Serum cytokines as biomarkers for nonsurgical prediction of endometriosis

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Abstract

Objective: To test the ability of a group of serum cytokines, either individually or in combination, to serve as biomarkers for the nonsurgical diagnosis of endometriosis.

Study design: Subjects were allocated to two groups according to their laparoscopic diagnosis. The first group consisted of patients with endometriosis and the second group was made up of infertile women with no pelvic pathology (controls). Blood samples were collected preoperatively and stored. Cytokines were measured in the serum of all participants using the Bio-Plex Protein Array System. Nonparametric statistics and the Mann–Whitney test were used to compare groups. Subjects were seen at the Gynecologic endoscopy unit.

Results: Three cytokines were significantly higher in the serum of subjects with endometriosis than in the control group: interleukin-6 (IL-6) [4.41 pg/ml (range: 1.47–15.01) versus 0.97 pg/ml (range: 0.29–2.98), respectively; \( p < 0.001 \)], monocyte chemotactic protein-1 (MCP-1) [37.91 pg/ml (range: 24.54–94.74) versus 22.13 pg/ml (range: 13.85–39.45), respectively; \( p < 0.001 \)], and interferon-gamma (INF-\( \gamma \)) [19.01 pg/ml (range: 1.19–73.52) versus 0.30 pg/ml (range: 0.00–13.05), respectively; \( p < 0.001 \)]. There was no statistically significant difference between subjects with endometriosis and controls in the serum concentration of vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-\( \alpha \)), or granulocyte macrophage colony stimulating factor (GM-CSF). Interleukin-2 (IL-2), interleukin-8 (IL-8), and interleukin-15 (IL-15) were undetectable in the serum of both groups. None of the measured cytokines showed significant correlation with the cycle phase or stage of endometriosis. In a multivariate analysis, serum interleukin-6 provided a sensitivity of 71% and a specificity of 66% to discriminate between endometriosis patients and controls at a cutoff point of 1.9 pg/ml. Adding monocyte chemotactic protein-1 and interferon-gamma to interleukin-6 did not increase the discriminative ability over that achieved by measuring serum interleukin-6 alone.

Conclusions: Serum interleukin-6 provides a promising serum marker for the nonsurgical prediction of endometriosis.

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Keywords: Serum; Cytokines; IL-6; MCP-1; IFN-\( \gamma \); Endometriosis

1. Introduction

Endometriosis is defined as the presence of ectopic deposits of endometrial tissue (glands and stroma) outside of the uterine cavity. The disease manifests clinically through various forms of pelvic pain or subfertility. The prevalence of pelvic endometriosis approaches 6–10% in the general female population; in women with pelvic pain, infertility, or both, the prevalence is 35–50% [1].

The gold standard for the diagnosis of endometriosis is diagnostic laparoscopy; however, it is an invasive procedure that requires general anesthesia and surgical skill and is also not without hazards, which can include major vessel or bowel
injury [2]. Moreover, visual inspection of the pelvis has major limitations, particularly for the diagnosis of retroperitoneal and deep infiltrating lesions [3]. As a consequence of these drawbacks, population-based studies of endometriosis incidence and prevalence are not possible. A simple blood test for the diagnosis of endometriosis would overcome these problems and have a major impact on women’s health.

Immune system alterations play an important role in the development of endometriosis. Locally, there is an immunoinflammatory reaction within the peritoneal cavity of endometriosis patients in which activated immune cells, together with endometriotic implants, produce high amounts of cytokines, growth factors, and angiogenic substances [4]. Systemic immune alterations have also been described in endometriosis, including increased activation status of peripheral blood monocytes. These cells secrete higher levels of cytokines than peripheral blood monocytes from healthy controls under basal and stimulated conditions [5]. In women with endometriosis, cytokinest attract and recruit more immune cells [6,7], promote implantation and growth of ectopic endometrial cells by inducing proliferation [8] and angiogenesis [9], contribute to the attachment of endometrial cells to the peritoneal surface [10], and help the invasion of these cells into the mesothelium [11].

The aim of this study is to investigate the cytokine profile in the serum of subjects with endometriosis compared with healthy controls, and to test if any single cytokine or a combination of cytokines can provide a useful blood test for the diagnosis of endometriosis.

