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Plasma Proteomic Signature of Endometrial Cancer in Patients with Diabetes

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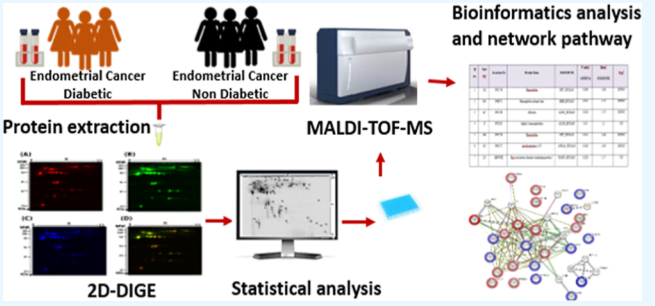
ABSTRACT: The incidence and mortality of endometrial cancer (EC) have increased in recent years. There is mounting evidence that diabetes may play a role in the greater incidence of EC. The molecular mechanisms of the interaction between type 2 diabetes and EC are not yet clearly understood yet. The present study was undertaken to investigate the plasma proteomics of EC patients with diabetes in comparison to those of EC patients without diabetes. Plasma samples were obtained from age-matched patients (EC diabetic and EC nondiabetic). Untargeted proteomic analysis was carried out using a two-dimensional differential gel electrophoresis coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Of the 33 proteins identified, which significantly differed in the plasma abundance between groups, 17 were upregulated and 16 were downregulated. The majority of the altered proteins are involved in the acute phase reaction, cholesterol metabolism, scavenging of heme from plasma, and plasma lipoprotein assembly and mobilization. α-2-macroglobulin, Ras association domain-containing protein 3, apolipoprotein A-I, α-1B-glycoprotein, and zinc-α-2-glycoprotein were significantly upregulated. The significantly downregulated proteins included haptoglobin, apolipoprotein A-IV, hemopexin, and α-1-antichymotrypsin. The differential expression of proteins found in patients who had EC and diabetes indicated severe disease and a poor prognosis. The protein interaction analysis showed dysregulation of cholesterol metabolism and heme scavenging pathways in these patients.



1. INTRODUCTION

Endometrial cancer (EC) is a malignant disease that originates from the inner epithelium of the uterus. It was the sixth most frequent cancer affecting women worldwide in 2020.[1] The incidence and mortality of EC have increased in recent years. The increases in the prevalence of risk factors such as obesity, physical inactivity, and the aging population are attributed to the increased incidence rate of EC.[1] The majority of the EC cases are of low-grade type I, which has a favorable prognosis, while 15% of cases are of type II invasive tumors.[2] Along with obesity and age, the other risk factors attributed to the increased incidences of EC are late-onset menopause, lower age of menarche, chronic anovulation, family history of EC, infertility due to the polycystic ovarian syndrome, long-term unbalanced estrogen therapy, tamoxifen treatment, and nulliparity.[3] Endometrial malignancies of the most prevalent type 1 low-grade endometrioid carcinoma predominantly depend on the continuous estrogenic environment without progesterone or progestins. The estrogen-secreting tumors and polycystic ovaries interfere with the ovulation and menstrual cycles, leading to an elevated free estrogen environment, and are associated with a high risk for EC.[4] Early menarche and late menopause enhance endogenous estrogen exposure throughout the course of a woman's lifetime and are linked to an elevated risk of EC.[5] Type 2 diabetes (T2D) and insulin resistance are also implicated as risk factors for EC.[4]

Diabetes mellitus (DM) is a common metabolic disorder that is characterized by the inability to utilize blood glucose due to insulin deficiency and/or insulin resistance. T2D is thought to be linked to a higher risk of developing certain malignancies. There is mounting evidence that diabetes may play a role in the greater incidence of EC. The risk of EC is elevated by 62% independent of obesity in women with T2D.[6,7] Hyperglycemia and hyperinsulinemia are hallmarks of diabetes, and these factors independently contribute to the increased risk of EC. Insulin resistance leads to increased insulin levels in patients with diabetes. Through the pro-

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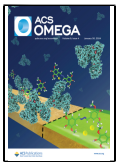

