

A PREDICTIVE VALUE OF NEURO-FIBROTIC SERUM SIGNATURE INTEGRATES NGF AND TGF- β 1 WITH MARKERS OF DEFECTIVE REPAIR IN ENDOMETRIOSIS OUTCOMES

PREDIKTIVNA VREDNOST NEUROFIBROTIČKOG SERUMŠKOG POTPISA
ZASNOVANOG NA NGF-U I TGF- β 1 UZ MARKERE POREMEĆENE REPARACIJE
ZA PREDVIĐANJE ISHODA ENDOMETRIOZE

Han Dong, Qun Zhang*

Medical Laboratory, Fengjie Affiliated Hospital of Chongqing Three Gorges Medical College,
Chongqing 404600, China

Summary

Background: Endometriosis recurrence after laparoscopic excision remains a significant clinical challenge, with no reliable serum biomarkers for prediction. Current biomarkers fail to capture the full spectrum of recurrence drivers, including neuroinflammation and aberrant tissue repair. This study aimed to determine if a novel panel integrating these pathogenic axes could predict recurrence within 24 months post-surgery.

Methods: A prospective cohort study was conducted on 118 women undergoing their first laparoscopic complete excision of endometriosis. Preoperative serum levels of a 10-biomarker panel were measured using multiplex immunoassays. This panel added Nerve Growth Factor (NGF) and Transforming Growth Factor-beta 1 (TGF- β 1) to an existing inflammatory/fibrotic core (IL-33, sST2, TNFR-I, TNFR-II, IL-1Ra, IL-4, IL-10, PDGF-BB). The primary outcome was clinically significant recurrence within 24 months, defined as the return of endometriosis-associated pain after an initial postoperative symptom-relief interval, together with transvaginal ultrasound evidence of a new endometriotic cyst or deep infiltrating lesion. Asymptomatic imaging-only findings were not classified as primary outcome events.

Results: Recurrence was observed in 29 patients (24.6%). Patients who had a recurrence possessed a unique pre-operative signature, characterised by neuroinflammation

Kratak sadržaj

Uvod: Endometrioza koja se ponovo javlja nakon laparoskopske ekscizije i dalje predstavlja značajan klinički izazov, a pouzdani serumski biomarkeri za njeno predviđanje ne postoje. Trenutno dostupni biomarkeri ne obuhvataju čitav spektar mehanizama koji dovode do ponovne pojave bolesti, uključujući neuroinflamaciju i poremećenu reparaciju tkiva. Cilj ove studije bio je da utvrdi da li novi panel biomarkera, koji integriše ove patogenetske osovine, može da predvidi ponovnu pojavu bolesti u periodu od 24 meseca nakon operacije.

Metode: Sprovedena je prospektivna kohortna studija koja je obuhvatila 118 žena podvrgnutih prvoj laparoskopskoj potpunoj eksciziji endometrioze. Preoperativni serumski nivoi panela od 10 biomarkera određeni su primenom multipleks imunotestova. Panel je proširen dodavanjem faktora rasta nerava (NGF) i transformišućeg faktora rasta beta 1 (TGF- β 1) postojećem inflamatorno-fibrotičkom jezgru koje su činili IL-33, sST2, TNFR-I, TNFR-II, IL-1Ra, IL-4, IL-10 i PDGF-BB. Primarni ishod bila je klinički značajna ponovna pojava bolesti u roku od 24 meseca, definisana kao povratak bola povezanog sa endometrizmom nakon početnog postoperativnog perioda poboljšanja simptoma, uz ultrazvučni dokaz novonastale endometriotične ciste ili duboko infiltrirajuće lezije. Asimptomatski nalazi na snimanju nisu smatrani događajima primarnog ishoda.

Address for correspondence:

Qun Zhang
Medical Laboratory, Fengjie Affiliated Hospital of Chongqing
Three Gorges Medical College, Chongqing
No. 2 Kangning Street, Yufu Subdistrict, Fengjie County,
Chongqing 404600, China
e-mail: 13668492972@163.com

(NGF elevated, $p=0.001$), a profibrotic tendency (TGF- β 1 elevated, $p<0.001$), and a dysregulated alarmin/repair pathway (sST2 elevated and PDGF-BB low, $p<0.001$). Five biomarkers were determined as independent predictors of recurrence via multivariate analysis: TGF- β 1 elevated (OR=2.90, $p=0.001$), NGF elevated (OR=1.35, $p=0.008$), sST2 elevated (OR=2.80, $p<0.001$), PDGF-BB low (OR=0.84, $p=0.01$), and TNFR-I elevated (OR=1.52, $p=0.04$).

Conclusion: NGF and TGF- β 1 combination defines a »neuro-fibrotic repair endotype,« which is a remarkably strong predictor of endometriosis recurrence. Such an endotype, characterised by an autoregulatory cycle of inflammation driven by neural mechanisms and resulting in fibrosis, constitutes a promising new treatment target.

Keywords: endometriosis recurrence, serum biomarkers, nerve growth factor, TGF- β 1, IL-33, sST2, PDGF-BB, personalised medicine

Introduction

Endometriosis, an estrogen-dependent chronic inflammatory disease defined by the presence of endometrial-like tissue outside the uterus, affects 10–15% of women of reproductive age (1). Laparoscopic excision is the gold-standard surgical treatment for symptomatic patients, aiming to restore pelvic anatomy and alleviate pain (2). However, its long-term success is compromised by a significant recurrence rate of 20–50% within 5 years post-surgery (3). Predicting which patients will experience recurrence remains a major unmet clinical need, as current practice relies on symptom reporting and post hoc imaging rather than pre-existing biological predispositions. A reliable, non-invasive predictive tool could revolutionise postoperative counselling and facilitate personalised adjuvant therapies, such as earlier hormonal suppression, to delay or prevent recurrence.

