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Increased expression of IL-37 correlates with TNF- α levels and disease stage in endometriosis patients

Dina Ragab^{1*} , Ahmed Abbas² and Ramy Salem¹

Abstract

Background: IL-37 is an anti-inflammatory cytokine that increases in several inflammatory diseases with the main inducing signal for its production being pro-inflammatory cytokines like TNF- α . We aimed to assess the correlation between peritoneal fluid levels of IL-37 and TNF- α in endometriosis patients and investigate their association with disease stage. Levels of IL-37 and TNF- α were assessed in peritoneal fluid of 50 patients with endometriosis and 23 endometriosis-free females using enzyme-linked immunosorbent assay. We also assessed serum levels of IL-37 using enzyme-linked immunosorbent assay and expression of IL-37 mRNA in peritoneal fluid cells using polymerase chain reaction.

Results: Peritoneal fluid levels of IL-37 and TNF- α were higher in endometriosis patients than in control females. Also, levels were higher in patients with late endometriosis than patients with early endometriosis. In addition, serum levels of IL-37, as well as IL-37 mRNA expression in peritoneal fluid cells, were higher in patients than controls. In endometriosis patients, peritoneal fluid levels of IL-37 positively correlated with levels of TNF- α .

Conclusion: Collectively, our results show increased levels of the anti-inflammatory cytokine IL-37 in endometriosis patients that correlate with levels of the pro-inflammatory cytokine TNF- α , one of the main signals for IL-37 production.

Keywords: IL-37, TNF- α , Peritoneal fluid, Endometriosis

Background

Endometriosis is a disease characterized by the presence of functioning endometrial tissue, both glandular epithelium, and stroma, outside of the uterus [1]. It is a common disease that affects around 2–10% of females [2]. Patients typically complain of abdominal pain, dysmenorrhea, dyspareunia, and infertility, however, some patients may be asymptomatic and are accidentally discovered during laparoscopy for any other reason [3]. Endometriosis can occur in a variety of locations, the most common of which are the ovary, rectovaginal

septum, Douglas pouch, and uterosacral ligaments. It can sometimes occur in the vagina, rectum, fallopian tubes, uterine cervix, or the urinary tract [4]. Symptoms associated with endometriosis have an impact on the patient's physical, psychological, and social well-being [5].

The exact etiology of endometriosis is not fully elucidated, however multiple theories have been proposed, among which are environmental factors, hormones, dysregulated immunity, inflammatory factors as well as genetic and epigenetic factors [6]. One widely accepted theory is retrograde menstruation followed by implantation of endometrial fragments. A growing body of literature indicates that the survival of endometrial tissue is enhanced by a concurrently altered immune surveillance that allows the survival of the implanted endometrial tissue [7].

*Correspondence: dinaragab@med.asu.edu.eg

¹ Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Full list of author information is available at the end of the article

Interleukin-37 (IL-37) is an anti-inflammatory cytokine and one of the members of the IL-1 family. IL-37 is mainly expressed in circulating monocytes, tissue macrophages, dendritic cells, tonsil B cells, and plasma cells [8]. IL-37's anti-inflammatory function is primarily achieved by suppressing innate immunity [9]. Expression and production of IL-37 are increased in response to exposure to various toll-like receptor ligands, as well as, pro-inflammatory cytokines including IL-1, TNF- α , IL-18, and IFN- γ [8]. Furthermore, data from multiple studies show that IL-37 plays a role in controlling the inflammatory process in a variety of inflammatory diseases [10–17].

As an anti-inflammatory cytokine, IL-37 is suggested to play a role in endometriosis. Women with endometriosis had higher IL-37 mRNA expression in ectopic and eutopic endometrium [18] as well as higher serum and intraperitoneal fluid levels [19]. In mice, IL-37 overexpression inhibited endometrial stromal cells proliferation and adhesion [20]. The relationship between IL-37 and proinflammatory cytokines in endometriosis patients has not been investigated before. Because pro-inflammatory cytokines, such as TNF- α , are considered to be the primary stimulus for increased IL-37 expression and production [21], herein, we sought to examine the relationship between levels of IL-37 and levels of TNF- α in peritoneal fluid of endometriosis patients. We also assessed serum IL-37 levels as well as the expression of IL-37 mRNA in peritoneal fluid cells of endometriosis patients.

