

Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial

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BACKGROUND: The objective of this prospective controlled trial was to investigate the ability of a group of serum and peritoneal fluid (PF) markers to predict, non-surgically, endometriosis. **METHODS AND RESULTS:** Serum and PF samples were obtained from 130 women while undergoing laparoscopy for pain, infertility, tubal ligation or sterilization reversal. Concentrations of six cytokines [interleukin (IL)-1 β , IL-6, IL-8, IL-12, IL-13 and tumour necrosis factor (TNF)- α] were measured in serum and PF, and reactive oxygen species (ROS) in PF, and levels were compared among women who were allocated to groups according to their post-surgical diagnosis. Fifty-six patients were diagnosed with endometriosis, eight with idiopathic infertility, 27 underwent tubal ligation or reanastomosis (control group) and 39 were excluded due to bloody PF. Only serum IL-6 and PF TNF- α could be used to discriminate between patients with and without endometriosis with a high degree of sensitivity and specificity ($P < 0.001$). A threshold of 15 pg/ml PF TNF- α provided 100% sensitivity and 89% specificity (positive likelihood ratio of 9.1 and negative likelihood ratio of 0). A threshold of 2 pg/ml for serum IL-6 provided a sensitivity of 90% and specificity of 67% (positive likelihood ratio of 2.7 and negative likelihood ratio of 0.14). **CONCLUSIONS:** By measuring serum IL-6 and PF TNF- α , it was possible to discriminate between patients with endometriosis and those without. Before these markers can be used as a non-surgical diagnostic tool, these data should be verified in a larger study.

Key words: cytokines/endometriosis/non-surgical diagnosis/reactive oxygen species

Introduction

Endometriosis is one of the most common benign gynaecological disorders, and is present in >10% of woman of reproductive age in the USA (Goldman and Cramer, 1989). Although endometriosis can be effectively treated, there is no easy way to diagnose the condition, and at present patients with suspected endometriosis must undergo laparoscopy. Although laparoscopy is a minimally invasive procedure, it requires general anaesthesia and surgical skills when carried out, and there are also potential complications and procedural costs. Thus, a non-surgical diagnostic approach would be of great benefit to both physicians and women alike.

The identification of such a non-surgical approach has led researchers to consider why endometriosis occurs. There is ample evidence to suggest that the immune system may be responsible, and in fact it is considered by some that altered immune responsiveness explains why some women develop endometriosis, whereas others do not (Ramey and Archer, 1993). Moreover, several studies have found that the immuno-

logical components in the peritoneal fluid (PF) of patients with endometriosis play an essential role in the pathogenesis and progression of the disease (Ramey and Archer, 1993). Increased macrophage numbers and activity in the PF also have been documented (Hill *et al.*, 1988).

The endometriosis-associated inflammatory response, tissue repair and neovascularization are dependent on the PF macrophages and their secretory products (cytokines) (Calhaz-Jorge *et al.*, 2000). Cytokines are diverse proteins that play a central role in regulating cell proliferation, activation, motility, adhesion, chemotaxis and morphogenesis. The cytokines—interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10 and tumour necrosis factor (TNF)- α —have been implicated in the pathogenesis of endometriosis (Keenan *et al.*, 1995; Arici *et al.*, 1996; Punnonen *et al.*, 1996; Ho *et al.*, 1997; McLaren *et al.*, 1997; Gazvani *et al.*, 1998; Koninckx *et al.*, 1998; K pker *et al.*, 1998; Zeyneloglu *et al.*, 1998; Garcia-Velasco and Arici, 1999; Iwabe *et al.*, 2000; Shimoya *et al.*, 2000; Mass *et al.*, 2001). Other components such as reactive oxygen species (ROS) have

been detected in the PF of endometriosis patients (Wang *et al.*, 1997), though their role in the disease progression has yet to be determined. The immunological alterations associated with endometriosis are not only local, but also systemic.

Other studies (Keenan *et al.*, 1995; Arici *et al.*, 1996; Punnonen *et al.*, 1996; Ho *et al.*, 1997; McLaren *et al.*, 1997; Wang *et al.*, 1997; Gazvani *et al.*, 1998; Zeyneloglu *et al.*, 1998; Garcia-Velasco and Arici, 1999; Calhaz-Jorge *et al.*, 2000; Iwabe *et al.*, 2000; Koga *et al.*, 2000) have analysed the potential link between cytokines and endometriosis, but were limited in that only one or two cytokines were evaluated, either in the PF or in the serum for a given cohort of patients in an uncontrolled fashion. In addition, none of the studies analysed the inter-relationships between local and systemic cytokine production for the same group of endometriosis patients. Finally, whether these cytokines have any predictive value in terms of non-surgical diagnosis of endometriosis was not determined.