The pathogenesis of recurrence is multifactorial, involving microscopic disease residue, de novo lesion formation, and a »permissive« pelvic environment (4). Central to this pathology is a profound dysregulation of the immune-inflammatory response. Interleukin-33 (IL-33), an alarmin released during cell damage, has been identified as a key modulator of type 2 immunity and fibrosis in endometriosis (5). Its decoy receptor, soluble ST2 (sST2), neutralises IL-33, and an elevated sST2/IL-33 ratio is a signature of chronic inflammatory states (6). Tumour necrosis factor- α (TNF- α) signalling, mediated through its receptors TNFR-I and TNFR-II, drives lesional cell proliferation, adhesion, and the creation of a neo-angiogenic environment (7). Paradoxically,

Rezultati: Ponovna pojava endometrioze zabeležena je kod 29 pacijentkinja (24,6%). Pacijentkinje kod kojih je došlo do ponovne pojave bolesti imale su karakterističan preoperativni biomarkerni profil, obeležen neuroinflamacijom (povišen NGF, $p=0,001$), profibrotičkom sklonošću (povišen TGF- β 1, $p<0,001$) i poremećenom alarminsko-reparativnom signalizacijom (povišen sST2 i snižen PDGF-BB, $p<0,001$). Multivarijantna analiza identifikovala je pet nezavisnih prediktora ponovne pojave bolesti: povišen TGF- β 1 (OR=2,90, $p=0,001$), povišen NGF (OR=1,35, $p=0,008$), povišen sST2 (OR=2,80, $p<0,001$), snižen PDGF-BB (OR=0,84, $p=0,01$) i povišen TNFR-I (OR=1,52, $p=0,04$).

Zaključak: Kombinacija NGF-a i TGF- β 1 definiše »neuro-fibrotički reparativni endotip«, koji predstavlja izuzetno snažan prediktor ponovne pojave endometrioze. Takav endotip, karakterisan autoregulatornim ciklusom inflamacije pokrenutim neuralnim mehanizmima i posledičnom fibrozom, predstavlja obećavajuću novu terapijsku metu.

Ključne reči: ponovna pojava endometrioze, serumske biomarkeri, faktor rasta nerava, TGF- β 1, IL-33, sST2, PDGF-BB, personalizovana medicina

endogenous anti-inflammatory regulators are also highly active. Interleukin-1 receptor antagonist (IL-1Ra) is elevated in the peritoneal fluid of patients and may contribute to progesterone resistance (8), while IL-4 and IL-10 are archetypal Th2 cytokines that promote antibody-mediated responses and suppress cell-mediated clearance of ectopic deposits (9, 10).

Perhaps most critically, the natural history and potential for recurrence of an endometriotic lesion are inseparable from the process of wound healing and fibrosis. Platelet-derived growth factor-BB (PDGF-BB) is the major serum mitogen for mesenchymal cells and is fundamental to tissue repair (11). Prior work established that a core profile of impaired tissue repair, characterised by a decoupled alarmin system (low IL-33, high sST2), persistent inflammation (high TNFR-I), and deficient mesenchymal repair capacity (low PDGF-BB), predicts recurrence. However, endometriosis is not just an inflammatory and fibrotic condition; it is fundamentally a neuroinflammatory disease. Lesions develop their own nerve supply, creating a bidirectional crosstalk between nerve fibres and inflammatory cells that sustains pain and lesional growth (12). Nerve Growth Factor (NGF), a neurotrophin central to this process, is elevated in endometriotic lesions and promotes sensory nerve ingrowth and neurogenic inflammation (13, 14). Its systemic levels as a predictor of surgical outcome remain unexplored.

Moreover, the master profibrotic cytokine Transforming Growth Factor-beta 1 (TGF- β 1) is proposed to underlie the concept of the »wound that never heals.« TGF- β 1 induces EMT, FMT, and the

deposition of excessive extracellular matrix, forming a scarred and adhesive pelvis (15, 16). We proposed that combining NGF and TGF- β 1 with an already established inflammatory fibrosis mediator panel would provide a biological snapshot of the »neuro-fibrotic repair endotype.« The purpose of this study was to evaluate this idea by testing whether a panel of these serum markers could predict recurrence within 2 years of follow-up.

Materials and Methods

Study design and patient population

This is a single-centre prospective cohort study undertaken at a tertiary referral centre for endometriosis. Patient recruitment and the initial collection of sera were conducted between March 2019 and January 2022, while the follow-up after surgery took place in January 2024. During the recruitment process, 176 premenopausal women undergoing surgery for possible or diagnosed endometriosis were enrolled in the study. 38 out of those patients were excluded from the study as they failed to meet the preset inclusion criteria: possible or confirmed adenomyosis from pre-surgical imaging findings ($n=12$), use of hormones within 3 months of surgery ($n=11$), autoimmune, neoplastic, or acute infections ($n=5$), prior surgical excision or planned incomplete excision ($n=6$), and age outside preset limit or not being pre-menopausal ($n=4$). Of the 138 participants, 14 were not enrolled in the study due to either refusal to participate ($n=8$) or inability to fulfil the post-surgery follow-up ($n=6$). Thus, a total of 124 patients underwent pre-surgical serum sample collection and were included in the study.

The study included 118 consecutive, premenopausal women aged 21–45 undergoing their first laparoscopic complete excision of endometriosis. Exclusion criteria were suspected or confirmed adenomyosis by preoperative imaging; any hormonal therapy (e.g., GnRH agonists, oral contraceptives) within three months prior to surgery; confirmed autoimmune, neoplastic, or acute infectious diseases; and refusal to forgo immediate postoperative hormonal therapy. The decision to prescribe postoperative hormonal treatment was standardised; it was only initiated upon clinical confirmation of recurrence. The study was approved by the Institutional Ethics Committee (Protocol No. FY2026-026), and all participants provided written informed consent.

Surgical procedure and follow-up

All surgeries were performed by two experienced laparoscopic surgeons (L.J.Y., L.H.C.). A complete excision of all visible endometriotic lesions was performed, and the disease was staged accord-

ing to the revised American Society for Reproductive Medicine (rASRM) classification (17).

