluted DNA sample was preserved in -200 for polymerase chain reaction.

### Sample collection for cytokine

In the same day of endometrial sample collection, patients were advised for lying in lithotomy position under aseptic precaution, an autoclaved speculam was placed on the vagina for visualizing the external os of the cervix. Then cotton were used to clean the cervix. A 2 ml syringe was connected with embryo transfer cannula and was allowed to pass through the external os without touching vulva or vagina. Suction was gradually applied by syringe. At the time of removal to avoid the contamination of cervical mucus, the outer sheath of cannula was kept in the cervix and inner catheter was then withdrawn through the outer sheath. Aspiration was transferred to sterile cryovial and placed as soon as possible into the -800.

### Multiplex PCR

PCR was carried out using extracted DNA sample. We used three different pair of primer for simultaneously detection of three target region of the Mycobacterium and non tubercular mycobacterium genome in a single reaction tube. Master mix preparation procedure, three set of primer and condition of PCR has been described in details (Bhattacharya et.al., 2001).

### Cytokine assay

Endometrial secretions were analyzed using commercially available solid phase sandwich Enzyme Linked Immune Sorbent Assay (ELISA) for detecting 8 soluble mediators from Diaclone, SAS, France. In our study we used eight inflammatory cytokines and two anti-inflammatory cytokines who are directly involve in embryo implantation or rejection process. All cytokines were eligible for inclusion in the panel where monoclonal antibody were available.

### Statistical analysis

Data were presented as mean±SD for continuous variables according to the distribution of variable. If the distribution was symmetric, mean±SD was used to describe the continuous variable. Histogram was used for graphical presentation of continuous one way ANOVA was used for comparisons of different measures.

## Result

A total 150 patients samples were included for endometrial cytokine assay. They were divided into three categories, as discussed in materials and methods section. We have performed six inflammatory mediators and two anti-inflammatory mediators for assay. Descriptive statistics showed in Table 1, revealed that mean value of all inflammatory cytokines were increased from normal range for category–A. In case of category-B, only four inflammatory cytokines (IL-6, IL-12, IFN-$\gamma$, TNF-$\alpha$) were increased from detectable range. Interestingly we observed, mean value of IL-6, IL-12 and TNF-$\alpha$ were increased for category-C but IFN-$\gamma$ was in normal range. Moreover, TNF-$\alpha$ has no significant different average values for all three categories of patients. Both anti inflammatory cytokines IL-10 and IL-13 have almost same average value for all three categories of patients.

As per manufacturer instruction, detectable range of cytokines were IL-1$\beta$<6.5 pg/ml, IL 2<7.0 pg/ml, IL 6<2.0 pg/ml, IL-10<5.0 pg/ml, IL-12<20.0 pg/ml, IL-13<3.0 pg/ml, IFN-$\gamma$<5.0 pg/ml, TNF-$\alpha$ < 8.0 pg/ml.

Following the ANOVA result, we have found that average values of IL-1β, IL-2, IL-6, IL-12, IFN-γ among the pro inflammatory cytokines were significantly differ among the three categories of patients at 1% level of significance. Among the anti-inflammatory cytokines only average values of IL-10 was significantly differ among the three categories of patients at 5% level of significance. From pro-inflammatory group average value of TNF-α and under antiinflammatory group average values of IL-13 were not significant among the three categories of patients. Figure 3-10 accordingly

**Table 1:** Distribution of descriptive statistics among three categories of patient.

| Cytokine | Pcr+ve without endometriosis(category–A) | | | pcr+ve with endometriosis(Category -B) | | | pcr-ve with endometriosis(Category-C) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Range | Mean | SD | Range | Mean | SD | Range |
| IL-1β | 138.95 | 27.54 | 102.30 | 4.35 | 1.94 | 6.90 | 4.35 | 1.94 | 6.90 |
| IL-2 | 14.37 | 2.46 | 9.40 | 5.33 | 1.78 | 8.70 | 5.33 | 1.78 | 8.70 |
| IL-6 | 231.07 | 30.79 | 155.86 | 123.03 | 35.84 | 157.42 | 40.71 | 26.34 | 83.67 |
| IL-10 | 6.31 | 2.87 | 11.50 | 6.31 | 2.87 | 11.50 | 4.43 | 1.84 | 7.10 |
| IL-13 | 1.32 | 0.89 | 3.20 | 1.32 | 0.89 | 3.20 | 1.32 | 0.89 | 3.20 |
| IL-12 | 46.29 | 24.74 | 93.60 | 33.09 | 19.92 | 57.70 | 16.57 | 4.59 | 20.40 |
| IFN-γ | 21.98 | 8.36 | 30.50 | 20.18 | 7.34 | 30.50 | 2.90 | 1.21 | 4.90 |
| TNF-α | 64.81 | 38.34 | 183.60 | 56.91 | 26.26 | 85.20 | 56.91 | 26.26 | 85.20 |

