Interleukin-6, interleukin-1β, and tumor necrosis factor α in menstrual effluents as biomarkers of chronic endometritis

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Objective: To assess the relationship between chronic endometritis (CE) and proinflammatory cytokine levels in menstrual effluents and to develop a simple noninvasive test for screening CE.
Design: Case-control study.
Setting: Academic center.
Patient(s): Sixty-four women referred to our center for infertility.
Intervention(s): Office hysteroscopy; endometrial biopsy; collection of menstrual blood at subsequent cycle.
Main Outcome Measure(s): Interleukin (IL) 6, IL-1β, and tumor necrosis factor (TNF) α concentrations in menstrual effluents.
Result(s): Thirty-six out of 64 infertile women had histologically proven CE. The remaining 28 women were included as controls. IL-6, IL-1β, and TNF-α levels were markedly higher in menstrual effluents of women with CE compared with control subjects. Receiver operating characteristic curve analysis revealed a good CE screening capacity for all of the cytokines. The combined evaluation of either IL-6/TNF-α or IL-6/IL-1β increased the diagnostic capacity of the test, which reached a 100% sensitivity and a negative predictive value of 100 when at least one cytokine was found to exceed its cutoff value; it also reached a 100% specificity and a positive predictive value of 100 in cases of positivity of both cytokines. Logistic regression analysis confirmed the IL-6/TNF-α–based model as a significant predictor of CE.
Conclusion(s): Proinflammatory cytokine levels are increased in menstrual effluents of women with CE. A test dosing IL-6 and TNF-α seems to have a high screening capacity for CE. (Fertil Steril® 2014;101:242–7. ©2014 by American Society for Reproductive Medicine.)
Key Words: Chronic endometritis, menstrual effluents, cytokines, IL-6, IL-1β, TNF-α

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Chronic endometritis (CE) is a chronic inflammation of the endometrial lining. It is a poorly investigated disorder whose clinical impact, diagnosis, and therapy have not yet been well defined. Clinically, CE is frequently asymptomatic or accompanied by only mild disturbances. Nevertheless, it may account for dysfunctional uterine bleeding (DUB), pelvic pain, and/or reproductive failure (1). With particular reference to this last point, it is worth mentioning that CE was diagnosed in 9.3% of patients with recurrent miscarriages (in 12.9% of patients with miscarriages of unknown etiology) (2) and in 30.3% of patients with repeated implantation failure after IVF-ET (3).

Histologic examination is considered to be the criterion standard for the diagnosis of CE, with plasma cells found in endometrial stroma being the hallmark of the disorder. The presence of lymphocytes, neutrophils, histiocytes, and eosinophils, on the other hand, is not diagnostic for CE, because they are normal components of the endometrial stroma, especially shortly before menses (1, 4).

We have previously demonstrated that fluid office hysteroscopy is a reliable and useful technique for

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diagnosing CE. Compared with the histologic diagnosis, fluid hysteroscopy showed a diagnostic accuracy of 93.4% (5–7). Furthermore, it is a well tolerated and safe procedure. Yet its invasive nature, the need for special skills, and the implied costs hinder its use as a screening technique for CE, which has a low prevalence in the general population.

Looking at different ways to screen CE, Yudin et al. (8) showed that finding vaginal neutrophils had a high sensitivity and negative predictive value (NPV) but a low specificity and a positive predictive value (PPV) for diagnosing upper genital tract infection, including CE. More recently, Kamiyama et al. (9) demonstrated that bacterial endotoxin levels detected in menstrual effluent were inversely correlated with pregnancy rates after IVF-ET.

Following Kamiyama et al.'s reasoning, we may speculate that menstrual effluents of women with CE contain inflammatory mediators that reflect this disorder. Inflammation is, indeed, associated with the release of a number of molecules that can amplify the immune response, either activating dormant cells or recruiting new cells to the area involved in the inflammatory process, including interleukin (IL) 6, IL-1β, and tumor necrosis factor (TNF) α. On the basis of the above data, the present study aimed at: 1) assessing the relationship between CE and proinflammatory cytokines in menstrual effluents; and 2) developing a simple noninvasive test for screening CE on the basis of cytokine levels measured in menstrual effluents.