The patients were evaluated at three, six, twelve, eighteen, and twenty-four months following surgery, or earlier if their symptoms recurred. The main criteria for success were the clinical occurrence of a recurrence, which involved moderate or severe pain associated with endometriosis, whether in the form of dysmenorrhea, dyspareunia, or pelvic pain not associated with menstruation, coupled with a transvaginal ultrasonography finding of a new endometriotic cyst of the ovary or deep endometriotic infiltrate. The assessment at 3 months after surgery was necessary to differentiate recurrences that occurred after early postoperative clinical improvement from those with persistent symptoms or partial response (18). Isolated sonographic findings without symptoms were not considered a recurrence event.

Postoperative symptom relief was assessed at the 3-month visit using a structured clinical assessment of endometriosis-associated pain domains. Symptom relief was defined as the absence of patient-reported moderate-to-severe dysmenorrhea, dyspareunia, or non-menstrual pelvic pain and no symptom-driven need for additional medical treatment during the first 3 postoperative months.

This study was approved by the Ethics Committee of Fengjie Affiliated Hospital of Chongqing Three Gorges Medical College (Approval No. FY2026-026). Written informed consent was obtained from all participants prior to enrolment. All procedures were conducted in accordance with the Declaration of Helsinki.

Serum sample collection and biomarker measurement

Sample collection and pre-analytical handling

Fasting venous blood samples (10 mL) were collected from each participant on the morning of surgery, prior to anaesthetic induction, to avoid any potential confounding effect of anaesthetic agents on cytokine levels. Blood was drawn via standard antecubital venipuncture using a 21-gauge butterfly needle into sterile, additive-free silicone-coated serum separator tubes (SST) (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). To allow for complete clot formation, samples were left undisturbed at room temperature (20–25 °C) for exactly 30 minutes, a duration that minimises ex vivo cytokine release or degradation by blood cells. Samples were then centrifuged at 2000 $\times$ g for 10 minutes at 4 °C (Sorvall ST 40R, Thermo Fisher Scientific, Waltham, MA, USA). The resulting supernatant serum was immediately aliquoted into sterile, DNase/

RNase-free 500 μ L polypropylene cryovials (Corning, Corning, NY, USA) to avoid repeated freeze-thaw cycles of the primary sample. Each patient's sample yielded six 200 μ L aliquots. All aliquots were flash-frozen in liquid nitrogen vapour phase for 30 seconds and then stored in a monitored -80 °C freezer (Thermo Scientific Forma 900 series) until batch analysis. The time from blood collection to final -80 °C storage was documented and did not exceed 90 minutes for any sample. A single freeze-thaw cycle was permitted at the time of assay; samples showing any evidence of turbidity or haemolysis upon thawing were excluded.

Selection rationale for the expanded 10-biomarker panel

The expanded panel was designed to capture four interconnected pathogenic axes of endometriosis recurrence. The alarmin and decoy receptor axis was assessed by Interleukin-33 (IL-33), an alarmin released during cell damage that initiates type 2 immune repair responses, and soluble Suppression of Tumorigenicity 2 (sST2), its high-affinity decoy receptor that neutralises IL-33 bioactivity. An elevated sST2/IL-33 ratio signifies a functionally silenced alarmin system. The pro-inflammatory signalling axis was represented by Tumour Necrosis Factor Receptor-I (TNFR-I) and TNFR-II, the cognate receptors mediating TNF- α -driven NF- κ B activation and cell survival, and by Interleukin-1 Receptor Antagonist (IL-1Ra), an endogenous competitive inhibitor of the IL-1 receptor that reflects chronic inflammatory counter-regulation and is implicated in progesterone resistance. The immune polarisation and evasion axis included Interleukin-4 (IL-4) and Interleukin-10 (IL-10), the canonical Th2 and immunoregulatory cytokines that suppress cell-mediated clearance of ectopic endometrial deposits and promote a tolerogenic M2-macrophage phenotype. The tissue repair and fibrosis axis was assessed by Platelet-Derived Growth Factor-BB (PDGF-BB), the major serum mitogen for mesenchymal cells (fibroblasts, smooth muscle cells, pericytes) essential for granulation tissue formation and functional wound closure. This axis was critically expanded with the two novel biomarkers: Nerve Growth Factor (NGF) and Transforming Growth Factor-beta 1 (TGF- β 1).

NGF was chosen as the sentinel marker of the neuroinflammatory axis. Endometriotic lesions are densely innervated by NGF-sensitive sensory and sympathetic nerve fibres, and NGF itself is produced by lesional stromal cells, mast cells, and activated macrophages within the endometriotic microenvironment. Systemically elevated NGF is not merely a correlate of pain but an active driver of neurogenic inflammation; it directly stimulates nerve ingrowth

into ectopic deposits, sensitises nociceptors, and promotes the degranulation of mast cells, which in turn release more NGF, creating a self-perpetuating loop. Its preoperative serum level was hypothesised to reflect the extent of established neuro-inflammatory wiring resistant to surgical excision. TGF- β 1 was chosen as the master regulator of the pro-fibrotic axis. It is the most potent known inducer of epithelial-mesenchymal transition (EMT), fibroblast-to-myofibroblast transdifferentiation (FMT), and excessive extracellular matrix (collagen, fibronectin) deposition. In the context of endometriosis, TGF- β 1 is released from platelets, macrophages, and lesional cells, driving the transformation of the pelvic peritoneum into a rigid, adhesive, and pro-implantation niche. High systemic TGF- β 1 was hypothesised to identify patients with an intrinsically fibrotic repair response who would heal with pathological scar formation rather than functional mesothelial restitution following surgical trauma.

Multiplex immunoassay methodology

All 10 biomarkers were measured simultaneously using a customised magnetic bead-based multiplex sandwich immunoassay panel (Human Premixed Multi-Analyte Kit, Luminex Discovery Assay, R&D Systems, Minneapolis, MN, USA) on the Luminex xMAP technology platform. This technology utilises differentially dyed paramagnetic polystyrene microspheres, each internally labelled with a unique red/infrared fluorophore ratio to define distinct spectral addresses corresponding to individual analytes. Antibodies for each target were covalently coupled to distinct bead regions following the manufacturer's proprietary protocol. The assay was performed according to a standardised, optimised protocol in a 96-well microplate format.

