Enhanced analysis of endometriosis patients' plasma using #Enzian annotation highlights potential biomarkers for early-stages of disease

Check for updates
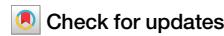
Daniel Rodriguez Gutierrez[1], Alina Astourian[1], Marianne Spalinger[1], Lucie Berclaz[1], Monique Hartmann[1], Julian Metzler[2], Isabell Witzel[2], Patrick Imesch[2], Valentina Vongrad[1] & Brigitte Leeners[1] ✉

Endometriosis is a chronic inflammatory condition marked by the presence of endometrial-like tissue outside the uterus, often co-occurring with leiomyoma and presenting a diagnostic challenge. We analyzed 96 plasma cytokines and inflammatory markers in 86 women undergoing surgery for suspected endometriosis, using multiplex assays and unsupervised clustering methods. Patients were classified using both rASRM and the more granular #Enzian system to assess lesion-specific and stage-specific biomarker patterns. We identified five distinct patient clusters reflecting disease heterogeneity, with improved resolution using the #Enzian classification. Notably, the presence of leiomyoma influenced cytokine profiles, potentially obscuring biomarker signals. Key biomarkers including perforin, TRAIL, sFasL, IL-17F, PDGF, VEGFA, and MCP-2 were associated with disease presence and stage. These findings highlight the value of advanced classification systems and emphasize the importance of accounting for comorbid conditions. Our results support the development of non-invasive biomarker panels for earlier and more accurate diagnosis of endometriosis.

Endometriosis (EM) is a chronic gynecological disorder characterized by the presence of endometrium-like tissue at ectopic locations, including the ovaries, recto-sigmoid, fallopian tubes, and pelvic peritoneum[1]. Although the exact incidence and prevalence of EM is difficult to determine, it is estimated to affect 5–10% of women of reproductive age worldwide, imposing a significant burden on patients' lives through chronic pelvic pain, fatigue, infertility, and diminished quality of life[2,3]. The most common types of pelvic EM are superficial peritoneal lesions (15–50% of EM patients), deep-infiltrating lesions (DE, 20%), and endometriomas (2–10% of EM patients and 50% of women treated for infertility)[4].

Historically, researchers have viewed endometriotic lesions as a single, uniform disease phenotype[5]. However, extensive heterogeneity has been demonstrated in endometriotic lesions, even among lesions from the same patient[6]. This diversity challenges traditional histological classifications, often rendering them ineffective in precise diagnosis but also in guiding treatment. It also highlights the need for more nuanced approaches to diagnosis, classification, and treatment of what accounts for the complex and variable nature of the disease. In clinical practice, better outcomes are often observed when endometriosis surgeries are performed by experienced teams in specialized centers, where comprehensive care and advanced surgical expertise contribute to more effective symptom relief and reduced recurrence[7].

Irrespective of its heterogeneity, EM is considered an inflammatory disease, marked by increased levels of activated macrophages and cytokines, such as interleukins, and tumor necrosis factor-alpha (TNF-α), in the peritoneal fluid of affected women[8]. Several inflammation-related biomarkers show elevated levels in the plasma of women with EM, including annexin A2 (ANXA2), fibronectin, collagen IV, C-reactive protein (CRP), and monocyte chemoattractant protein-1 (MCP-1)[9–12]. Moreover, this was also the case for a wide range of other molecules, such as glycoproteins, growth factors, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and various proteins associated with angiogenesis and immune response pathways[13].

With the current lack of efficient biomarkers, the gold-standard for diagnosing EM is laparoscopic pathological biopsy when imaging studies are unremarkable[14]. Furthermore, there is currently no treatment available

[1]Department of Reproductive Endocrinology, University Hospital Zurich, Zurich, Switzerland. [2]Department of Gynecology, University Hospital Zurich, Zurich, Switzerland. ✉e-mail: Brigitte.Leeners@usz.ch

to entirely cure EM. Hormonal therapies fail in up to 30% of cases[15], recurrence is common after surgical resection (30% of cases)[13], and, for 55% of EM patients, current medications offer only limited symptom relief in the long term[16,17], underscoring the urgent need for novel diagnostic and therapeutic targets. Ideally, a noninvasive biomarker for diagnosing EM would be derived from serum or plasma, as well as saliva, urine, or menstrual effluent, offering a valuable tool for the differentiated diagnosis of different endometriotic lesions and therapeutic monitoring of patients alongside standard clinical evaluations.

Emerging technologies such as proteomics, metabolomics, and genomics, allow exploration of extensive panels of molecules or gene profiles. These technologies represent a significant advancement in the field, potentially eliminating the need for invasive procedures like laparoscopies for diagnostic purposes. Despite the promise of these advanced approaches, the clinical translation of biomarkers for EM remains challenging. To date, no single biomarker or panel has been validated with the specificity and sensitivity required for routine clinical diagnostic use[9].

Additionally, comorbid conditions and medications can confound proteomic and metabolic profiles, making it challenging to distinguish disease-specific signals from those arising due to coexisting conditions. Adjusting models for comorbid conditions and medication has been shown to result in a notable reduction of significant associations and unique predictors, particularly in diseases with high comorbidity rates[18–20]. These findings emphasize the necessity for research to incorporate both comorbidity profiles and medication regimens as covariables to enhance the precision of disease-specific risk factor identification, ultimately aiding the development of more effective diagnostic and treatment strategies for chronic diseases.

Therefore, the aim of the present study was to evaluate whether plasma cytokines and inflammatory markers could facilitate the diagnosis of EM based on the most recent annotation systems rASRM and #Enzian. We also investigated whether specific biomarkers were associated with (i) disease stage, (ii) specific lesion types according to #Enzian annotation, and how (iii) potential confounders such as myoma or medication influence biomarker findings.

Results

Lack of distinct cytokine profile differences between EM patients and controls without clustering

The comparison of 96 cytokine markers of women diagnosed with EM with those of control women without considering the presence of myoma did not reveal any clear group separation nor did it identify significantly regulated markers, even when all patients' metadata were incorporated in the analysis (Fig. 1a and b, Supplementary Table 3). Although we observed significant differences between women presenting with EM and control women with regard to age and parity (Table 1), these did not result in any differences between biomarker profiles.

Comparison of biomarkers in women with and without EM and/or myoma: Myoma obscures the differences between healthy subjects and EM patients

In our cohort, among the 65 patients diagnosed with EM, 18 (27.7%) were also positive for myoma. In contrast, within the control (no EM) group, 11 out of 21 individuals (52.4%) presented with myoma (Fig. 1c). To explore the impact of myoma on plasma marker levels, we compared controls without myoma with those in whom myoma had been detected, as well as EM patients with and without myoma (Figure 1d–h, and Supplementary Tables 1 and 4). In patients with myoma as a single condition, we observed a significant reduction in the plasma levels of perforin, IL-31, CXCL16, and ENA-78 compared to controls (Fig. 1d). Perforin was also significantly reduced in EM patients without myoma (Fig. 1e). In cases where EM and myoma coexisted, a combined effect of both conditions was evident, with significant reductions in perforin, CXCL16, and TRAIL when compared with controls without myoma (Fig. 1f). The overlapping impact of these two conditions on plasma markers was further highlighted by the absence of

differentially expressed markers when patients with EM alone were compared with those with myoma alone (Fig. 1g). These results may explain the masking of potential EM markers by including patients diagnosed with myoma among the control group. Use of hormonal medication did not significantly affect the plasma levels of the analyzed markers (Supplementary Fig. 1).

Association between biomarkers and disease stage: IL-17F, PDGF-AB/BB, VEGFA, MCP-2, and MPI-1β plasma levels were increased in early stages of EM

The unsupervised clustering presented here successfully grouped patients into manageable clusters based on their EM heterogeneity and severity without introducing experimenter bias and consequently generated a reliable basis for the evaluation of stage-specific biomarkers (Fig. 2a–c, Supplementary Fig. 2). In contrast, rASRM groups often included patients with very different degrees of heterogeneity and severity (Fig. 2d, asterisks). For example, the rASRM II group, composed mostly of patients with mild peritoneal lesions, also includes a patient with P2, T3, A2, B2, C2 lesions and patent tubes.

We then analyzed whether our #ENZIAN clustering approach outperforms the traditional rASRM classification in highlighting potential biomarkers for different stages of EM (Fig. 3). Using the rASRM classification, we observed a significant reduction of perforin plasma levels across various EM stages and in myoma patients compared to the control group (Fig. 3a). Other markers, such as sFasL, TRAIL, CXCL16, and PDGF-AB/BB, were differentially expressed in one or more stages, especially in the more severe stages of the disease.

Using the #ENZIAN annotation, our clustering approach confirmed the reduction of perforin among cluster #II to #V as well as the reduction of CTACK in the most severe cluster, and sFasL in intermediate stages of the disease (Fig. 3b). Most importantly, this approach allowed the identification of an initial stage cluster #I, where IL-17F, PDGF-AB/BB, VEGFA, MCP-2, and MPI-1β plasma levels were significantly elevated. These elevations were unique to the earliest stages of EM and were not apparent with the rASRM classification.

