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PROFILING SERUM CYTOKINES AS STAGE-SPECIFIC BIOMARKERS FOR ENDOMETRIOSIS: A RETROSPECTIVE STUDY FOR NON-INVASIVE STAGING

PROFILISANJE SERUMSKIH CITOKINA KAO STADIJUMSKI SPECIFIČNIH BIOMARKERA ZA ENDOMETRIOZU: STUDIJA PRESEKA ZA NEINVAZIVNO ODREĐIVANJE STADIJUMA BOLESTI

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Summary

Background: Endometriosis staging according to the revised American Society for Reproductive Medicine (rASRM) criteria relies on invasive laparoscopic surgery. The development of a reliable, non-invasive method to assess disease severity remains a critical unmet clinical need. To identify and validate a panel of serum cytokines capable of distinguishing between different rASRM stages of endometriosis and differentiating patients from disease-free controls.

Methods: In this retrospective study, we enrolled 180 participants: 120 women with surgically and histologically confirmed endometriosis (stratified into Stage I–II, n=60; Stage III–IV, n=60) and 60 healthy, age-matched controls. A multiplex bead-based immunoassay was used to quantify 40 cytokines in serum samples. Machine learning algorithms, primarily Random Forest, were employed for feature selection and the development of a discriminatory biomarker panel.

Results: Univariate analysis identified eight cytokines with significant differential expression across groups: IL-8, TNF-α, VEGF-A, IL-1β, MCP-1, IL-10, IL-17A, and IFN-γ. In internal validation, a combined panel of IL-8, VEGF-A, and TNF-α demonstrated promising discriminatory performance power. This panel differentiated controls from all endometriosis patients with an area under the curve (AUC) of 0.94 (95% CI: 0.90–0.98). Most notably, it distinguished early-stage (I–II) from advanced-stage (III–IV) disease with an AUC of 0.87 (95% CI: 0.81–0.93). Serum

Kratak sadržaj

Uvod: Stadijum endometrioze se prema revidiranim kriterijumima Američkog društva za reproduktivnu medicinu (rASRM) zasniva na invazivnoj laparoskopskoj hirurgiji. Razvoj pouzdane, neinvazivne metode za procenu težine bolesti i dalje predstavlja značajnu kliničku potrebu koja nije zadovoljena. Cilj ovog istraživanja je bio da se identifikuje i validira panel serumskih citokina sposoban da razlikuje različite stadijume endometrioze prema rASRM klasifikaciji, kao i da razlikuje obolele pacijentkinje od zdravih kontrolnih.

Metode: U ovu retrospektivnu studiju je uključeno 180 ispitanica: 120 žena sa hirurški i histološki potvrđenom endometriozom (podeljenih na stadijum I–II, n=60; i stadijum III–IV, n=60) i 60 zdravih kontrolnih subjekata usklađenih po starosti. Za kvantifikaciju 40 citokina u uzorcima seruma korišćen je multipleks imunoesej zasnovan na mikročesticama. Za selekciju karakteristika i razvoj diskriminacionog biomarkera su primenjeni algoritmi mašinskog učenja, prvenstveno »Random Forest«.

Rezultati: Univarijantna analiza je identifikovala osam citokina sa značajno različitom ekspresijom između grupa: IL-8, TNF-α, VEGF-A, IL-1β, MCP-1, IL-10, IL-17A i IFN-γ. U internoj validaciji, kombinovani panel IL-8, VEGF-A i TNF-α je pokazao obećavajuću diskriminativnu moć. Ovaj panel je razlikovao kontrolnu grupu od svih pacijentkinja sa endometriozom sa površinom ispod krive (AUC) od 0,94 (95% CI: 0,90–0,98). Najznačajnije, razlikovao je rani stadijum (I–II) od uznapredovalog stadijuma (III–IV)

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levels of VEGF-A and IL-8 showed strong positive correlations with total rASRM scores.
Conclusion: We identified an internally validated three-cytokine serum signature (IL-8, VEGF-A, TNF-α) with promising diagnostic performance for endometriosis detection and stage discrimination. This serum-based biomarker approach may support future non-invasive triage strategies, pending external validation.

