

Gynecology & Reproductive Health

Differential Protein Expression of mild to severe forms of Endometriosis in South Indian Ethnic patients

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Received: 05 Sep 2023; **Accepted:** 12 Oct 2023; **Published:** 20 Oct 2023

Citation: Rozati R, Mohiuddin N, Ayapati VA, et al. Differential Protein Expression of mild to severe forms of Endometriosis in South Indian Ethnic patients. *Gynecol Reprod Health*. 2023; 7(5): 1-11.

ABSTRACT

Introduction: Endometriosis is a prevalent, benign, estrogen-dependent, chronic gynecological disorder associated with pelvic pain and infertility. Despite its prevalence and impact on reproductive health, the underlying molecular mechanisms and differential protein expression patterns associated with different disease severity levels remain poorly understood. Key components such as extracellular matrix (ECM) components (laminins, fibronectins), adhesion molecules, cytokines, and growth factors have been shown to contribute to the pathogenesis of Endometriosis. However, the precise nature of these molecular networks and Signalling pathways remain incompletely understood, necessitating further investigation. In our study, we employed proteome profiling techniques to analyze endometrial tissue samples from women with severe forms of endometriosis, comparing them with samples from normal controls.

Objective: It is to identify alterations and dysregulations in specific proteins and pathways associated with endometriosis. We utilized high-resolution two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for protein expression analysis and identification during the mid-secretory phase.

Findings: We identified thirty differentially expressed proteins, including ZC3H13, Tax1bp1, ANKRD36, ZNF658B, MALRD1, and PRRC2A. Notably, a strong association was observed among genes encoding three metabolic enzymes. Enrichment analysis revealed numerous pathways related to endometriosis-related morbidity in KEGG, WiKi, and Reactome databases, underscoring the significant role of these proteins.

Conclusion: This study employed proteome profiling techniques to analyze endometrial tissue samples from women with severe forms of endometriosis, comparing them with samples from normal controls. The research identified thirty differentially expressed proteins, including ZC3H13, Tax1bp1, ANKRD36, ZNF658B, MALRD1, and PRRC2A. Notably, a strong association was observed among genes encoding three metabolic enzymes. Enrichment analysis revealed numerous pathways related to endometriosis-related morbidity, emphasizing the significant role of these proteins.

This research enables the development of accurate diagnostic tools and personalized treatments by identifying specific proteins and pathways associated with endometriosis. The identified proteins also have the potential to serve as biomarkers for disease progression, aiding in monitoring and assessing treatment efficacy.

Keywords

Differentially Expressed Proteins (DEPs), Endometriosis, Proteomics.

Introduction

Endometriosis is a debilitating gynecological disease characterized by the ectopic growth of endometrial-like tissue outside the uterus [1]. It is associated with significant molecular alterations, including changes in gene expression, protein activity, and signaling pathways [2]. As inflammation is a central feature of endometriosis, it is driven by immune cell activation, cytokine release, and the recruitment of inflammatory mediators [3]. The dysregulated immune response not only perpetuates the inflammatory milieu but also contributes to tissue damage, fibrosis, and the formation of adhesions [4]. Moreover, estrogen signaling, which normally regulates endometrial tissue growth and shedding, is altered in endometriosis. Dysfunctional estrogen metabolism and responsiveness promote the survival and growth of ectopic endometrial cells, contributing to disease progression [5].

Understanding the molecular milieu and pathways involved in endometriosis is crucial for unraveling its complex pathogenesis. The disease is characterized by chronic inflammation, and various molecular factors contribute to the establishment and persistence of endometriotic lesions. Dysregulated gene expression, epigenetic modifications, and aberrant protein activity play roles in promoting the survival, attachment, invasion, and angiogenesis of ectopic endometrial cells [6]. These molecular changes affect crucial cellular processes, including cell proliferation, apoptosis resistance, immune response dysregulation, hormonal signaling disruption, and extracellular matrix remodeling [6]. Additionally, the aberrant activation of various signaling pathways has been implicated in endometriosis [7,8]. The PI3K/AKT/mTOR pathway, MAPK/ERK pathway, Wnt/ β -catenin pathway, and NF- κ B pathway, among others, exhibit dysregulated activity in endometriotic lesions. These pathways regulate cellular processes such as cell survival, proliferation, migration, angiogenesis, and immune responses, thereby influencing the pathophysiology of endometriosis [9]. Despite the substantial impact of endometriosis on patients' lives, diagnosing the condition remains challenging due to the lack of specific biomarkers [10]. Efforts to identify molecular biomarkers for endometriosis, such as altered protein expression patterns, are ongoing and hold promise for improving early detection and noninvasive diagnostic approaches.

Proteomics, a branch of molecular biology focused on studying the temporal and spatial expression of proteins in cells or tissues, provides a promising avenue for understanding the pathophysiology of endometriosis and identifying potential diagnostic or therapeutic targets [11-13]. High-resolution two-dimensional gel electrophoresis (2-DE) analysis, coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), enables the comparison of protein expression patterns and the identification of differentially expressed proteins. This approach offers insights into the intricate

molecular alterations associated with endometriosis and can help unravel the underlying mechanisms contributing to disease progression and therapeutic resistance.

To achieve a comprehensive understanding of endometriosis, it is crucial to investigate the different stages of the disease and identify potential biomarkers that could aid in early detection and personalized treatment strategies. Therefore, our study aimed to assess the proteome analysis of mild to severe forms of endometriosis, focusing on differential protein expression. By analyzing paired samples from six endometrial tissue specimens representing different stages of endometriosis during the mid-secretory phase (LH +7), we aimed to uncover the specific proteins and pathways associated with disease severity. Through this research, we anticipate shedding light on the key proteins overexpressed in endometriosis and their role in pathogenesis and invasiveness. Identifying these proteins and elucidating their underlying mechanisms could pave the way for the development of novel definitive biomarkers and targeted therapies for endometriosis. Additionally, the integration of proteomics with other omics technologies, such as genomics and metabolomics, offers a holistic approach to understanding this complex condition [14]. Our study involved the acquisition of paired samples from six endometrial tissue specimens obtained from different stages of endometriosis during the mid-secretory phase (LH +7). These samples underwent two-dimensional gel electrophoresis (2-DE) and mass spectrometry to identify upregulated proteins (Figure 1 provides an overview of our study's workflow). Subsequently, we examined the physiological pathways associated with the identified proteins to assess the impact of specific genes on the protein expression profile observed in patients with severe forms of endometriosis.