The present study was conducted in an attempt to overcome the limitations of the previous studies and to assess whether endometriosis can be diagnosed non-surgically. A non-surgical screening test would enable physicians to prescribe medical treatment without their patients having to undergo laparoscopy in order to establish a diagnosis. The objectives of this study were to: (i) evaluate a group of cytokines both in the PF and serum of endometriosis patients and in control patients without endometriosis throughout the menstrual cycle; (ii) evaluate ROS in the PF of endometriosis patients and controls; and (iii) assess whether any of these markers can, non-surgically, discriminate between patients with endometriosis and those without.

Materials and methods

Patient enrolment

The study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. Written informed consent was obtained from each patient. The study included patients undergoing laparoscopy for pain, infertility, tubal ligation or sterilization reversal at a minimally invasive surgery unit in a tertiary care referral centre in the mid-West USA. Enrolment took place between 1998 and 2000. Patients with blood-contaminated PF were excluded, and the remaining patients were allocated to groups depending on their post-surgical diagnosis. Blood samples were collected from each patient pre-operatively.

In patients with endometriosis, the severity of the disease was graded according to the revised four-stage American Fertility Society scoring system (American Society for Reproductive Medicine, 1996).

Preparation of serum

Venous blood was withdrawn aseptically into sterile 10 ml tubes containing 0.2 ml heparin at a concentration of 1000 IU/ml. The collected blood samples were centrifuged at 300 *g* for 7 min, and the clear serum was stored at -70°C until taken for analysis.

Preparation of PF

PF was aspirated from the peritoneal cavity from an abdominal port during laparoscopy. ROS levels were measured in the unprocessed sample. The cellular constituents of the PF were removed by centrifugation at 300 *g* for 20 min, after which the supernatants were

removed and stored in aliquots at -70°C until cytokine concentrations were determined.

Measurement of ROS levels

ROS levels were measured in the PF using a Berthold luminometer (Autolumat LB 953; Wallac Inc., Gaithersburg, MD, USA). Aliquots of 400 μl of unprocessed specimens were prepared in duplicate along with a blank and a control. ROS levels were determined with a chemiluminescence assay using luminol (5-amino-2,3 dehydro-1,4 phthalazinedione; Sigma Chemical Co., St Louis, MO, USA) as the probe (Wang *et al.*, 1997). Following the addition of 10 μl of luminol [5 mmol/l; dissolved in dimethyl sulphoxide (Sigma)] to the specimens, measurements were recorded for 15 min in the integration mode. Results were expressed as $\times 10^4$ counted photons per minute (c.p.m.).

Measurement of serum and PF cytokines

Concentrations of IL-1 β , IL-6, IL-8 (PF only), IL-12, IL-13 and TNF- α were measured in serum PF using commercially available, cytokine-specific, enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Inc., Minneapolis, MN, USA). Frozen serum PF samples were thawed and then analysed. Samples from each patient group were always measured in parallel and in duplicate in order to avoid inter-assay variance. The sensitivities of the IL-1 β , IL-6, IL-8, IL-12, IL-13 and TNF- α ELISAs were 1.0, 0.7, 10.0, 5.0, 32.0 and 4.4 pg/ml respectively, with standard curve ranges of 3.9–250, 3.12–300, 31.2–2000, 7.8–500, 62.5–4000 and 15.6–1000 pg/ml respectively.

Statistical analysis

The demographic variables, serum measurements and PF measurements were compared across patient groups with Kruskal–Wallis tests. Pair-wise comparisons between groups were performed using the Wilcoxon's rank-sum test. Results of these analyses were reported using the median and interquartile range (IQR: 25–75th percentile). Comparisons of percentages among the groups were performed with Fisher's Exact tests.