We evaluated the discriminative power of the identified plasma biomarkers in classifying individuals into their respective groups based on their plasma marker profiles and predicted their optimal threshold in plasma (Fig. 4 and Supplementary Table 5). Our analysis revealed discriminative performance of various of these plasma markers when comparisons of control group with EM and/or myoma groups were made (Fig. 4a). For the control group without EM or myoma, biomarkers such as perforin (AUC = 0.82, predicted cutoff = >7.64 ng/ml), TRAIL (AUC = 0.75, predicted cutoff = >68.73 pg/ml), and CXCL16 (AUC = 0.77, predicted cutoff = >802.25 pg/ml) demonstrated high discriminative ability, characterized by strong sensitivity and relatively low false positive rates. In the myoma group, ENA-78 and IL-34 showed notable sensitivity values (1.00 and 0.91, respectively) but were associated with higher false positive rates, indicating potential limitations in specificity. Focusing on the EM group, IL-31, GRO-alpha, and LIF exhibited moderate performance, with sensitivity values ranging from 0.70 to 0.91 and AUCs between 0.61 and 0.67. While their overall classification ability was less robust, these biomarkers may still have diagnostic relevance in specific contexts. The subgroup of individuals with both EM and leiomyoma demonstrated moderate discriminative performance for biomarkers like TARC (AUC = 0.68) and IL-17F (AUC = 0.67).

The biomarker analysis revealed notable differences in diagnostic performance across the #Enzian clusters, with specific biomarkers showing promise in distinguishing early stages of EM (clusters #I and #II) from controls (Fig. 4b, Supplementary Table 5). Regarding cluster #I, IL-17F and PDGF-AA demonstrated strong classification performance, with AUCs of 0.75 and 0.84, respectively, combined with balanced sensitivity (0.80 and 0.80) and specificity (0.80 and 0.83). Similarly, VEGF-A exhibited robust predictive performance for cluster #I with an AUC of 0.83 and a sensitivity of 1.00, albeit poor specificity (0.58). In cluster #II, IL-31 exhibited medium
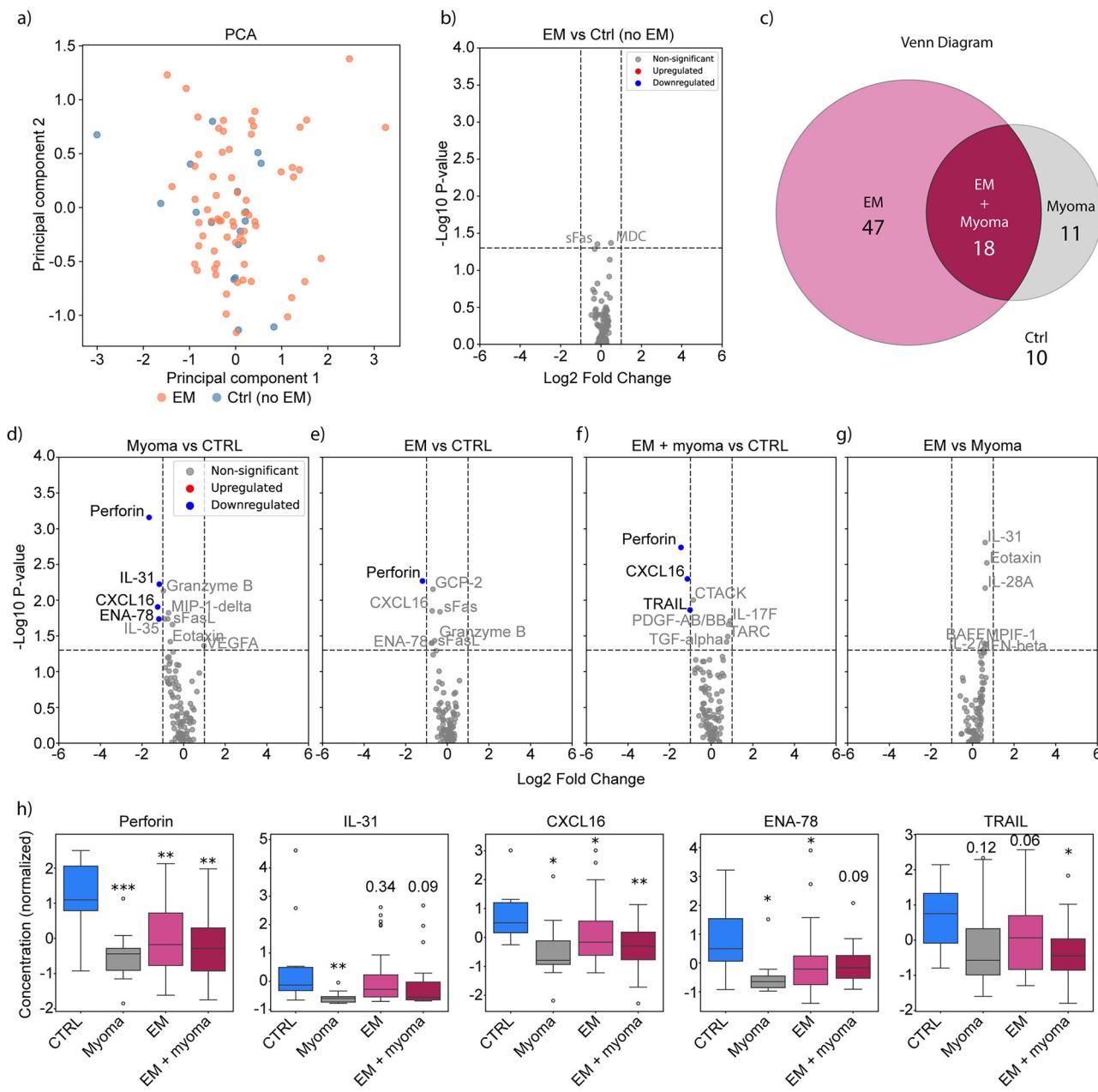
Fig. 1 | Patient clustering and biomarker analysis in endometriosis. a PCA using cytokine data showing limited separation between endometriosis (EM) and control (CTRL) groups. **b** Volcano plot showing significantly altered markers in EM vs. controls; no markers were significantly upregulated. **c** Venn diagram showing leiomyoma (Myoma) co-occurrence: 18/65 EM patients and 11 controls had myoma. **d–g** Volcano plots comparing biomarker expression in patients with myoma (**d**), EM (**e**), and EM+myoma (**f**) against healthy controls without either condition, and EM alone vs. myoma (**g**). **h** Boxplots showing normalized expression of selected markers; whiskers indicate 1.5× IQR. *P*-values (t-test or Mann–Whitney U test) are indicated above each box; *$p < 0.05$, **$p < 0.01$; non-significant results are labeled.

performance, with an AUC value of 0.66, while its sensitivity and specificity values were moderate (0.64 and 0.69, respectively). For prediction of later-stage clusters (#III, #IV, and #V), biomarkers generally displayed lower discriminative power compared to those in early stages. Across all groups, biomarkers such as CXCL16, TRAIL, and perforin demonstrated moderate-to-high diagnostic performance, with AUCs ranging from 0.77 to 0.82, and moderate to high sensitivity and specificity, underscoring their utility in distinguishing healthy controls from EM cases. The diagnostic potential of biomarkers such as TARC, GCP-2, and IL-17F in combined conditions (EM + myoma) was mostly limited, with AUCs of 0.64–0.68.

Additionally, we explored the presence of trends among all markers analyzed and the #Enzian clusters in order to highlight potentially affected mechanisms (Supplementary Fig. 3a). The expression trend of

perforin, with reduced levels in myoma-only patients and a gradual reduction in EM stages, was unique and grouped individually. However, several markers followed the sFasL trend, with a reduced expression in the more severe clusters and myoma. Among this group, we found Granzyme B, IL-7, IL-35, IL-16, HMGB1, and eotaxin 3. In contrast, CXCL16 and ENA-78 markers were significantly reduced in myoma patients but not in those with EM. Interestingly, we observed multiple markers specifically elevated in #I cluster, including the significant VEGFA, MIP-β, MCP-2, PDGF-AB/BB, and IL-17F, as well as the non-significant INF-α2, TGF-α, CCL28, FGF-2, granzyme B, I-309, MCP-4, GM-CSF, and interleukins 1α, 17A, 34, 15, and 2. Additionally, CTACK, TRAIL, and GCP-2 were similarly decreased in EM and myoma patients, among eotaxins 1 and 2, and SCF markers.

Table 1 | Descriptive statistics

		Missing	Overall	CTRL (no EM)	EM	P-Value	
n			86	21	65		
Age (years)			37.3 (6.3)	39.9 (5.2)	36.5 (6.5)	0.021	*
BMI		8	24.7 (5.7)	25.3 (5.7)	24.6 (5.7)	0.699	ns
# cycles during last year		4	10.2 (2.5)	10.6 (2.3)	10.1 (2.5)	0.487	ns
Ever pregnant, *n* (%)	Yes		38 (44.2)	14 (66.7)	24 (36.9)	0.033	*
	No		48 (55.8)	7 (33.3)	41 (63.1)		
Main symptom, *n* (%)	Infertility		14 (16.3)	3 (14.3)	11 (16.9)	0.049	*
	Pain		57 (66.3)	10 (47.6)	47 (72.3)		
	Bleeding dis.		7 (8.1)	3 (14.3)	4 (6.2)		
	Other		4 (4.7)	2 (9.5)	2 (3.1)		
	No symptoms		2 (2.3)	1 (4.8)	1 (1.5)		
	Unknown		2 (2.3)	2 (9.5)			
Relatives with endometriosis, *n* (%)	Sister		2 (2.3)	0 (0.0)	2 (3.1)	0.705	ns
	Mother		15 (17.4)	5 (23.8)	10 (15.4)		
	Grandmother		1 (1.2)	0 (0.0)	1 (1.5)		
	Aunt		2 (2.3)	1 (4.8)	1 (1.5)		
	Cousin		2 (2.3)	1 (4.8)	1 (1.5)		
	Other		2 (2.3)	0 (0.0)	2 (3.1)		
	None		62 (72.1)	14 (66.7)	48 (73.8)		
Hormonal treatment, *n* (%)	Combined Oral Contraceptives		4 (4.7)	1 (4.8)	3 (4.6)	0.831	ns
	Progesterone only		16 (18.6)	3 (14.3)	13 (20.0)		
	GnRH agonist		1 (1.2)		1 (1.5)		
	IUDs (copper)		2 (2.3)		2 (3.1)		
	None		63 (73.3)	17 (81.0)	46 (70.8)		

Data are presented as mean (SD) or *n* (%) when indicated. For continuous variables, t-test was used to compare the means of the two groups. For categorical variables with two categories, the two-proportion z-test was used to test for differences in proportions. For categorical variables with more than two categories, the chi-square test was used to evaluate associations between the groups. *$p < 0.05$.