Materials and Methods

Participants: This investigation received ethical approval from the Ethics Committees of the Medical Health and Research Institute. Before surgical procedures and sample collections, written informed consent was obtained from each patient. The study included patients who were admitted to the Medical Health and Research Institute between February 1, 2021, and February 28, 2023. A total of ten patients (aged 22-45 years) diagnosed with stage III/IV endometriosis, according to the Revised American Society for Reproductive Medicine classification system, 1997 (rASRM), underwent laparoscopic and histological examinations. From these patients, samples of ovarian endometrioma cyst walls (ectopic endometrium, EcEM) and matched endometrium (EuEM) were collected. In addition, normal endometrial tissue specimens (control endometrium, cEM) were obtained from five patients (aged 23-42 years) without endometriosis. All participants had regular menstrual cycles and had not received gonadotropin-releasing hormone analogues (GnRHa) or other hormonal medications within six months before surgery. Clinical characteristics such as age, BMI, gravidity and parity did not differ statistically between both the groups. After removal, tissue samples were promptly frozen in liquid nitrogen and stored at -80°C for subsequent experiments.

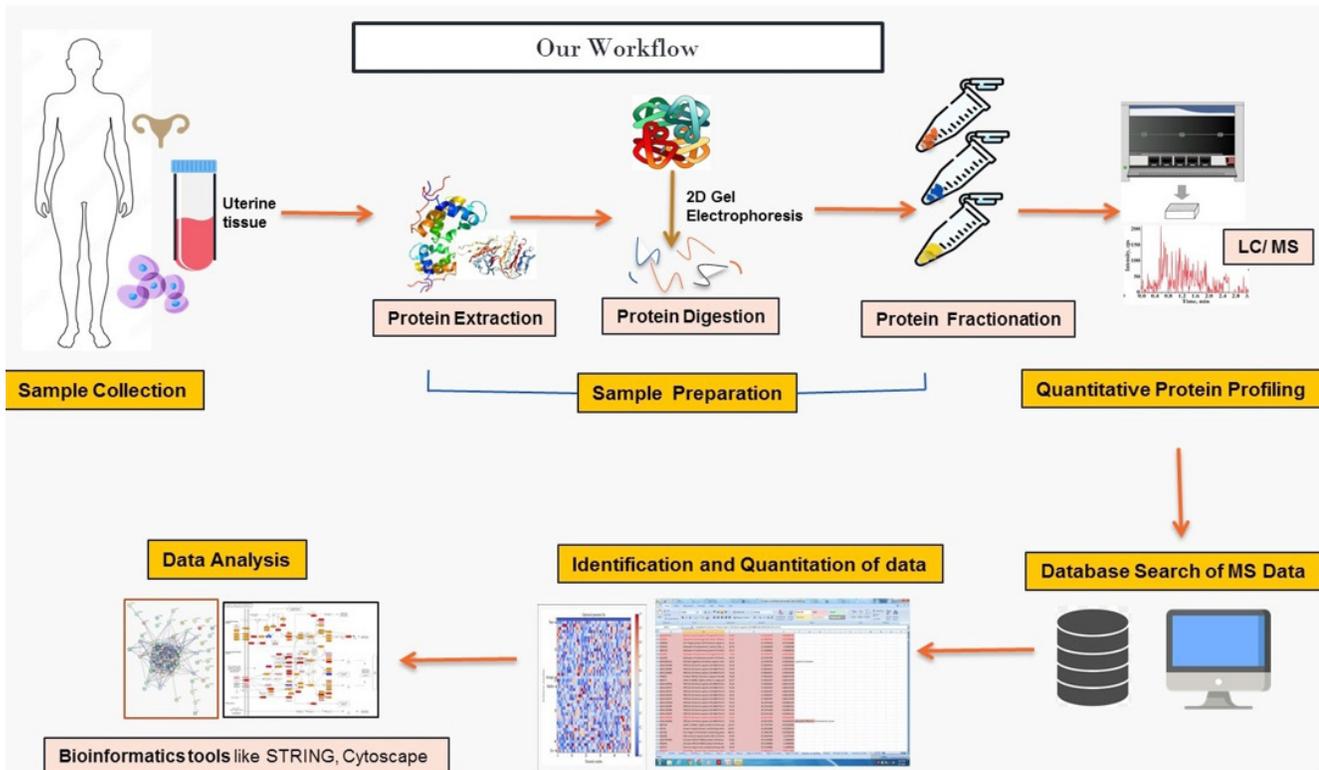


Figure 1: Workflow Overview.

Sample Preparation: Biopsies of endometrial tissue were obtained during the mid-secretory phase of the menstrual cycle (LH +7). Endometrial samples were collected under sterile conditions using a pipette catheter and stored at -80°C for proteome analysis. To deplete serum samples, the ProteoPrep Blue Albumin and IgG Depletion Kit (SIGMA-ALDRICH) was employed, and protein content was measured using the Bradford test. Frozen endometrial tissue samples (40 mg) were homogenised using a handheld homogenizer (POLYTRON® System PT 1200 E) with 400 μl of urea lysis solution (8 M urea, 65 mM CHAPS, 2 M thiourea, 33 mM Tris, 6 mM PMSF, 65 mM DTT, and 1% protease cocktail inhibitor). The homogenate was then vortexed for 30 seconds at 10-minute intervals (three times) and sonicated on ice using an ultrasonic cleaner in 10-second bursts at 10-second intervals ten times. Subsequently, the sample was centrifuged at 16,000 g for 10 minutes using an Eppendorf centrifuge, and the clear supernatant was collected and stored at -80°C for further analysis.

2-DE and Image Analysis: For the first dimension, pH 3-10 13 cm IPG strips from GE Healthcare, Uppsala, Sweden, were utilised, and active/passive rehydration was performed. The proteins were focused on an IPGPhor III (GE Healthcare, Uppsala, Sweden) apparatus using the following IEF conditions: 100 V gradient for 1 hour, 300 V gradient for 2 hours, 1000 V gradient for 1 hour, 5000 V gradient for 5 hours, and 5000 V step, followed by a 5000 V step held for 7 hours at a constant temperature of 20°C . Each IPG strip underwent isoelectric focusing (IEF), followed by

equilibration with 2% DTT and incubation with a different buffer containing 2.5% iodoacetamide in place of DTT. The second dimension PAGE (12.5%) was performed using the SE600 system (GE Healthcare, Uppsala, Sweden) at 1W/gel for 1 hour and 13W/gel for 3 hours. The protein spots were stained with colloidal Coomassie blue G-250 and scanned using a high-precision scanner (ScanMaker 9700XL, Microtek). The gel images were analysed using the gel image analysis tool PDQuest 8.01 (Bio-Rad). Mass spectrometric analysis was performed on the target protein spots excised from the gel after trypsin digestion. Gel fragments were rinsed with Milli-Q water and treated with a decolorizing solution containing 50% acetonitrile and 25% ammonium bicarbonate. After dehydration in 100% acetonitrile (ACN) for 10 minutes, the decolorized gel particles were vacuum dried for 30 minutes.