On the day of the assay, patient serum aliquots were thawed once on ice (4 °C) and gently vortexed for 5 seconds to ensure homogeneity. Samples were centrifuged at 10,000 $\times$ g for 5 minutes at 4 °C to remove any precipitated protein or debris, and the clarified supernatant was used immediately. A 50 μ L aliquot of each undiluted serum sample, standard, or quality control was added to the pre-wetted assay plate. The premixed magnetic bead cocktail (50 μ L per well), containing 5000 beads per analyte per well, was added. The plate was sealed with an adhesive foil plate sealer and incubated on a microplate shaker (800 $\pm$ 50 rpm) for 2 hours at room temperature (20–25 °C) in the dark. Following incubation, the plate was placed on a handheld magnetic block for 60 seconds to capture the beads, and the supernatant was decanted. Wells were washed three times with 200 μ L of Wash Buffer (0.05% Tween-20 in PBS, pH 7.4) using a magnetic wash station. A 50 μ L aliquot of the biotinylated detection antibody

cocktail, consisting of polyclonal antibodies specific to distinct epitopes of each target analyte, was added and incubated with shaking for 1 hour at room temperature. After another three washes, 50 μ L of Phycoerythrin (PE)-conjugated streptavidin (SA-PE) reporter solution was added and incubated for 30 minutes. A final three washes were performed, after which the beads were resuspended in 100 μ L of Luminex Sheath Fluid.

Data acquisition and quality control

Plates were read immediately on a MAGPIX CCD-based imaging system (Luminex Corp, Austin, TX, USA) running xPONENT software version 4.3. A minimum of 100 beads per analyte per well were acquired. Median fluorescence intensity (MFI) values were generated for each bead population. Standard curves for each of the 10 analytes were constructed using a five-parameter logistic (5PL) non-linear regression fit from serial dilutions of lyophilised recombinant protein standards reconstituted in a serum-based matrix diluent to control for matrix effects. All standards and controls were assayed in duplicate.

The analytical performance of the multiplex panel in our hands was rigorously validated. For each analyte, the lower limit of quantification (LLOQ) was defined as the lowest standard concentration with a back-calculated recovery of 80–120% and a coefficient of variation (CV) < 20%. Intra-assay precision (repeatability) was assessed by measuring three serum control samples of low, medium, and high concentration (representing the range of patient values) in 20 replicates on a single plate. Inter-assay precision (reproducibility) was determined by measuring the same three control samples in duplicate across 12 independent runs performed on different days by two operators. The intra- and inter-assay CVs for all 10 analytes were below 8% and 14%, respectively. Accuracy was confirmed by spike-and-recovery experiments in pooled normal human serum, with recoveries ranging from 88% to 112% for all analytes. Linearity of dilution was verified by serially diluting five high-concentration patient samples from 1:2 to 1:16; observed versus expected values showed correlation coefficients (r) > 0.99 for all analytes. No significant cross-reactivity was detected between the 10 antibody pairs in the multiplex format, as verified by single-analyte spikes. All sample measurements were performed in a single batch to eliminate inter-batch variability, and operators were blinded to the patient's recurrence status. Results are reported in pg/mL, calculated from the 5PL regression fit.

Analyte-specific lower limits of quantification (LLOQs) were derived from the standard curves and assay-level quality-control data for the custom-

ised multiplex panel. The LLOQ for each biomarker was defined as the lowest calibrator concentration with acceptable back-calculated recovery within 80–120% and CV < 20%, consistent with commonly accepted bioanalytical validation criteria for quantification at the lower limit. The LLOQ values for all 10 analytes are provided in *Supplementary Table S2*.

Assay completeness was assessed before statistical analysis. A valid biomarker result was defined as a concentration within the reportable range of the assay or a value above the LLOQ after 5PL interpolation with acceptable bead count and replicate precision according to the manufacturer's quality-control criteria. All 118 patients included in the final analytic cohort had valid preoperative serum measurements for all 10 biomarkers after assay-level quality control. Therefore, no biomarker values were missing in the final dataset, and no laboratory measurement was excluded because of failed bead acquisition, unacceptable duplicate variability, haemolysis, turbidity, or out-of-range signal after repeat verification.

Statistical Analysis

Sample size considerations were based on both diagnostic-discrimination and prognostic-modelling requirements. Initially, we used the framework described by Hajian-Tilaki for diagnostic accuracy studies to ensure that the expected number of recurrence events would be sufficient to estimate an AUC exceeding 0.80 with acceptable precision. Assuming an anticipated 24-month recurrence rate of approximately 27%, a cohort of at least 100 participants was expected to yield at least 25 recurrence events. However, because the present study also developed a multivariable prognostic model, we considered the number of outcome events in relation to the number of candidate predictor parameters to minimise overfitting. Given the modest number of recurrence events, the multivariable model was restricted to clinically and biologically prespecified predictors, and the final model was interpreted as an exploratory derivation model requiring external validation before clinical implementation (19).

Missing data were assessed separately for clinical variables, follow-up outcomes, and serum biomarker measurements. Patients were included in the final analytic cohort only if they had a complete 24-month outcome assessment and valid measurements for all 10 serum biomarkers. Because no biomarker values were missing among the 118 patients included in the final analysis, all univariate comparisons, multivariable regression analyses, and ROC analyses were performed as complete-case analyses without imputation.

Continuous data were tested for normality using the Shapiro-Wilk test. Comparisons between the non-recurrence and recurrence groups were performed using the Mann-Whitney U test or unpaired t-test as appropriate. Categorical variables were compared using the χ^2 test. Biomarker levels with $p < 0.10$ in univariate analysis were entered into a backward stepwise multivariate logistic regression model to identify independent predictors of recurrence. Receiver operating characteristic (ROC) curves were generated, and the predictive performance of individual biomarkers, the original 3-biomarker model, and the new expanded 5-biomarker model was assessed by the AUC. The DeLong test was used for paired AUC comparisons. A p -value < 0.05 was considered statistically significant. Analyses were performed using SPSS v28.0 (IBM Corp., Armonk, NY) and MedCalc version 20.0 (MedCalc Software, Ostend, Belgium).