Association between biomarkers and lesion types: IL-17F and PDGF-AB/BB marker levels are associated with specific #Enzian-based lesion types

Thanks to the versatility of #Enzian classification, we were also able to zoom into different lesion types (Supplementary Figs. 3b and 4). Although we could capture decline tendencies of perforin plasma levels in patients with tubule-ovarian lesions, as well as grade 3 ovarian lesions, those tendencies did not reach significance. However, IL-17F levels were significantly elevated in EM patients without peritoneal lesions, with tubulo-ovarian lesions of grade 2 and initial-stage lesions in the rectum location. PDGF-AB/BB levels were also significantly higher in non- and grade 1 peritoneal, as well as grade 3 ovarian lesions. These findings suggest a lesion-type-dependent influence on levels of certain cytokines and other plasma markers in EM patients.

Combined effect of EM and concomitant myoma on plasma markers

Since the presence of myoma was also associated with a downregulation of several of the analyzed markers, we tested whether this association was uniform among the defined #Enzian clusters of EM patients. We observed that the plasma levels of perforin tend to be lower for patients with concomitant EM and myoma compared with the levels of those presenting exclusively with EM (Fig. 5). This effect was also present in most of the clusters for sFasL, TRAIL, Granzyme B, and CTACK, albeit not significant. IL-17F, PDGF-AB/BB, VEGFA, and MCP-2 elevation in #I cluster was significant for patients with EM only compared to levels for the control group. Additionally, within the same cluster, IL-17F levels were more pronounced only in the presence of concomitant myoma. This effect was also observed in MIP-1β levels in #I cluster.

Discussion

EM and myoma are hormonally responsive conditions that often co-occur, posing significant challenges for biomarker research. We identified potential key immune markers, including perforin, TRAIL, and sFasL, whose levels were reduced in both EM and myoma patients, suggesting impaired apoptotic and cytotoxic responses that may contribute to lesion persistence. Additionally, elevated IL-17F, PDGF-AB/BB, VEGFA, and MCP-2, levels in EM patients highlight their potential as early-stage biomarkers and therapeutic targets.

In patients with myoma only, many markers were significantly altered, showing a reduction similar to that observed in EM patients. This overlap complicated the identification of EM-specific biomarkers, as the presence of myoma in the control group introduced substantial variability. In addition, uncontrolled factors such as hormonal treatment and menstrual cycle phase likely contributed further to biomarker variability across groups. Our study underscores the importance of precise classification methodologies in differentiating EM heterogeneity and the need for meticulous control group selection in biomarker analyses for their relevance in the identification of treatment targets for EM and myoma. While most studies rely on laparoscopy-confirmed EM-negative women[21–25], this approach often introduces bias, as the indication for surgery typically involves other gynecological disorders such as myoma. In our cohort, 69% of EM-negative women presented with myoma, reflecting its high global prevalence (77%) among adult women, with a substantial proportion remaining asymptomatic[26]. This observation aligns with recent proteomics studies[27], which emphasize that differences between study groups and inclusion of gynecological comorbidities in control groups may substantially bias biomarker discovery.

a) #Enzian annotation coding / Dimensionality reduction Model: Kernel-PCA / Clustering Model: K-means (clusters named according mean severity score)

b)

c)

d) #Enzian clusters / rASRM groups / #Enzian Severity Index (ESI)
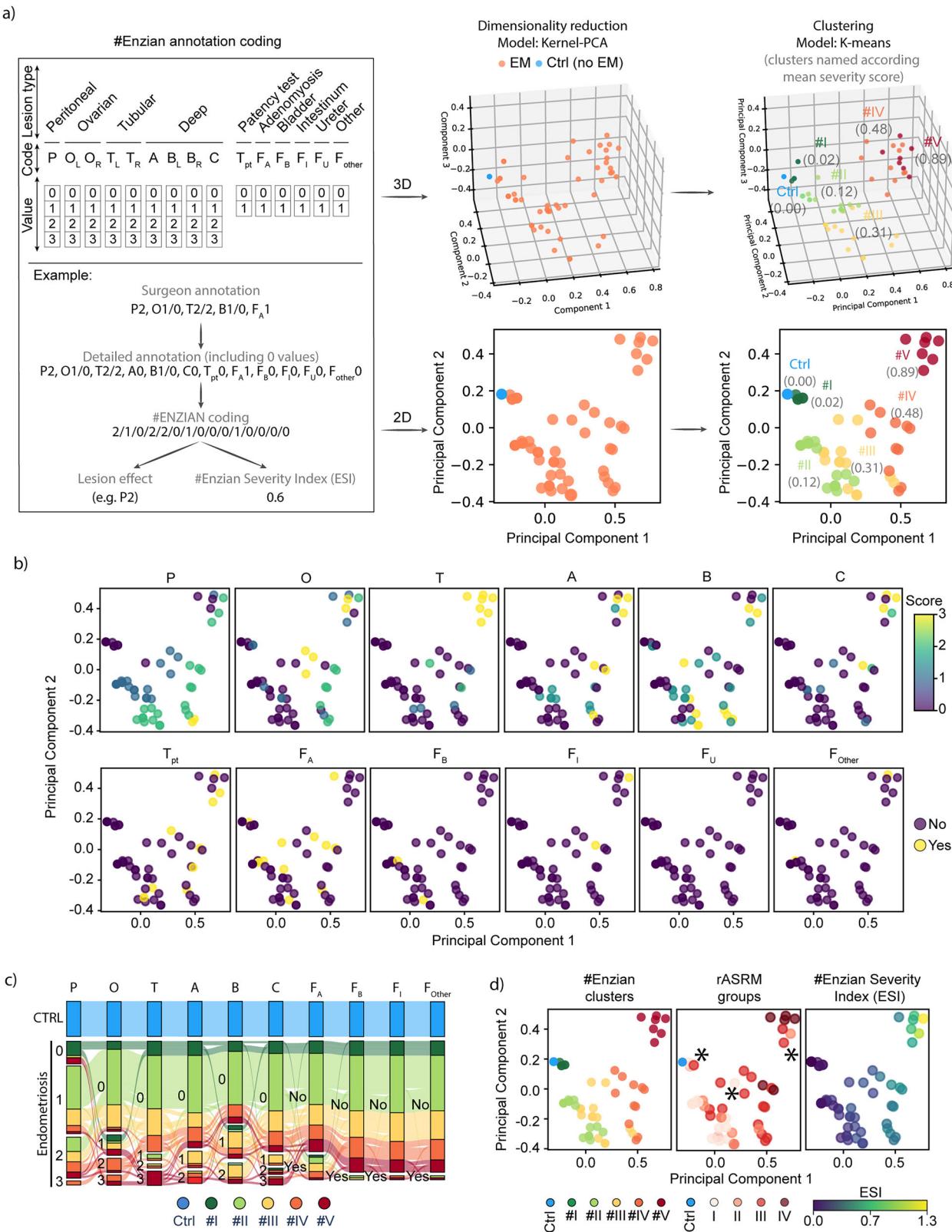
In our study, we observed a significant decrease in plasma levels of perforin, TRAIL, and sFasL in patients with EM and/or myoma compared to healthy controls. These molecules play central roles in immune surveillance through cytotoxic T cell and natural killer (NK) cell-mediated apoptosis.

Perforin, a critical pore-forming protein released by cytotoxic lymphocytes, plays a pivotal role in the cytotoxic activity of CD8 + T and NK cells, facilitating granzyme entry into target cells to induce apoptosis[28]. The observed decrease in perforin, together with lower granzyme B levels, suggests functional impairment of cytotoxic T lymphocytes and NK cells. Previous studies have implicated cytotoxic T cells in the pathogenesis of EM, reporting a defective T cell response and reduced cytotoxicity toward autologous endometrial cells[29].

Fig. 2 | EM patient clustering based on the #Enzian classification system. EM patient clustering according to #Enzian classification. **a** Schematic representation of the #Enzian classification coding system. Possible values and lesion types for different anatomical regions. Example annotations are provided to illustrate how the coding is applied from surgeon's annotation, decomposed and translated into a severity score (SC) derived from the lesion types and their respective severity levels (0-3). The dimensionality reduction model (Kernel-PCA) is presented in 2D and 3D. EM patients (orange) and controls (blue) are plotted. Unsupervised clustering (K-means) was used for classifying patients. Clusters were named according to the mean Enzian Severity Index (mESI) of each group as control (Ctrl, 0.00), #I (0.02), #II (0.12), #III (0.31), #IV (0.48), and #V (0.89) respectively. **b** Principal Component Analysis (PCA) showing the distribution of each lesion classifier from #Enzian among the different clusters. **c** Parallel categories plot representing the #Enzian classification across different patient clusters. This plot visualizes the distribution of lesion types and severities. The categories show the relationship between the extent of disease and patient groups, providing a comprehensive overview of the classification. **d** PCA showing the #Enzian cluster, rASRM group, and ESI generated for each patient. Asterisks: patients showing dissociation between rASRM classification and ESI. A: retrovaginal space, B: sacrouterine ligaments, C: rectum, CTRL: control, EM: endometriosis, FA: adenomyosis, FI: intestinum, Fother: other, FU: ureter, O: ovarian, P: peritoneal, T: tubo-ovarian, Tpt: patency test.