Methods

Study participants

The study included 73 females recruited from the Gynecology and Obstetrics Department at the Faculty of Medicine, Ain Shams University. All study participants had regular menstrual cycles and were in the menstrual cycle's proliferative phase (according to the last menstrual period date). Females who had received hormonal treatment in the previous 6 months or who had any concomitant autoimmune disease, endometrial hyperplasia, endometritis, endometrial cancer, or adenomyosis were excluded. The patients' group included 50 endometriosis patients who were diagnosed through histopathological examination following laparoscopy. The revised American Fertility Society (rAFS) classification of endometriosis was used to assess endometriosis staging and scoring. The rAFS score was used to categorize patients into four stages: Stage I (minimal; rAFS score 1–5), Stage II (mild; rAFS score 6–15), Stage III (moderate; rAFS score 16–40) and Stage IV (severe; rAFS score >40). Endometriosis patients in stages I and II were grouped as “early endometriosis group” while those in stage III and stage IV were grouped as “late endometriosis group”. As a control

group, 23 age-matched females, who underwent laparoscopy for diagnostic reasons, tubal disconnection, ovarian cystectomy, ovarian drilling, and who were confirmed to not have endometriosis during laparoscopy \pm histological examination if needed, were recruited to the study. Before taking part in the study, each participant provided written informed consent.

Samples

Two milliliters (2 mL) of venous blood were collected into vacutainer tubes (gel type), and the serum was centrifuged at 3000 rpm for 15 min before being stored at -80°C until it was used later to measure serum IL-37 levels. Peritoneal fluid (2 mL) was collected at the beginning of the laparoscopy from the pouch of Douglas in a sterile centrifuge tube. After centrifuging the sample at 3000 rpm for 15 min, the supernatant was stored at -80°C until used to assess peritoneal fluid IL-37 and TNF- α levels, while cell pellets were stored in RNA later (Qiagen, Germany) at -80°C till used later in mRNA extraction.

Assessment of IL-37 and TNF- α levels

Levels of IL-37 in both serum and peritoneal fluid were measured using an enzyme-linked immunosorbent assay (ELISA) kit provided by Elabscience, USA, while levels of TNF- α in the peritoneal fluid were measured using an ELISA kit provided by R&D Systems, UK.

Assessment of IL-37 mRNA expression

mRNA extraction

RNeasy Mini Kit (Qiagen, Germany) was used to extract RNA from peritoneal fluid cells. Reverse-transcription of RNA into complementary DNA was done using QuantiTect Reverse Transcription kit (Qiagen, Germany) by incubating a total volume of 14 μL containing 1 μg RNA, 2 μL gDNA wipe-out buffer, and RNase free water for 2 min at 42°C . Then 1 μL reverse transcriptase, 4 μL RT buffer, and 1 μL RT primer mix were added to reach a total volume of 20 μL . The mixture was incubated for 15 min at 42°C followed by 3 min at 95°C .

Real-time PCR

TaqMan Gene Expression Master Mix (Applied Biosystems, USA) and TaqMan Gene Expression Assay sets (Applied Biosystems, USA) were used for gene expression PCR analysis of IL-37 and GAPDH as the normalizing house-keeping gene. The reaction volume (total 20 μL) included 10 μL Master Mix, 1 μL Taqman assay, 7 μL nuclease-free water, and 2 μL cDNA. Rotorgene QPCR System was used for amplification according to the following protocol: initial activation for 10 min at 95°C , followed by 40 cycles of denaturation for 15 s at

95 °C followed by annealing and extension for 1 min at 60 °C. The comparative CT method (also known as the $2^{-\Delta CT}$ method) was used to calculate IL-37 mRNA relative expression by normalizing to GAPDH.

Statistical analysis

For statistical analysis, Graph Pad Prism 5 (San Diego, CA, USA) was used. The median and interquartile range were used to present quantitative data, while number and percentage were used to present categorical data. To compare groups, the Mann–Whitney test was used. Spearman's correlation was used to determine the degree of correlation between parameters. A p value of <0.05 was deemed statistically significant.

Results

Clinical characteristics of study participants

The study included 50 patients with endometriosis, with a mean age of 29.7 ± 6.2 years. Patients were classified according to their rAFS stage: seven had stage I endometriosis, nineteen had stage II endometriosis, nineteen had stage III endometriosis, and five had stage IV endometriosis. Control samples were collected from 23 women who did not have endometriosis and had mean age of 30.9 ± 5.1 years (Table 1).