Results

Baseline patient characteristics

Of 176 women screened for eligibility, 138 met the prespecified eligibility criteria, and 124 were enrolled with baseline serum sampling before

surgery. Six enrolled patients did not complete the full 24-month follow-up and were excluded from the final analysis, yielding a final analytic cohort of 118 patients with complete biomarker and outcome data. Follow-up completion was 100.0% at 3 months, 99.2% at 6 months, 98.4% at 12 months, 96.8% at 18 months, and 95.2% at 24 months among enrolled patients. The participant flow and reasons for exclusion or attrition are shown in *Figure 1*. Clinically significant recurrence, defined by renewed pain symptoms with sonographic evidence of a new endometriotic lesion, was observed in 29 of the 118 analysed patients (24.6%). Clinically significant recurrence, defined by renewed endometriosis-associated pain together with sonographic evidence of a new lesion, was observed in 29 patients (24.6%). This estimate should therefore be interpreted as the 24-month rate of symptomatic imaging-confirmed recurrence rather than the rate of all anatomical or biological lesion recurrence. The median time to diagnosis of recurrence was 14 months post-surgery (interquartile range [IQR] 11–19). No significant differences were observed between the recurrence and non-recurrence groups regarding age (34.1 ± 5.4 vs 32.8 ± 5.1 years, $p = 0.24$) or body mass index (22.9 ± 3.8 vs 23.6 ± 4.1 kg/m², $p = 0.41$).

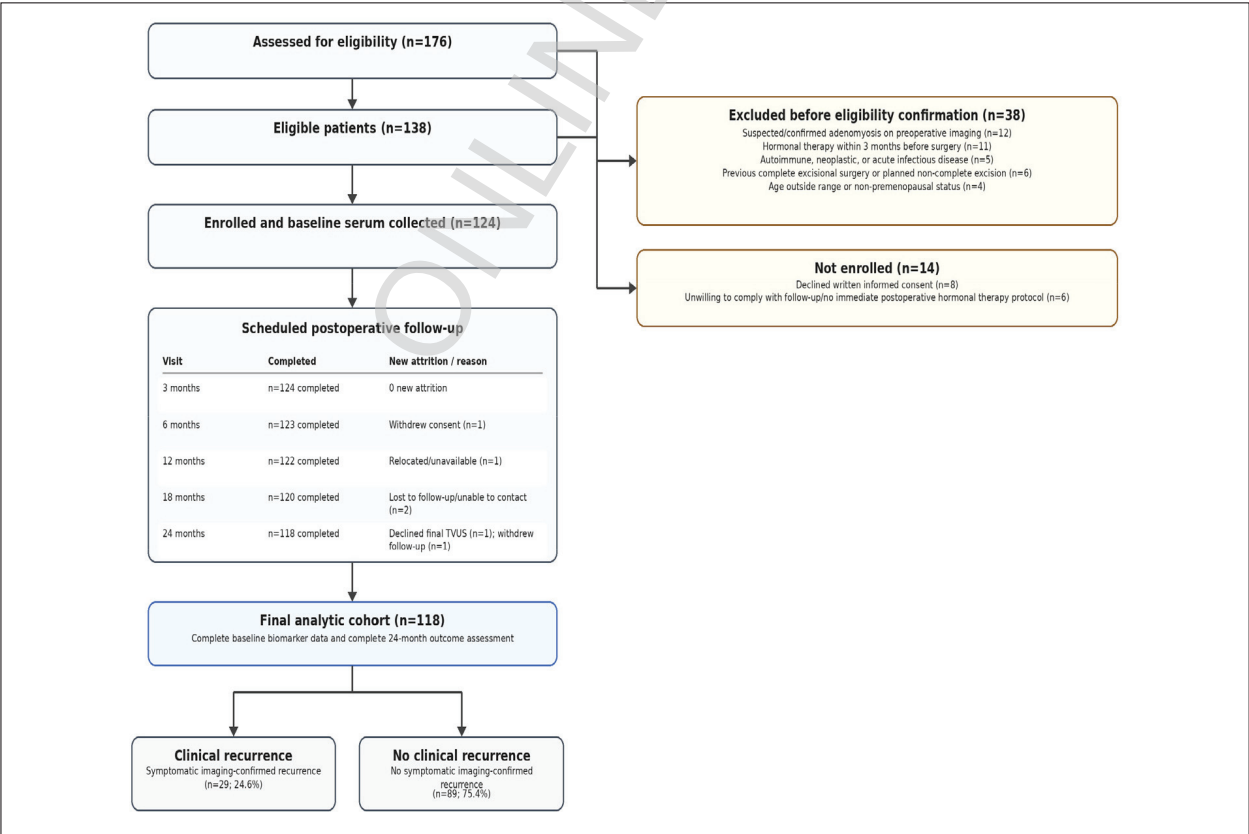


Figure 1 STROBE/CONSORT-style participant flow diagram.

Table I Baseline demographic and clinical characteristics of the study cohort by recurrence status at 24 months.

Variable	Non-Recurrence (n=89)	Recurrence (n=29)	p-value
Demographics			
Age (years), mean $\pm$ SD	32.8 $\pm$ 5.1	34.1 $\pm$ 5.4	0.24
BMI (kg/m ²), mean $\pm$ SD	23.6 $\pm$ 4.1	22.9 $\pm$ 3.8	0.41
Surgical History			
Prior Surgery for Endometrioma, n (%)	5 (5.6)	7 (24.1)	0.008
Intraoperative Findings			
rASRM Stage, n (%)			0.002
Stage I–II	45 (50.6)	5 (17.2)	
Stage III–IV	44 (49.4)	24 (82.8)	
Complete Cul-de-sac Obliteration, n (%)	12 (13.5)	11 (37.9)	0.006
Coexisting Leiomyoma, n (%)	14 (15.7)	4 (13.8)	0.99

rASRM, revised American Society for Reproductive Medicine; BMI, body mass index. P-value <0.05 considered significant

Intraoperative findings proved to be highly discriminating. The recurrence group demonstrated significantly more severe disease, with 82.8% (24/29) classified as rASRM stage III or IV, compared to 49.4% (44/89) in the non-recurrence group ($p=0.002$). Similarly, complete cul-de-sac obliteration, a marker of severe deep infiltrating disease, was present in 37.9% (11/29) of recurrence patients versus only 13.5% (12/89) of those without recurrence ($p=0.006$). A history of prior surgical intervention for an endometrioma was also significantly more prevalent in the recurrence group (24.1% vs 5.6%, $p=0.008$). The full baseline demographic and clinical characteristics are detailed in *Table I*.