Both perforin and granzyme B have also been implicated in the shedding of human endometrium, contributing to endometrial menstruation[30,31]. A deficiency in perforin-mediated cytotoxicity may therefore play a role in lesion persistence in EM. Further, Yang et al.[32] proposed that interactions between endometriotic stromal cells and macrophages produce IL-10 and TGF-β, impairing NK and CD8+ T cell cytotoxicity in EM and promoting immune evasion.

Perforin-mediated cytotoxicity is considered a rapid mechanism of action for cytotoxic T lymphocytes[33]. However, CD8+ T and NK cells can also utilize a slower cytotoxic mechanism involving the Fas ligand (FasL) pathway, which triggers programmed cell death in target cells through the interaction of Fas expressed on the target cell surface with FasL on T cells. The triggering of Fas by FasL results in induction of programmed cell death in susceptible Fas-bearing cells[34]. In EM, a stage-dependent reduction in FasL expression has been observed in ectopic endometrial tissue and peritoneal fluid, possibly mediated by macrophage-derived factors such as TGF-β and PDGF[35,36].

In addition, cytotoxic T and NK cells can kill target cells through the TNF-related apoptosis-inducing ligand (TRAIL) pathway[37]. TRAIL is a TNF family member that induces apoptosis upon binding to its receptors. Decreased levels of granzyme B, perforin, and TRAIL have been reported in the peritoneal fluid of EM patients, indicating functionally defective NK cells[38]. Further, TRAIL antagonists such as osteoprotegerin (OPG) are elevated in EM peritoneal fluid, interfering with TRAIL-induced apoptosis[39].

The impairment of these apoptosis-inducing pathways collectively weakens the immune response of local NK and CD8 + T cells, potentially allowing ectopic lesions to survive and grow[39,40]. These mechanisms may be promoted by elevated TGF-β and estradiol levels[41], which are known to suppress immune cell activity. In mouse and human studies, increasing TGF-β expression has been associated with decreased cytotoxic responses and enhanced lesion survival and invasion[42].

Interestingly, early-stage EM (cluster #I) displayed a distinct profile, with elevated levels of immune and angiogenic markers, including IL-17F, PDGF-AB/BB, VEGFA, MCP-2, and MIP-1β. These elevations suggest early inflammatory and angiogenic responses supporting lesion establishment.

Among the immune markers, IL-17F plays a significant role. IL-17F shares strong homology with IL-17A and is produced mainly by Th17 cells[43]. Sisnett et al.[44] hypothesized that IL-17 produced by Th17 cells exacerbates EM by recruiting immune cells to lesion sites and enhancing lesion establishment. Elevated IL-17 levels have been consistently observed in EM, especially in its early stages[45–47]. Moreover, increased RNA expression of IL-17A, IL-17F, IL-12B, and TGF-β1 in ectopic tissues suggests coordinated upregulation of inflammatory and immune-modulatory pathways[44,48].

The macrophage inflammatory protein MIP-1β (CCL3), expressed in the endometrium, is correlated with NK cell recruitment to the endometrial zone, as indicated by a strong correlation between the endometrial MIP-1β concentration and the number of endometrial NK cells[49]. In vitro studies demonstrated higher secretion of MIP-1β by lymphocytes from women with EM, indicating a role in the altered immune environment[50]. Our data show that MIP-1β is elevated particularly in patients with both myoma and

EM at early stages, suggesting that myoma may amplify inflammatory chemokine responses. MCP-2, a C-C chemokine subfamily member, activates basophils, mast cells, and NK cells. Its increased expression in endometriotic lesions further suggests a role in chronic inflammation[51].

Angiogenesis is another critical mechanism in the establishment of early EM lesions, with VEGFA and PDGF playing central roles. VEGFA promotes vascularization and is upregulated in the peritoneal fluid of EM patients, with enhanced regulation during menstruation[52,53]. In vitro studies, inhibition of VEGFA reduced ectopic endometrial mesenchymal stem cell proliferation, motility, and angiogenesis[54,55]. According to our results, increased levels of VEGFA might also be involved in the stimulation of proliferation, motility, and angiogenesis in lesions at early stages of the disease.

Platelet-derived growth factor isoforms (PDGF-AB/BB) exhibit angiogenic effects by stimulating endometrial stromal cell proliferation, migration, and invasion[56]. Both VEGFA and PDGFs not only contribute to neovascularization but also modulate immune responses, potentially creating a permissive environment for early lesion growth.

Altogether, our results suggest that early EM development is characterized by a dual imbalance: weakened immune clearance through impaired cytotoxicity and enhanced inflammatory-angiogenic signaling. Defective apoptosis pathways, reflected by reduced plasma levels of perforin, TRAIL, and sFasL, may allow survival and implantation of ectopic endometrial fragments, while elevated inflammatory and angiogenic markers such as IL-17F, MCP-2, MIP-1β, VEGFA, and PDGF-AB/BB promote lesion establishment, vascularization, and persistence. This coordinated disruption of immune surveillance and tissue remodeling highlights the complex pathophysiology underlying EM and may explain its frequent progression from early, asymptomatic stages to more severe, chronic disease. Understanding this provides a mechanistic framework for identifying reliable non-invasive biomarkers, guiding the development of diagnostic panels, and identifying new therapeutic targets focused on restoring immune function and limiting angiogenesis in EM patients. In line with this, Schoeman et al. results[27] further support the need to integrate immunological, metabolic, and angiogenic axes into future biomarker research and explore the potential of these biomarkers for stratifying patients based on disease stage, lesion subtype, and comorbidities to enhance clinical applicability and relevance.

Our analysis revealed the potential of several biomarkers, such as perforin, IL-17F, and PDGFs, for application in the early diagnosis of EM and related conditions. A decline in perforin plasma levels below 7.64 ng/ml may reflect cytotoxic dysfunction, consistent with the immune evasion mechanisms described earlier. Conversely, elevated levels of IL-17F (>40.09 pg/mL) and PDGF-AA (> 1.78 ng/mL) may reflect active inflammatory and angiogenic signaling during early lesion establishment. The identification of these patterns in mild or early-stage EM highlights the potential for plasma biomarker-based tools to aid in early, non-invasive diagnosis, a major unmet clinical need. Nonetheless, further validation is required in larger, prospective cohorts with careful stratification by disease stage, hormonal treatment, and comorbidities such as myoma.

The diversity of EM lesions complicates patient classification, diagnosis, and treatment monitoring. Understanding the clinical impact of individual
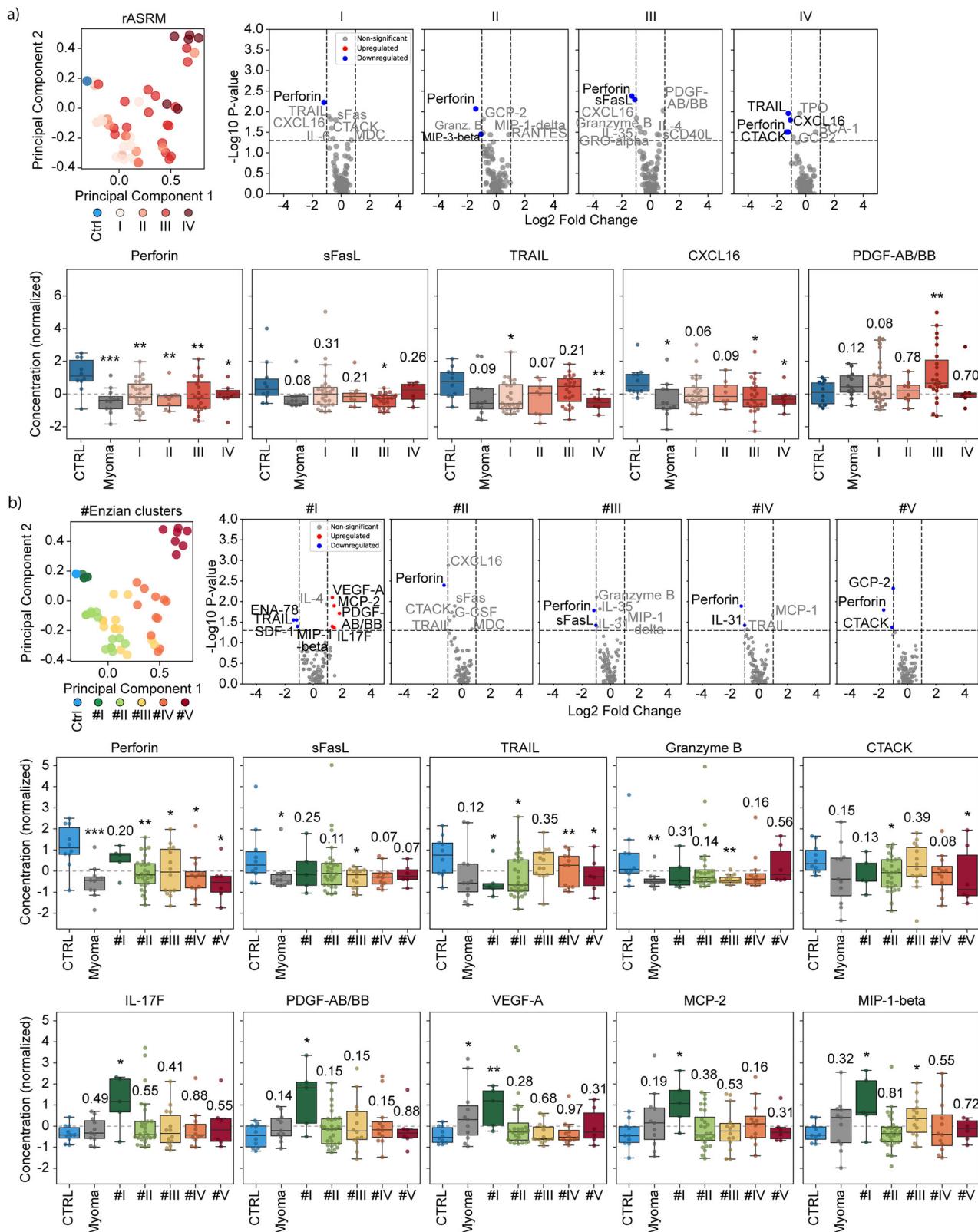
Fig. 3 | Plasma biomarker analysis by #Enzian cluster and myoma status.
a Comparison of revised American Society for Reproductive Medicine (rASRM) classification (groups I–IV) and #Enzian-based classification (groups #I–#V). Volcano plots compare each cluster to the control (Ctrl) group without leiomyoma (myoma). Labels for significantly reduced (blue) and significantly elevated (red) markers are shown. Markers for which the p-value was < 0.05, but the fold change was lower than 2, are labelled in gray. Boxplots display the normalized expression of significantly expressed markers across different groups. The central box shows the