MATERIALS AND METHODS

Patients

Sixty-four women, referred to the Endoscopic Unit of the Gynecology, Obstetrics, and Neonatology Division of the University of Bari for infertility, were enrolled in this case-control study, which was approved by an Institutional Review Board, after signing informed consents. All women had been trying to conceive for more than 1 year without undergoing any hormonal treatment. All of the women were free from diseases affecting the immune system, cancer, and systemic infections and were not taking any antiinflammatory drugs or corticosteroids. In all of the women gynecologic evaluation was normal; fat and hair distribution, exploration of external genitalia, and speculum examination were normal. Also at bimanual examination, no abnormalities were found. In all of the women chlamydia and gonorrhea testing on cervical swabs was negative. Transvaginal echography was normal as well, and endometriosis had been excluded after diagnostic laparoscopy. Because of the specific expertise achieved by our unit in the field of diagnosis and treatment of CE, 19 out of 64 women were referred to our center following a hysteroscopic diagnosis of CE carried out in other hospitals.

CE was investigated by means of office hysteroscopy and histology in the follicular phase between days 7 and 10. During office hysteroscopy, 0.9% NaCl was used to distend the uterine cavity, with the use of gravity-generated pressure (1 meter above the patient). A lens-based 2.9-mm hysteroscope, a 300-W light source with a xenon bulb, and a 3-CCD digital camera (Karl Storz) were used. Exploration of the uterine cavity consisted of a panoramic view of the cavity followed by examination of both cornua, tubal ostia, and anterior and posterior walls and thorough assessment of the endometrial mucosa by moving the hysteroscope near to each uterine wall to get a view that paralleled the endometrial surface to identify any irregularity of the mucosa. All hysteroscopies were performed by the same operator. The CE diagnosis relied on the demonstration of specific signs such as micropolyps, stromal edema, and focal or diffuse hyperemia (5–7).

Immediately after the complete hysteroscopy, all women underwent an endometrial biopsy with the use of a 3-mm Novak curette connected to a 20-mL syringe. Endometrial samples for histologic examination were fixed in neutral formalin and later embedded in paraffin according to routine histologic procedure. Five microsections were stained with hematoxylin-eosin. A single operator, blinded to the hysteroscopic findings, performed a histologic examination following the diagnostic criteria described in literature (6, 10). Specifically, superficial stromal edema, increased stromal density, pleomorphic stromal inflammatory infiltrate (lymphocytes, neutrophils, eosinophils, and histiocytes) associated with signs of epithelium and/or vessel aggression, and the presence of plasma cells were considered to be signs of CE.

Thirty-six out of 64 infertile women were diagnosed with CE at histology. The remaining 28 women, in whom endometrial biopsy did not reveal any sign of CE, were included as control subjects. In all cases, hysteroscopic findings matched histologic diagnosis. Characteristic features of patients and control subjects are summarized in Table 1.

Menstrual Effluent Sample Collection

All women were asked to return on the second–third day of the subsequent menstrual phase for collection of a menstrual effluent sample. Aspiration of the menstrual blood was performed by inserting a 16-gauge teflon cannula connected to a 10-mL syringe into the cervical canal up to the internal cervical ostium and was absolutely painless. Aspiration was continued until $\geq$0.5 mL menstrual blood was sampled. Samples were immediately centrifuged at 1,500 rpm for 30 minutes and the supernates frozen at -80°C before being used for functional assays.

Cytokine Analysis

IL-6, IL-1β, and TNF-α concentrations were determined by ELISA (Euroclone Life Sciences). To this aim, 96-well flat-bottomed microtiter plates (EIA/RIA plates; Corning Costar) were coated with capture monoclonal antibody in coating buffer (1$\times$ phosphate-buffered saline solution [PBS], pH 7.2-7.4) and incubated overnight at 4°C. After washing with PBS–0.05% Tween-20 (wash buffer), wells were blocked with PBS–5% bovine serum albumin [BSA]–0.1% Tween-20 (blocking buffer) for 2 hours; afterward they were emptied and left to dry for 24 hours. One hundred microliter per well of standards or samples, serially diluted in diluent buffer (PBS–1% BSA–0.1% Tween-20), plus 50 μL/well of biotinylated monoclonal antibody were then dispensed and plates were incubated for 1 hour at room temperature. After

TABLE 1

Characteristics of infertile women with and without a diagnosis of chronic endometritis (CE).