Protein Identification and Data Analysis: Protein spots that were excised from the gel were decolorized, digested, and extracted following the protocol described by [15]. The peptide samples were analyzed using Matrix-assisted Laser Desorption Ionization (MALDI-TOF/TOF) Mass Spectrometry and LTQ-Orbitrap XL (Bruker Daltonics, Bremen, Germany). Protein identification was performed using the BioTools 3.0 software on MASCOT (V2.1, Matrix Science, UK) based on the peptide mass fingerprint data. Proteins with a MASCOT score greater than 64 and more than four peptide matches were considered significant ($P < 0.05$). Proteins exhibiting a minimum 1.5-fold change between groups and a P-value of 0.05 were subjected to further bioinformatic analysis.

Table 1: Protein expression levels measured in the unexplained infertile and the fertile proteome as identified by MALDI-TOF/TOF or LTQ-Orbitrap XL mass. Only the most expressed proteins are reported.

Accession Number	Protein Description	Gene Symbol	Subcellular localization	Protein Score	Fold Change	Ratio	p value
Q96C95	Afadin	AFDN	Cell Junction, Adherens junction	46.29	2.2	1.2	0.012
A0A3B3ITG1	Laminin subunit alpha 3	LAMA3	extracellular membrane, extracellular matrix, basement membrane	45.84	3.8	14.7	0.01
Q32Q34	zinc finger CCCH-type containing 13	ZC3H13	Nucleoplasm, nucleoplasm	98.94	-0.1	0.9	0.03
A0JP65	signal peptide, CUB domain and EGF like domain containing 1	SCUBE1	Cell Membrane	87.83	2.7	1.7	0.12
Q5TZA2	Rootletin	CROCC	Cytoplasm, Centriole, Cytoskeleton, Centrosome	92.72	5.7	52.9	0.017
A0A4D6DSY5	Tax1-binding protein 2 isoform 2	Tax1bp1	Cytoplasm, Mitochondrion, Cytoplasmic vesicle	40.66	5.7	52.9	0.023
H3BSK8	RB binding protein 6, ubiquitin ligase	RBBP6	Nucleus, Nucleolus, Cytoskeleton, Cytoplasm	241.3	7.0	17.9	0.06
AAB41287	Tenascin-X protein	Tenascin-X	Extracellular Matrix	4.01	4.06	16.7	0.09
A0A0K2BMD8	Mutant hemoglobin alpha 2 globin chain	HBA2	Cytosol	258	1.6	0.32	0.021
A6QL64	Ankyrin repeat domain-containing protein 36A	ANKRD36	-	385.14	7.2	144	0.034
A0A804HKE7	Microtubule-associated protein	MAP4	Cytoplasm, Cytoskeleton	53.85	5.6	50.4	0.041
A0A024R7N1	GEM interacting protein, isoform CRA_b	GMIP	Cytosol, Nucleoplasm, Plasma Membrane	168.45	3.3	10	0.02
Q59FK4	Tyrosine-protein kinase	EIF2AK2	Cytoplasm	149.27	2.2	15.2	0.03
Q6PDB4	Zinc finger protein 880	ZNF880	Nucleus	138.16	3.2	9.2	0.05
P48634	Protein PRRC2A	PRRC2A	Cytoplasm, Nucleus	76.99	4.8	27.9	0.037
Q9H5P4	PDZ domain-containing protein 7	PDZD7	cell projection (cilium), Nucleus	66.9	2.9	3.2	0.016
Q5VYJ5	MAM and LDL-receptor class A domain-containing protein 1	MALRD1	Cytoplasmic vesicle membrane	56.21	5.3	41.2	0.036
Q9NXG0	Centlein	CNTLN	Cytoplasm, Cytoskeleton, microtubule organizing center, centrosome, centriole	51.24	3.5	11.5	0.021
A0A3B3ITG1	Laminin subunit alpha 3	LAMA3	extracellular membrane, extracellular matrix, basement membrane	45.84	3.8	14.7	0.025
Q5JSZ5	Protein PRRC2B	PRRC2B	-	76.99	1.8	0.5	0.043
A0A7P0T9G4	ERCC excision repair 6, chromatin remodeling factor	ERCC6	-	32.61	3.9	15.4	0.041
Q7Z4V5	Hepatoma-derived growth factor-related protein 2	HDGFL2	Nucleus, Cytoplasm	32.61	3.9	15.4	0.021
Q16777	Histone H2A type 2-C	H2AC20	Nucleus	124.9	2.2	13.3	0.012
Q5TZA2	Rootletin	CROCC	-	87.35	1.9	0.71	0.043
A0A7P0Z4H1	C2 domain containing 3 centriole elongation regulator	C2CD3	-	177.9	1.7	2.5	0.044
A0A1B0GV46	Mucin like 3	MUCL3	Membrane	118.3	2.1	0.93	0.033
Q08AD1	Calmodulin-regulated spectrin-associated protein 2	CAMSAP2	Cytoplasm, Cytoskeleton, Gogi Complex	116	1.8	1.2	0.012
A0A0J9YWL9	Putative testis-expressed protein 13C	TEX13C	Cytoplasm	18.3	1.9	0.73	0.015
Q9P2N4	A disintegrin and metalloproteinase with thrombospondin motifs 9	ADAMTS9	Extracellular space, Extracellular matrix, Endoplasmic Reticulum	117	1.7	4.5	0.043
A0A1B0GU80	Cilia and flagella associated protein 54	CFAP54	-	296	2.1	3.4	0.012

Statistical Analysis

Clinical and experimental data were analyzed using Student's t-tests conducted in SPSS for IBM Statistics version 20 (IBM Corp., Armonk, NY, USA). Only proteins that showed consistent and significant changes (either increased or decreased) were included for subsequent bioinformatics analysis. P-values were calculated using right-tailed Fisher's exact tests, when appropriate, to identify statistically significant pathways and networks associated with the identified proteins. A significance level of $P < 0.05$ was used for all analyses.

Results

Quantitative Protein Profiling

We performed a comparative proteomic analysis of uterine tissues from both groups to generate 2-DE reference maps and identify upregulated proteins. The quantitative analysis utilizing Spectrum Mill and Mascot search engines resulted in the identification of over 55 proteins. Among them, 30 proteins exhibited an upregulation of >1.5 -fold in cases of severe endometriosis. On average, 1200 spots were detected in the gels for both proteomes. Comparative analysis with normal endometrium revealed a significant elevation

of 30 protein locations in the endometriosis group ($p < 0.05$). All 30 proteins exhibited a fold change of up to 1.5-fold in expression. Additionally, we identified four down-regulated proteins (Haemoglobin beta isoforms), although the differences were not significant and were not further investigated. Protein spots were identified using MALDI-TOF/TOF and LTQ-Orbitrap XL, with MS/MS data searched against the human section of the UniProt database (Version 20140709, 88,993 sequences) (Table 1).