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proliferative and antiapoptotic effects of insulin on endometrial cells, hyperinsulinemia increases the risk of EC.[7] Insulin can also indirectly promote tumorigenesis by modulating insulin-like growth factors (IGFs), sex hormones, and adipokines.[8] Hyperglycemia is found to be a risk factor for EC independent of obesity.[9] Blood glucose levels were found higher in morbidly obese women with type I EC than those without EC while their estrogen and insulin levels were found to be similar.[10] Persistently high blood glucose levels promote tumor growth by serving as a carbon supply for a variety of metabolic processes that are essential for proliferation. The molecular mechanisms of the interaction between T2D and EC are not clearly understood yet. Our previous study on proteomic analysis of EC tissues from patients with T2D showed that proteins linked with cancers and metastasis were found to be upregulated.[11] However, there are no studies on plasma proteomics comparing EC patients with and without diabetes. Untargeted proteomic analysis on such a population can reveal the overall molecular changes due to the presence of diabetes in EC patients. The present study was undertaken to investigate the plasma proteomics of EC patients with diabetes in comparison to EC patients without diabetes.

2. MATERIALS AND METHODS

2.1. Ethical Approval and Consent to Participate. All procedures for the study were carried out after obtaining consent from the Institutional review board, College of Medicine, King Saud University. The participants included in the study provided written informed consent (IRB number: E-193622). The study related procedures were carried out at the Proteomics Resource Unit, Obesity Research Center, College of Medicine and King Khalid University Hospital (KKUH), King Saud University, Riyadh, Saudi Arabia.

2.2. Study Design and Patient Selection. The study included patients between the ages of 46 and 75 who presented to the outpatient clinics of the Obstetrics and Gynecology-Oncology Department at King Khalid University Hospital, College of Medicine, King Saud University. Clinical appointments were used to carry out the initial evaluation, and 16 women in total were recruited. Patients who agreed to take part in the trial were enlisted, and informed consent was obtained from each patient. The patients were subdivided into two groups, endometrial cancer diabetic (ECD = 8) and endometrial cancer nondiabetic (ECND = 8), based on the following inclusion criteria: ECD: women diagnosed with diabetic and EC with atypia tended toward metastatic changes, undergoing total hysterectomy; ECND: nondiabetic women diagnosed with EC with atypia tended toward metastatic changes, undergoing total hysterectomy. The criteria used to determine diagnosis of DM was considered when at least one of the following criteria was met: (1) documented DM in medical records; (2) the presence of analytical studies complying with the DM diagnostic criteria of the 2020 American Diabetes Association guidelines; and (3) if the patient was on antidiabetic medication. The pathological features of the 16 patients were collected from the clinical records and from the files of the Department of Pathology. To avoid confounding results and selection biases, the selection of DM and non-DM patients was performed rigorously by creating two groups of patients with an equivalent female ratio, median age of diagnosis, tumor stage, and histological type.

Progenesis SameSpots nonlinear dynamics statistical software was used to calculate sample size and determine the minimum number of biological replicates. All patients, including those with diagnoses of ECD and ECND, had blood collected in EDTA-containing tubes. The samples were collected and centrifuged at 2500g for 20 min at 4 °C. Aliquots of the plasma samples were placed in various cryotubes and kept at 80 °C pending use.

2.3. Highly Abundant Protein Depletion. The highly abundant proteins that may interfere with MS analysis and biomarker detection were depleted for all of the plasma samples prior to any proteomics experiments. According to the manufacturer's instructions and utilizing a multiple affinity removal technique, Top-20 Depletion ProteoPrep spin columns (Sigma), we depleted the most abundant proteins (immunoglobulins, albumin, α-1 antitrypsin, and transferrin).[12]

2.4. Protein Precipitation. To remove interfering compounds and minimize plasma protein degradation, TCA/acetone precipitation was performed. The ice-cold acetone/TCA (10% w/v) in a ratio of 1:4 was mixed with depleted plasma proteins and were vortexed for 15 s. The mixture was kept at −20 °C for protein precipitation. After overnight incubation, the mixture was centrifuged at 2000g for 15 min at 4 °C. The supernatant was discarded without disturbing the pellet sediment at the bottom of the tube.[13]

