AJRI American Journal of Reproductive Immunology
Official Journal of the American Society for Reproductive Immunology

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Journal:	*American Journal of Reproductive Immunology*
Manuscript ID	AJRI-04-20-101
Manuscript Type:	Short Communication
Date Submitted by the Author:	29-Apr-2020
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Keywords:	Endometriosis, Infertility, Immune checkpoint, PD-1/PD-L1, CTLA-4, Biomarker, HLA-G

Short Communication

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Soluble immune checkpoints CTLA-4, HLA-G, PD-1 and PD-L1 are associated with endometriosis-related infertility

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Funding Information :

This work was supported by internal research grant of Faculty of Medicine, Airlangga University, Surabaya Indonesia (ID No. 2158/UN3/2019)

Abstract

Problem: Soluble immune checkpoint molecules are relatively novel discovered immune regulatory mediators. Their role in the pathogenesis of endometriosis remains elusive. Therefore, we aimed to investigate the relationship between the clinical manifestation of endometriosis-associated infertility and the level of four soluble immune checkpoints: sCTLA4, sHLA-G, sPD-1, and sPD-L1.

Method of study: The soluble immune checkpoint concentrations in serum and peritoneal fluid were evaluated by the enzyme-linked immunosorbent assay (ELISA) from 88 patients who underwent laparoscopy. Clinical and hematological parameters were documented and analyzed.

Results: Endometriosis cases were found to have significantly higher levels of serum sPD-L1 and all four molecules in peritoneal fluid compared to non-endometriosis control. Contrary, no significant differences were found in the concentration of serum sCTLA-4, sHLA-G and, sPD-1 between endometriosis and control group. There were significant positive correlations between serum and peritoneal fluid concentrations of sCTLA-4, sPD-L1, and sHLA-G. Serum sPD-L1 could discriminate the endometriosis-related infertility to other pathological control. At a cutoff of 14,61 pg/mL, serum sPD-L1 had sensitivity of 77% and specificity of 83%. Moreover, sPD-L1 level showed positive correlations with pelvic adhesion score and myeloid cell count.

Conclusion: The elevated level of sPD-L1 in serum and immune checkpoint molecules in the peritoneal fluid could represent the hallmark of immune regulation in endometriosis cases. Serum sPD-L1 could be used as a noninvasive biomarker of endometriosis. Also, the immune compartment related to the local immune checkpoint molecules may be implicated in the mechanism involved in endometriosis-related infertility.

Keywords

Endometriosis-related infertility, sCTLA4, sHLA-G, sPD-1, sPD-L1

INTRODUCTION

Endometriosis is a common gynecological estrogen-dependent disorder. It affects approximately one-tenth of women in their reproductive age and characterized by the presence of endometrial-like tissue implants outside the uterine cavity[1]. The major symptoms of endometriosis are pelvic pain, infertility, and dysmenorrhea. Endometriosis can be found in 30-50% of women with infertility[2,3]. The revised American Fertility Society (rAFS) and American Society for Reproductive Medicine (rASRM) score system are the most widely used by the clinician to classify the severity of endometriosis patients[4]. Besides, Endometriosis Fertility Index (EFI) score was used to complement the rAFS/rASRM and better predict the pregnancy outcome[5].

Over the past three decades, the pathogenesis of endometriosis has been under investigation. It is known as a benign pathology and chronic inflammatory disorder. The cause of endometriosis is still elusive[1]. The retrograde menstruation hypothesis by Sampson is among the most accepted theory[6,7]. Genetic factors and dysregulation of immune homeostasis may contribute to the development of endometriosis. The studies of immune components in the peritoneum of endometriosis indicate an immune tolerance state and defective immune effector function towards endometrial-related antigens allowing the lesion development[8].