interquartile range (IQR) and median (horizontal line inside the box). The "whiskers" extend to the smallest and largest values within 1.5 times the IQR, respectively. **b** Expression of significantly differenciated markers among the clustering groups. P-values from the statistical tests (t-test or Mann-Whitney U test) comparing each group with the control group are shown on top of each group's box. Significant p-values are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001), while non-significant p-values are represented with their exact values.
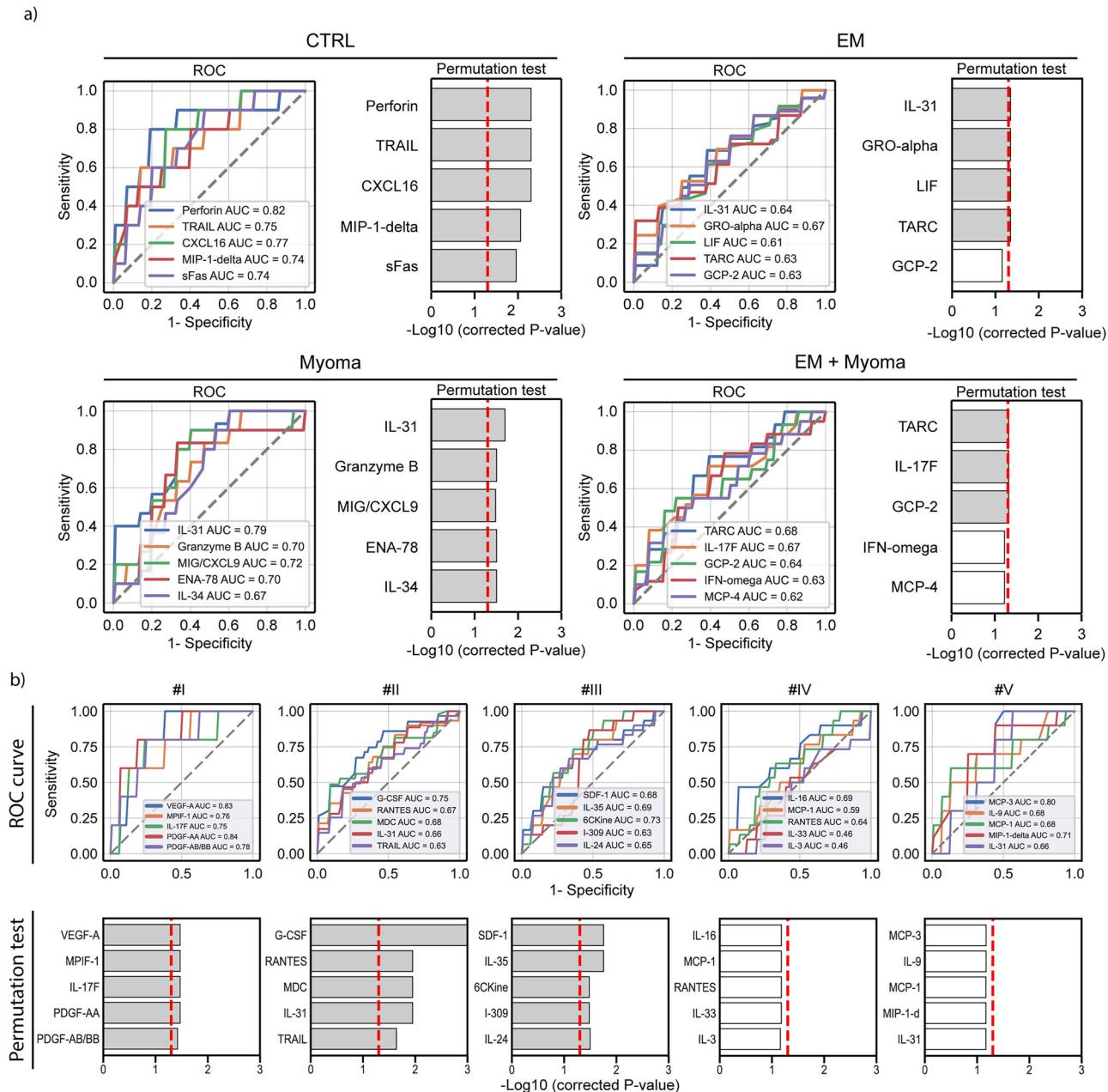
Fig. 4 | Evaluations of biomarkers as diagnostic predictors of disease presence and cluster-based classification. a Receiver Operating Characteristic (ROC) curves and permutation test results for the top 5 biomarkers in distinguishing control (CTRL) groups from various disease conditions, including leiomyoma (Myoma), endometriosis (EM), and EM + Myoma. **b** Analysis of biomarker performance within clusters generated based on #Enzian annotations (groups #I–#V), reflecting disease subtypes. The ROC curves illustrate the diagnostic accuracy of the top five biomarkers for each cluster/condition, with Area Under the Curve (AUC) values indicating their predictive power. Permutation test results are shown as −log10(p-value), with Bonferroni correction applied for multiple testing. The significance threshold after Bonferroni correction is marked with a red dotted line.

lesions is essential to address these challenges. The #Enzian system provides a comprehensive user-friendly mapping of EM, accounting for anatomic location, lesion size, adhesions, and the degree of involvement of adjacent organs[57,58] with excellent inter- and intra-observer agreement in MRI-based diagnosis of EM[59]. Using this classification system, we generated coherent patient clusters with similar EM heterogeneity and severity, revealing biomarkers, such as IL-17F, that may remain undetected when using other classification systems, particularly in the early stages of the disease.

The #Enzian annotation enables us to categorize patients based on a specific lesion type, facilitating the identification of trends in plasma biomarkers associated with that type. However, this approach cannot fully isolate the effects of the selected lesion type from the influence of other coexisting lesions. Consequently, the observed trends may be masked or diluted by the "noise" generated by these additional lesions. For instance, the trends of IL-22 and IL-24 in patients with peritoneal lesions, as well as IL-3, I-Tac, APRIL, and TPO in patients with tubo-ovarian lesions, underscore this limitation. These insights highlight the need for larger and more granular studies of lesion-specific biomarkers. Such efforts could significantly enhance our understanding of EM biology and pave the way for more effective, tailored therapeutic approaches.

One of the main limitations of our study is the small number of patients in the control group, which is largely due to the stringent criteria required for accurate diagnosis. Identifying control patients who are definitively negative for EM necessitates invasive laparoscopy, and further distinguishing those
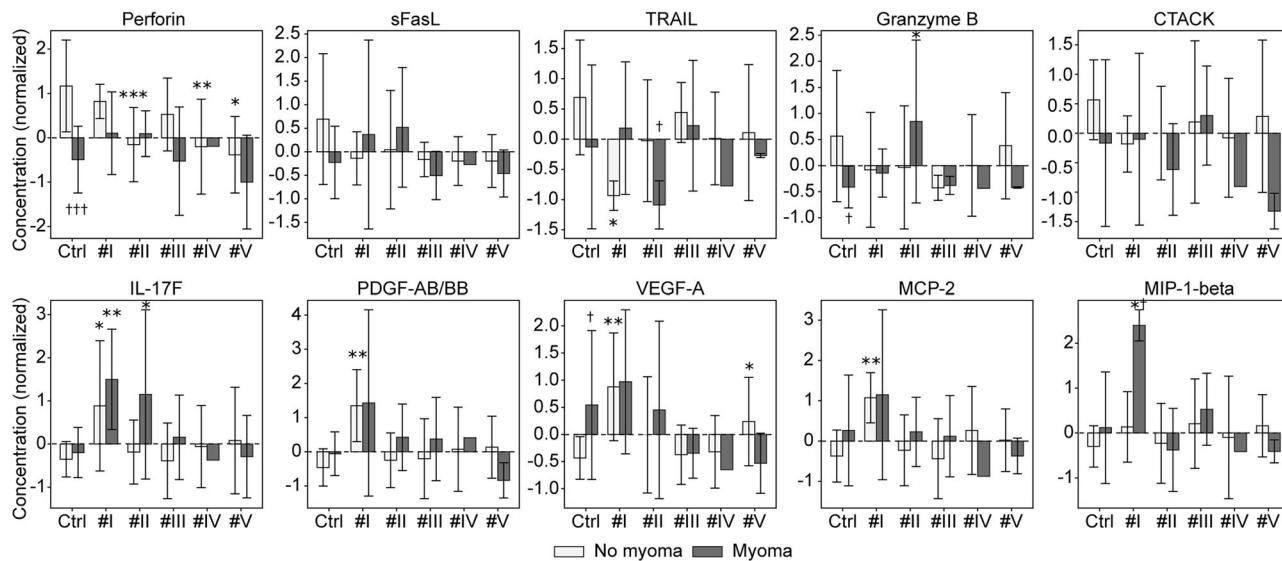
Fig. 5 | Combined effect of EM and myoma on significant markers in the previous analyses. Normalized expression of these markers among the clustering groups, discriminating by the presence (grey) or absence (white) of myoma. Data are represented as the mean ± SD. Asterisks (*) represent statistical significance for comparisons of clusters generated based on #Enzian annotations (groups #I–#V) vs its corresponding control (Ctrl). Daggers (†) represent statistical significance for comparisons between no myoma and myoma conditions within each cluster. A *p*-value < 0.05 was considered significant.