	Positive for CE (n = 36)	Negative for CE (n = 28)	P value
Female age, y[a]	29.3 ± 2.8	30.0 ± 2.7	.301[c]
Male age, y[a]	32.7 ± 3.3	32.3 ± 3.4	.604[c]
Duration of infertility, mo[a]	16.8 ± 2.9	17.1 ± 2.9	.716[c]
Female FSH, IU/L	7.5 ± 1.5	7.0 ± 1.7	.187[c]
Female BMI, kg/m^2	22.5 ± 1.5	22.4 ± 1.4	.906[c]
TMSC, $\times 10^{6}$[b]	103.4 ± 29.5	97.5 ± 22.9	.386[c]
Obstetric history, n			1[d]
Primary infertile	36	28	
Secondary infertile	0	0	
Smoker (female), n			.622[d]
Yes	12	11	
No	24	17	

Note: BMI = body mass index; TMSC = total motile sperm count.
[a] Ages and duration of infertility at time of first visit.
[b] TMSC of the first semen analysis.
[c] Student *t* test for unpaired samples.
[d] χ^2 test for contingency tables.

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extensive washing, 100 μL streptavidin–horseradish peroxidase (HRP; 1:100 diluted in HRP-Streptavidin Diluent Buffer [PBS–0.1% Tween-20–1% BSA]) was added to each well for 20 minutes at room temperature. The plates were washed again and developed with 3,3′,5,5′-tetramethylbenzidine in the dark for 15 minutes at room temperature. The reaction was then stopped by adding 100 μL 1 mol/L H_2SO_4, and the absorbance at 450 nm (reference filter set to 630 nm) was read on the microplate reader ETI-System Fast Reader S800 (Sorin Biomedica).