Bioinformatic Analysis

To elucidate the biological function, pathways, and interaction networks associated with the collected data, we employed various bioinformatic tools and software, including UniProt, Genecards (v.4.8.2), KEGG, Reactome, and STRING (version 10.5). The genes corresponding to differentially expressed proteins were mapped to multiple gene annotation data and biological pathways using PANTHERS 14.0. The categorization of genes was based on molecular function, biological process, route, cellular location, and protein class.

Protein-Protein Interaction and Co-Expression Study

In our study, we utilized the web-based visualization tool STRING to construct and explore the protein-protein interaction (PPI) network. At the same time, Cytoscape (v3.8.2) was employed for additional assessment. The PPI network was an undirected graph, with proteins as nodes and their connections as edges (Figure 2). Coexpression analysis revealed a strong association among the genes of three metabolic enzymes, including Centlein (CNTLN) and Ciliary rootlet coiled-coil, rootletin (CROCC); (combined score: 0.754), as well as between DNA excision repair protein ERCC-6 (ERCC6) and Histone cluster 2 h2a family member c (HIST2H2AC) (combined score: 0.781), E3 ubiquitin-protein ligase (RBBP6) and Zinc finger CCCH domain-containing protein

13 (ZC3H13) (combined score: 0.446). We further analysed co-expression mapping using the STRING database (Figure 3). We found the most significant co-expression between ZC3H13 and HDGFRP2. Additionally, RBBP6 co-expressed with ZC3H13, PRRC2A co-expressed with PRRC2B (co-expression score: 0.179), C2CD3 co-expressed with ANKRD36, and MAP4 co-expressed with CAMSAP2. Notably, there is a potential link between ZC3H13, HDGFRP2, ZC3H13, and Proline-rich coil isoforms and severe endometriosis (Figure 3).

Pathway and Functional Enrichment Analysis

We conducted pathway and functional enrichment analysis using the web-based gene set enrichment analysis program EnrichR, which utilises all differentially expressed common genes to identify significant molecular pathways. We employed KEGG, WiKi, and the Reactome pathway databases to analyse the signalling pathways and gene ontology of the differentially expressed genes in our cohort of unexplained female infertility. Signaling pathways and gene ontology (GO) categories were used as annotation sources, including biological processes, molecular function, and cellular components. Statistical significance was determined based on the adjusted p-value to obtain enhanced results. Using the PANTHER Database, we characterized the upregulated proteins based on their biological processes, cellular components, and protein classes. We represented the gene ontology of the upregulated proteins using a pie chart (Figure 4a), which revealed that the majority (32.4%) of these genes belong to the category of Basement Membrane, closely followed by the Ribosome category (25.4%). Regarding the influence of biological processes, we observed derangements in microtubule minus-end binding, protein tyrosine kinase activator activity, and kinesin binding activities of the cells (Figure 4b). Most proteins (35.5%) did not fall into any specific Panther class, indicating a lack of research in this area (Figure 4c). Among the

node1	node2	score
CNTLN	CROCC	<u>0.754</u>
CROCC	CNTLN	<u>0.754</u>
ERCC6	HIST2H2AC	<u>0.52</u>
HIST2H2AC	ERCC6	<u>0.52</u>
RBBP6	ZC3H13	<u>0.446</u>

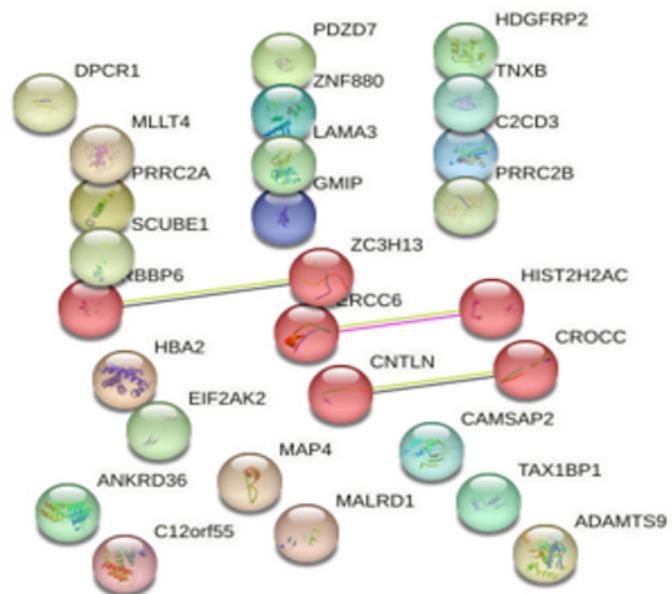


Figure 2: Protein-protein interaction network between the annotated genes using String Database based on Confidence Prediction. (Significant Interactions with high confidence indicated in red).

