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PROSPECTIVE COMPARATIVE STUDY OF DIFFERENTIAL PROTEIN EXPRESSION IN TISSUE AND BLOOD SAMPLES OF MILD TO SEVERE FORMS OF ENDOMETRIOSIS UTILIZING PROTEOMICS

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ABSTRACT

Endometriosis is a complex condition characterized by cellular transformations in the body which plays a pivotal role in the pathogenesis and advancement of the condition. The integration of high-resolution two-dimensional gel electrophoresis (2-DE) analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) facilitates the identification of proteins with differential expression in mild to severe forms of disease. **Objective:** This is the first study conducted in South Indian ethnic patients to compare the differential protein expression in mild to severe forms of endometriosis found in tissue and blood samples of the patients utilizing proteomics as its fundamental methodology. **Methods:** Twelve women with infertility as an issue have undergone laparoscopic procedures. The analysis included blood samples from six women diagnosed with EM (endometriosis) and six without EM during surgery, both groups having undergone biopsies. The blood sample was collected on the same day of surgery, at a fasting state, and was sent to the laboratory for further analysis of the protein expression in different groups i.e. Endometriosis and control group. **Results:** We identified seventeen proteins commonly expressed in blood and tissue samples of Endometriosis patients when compared with healthy controls and propose a panel of (A0PJJ2) methyltransferase zinc finger CCCH-type containing 13 (ZC3H13), Signal peptide, CUB, and EGF-like domain-containing proteins (SCUBE 1), Afadin (P55196), proline-rich coiled-coil 2A (PRRC2A) for differentiating women with unexplained Endometriosis from controls by using a decision tree model (accuracy: 0.97). **Conclusion:** The significantly upregulated proteins found in our study might pave the way in the field of endometriosis research further as potential biomarkers for early identification of the disease and for designing targeted and personalized treatment strategies. We propose that conducting diagnostic tests for both blood and tissue markers could enhance the precision of diagnosing Endometriosis.

KEYWORDS: Endometriosis, Protein expression, Laparoscopy, Proteomics.

INTRODUCTION

Endometriosis is an estrogen dependent, gynecological disorder that has an unknown origin. Endometriosis represents a distressing gynecological condition marked by the abnormal growth of tissue similar to the endometrium outside the uterus.[1] It is clinically manifested as dysmenorrhea, dyspareunia, and/or pelvic pain, significantly impacting the overall quality of life for women affected by it[2] and subsequently affecting the fertility of couples. The precise mechanism through which it impacts fertility remains incompletely understood.[3] At present, laparoscopy stands as the most specific and sensitive technique available for assessing and monitoring endometriosis. Despite its efficacy, microscopic or occult endometriosis might be misdiagnosed due to the challenge in visualizing these lesions during laparoscopy. Moreover, invasive procedures have been linked to the accidental induction of endometriosis. The persistence or recurrence of endometriosis commonly occurs even after standard treatments. Consequently, the advantages of noninvasive, biochemical diagnostic markers, particularly in serum, for detecting endometriosis are becoming increasingly apparent.[4]

Utilizing a blood test for diagnosing endometriosis remains challenging but can offer several advantages, including its minimal invasiveness, ready availability, acceptance among women, quick results, and cost-effectiveness in comparison to surgery. Various studies have assessed markers associated with these pathophysiological processes in blood samples, either as a single test or a combination of multiple biomarkers. However, most blood-based tests have only undergone evaluation in a limited number of small studies, each employing varying methods and laboratory techniques.[5] Despite the significant toll that endometriosis takes on patients' lives, diagnosing the condition remains challenging primarily because of the absence of specific biomarkers.[6]

Recent studies have uncovered a distinct expression profile of circulatory microRNAs (miRNAs) in endometriosis tissues when compared to the eutopic endometrium of women with endometriosis and controls.[7,8,9] Given that miRNAs serve as post-transcriptional regulators of gene expression and exhibit stability over time in body fluids and tissues, they are increasingly regarded as highly promising diagnostic biomarkers. In another work, we have found that circulatory onco-miR21 can be used as a potential biomarker for diagnosing endometriosis.[10]

Proteomics, the investigation of proteins' spatial and temporal expression in cells or tissues, allows for the evaluation of both quantitative and qualitative cellular responses concerning particular proteins. The use of high-resolution two-dimensional gel electrophoresis (2-DE) offers a robust method to compare protein expression patterns. Additionally, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) assists in pinpointing protein spots on 2-DE gels. In our research, we collected samples from twelve endometrial tissue specimens and blood samples obtained from infertile women during the mid-secretory phase from diverse grades of Endometriosis. These samples underwent two-dimensional gel electrophoresis and mass spectrometry to identify increased protein expression levels.