2.5. Cye Dye Labeling, Two-Dimensional (2D) Electrophoresis, and Image Scanning. The derived protein pellet was resuspended in an appropriate buffer containing 7 M urea, 2 M thiourea including 4% CHAPS, and 30 mM Tris. 2D-Quantkit (GE Healthcare, Sweden) was used to determine the protein concentrations in triplicates. As previously described by our group, the proteins were labeled with CyDyeTM DIGE Fluor minimal dye (400 pmol/50 g) (GE Healthcare, Sweden) in accordance with the manufacturer's instructions.[14−18] Prior to labeling the pH of all the samples was checked. In brief, for each sample, 50 μg of protein was mixed with 400 pmol of freshly dissolved Cy-3 or Cy-5 in anhydrous dimethylformamide for 30 min on ice in the dark. By adding lysine (1.0 L, 10 mM, and 10 min on ice in the dark), the reaction was stopped. Samples were labeled with, either Cy3 or Cy5 covalently, the fluorophore, while Cy2 labeled protein samples (50 μg, from all 16 samples) were used as the pooled internal standard. The labeled samples were combined according to the experimental design (Table S1) as mentioned.[14−18] After being passively rehydrated (30 V, 12 h), the eight Immobiline DryStrips (24 cm, pH 3−11; GE Healthcare, Sweden) were used to carry out the one-dimensional analytical gel electrophoresis. After that, isoelectric focusing was performed using a GE Healthcare Ettan IPGphor IEF machine, as previously discussed.[14−18] The steps and holding order are as follows: for focusing at 20 °C and 50 A per strip, the following voltages were used: (1) 500 V for 1 h; (2) 1000 V for 1 h; (3) 8000 V for 3 h; and (4) 8000 V for 45,000 Vh and stored at −80 °C. The strips were equilibrated at the following conditions (15 min, RT, gentle stirring, 5 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 65 mM DTT) prior to running the two-dimensional gel electrophoresis. In the same solution containing 250 mM iodoacetamide, a further equilibration lasting 15 min was carried out. On low fluorescence glass, polyacrylamide gradient gels (5−20%) were created. With the use of six vertical Ettan DALT units from GE Healthcare in Sweden, two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 15 °C with 1 W per gel for 1 h and 2 W per gel after that until the bromophenol blue dye

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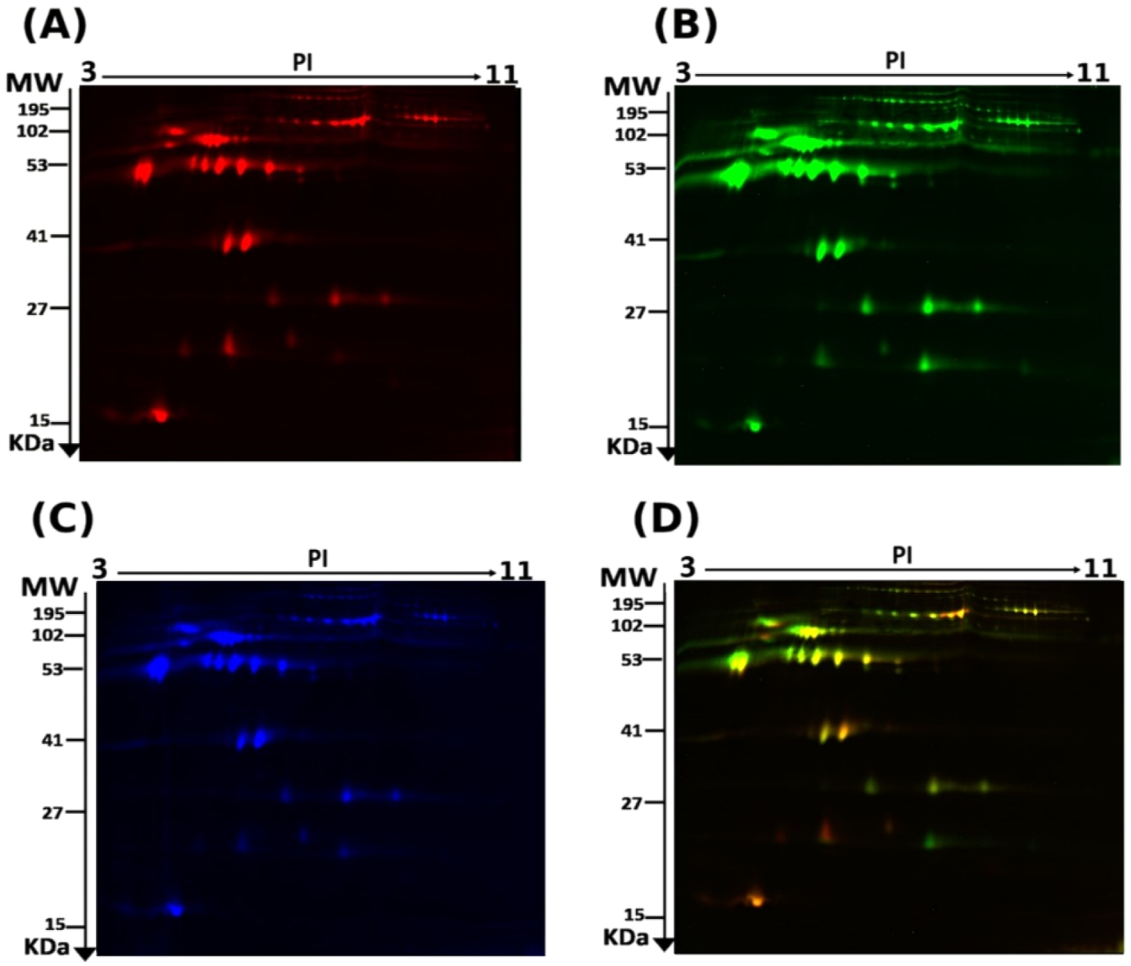