**Table 2**: Distribution of ANOVA result of various cytokines.

| Parameter | F value | *P*-value |
|---|---|---|
| IL-1β | 591.43 | 0.000*** |
| IL-2 | 164.49 | 0.000*** |
| IL-6 | 233.60 | 0.000*** |
| IL-10 | 4.46 | 0.015** |
| IL-13 | 0.00 | 1.000* |
| IL-12 | 16.15 | 0.000*** |
| IFN-γ | 66.43 | 0.000*** |
| TNF-α | 0.55 | 0.581* |

*** significant at 1%, ** significant at 5%, * not significant

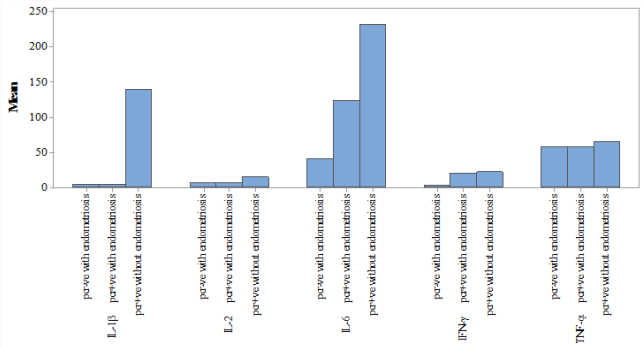


**Figure 1:** Distribution the mean value of IL-1β, IL-2, IL-6, IFN-γ and TNF-α as a bar diagram.

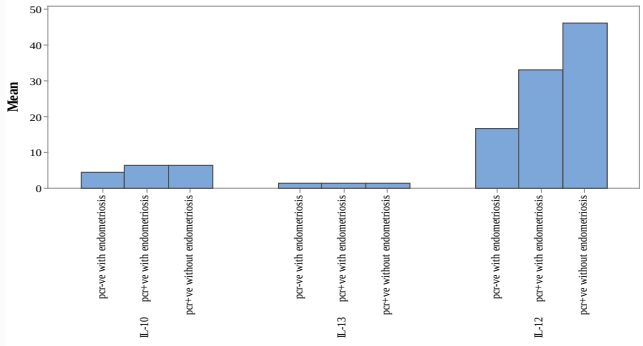


**Figure 2:** Distribution the mean value of IL-10, IL-12, and IL-13 as a bar diagram.

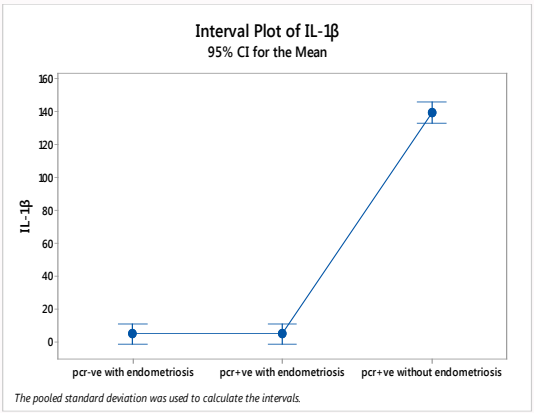


**Figure 3:** Distribution of 95% confidential 95% confidential interval plot of IL-1β.

showed 95% confidential interval plot of eight different cytokines against three categories.

## Discussion

Clinical experience suggests that there is an association between the extent of disease and the degree of reduced spontaneous fertility in endometriosis, although the strength of this association is variable [9]. Among women with minimal/mild endometriosis, approximately 50% will be able to conceive without treatment, whereas in women with moderate disease, only 25% will conceive spontaneously, and few spontaneous conceptions occur in the case of severe disease [10]. Endometriotic peritoneal implants induce an acute inflammatory reaction, which is associated with recruitment and activation of T helper and regulatory T (Treg) cell subsets. After resolution of the acute phase, monocytes/macrophages maintain a chronic inflammation contributing to adhesion formation and angiogenesis [11].