Elevated IL-37 levels in endometriosis patients

We first wanted to look into IL-37 levels and expression in Egyptian endometriosis patients because they had never been investigated before. And, before assessing the correlation of IL-37 with TNF- α , we wanted to find out if IL-37 levels were higher in endometriosis patients

compared to control females. Endometriosis patients showed significantly higher serum IL-37 levels compared to controls ($p < 0.0001$) (Fig. 1A). Furthermore, peritoneal fluid samples of endometriosis patients showed significantly higher IL-37 levels compared to the peritoneal fluid of controls ($p < 0.0001$) (Fig. 1B).

Higher IL-37 levels in late endometriosis stages

To evaluate if higher IL-37 levels were related to endometriosis stage, patients were divided into two groups based on their r-AFS stage: early endometriosis (stages I and II) and late endometriosis (stages III and IV), and IL-37 levels were compared between both groups. Patients with late stages of endometriosis ($n = 24$) had significantly higher serum IL-37 levels compared to patients with early stages of endometriosis ($n = 26$) ($p = 0.0018$) (Fig. 2A). Furthermore, IL-37 levels in peritoneal fluid were significantly higher in patients with late stage endometriosis compared to patients with early stage endometriosis ($p = 0.0001$) (Fig. 2B).

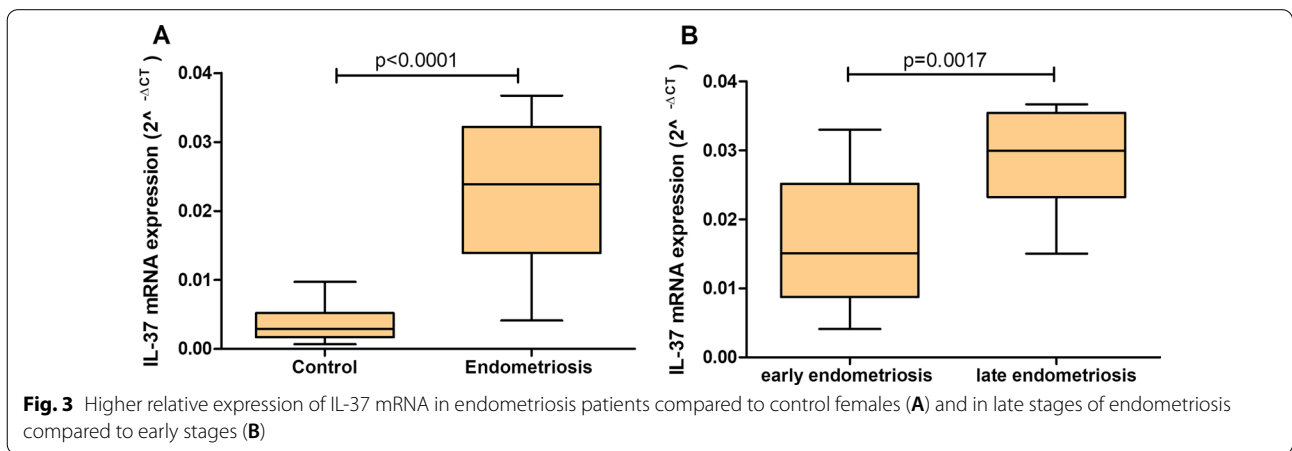
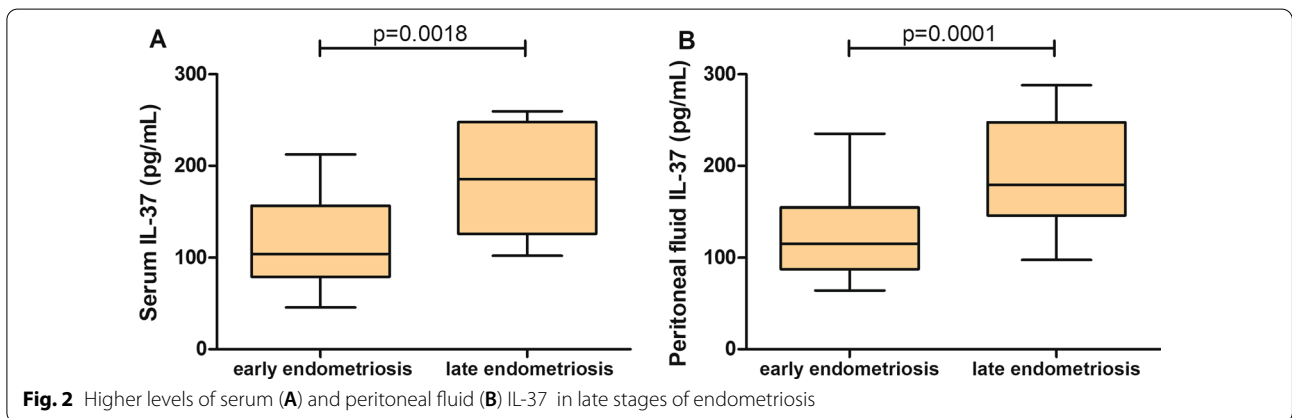
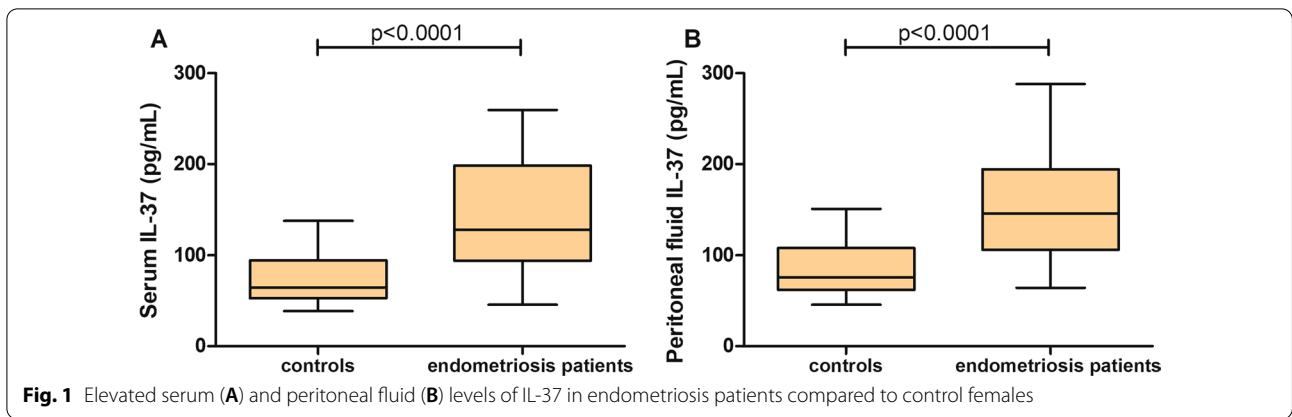
Increased IL-37 mRNA expression in endometriosis patients