Immune checkpoint molecules act as an inhibitory signaling pathways to maintain immune tolerance, especially in the adaptive immune compartment[9]. Over the past decade, there are known immune checkpoint molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA4), human leukocyte antigen G (HLA-G), programmed cell death protein 1 (PD-1), and programmed cell death ligand 1 (PD-L1)[10,11]. These molecules regulate T-cell homeostasis, inhibit autoreactive T cells, drive peripheral tolerance in malignancy, pregnancy, and sepsis[12,13]. Based on their location, there are two forms of these molecules: membrane-bound and cell-free soluble. The transmembrane molecules promote T regulatory (Treg) cell development and function. It also inhibits effector T-cell differentiation and cytokine production leading to immunosuppression[10-13]. The soluble forms of immune checkpoint molecules were discovered later, and their biological functions have been described[14]. The immune regulatory effect of soluble PD-L1 (sPD-L1), sCTLA-4, and sHLA-G trigger Treg differentiation and T cells apoptosis due to retention of their receptor[15].

In contrast, sPD-1 may act as a decoy receptor inhibiting the effect of sPD-L1[15]. In cancer research, the release of such immune regulatory molecules may represent an

unanticipated contributing factor to the overall immune regulatory state in the tumor microenvironment and systemic circulation[15]. The role of the PD-1/PD-L1 pathway and HLA-G is also essential for the maintenance of a healthy pregnancy[12].

In the past few years, immune checkpoint molecules have become the focus of reproductive immunology and inflammation[16]. Considering the above functions of soluble immune checkpoint molecules on immune regulation, it seems that they act as a crucial factor in the pathogenesis of endometriosis. Recently, the membrane-bound PD-1/PD-L1 expressed lymphocytes and endometrial tissues were recently described in endometriosis[17,18,19]. In the current study, we hypothesize that soluble immune checkpoint molecules in peripheral circulation and peritoneal fluid will be elevated during endometriosis in infertile women.

METHOD OF THE STUDY

Study Design

This study was approved by the Ethical Review Committee Faculty of Medicine, Airlangga University, Surabaya, Indonesia (No. 62/EC/KEPK/FKUA/2020). A total of 88 patients who underwent laparoscopy were recruited under informed consent. The group study included 44 patients of non-endometriosis with infertility. All subjects were ranged in reproductive age from 24 to 41 years with a median age of 30. The inclusion criteria were a case group that underwent a laparoscopic with the diagnosis of endometriosis and non-endometriosis. The control group consisted of non-endometriosis women with single benign gynecologic disorder-related to the fallopian tubes, ovaries, or fibroids. The exclusions criteria were as follows: (1) patients with hormonal medications and immunosuppressive drugs; (2) patients with unexplained infertility and presented more than single gynecological disorders. All subjects had no known infections, autoimmune diseases, diabetes mellitus, and malignancy before infertility diagnosis. The primary and secondary infertile women were 84.2% and 14.8%, respectively. The year of infertility was between 1-16 years. The characteristics of the study and the control groups are shown in Table 1.

Collection of Peritoneal Fluid

The peritoneal fluid was isolated from the Douglas pouch area or perivesical space and collected in the early step of laparoscopy by aspiration needle with a laparoscopic direct vision before any manipulation. The sample was placed in a 15 mL sterile centrifuge tube. Further, the tube was spun at 3000 rpm, 4°C for 10 min. The supernatant was aspirated and placed into 1.5 mL centrifuge tubes. The sample was stored at -80°C for further analysis.

Collection of Serum

Blood samples were obtained before the laparoscopic examination. The samples of 5 ml were placed into BD Vacutainer tubes and centrifuged at 3000 rpm, 4 °C for 10 min. Serum was isolated in the upper phase and stored at −80 °C until used.