These samples underwent comprehensive proteome analysis using high-throughput techniques. The findings demonstrated distinct proteomic variations between mild and severe forms of endometriosis. This underscores the necessity to delve deeper into cellular and molecular alterations occurring at the level of cell-free circulation. Understanding these changes could potentially eliminate the requirement for minimally invasive procedures like laparoscopy, but also to assist in identifying potential biomarkers. These biomarkers could be instrumental in early detection and the development of personalized medicine strategies for endometriosis. Recent studies have highlighted

the significance of analyzing differential protein expression in comprehending disease progression, particularly in the realm of cancer research. Utilizing bioinformatics, we now can design novel drug targets based on this enhanced understanding.[11]

MATERIALS AND METHODS

The research is conducted at the Medical Health and Research Institute focusing on women aged 17 to 35 years. The study received approval from the Institutional Review Board (IRB) at the same institute in Hyderabad, Telangana. Before sample collection, participants provided Signed Informed Consent forms, and their demographic details were recorded using a sample collection proforma. The study involved women admitted for various reasons such as pelvic masses, pelvic pain, Endometriosis, and other conditions, all of whom underwent laparoscopic and histological examinations. Each participant had regular menstrual cycles, had not undergone hormone therapy (such as gonadotropin-releasing hormone agonists or contraceptive tablets) before surgery, and refrained from using anti-inflammatory medications for at least 3 months before the laparoscopy (Xiaon et al). Post-laparoscopy, a comprehensive examination of the peritoneal cavity was conducted to identify any abnormal or typical endometriotic implants and scarring. Detected endometriotic lesions were excised and sent for analysis by a pathologist. Upon confirmation of endometrial lesions through laparoscopic and histological examinations, patients with endometriosis were diagnosed and categorized using the American Society of Reproductive Medicine Revised System standards to determine the severity of their condition. All patients have undergone hysteroscopy and endometrial biopsy.

Pathology reports were used to categorize the women in the study into different endometriosis groups (I, II, III, IV, and Chocolate Cyst) or an endometriosis-free control group. Participants were classified as endometriosis cases or non-endometriosis cases based on the laparoscopy findings. The study cohort was divided into two groups: Group 1 comprised patients diagnosed with endometriosis, while Group 2 included patients with other conditions related to Endometriosis, such as tubal factor, diminished ovarian reserve, male factor, and other reasons, without a history of endometriosis. Exclusions from the study encompassed individuals who had undergone menopause, used hormones within the past three months, had adenomyosis, or had cancer. All the patients have undergone laparoscopy and hysteroscopy. Laparoscopic biopsy endometrial tissue and Blood samples were collected from all the participants at clinical visit in fasting state and these samples were sent to laboratory for further analysis.

Sample collection

In this section of the study, twelve women with infertility as an issue have undergone laparoscopic procedures. The relationship between the groups concerning baseline characteristics like age, BMI, gravidity, and parity is shown in Table 1. The analysis included tissue samples from biopsy and blood samples from six women diagnosed with EM (endometriosis) and six without EM during surgery, both groups having undergone biopsies. The blood sample was collected on the same day of surgery, in a fasting state. Each woman underwent a single endometrial biopsy during the mid-secretory phase of their cycle.

2-DE and Image analysis

In the initial phase, GE Healthcare's pH 3-10 13 cm IPG strips from Uppsala, Sweden, were used, employing both active and passive rehydration methods. Protein focusing was performed on an IPGPhor III apparatus, also manufactured by GE Healthcare in Uppsala, Sweden, following specific IEF (isoelectric focusing) conditions: a gradient of 100 V for 1 hour, 300 V for 2 hours, 1000 V for 1 hour, 5000 V for 5 hours with a subsequent step,

maintaining this voltage for 7 hours, at a constant temperature of 20°C. Each IPG strip underwent IEF, succeeded by equilibration with 2% DTT (dithiothreitol), and followed by incubation in a different buffer solution containing 2.5% iodoacetamide, replacing the DTT. For the second dimension, polyacrylamide gel electrophoresis (PAGE) with a concentration of 12.5% was carried out utilizing GE Healthcare's SE600 system from Uppsala, Sweden. The process included applying 1W/gel for 1 hour, followed by 13W/gel for 3 hours. Subsequently, protein spots were stained with colloidal Coomassie blue G-250 and scanned using the high-precision MicrotekScanMaker 9700XL scanner. Gel images were analyzed using Bio-Rad's gel image analysis tool, PDQuest 8.01.