Real-time PCR was used to look at the expression of IL-37 mRNA in peritoneal fluid cells. IL-37 mRNA relative expression was higher in peritoneal fluid cells of endometriosis patients compared to cells from controls ($p < 0.0001$) (Fig. 3A). In addition, late endometriosis patients had significantly higher IL-37 mRNA relative expression than early endometriosis patients ($p = 0.0017$) (Fig. 3B).

Table 1 Demographical and clinical characteristics of endometriosis patients and controls

	Endometriosis patients ($n = 50$)	Controls ($n = 23$)
Age in years, mean \pm SD	29.7 \pm 6.2	30.9 \pm 5.1
Parity, n (%)		
P0	38 (76%)	15 (65.2%)
P1	6 (12%)	5 (21.7%)
P2	6 (12%)	3 (13.1%)
rAFS score for endometriosis, median (IQR)	14 (10–26)	NA
rAFS stage for endometriosis, n (%)		
Stage I	7 (14%)	NA
Stage II	19 (38%)	
Stage III	19 (38%)	
Stage IV	5 (10%)	
Serum IL-37 (pg/mL), median (IQR)	128.1 (93.9–198.7)	64.6 (52.7–94.3)
Peritoneal fluid IL-37 (pg/mL), median (IQR)	146.2 (106.1–194.4)	75.5 (61.8–108.2)
IL-37 mRNA expression, ($2^{-\Delta CT}$)	0.0239 (0.01393–0.03218)	0.0029 (0.0017–0.0052)
Peritoneal fluid TNF- α (pg/mL), median (IQR)	55.55 (39.7–63.5)	15.6 (12.8–19.3)

IQR interquartile range, NA not applicable, rAFS revised American Fertility Society