Soluble Immune Checkpoint Assay

The concentrations of immune checkpoints in peritoneal fluid and serum were detected by Enzyme-Linked Immunosorbent Assay (ELISA). The kits of sCTLA4 (Thermo Fischer Scientific, Erlangen, Germany), sHLA-G (Exbio, Prague, Czech Republic), sPD-1 and sPD-L1 (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer's instructions. The assay's concentration ranges of sCTLA4, sHLA-G, sPD-1 and sPD-L1 were 10-1000 pg/mL, 3.2-125 U/ml, 0.046-10 ng/mL, and 0.32-625 pg/mL, respectively. Plates were developed using tetramethylbenzidine substrate (ThermoFisher), stopped using 2 N H_2SO_4, and read at a dual-wavelength of 450 nm in a microplate reader (BIO-RAD, USA) to obtain the optical density (OD) and concentration values.

Statistical Analysis

All statistical calculations were performed using GraphPad Prism version 8.01. The data distribution was assessed by the Shapiro-Wilk normality test and quantile-normal plots analysis. The two-tailed Mann-Whitney U test was used to determine the differences between the endometriosis group and the control group. The three or more groups in this study were compared using the Kruskal-Wallis rank test. Correlation analysis was also conducted using Spearman's rank test represented as rho (r) between two continuous variables. Rho value indicates the strength of correlations: $r \geq 0.4$, strong; $0.4 > r \geq 0.2$, moderate; and $r < 0.2$, weak. A *p-value* below 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity of significant soluble immune checkpoint molecules in serum.

RESULT & DISCUSSION

Clinical studies involving the detection of immune checkpoints have demonstrated numerous roles for this protein family in malignancy, cancer, pregnancy, and inflammation[10-15]. Our novel data have supported the hypothesis that these proteins with immune regulatory function may be important in the pathogenesis of endometriosis. In blood

serum, we detected an increased concentration of serum sPD-L1 in endometriosis patients (Figure 1A). The serum level of sPD-L1 in endometriosis-related infertility cases was statistically different and nearly twice higher compared to control (19.33 pg/mL, 14.47-49.77 vs. 10.45 pg/mL, 5.48-14.40 $p<0.0001$). In contrast, we did not observe a difference in serum sCTLA-4, sHLA-G and sPD-1 between the two groups ($p=0.1357$, $p=0.2586$, $p=0.5649$, respectively). Although we did not observe any significant difference in serum sCTLA-4 between endometriosis and control. The late stage of endometriosis (stage III and stage IV) showed significantly higher serum sCTLA-4 compared to early-stage endometriosis and control group with p values of 0.0063 and 0.0192, respectively (Figure 1B).

On the other hand, the significant difference serum concentration of sPD-L1 between endometriosis and control group was determined only by the late-stage endometriosis ($p<0.0001$), not by the early stage. These results might indicate the role of sPD-L1 and sCTLA-4 on systemic tolerogenic effect on immune function in endometriosis patients, which is analogous to the membrane-bound PD-L1[15,20]. Previous studies have reported a systemic anti-inflammatory state in endometriosis patients with elevated concentration of serum IL-10, which is known as one of the effector molecules in immune checkpoint signaling pathway[21,22]. However, the significantly higher level of sCTLA-4 and sPD-L1 in the sera of advanced-stage endometriosis might also be the result of increased proteolytic cleavage of membrane forms from the local endometrial-like cell lesions, which then circulate into the periphery[17,18,20].

We further analyzed the protein concentrations in the peritoneal fluid. As expected, we observed higher concentrations of the four molecules in endometriosis cases compared to their concentrations in serum, including sCTLA-4 (202.80 pg/ml) and sPD-L1 (31.99 pg/ml). Interestingly, elevations of all four molecules were evident in the peritoneal fluid of women with endometriosis-related infertility group compared to gynecologic control. The concentrations of peritoneal sCTLA-4 and sPD-L1 in endometriosis cases were significantly higher compared to control group with median concentrations at 202.8 pg/mL vs 76.36 pg/mL, $p=0.0002$ and 31.99 pg/mL vs 23.50 pg/mL, $p=0.0149$, respectively (Figure 1C). Correspondingly, the median concentrations of sHLA-G in endometriosis and control was 10.74 U/mL vs 3.69 U/mL, $p<0.0001$ while the levels of sPD-1 of the two groups were 0.634 ng/mL vs 0.010 ng/mL, $p<0.0001$ (Figure 1C). On the other hand, the peritoneal concentration of sPD-L1 had shown no statistical differences between the endometriosis and