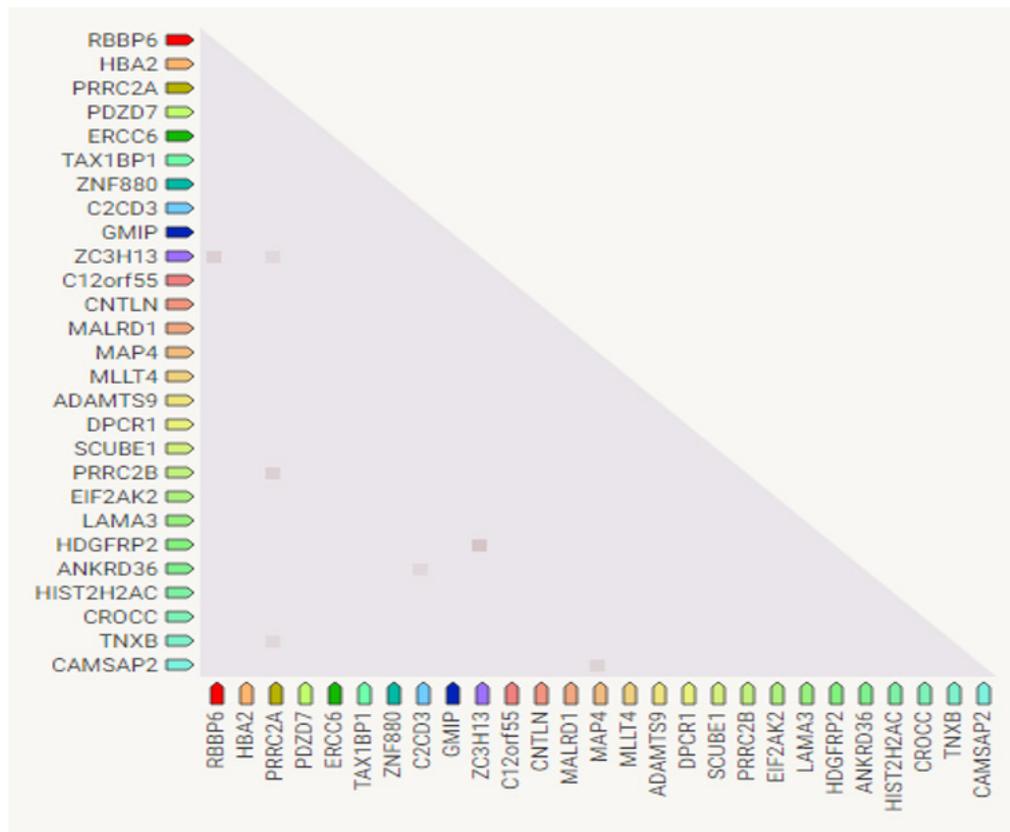


Figure 3: In the triangle matrix below, the intensity of the color indicates the confidence level that two proteins are functionally associated, given the overall expression data in the organism.

known classes, the Gene-specific transcriptional regulator protein class (Panther Category ID: PC00264) exhibited the highest upregulation in our study.

The enrichment study identified 26, 20, and 125 pathways in the KEGG, WiKi, and Reactome databases. We considered only the top ten significant pathways from each pathway database. We found several significant pathways, including Proteoglycans in cancer (hsa05205), Notch Signalling (hsa04330), PPAR signaling pathway (hsa03320), Pathways in cancer (hsa05200), Diseases of signal transduction (R-HSA-5663202), CD28 dependent PI3K/Akt signaling (R-HSA-389357), ABC transporters in lipid homeostasis (R-HSA-1369062), VEGF-VEGFR2 Signalling Pathway (hsa04370), and Thyroid hormone signaling pathway (hsa04919). These pathways and other significant pathways are depicted in Figure 5a–4c.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Discussion

Endometriosis is a complex gynecological disorder characterized by the presence of endometrial tissue outside the uterus, leading to symptoms such as pelvic pain and infertility [16]. Despite its

prevalence and impact on reproductive health, the underlying molecular mechanisms and differential protein expression patterns associated with different disease severity levels remain poorly understood [17].

In this study, we aimed to address this knowledge gap by conducting a proteomic analysis of endometrial tissue samples from women with severe forms of endometriosis, comparing them with samples from normal controls. Our study employed proteome profiling techniques, specifically high-resolution two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), to analyze the protein expression patterns in the mid-secretory phase of the menstrual cycle. By comparing the protein profiles of endometriosis samples with those of normal controls, we aimed to identify alterations and dysregulations in specific proteins and pathways associated with endometriosis. The proteomic analysis revealed thirty differentially expressed proteins in endometriosis samples compared to normal controls. Among these proteins, several notable candidates were identified, including ZC3H13, Tax1bp1, ANKRD36, ZNF658B, MALRD1, PRRC2A, and tenascin. Tenascin is an extracellular matrix (ECM) glycoprotein that plays a crucial role in tissue remodeling, cell adhesion, and migration [18]. Accumulating evidence suggests that tenascin is involved in the development and progression of endometriosis

Figure 4 (A-C): Gene Ontology of the annotated Proteins using PANTHER Database

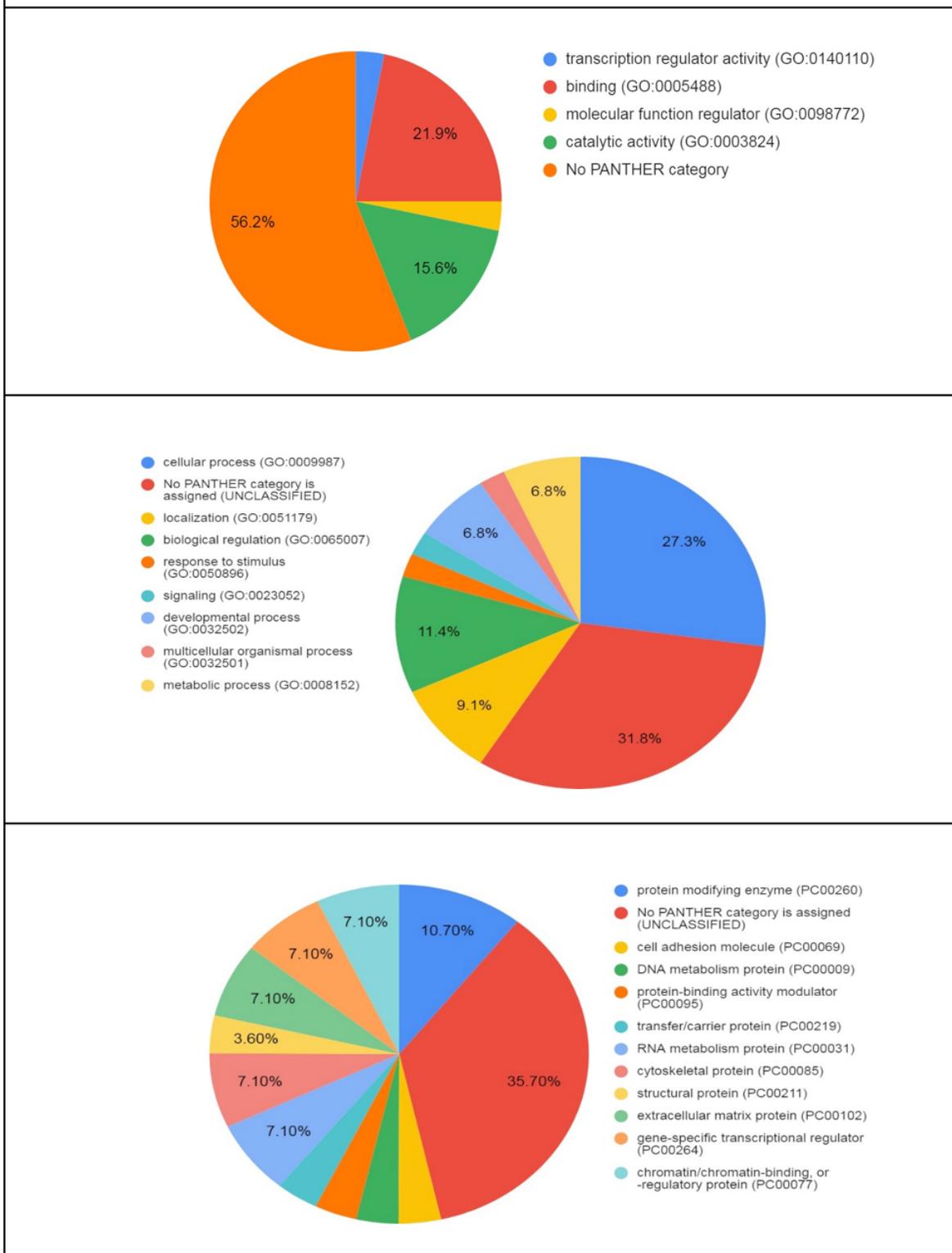
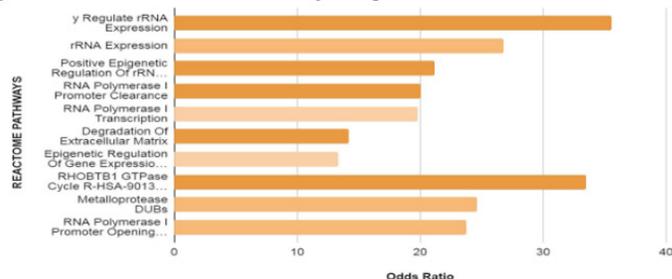