Keywords: endometriosis, biomarker, cytokine, inflammation, non-invasive diagnosis, rASRM staging, IL-8, TNF-α, VEGF-A

sa AUC od 0,87 (95% CI: 0,81–0,93). Serumski nivoi VEGF-A i IL-8 pokazali su snažnu pozitivnu korelaciju sa ukupnim rASRM skorovima.
Zaključak: Identifikovan je interno validiran tročlani serumski citokinski potpis (IL-8, VEGF-A, TNF-α) sa dijagnostičkim performansama za detekciju endometrioze i razlikovanje stadijuma bolesti koje obećavaju. Ovaj pristup zasnovan na serumskim biomarkerima može doprineti razvoju budućih neinvazivnih strategija trijaže, uz potrebu za eksternom validacijom.

Ključne reči: endometrioza, biomarker, citokini, inflamacija, neinvazivna dijagnoza, rASRM stadijum, IL-8, TNF-α, VEGF-A

Introduction

Endometriosis is a chronic, inflammatory gynaecological condition affecting approximately 10% of women of reproductive age, characterised by the presence of endometrial-like tissue outside the uterine cavity (1). The current gold standard for definitive diagnosis and staging remains invasive laparoscopic surgery with histological confirmation (2). The revised American Society for Reproductive Medicine (rASRM) classification system (Stages I–IV) is the most widely used staging tool. Still, it has been criticised for its poor correlation with patient-reported pain and quality-of-life outcomes. This discordance reflects the biological heterogeneity of endometriosis, including variation in lesion phenotype, anatomical distribution, inflammatory activity, immune dysregulation, angiogenic potential, fibrosis, and neural involvement. Thus, anatomical stage alone may not fully capture the underlying biological activity of the disease (3, 4).

A hallmark of endometriosis is localised and systemic inflammation (5). Cytokines, as pivotal signalling molecules, orchestrate inflammatory responses, angiogenesis, cell proliferation, and immune cell recruitment – all processes central to the establishment and maintenance of endometriotic lesions (6). While numerous studies have investigated cytokine alterations in peritoneal fluid (7), the peritoneal compartment represents a local microenvironment that is not easily accessible for routine clinical assessment. In contrast, serum cytokines reflect a systemic, integrated inflammatory state and offer a practical avenue for the discovery of non-invasive biomarkers (8).

There is a biological rationale for investigating cytokines in relation to anatomical disease severity. The rASRM staging system is based on the location, extent, and depth of endometriotic implants, the presence of ovarian endometriomas, and the severity of adhesions. These features are not merely anatomical findings but reflect underlying biological processes, including inflammatory cell recruitment, angiogenesis, extracellular matrix remodelling, fibrosis, and immune dysregulation. Cytokines and chemokines are central mediators of these processes. For example, IL-8 and MCP-1 promote leukocyte recruitment and inflammatory amplification; TNF-α and IL-1β enhance inflammatory signalling, adhesion, and lesion survival; and VEGF-A supports neovascularisation required for the persistence and expansion of ectopic implants. Therefore, increasing anatomical burden may be accompanied by a more pronounced systemic inflammatory and angiogenic cytokine profile.

Previous research has explored serum cytokines primarily for the binary task of disease detection versus no disease (9, 10). Several factors have limited translation into clinical use, including small or heterogeneous cohorts, variable control-group definitions, inconsistent timing of blood sampling across menstrual cycles, limited single-marker or small-panel approaches, lack of correction for confounders, and insufficient validation. Importantly, most prior studies did not evaluate whether serum inflammatory profiles could discriminate between early and advanced rASRM stages (11). Therefore, the ability of a multiplex serum cytokine profile to predict anatomical disease severity preoperatively remains insufficiently investigated (12).

The present study was designed to address this gap by applying a 40-analyte multiplex cytokine platform to a surgically and histologically confirmed cohort, stratifying patients by rASRM stage, standardising blood collection to the early proliferative phase before anaesthesia and surgical manipulation, and evaluating both individual cytokines and a combined cytokine panel for stage discrimination. This study aimed to evaluate whether serum cytokine profiles differ across laparoscopically and histologically confirmed rASRM stages of endometriosis and to identify a candidate cytokine panel capable of distinguishing women with endometriosis from disease-free controls and differentiating early-stage (Stage I–II) from advanced-stage (Stage III–IV) disease.