without myoma adds an additional layer of complexity. This constraint resulted in a limited sample size that may impact the generalizability of our findings. The significant age difference between EM patients and women without EM (p = 0.021) might have influenced findings. Additionally, the menstrual cycle phase was not included as a variable in the analysis due to the inconsistent availability of this information in routine clinical documentation. While all patients were recruited during standard preoperative assessments, cycle phase data were not uniformly recorded across sites. As such, we cannot exclude the possibility that physiological variation across the cycle may have contributed to biomarker variability.

Our findings revealed a significant reduction in key immune markers, including perforin, TRAIL, and sFasL, in both EM and myoma patients, indicating impairments in apoptotic and cytotoxic immune responses that may contribute to lesion persistence. Patient clustering using #Enzian annotation identified elevated levels of IL-17F, PDGF-AB/BB, VEGFA, and MCP-2 in early-stage EM, highlighting their potential as early biomarkers and therapeutic targets. The interplay between immune suppression and angiogenesis in early-stage EM suggests promising avenues for combination therapies targeting these pathways. Advanced classification systems and precise biomarker analyses are essential for gaining deeper insights into EM pathophysiology. At the same time, identifying early-stage biomarkers offers hope for more accurate and timely diagnosis, enabling earlier interventions. These findings could drive the development of personalized and effective treatments, ultimately enhancing the quality of life for individuals affected by this challenging condition.

Methods
Study design
Patient recruitment and enrollment through senior gynecologists, supported by a study nurse, took place between 2021 and 2023 at the Departments of Reproductive Endocrinology and Gynecology at the University Hospital Zurich, Switzerland. The study was approved by the national medical ethics committee (BASEC 2020-02117), and all participants provided written informed consent prior to inclusion. All procedures involving human participants were conducted in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments.

Inclusion criteria for the study comprised 18- to 50-year-old women undergoing surgery (including hysterectomy) for EM, as well as women without EM undergoing surgical management of uterine leiomyoma, or sterilization. Exclusion criteria for the clinical study were current pregnancy, breastfeeding, a positive diagnosis of immunodeficiency or autoimmune diseases, or increased risk of bleeding. Participants were interviewed regarding their lifestyle (i.e., alcohol consumption, smoking status, sports activity) and medical history, especially with regard to pain perception, as well as medication intake during the week prior to surgery. A meticulous investigation of the patients' history, plus general and gynecological exams served to identify any additional pathology before the surgical procedure. All baseline demographic and clinical data were collected as metadata information. Myomas were assessed by transvaginal ultrasound and palpation, and further classified according to the FIGO (International Federation of Gynecology and Obstetrics) uterine fibroid classification system, including size, type, and location. The reference test for the diagnosis of EM was laparoscopy with visualization of typical lesions and histological evaluation, performed by expert surgeons with at least ten years of experience. EM was classified according to the revised American Society for Reproductive Medicine (rASRM) and the #Enzian classification by the surgeon immediately after surgery.

Patients were included in further analysis if they had clear and comprehensive information regarding EM and/or uterine leiomyoma, including detailed rASRM and #Enzian annotations, and sufficient plasma for biomarker analysis. Of 89 initially selected patients, only three were excluded based on these criteria. Patients diagnosed with EM were assigned to the EM group, while those without EM were included in the control (no EM) group, regardless of the presence of leiomyoma.

Plasma sample collection
EDTA-blood was collected from patients on the day of the surgery according to standard operating procedure. Blood samples of a minimum 9 ml were taken into EDTA-coated tubes for plasma collection.

All blood specimens were collected immediately upon transfer from the ward, and prior to the induction of anesthesia, the induction of anesthesia was initiated only after blood collection to avoid confounding effects. Samples were kept at 4 °C until processing. HAV IgG/IgM Combo and HBsAg /HCV /HIV /Syphilis Combo Rapid Test Cassettes (CiTest Diagnostics, Canada) were used to measure major infections. Within one hour after collection, the samples were centrifuged at 2000 G at 4 °C for 10 min. The plasma was aspirated, aliquoted into 500 µl volumes, and stored at −80 °C until analysis.

Dimensionality reduction and unsupervised clustering

The actual rASRM classification score alone does not provide a detailed description of the heterogeneity or the full extent of multiple lesions and cannot map DE[57,60,61]. Thus, we also used the #Enzian annotation, which allows a comprehensive mapping of EM, including anatomical location, lesion size, adhesions, and involvement of adjacent organs, using pictograms to increase reliability and convenience of scoring[57]. The #Enzian annotation was decomposed to single lesion type variables (P, O left and right, T left and right, A, B left and right, C, Fa, Fb, Fi, Fu, Fother, and patency test) (Fig. 1), or combined to obtain an #Enzian Severity Index (ESI) by averaging the scores from all #Enzian variables. An ESI score can be used to indicate the overall stage of the disease (Fig. 1a). The dimensionality reduction of these 15 variables distributed the patients according to the combined weight of every lesion-type severity, using a kernel PCA algorithm to visualize the distribution of patients according to those variables in 3D and 2D space[62]. For control patients, we assigned an #Enzian annotation with a 0 value on every variable. Theoretically, ESI values can range from 0 (no EM) to 2.2 (assuming the maximum value in all #Enzian variables). To identify the optimal number of clusters, we performed silhouette analysis, which evaluates the consistency within clusters and the separation between them (Supplementary Fig. 2). Based on the silhouette scores, we selected five as the optimal number of clusters for K-means unsupervised clustering of the resulting distribution. This approach generated 5 distinct clusters. We named each cluster after its mean ESI (mESI), using similar terminology to that of the rASRM classification and adding a # to indicate the #Enzian origin (Fig. 1a and c). Thus, we distinguished between #I (mESI = 0.02), #II (mESI = 0.12), #III (mESI = 0.31), #IV (mESI = 0.43), and #V (mESI = 0.89). We manually separated the control group (mESI = 0.00) from the #I cluster. Cluster #I ($n = 5$) was composed of patients with one unique lesion of grade 1 or 2 and no peritoneal or tubule-ovarian lesions. Cluster #II ($n = 28$) was mainly composed of grade 1 and 2 peritoneal lesions and few low-grade ovarian and deep lesions at the sacrouterine ligaments. #III cluster ($n = 14$) comprised mainly patients with peritoneal and deep lesions. #IV cluster ($n = 11$) contained patients with multiple lesions of medium to high grade, including peritoneal, ovarian, and deep lesions from multiple locations. #V ($n = 7$) was considered the most severe cluster, composed of patients with grade 3 tubulo-ovarian lesions coexisting with peritoneal, ovarian, and deep lesions.

Multiplex analysis of biomarker measurements

All methods were carried out in accordance with the relevant guidelines and regulations. A total of 500 µl of EDTA-plasma samples were aliquoted and sent to Eve Technologies Corp. (Calgary, Alberta, Canada). Multiplexing analysis was performed using the Luminex™ 200 system (Luminex, Austin, TX, USA). Ninety-six markers were simultaneously measured in the samples using Eve Technologies' Human Cytokine 96-Plex Discovery Assay®, which consists of two separate kits, the Panel A 48-plex and the Panel B 48-plex (MilliporeSigma, Burlington, Massachusetts, USA). The assay was run according to the manufacturer's protocol. The Panel A 48-plex consisted of sCD40L, EGF, eotaxin, FGF-2, FLT-3 ligand, fractalkine, G-CSF, GM-CSF, GROα, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, IP-10, MCP-1, MCP-3, M-CSF, MDC, MIG/CXCL9, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGFα, TNF-α, TNF-β, and VEGF-A. The Panel B 48-plex consisted of 6CKine, APRIL, BAFF, BCA-1, CCL28, CTACK, CXCL16, ENA-78, eotaxin-2, eotaxin-3, GCP-2, granzyme A, granzyme B, HMGB1, I-309, I-TAC, IFNβ, IFNω, IL-11, IL-16, IL-20, IL-21, IL-23, IL-24, IL-28A, IL-29, IL-31, IL-33, IL-34, IL-35, LIF, lymphotactin, MCP-2, MCP-4, MIP-1δ, MIP-3α, MIP-3β, MPIF-1, perforin, sCD137, SCF, SDF-1, sFAS, sFASL, TARC, TPO, TRAIL, and TSLP. All shared sample information was fully anonymized, and the Eve Technologies Corp. personnel conducting the assays were blinded to patient-identifying information and diagnoses. Estimated concentrations were presented in pg/ml.