Further analysis involved mass spectrometric examination of specific protein spots of interest excised from the gel after trypsin digestion. Gel fragments underwent washing with Milli-Q water, followed by treatment with a decolorizing solution comprising 50% acetonitrile and 25% ammonium bicarbonate. The fragments were dehydrated in 100% acetonitrile (ACN) for 10 minutes, followed by vacuum-drying the decolorized gel particles for 30 minutes.[11]

Identification of protein and data analysis

The protein spots excised from the gel were subjected to a decolorization process, followed by digestion and extraction, following the protocol outlined by[12] Peptide specimens underwent analysis using Matrix-assisted Laser Desorption Ionization (MALDI-TOF/TOF) Mass Spectrometry and LTQ-Orbitrap XL (BrukerDaltonics, Bremen, Germany). The identification of proteins was carried out utilizing BioTools 3.0 software within MASCOT (V2.1, Matrix Science, and UK), relying on data obtained from the peptide mass fingerprint. Proteins with a MASCOT score surpassing 64 and more than four peptide matches were considered significant ($P<0.05$). Furthermore, proteins that exhibited a minimum 1.5-fold change between groups and had a P-value of 0.05 were considered significant and underwent bioinformatics analysis.[11] We further utilized the bioinformatic tool i.e., Panther classification system to analyze the genes associated with proteins showing differential expression by mapping them onto various gene annotation datasets and biological pathways via PANTHERS 14.0. The categorization of these genes was determined according to their molecular function, biological process, pathway involvement, cellular localization, and protein class.

Statistical analysis

The clinical and experimental data were subjected to analysis using Student's t-tests within SPSS for IBM Statistics version 20 (IBM Corp., Armonk, NY, USA). Categorical variables were analyzed using the Chi-square test. We specifically focused on proteins showing consistent and noteworthy alterations, whether an increase or decrease, for further examination through bioinformatics analysis. For determining statistically significant pathways and networks associated with these identified proteins, p-values were computed using right-tailed student's t-tests when applicable. Throughout all analyses, a significance threshold of $P<0.05$ was consistently applied.

RESULTS

The study analysis comprised twelve patient samples. The analysis included six patient's endometrial tissue and blood samples diagnosed with EM (endometriosis) and six patient's tissue and blood samples without EM (endometriosis) during surgery. The baseline characteristics of the patients are presented in Table 1. The age of the patients in the control group was higher than the endometriosis group. Both groups had more Asian patients when compared to other ethnicities. Other clinical characteristics like parity, gravidity, rARSM stage of endometriosis patients, and clinical diagnosis in the control group are mentioned in Table 1. The extended duration of infertility among individuals with Endometriosis compared to those without is a notable concern. In this study, our focus was to assess this circumstance

by analyzing the protein expression patterns implicated in the development of Endometriosis, and their potential role as crucial biomarkers for the early identification and treatment of this condition.

Table 1: Baseline characteristics of endometriosis and control group.[13]

Baseline parameter	Endometriosis group	Control group	p-value
Number of women	6	6	NA
Age (years, mean ± S.D)	34±0.3	35.4 ±0.8	0.007[a]
Body mass index (mean ± S.D)	23.5 ±0.4	24.0 ±0.3	0.03[a]
Height (cm)	169±0.7	168±1	0.07[a]
Weight (kg)	65.6±8.0	64±7.0	0.72[a]
Ethnicity, n (%)			
Asian	3 (50%)	4 (66.66%)	0.55[b]
African American	2 (33.33%)	2(33.33%)	>0.99[b]
Unknown	1 (16.66%)	--	--
Parity, n (%)			
0	4 (66.66%)	3 (50%)	0.55[b]
≥1	2 (33.33%)	2 (33.33%)	>0.99[b]
Unknown	--	1 (16.66%)	--
Gravidity, n (%)			
0	2 (33.33%)	1 (16.66%)	0.50[b]
≥1	4 (66.66%)	5 (83.33%)	0.50[b]
Unknown	--		
Duration of infertility in years	12 ±2.5	11 ±3.1	0.22[a]
rARSM Stage, n (%)			
Stage I	1 (16.66%)	NA	NA
Stage II	1 (16.66%)	NA	NA
Stage III	2 (33.33%)	NA	NA
Stage IV	2 (33.33%)	NA	NA
Diagnosis in Non-endometriosis women, n (%)			
Healthy	NA	2 (33.33%)	NA
Ovarian cysts	NA	1 (16.66%)	NA
Polyps	NA	2 (33.33%)	NA
Other gynecological problems	NA	1 (16.66%)	NA

NA=Not Applicable

a. Students t-test

b. Chi-square test

Quantitative Protein Profiling

We conducted a comparative proteomic analysis of uterine tissues from both groups, creating 2-DE reference maps to pinpoint upregulated proteins. Employing quantitative analysis through Spectrum Mill and Mascot search engines, we identified over 55 proteins as seen in Table 1. Among these, 32 proteins showed an increase of more than 1.5-fold in severe endometriosis cases, and over 16 proteins showed an increase of more than 2-fold change in severe endometriosis as shown in Figure 1. On average, 1200 spots were detected in the gels for both proteomes. Protein spots were identified through MALDI-TOF/TOF and LTQ-Orbitrap XL methods, with MS/MS data searched against the human section of the UniProt database (Version 20140709, consisting of 88,993 sequences). [11] The protein expression levels measured in the unexplained infertile proteome found in blood samples as identified by MALDI-TOF/TOF or LTQ-Orbitrap XL mass are shown in Table 2. Only the most expressed and significant proteins have been reported. We also determined protein expression levels measured in the unexplained infertile proteome found in endometrial tissue samples, identified by MALDI-TOF/TOF or LTQ-Orbitrap XL mass as shown in Table 3.