Figure 4: (A-C) Gene ontology of the annotated proteins using PANTHER Database.

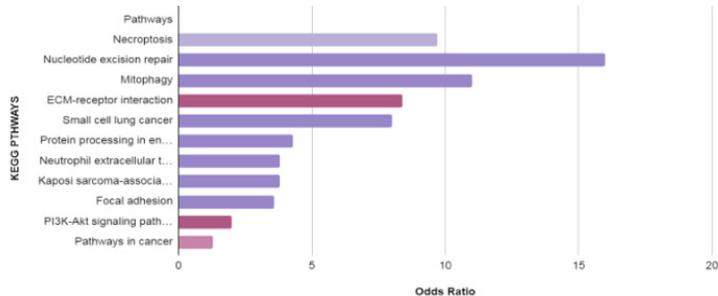
1. Molecular Function
2. Biological process
3. Protein Class

a) The Signalling pathways from KEGG enrichment analysis are shown by the bar diagram with odds ratio and table with adjusted p-value

KEGG Pathways	Adjusted p-value	Genes in Pathway
Necroptosis	0.31	H2AC20;EIF2AK2
Nucleotide excision repair	0.31	ERCC6
Mitophagy	0.31	TAX1BP1
ECM-receptor interaction	0.31	LAMA3
Small cell lung cancer	0.31	LAMA3
Protein processing in endoplasmic reticulum	0.31	EIF2AK2
Neutrophil extracellular trap formation	0.31	H2AC20
Kaposi sarcoma-associated herpes virus infection	0.31	EIF2AK2
Focal adhesion	0.31	LAMA3
PI3K-Akt signaling pathway	0.44	LAMA3
Pathways in cancer	0.52	LAMA3

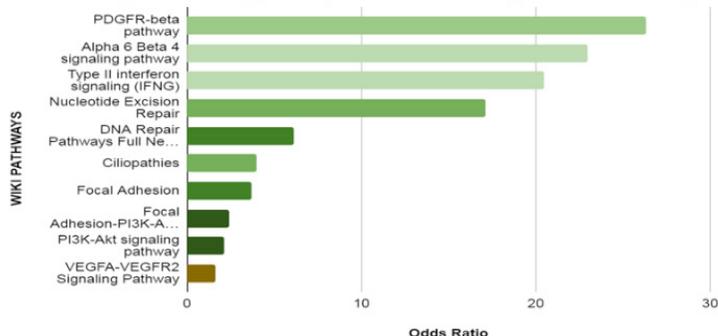


b) The top ten signaling pathways from Reactome enrichment analysis are shown by the bar diagram with p-values



Reactome Pathway	Adjusted P-value	Genes in the pathway
rRNA Expression R-HSA-427389	0.13	H2AC20;ERCC6
B-WICH Complex Positively Regulates rRNA Expression R-HSA-5250924	0.13	H2AC20;ERCC6
Positive Epigenetic Regulation Of rRNA Expression R-HSA-5250913	0.13	H2AC20;ERCC6
RNA Polymerase I Promoter Clearance R-HSA-73854	0.13	H2AC20;ERCC6
RNA Polymerase I Transcription R-HSA-73864	0.13	H2AC20;ERCC6
Degradation Of Extracellular Matrix R-HSA-1474228	0.2	SCUBE1;ADAMTS9
Epigenetic Regulation Of Gene Expression R-HSA-212165	0.2	H2AC20;ERCC6
RHOBTB1 GTPase Cycle R-HSA-9013422	0.22	RBBP6
Metalloprotease DUBs R-HSA-5689901	0.22	H2AC20
RNA Polymerase I Promoter Opening R-HSA-73728	0.22	H2AC20

c) The top 20 signaling pathways from WikiPathways enrichment analysis are shown by the bar diagram with p-values.



Wiki Pathways	Adjusted P-value	Genes associated with the pathways
PDGFR-beta pathway	0.14	EIF2AK2
Alpha 6 Beta 4 signaling pathway	0.14	LAMA3
Type II interferon signaling (IFNG)	0.14	EIF2AK2
Nucleotide Excision Repair	0.14	ERCC6
DNA Repair Pathways Full Network	0.22	ERCC6
Ciliopathies	0.28	C2CD3
Focal Adhesion	0.28	LAMA3
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	0.38	LAMA3
PI3K-Akt signaling pathway	0.4	LAMA3
VEGFA-VEGFR2 Signaling Pathway	0.45	ADAMTS9

Figure 5: The top ten signaling pathways from GO biological process enrichment analysis are shown by the bar diagram with p-Values.

[19,20]. This protein is also expressed during embryogenesis, suggesting a role in the regulation of epithelial-mesenchymal interactions [21]. During the menstrual cycle, tenascin expression in the human endometrium changes physiologically. Depositions of tenascin occur mostly in the stroma surrounding endometrial glands during proliferative phases of the menstrual cycle [22]. Studies have shown that tenascin expression is significantly increased in the ectopic endometrial lesions of women with endometriosis compared to the eutopic endometrium of healthy individuals [22,23]. This upregulation of tenascin in Endometriosis may thus contribute to the adhesion, invasion, angiogenesis, immune modulation, and tissue remodeling processes observed in endometriosis [24]. Thus, ECM proteins are emerging as a potential therapeutic target for Endometriosis [24].