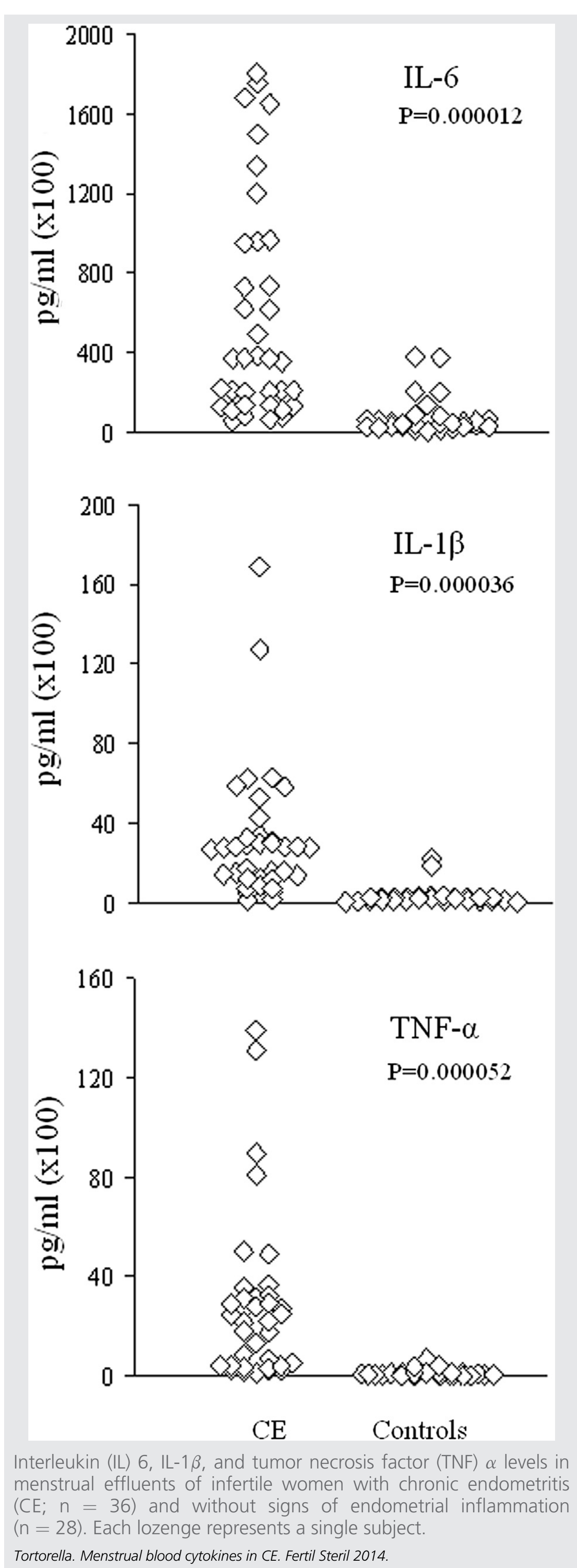
Statistical Analyses

Comparisons between groups were performed by means of the unpaired Student *t* test and χ^2 test for continuous and categoric data, respectively. Receiver operating characteristic (ROC) curve analysis was used to evaluate the role of cytokines in menstrual effluents as a tool for screening or diagnosing CE and to identify a hypothetical cutoff for each cytokine. Relations between cytokines were assessed by Pearson correlation index. Logistic regression analysis was performed to assess the diagnostic power of the single cytokines and their combination, and to achieve a bias-adjusted classification table by means of the jacknifing method. SAS 9.2 and Medcalc 9.2.0.1 were used for the statistic analyses.

RESULTS

Concentrations of IL-6, IL-1β, and TNF-α in menstrual effluents were markedly higher in infertile women with CE compared with control subjects (IL-6: 58,719 ± 55,617 pg/mL vs. 8,099 ± 9,720 pg/mL; t = 4.75; P=.000012; IL-1β: 3,219 ± 3,358 pg/mL vs. 346 ± 491 pg/mL; t = 4.46; P=.000036; TNF-α: 2,885 ± 3,329 pg/mL vs. 141 ± 161 pg/mL; t = 4.35; P=.000052, respectively; Fig. 1). The possibility of using cytokine levels in menstrual effluents to set up a screening test for CE was then evaluated by means of the

FIGURE 1



Interleukin (IL) 6, IL-1β, and tumor necrosis factor (TNF) α levels in menstrual effluents of infertile women with chronic endometritis (CE; n = 36) and without signs of endometrial inflammation (n = 28). Each lozenge represents a single subject.

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ROC curve analysis. The results, illustrated in Figure 2A, show a good performance of all the cytokines, without significant differences among them (AUCs 0.942, 0.917, and 0.957 for IL-1β, IL-6, and TNF-α, respectively). Figure 2B shows the best cutoff determined by ROC curves for each cytokine, as well as the corresponding measures of diagnostic accuracy.

A more detailed analysis of cytokine levels in menstrual effluents collected from women with CE showed a significant correlation between IL-1β and TNF-α levels (r = 0.65; P=.00001), whereas no correlation was detected between IL-6 and IL-1β or between IL-6 and TNF-α levels (r = 0.13 [P=.46] and $r = -0.12$ [P=.48], respectively).

On the basis of the above data, we evaluated the possibility to improve the screening or the diagnostic capacity of the test by combining the values of two cytokines with reference to the cutoffs reported in Figure 2B. Results achieved with this approach are summarized in Table 2. The simultaneous evaluation of two cytokines ruled out the possibility that the test was actually negative in the presence of CE when at least one cytokine exceeded its cutoff value. In this condition, in fact, the test reached a 100% sensitivity and an NPV of 100, a result observed for all cytokine combinations. Moreover, in cases of positivity of both cytokines the test showed a striking increase in its diagnostic capacity. This finding was particularly evident when considering the IL-6/TNF-α and the IL-6/IL-1β combinations, both associated with a 100% specificity and a PPV of 100 (Table 2).