Table – 2: Protein expression levels assessed in the unexplained infertile and fertile proteome found in Blood samples as identified by MALDI-TOF/TOF or LTQ-Orbitrap XL mass. Only the most expressed proteins are reported.

Accession number	Description	Score	Severe Vs Mild Ratio	Log 2 Fold Change
A0PJJ2	ZC3H13 protein (Fragment)OS=Homo sapiens OX=9606 GN=ZC3H13 PE=2 SV=1	165.38	2.033991	1.02431
HOYLN8	Non- specific serine/threonine protein kinase OS=Homo sapiens OX=9606 GN=TRPM7 PE=1 SV=1	81.03	116.746	6.86722
A0AO24R5V1	Non- specific serine/threonine protein kinase OS=Homo sapiens OX=9606 GN=TRPM7 PE=3 SV=1	81.03	109.9472	6.78066
Q96QT4	Transient receptor potential cation channel subfamily M MEMBER 7 OS=Homo sapiens OXX=9606 GN =TRPM7 PE= SV=1	89.92	114.4342	6.83837
P55196 A0AU9X998	Afadin OS=Homo sapiens OX=9606 GN=AFIDIN PE=1 SV=3 PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	163.96 74.16	40.04485 28.50273	5.32354 4.83302
A0A1U9X9A5 A0A1U9X987	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1 PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16 74.16	28.50273 28.21912	4.83302 4.81860
A0A1U9X996 A0A1U9X999	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1 PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16	28.21912 28.21912	4.81860 4.81860
A0A1U9X9A9	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16	28.21912	4.81860
A0A1U9X972	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16	27.93834	4.80417
A0A1U9X977	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16	27.93834	4.80417
A0A1U9X975	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16	27.93834	4.80417
A0A1U9X974	PRRC2A OS=Homo sapiens OX=9606 PE=4 SV=1	74.16	27.93834	4.80417
Q81WY4	Signal peptide, CUB and EGF-like domain-containing protein 1 OS=Homo sapiens OX=9606 GN=SCUBE1 PE=1 SV=3	89.28	1.665291	0.73577

Table 3: Protein expression levels assessed in the unexplained infertile and fertile proteome found in Tissue samples as identified by MALDI-TOF/TOF or LTQ-Orbitrap XL mass. Only the most expressed proteins are reported.[11]

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 alpha3	LAMA3	extracellular membrane, extracellular matrix, basement membrane	45.84	3.8	14.7	0.01
Q32Q34	Zinc finger CCCH-type containing 13	ZC3H13	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	HBA2	Cytosol	258	1.6	0.32	0.021

	globin chain						
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	ell 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 organising 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 like3	MUCL3	Membrane	118.3	2.1	0.93	0.033
Q08AD1	Calmodulin-regulated spectrin-associatedprotein2	CAMSAP2	Cytoplasm, Cytoskeleton, GolgiComplex	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

The comparative proteomic analysis using Protein Lynx global servers (PLGS) in severe endometriosis as compared with mild endometriosis was done and the highly expressed proteins were found to be more than 2-fold increased. The log fold changes along with accession number of proteins reported in blood and endometrial tissue samples are depicted in Figure 1A and 1B.

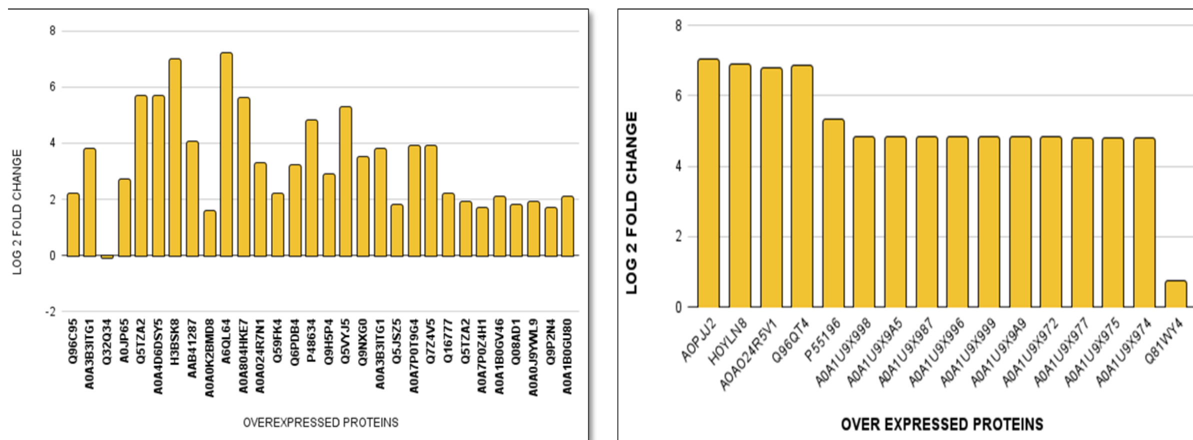

Figure 1: A graphical representation depicting the proteins that are significantly overexpressed, exerted on log fold change in severe form of endometriosis found in:

1A: Tissue samples; 1B: Blood samples

Using the PANTHER Database, we characterized the upregulated common proteins found in blood and tissue samples of endometriosis patients and the control group based on their biological processes, cellular components, and protein classes. We represented the gene ontology of the upregulated proteins found in blood and tissue samples using a pie chart which revealed that in terms of biological process majority of proteins had no Panther category assigned (26%) and cellular process (26%) followed by a smaller proportion of proteins belonged to process like developmental process (14.3%), biological regulation (14.3%) and response to stimulus (14.3%). Regarding the influence of cellular components, we observed majority of proteins had no panther category assigned (75%) followed by 25% proteins belonging to cellular anatomical entity (25%). In terms of protein class, 25% proteins were found to have no panther category assigned indicating a lack of research in this area, 25% proteins belonged to RNA metabolism proteins, 25% proteins belonged to cytoskeletal protein and 25% proteins belonged to extracellular matrix protein as shown in Figure 2.

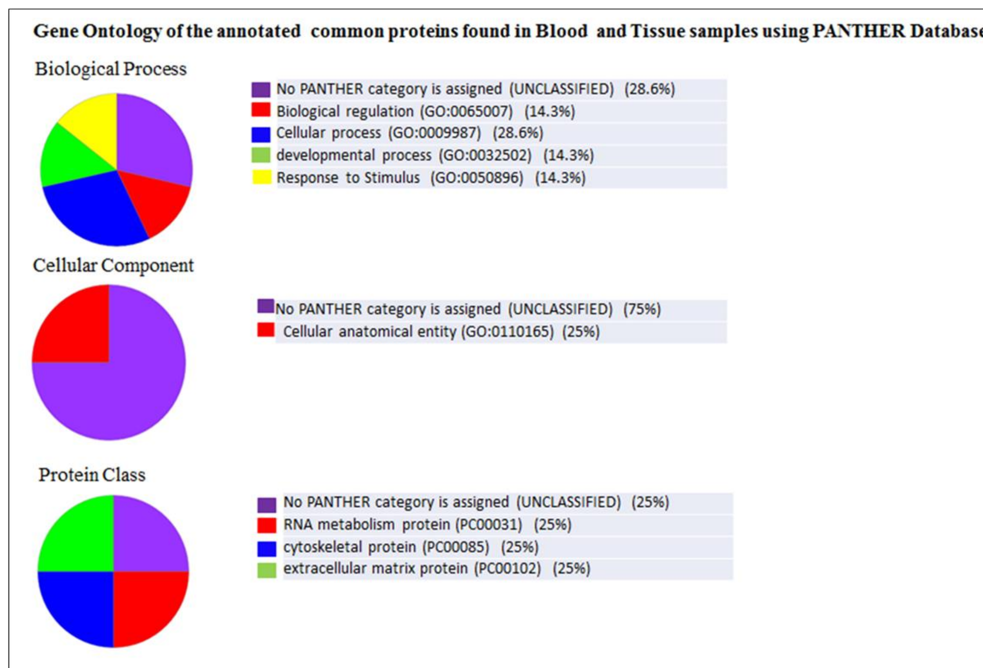

Figure 2: Gene Ontology of common proteins found in blood and tissue samples using PANTHER database.

In our study, we identified seventeen proteins commonly expressed in blood and tissue samples of Endometriosis patients when compared with healthy controls and propose a panel of the following:
(A0PJJ2) methyltransferase zinc finger CCCH-type containing 13 (ZC3H13), Signal peptide, CUB, and EGF-like domain-containing proteins (SCUBE 1), Afadin (P55196), proline-rich coiled-coil 2A (PRRC2A) for differentiating women with unexplained Endometriosis from controls by using a decision tree model (accuracy: 0.97). More than ten proteins showed a log 2-fold increase (upregulated) in the severe form of Endometriosis. This could mean that these genes encoding proteins have a significant role in the pathogenesis of Endometriosis and can be used as effective biomarkers for the detection and treatment of severe stage of Endometriosis.

DISCUSSION

Endometriosis, a common, non-malignant gynecological condition, is reliant on estrogen and characterized by chronic pelvic pain and fertility issues. Despite its widespread occurrence and significant effects on reproductive health, the specific molecular mechanisms and varied protein expression patterns linked to varying levels of disease severity are still not thoroughly comprehended.