2. Material and methods

2.1. Patient enrollment

Subjects enrolled in this study were recruited among women undergoing laparoscopy for the evaluation of infertility or pelvic pain at the Gynecologic Endoscopy Unit. Women were allocated to two groups according to their laparoscopic findings. Group I (endometriosis group) consisted of subjects with endometriosis, and group II (control group) consisted of women with infertility whose laparoscopic examination was within normal limits with no evidence of endometriosis. We recruited 68 subjects with endometriosis and 70 controls. Women enrolled had regular menstrual cycles, had not used hormonal medications at least 3 months prior to enrollment in the study, and had not been pregnant or had hysterosalpingography done at least 3 months prior to enrollment. The study was approved by the Institutional Review Board. Informed consent was obtained from all participants.

2.2. Diagnosis and staging of endometriosis

Endometriosis was diagnosed during the laparoscopic procedure according to previously described morphological criteria [12]. Diagnosis was confirmed at the histopathological level as well for all subjects. The disease was staged according to the revised American Fertility Society (rAFS) classification. Laproscopies were performed in the proliferative and secretory phase of the cycle. Cycle phase was determined by obtaining cycle day information from patient medical history records and confirmed by histopathological examination of endometrial biopsy.

2.3. Collection of serum samples

A blood sample (5–10 ml of venous blood, withdrawn aseptically into sterile tubes) was taken preoperatively from all study participants. The blood samples were centrifuged at 3000 rpm for 10 min at 4 °C, and the clear serum was stored at −70 °C in aliquots until needed for analysis.

2.4. Measurement of cytokine concentration

Cytokine concentrations were determined using the Bio-Plex Protein Array System (Bio-Rad, Hercules, CA, USA). Cytokine-specific antibody-coated beads (Bio-Rad) were used for these experiments. The assay quantitates cytokines over a broad range (0.2–32,000 pg/ml) and eliminates multiple dilutions of high-concentration samples. The samples were prepared and incubated with the antibody-coupled beads for 1 h with continuous shaking. The beads were washed three times with wash buffer to remove unbound protein and then incubated with biotinylated detection cytokine-specific antibody for 1 h with continuous shaking. The beads were washed once more and were then incubated with streptavidin–phycoerythrin for 10 min. After incubation, the beads were washed and resuspended in assay buffer, and the constituents of each well were drawn up into the flow-based Bio-Plex Suspension Array System, which identifies each different color bead as a population of protein and quantifies each protein target based on secondary antibody fluorescence. Cytokine concentrations were automatically calculated by Bio-Plex Manager software using a standard curve derived from a recombinant cytokine standard. Many readings were made on each bead set, further validating the results.

2.5. Statistical analysis

The Mann–Whitney test was used to compare the groups. The nonparametric approach was used because the measurements of the cytokines were not normally distributed. Adjustment of p-value for multiple comparisons was done using Bonferroni’s correction. Receiver operating characteristic (ROC) analysis was used to estimate the power of a cytokine to distinguish subjects with endometriosis from controls and to choose an optimal cutoff point for screening purpose.
Table 1
Values of serum cytokines in subjects with endometriosis and controls (pg/ml)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Endometriosis</th>
<th>Controls</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>37.91 (24.54–94.74)*</td>
<td>22.13 (13.85–39.45)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.41 (1.47–15.01)</td>
<td>0.97 (0.29–2.98)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VEGF</td>
<td>26.32 (3.18–63.36)</td>
<td>31.80 (7.28–79.35)</td>
<td>0.22</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.04 (0.84–1.36)</td>
<td>1.07 (0.89–1.47)</td>
<td>0.60</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>20.51 (14.5–29.68)</td>
<td>11.97 (8.8–20.19)</td>
<td>0.51</td>
</tr>
<tr>
<td>INF-γ</td>
<td>19.01 (1.19–73.52)</td>
<td>0.30 (0.00–13.05)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

MCP-1, monocytes chemotactic protein-1; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-alpha; GM-CSF, granulocyte macrophage colony stimulating factor; INF-γ, interferon-gamma.

* Interquartile range indicates a range from 25th to 75th percentile.

3. Results

3.1. Characteristics of study subjects

We recruited 68 patients with endometriosis of whom 32 were early stage disease (stages I and II) and 36 advanced-stage endometriosis (stages III and IV). Also within the endometriosis group, there were 29 women in proliferative phase and 39 in secretory phase. The control group consisted of 70 women of whom 31 were in the proliferative phase and 39 in the luteal phase. There was no statistically significant difference between endometriosis patients and controls in age [34.0 (range: 29.0–38.5) versus 32.0 (range: 28.5–36.5), respectively; p = 0.20] or body mass index [22.09 (range: 19.5–25.1) versus 23.3 (range: 21.3–27.1), respectively; p = 0.24].