Preoperative serum biomarker levels

All 118 patients in the final analytic cohort had valid serum measurements for each of the 10 biomarkers. Thus, biomarker comparisons and prediction models were based on the same 118 complete cases, with no biomarker-specific exclusions or imputation. Assay completeness for each biomarker is summarised in *Supplementary Table S1*.

A profoundly dysregulated systemic environment was confirmed and expanded upon in the recurrence group. All ten measured biomarkers showed significant or directionally consistent differential expression, adding a neuroinflammatory and a fibrotic pillar to the previously described model.

Supplementary Table S1 Completeness of serum biomarker measurements in the final analytic cohort.

Biomarker	Final analytic cohort, n	Successfully assayed, n (%)	Missing values, n (%)	Handling in analysis
NGF	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
TGF- β 1	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
IL-33	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
sST2	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
PDGF-BB	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
TNFR-I	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
TNFR-II	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
IL-10	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
IL-1Ra	118	118 (100.0)	0 (0.0)	Complete-case; no imputation
IL-4	118	118 (100.0)	0 (0.0)	Complete-case; no imputation

A valid assay result was defined as a measurable concentration passing assay-level quality-control checks for bead acquisition, standard-curve interpolation, and duplicate precision. No biomarker values were missing among the 118 patients included in the final analytic cohort.

Supplementary Table S2 Lower limits of quantification for the 10-biomarker multiplex serum panel.

Analyte	Abbreviation	Unit	LLOQ	Source of LLOQ	Acceptance criterion
Nerve Growth Factor	NGF	pg/mL	3.13	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Transforming Growth Factor-beta 1	TGF- β 1	pg/mL	31.25	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Interleukin-33	IL-33	pg/mL	1.56	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Soluble Suppression of Tumorigenicity 2	sST2	pg/mL	15.63	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Platelet-Derived Growth Factor-BB	PDGF-BB	pg/mL	15.63	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Tumor Necrosis Factor Receptor-I	TNFR-I	pg/mL	7.81	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Tumor Necrosis Factor Receptor-II	TNFR-II	pg/mL	15.63	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Interleukin-10	IL-10	pg/mL	0.78	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Interleukin-1 Receptor Antagonist	IL-1Ra	pg/mL	7.81	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%
Interleukin-4	IL-4	pg/mL	0.39	Study-specific assay validation / CoA	Recovery 80–120%; CV <20%

LLOQ was defined as the lowest analyte-specific calibrator concentration on the 5PL standard curve that met predefined acceptance criteria for back-calculated recovery and precision. Values are reported in pg/mL.

The neuro-fibrotic axis (NGF and TGF- β 1)

The most striking new findings were the dramatic elevations in the two novel biomarkers. NGF was significantly higher in patients who would recur (median 135 pg/mL, IQR 85–210) compared to the non-recurrence group (median 72 pg/mL, IQR 48–115; $p=0.001$). Similarly, TGF- β 1 was profoundly elevated in the recurrence group (median 18,500 pg/mL, IQR 12,800–26,100) versus the non-recurrence group (median 9,400 pg/mL, IQR 7,100–13,200; $p<0.001$). This pair defines a systemic loop where NGF-driven neuroinflammation can promote TGF- β 1-mediated fibrosis, and vice versa.

The dysregulated alarmin and repair axis (IL-33/sST2, PDGF-BB)

The previously observed reciprocal dysregulation persisted. sST2 remained profoundly elevated in the recurrence group (median 1250 pg/mL, IQR 880–1810) compared to the non-recurrence group (median 710 pg/mL, IQR 412–1050; $p<0.001$). In contrast, levels of the alarmin IL-33 were significantly depressed in patients who would later recur (median 45 pg/mL, IQR 25–92 vs 82 pg/mL, IQR 48–155; $p=0.005$). The median sST2/IL-33 ratio was approximately five-fold higher in the recurrence

group (28.9 vs 8.7), quantifying a functionally decoupled system where the danger signal is effectively neutralised. This profile suggests a chronic, smouldering inflammatory state resistant to resolution rather than an acute, wound-healing response. PDGF-BB was again dramatically low in the recurrence group (median 480 pg/mL, IQR 290–880) compared to the non-recurrence group (median 1250 pg/mL, IQR 780–2100; $p<0.001$), confirming a fundamental deficiency in mesenchymal repair capacity.

The inflammatory milieu (TNF Receptors, IL-10)

The pro-inflammatory and immunosuppressive signatures were maintained. TNFR-I concentrations were 1150 pg/mL (IQR 790–1520) in patients who recurred versus 820 pg/mL (IQR 590–1100) in those who remained disease-free ($p=0.004$). Similarly, TNFR-II levels were 2520 pg/mL (IQR 1780–3900) in the recurrence group compared to 1950 pg/mL (IQR 1450–2650; $p=0.008$). The anti-inflammatory cytokine IL-10 was significantly elevated in the recurrence group (median 41 pg/mL, IQR 19–75) compared to the non-recurrence group (median 25 pg/mL, IQR 12–42; $p=0.03$). No statistically significant differences were detected for

Table II Comparison of preoperative serum biomarker concentrations between the non-recurrence and recurrence groups (expanded 10-biomarker panel).