This study sought to bridge this informational void by performing a transcriptomic and proteomic investigation on endometrial tissue samples and blood samples obtained from women experiencing severe endometriosis. We compared these tissue samples with those of blood and found more than ten proteins of more than 2-fold increase in tissues and blood. In our research, we utilized proteome profiling methodologies, specifically employing high-resolution two-dimensional gel electrophoresis (2-DE) and Protein Lync Global server (PLGS). These techniques were employed to scrutinize the expression patterns of proteins during the mid-secretory phase of the menstrual cycle. By comparing the protein profiles of endometriosis tissue samples with those of blood samples of endometriosis patients, we aimed to identify alterations and dysregulations in specific proteins and pathways associated with endometriosis.[11]

We found certain proteins that were commonly over-expressed in **"Mild", "Severe" and "Severe OE" like AOPJJ2, SCUBE 1, P55196, and PRRC2A.** These proteins were studied for their role in endometriosis. The existing data have shown that the proteins were highly expressed in severe endometriosis cases as compared with mild cases. Interestingly, the results show that (A0PJJ2) methyltransferase zinc finger CCCH-type containing 13 (ZC3H13) was expressed as significantly high in the severe form of endometriosis.[14] This protein is known to contribute to the m6A RNA methylation process, and other functions as methyltransferases catalyze the addition of m6A marks on RNA. In the realm of oncology, there has been a notable shift in attention towards the role of m6A methyltransferases. Various studies have yielded significant breakthroughs in comprehending and addressing their function in both understanding and treating cancer. The impact of m6A methyltransferases on the proliferation, invasion, and metastasis of diverse tumors exhibits considerable variation.[15]

The second over-expressed proteins are (H0YLN8, A0A024R5V1, Q96QT4), the transient receptor potential melastatin-subfamily member 7 (TRPM7) is a divalent cations permeant channel but also has intrinsic serine/threonine kinase activity. Its widespread expression in normal tissues has been well-established, and research suggests its involvement in crucial physiological and pharmacological mechanisms via its channel-kinase activity. These include the regulation of calcium/magnesium homeostasis and the phosphorylation of proteins associated with embryogenesis or fundamental cellular processes.[16]

P55196 (AFADIN) ROLE

The AFADIN (P55196) protein belongs to the cadherin superfamily and plays key roles in intercellular adhesion. An emerging intercellular adhesion system, consisting of afadin along with nectin, also has roles in the organization of a variety of intercellular junctions either in cooperation with or independently of, adherin. The adhesion system is furthermore involved in the formation of synapses in neurons and the organization of heterotypic junctions between Sertoli cells and spermatids in the testis.[17] In a study conducted by Ikeda, W et al, Afadin molecule has been widely studied in mice. The study showed that in wild-type mice between embryonic days 6.5–8.5, afadin exhibited significant expression levels within the embryonic ectoderm and mesoderm, while its presence was notably scarce in extra-embryonic regions like the visceral endoderm. However, in Afadin−/− mice, developmental abnormalities surfaced, particularly during and after gastrulation.[18] However, the exact mechanisms by which afadin influences Endometriosis are still under investigation, and further research is necessary to fully elucidate its precise role in reproductive health and fertility.

PRRC2A GENE ROLES

CNS: The proline-rich coiled-coil 2A (PRRC2A) gene is located at the MHC class III region and its function is largely unknown. At present, the PRRC2A gene has been widely studied in other immune-related diseases, especially accumulative data completely defining the increased risk for the development of RA. Studies also shed light on the significance of Prrc2a in the development and function of oligodendrocytes, offering insights into potential therapeutic targets for conditions associated with impaired myelination or oligodendrocyte-related disorders. Research has shown that Prrc2a functioning as a novel m^6A reader plays a crucial role in oligodendrocyte specification and myelination, suggesting that PRRC2A may modify the risk of demyelinating diseases of the CNS.[19]

SPERMATOGENESIS: The PRRC2A gene has been identified as being prominently expressed in the testes, playing a crucial role in male fertility. Observations have shown that PRRC2A protein expression is notably heightened during the initial phase of spermatogenesis, particularly during the period between postnatal days 14 to 22 (P14-22). Studies suggest that PRRC2A's role in male meiosis I am essential for the completion of this stage by positively influencing the degradation or translational efficiency of mRNA targets involved in this process. This suggests that PRRC2A may have a wider role in regulating various diseases, making it a potential target for clinical treatment.[19] However, further investigations are warranted to uncover the full extent of PRRC2A's involvement in other physiological and pathological processes beyond male fertility and cancer.