Materials and Methods

Study population and ethical approval

This retrospective study was conducted using data and archived serum samples from patients treated between January 2024 and December 2025. The Institutional Review Board approved the study protocol, and written informed consent was obtained from all participants. A total of 180 women were were identified from the medical records of the Department of Reproductive Medicine.

Endometriosis group (n=120): Patients scheduled for diagnostic or operative laparoscopy for chronic pelvic pain and/or infertility, with subsequent histological confirmation of endometriosis. Staging was performed during laparoscopy by two independent surgeons using the rASRM scoring system (13). This group was stratified into minimal/mild (Stage I–II, n=60) and moderate/severe (Stage III–IV, n=60).

Control group (n=60): Healthy, age-matched women (±3 years) with no history of chronic pelvic pain, normal gynaecological examination, and no evidence of pelvic pathology on transvaginal ultrasound. All control participants underwent laparoscopic tubal sterilisation for permanent contraception. During the procedure, the pelvis was systematically inspected, and controls were included only if laparoscopic visualisation confirmed the absence of visible endometriotic lesions, adhesions, endometriomas, or other pelvic pathology.

Exclusion criteria: Applied to all participants and included: current pregnancy or lactation; active pelvic inflammatory disease or systemic infection; diagnosis of any autoimmune, inflammatory, or malignant disease; use of hormonal medications (e.g., GnRH agonists, combined oral contraceptives, progestins) within the 3 months before enrolment; use of anti-inflammatory drugs (NSAIDs, corticosteroids) within 2 weeks of sample collection. To minimise potential metabolic and inflammatory confounding of serum cytokine concentrations, participants with known diabetes mellitus, metabolic syndrome, obesity-related systemic inflammatory disease, autoimmune disease, chronic inflammatory disorders, malignancy, active systemic infection, or pelvic inflammatory disease were excluded.

Sample collection and processing

Peripheral venous blood (10 mL) was collected from all participants during the early proliferative phase of the menstrual cycle (days 2–5), before induction of anaesthesia and before any surgical incision or pelvic manipulation (14). For this retrospective analysis, peripheral venous blood (10 mL) had been previously collected from all participants during the early proliferative phase of the menstrual cycle (days 2–5), before induction of anaesthesia and before any surgical incision or pelvic manipulation, and the serum samples were stored at -80 °C. This timing was selected to minimise the potential influence of anaesthesia- or surgery-induced inflammatory changes on circulating cytokine concentrations. Blood was collected in serum separator tubes, allowed to clot at room temperature for 30 minutes, and centrifuged at 2000 × g for 15 minutes. After centrifugation, serum samples were visually inspected for haemolysis and lipemia. Samples showing visible haemolysis or lipemia were excluded from analysis. The separated serum was aliquoted and immediately stored at -80 °C until batch analysis.

Demographic and clinical variables, including age, body mass index (BMI), parity, pain score, infertility status, and rASRM score, were recorded for all participants. BMI was calculated as weight in kilograms divided by height in meters squared and was compared across study groups to assess baseline comparability.

Cytokine measurement

Serum concentrations of 40 cytokines, chemokines, and growth factors were simultaneously quantified using a commercially available validated magnetic bead-based multiplex immunoassay (Human Cytokine/Chemokine/Growth Factor Panel A, Milliplex® MAP, Merck Millipore). The analysed panel included IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IFN-γ, TNF-α, TNF-β, VEGF-A, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Eotaxin/CCL11, IP-10/CXCL10, G-CSF, GM-CSF, FGF-2, PDGF-AA, PDGF-AB/BB, EGF, HGF, NGF-β, SCF, SDF-1α/CXCL12, GRO-α/CXCL1, Fractalkine/CX3CL1, IFN-α2, FLT3 ligand, and TGF-α. Calibration standards were included on each assay plate to generate standard curves for analyte quantification, and manufacturer-provided internal quality-control samples were run on each plate to monitor assay performance. Plates were accepted only if standard curves showed acceptable fit and quality-control values were within the manufacturer's specified ranges. Intra- and inter-assay coefficients of variation were <10% and <15%, respectively.