control group. In Figure 1D, we further compared the severity stages and control group. As expected, three out of four molecules (sCTLA-4, sHLA-G, sPD-1) had significantly higher concentrations in late-stage endometriosis compared to control ($p=0.0003$, $p=0.0081$ and $p<0.0001$, respectively). In contrast, the early stage of endometriosis had increased peritoneal sPD-L1 concentrations compared to the late-stage endometriosis ($p=0.0057$) and the control group ($p=0.0003$). These results might indicate that the local immune regulatory role of soluble immune checkpoint molecules both at the early stage and advanced endometriosis. Moreover, sCTLA-4 and sPD-L1 that were observed in the patient's sera in this study might be originated from the local peritoneal cavity. We indeed found significant positive correlations between serum and peritoneal concentrations of sCTLA-4 ($r=0.377$, $p=0.002$), sPD-L1 ($r=0.306$, $p=0.013$), sHLA-G ($r=0.406$, $p=0.001$), but not in sPD-1 concentrations ($r=0.110$, $p=0.384$). Moreover, the significantly increased levels of local immune checkpoint molecules in the peritoneum could be due to production by the ectopic lesions during chronic disease progression, as previously reported for sHLA-G in endometriosis[17] and sPD-L1 in Hodgkin lymphoma cases[23]. Their increased levels in the local tissue might be due to expression by endometrial lesions and local immune cells. These findings give us the first insight of their involvement in the pathogenesis of endometriosis, leading to infertility phenotype.

Next, of those significantly higher molecules in serum and peritoneal fluid, we further evaluated which of the three patient populations within the control group –fallopian, ovary, or myoma-related infertility– that significantly different from the severity of endometriosis. As shown in Figure 2A-B, fallopian- and myoma- related infertility had shown significantly lower concentrations of serum sCTLA4 ($p<0.05$) and sPD-L1 ($p<0.001$) compared to the late-stage endometriosis (stages III and IV). Also, the late-stage serum sPD-L1 was evident to have higher concentration compared to the ovary-related infertility cases ($p=0.0005$) (Figure 2B). Contrary, the serum sCTLA-4 and sPD-L1 of the early-stage endometriosis group (stages I and II) had shown no differences compared to the three control groups. In agreement with data in Figure 1D, the peritoneal sCTLA-4, sHLA-G and sPD-1 of advanced endometriosis had significantly increased concentrations compared to the fallopian- and myoma- related control with $p< 0.01$, $p<0.05$ and $p<0.01$, respectively (Figure 2C-E).

Interestingly, the early-stage group had also increased levels of peritoneal sHLA-G and sPD-L1 compared to the levels in those infertile women with fallopian- and myoma-related pathology (Figure 2D and 2F). In contrast, no significant difference was found in the level of peritoneal sPD-L1 in the late endometriosis group and the three control populations (Figure 2F). The results suggest a distinct immunological determinant governed by immune checkpoint molecules based on the gynecological organ-related disorders. The presence of soluble molecules in the peritoneal cavity not only highlights their involvement in the underlying disease but also may emphasize the cellular sources of the four molecules, which might not be abundant in the gynecological organs, especially in fallopian and uterus. Additionally, the differences in immune checkpoint profiles in the early and late-stage might indicate their interplay in the development of endometriosis.