The Signal peptide, CUB, and EGF-like domain-containing proteins (SCUBE) constitute secretory cell surface glycoproteins pivotal in various developmental processes. Specifically, SCUBE1 resides within platelet α-granules and is subsequently transferred to the cell surface and released through proteolysis following post-activation. SCUBE1's implications are frequently linked to pathological conditions, including thrombus formation, atherosclerotic plaque development, inflammation, and disorders associated with hypoxia.[20]

This study sheds light on the intricate profile of proteins that exhibit significant expression across various stages of endometriosis. In our study, we identified seventeen proteins commonly expressed in blood and tissue samples of Endometriosis patients when compared with healthy controls and propose a panel of (A0PJJ2) methyltransferase zinc finger CCCH-type containing 13 (ZC3H13), Signal peptide, CUB, and EGF-like domain-containing proteins (SCUBE 1), Afadin (P55196), proline-rich coiled-coil 2A (PRRC2A) for differentiating women with unexplained Endometriosis

from controls by using a decision tree model (accuracy: 0.97). More than ten proteins showed log 2-fold increase (upregulated) in the severe form of Endometriosis. This could mean that these genes encoding proteins have a significant role in the pathogenesis of Endometriosis and can be used as effective biomarkers for the detection and treatment of severe stage of Endometriosis. More than ten proteins showed a log 2-fold increase (upregulated)in the severe form of Endometriosis. Moreover, we analyzed the gene ontology to identify and describe the functions of gene products in the organism, which could help to understand the relationship between the functionality of the proteins and the clinical progression of the disease. Our study's findings represent a significant stride in the field of endometriosis research, providing a comprehensive perspective on the protein expression dynamics throughout the different stages of the condition and paving the way for targeted and personalized approaches toward better management and treatment strategies.

LIMITATIONS

It is crucial to acknowledge the limitations of our study, including the relatively small sample size and potential confounding factors. To validate these findings and provide a more comprehensive understanding of different proteins expressed in severe forms of endometriosis, larger and more diverse cohorts should be investigated.

CONCLUSION

Our comparative study between blood and endometrial tissue samples of different grades of endometriosis sheds light on the complex proteins expressed significantly in different stages of Endometriosis. The significant upregulated proteins might pave the way in the field of endometriosis research further as potential biomarkers for early identification of the disease and for designing targeted and personalized treatment strategies. Identifying these significant proteins offers promising prospects for advancements in diagnostic methods, treatments, and potential interventions tailored to specific stages of endometriosis. Exploring additional blood markers in research could potentially decrease the necessity for more invasive procedures such as laparoscopy. Furthermore, tissue biomarkers have exhibited a higher probability of enhanced diagnostic accuracy compared to markers detected in the bloodstream. Yet, according to our research, we propose that conducting diagnostic tests for both blood and tissue markers could enhance the precision of diagnosing Endometriosis. In other words, it could increase the diagnostic accuracy.

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Authors' Contributions

All authors contributed equally to this work; RR, SSA, AAK, VAA designed the study; AAK, SB conducted the laboratory work; RR, SSA, WT, AAK wrote the manuscript. SSA performed the statistical analysis. All authors reviewed and approved the final manuscript.