Statistical analysis

Statistical analysis was performed using R software (version 4.2.1). Continuous variables are presented as median [interquartile range]. The Kruskal-Wallis test with Dunn's post-hoc correction for multiple comparisons was used to analyse differences in cytokine levels across the three groups

(Control, Stage I–II, Stage III–IV). Spearman's rank correlation coefficient (r) was calculated to assess the relationship between individual cytokine levels and total rASRM scores.

Baseline demographic and clinical variables, including BMI, were compared across the three study groups. Continuous variables were analysed using the Kruskal-Wallis test, and categorical variables were analysed using the chi-square test or Fisher's exact test, as appropriate. Because BMI was not significantly different across groups, it was not included as a covariate in the primary biomarker model.

All 40 measured cytokines, chemokines, and growth factors were included in the initial univariate screening analysis. Differences across the three groups were assessed using the Kruskal-Wallis test. To account for multiple testing, p-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure. Cytokines with an adjusted p-value <0.05 were considered statistically significant and were further evaluated using Dunn's post-hoc test for pairwise group comparisons. Cytokines that remained significant after FDR correction were presented in the main results table, whereas the complete dataset for all 40 analytes, including non-significant markers, is provided in Supplementary *Table IV*.

The diagnostic performance of individual cytokines and the combined three-cytokine panel was evaluated using receiver operating characteristic (ROC) curve analysis. Individual ROC curves were generated for IL-8, VEGF-A, and TNF-α separately and compared with the combined logistic regression-derived cytokine score. Analyses were performed for two diagnostic comparisons: all endometriosis patients versus controls, and Stage I–II versus Stage III–IV disease.

The optimal cut-off points for each marker and the combined panel were determined using the Youden index, calculated as sensitivity + specificity -1. Sensitivity, specificity, positive predictive value, and negative predictive value were reported at the cut-off that maximised the Youden index. To reduce overfitting, AUC values for the combined panel were estimated using held-out predictions from repeated stratified cross-validation, with feature selection performed only within the training folds.

Feature selection and predictive modelling were conducted using a Random Forest classifier with 10-fold stratified cross-validation (15). In each repeat, 80% of the data were used for model training, and 20% were held out for validation, with stratification by diagnostic group. Importantly, cytokine feature selection using Random Forest Gini importance was performed within each training fold only. The held-out fold was used exclusively for model evaluation. This nested approach was used to avoid information leakage between feature selection and performance assessment. Features that consistently ranked among the top predictors across cross-validation iterations were selected for the final minimal panel. A logistic regression model incorporating IL-8, VEGF-A, and TNF-α was then used to generate the combined cytokine score, and diagnostic performance was estimated from the held-out validation predictions pooled across cross-validation iterations.

Spearman's rank correlation analysis was performed to evaluate the association between each measured cytokine and total rASRM score among patients with surgically confirmed endometriosis. Correlation analyses were performed for all 40 analytes. To account for multiple testing, p-values were adjusted using the Benjamini-Hochberg false discovery rate method.

Results

Participant characteristics

The demographic and clinical characteristics of the study participants are summarised in *Table I*. The three groups were well-matched for age and

Table I Demographic and clinical characteristics of study participants.

Characteristic	Control (n=60)	Endometriosis Stage I–II (n=60)	Endometriosis Stage III–IV (n=60)	p-value
Age (years), median [IQR]	30 [27–34]	31 [28–35]	32 [29–36]	0.215
BMI (kg/m^2), median [IQR]	22.5 [20.8–24.3]	22.1 [20.5–24.0]	23.0 [21.2–25.1]	0.187
Nulliparous, n (%)	15 (25.0)	42 (70.0)*	35 (58.3)*	<0.001
Visual Analogue Scale for Pain (0–10), median [IQR]	1 [0–2]	7 [6–8]*	8 [7–9]*†	<0.001
Primary Infertility, n (%)	0 (0)	28 (46.7)*	22 (36.7)*	<0.001
rASRM Total Score, median [IQR]	-	12 [8–16]	48 [36–64]†	<0.001

IQR, interquartile range; BMI, body mass index.
*p<0.05 vs Control group (Dunn's post-hoc test)
†*p<0.05 vs Stage I–II group (Dunn's post-hoc test)

Table II Serum concentrations of significantly dysregulated cytokines across study groups.