Since serum sCTLA-4 and sPD-L1 were elevated in endometriosis, we examined their diagnostic values as a noninvasive biomarker. As shown in Figure 3A-B, the area under the curve (AUC) from the ROC analysis of the two molecules have revealed that the concentration of serum sPD-L1 has good sensitivity and specificity to determine endometriosis-related infertility (AUC = 0.7668, $p<0.0001$) and late-stage endometriosis (AUC=0.8241, $p<0.0001$). Diagnostic accuracy was promising for increasing concentration of sPD-L1 in discriminating endometriosis and other benign gynecologic diseases with a sensitivity of 77.27%, a specificity of 82.76%, and cut-off concentration of 14.61 pg/mL. Moreover, sCTLA-4 had lower diagnostic value with sensitivity of 70.45%, specificity of 55.17% and cut-off concentration of 75.53 pg/mL. The parameters of two local soluble molecules sHLA-G and sPD-1 in the peritoneal fluid also showed good diagnostic accuracy; however, the invasive biomarker is less favorable in clinical settings. This result warrants further investigation on serum sPD-L1 in a larger population of gynecological disorder-related infertility.

Additionally, we also performed an association analysis between laboratory or clinical parameters and serum sCTLA-4 or sPD-L1 levels. Interestingly, there were evident moderate positive correlations between blood monocyte count and sCTLA-4 ($r=0.268$, $p=0.020$) as well as sPD-L1 ($r=0.345$, $p=0.0024$) suggesting a possible cellular source of sPD-L1. sPD-L1 is known to be secreted by myeloid cells such as monocytes[24,25]. We also found that Endometriosis Fertility Index (EFI) score elements such as pelvic adhesion total score ($r=0.335$, $p=0.0014$) and rAFS endometriosis score ($= -0.431$, $p<0.0001$), and the presence of dysmenorrhea ($p=0.0327$) were associated with serum sPD-L1. In addition, serum sCTLA-4

were associated with total EFI score (r= -0.264, p=0.013), rAFS endometriosis score (= -0.411, p<0.0001) and dysmenorrheal symptom (p=0.006). The relationship between sCTLA-4 and sPD-L1 with clinical parameters – related to the infertility diagnosis points out to the possible immune regulatory mechanism during clinical symptom development in endometriosis.

The current work highlights an emerging role of soluble immune checkpoint molecules, especially sPD-L1, as a candidate biomarker in endometriosis-related infertility. Moreover, our study also suggests, for the first time, possible involvement of local immune checkpoint molecules as immune regulators in the pathogenesis of endometriosis. Hence, further investigation on the determination of immunological interactions and biological functions by immune checkpoints between immune and endometrial cells is needed to deepen our understanding of the underlying disease mechanism in our quest for the targeted immunotherapy for endometriosis.

ACKNOWLEDGEMENT

This work was supported by internal research grant of Faculty of Medicine, Airlangga University. We thank Prof. Dr. Inge Maria Lusida and Mochammad Amin from Institute of Tropical Diseases for additional laboratory space during our sample collection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Table 1. Patients Characteristics

Variables	Gynecologic control (n=44)	Endometriosis (n=44)	*p* value
Age (years)[a]	30 (24 – 41)	30 (24 – 38)	0.615[1]
Type of infertility[b]			>0.999[1]
- Primary	37 (84.0%)	38 (86.4%)	
- Secondary	7 (14.0%)	6 (13.6%)	
Year of infertility[a]	4 (1 – 16)	4 (1 – 15)	0.339[1]
Least function (LF) score[a]	4 (0 – 8)	4 (0 – 8)	0.336[1]
rAFS adhesion score[a]	0 (0 – 22)	9.5 (0 – 27)	<0.0001[1]
Dysmenorrhea[b]	23 (52.3%)	24 (54.5%)	>0.999[1]
rAFS EFI score[a]	N/A	6 (2 – 9)	N/A

Data shown as [a]median (minimum-maximum range) and [b]frequency (percentage);

[1]Statitistical analysis performed with two-tailed Mann-Whitney U test; N/A : not applicable