In order to determine which serum marker could best distinguish the patients with endometriosis from those without, a multivariate logistic regression was performed. Sensitivity and specificity were assessed with receiver operating characteristic (ROC) curves. The area under the curve was approximately the percentage correctly classified if the test was used as a diagnostic tool. The same analyses were performed with the PF measures. Positive (sensitivity/100 – specificity) and negative (100 – sensitivity/specificity) likelihood ratios (LRs) were also calculated. Statistical computations were performed with SAS version 8.1 (SAS Institute, Cary, NC, USA), and statistical significance was assessed using two-tailed tests and an alpha level of $P < 0.05$.

Results

Patient demographics

A total of 130 patients between the ages of 18–44 years was enrolled; 39 were excluded because of blood-contaminated PF. Of the remaining 91 patients, 56 were diagnosed with endometriosis, eight with idiopathic infertility, and 27 had undergone tubal ligation or reanastomosis (control group). Each group included patients in the proliferative and luteal phase of the menstrual cycle. Of the 56 patients with endometriosis, 34 had early disease (stage I and II) and 22 had late disease (stages III and IV). The median age of the study cohort

Table I. Serum and peritoneal fluid (PF) cytokine levels in women with endometriosis and idiopathic infertility compared with tubal ligation/reanastomosis (control) group

Variable	Endometriosis			Idiopathic infertility			Controls			<i>P</i> -value ^a			
	<i>n</i>	Median	Percentile 25 75 ^b	<i>n</i>	Median	Percentile 25 75 ^b	<i>n</i>	Median	Percentile 25 75 ^b	Overall	1 versus 2	1 versus 3	2 versus 3
PF ROS ×10 ⁴ cpm	34	124.70	(27.81, 498.78)	8	201.80	(109.97, 596.28)	20	73.85	(24.67, 145.59)	0.08	NS	NS	0.02
Serum cytokines (pg/ml)													
IL-6	20	21.58	(8.22, 60.36)	4	4.37	(1.68, 11.05)	11	0.00	(0.00, 0.28)	< 0.001	0.07	0.001	NS
TNF-α	21	8.33	(1.37, 69.22)	7	1.37	(1.37, 3.98)	22	1.37	(1.37, 1.37)	0.004	NS	0.003	0.07
IL-13	21	44.57	(44.57, 49.87)	7	44.57	(44.57, 44.57)	25	44.57	(44.57, 44.57)	0.34	NS	NS	NS
IL-12	22	0.00	(0.00, 0.00)	7	48.17	(0.00, 96.34)	25	0.00	(0.00, 31.32)	0.43	NS	NS	NS
IL-1β	22	10.98	(8.65, 18.52)	7	15.11	(1.62, 15.46)	23	8.97	(3.97, 15.57)	0.45	NS	NS	NS
Peritoneal fluid cytokines (pg/ml)													
IL-1β	35	3.53	(0.00, 35.96)	8	4.95	(3.77, 97.06)	25	0.00	(0.00, 8.14)	0.12	NS	NS	NS
IL-8	37	14.59	(9.57, 28.80)	7	10.40	(5.02, 39.91)	25	1.04	(0.00, 9.02)	0.004	NS	0.002	NS
L-13	28	1.20	(0.00, 16.73)	7	2.39	(0.00, 4.78)	20	0.00	(0.00, 0.00)	0.15	NS	NS	NS
IL-6	28	39.32	(17.00, 83.96)	7	24.88	(21.92, 27.88)	22	21.94	(15.29, 34.02)	0.21	NS	NS	NS
TNF-α	28	54.83	(26.25, 63.46)	7	11.12	(0.00, 14.89)	12	0.00	(0.00, 0.00)	< 0.001	< 0.001	< 0.001	0.02

^aKruskal–Wallis tests were used to calculate the overall *P*-values, which tested the null hypothesis that all three groups were equivalent; Wilcoxon rank-sum test was used to perform the pairwise comparisons.

^b25, 75 indicates the 25th and 75th percentile.

NS = not significant; ROS = reactive oxygen species.

was 32.5 years (IQR: 29.0, 38.0), median body mass index (BMI) was 24.0 (IQR: 20.8, 27.8), and median parity was 1 (IQR: 0, 2). No significant differences were seen in age, parity and BMI among the three patient groups.

Serum cytokines

Serum IL-6 levels were significantly higher in the endometriosis group than in the control group ($P = 0.001$), and approached significance when compared with the idiopathic infertility group ($P = 0.06$) (Table I). There were no significant differences in the percentage of patients with insufficient serum to obtain a serum IL-6 among the three groups. Serum IL-1β, IL-12 and IL-13 levels were similar in all three groups. Patients in the endometriosis group had higher serum TNF-α levels than the control group ($P = 0.003$), but had comparable levels with the idiopathic infertility group. There was no significant difference in cytokine levels between the follicular and luteal phases of the menstrual cycle. For the endometriosis group, the stage of the disease (early versus late) did not influence cytokine levels (Table I).