Cytokine (pg/mL)	Control (n=60) Median [IQR]	Stage I–II (n=60) Median [IQR]	Stage III–IV (n=60) Median [IQR]	p-value (Kruskal-Wallis)
IL-8	5.2 [3.8–7.1]	12.5 [9.4–16.8]*	25.6 [19.3–35.2]*†	<0.001
TNF-α	8.1 [6.5–10.0]	11.8 [9.2–14.5]*	18.9 [14.1–23.0]*†	<0.001
VEGF-A	85.3 [62.1–110.5]	152.0 [120.8–198.4]*	320.5 [245.0–410.7]*†	<0.001
IL-1β	0.4 [0.3–0.6]	0.8 [0.5–1.2]*	1.5 [1.0–2.1]*†	<0.001
MCP-1	120.5 [95.3–150.2]	185.4 [152.0–230.0]*	280.3 [225.6–355.1]*†	<0.001
IL-10	3.5 [2.6–4.5]	3.1 [2.3–4.0]	2.0 [1.5–2.8]*†	<0.001
IL-17A	1.2 [0.8–1.8]	2.5 [1.8–3.5]*	4.8 [3.5–6.5]*†	<0.001
IFN-g	5.0 [3.5–7.2]	9.8 [7.1–13.0]*	7.5 [5.2–10.1]†	<0.001

IQR, interquartile range.
*p<0.05 vs Control group (Dunn's post-hoc test)
†*p<0.05 vs Stage I–II group (Dunn's post-hoc test)

Table III Diagnostic performance of the three-cytokine panel (IL-8, VEGF-A, TNF-α).

Diagnostic task	Marker	AUC (95% CI)	Optimal cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
All endometriosis vs control	IL-8	0.91 (0.86–0.96)	8.3 pg/mL	85	82	90	73
All endometriosis vs control	VEGF-A	0.90 (0.85–0.95)	121.5 pg/mL	82	83	91	70
All endometriosis vs control	TNF-a	0.83 (0.76–0.89)	10.9 pg/mL	75	78	87	61
All endometriosis vs control	Combined panel	0.94 (0.90–0.98)	0.62	90	88	93	83
Stage I–II vs Stage III–IV	IL-8	0.82 (0.74–0.89)	18.1 pg/mL	78	77	77	78
Stage I–II vs Stage III–IV	VEGF-A	0.84 (0.77–0.91)	220.0 pg/mL	80	78	78	80
Stage I–II vs Stage III–IV	TNF-α	0.78 (0.70–0.86)	15.6 pg/mL	73	72	72	73
Stage I–II vs Stage III–IV	Combined panel	0.87 (0.81–0.93)	0.55	83	82	83	82

AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

body mass index (BMI). The prevalence of nulliparity differed significantly across groups and was higher in both endometriosis groups than in controls. Pelvic pain scores differed significantly across groups and were higher in both endometriosis groups than in controls. BMI was analysed as part of the baseline demographic comparison and did not differ significantly among controls, Stage I–II patients, and Stage III–IV patients (p=0.187), suggesting that BMI imbalance was unlikely to account for the observed cytokine differences.

Differential serum cytokine expression

Initial screening of the 40-plex data revealed significant dysregulation of multiple cytokines. *Table II* presents the serum concentrations of the eight cytokines that showed statistically significant differential expression across the Control, Stage I–II, and Stage III–IV groups. Serum concentrations of IL-8, TNF-α, VEGF-A, IL-1β, MCP-1, and IL-17A increased across the three groups, with the lowest median values in controls and the highest in Stage III–IV patients. In contrast, the immunoregulatory cytokine IL-10 was significantly lower in Stage III–IV patients compared to both controls and Stage I–II

Table IV Spearman correlations between serum cytokines and total rASRM score.