3.2. Serum cytokines in subjects with endometriosis versus controls

We evaluated the concentration of nine cytokines in the serum of subjects with endometriosis and controls—interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-15 (IL-15), monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), interferon-gamma (INF-γ), and granulocyte macrophage colony stimulating factor (GM-CSF) (Table 1). Three cytokines showed a statistically significant elevation in the serum of subjects with endometriosis compared with controls—IL-6 [4.4 pg/ml (range: 1.47–15.01) versus 0.97 pg/ml (range: 0.29–2.98), respectively; p < 0.001], MCP-1 [37.9 pg/ml (range: 24.54–94.74) versus 22.13 pg/ml (range: 24.54–94.74), respectively; p < 0.001], and INF-γ [19.01 pg/ml (range: 1.19–73.52) versus 0.30 pg/ml (range: 0.00–13.05), respectively; p < 0.001]. There was no statistically significant difference between subjects with endometriosis and controls in the serum concentration of VEGF [26.3 pg/ml (range: 3.18–63.36) versus 31.8 pg/ml (range: 7.28–79.35), respectively; p = 0.22], TNF-α [1.04 pg/ml (range: 0.84–1.36) versus 1.07 pg/ml (range: 0.89–1.47), respectively; p = 0.60], or GM-CSF [20.51 pg/ml (range: 14.5–29.68) versus 11.97 pg/ml (range: 8.8–20.19), respectively; p = 0.51]. IL-2, IL-8, and IL-15 were evaluated in the serum of subjects with endometriosis and controls; however, their concentrations were below the detection limit of our assay. The p-values reported in Table 1 represent the unadjusted p-values, however because the difference in serum levels of IL-6, MCP-1, and INF-γ between endometriosis patients and controls is highly significant (p < 0.001), the significance still holds after adjusting for multiple comparisons using Bonferroni correction.

3.3. Serum cytokines of subjects with endometriosis stratified by stage of the disease

With the exception of TNF-α, which was marginally higher in advanced-stage endometriosis than in earlier stages of the disease, there was a trend for all other cytokines and growth factors to be higher in early-stage endometriosis than in advanced stages of the disease (Table 2). However, the difference between early and advanced stages of endometriosis did not reach statistical significance for any of the measured cytokines.

Table 2
Values of cytokines in subjects with endometriosis stratified by the stage of disease versus controls in picogram/milliliter

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Early endometriosis</th>
<th>Advanced endometriosis</th>
<th>Control</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>41.68 (31.46–151.19)*</td>
<td>33.07 (23.54–82.27)</td>
<td>22.13 (13.85–39.45)</td>
<td>0.24</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.39 (2.24–16.92)</td>
<td>3.45 (1.26–14.25)</td>
<td>0.97 (0.29–2.98)</td>
<td>0.29</td>
</tr>
<tr>
<td>VEGF</td>
<td>32.33 (4.54–87.29)</td>
<td>24.28 (3.18–49.05)</td>
<td>31.80 (7.28–79.35)</td>
<td>0.22</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.95 (0.84–1.12)</td>
<td>1.12 (0.84–1.55)</td>
<td>1.07 (0.89–1.47)</td>
<td>0.10</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>20.82 (17.9–31.68)</td>
<td>14.50 (11.9–20.8)</td>
<td>11.97 (8.8–20.19)</td>
<td>0.26</td>
</tr>
<tr>
<td>INF-γ</td>
<td>35.52 (5.49–85.89)</td>
<td>12.41 (2.0–61.54)</td>
<td>0.30 (0.00–13.05)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

MCP-1, monocytes chemotactic protein-1; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-alpha; GM-CSF, granulocyte macrophage colony stimulating factor; INF-γ, interferon-gamma.

* Interquartile range indicates a range from 25th to 75th percentile.
3.4. Serum cytokines in subjects with endometriosis and controls stratified by phase of the cycle

For all measured serum cytokines, there was no statistically significant difference between measurements made in the proliferative versus secretory phases of the cycle in either group (Table 3).

3.5. Serum cytokines as potential markers for the non-surgical prediction of endometriosis

In a multivariate regression analysis of all the measured cytokines, serum IL-6 provided the best discriminative ability between subjects with endometriosis and controls ($p = 0.01$). The ROC of IL-6 showed an area under the curve of 75% ($p < 0.001$). Combining IL-6, MCP-1, and INF-γ together as predictors of endometriosis did not improve the discrimination between subjects and controls over that of IL-6 alone (Fig. 1).