According to previous literature, increased concentration of Interleukin-1b (IL-1b), IL-8, IL-10 and tumor necrosis factor-α in follicles adjacent to endometriomas has been associated with reduced ovarian response [12]. The level of IL-6 in peritoneal fluid from women with endometriosis has been documented to be elevated and this cytokine may inhibit sperm motility [13,14]. Similarly in our study women with endometriosis has high level of IL-6 and TNF-α but IFN-γ was in normal range. These inflammatory mediators also contribute to sperm DNA fragmentation [15]. Moreover oxidative stress, prostaglandins and cytokines may interfere with oocyte–sperm interactions, impair embryo development and hinder implantation [16]. The level of pro- inflammatory cytokines IFN-γ and TNF-α followed by IL-1β, IL-2 and IL-6 were significantly increased in

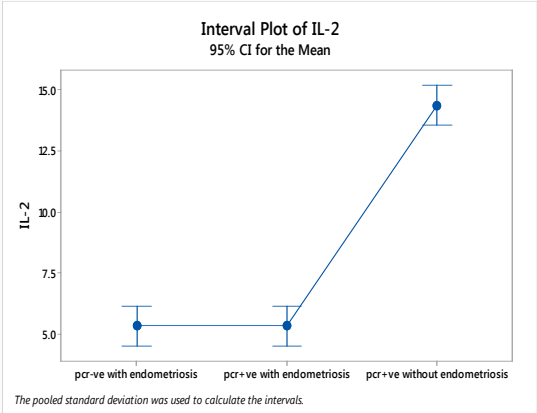


**Figure 4:** Distribution of interval plot of IL-2.

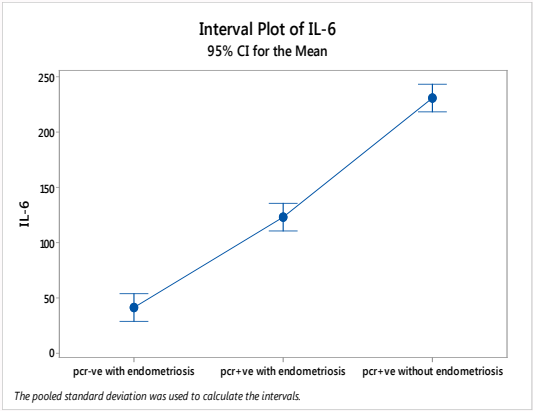


**Figure 5:** Distribution of 95% confidential 95% confidential interval plot of IL-6.

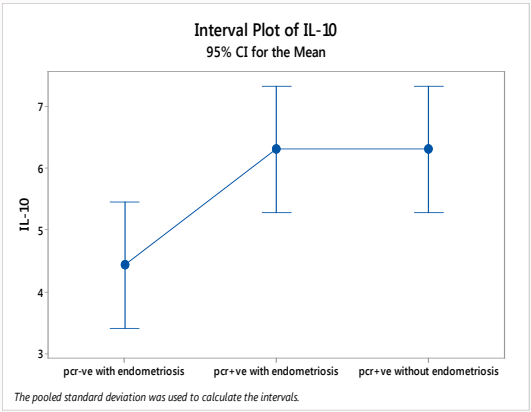


**Figure 6:** Distribution of interval plot of IL-10.

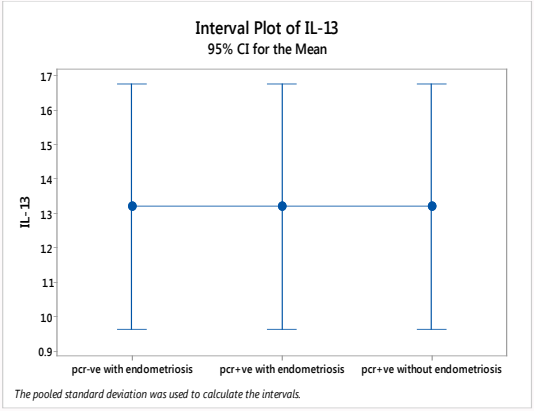


**Figure 7:** Distribution of 95% confidential 95% confidential interval plot of IL-13.

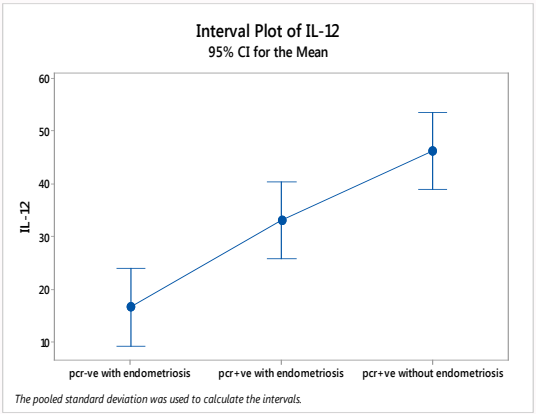


**Figure 8:** Distribution of interval plot of IL-12.

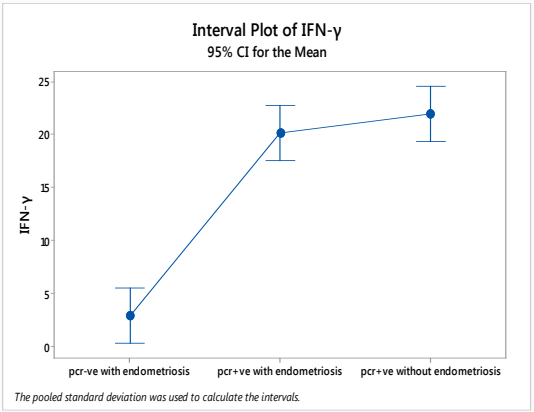


**Figure 9:** Distribution of 95% confidential 95% confidential interval plot of IFN-γ.

women with latent genital tuberculosis, in the present study. This finding correlates with the study by Chou CH et al., which states that use of IFN-γ shorten the time to diagnosis of extrapulmonary tuberculosis than conventional culture method [17]. IGRAs are *in vitro* assays that detect the presence of cellular immune responses toward MTB-specific antigens. They measure IFN-γ release in response to the region of difference 1-encoded (genomic region of difference) immunodominant antigens such as early secretory antigenic target-6, culture filtrate protein 10, and the TB7.7 antigens. In contrast to the TST, the antigens used in IGRAs are absent in most of the NTM (with the exception of Mycobacterium