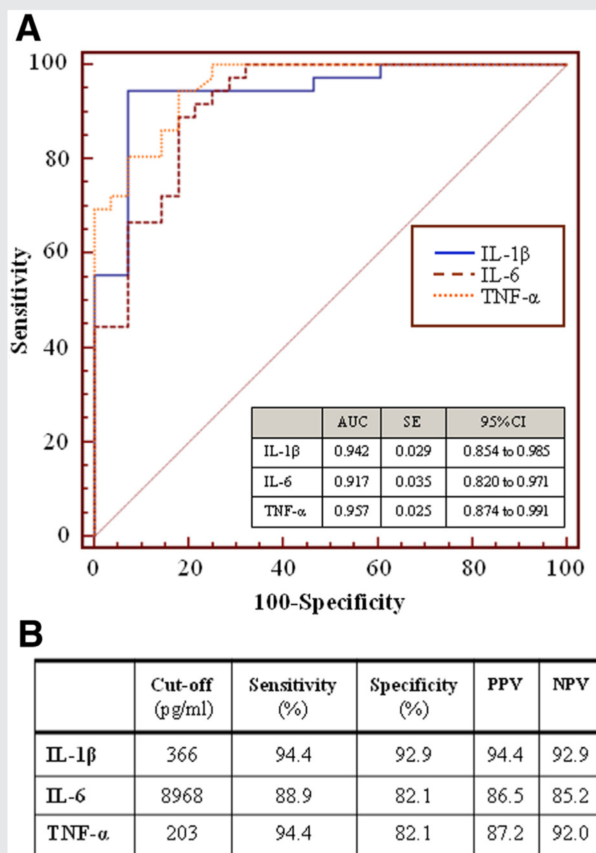
Logistic regression analysis confirmed the role of the cytokines in predicting CE. Applying this method, the IL-6/TNF-α–based model proved to be the best predictor of CE (likelihood ratio test: 72.63; $P<.001$; AUC = 0.989), reaching a sensitivity of 91.7% and a specificity of 96.4%.

DISCUSSION

Owing to its hidden nature, CE is an underdiagnosed pathology. Consequently, the prevalence of CE in women affected by infertility is quite variable, ranging from 3% to >40% (6, 11–14). In our population, CE was diagnosed in 56% of infertile women, such a high prevalence relying on the acquisition of specific diagnostic skills as well as on patient selection. Regarding the latter point, 19 of the 64 infertile women, diagnosed with CE in another center, were referred to our unit for diagnostic confirmation and treatment.

The levels of proinflammatory cytokines, specifically IL-6, IL-1β, and TNF-α, were significantly elevated in

FIGURE 2



	AUC	SE	95%CI
IL-1β	0.942	0.029	0.854 to 0.985
IL-6	0.917	0.035	0.820 to 0.971
TNF-α	0.957	0.025	0.874 to 0.991

	Cut-off (pg/ml)	Sensitivity (%)	Specificity (%)	PPV	NPV
IL-1β	366	94.4	92.9	94.4	92.9
IL-6	8968	88.9	82.1	86.5	85.2
TNF-α	203	94.4	82.1	87.2	92.0

(A) Receiver operating characteristic curve analysis of IL-6, IL-1β, and TNF-α measured in menstrual effluents of infertile women with and without CE. (B) Cutoff values of IL-1β, IL-6, and TNF-α in menstrual effluents and corresponding sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for a CE diagnosis. Other abbreviations as in Figure 1.

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TABLE 2

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for a chronic endometritis diagnosis obtained by combining the values of two cytokines measured in menstrual effluents.

	Sensitivity (%)	Specificity (%)	PPV	NPV
1 or 2 positive vs. both negative				
IL-6 and TNF-α	100	64.3	78.3	100
IL-6 and IL-1β	100	75.0	83.7	100
Both positive vs. 1 or 2 negative				
IL-6 and TNF-α	83.3	100	100	82.4
IL-6 and IL-1β	83.3	100	100	82.4

Note: Il = interleukin; TNF = tumor necrosis factor.

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menstrual effluents of infertile women with CE compared with women with no evidence of CE. These data are in accordance with those of Boomsma et al., who found a significant positive association between elevated levels of IL-1β in endometrial secretions and the presence of bacterial vaginosis (15). These findings are also in keeping with earlier studies indicating that cytokine production in the endometrium may contribute to DUB and reduced endometrial receptivity. In this regard, DUB in levonorgestrel users has been correlated with an altered endometrial expression of IL-13 and IL-15, two key cytokines in inflammatory and immune cell trafficking (16).