PF cytokines

PF levels of IL-1β, IL-6, IL-12 and IL-13 were comparable in all three groups, irrespective of the phase of the menstrual cycle (Table I). IL-8 levels were significantly higher in the endometriosis group than in the control group ($P = 0.002$) (Table I). No significant differences were found between the endometriosis and idiopathic infertility groups. TNF-α levels in the endometriosis group were significantly higher than in the control group ($P < 0.001$) and the idiopathic infertility group ($P < 0.001$). There were no significant differences in the percentage of patients with insufficient TNF-α measures among the three groups. Again, the stage of endometriosis did not affect cytokine levels (Table I).

PF ROS concentrations

ROS levels in the PF of endometriosis patients were not significantly higher than those of the control group, but were significantly higher in the idiopathic infertility group compared with controls ($P = 0.02$). ROS levels were stable throughout the menstrual cycle in all groups. For the endometriosis group, ROS levels did not correlate with the stage of the disease (early versus late) (Table I).

Serum and PF cytokines in predicting endometriosis

Among the serum markers, significant differences were observed with IL-6 and TNF-α. The endometriosis group had significantly higher serum IL-6 ($P = 0.001$) and TNF-α ($P = 0.003$) than the control group. Among the PF measures, endometriosis patients had higher TNF-α levels than both the idiopathic group ($P < 0.001$) and the control group ($P < 0.001$). In addition, the idiopathic group had significantly higher PF TNF-α levels than the control group ($P = 0.02$). Moreover, endometriosis patients had significantly higher PF IL-8 levels ($P = 0.002$) than did controls.

When the idiopathic and control patients were combined (non-endometriosis group; $n = 35$), patients with endometriosis had significantly higher serum IL-6 ($P < 0.001$) and TNF-α ($P = 0.002$), and PF TNF-α ($P < 0.001$) and IL-8 ($P = 0.01$) levels (Table II). In multivariate analyses using all the serum measures, only IL-6 levels could be used to discriminate between patients with endometriosis and those without. None of the other serum measures improved this discrimination. The distribution of serum IL-6 levels among the patient groups is shown in Figure 1. Among the PF measures, multivariate logistic regression analysis showed that only TNF-α could be used to discriminate between patients with endometriosis and those without. The PF levels of TNF-α in the three patient groups is shown in Figure 2.

Table II. Serum and peritoneal fluid (PF) cytokines in endometriosis and non-endometriosis groups

Variable	Non-endometriosis ^a			Endometriosis			P-value
	n	Median	Percentile 25 75 ^c	n	Median	Percentile 25 75 ^c	
Age	35	32.50	(29.00, 38.00)	56	33.00	(29.00, 37.00)	NS
Parity	35	1.00	(0.00, 2.00)	56	0.00	(0.00, 1.00)	NS
BMI	35	26.90	(26.32, 29.02)	56	23.92	(20.76, 27.68)	NS
PF ROS × 10 ⁴ cpm	28	103.73	(38.59, 226.12)	34	124.70	(27.81, 498.78)	NS
Serum cytokines (pg/ml)							
IL-6 ^b	15	0.00	(0.00, 3.37)	20	21.58	(8.22, 60.36)	< 0.001
TNF-α	29	1.37	(1.37, 1.37)	21	8.33	(1.37, 69.22)	0.002
IL-13	32	44.57	(44.57, 44.57)	21	44.57	(44.57, 49.87)	NS
IL-12	32	0.00	(0.00, 31.32)	22	0.00	(0.00, 0.00)	NS
IL-1β	30	9.70	(3.97, 15.46)	22	10.98	(8.65, 18.52)	NS
Peritoneal fluid cytokines (pg/ml)							
IL-1β	33	4.30	(0.00, 13.67)	35	3.53	(0.00, 35.96)	NS
IL-8	32	6.06	(0.00, 10.95)	37	14.59	(9.57, 28.80)	0.01
IL-13	27	0.00	(0.00, 3.59)	28	1.20	(0.00, 16.73)	NS
IL-6	29	24.88	(15.42, 32.47)	28	39.32	(17.00, 83.96)	NS
TNF-α ^b	19	0.00	(0.00, 9.61)	28	54.83	(26.25, 63.46)	< 0.001

^aNon-endometriosis group consists of idiopathic infertility and control group.