REFERENCES

1. Smolarz, B., Szyłło, K., & Romanowicz, H., Endometriosis: Epidemiology, Classification, Pathogenesis, Treatment and Genetics (Review of Literature). *International journal of molecular sciences*, 2021; *22*(19): 10554. https://doi.org/10.3390/ijms221910554
2. Klemmt, P. A. B., & Starzinski-Powitz, A., Molecular and Cellular Pathogenesis of Endometriosis. *Current women's health reviews*, 2018; *14*(2): 106–116. https://doi.org/10.2174/1573404813666170306163448
3. Macer, M. L., & Taylor, H. S., Endometriosis and infertility: a review of the pathogenesis and treatment of endometriosis-associated infertility. *Obstetrics and gynecology clinics of North America*, 2012; *39*(4): 535–549. https://doi.org/10.1016/j.ogc.2012.10.002
4. Zhang, H., Niu, Y., Feng, J., Guo, H., Ye, X., & Cui, H., Use of proteomic analysis of endometriosis to identify different protein expression in patients with endometriosis versus normal controls. *Fertility and sterility*, 2006; *86*(2): 274–282. https://doi.org/10.1016/j.fertnstert.2006.01.028
5. Nisenblat, V., Bossuyt, P. M., Shaikh, R., Farquhar, C., Jordan, V., Scheffers, C. S., Mol, B. W., Johnson, N., & Hull, M. L., Blood biomarkers for the non-invasive diagnosis of endometriosis. *The Cochrane database of systematic reviews*, 2016; *2016*(5): CD012179. https://doi.org/10.1002/14651858.CD012179
6. Anastasiu, C. V., Moga, M. A., Elena Neculau, A., Bălan, A., Scârneciu, I., Dragomir, R. M., Dull, A. M., & Chicea, L. M., Biomarkers for the Noninvasive Diagnosis of Endometriosis: State of the Art and Future Perspectives. *International journal of molecular sciences*, 2020; *21*(5): 1750. https://doi.org/10.3390/ijms21051750
7. Laudanski, P., Charkiewicz, R., Tolwinska, A., Szamatowicz, J., Charkiewicz, A., & Niklinski, J., Profiling of Selected MicroRNAs in Proliferative Eutopic Endometrium of Women with Ovarian Endometriosis. *BioMed research international*, 2015; *2015*: 760698. https://doi.org/10.1155/2015/760698
8. Cosar, E., Mamillapalli, R., Ersoy, G. S., Cho, S., Seifer, B., & Taylor, H. S., Serum microRNAs as diagnostic markers of endometriosis: a comprehensive array-based analysis. *Fertility and sterility*, 2016; *106*(2): 402-409.
9. Haikalis, M. E., Wessels, J. M., Leyland, N. A., Agarwal, S. K., & Foster, W. G., MicroRNA expression pattern differs depending on endometriosis lesion type. *Biology of reproduction*, 2018; *98*(5): 623-633.
10. Rozati, R., Khan, A. A., Mehdi, A. G., Tabasum, W., & Begum, S., Circulating oncomiR-21 as a potential biomarker of mild and severe forms of endometriosis for early diagnosis and management. *Gene Reports*, 2023; *30*: 101725.
11. Rozati, R., Mohiuddin, N., & Ayapati, V. A., Differential Protein Expression of mild to severe forms of Endometriosis in South Indian Ethnic patients. Gynecol Reprod Health, *Correspondence: Roya Rozati, Medical and Research Director, Maternal Health and Research Trust, Banjara Hills, Hyderabad, India. Received,* 2023; 7(5): 1-11.
12. Liu, A. X., Jin, F., Zhang, W. W., Zhou, T. H., Zhou, C. Y., Yao, W. M., Qian, Y. L., & Huang, H. F., Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. *Biology of reproduction*, 2006; *75*(3): 414–420. https://doi.org/10.1095/biolreprod.105.049379
13. Bahramy, A., Zafari, N., Izadi, P., Soleymani, F., Kavousi, S., & Noruzinia, M., The Role of miRNAs 340-5p, 92a-3p, and 381-3p in Patients with Endometriosis: A Plasma and Mesenchymal Stem-Like Cell Study. *BioMed research international*, 2021; *2021*: 5298006. https://doi.org/10.1155/2021/5298006

14. *ZC3H13 zinc finger CCCH-type containing 13 [Homo sapiens (human)] - Gene - NCBI*. (n.d.). https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=23091
15. Gu, J., Zhan, Y., Zhuo, L., Zhang, Q., Li, G., Li, Q., Qi, S., Zhu, J., Lv, Q., Shen, Y., Guo, Y., Liu, S., Xie, T., & Sui, X., Biological functions of m^6A methyltransferases. *Cell & bioscience*, 2021; *11*(1): 15. https://doi.org/10.1186/s13578-020-00513-0
16. Nadezhdin, K.D., Correia, L., Narangoda, C. *et al.* Structural mechanisms of TRPM7 activation and inhibition. *Nat Commun*, 2023; 14: 2639. https://doi.org/10.1038/s41467-023-38362-3
17. Takai, Y., & Nakanishi, H., Nectin and afadin: novel organizers of intercellular junctions. *Journal of cell science*, 2003; *116*(Pt 1): 17–27. https://doi.org/10.1242/jcs.00167
18. Ikeda, W., Nakanishi, H., Miyoshi, J., Mandai, K., Ishizaki, H., Tanaka, M., Togawa, A., Takahashi, K., Nishioka, H., Yoshida, H., Mizoguchi, A., Nishikawa, S., & Takai, Y., Afadin: A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis. *The Journal of cell biology*, 1999; *146*(5): 1117–1132. https://doi.org/10.1083/jcb.146.5.1117
19. Wu, R., Li, A., Sun, B., Sun, J. G., Zhang, J., Zhang, T., Chen, Y., Xiao, Y., Gao, Y., Zhang, Q., Ma, J., Yang, X., Liao, Y., Lai, W. Y., Qi, X., Wang, S., Shu, Y., Wang, H. L., Wang, F., Yang, Y. G., … Yuan, Z., A novel m^6A reader Prrc2a controls oligodendroglial specification and myelination. *Cell research*, 2019; *29*(1): 23–41. https://doi.org/10.1038/s41422-018-0113-8.
20. Kumar, S., Prajapati, K. S., & Gupta, S., The Multifaceted Role of Signal Peptide-CUB-EGF Domain-Containing Protein (SCUBE) in Cancer. *International journal of molecular sciences*, 2022; *23*(18): 10577. https://doi.org/10.3390/ijms231810577.