Confounders

When using plasma as a source of biomarkers for a specific disease, it is crucial to highlight that analyses represent bulk measurements, influenced by all the conditions of the patient. In EM, most patients present with multiple lesions that often coexist with other conditions, such as myoma. We hypothesized that the presence of additional conditions could obscure the detection of differences between control subjects and those with EM. Myoma was of particular interest, given its high prevalence in affecting more than 70% of reproductive-aged women worldwide, its significant overlap with EM, and the role of inflammation in the pathogenesis of both conditions[26,63]. Furthermore, concomitant medication may influence findings in biomarkers[64]. Therefore, we controlled our findings for the presence of myoma as well as the potential influence of current treatments, including combined oral contraceptives, progesterone therapy, GnRH agonists, and copper IUDs, on inflammatory biomarkers levels.

Statistics

Altogether, data from 65 women with endometriosis and 21 controls were available for analysis. The data were processed using Python programming language with open-source packages such as pandas, scikit-learn, scipy, seaborn, and matplotlib[65–68]. Results of the descriptive analysis (i.e. patient's clinical data) were presented as mean ± standard deviation (SD) while the concentrations of the measured proteins were presented as mean ± SD when variables were normally distributed and as mean and quartiles (Q1, Q3) when non-normally distributed (Table 1). Values that exceeded the mean ± 3xSD threshold were considered outliers and removed from the analysis[69]. Fisher's exact and Chi-square tests were used for comparison of categorical variables. For continuous variables, t-test was used to compare the means of two groups and ANOVA for multiple groups. For categorical variables with two categories, the two-proportion z-test was used to test for differences in proportions. For categorical variables with more than two categories, the Chi-square test was used to evaluate associations between the groups. The normal distribution of every marker was tested using the Shapiro-Wilk test. For markers with a normal distribution, ANOVA was used to compare the means between groups. For markers that did not follow a normal distribution, the Kruskal-Wallis test was applied. Apart from single proteins, additional variables were constructed representing ratios of the proteins' concentrations. The expression data were normalized using mean-centering and standard deviation normalization. In brief, for each cytokine marker, the mean expression level across all samples was calculated. This mean was then subtracted from each individual sample's expression level, resulting in a distribution centered around zero. After mean-centering, each marker's expression values were divided by the standard deviation of the expression levels across all samples, thus scaling the data such that each marker has a standard deviation of one, and ensuring comparability across different markers. Corrected P-values of <0.05 were considered significant.

After a first comparison of biomarker profiles in women diagnosed with EM compared with control women, we added the presence of myoma as well as concomitant medication as additional factors in our analysis. Biomarker evaluation was performed using a logistic regression model for each biomarker independently, following a one-vs-rest strategy for multi-class classification. Each biomarker's discriminative ability was quantified using the Area Under the Receiver Operating Characteristic Curve (AUC), calculated via 5-fold stratified cross-validation to ensure robust and unbiased performance estimates. For each cluster/condition, biomarkers were ranked by their mean AUC scores, and the top five biomarkers with the highest AUCs were selected for further analysis. To assess the statistical significance of the observed AUC values, permutation testing was conducted by randomly shuffling the class labels 1000 times to generate a null distribution of AUCs under the hypothesis of no association. P-values were calculated as the proportion of permuted AUCs that exceeded the observed AUC. To correct for multiple comparisons, Bonferroni correction was applied to the permutation-derived p-values, with statistical significance

defined as an adjusted *p*-value < 0.05. The results were presented as −log10(*p*-value). Youden's J statistic was used to identify the optimal cutoff for the prediction of either EM and/or myoma or a specific #Enzian category by the selected biomarkers. These cutoffs represent the biomarker plasma concentration at which the balance between sensitivity and specificity is maximized, ensuring robust discrimination between the studied classes.

Data availability
The data underlying this article are available in the article and in its online supplementary material.

References

1. Johnson, N. P. et al. World Endometriosis Society consensus on the classification of endometriosis. *Hum. Reprod.* **32**, 315–324 (2017).
2. Richter, M. et al. From donor to the lab: a fascinating journey of primary cell lines. *Front Cell Dev. Biol.* **9**, 711381 (2021).
3. Ramin-Wright, A. et al. Fatigue - a symptom in endometriosis. *Hum. Reprod.* **33**, 1459–1465 (2018).
4. Imperiale, L., Nisolle, M., Noël, J. C. & Fastrez, M. Three Types of Endometriosis: Pathogenesis, Diagnosis and Treatment. State of the Art. *J. Clin. Med.* **12**. https://doi.org/10.3390/jcm12030994 (2023).
5. Tan, Y. et al. Single-cell analysis of endometriosis reveals a coordinated transcriptional programme driving immunotolerance and angiogenesis across eutopic and ectopic tissues (vol 24, pg 1306, 2022). *Nat. Cell Biol.* **24**, 1679–1679 (2022).
6. Colgrave, E. M. et al. Extensive heterogeneity in the expression of steroid receptors in superficial peritoneal endometriotic lesions. *Reprod. Biomed. Online* **48**, 103409 (2023).
7. Bougie, O. et al. Impact of surgeon characteristics on endometriosis surgery outcomes. *J. Minim. Invasive Gynecol.* **32**, 709–717.e706 (2025).
8. Mu, F. et al. A Prospective study of inflammatory markers and risk of Endometriosis. *Am. J. Epidemiol.* **187**, 515–522 (2018).
9. Anastasiu, C. V. et al. Biomarkers for the Noninvasive Diagnosis of Endometriosis: State of the Art and Future Perspectives. *Int. J. Mol. Sci.* **21**. https://doi.org/10.3390/ijms21051750 (2020).
10. Vodolazkaia, A. et al. A high sensitivity assay is more accurate than a classical assay for the measurement of plasma CRP levels in endometriosis. *Reprod. Biol. Endocrinol*. **9**. https://doi.org/10.1186/1477-7827-9-113 (2011).
11. Warzecha, D. et al. Plasma and Peritoneal Fluid Fibronectin and Collagen IV Levels as Potential Biomarkers of Endometriosis. *Int. J. Mol. Sci*. **23**. https://doi.org/10.3390/ijms232415669 (2022).
12. Zhang, T. et al. MDSCs drive the process of endometriosis by enhancing angiogenesis and are a new potential therapeutic target. *Eur. J. Immunol.* **48**, 1059–1073 (2018).
13. Ghafouri-Fard, S., Shoorei, H. & Taheri, M. Role of non-coding RNAs in the Pathogenesis of Endometriosis. *Front. Oncol.* **10**, 1370 (2020).
14. Gratton, S. M. et al. Diagnosis of Endometriosis at Laparoscopy: A validation study comparing surgeon visualization with histologic findings. *J. Obstet. Gynaecol. Ca* **44**, 135–141 (2022).
15. Wang, Y. N. et al. Distinct subtypes of endometriosis identified based on stromal-immune microenvironment and gene expression: implications for hormone therapy. *Front. Immunol*. **14**. https://doi.org/10.3389/fimmu.2023.1133672 (2023).
16. Zakhari, A. et al. Endometriosis recurrence following post-operative hormonal suppression: a systematic review and meta-analysis. *Hum. Reprod. Update* **27**, 96–107 (2021).
17. Drechsel-Grau, A. et al. Long-term follow-up regarding pain relief, fertility, and re-operation after surgery for deep Endometriosis. *J. Clin. Med.* **13**, 5039 (2024).
18. Jaagura, M. et al. Comorbidities confound metabolomics studies of human disease. *Sci. Rep.* **14**, 24810 (2024).
19. Pusic, M. et al. Antibody arrays identified cycle-dependent plasma biomarker candidates of peritoneal Endometriosis. *J. Pers. Med* **12**, 852 (2022).
20. Rocha, A. L., Vieira, E. L., Maia, L. M., Teixeira, A. L. & Reis, F. M. Prospective evaluation of a panel of plasma cytokines and chemokines as potential markers of pelvic Endometriosis in symptomatic women. *Gynecol. Obstet. Invest.* **81**, 512–517 (2016).
21. Mihalyi, A. et al. Non-invasive diagnosis of endometriosis based on a combined analysis of six plasma biomarkers. *Hum. Reprod.* **25**, 654–664 (2010).
22. Starodubtseva, N. et al. Identification of potential endometriosis biomarkers in peritoneal fluid and blood plasma via shotgun lipidomics. *Clin. Mass Spectrom.* **13**, 21–26 (2019).
23. Vanhie, A. et al. Plasma miRNAs as biomarkers for endometriosis. *Hum. Reprod.* **34**, 1650–1660 (2019).
24. Waelkens, E. et al. Discovery of Endometriosis biomarkers in menstrual plasma samples using a proteomics approach. *Fertil. Steril.* **106**, E275–E275 (2016).
25. Zafari, N. et al. A Panel of Plasma miRNAs 199b-3p, 224-5p, and Let-7d-3p as non-invasive diagnostic biomarkers for Endometriosis (Jan, 10.1007/s43032-020-00415-z, 2021). *Reprod. Sci.* **28**, 1000–1000 (2021).
26. Giuliani, E., As-Sanie, S. & Marsh, E. E. Epidemiology and management of uterine fibroids. *Int. J. Gynaecol. Obstet.* **149**, 3–9 (2020).
27. Schoeman, E. M. et al. Identification of plasma protein biomarkers for endometriosis and the development of statistical models for disease diagnosis. *Hum. Reprod.* **40**, 270–279 (2025).
28. Osinska, I., Popko, K. & Demkow, U. Perforin: an important player in immune response. *Cent. Eur. J. Immunol.* **39**, 109–115 (2014).
29. Osuga, Y. et al. Lymphocytes in Endometriosis. *Am. J. Reprod. Immunol.* **65**, 1–10 (2011).
30. Male, V. et al. Immature NK cells, capable of producing IL-22, are present in human uterine Mucosa. *J. Immunol.* **185**, 3913–3918 (2010).
31. Konno, R. et al. Apoptosis of human endometrium mediated by perforin and granzyme B of NK cells and cytotoxic T lymphocytes. *Tohoku J. Exp. Med.* **187**, 149–155 (1999).
32. Yang, H. L. et al. The crosstalk between endometrial stromal cells and macrophages impairs cytotoxicity of NK cells in endometriosis by secreting IL-10 and TGF-β. *Reproduction* **154**, 815–825 (2017).
33. Hassin, D., Garber, O. G., Meiraz, A., Schiffenbauer, Y. S. & Berke, G. Cytotoxic T lymphocyte perforin and Fas ligand working in concert even when Fas ligand lytic action is still not detectable. *Immunology* **133**, 190–196 (2011).
34. Gogacz, M. et al. Fas-related apoptosis of peritoneal fluid macrophages in Endometriosis patients: understanding the disease. *J. Immunol. Res.* **2017**. https://doi.org/10.1155/2017/3175394 (2017).
35. Garcia-Velasco, J. A., Arici, A., Zreik, T., Naftolin, F. & Mor, G. Macrophage derived growth factors modulate Fas ligand expression in cultured endometrial stromal cells: a role in endometriosis. *Mol. Hum. Reprod.* **5**, 642–650 (1999).
36. Sturlese, E. et al. Dysregulation of the Fas/FasL system in mononuclear cells recovered from peritoneal fluid of women with endometriosis. *J. Reprod. Immunol.* **92**, 74–81 (2011).
37. Morales-Kastresana, A. et al. Essential complicity of perforin-granzyme and FAS-L mechanisms to achieve tumor rejection following treatment with anti-CD137 mAb. *J. Immunother. Cancer* **1**, 3 (2013).
38. Jeung, I., Cheon, K. & Kim, M. R. Decreased Cytotoxicity of peripheral and peritoneal natural killer cell in Endometriosis. *Biomed. Res. Int.-Uk* **2016**. https://doi.org/10.1155/2016/2916070 (2016).