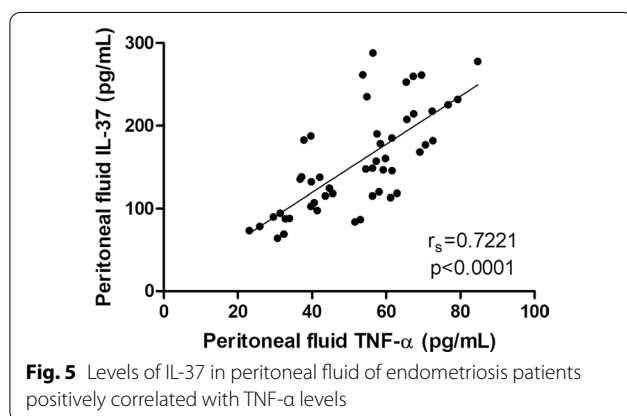
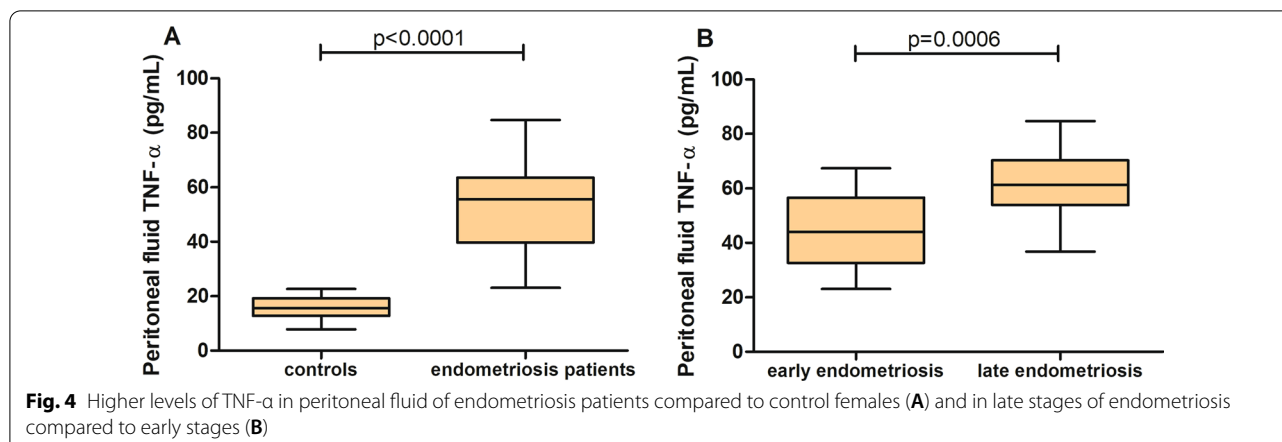
High peritoneal fluid TNF- α levels in endometriosis patients

Endometriosis patients showed significantly higher TNF- α levels in their peritoneal fluid compared to controls ($p < 0.0001$) (Fig. 4A). Moreover, patients with late stages of endometriosis showed significantly higher

peritoneal fluid TNF- α levels compared to patients with early stages of endometriosis ($p = 0.0006$) (Fig. 4B).

IL-37 levels correlate with TNF- α levels

TNF- α , among other pro-inflammatory cytokines, effectively induces the expression of IL-37 [20]. The



relationship between IL-37 and TNF- α in endometriosis patients has not been previously investigated. To address this point, we examined the correlation between levels of IL-37 and levels of TNF- α in the peritoneal fluid of endometriosis patients. IL-37 levels positively correlated with TNF- α levels in peritoneal fluid ($r_s = 0.7221$, $p < 0.0001$) (Fig. 5).

Discussion

According to emerging evidence, immune cells and cytokines play a profound role in the immuno-pathogenesis of endometriosis. Endometriosis is, in fact, thought to be an estrogen-dependent chronic inflammatory condition [7, 22]. Supporting this concept are studies focusing on macrophages and natural killer cells in both ectopic endometrial tissue and the peritoneal fluid, suggesting that ectopic endometrial tissue contains an increased number of immune cells that produce a variety of products, including several cytokines and growth factors that enhance ectopic tissue implantation and contribute to the inflammation and fibrosis that result from endometriosis [23, 24]. Cytokines are considered among the key players that can participate in modulating the

implantation milieu and participating in the inflammatory process that is associated with endometriosis [25].