Cytokine	Spearman's r	Raw p-value	FDR-adjusted p-value	Direction of association
VEGF-A	0.72	<0.001	<0.001	Positive
IL-8	0.65	<0.001	<0.001	Positive
TNF-a	0.58	<0.001	<0.001	Positive
MCP-1	0.54	<0.001	<0.001	Positive
IL-17A	0.51	<0.001	<0.001	Positive
IL-1b	0.49	<0.001	<0.001	Positive
IL-10	-0.43	<0.001	0.002	Negative
IFN-g	0.18	0.051	0.170	Weak positive
RANTES/CCL5	0.12	0.192	0.480	Weak positive
Eotaxin/CCL11	0.10	0.276	0.590	Weak positive
G-CSF	0.16	0.081	0.250	Weak positive
GM-CSF	0.11	0.231	0.520	Weak positive
FGF-2	0.09	0.328	0.640	Weak positive
PDGF-AA	0.13	0.156	0.430	Weak positive
PDGF-AB/BB	0.14	0.128	0.400	Weak positive
IL-1RA	0.08	0.386	0.690	Weak positive
IL-2	0.06	0.514	0.780	Weak positive
IL-4	-0.05	0.587	0.820	Weak negative
IL-5	0.07	0.446	0.730	Weak positive
IL-6	0.20	0.029	0.120	Weak positive
IL-7	0.05	0.589	0.820	Weak positive
IL-9	0.04	0.663	0.850	Weak positive
IL-12p40	0.09	0.326	0.640	Weak positive
IL-12p70	0.07	0.448	0.730	Weak positive
IL-13	-0.04	0.665	0.850	Weak negative
IL-15	0.15	0.102	0.330	Weak positive
TNF-β	0.12	0.194	0.480	Weak positive
MIP-1α/CCL3	0.17	0.064	0.210	Weak positive
MIP-1β/CCL4	0.15	0.102	0.330	Weak positive
IP-10/CXCL10	0.16	0.081	0.250	Weak positive
EGF	0.10	0.276	0.590	Weak positive
HGF	0.18	0.051	0.170	Weak positive
NGF-β	0.11	0.231	0.520	Weak positive
SCF	0.09	0.328	0.640	Weak positive
SDF-1a/CXCL12	0.12	0.192	0.480	Weak positive
GRO-a/CXCL1	0.19	0.038	0.130	Weak positive
Fractalkine/CX3CL1	0.14	0.128	0.400	Weak positive
IFN-α2	0.08	0.386	0.690	Weak positive
FLT3 ligand	0.06	0.514	0.780	Weak positive
TGF-α	0.07	0.448	0.730	Weak positive

Table V Serum concentrations of cytokines without significant differential expression across study groups.

Cytokine (pg/mL)	Control (n=60) Median [IQR]	Stage I–II (n=60) Median [IQR]	Stage III–IV (n=60) Median [IQR]	p-value (Kruskal-Wallis)
RANTES (CCL5)	8500 [7200–10200]	8900 [7500–10500]	9100 [7800–11000]	0.42
Eotaxin (CCL11)	45 [32–58]	48 [35–62]	50 [38–65]	0.31
G-CSF	22 [15–30]	25 [18–33]	28 [20–36]	0.18
GM-CSF	1.5 [1.0–2.2]	1.6 [1.1–2.4]	1.8 [1.3–2.6]	0.21
FGF-2	12 [8–16]	13 [9–17]	14 [10–18]	0.39
PDGF-AA	1800 [1500–2200]	1900 [1600–2300]	2000 [1700–2400]	0.25
PDGF-AB/BB	4200 [3500–5000]	4400 [3700–5200]	4600 [3900–5400]	0.28

patients. IFN-γ concentrations were higher in Stage I–II patients than in controls and Stage III–IV patients.

Identification of a predictive cytokine panel

Random Forest analysis identified IL-8, VEGF-A, and TNF-α as the top three most important features for classifying patients into Control, Stage I–II, and Stage III–IV groups, based on the Gini importance metric. These cytokines were selected for inclusion in a minimal diagnostic panel.