39. Harada, M. et al. Concentration of osteoprotegerin (OPG) in peritoneal fluid is increased in women with endometriosis. *Hum. Reprod.* **19**, 2188–2191 (2004).

40. Bersinger, N. A. et al. PAPP-A and osteoprotegerin, together with interleukin-8 and RANTES, are elevated in the peritoneal fluid of women with endometriosis. *Am. J. Obstet. Gynecol.* **195**, 103–108 (2006).

41. Shen, Z., Rodriguez-Garcia, M., Patel, M. V. & Wira, C. R. Direct and Indirect endocrine-mediated suppression of human endometrial CD8+T cell cytotoxicity. *Sci. Rep.* **11**, 1773 (2021).

42. Young, V. J., Ahmad, S. F., Duncan, W. C. & Horne, A. W. The role of TGF-β in the pathophysiology of peritoneal endometriosis. *Hum. Reprod. Update* **23**, 548–559 (2017).

43. Chang, S. H. & Dong, C. IL-17F: Regulation, signaling and function in inflammation. *Cytokine* **46**, 7–11 (2009).

44. Sisnett, D. J. et al. The Dysregulated IL-23/TH17 Axis in Endometriosis Pathophysiology. *J. Immunol.* **212**, 1428–1441 (2024).

45. Shi, J. L. et al. IL-17: an important pathogenic factor in endometriosis. *Int. J. Med. Sci.* **19**, 769–778 (2022).

46. Miller, J. E. et al. IL-17A modulates peritoneal macrophage recruitment and M2 Polarization in Endometriosis. *Front. Immunol*. **11**. https://doi.org/10.3389/fimmu.2020.00108 (2020).

47. Sikora, J., Smycz-Kubanska, M., Mielczarek-Palacz, A., Bednarek, I. & Kondera-Anasz, Z. The involvement of multifunctional TGF-β and related cytokines in pathogenesis of endometriosis. *Immunol. Lett.* **201**, 31–37 (2018).

48. Soni, U. K. et al. A high level of TGF-B1 promotes endometriosis development via cell migration, adhesiveness, colonization, and invasiveness. *Biol. Reprod.* **100**, 917–938 (2019).

49. Kitaya, K. et al. Expression of macrophage inflammatory protein-1β in human endometrium:: Its role in endometrial recruitment of natural killer cells. *J. Clin. Endocrinol. Metab.* **88**, 1809–1814 (2003).

50. Sadlocha, M., Toczek, J., Czuba, Z. & Stojko, R. Effects of macrophage inflammatory Protein 1 Alpha and Beta Chemokine concentrations in the progression of Endometriosis. *Clin. Exp. Obstet. Gyn.* **49**. https://doi.org/10.31083/j.ceog4907153 (2022).

51. Aksak, T., Gümürdülü, D., Çetin, M. T. & Polat, S. Expression of monocyte chemotactic protein 2 and tumor necrosis factor alpha in human normal endometrium and endometriotic tissues. *J. Gynecol. Obstet. Hum.* **50**, 101971 (2021).

52. Young, V. J., Ahmad, S. F., Brown, J. K., Duncan, W. C. & Horne, A. W. Peritoneal VEGF-A expression is regulated by TGF-β1 through an ID1 pathway in women with endometriosis. *Sci. Rep.* **5**, 16859 (2015).

53. Danastas, K., Miller, E. J., Hey-Cunningham, A. J., Murphy, C. R. & Lindsay, L. A. Expression of vascular endothelial growth factor A isoforms is dysregulated in women with endometriosis. *Reprod. Fert. Dev.* **30**, 651–657 (2018).

54. Li, H. et al. FGA controls VEGFA secretion to promote Angiogenesis by activating the VEGFR2-FAK signalling pathway. *Front. Endocrinol.* **13**. https://doi.org/10.3389/fendo.2022.791860 (2022).

55. Zubrzycka, A., Migdalska-Sek, M., Jedrzejczyk, S. & Brzezianska-Lasota, E. Circulating miRNAs related to Epithelial-Mesenchymal Transitions (EMT) as the new molecular markers in Endometriosis. *Curr. Issues Mol. Biol.* **43**, 900–916 (2021).

56. Matsumoto, H. et al. Regulation of proliferation, motility, and contractility of human endometrial stromal cells by platelet-derived growth factor. *J. Clin. Endocrinol. Metab.* **90**, 3560–3567 (2005).

57. Keckstein, J. et al. The #Enzian classification: A comprehensive non-invasive and surgical description system for endometriosis. *Acta Obstet. Gynecol. Scand.* **100**, 1165–1175 (2021).

58. Forschung, S. E. Vol. IOS, Android (ed Stiftung Endometriose Forschung) (App Store).

59. Harth, S. et al. Application of the #Enzian classification for endometriosis on MRI: prospective evaluation of inter- and intraobserver agreement. *Front Med.* **10**, 1303593 (2023).

60. Vercellini, P. et al. Reproductive performance, pain recurrence and disease relapse after conservative surgical treatment for endometriosis: the predictive value of the current classification system. *Hum. Reprod.* **21**, 2679–2685 (2006).

61. Vercellini, P. et al. Endometriosis and pelvic pain: relation to disease stage and localization. *Fertil. Steril.* **65**, 299–304 (1996).

62. Colgan, R. E., Gutierrez, D. E., Sundram, J., Bhaskar, T. G. Analysis of medical data using dimensionality reduction techniques. *Proceedings of the Conference: AMALTHEA –2013. Melbourne*, https://doi.org/10.13140/2.1.2270.1762 (2013).

63. Lou, Z. et al. Global, regional, and national time trends in incidence, prevalence, years lived with disability for uterine fibroids, 1990-2019: an age-period-cohort analysis for the global burden of disease 2019 study. *BMC Public Health* **23**, 916 (2023).

64. Wehbe, E. et al. Therapeutic Drug Monitoring and Biomarkers: towards Better Dosing of Antimicrobial Therapy. *Pharmaceutics* **16**. https://doi.org/10.3390/pharmaceutics16050677 (2024).

65. McKinney, W. Data Structures for Statistical Computing in Python. *Proceedings of the Python in Science Conference*, 56–61. https://doi.org/10.25080/majora-92bf1922-00a (2010).

66. Pedregosa, F. et al. Scikit-learn: Machine Learning in Python. *J. Mach. Learn. Res.* **12**, 2825–2830 (2011).

67. Virtanen, P. et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* **17**, 261–272 (2020).

68. mwaskom/seaborn: v0.8.1 (2017).

69. Kwak, S. K. & Kim, J. H. Statistical data preparation: management of missing values and outliers. *Korean J. Anesthesiol.* **70**, 407–411 (2017).

Author contributions
D.R.G., B.L., and V.V. were involved in the study design and conceptualization. D.R.G., M.S., A.A., and L.B. performed the statistical analysis. All authors were involved in data interpretation. M.H., I.W., P.I., and J.M. collected human samples and clinical data. D.R.G. drafted the original manuscript. All authors contributed to the writing of the manuscript, made critical comments, and approved the final version.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s44294-025-00099-3.

Correspondence and requests for materials should be addressed to Brigitte Leeners.

Reprints and permissions information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.