Furthermore, emerging research has highlighted the role of epigenetic modifications, such as DNA methylation, in the etiology of endometriosis [25]. Epigenetic changes can regulate gene expression patterns without altering the DNA sequence and

have been implicated in various diseases, including endometriosis. Aberrant DNA methylation patterns have been observed in the eutopic and ectopic endometrium of women with endometriosis. These epigenetic alterations can affect the expression of genes involved in crucial biological processes, including hormone signaling, immune response, inflammation, and tissue remodeling in Endometriosis [26]. Methylation changes in genes associated with estrogen metabolism, inflammation, and ECM remodeling have been particularly implicated in endometriosis pathogenesis. Thus the integration of proteomic analysis and epigenetic studies can provide a comprehensive understanding of the molecular alterations associated with endometriosis. The identification of differentially expressed proteins, including tenascin, coupled with the characterization of epigenetic changes, such as DNA methylation, contributes to a better understanding of the molecular mechanisms underlying endometriosis. These findings may have implications for the development of diagnostic biomarkers, therapeutic targets, and potential treatment strategies for endometriosis. Currently, proteomic Research focuses on establishing 2-DE reference maps

from organs, tissues, and cells, as well as analyzing special function proteins using proteomic comparison to improve disease molecular classification. It aims to improve disease molecular classifications and to identify sensitive biomarkers for diagnosis, treatment, and prognosis [27]. This study aimed to investigate molecular changes in uterine tissues associated with severe infertility compared to normal endometrium. Comparative proteomic analysis using two-dimensional gel electrophoresis (2-DE) identified over 55 proteins with differential expression patterns. MALDI-TOF/TOF and LTQ-Orbitrap XL instruments were used to identify protein spots, and UniProt database search enhanced result reliability. Comparative analysis revealed significant elevation of 30 protein locations in the endometriosis group, indicating a distinct proteomic profile in uterine tissues associated with severe infertility ($p < 0.05$). Considering that even minor alterations in protein expression can yield substantial functional consequences, we incorporated a fold change threshold of > 1.5 to account for their significance.

In our study, tissue profiling revealed numerous proteins (including the upregulation of the Laminin subunit alpha 3 (LAMA3) protein) whose differential expression is associated with the implantation of endometriotic tissue outside the endometrium and/or the progression of the disease. Notably, despite being histologically benign, endometriotic cells exhibit peculiar invasive properties. Previous studies [28-30], have indicated that this invasiveness may result from the enrichment of multiple proteins involved in the cytoskeletal, extracellular matrix (ECM), and cell matrix interactions of the endometrium. According to the Gene Ontology results from our study, approximately 20% of the identified proteins belonged to these protein classes, and about 43% of the annotated proteins were implicated in the disruption of the cellular process (GO:0009987) of the endometrium. Apart from altered cell adhesion and migration, dysregulated cellular processes involved in endometriosis include abnormal cell proliferation, inflammatory responses, angiogenesis, hormonal imbalance, and immune system dysfunction. These processes contribute to the growth, survival, and spread of endometrial tissue outside the uterus, leading to the characteristic symptoms and complications of endometriosis. While there were certain uncertainties in the enrichment analysis, our study identified numerous proteins involved in the organization of the cytoskeleton and extracellular matrix (ECM), as well as in facilitating cell-matrix interactions. These proteins were found to be enriched. The attachment of endometrial cells to the peritoneal ECM and the invasion of retrograde-shed endometrial cells are essential steps in the implantation of ectopic endometrial cells. It is intriguing to consider that the adhesion/ECM proteins identified in our study likely play important roles in facilitating this process. To annotate the differentially expressed proteins, we utilized databases such as UniProt, Genecards, PANTHER, KEGG, Reactome, and STRING, enabling exploration of functional annotations, biological pathways, and protein-protein interactions. Co-expression mapping in the STRING database highlighted strong co-expression between Zinc finger CCCH domain-containing protein 13 (ZC3H13) and HDGFRP2, suggesting their potential functional relationship in severe endometriosis.

In addition to the identified differentially expressed genes and proteins, investigating the molecular pathways associated with endometriosis can provide further insights into the disease pathogenesis. Pathway enrichment analysis of the differentially expressed proteins in our study highlighted several molecular pathways that are known to play significant roles in endometriosis-related morbidity. Extracellular Matrix (ECM) Remodeling: Endometriosis is characterized by abnormal ECM remodeling, which is crucial for the establishment and growth of ectopic lesions. The dysregulated expression of ECM components, such as tenascin, laminins, and fibronectins, can disrupt the normal tissue architecture and contribute to the invasive and adhesive properties of endometrial cells [31]. Pathways involved in ECM remodeling, such as focal adhesion, integrin signaling, and TGF- β signaling, were found to be enriched in our analysis, emphasizing the importance of ECM dysregulation in endometriosis.

Angiogenesis and Vascular Remodeling: Angiogenesis, the formation of new blood vessels, is critical for the survival and growth of endometriotic lesions [32]. The differential expression of proteins from our study associated with angiogenesis, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), suggests their involvement in promoting the neovascularization observed in endometriosis. Enriched pathways related to angiogenesis, such as VEGF signaling, HIF-1 signaling, and endothelial cell migration, support the significance of angiogenic processes in the pathophysiology of endometriosis.

Immune Dysfunction and Inflammation: Endometriosis is also characterized by an altered immune microenvironment and chronic inflammation [33]. Dysregulation of immune cell function, impaired immune surveillance, and pro-inflammatory cytokine production contribute to the pathogenesis of endometriosis. Pathways associated with immune dysfunction, including cytokine-cytokine receptor interaction, Toll-like receptor signaling, and NF- κ B signaling, were enriched in our analysis. These findings underscore the crucial role of immune dysregulation and inflammation in the development and progression of endometriosis.

Hormone Signaling: Endometriosis is an estrogen-dependent disorder, and dysregulation of hormone signaling pathways is implicated in the disease. Estrogen stimulates the growth and survival of endometriotic lesions. Pathways related to hormone signaling, such as estrogen signaling, progesterone signaling, and MAPK signaling, were found to be enriched in our analysis. These findings support the involvement of hormone-dependent signaling pathways in the aberrant growth and survival of endometriotic tissue.

Epigenetic Modulation: Epigenetic modifications, including DNA methylation, play a crucial role in gene expression regulation and have been implicated in endometriosis. Aberrant DNA methylation patterns in genes associated with estrogen metabolism, inflammation, and ECM remodeling have been observed in endometriosis. Altered DNA methylation can contribute to

the dysregulation of key molecular pathways involved in the pathogenesis of endometriosis.