^bAmong serum variables, only IL-6 was significant, and among peritoneal fluid variables, only TNF-α was significant in multivariate logistic regression.

^c25, 75 indicates the 25th and 75th percentile.

BMI = body mass index; ROS = reactive oxygen species; NS = not significant.

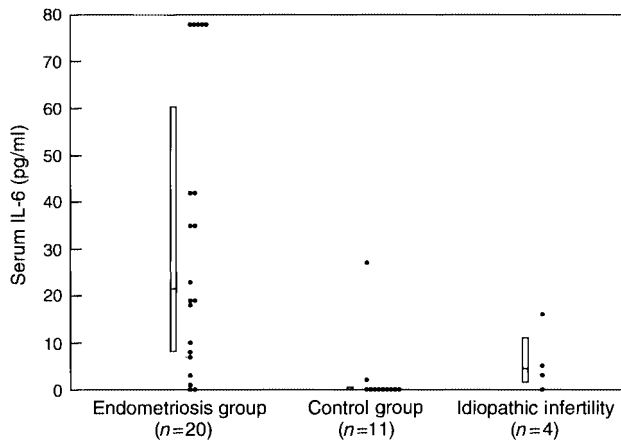


Figure 1. The distribution of serum interleukin (IL)-6 in patients with endometriosis and idiopathic infertility, and in the control group.

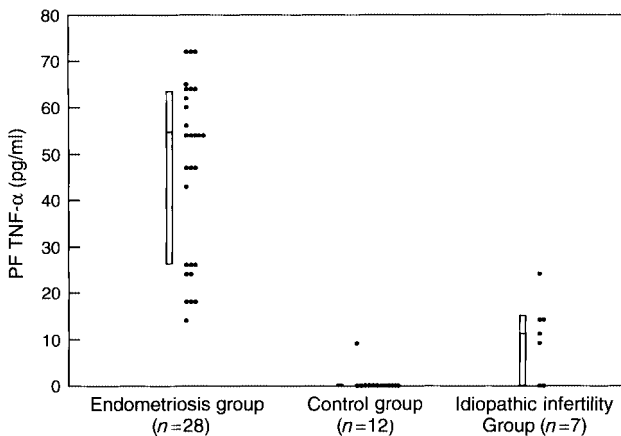


Figure 2. The distribution of peritoneal fluid (PF) tumour necrosis factor (TNF)-α in patients with endometriosis and idiopathic infertility, and in the control group.

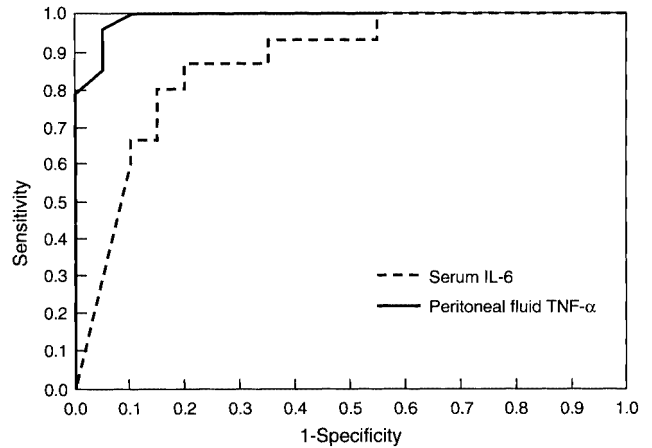


Figure 3. Receiver-operating characteristic curves of the endometriosis versus non-endometriosis groups. The area under the curve for the peritoneal fluid TNF-α was 99%, and that for serum IL-6 was 87%.

PF TNF-α had an exceptional 99% area under the curve (95% CI: 97–100%), indicating a very high discrimination ability (Figure 3). A threshold of 15 pg/ml provided 100% sensitivity and 89% specificity [positive likelihood ratio (LR+) of 9.1 and negative likelihood ratio (LR-) of 0]. A threshold of 20 pg/ml yielded 96% sensitivity and 95% specificity (LR+ 19.2 and LR- 0.04).