Diagnostic performance of the cytokine panel

The combined cytokine score was calculated using logistic regression models based on log-transformed serum concentrations of IL-8, VEGF-A, and TNF-α. For discrimination of all endometriosis patients from controls, the model equation was:

$$\text{Logit}(P) = \beta_0 + \beta_1 \times \log(\text{IL-8}) + \beta_2 \times \log(\text{VEGF-A}) + \beta_3 \times \log(\text{TNF-}\alpha),$$

where P represents the predicted probability of endometriosis.

For discrimination of Stage I–II from Stage III–IV disease, the same three-marker structure was used, with P representing the predicted probability of advanced-stage disease.

For all endometriosis versus controls, the final model was:

$$\text{Logit}(P) = [\text{intercept}] + [\beta\text{IL-8}] \times \log(\text{IL-8}) + [\beta\text{VEGF-A}] \times \log(\text{VEGF-A}) + [\beta\text{TNF-}\alpha] \times \log(\text{TNF-}\alpha)$$

For Stage I–II versus Stage III–IV disease, the final model was:

$$\text{Logit}(P) = [\text{intercept}] + [\beta\text{IL-8}] \times \log(\text{IL-8}) + [\beta\text{VEGF-A}] \times \log(\text{VEGF-A}) + [\beta\text{TNF-}\alpha] \times \log(\text{TNF-}\alpha)$$

The diagnostic utility of the three-cytokine panel was evaluated using ROC curve analysis (*Table III*). A combined score derived from logistic regression was used.

ROC analysis demonstrated that each of the three selected cytokines had discriminatory ability, although the combined model outperformed individual markers. For distinguishing all endo-metriosis patients from controls, IL-8, VEGF-A, and TNF-α achieved AUCs of 0.91, 0.90, and 0.83, respectively, whereas the combined three-cytokine panel achieved the highest AUC of 0.94. For distinguishing Stage I–II from Stage III–IV disease, VEGF-A showed the strongest individual performance, with an AUC of 0.84, followed by IL-8 (0.82) and TNF-α (0.78). The combined panel improved discriminatory performance, achieving an internally validated AUC of 0.87. For discriminating between all endometriosis patients (Stages I–IV) and healthy controls, the panel achieved an excellent AUC of 0.94 (95% CI: 0.90–0.98), with a sensitivity of 90% and specificity of 88% at the optimal cut-off point. For the more clinically challenging task of discriminating early-stage (I–II) from advanced-stage (III–IV) disease, the panel maintained strong performance with an AUC of 0.87 (95% CI: 0.81–0.93), sensitivity of 83%, and specificity of 82%.

Correlation with surgical disease severity

Spearman's rank correlation analysis was performed between the total rASRM score and all 40 measured cytokines among patients with endometriosis. After FDR correction, VEGF-A, IL-8, and TNF-α showed the strongest positive correlations with total rASRM score. VEGF-A demonstrated the strongest correlation (Spearman's $r=0.72$, FDR-adjusted $p<0.001$), followed by IL-8 ($r=0.65$, FDR-adjusted $p<0.001$) and TNF-α ($r=0.58$, FDR-adjusted $p<0.001$).

Other cytokines with non-significant variations

In addition to the eight cytokines highlighted above, the multiplex assay quantified the remaining 32 analytes: IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-12p40, IL-12p70, IL-13, IL-15, TNF-β, RANTES/CCL5, Eotaxin/CCL11, IP-10/CXCL10, MIP-1α/CCL3, MIP-1β/CCL4, G-CSF, GM-CSF, FGF-2, PDGF-AA, PDGF-AB/BB, EGF, HGF, NGF-β, SCF, SDF-1α/CXCL12, GRO-α/CXCL1, Fractalkine/CX3CL1, IFN-α2, FLT3 ligand, and TGF-α. These analytes did not show statistically significant differences across the three study groups in univariate analysis after correction for multiple comparisons. They were not highly variable in the Random Forest feature selection process.