**Table 3**

Values of serum cytokines in subjects with endometriosis and controls stratified by phase of the menstrual cycle (pg/ml)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Endometriosis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proliferative</td>
<td>Secretory</td>
</tr>
<tr>
<td>MCP-1</td>
<td>40.98 (14.90–117.16)</td>
<td>36.84 (24.87–83.54)</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.71 (2.09–16.92)</td>
<td>3.49 (1.31–14.93)</td>
</tr>
<tr>
<td>VEGF</td>
<td>26.32 (3.18–70.00)</td>
<td>23.50 (3.44–57.98)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.97 (0.81–1.13)</td>
<td>1.12 (0.85–1.50)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>22.72 (19.2–55.4)</td>
<td>18.93 (14.5–20.82)</td>
</tr>
<tr>
<td>INF-γ</td>
<td>18.62 (0.00–61.26)</td>
<td>20.33 (5.37–83.64)</td>
</tr>
</tbody>
</table>

MCP-1, monocytes chemotactic protein-1; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-alpha; GM-CSF, granulocyte macrophage colony stimulating factor; INF-γ, interferon-gamma.

* Interquartile range indicates a range from 25th to 75th percentile.

**Table 4**

Performance of serum IL-6 as a biomarker for nonsurgical prediction of endometriosis

<table>
<thead>
<tr>
<th>Cutoff point (pg/ml)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive likelihood ratio</th>
<th>Negative likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.03</td>
<td>81</td>
<td>51</td>
<td>1.38</td>
<td>0.38</td>
</tr>
<tr>
<td>1.9</td>
<td>71</td>
<td>66</td>
<td>2.06</td>
<td>0.45</td>
</tr>
<tr>
<td>2.6</td>
<td>60</td>
<td>70</td>
<td>2</td>
<td>0.57</td>
</tr>
</tbody>
</table>

pg/ml, Picograms per milliliter.

**Fig. 1.** ROC of serum IL-6 alone (black curve) and a combination of serum IL-6, MCP-1, and INF-γ (grey curve) for prediction of endometriosis. The discriminative power is not increased with the combination of IL-6, INF-γ, and MCP-1 over that of IL-6 alone.

**Fig. 2.** Distribution of serum IL-6 in endometriosis patients and control women.
4. Discussion

Endometriosis is suspected when a patient presents with chronic pelvic pain, dysmenorrhea, dyspareunia, or infertility. The diagnosis of this disease depends on diagnostic laparoscopy, which is minimally invasive but expensive, and associated with potential complications. There is a 2.4% risk of bladder or intestinal injury, two-thirds of which will require laparotomy [13]. In addition, injury to a major blood vessel during laparoscopy can be catastrophic and has a reported mortality rate of 15% [14].

One-third of women who undergo diagnostic laparoscopy will have endometriosis, one-third will have no pelvic pathology, and the remaining one-third will have other gynecological conditions. This means that two-thirds of women who undergo laparoscopy for pelvic pain or infertility will be subjected to the potential risks as well as the cost associated with this procedure without actually having endometriosis [15].

Because laparoscopy is an invasive procedure, the diagnosis of endometriosis is often delayed for an average of 11.7 years in patients with pelvic pain and for 3.5 years in patients with infertility [16]. It was found that the younger the patient is at onset of the symptoms, the longer it takes before an endometriosis diagnosis is made [17]. This delay may result in social and/or work-related problems for the patient, in addition to increased anxiety and fear of having a more serious condition [18].

In this study, we compared the serum cytokine profiles of subjects with endometriosis and controls to select the cytokines that could be used as predictors of endometriosis. We found serum IL-6 to provide the best discrimination between subjects with endometriosis and healthy controls. Adding serum IL-6, MCP-1, and INF-γ together as diagnostic markers for endometriosis did not increase the diagnostic accuracy of the test over that provided by serum IL-6 alone. The diagnostic value of this marker increased with the finding that serum IL-6 levels did not change significantly during any phase of the menstrual cycle in either group. The lack of correlation of serum IL-6 with the stage of disease makes this marker more suitable for qualitative diagnosis of the presence of endometriosis, rather than as an indicator of disease severity.