Serum IL-6 achieved a relatively high diagnostic value, with an area under the curve of 87% (95% CI: 75–99%; Figure 3). A serum IL-6 threshold of 2 pg/ml provided a sensitivity of 90% and specificity of 67% (LR+ 2.7 and LR- 0.14). A threshold of 4 pg/ml provided sensitivity of 85% and specificity of 80% (LR+ 4.3 and LR- 0.19), and a threshold of 7.5 pg/ml provided sensitivity of 80% and specificity of 87% (LR+ 6.2 and LR- 0.23).

Discussion

The results of the present study indicate that serum IL-6 and PF TNF- α levels can be used to discriminate between patients with or without endometriosis, and with a high degree of sensitivity and specificity. The finding that these measures were not significantly affected by the phase of the menstrual cycle makes them more convenient to apply as reliable diagnostic tests in clinical practice. In addition, neither measure was affected by the stage of the disease, and hence they should be viewed as qualitative diagnostic tests rather than quantitative tests of severity.

IL-6 is a pleiotropic cytokine that is produced by a variety of cell types, including monocytes, lymphocytes, fibroblasts, endothelial cells, keratinocytes and mesangial cells (Ray *et al.*, 1997). The cytokine, which appears to mediate numerous physiological and pathogenic processes, acts on a wide variety of cells and regulates immune responses, acute-phase responses of the liver, haematopoiesis, neuronal functions and osteoclastogenesis (Gorospe *et al.*, 1992). The cytokine may also have important functions in reproductive physiology, including the regulation of ovarian steroid production, folliculogenesis and early events related to implantation (Jacobs *et al.*, 1992; Akoum *et al.*, 1996). Previous studies (Akoum *et al.*, 1996; Tseng *et al.*, 1996) have indicated that both eutopic and ectopic endometrium produce IL-6, and that this may limit the value of serum IL-6 as an independent tool for predicting the presence of endometriosis.

TNF- α is secreted by activated macrophages (Halme, 1989), and has potent inflammatory, cytotoxic and angiogenic properties (Mori *et al.*, 1991). It may also play a role in the progression of endometriosis and its associated infertility. Several studies have shown that TNF- α levels are increased in the PF of women with endometriosis (Eisermann *et al.*, 1988; Mori *et al.*, 1991; Taketani *et al.*, 1992; Overton *et al.*, 1996; Rana *et al.*, 1996). However, other studies have been unable to confirm whether differences occur in TNF- α levels in women with and without endometriosis (Vercellini *et al.*, 1993; Harada *et al.*, 1997). These apparently conflicting results may be due to: (i) differences in the techniques used to assess TNF- α (earlier studies used bioassays based on low-sensitivity kits); (ii) variations in the definition of the studied populations; and (iii) the heterogeneity of the endometriosis disease itself.

In the present study, a marked elevation in TNF- α level was apparent in the PF of patients with endometriosis. The positive and negative LR_s were so good that it is possible that ultrasonographically guided transvaginal aspiration of the PF from the cul-de-sac might serve as a basis for the non-surgical diagnosis of endometriosis.

ROS exert their cytotoxic effects mainly by causing peroxidation of membrane phospholipids, which in turn leads to increased membrane permeability, degraded membrane integrity, inactivated enzymes and structural damage of the DNA; cell death rapidly follows (Halliwell, 1994). ROS levels were not significantly elevated in patients with endometriosis compared with the control and idiopathic infertility groups. In addition, the levels of ROS produced by the PF varied widely, being significantly higher in idiopathic infertility patients than

in controls. This difference indicated that high levels of ROS may contribute to infertility in patients with idiopathic infertility, and consequently, ROS cannot be used as a marker to predict endometriosis.

The present study had two main limitations. First, although it was not possible to obtain sufficient serum and PF to measure all cytokines (including IL-6 and TNF- α) in all of the study population, the complete range of target cytokines was measured in a sufficient number of patients. Second, all bloody PF samples were excluded because cytokine levels might have been affected by blood contamination; consequently, the present findings are not applicable to patients with such PF.

In summary, serum IL-6 and PF TNF- α may be good markers of endometriosis, and permit non-surgical diagnosis. However, such findings must be verified in a larger group of patients and controls before being applied within the clinical situation.

Acknowledgement

This study was supported by a research grant from the Minimally Invasive Surgery Center (MISC) of the Cleveland Clinic Foundation (RPC#2156).

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Submitted on June 14, 2001; accepted on September 28, 2001