In the present study, we report higher serum and peritoneal fluid IL-37 levels in endometriosis patients with higher levels in later endometriosis stages compared to early stages. Similar findings were reported by Kaabachi et al. [19] who studied levels of IL-37 in 30 Tunisian endometriosis patients and found higher IL-37 levels in both serum as well as peritoneal fluid samples. Also, Jiang et al. [26] found comparable results in a group of 40 Chinese females with endometriosis compared to 32 controls. Meanwhile, in a study involving 27 Chinese endometriosis patients and 36 controls, Fan et al. [27] reported higher serum IL-37 in endometriosis patients but no difference in peritoneal fluid IL-37 levels. IL-37 tends to increase in inflammatory conditions in an attempt to dampen the inflammatory process [28]. Administration of recombinant IL-37 in endometriosis mice resulted in a significant decrease in endometriotic lesion size and weight and also lowered pro-inflammatory cytokine expression as well as vascular endothelial growth factor and soluble adhesion molecules, in peritoneal fluid of endometriosis mice [20]. Furthermore, in-vitro transfection of endometrial stromal cells with IL-37 RNA was reported to suppress endometrial stromal cell proliferation, adhesion, migration, and invasion [20]. Consequently, it is possible to postulate that IL-37 might play a potential role in the immune response that occurs in endometriosis.

Evidence is accumulating that IL-37 plays a role in limiting excessive inflammation. IL-37 levels have been found to rise in a variety of inflammatory conditions including osteoarthritis [10], rheumatoid arthritis [11], systemic lupus erythematosus [12], atopic dermatitis [13], and inflammatory bowel diseases [16]. Furthermore, the potential use of IL-37 as an anti-inflammatory agent in chronic inflammatory conditions has been investigated

in animals models, where treatment with IL-37 in allergic rhinitis murine models alleviated allergic inflammation through repressing STAT6 and STAT3 signalling pathways [29]. Treatment with recombinant IL-37 also suppressed joint inflammation in mouse models of arthritis [30]. IL-37b gene transfer enhanced the therapeutic efficacy of mesenchymal stem cells in dextran sulfate sodium-induced colitis mice through inducing regulatory T cells and myeloid-derived suppressor cells and regulating cytokine production [31].

Our results show higher expression of IL-37 mRNA in endometriosis patients compared to controls, with higher levels in later endometriosis stages compared to early stages. IL-37 expression increases in response to the pro-inflammatory cytokines e.g., IL-1, IL-18, TNF- α , and IFN- γ [21]. However, the relationship between these cytokines and IL-37 in endometriosis patients has not been studied before. Our results show higher levels of TNF- α in peritoneal fluid of endometriosis patients with higher levels in late endometriosis compared to early endometriosis explaining the high IL-37 levels and expression observed in endometriosis patients in our study. Furthermore, in our study, IL-37 levels in peritoneal fluid were found to positively correlate with TNF-levels. Several studies have reported increased TNF- α in the peritoneal fluid of endometriosis patients [32–34]. Meanwhile, increased IL-37 mRNA expression in peritoneal fluid cells was also reported by Kaabachi et al. [19]. Nevertheless, Jiang et al. [18] reported higher mRNA expression in ectopic endometrial biopsies as well as eutopic endometrial biopsies in endometriosis patients compared to eutopic endometrium from females without endometriosis.

To the best of our knowledge, this is the first study to look at the relationship between TNF- α and IL-37 levels in endometriosis patients. This is also the first study to assess IL-37 levels and expression in Egyptian endometriosis patients. One limitation of the present study is the relatively small number of patients included. Further research on a larger scale of patients as well as further follow-up studies are needed to assess the levels of IL-37 during endometriosis progression and the possible correlation of IL-37 levels with levels of TNF- α and other cytokines throughout the course of the disease.

Conclusions

In conclusion, we report higher serum and peritoneal fluid IL-37 levels as well as higher peritoneal fluid TNF- α levels in endometriosis patients with a positive correlation between TNF- α and IL-37 levels. Our data in addition to previous reports highlight the potential importance of IL-37 in the immune process during endometriosis.

Abbreviations

IL-37: Interleukin 37; rAFS: Revised American Fertility Society; TNF- α : Tumor necrosis factor α .

Acknowledgements

Not applicable.

Authors' contributions

DR, AA designed the study. AA collected the samples, collected patients' clinical data and staged the patients. DR, RS carried out the laboratory work, analyzed the data and carried out statistical analysis. DR, RS drafted the paper. AA revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the ethical committee of the Faculty of Medicine, Ain Shams University. Ethical approval reference number (FWA 000017585). Written informed consent was taken from all subjects before participation in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

²Obstetrics and Gynecology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

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