flavescens, Mycobacterium marinum, Mycobacterium Kansasii, and Mycobacterium szulgai), as well as from BCG strains [18,19]. This can be beneficiary in intervening earlier and preventing further damage to the pelvic organs and hence improving reproductive health in women. Low IFN-γ value occurs when there is clearance or resolution of infection. Hence multiple tests rather than a single diagnostic test may be useful for accurate diagnosis and has been suggested by some [20].

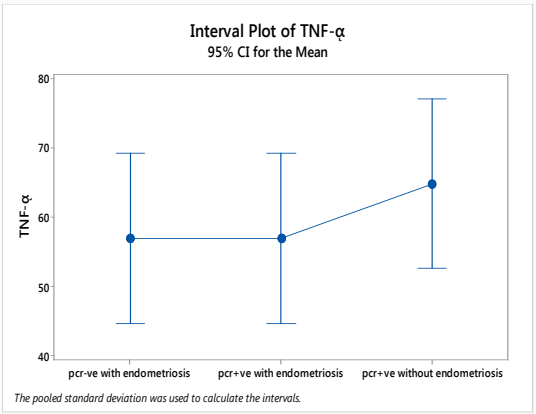


**Figure 10:** Distribution of interval plot of TNF-α.

The clinical finding of association of endometriosis with LGTB is a recent one, as mentioned in our study. The ill-effect of endometriosis on fertility has been so long discussed and well known. But the presence of tubercular bacilli in genital tract along with endometriosis probably worsens the situation. The inflammatory component of endometriosis is probably exaggerated by presence of tubercular bacilli, as during laparoscopy, tubal wall edema and inflammatory look of the pelvis is much more in the presence of LGTB. Though endometriosis induces aseptic inflammation of reproductive organs, the presence of LGTB exaggerates it, as it is indicated by the presence of IFN-¥ and TNF-α together. Those patients who have endometriosis only without LGTB, the presence of TNF-α in high concentration is found and in association with LGTB, both IFN-¥ and TNF-α are observed to be high. It is also interesting to find that in absence of endometriosis, the presence of LGTB induces rising levels of IFN-¥ only.

## Conclusion

Endometrial secretion cytokine assay will be novel, cost effective and invasive diagnostic screening method to identify lgtb, endometriosis and both. Local cytokines analysis will help to differentiate lgtb and endometriosis (Table 1and 2; Figure 1 and 2).

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## Author Contribution

A.D, B.B, S.C-Participated in study design, data collection and evaluation, drafting.

A.D, B.B-Conducted molecular experiments and RT-qPCR analysis.

S.C, B.B, RGC-Contributed to conception and design.

All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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