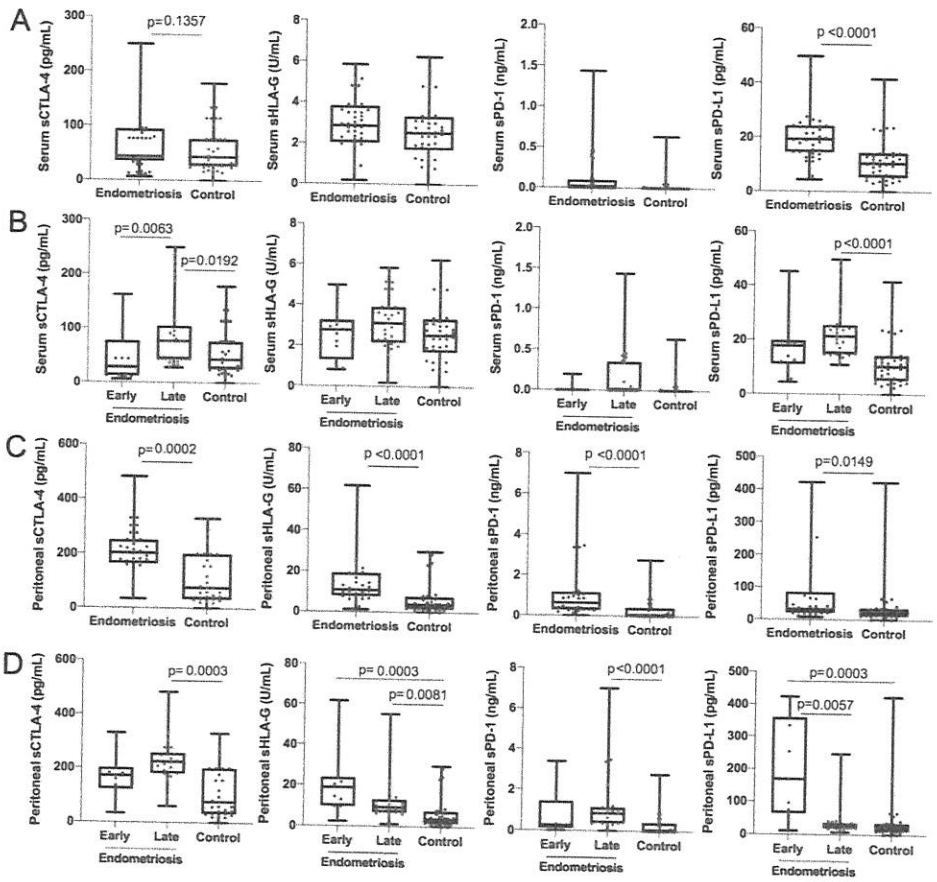

Figure 1. The concentrations of sCTLA4, sHLA-G, sPD-1, and sPD-L1 in serum (A) and peritoneal fluid (C) derived from endometriosis-related infertility and control group. Box plot graphs presented the four molecules from serum (B) and peritoneal fluid (D) in the early and late stage of endometriosis and control group. The p-values are shown.