Investigating these molecular pathways provides valuable insights into the underlying mechanisms of endometriosis. Further research into the specific genes, proteins, and interactions within these pathways will contribute to a deeper understanding of the disease and potentially facilitate the development of targeted therapeutic strategies for endometriosis.

In conclusion, our comparative proteomic analysis of uterine tissues from individuals with severe endometriosis and normal endometrium has revealed significant alterations in the expression levels of several proteins. The upregulated proteins identified in this study provide potential targets for further investigations into the underlying molecular mechanisms contributing to severe infertility. The observed differences in protein expression between the two groups highlight the complexity of the uterine proteome and emphasize the importance of understanding these changes in the context of female reproductive health.

Funding

The work was supported by the Indian Council of Medical Research.

Acknowledgments

We acknowledge all the authors of the manuscript.

References

1. Kao LGL, Giudice LC. Endometriosis. *Lancet*. 2004; 364: 1789-1799.
2. Laganà AS, Garzon S, Götte M, et al. The pathogenesis of endometriosis: molecular and cell biology insights. *Int J Mol Sci*. 2019; 20: 5615.
3. Machairiotis N, Vasilakaki S, Thomakos N. Inflammatory mediators and pain in endometriosis: a systematic review. *Biomedicines*. 2021; 9: 54.
4. Abramiuk M, Grywalska E, Małkowska P, et al. The role of the immune system in the development of endometriosis. *Cells*. 2022; 11: 2028.
5. Chantalat E, Valera MC, Vaysse C, et al. Estrogen receptors and endometriosis. *Int JMol Sci*. 2020; 21: 28.
6. Houshdaran S, Nezhad CR, Vo KC, et al. Aberrant endometrial DNA methylome and associated gene expression in women with endometriosis. *Biol Reprod*. 2016; 95: 93-101.
7. Kim BG, Yoo JY, Kim TH, et al. Aberrant activation of signal transducer and activator of transcription-3 (STAT3) signaling in endometriosis. *Hum Reprod*. 2015; 30: 1069-1078.
8. Guo B, Fang Y, Zhang J, et al. Pattern-recognition receptors in endometriosis: A narrative review. *Front Immunol*. 2023; 14: 1161606.
9. Stefani C, Miricescu D, Stanescu-Spinu, et al. Growth factors, PI3K/AKT/mTOR and MAPK signaling pathways in colorectal cancer pathogenesis: where are we now? *Int J Mol Sci*. 2021; 22: 10260.
10. Anastasiu CV, Moga MA, Elena Neculau A, et al. Biomarkers for the noninvasive diagnosis of endometriosis: state of the art and future perspectives. *Int J Mol Sci*. 2020; 21: 1750.
11. Ferrero S, Gillott DJ, Remorgida V, et al. Proteomics technologies in endometriosis. *Proteomics Clin Appl*. 2008; 5: 705-714.
12. Siva AB, Srivastava P, Shivaji S. Understanding the pathogenesis of endometriosis through proteomics: recent advances and future prospects. *Proteomics Clin Appl*. 2014; 8: 86-98.
13. Ferrero S. Proteomics in the diagnosis of endometriosis: opportunities and challenges. *Proteomics Clin Appl*. 2019; 13: 1800183.
14. Breuer EKY, Murph MM. The role of proteomics in the diagnosis and treatment of women's cancers: current trends in technology and future opportunities. *International Journal of Proteomics*. 2011.
15. Liu A, Jin X, Zhang F, et al. Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. *Biol Reprod*. 2006; 75: 414-420.
16. Parasar P, Ozcan P, Terry KL. Endometriosis: epidemiology, diagnosis and clinical management. *Curr Obstet Gynecol Rep*. 2017; 6: 34-41.
17. Khan MJ, Ullah A, Basit S. Genetic basis of polycystic ovary syndrome (PCOS): current perspectives. *Appl Clin Genet*. 2019; 12: 249-260.
18. Lu P, Takai K, Weaver, et al. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol*. 2011; 3: a005058.
19. Simón-Gracia L, Kiisholts K, Petrikaitė V, et al. Homing peptide-based targeting of tenascin-C and fibronectin in endometriosis. *Nanomaterials*. 2021; 11: 3257.
20. Ozkan ZS, Cilgin H, Atilgan R, et al. Investigation of Tenascin Expression in Endometriosis. *ISRN Pathology*. 2012.
21. Degen M, Tucker R. The Expression and Possible Functions of Tenascin-W during Development and Disease. *Front Cell Dev Biol*. 2019; 7: 53.
22. Vollmer G, Siegal GP, Chiquet-Ehrismann R, et al. Tenascin expression in the human endometrium and in endometrial adenocarcinomas. *Lab Invest*. 1990; 62: 725-730.
23. Tan O, Ornek T, Seval Y, et al. Tenascin is highly expressed in endometriosis and its expression is upregulated by estrogen. *Fertil Steril*. 2008; 89: 1082-1089.
24. Lu P, Takai K, Weaver, et al. Extracellular matrix degradation and re modeling in development and disease. *Cold Spring Harb Perspect Biol*. 2011; 3: a005058.
25. Zubrzycka A, Zubrzycki M, Perdas E, et al. Genetic, epigenetic, and steroidogenic modulation mechanisms in endometriosis. *J Clin Med*. 2020; 9: 1309.

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26. Szukiewicz D. Epigenetic regulation and T-cell responses in endometriosis—something other than autoimmunity. *Front Immunol.* 2022; 13: 943839.
 27. Steel LF, Haab BB, Hanash SM. Methods of comparative proteomic profiling for disease diagnostics. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2005; 815: 275-284.
 28. Irungu S, Mavrelos D, Worthington J, et al. Discovery of non-invasive biomarkers for the diagnosis of endometriosis. *Clin Proteom.* 2019; 16: 1-16.
 29. Regidor PA, Vogel C, Regidor M, et al. Expression pattern of integrin adhesion molecules in endometriosis and human endometrium. *Human reproduction update.* 1998; 4: 710-718.
 30. Umezawa M, Saito Y, Tanaka-Hattori N, et al. Expression profile of extracellular matrix and adhesion molecules in the development of endometriosis in a mouse model. *Reprod Sci.* 2012; 19: 1365-1372.
 31. Itoh H, Mogami H, Nemer LB, et al. Endometrial stromal cell attachment and matrix homeostasis in abdominal wall endometriomas. *Hum Reprod.* 2018; 33: 280-291.
 32. Rocha ALL, Reis FM, Taylor RN. Angiogenesis and endometriosis. *Obstet Gynecol Int.* 2013.
 33. Donnez J, Cacciottola L. Endometriosis: an inflammatory disease that requires new therapeutic options. *Int J Mol Sci.* 2022; 23: 1518.