Discussion

This study provides preliminary internally validated evidence for a distinct serum cytokine signature associated with the surgical stage of endometriosis. We identified a minimal panel of three cytokines, IL-8, VEGF-A, and TNF-α, that showed promising internally validated performance for detecting endometriosis and for distinguishing between early and advanced rASRM stages.

The biological relevance of this panel is firmly rooted in the pathogenesis of endometriosis. IL-8 is a potent neutrophil chemoattractant and activator that promotes angiogenesis and the proliferation of endometriotic stromal cells (17). Its stepwise elevation aligns with increasing inflammatory infiltrate in lesions. VEGF-A is the master regulator of angiogenesis, essential for the vascularisation and survival of ectopic implants (18, 19). The strong correlation between serum VEGF-A and rASRM score ($r=0.72$) likely mirrors the increasing neoangiogenesis required for larger, deeper, and more vascular lesions characteristic of advanced stages (20). TNF-α, a key pro-inflammatory cytokine, drives a cascade of inflammatory mediators, enhances cell adhesion, and promotes the establishment of endometriotic lesions (21, 22). The coordinated upregulation of these three factors in serum reflects the synergistic processes of inflammation (IL-8, TNF-α) and angiogenesis (VEGF-A, IL-8) that fuel disease progression (23).

The lower serum IL-10 levels observed in Stage III–IV patients are also noteworthy. IL-10 is an immunoregulatory cytokine that helps limit excessive inflammatory responses (24). In advanced endometriosis, reduced IL-10 levels may reflect an imbalance between pro-inflammatory and regulatory immune signalling (25). One possible hypothesis is that chronic inflammatory stimulation may impair compensatory anti-inflammatory responses; however, the present study cannot establish immune exhaustion or define the underlying cellular mechanism (26). Functional immune-cell studies and longitudinal analyses are required to determine whether reduced IL-10 reflects impaired regulatory activity, disease progression, treatment effects, or other immunological processes.

Our findings have direct clinical implications. If confirmed in independent external cohorts, the proposed panel may have potential as a pre-operative triage tool in symptomatic women. A high panel score could prompt earlier surgical planning for suspected advanced disease, while a low score might support a trial of empirical medical management or more conservative surgery for suspected minimal disease (27). This serum-based biomarker approach may support future non-invasive triage strategies for endometriosis, pending external validation in independent cohorts (28). It also introduces the possibility of biological phenotyping, identifying patients with a »high-inflammatory/angiogenic« signature who might be candidates for novel targeted therapies, such as anti-angiogenic or immunomodulatory agents (29).

The strengths of this study include its well-annotated retrospective cohort, rigorous phenotyping with surgical/histologic confirmation, phase-specific sampling, use of a broad multiplex platform, and application of machine learning for unbiased panel selection (30). However, several limitations must be acknowledged. This is a single-centre study, and external validation in an independent, multi-ethnic cohort is essential before clinical implementation (31). The retrospective design prevents assessment of the panel's utility for longitudinal monitoring, which should be the focus of future research. Although repeated stratified cross-validation was used to reduce overfitting and provide internal validation, no independent external validation cohort was included. Therefore, the reported diagnostic performance should be interpreted as preliminary and may overestimate performance in broader clinical populations. External validation in larger, multi-centre, ethnically diverse cohorts with pre-specified model coefficients and cut-off values is essential before clinical implementation. Finally, while we controlled for major confounders, the potential influence of subclinical comorbidities or genetic background on cytokine levels cannot be entirely ruled out.

Conclusion

In conclusion, we identified an internally validated three-cytokine serum signature (IL-8, VEGF-A, TNF-α) that shows promise as a non-invasive biomarker candidate for endometriosis detection and stage discrimination. This panel

captures key pathogenic pathways and correlates with surgical disease severity. By characterising the systemic inflammatory footprint of endometriosis, this work provides a basis for future validation studies aimed at developing clinically actionable blood-based triage tools.

Ethics statement

This study was approved by the Ethics Committee of Ganzhou People's Hospital (Approval No. PJB2026-093-01). Individual written informed consent was exempted because only anonymized leftover serum and archived medical records were analyzed. All procedures were conducted in accordance with the Declaration of Helsinki.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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