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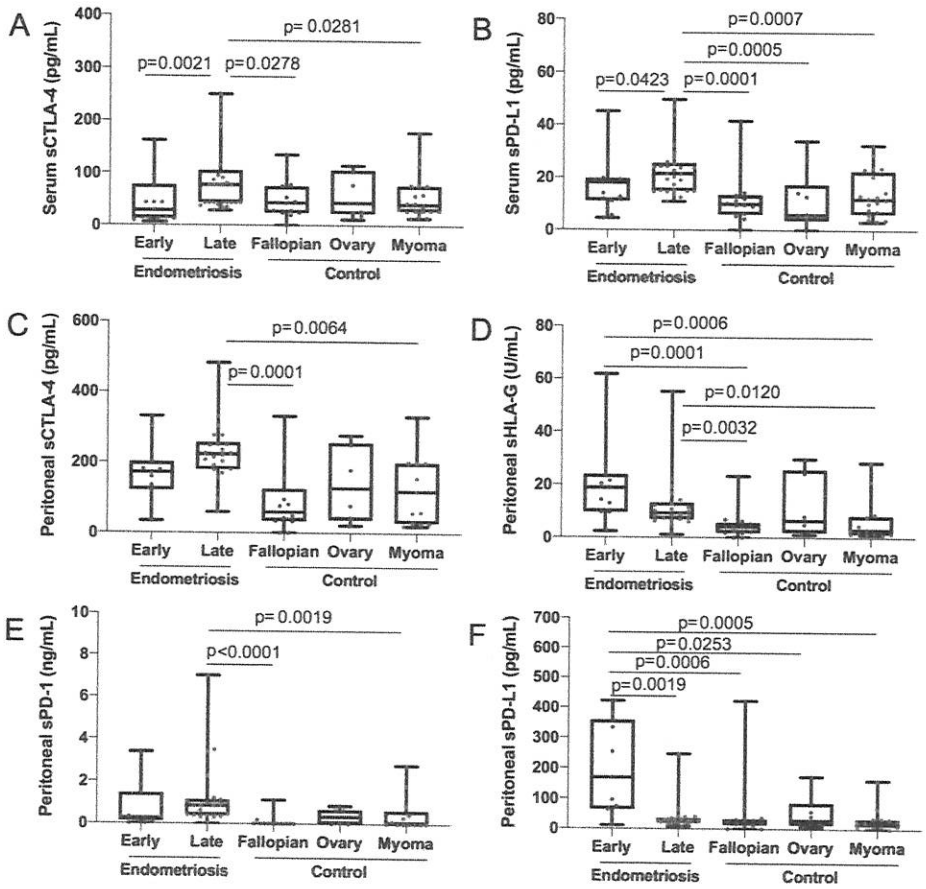

Figure 2. The levels of immune checkpoint molecules serum derived from the early and late stage endometriosis group and three control groups with fallopian-, ovary-, or myoma-related infertility for serum sCTLA-4 (A), serum sPD-L1 (B) and 4 molecules from peritoneal fluid : sCTLA4 (C), sHLA-G (D), sPD-1 (E) and sPD-L1 (F). The Kruskal-Wallis with Dunn's test was used to compare the 5 groups and the significant p-values are indicated.

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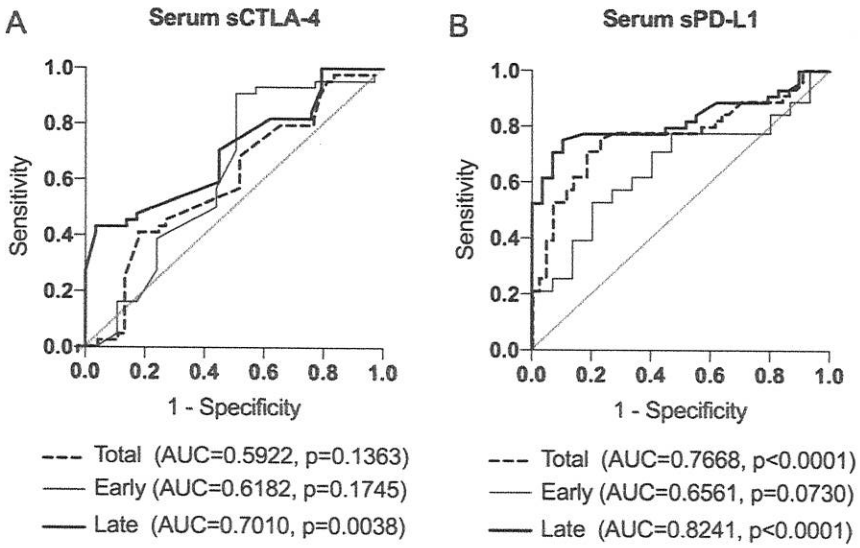

Figure 3. Receiver operating characteristics (ROC) curve to plot the sensitivity and specificity values of soluble immune checkpoints sCTLA-4 (A) and sPD-L1 (B) between control subject and all endometriosis cases (total), early and late stage endometriosis-related infertility compared to control group. The area under curve (AUC) and p-values are shown.

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