Regarding endometrial receptivity, it is worth noting that the endometrium is physiologically colonized by a pleiotropic population of immune-competent cells. The role of these cells is not only to ensure local defense, but also to allow embryo implantation and maintenance during pregnancy by modulating the host reaction against the embryo. In fact, successful implantation and maintenance of pregnancy are the result of a delicate balance between the embryo and endometrium and both seem to be closely dependent on the prevalence of a T_H2 versus a T_H1 cytokine profile at endometrial level (17). Accordingly, any condition affecting this balance may impair endometrial receptivity and fertility.

In this context, CE appears to affect the endometrial microenvironment significantly. During CE, in fact, not only the number but also the quality, type, and distribution of leukocytes in the endometrial tissue is remarkably altered. In a previous study, we found that the secretory endometrium of patients with CE displayed significantly lower percentages of $CD56^+CD16^-$ and $CD56^{bright}CD16^-$ cells and significantly higher percentage of $CD3^+$ cells (18). The increase in natural killer cells shifts the cytokine balance to a prevalence of the T_H1 pathway, with negative effects on implantation and/or trophoblast invasion, thus determining a higher susceptibility to miscarriage in the earlier stages of pregnancy (17, 18).

Our data showing very high levels of proinflammatory cytokines in menstrual effluents of women diagnosed with CE suggest that the endometrial production of these molecules is overexpressed as well. This is likely to be a reflection of a T_H1/T_H2 cytokine imbalance at the endometrial level, where a microenvironment similar to that occurring in other chronic inflammatory diseases, such as rheumatoid arthritis (19, 20) or inflammatory bowel diseases (21), might be present. The prevalence of a T_H1 cytokine profile, in fact, empowers the recruitment and activation of endometrial macrophages with an increased release of IL-1, TNF-α, and IL-6 that are, in turn, able to sustain a chronic inflammatory process. Moreover, the long-term consequences of this condition at the endometrial level are still unknown. Salama et al. (22) showed that in endometrial glandular cells exposed to TNF-α, a rise in aromatasic activity takes place, with a consequent increase in estrogen synthesis at endometrial level, this event being responsible for both functional changes and an enhanced susceptibility to cancer.

Investigating correlations between cytokines, we found that IL-1β and TNF-α levels were correlated with each other but not with IL-6 concentrations. These findings suggest that IL-6 responds to different factors compared with the other two proinflammatory cytokines. We may speculate that IL-6 identifies late stages of inflammation or that elevated levels of the cytokine are associated with unique inflammatory processes occurring as a result of peculiar pathogenic noxae. Regardless of the underlying mechanism, high local levels of IL-6, a well known differentiation factor of mature B lymphocytes (23), might largely account for the presence of plasma cells that is considered to be a histologic hallmark of CE.

The study also aimed at assessing whether measuring cytokines in menstrual effluents might provide a simple noninvasive tool for screening CE. In this regard, the results appear to be quite promising, especially when IL-6 and TNF-α or IL-6 and IL-1β are evaluated together. With both combinations, in fact, if at least one cytokine exceeds its cutoff value a sensitivity of 100% and a NPV of 100 are attained, thus ruling out the possibility of a false negative test. In these conditions, the test still has a good specificity and PPV and may thus be regarded as a screening test. Accordingly, women should be addressed to second-level surveys, such as fluid hysteroscopy combined with endometrial biopsy, for a definitive diagnosis. Notably, this step could even be omitted should both cytokines measured in menstrual effluents result positive. When this condition occurs, in fact, the test might acquire a high diagnostic value and only microbiologic analysis would be eventually necessary to address therapy. Although any test needs to be applied in an independent similar population to be definitively validated (24), logistic regression analysis may overcome the bias due to the use of the same data. Applying this method, we confirm the role of cytokines measured in menstrual effluents in predicting CE, the best screening for CE being achieved with the model based on the combined evaluation of IL-6 and TNF-α.

In conclusion, this study provides evidence that CE is associated with an altered endometrial paracrine milieu, characterized by a predominantly T_H1 cytokine profile sustaining significant levels of proinflammatory cytokines in the menstrual blood. The results of this pilot study may represent a first step for developing a test based on the combined dosage of IL-6 and TNF-α in menstrual effluents for screening CE in women complaining of infertility.

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