Serum Biomarker (pg/mL)	Non-Recurrence (n=89)	Recurrence (n=29)	p-value
Novel Neuro-Fibrotic Markers			
NGF	72 (48–115)	135 (85–210)	0.001
TGF- β 1	9,400 (7,100–13,200)	18,500 (12,800–26,100)	<0.001
Alarmin & Repair Markers			
IL-33	82 (48–155)	45 (25–92)	0.005
sST2	710 (412–1,050)	1250 (880–1810)	<0.001
PDGF-BB	1250 (780–2,100)	480 (290–880)	<0.001
Inflammatory Mediators			
TNFR-I	820 (590–1,100)	1150 (790–1,520)	0.004
TNFR-II	1,950 (1,450–2,650)	2520 (1,780–3,900)	0.008
IL-10	25 (12–42)	41 (19–75)	0.03
IL-1Ra	340 (210–550)	390 (250–620)	0.29
IL-4	15 (8–28)	18 (10–31)	0.41

Data are presented as median (interquartile range). Between-group comparisons were performed using the Mann–Whitney U test. NGF, nerve growth factor; TGF- β 1, transforming growth factor-beta 1; IL-33, interleukin-33; sST2, soluble suppression of tumorigenicity 2; PDGF-BB, platelet-derived growth factor-BB; TNFR-I, tumour necrosis factor receptor-I; TNFR-II, tumour necrosis factor receptor-II; IL-10, interleukin-10; IL-1Ra, interleukin-1 receptor antagonist; IL-4, interleukin-4.

IL-1Ra ($p=0.29$) or IL-4 ($p=0.41$). The complete comparison of the 10-biomarker panel is presented in *Table II*.

Independent predictors of endometriosis recurrence

To determine if these biomarkers could predict outcome independently of disease severity, a multivariate logistic regression model was constructed. Variables with $p<0.10$ from univariate analyses were entered, including the significant biomarkers (NGF, TGF- β 1, IL-33, sST2, PDGF-BB, TNFR-I, TNFR-II, IL-10) and the clinical confounders of rASRM stage and prior surgical history.

The analysis revealed that a final five-biomarker neuro-fibrotic signature emerged as independently predictive. The strongest predictors were a 500 pg/mL increase in TGF- β 1 (aOR 2.90, 95% CI 1.55–5.42, $p=0.001$) and a 100 pg/mL increase in sST2 (aOR 2.80, 95% CI 1.52–5.17, $p<0.001$). This was followed by a 10 pg/mL increase in NGF (aOR 1.35, 95% CI 1.08–1.69, $p=0.008$), a 100 pg/mL decrease in PDGF-BB (aOR 0.84, 95% CI

0.73–0.97, $p=0.01$), and a 50 pg/mL increase in TNFR-I (aOR 1.52, 95% CI 1.02–2.27, $p=0.04$). IL-10, TNFR-II, and IL-33 lost independent significance; their variance is now captured more precisely by this core neuro-fibrotic network. Notably, the rASRM stage did not emerge as an independent predictor when the biomolecular profile was accounted for (aOR 1.64, 95% CI 0.50–5.41, $p=0.41$), underscoring that the systemic biological risk captured by the blood test supersedes the purely anatomical classification. The full regression model, which had a Nagelkerke R^2 of 0.68, is detailed in *Table III*.

Predictive performance of the biomarker panels

The diagnostic accuracy of the final five-biomarker model was assessed using Receiver Operating Characteristic (ROC) analysis and compared to the original three-biomarker core model (sST2, PDGF-BB, TNFR-I). The new 5-biomarker neuro-fibrotic panel demonstrated outstanding discriminative power for predicting 24-month clinical recurrence, yielding an area under the ROC curve (AUC) of 0.93 (95% CI 0.88–0.98). This performance was

Table III Multivariate logistic regression analysis for independent prediction of endometriosis recurrence (5-biomarker neuro-fibrotic signature).

Predictor Variable (Scale)	Adjusted Odds Ratio	95% Confidence Interval	p-value
TGF- β 1 (per 500 pg/mL increase)	2.90	1.55–5.42	0.001
sST2 (per 100 pg/mL increase)	2.80	1.52–5.17	<0.001
NGF (per 10 pg/mL increase)	1.35	1.08–1.69	0.008
PDGF-BB (per 100 pg/mL decrease)	0.84	0.73–0.97	0.01
TNFR-I (per 50 pg/mL increase)	1.52	1.02–2.27	0.04
rASRM Stage (III/IV vs I/II)	1.64	0.50–5.41	0.41
Previous Surgery (Yes vs No)	2.45	0.58–10.30	0.22

Variables with $p < 0.10$ from univariate analyses were entered into the model. The rASRM stage and prior surgical history were included as clinical confounders. Model Nagelkerke $R^2 = 0.68$.

significantly superior to the original 3-biomarker model, which had an AUC of 0.88 (95% CI 0.81–0.95; DeLong test $p = 0.02$). For individual biomarker comparison, the AUC for TGF- β 1 alone was 0.82 (95% CI 0.73–0.91), for NGF alone was 0.74 (95% CI 0.63–0.84), for sST2 alone was 0.78 (95% CI 0.68–0.88), and for PDGF-BB alone was 0.79 (95% CI 0.70–0.89).

To evaluate clinical utility, a risk score cut-off was selected to maximise both sensitivity and specificity on the ROC curve.

At the optimal threshold, the 5-biomarker panel achieved a sensitivity of 89.7%, specificity of 88.8%, positive predictive value of 72.2%, and negative predictive value of 96.3% for symptomatic imaging-confirmed recurrence. These estimates indicate that the model was particularly strong in identifying patients at low risk of clinically significant recurrence within 24 months. However, because asymptomatic imaging-only recurrence was not included in the primary endpoint, the negative predictive value should not be interpreted as excluding all biological or anatomical recurrence.

These performance metrics confirm that the integration of NGF and TGF- β 1 with sST2, PDGF-BB, and TNFR-I captures synergistic predictive information that is not accessible from any single biomarker, from the original 3-biomarker model, or from conventional clinical staging alone.

Discussion

This prospective study demonstrates that by integrating NGF and TGF- β 1, the predictive model is elevated from a »defective repair endotype« to a more comprehensive »neuro-fibrotic repair

endotype« for endometriosis recurrence. The 5-biomarker panel – high TGF- β 1, NGF, sST2, TNFR-I, and low PDGF-BB – paints a picture of a system primed for lesion re-formation through a vicious cycle of nerve-driven inflammation and pathological scarring that outlasts surgical excision.