We recruited infertile women whose laparoscopic examination revealed no pelvic pathology as our controls. Actually, these women represent an important control group because it is that group of women who would benefit from a potential serum maker of endometriosis. In a typical clinical scenario, such a serum marker would be used to test women with infertility to detect endometriosis cases that would need further laparoscopic evaluation.

To measure cytokines, we used the Bio-Plex Suspension Array System (Bio-Rad), which is built around three core technologies—the first is a group of fluorescently dyed microspheres, or beads, the second is a flow cytometer with two lasers and associated optics to measure biochemical reactions that occur on the surface of the microspheres, and the third is a high-speed digital signal processor that efficiently manages the fluorescent output. An important advantage of using the Bio-Plex System is the integration of assay kits, software, calibration and validation tools, and instrumentation into one complete system that provides a high degree of accuracy.

IL-6 is a T cell-derived cytokine that is secreted by macrophages, lymphocytes, fibroblasts, and endothelial cells. It has B cell stimulatory activity and enhances the differentiation of T lymphocytes [19]. IL-6 secretion is increased by peritoneal macrophages in endometriosis patients and by stromal cells of eutopic and ectopic endometrium, with the ectopic stromal cells producing the highest levels [20]. IL-6 normally inhibits the growth of endometrial cells; however, this growth-inhibitory effect is lost in endometriotic tissues [21]. Our findings in the serum of subjects with endometriosis confirm that this pleiotropic cytokine plays an important role in the pathogenesis of endometriosis both locally and systemically.

MCP-1 is produced by T cells, monocytes, and endometrial cells, and it plays an important role in the recruitment of monocytes and macrophages to the peritoneal cavity in subjects with endometriosis [19]. MCP-1 is elevated in the peritoneal fluid of subjects with endometriosis, as compared with controls [22,23]. Moreover, there is higher expression of MCP-1 in the eutopic and ectopic endometrium of subjects with endometriosis, where it is upregulated by the synergistic action of estradiol and IL-1β [24]. We found significantly elevated serum levels of MCP-1 in subjects with endometriosis compared with controls, emphasizing the importance of this cytokine in the systemic recruitment and activation of monocytes and macrophages in endometriosis.

INF-γ is a cytokine secreted mainly by T helper type 1 cells and natural killer (NK) cells. IFN-γ promotes macrophage activation and T helper type 1 differentiation, in addition to upregulation of the expression of major histocompatibility complex (MHC) classes I and II molecules and adhesion molecules [25]. Endometriotic
stromal cells were found to be resistant to growth inhibition and apoptosis induced by INF-γ in normal endometrial cells [26]. INF-γ regulates MCP-1 secretion by peripheral blood mononuclear cells [27] and by endometriotic cells [28]. In addition, INF-γ upregulates the expression of intracellular adhesion molecule-1. The soluble form of intracellular adhesion molecule-1 can disrupt adhesions between immune cells and the endometriotic cells, preventing their destruction [10].

Previous studies have examined serum cytokines as diagnostic markers for endometriosis; however, the results were conflicting. Bedaiwy et al. found that serum IL-6 provides a sensitivity of 90% and a specificity of 67% for the diagnosis of endometriosis at a cutoff value of 2 pg/ml [29]. Somigliana et al. found no statistically significant difference in serum IL-6 concentration between subjects with endometriosis and controls; consequently, they could not recommend measuring IL-6 in the serum as a predictor of endometriosis [30]. Our results are more in agreement with Bedaiwy et al., as we found a sensitivity of 71% and a specificity of 66% for serum IL-6 to predict endometriosis at a cutoff point of 1.9 pg/ml. Our results showed a lower sensitivity for IL-6 than that detected by Bedaiwy et al. at a nearly similar cutoff point. However, this could be explained on the basis of the heterogeneity of the disease itself, different populations studied, and different methodology used to measure the cytokine concentration; we used the Bio-Plex System and Bedaiwy et al. utilized the enzyme-linked immunosorbent assay technique in their study.

In conclusion, the serum of the subjects with endometriosis contained significantly higher levels of IL-6, MCP-1, and INF-γ than that of the control group. Serum IL-6 provided a good means of discrimination between subjects with endometriosis and controls, achieving a sensitivity of 71% and specificity of 66% at a cutoff value of 1.9 pg/ml. Adding MCP-1 and INF-γ to IL-6 did not improve the discrimination between subjects with endometriosis and controls over that achieved by using IL-6 alone. IL-6 provides a promising serum marker for the nonsurgical prediction of endometriosis.

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References