The independent association of high NGF with recurrence is a pivotal finding. NGF is the master link between inflammation and neurogenesis in endometriosis, stimulating sensory nerve sprouting into lesions (13). This neurogenic inflammation releases pro-inflammatory and pro-fibrotic mediators, creating a feed-forward loop. Our data suggest that high systemic NGF identifies patients whose »pain wiring« and neuro-inflammatory drive are already established, a process not erased by simply removing the lesions. This aligns with clinical observations that pain recurrence does not always correlate with the extent of visible disease, as the neural architecture of pain may be fundamentally altered in these patients.

The emergence of TGF- β 1 as a top-tier predictor solidifies the central role of fibrosis in recurrence. TGF- β 1 orchestrates the pathological transformation of pelvic mesothelium into a scarred, adhesive substrate ripe for re-implantation (15). Its elevation, alongside the deficiency in PDGF-BB – a mediator of functional wound closure – indicates a profound imbalance. The system is skewed away from regenerative healing and toward a TGF- β 1-dominant, fibrotic repair process. This combination creates the »wound that never heals,« where a sclerotic peritoneum fosters recurrence (20–22).

The high sST2/IL-33 ratio fits into this neuro-fibrotic model. By neutralising IL-33's alarmin function, excess sST2 may cripple a crucial homeostatic pathway required for non-fibrotic tissue repair,

leaving unchecked TGF- β 1 activity (23). In a homeostatic response, nuclear IL-33 is released as an alarmin, promoting a type 2 immune response and activating PDGF receptor- α + fibroblasts. In our study, the recurrence group showed a systemically »decoupled« state, with low free IL-33 and an excess of its decoy receptor, sST2. This likely indicates a failed alarmin response, where IL-33 is consumed or neutralised before it can drive a productive repair process, leaving behind a silent, fibrotic environment (24, 25). The core inflammatory drive, marked by TNFR-I, synergises with this environment, sustaining the NF- κ B activation that fuels both lesional cell survival and neuroinflammation (26–29). Together with elevated IL-10, a potent immunosuppressive cytokine produced by T-regs and macrophages, our data paint a picture of a dominant type 2/immunosuppressive repair response that is inherently fibrotic and permissive for the re-establishment of ectopic endometrial tissue (30–33).

The clinical implications of these findings are significant. The high AUC of 0.93, with a specificity of 88.8% and a negative predictive value of 96.3%, suggests that a single preoperative blood test could effectively triage patients' risk after surgery. For a patient deemed high-risk by the 5-biomarker panel, immediate postoperative hormonal suppression with dienogest, which has been shown to reduce recurrence (34, 35), could be strongly recommended rather than taking a »wait-and-see« approach. The utility is clear when an observational strategy can be selected for low-risk patients with a 96.3% confidence that they will not recur within 24 months. The independence of our biomarker panel from the rASRM stage as a predictor is particularly important, as it captures a biological risk profile that anatomy alone does not. This suggests that the systemic neuro-fibrotic predisposition is a more powerful determinant of outcome than the surgically assessed disease burden (36).

The strengths of this study include the prospective design with strict inclusion and exclusion criteria, eliminating the confounding effect of immediate postoperative hormonal therapy. The measurement of a rationally designed, hypothesis-driven panel that spans neuroinflammatory, fibrotic, alarmin, and inflammatory axes is both a strength and a limitation, as other potentially relevant mediators like activin A or other TGF- β superfamily members were not assessed (30). The 24-month follow-up, while practical, may underestimate the total recurrence rate, which continues to accumulate with time (31). Additionally, the single-centre design and relatively modest sample size, while adequately powered for the primary analysis, necessitate external validation in larger, multi-ethnic cohorts before clinical integration. Future studies should also assess the stability of this neuro-fibrotic signature across the menstrual

cycle and its predictive value in patients who receive immediate postoperative medical therapy. A further statistical limitation is the limited number of recurrence events relative to the number of candidate biomarkers considered during model development. Although the observed event count was sufficient for the planned exploratory assessment of discriminatory performance, it may still permit optimism in multivariable regression coefficients and apparent AUC estimates. Therefore, the proposed five-biomarker signature should be interpreted as a derivation-stage prognostic model rather than a ready-to-use clinical test, and its calibration, discrimination, and clinical utility must be confirmed in an independent external cohort (37, 38).

The definition of recurrence used in this study should also be considered when interpreting the model. We intentionally selected symptomatic imaging-confirmed recurrence as the primary endpoint because it represents a clinically actionable event that is directly relevant to postoperative counselling and treatment decisions. However, this conservative definition does not capture asymptomatic imaging-only recurrence, which has been reported after endometriosis surgery and may represent biological or anatomical lesion recurrence before symptom relapse. Patients with asymptomatic sonographic recurrence who were classified as non-recurrence events in the present analysis could have introduced outcome misclassification. If such patients shared the same neuro-fibrotic biomarker profile as symptomatic recurrence cases, the associations observed in this study may be conservative, and the model may underestimate risk for broader biological recurrence. Conversely, if NGF-driven neuroinflammatory activity is more closely related to pain recurrence than to silent lesion reappearance, the current model may be more specific for clinically significant symptomatic recurrence than for anatomical recurrence alone. Therefore, the reported recurrence rate of 24.6% and the predictive performance metrics should be interpreted as applying to symptomatic imaging-confirmed recurrence within 24 months, not to all forms of postoperative endometriosis recurrence. Future validation cohorts should prespecify both a primary clinical recurrence endpoint and a secondary broader endpoint that includes asymptomatic imaging-confirmed recurrence, allowing sensitivity analyses to test whether the five-biomarker signature performs similarly across clinical and anatomical recurrence definitions.

Conclusion

The integration of NGF and TGF- β 1 defines a »neuro-fibrotic repair endotype« that is a powerful, independent predictor of endometriosis recurrence after laparoscopic excision. This preoperative 5-bio-

marker serum signature – high TGF- β 1, NGF, sST2, TNFR-I, and low PDGF-BB – reflects a self-perpetuating interplay of neuroinflammation and pathological scarring that surgical excision alone cannot cure. With an AUC of 0.93 and a negative predictive value of 96.3%, this panel offers a precise tool for personalised postoperative management, enabling clinicians to confidently triage patients to immediate

adjuvant therapy or expectant observation based on their biological risk profile.

Conflict of interest statement

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

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