Figure 1. Representative fluorescent protein profiles of a 2D-DIGE gel containing: (A) ECND samples labeled with Cy5, (B) ECD samples labeled with Cy3, (C) pooled control samples labeled with Cy2, and (D) representative overlay of Cy3/Cy5/Cy2 images of ECND and ECD. The one-dimensional separation of proteins was carried out on an IPG strip (pH 3−11), followed by two-dimensional separation [polyacrylamide gel electrophoresis (12.5%)]. Sapphire Bimolecular Imager (Azure Bio systems, Dublin, OH, USA) was used to capture protein spot images.

front reached the bottom of the gel. After SDS PAGE electrophoresis, the gels were digitalized using the image analysis program Sapphire Capture system (Azure Biosystems, Dublin, OH, USA) after being scanned with the Sapphire Biomolecular Imager (Azure Biosystems, Dublin, OH, USA). For the preparative gels, total protein (1 mg) from a pool of equal protein levels from the 16 plasma samples (8 ECD and 8 ECND) was employed. Gels were stained for 5 days, and after being briefly rinsed with Milli-Q water, the stained gels were kept until the spots could be picked out and identified using MS, as described earlier.[14−18]

2.6. Statistical Analysis. Progenesis SameSpots software (Nonlinear Dynamics, UK) was used for examining two-dimensional differential gel electrophoresis (2D-DIGE) gel images. The software detected the spots by automatic detection using the comparison modules, which also included DIGE normalization. To ensure that no information was lost, all gel images were aligned with the reference gel and overlaid. The software determined the normalized volume (NV) of each spot on each gel from the Cy3 (or Cy5) to the Cy2 spot volume ratio. The spot volumes underwent a log transformation to produce normally distributed data. Differential expression was measured using log-normalized volume (LNV). A direct comparison of the ECD and ECND groups was carried out, and fold difference values and *p*-values were calculated using one-way analysis of variance. Prior to using statistical criteria, each spot was prefiltered and personally examined (ANOVA, p 0.05). Instead of using spot intensities, normalized spot volumes were used for statistical processing. Only those spots that satisfied the aforementioned statistical criteria were taken into account for MS analysis.

2.7. Protein Identification Using MALDI-TOF-MS. The gel spots stained with Coomassie were manually removed, cleaned, and digested using the techniques previously outlined.[14−18] A MALDI target (384 MTP Anchorchip; 800 m Anchorchip; Bruker Daltonics, Bremen, Germany) was spotted with a mixture of tryptic peptides (0.8 μL) generated from identified proteins. According to previous descriptions,[14−18] matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectra were acquired using an Ultra-flexTerm TOF mass spectrometer outfitted with a LIFT-MS/MS device (Bruker Daltonics) at reflector and detector voltages of 21 kV and 17 kV, respectively. The peptide calibration standard II from Bruker Daltonics was used to calibrate peptide mass fingerprints (PMFs). The Flex Analysis software (Bruker Daltonics, version 2.4) was used to evaluate the PMFs. Utilizing BioTools v3.2 (Bruker Daltonics), the data for MS were analyzed. The Mascot search algorithm (v2.0.04, updated on 09/05/2021; Matrix Science Ltd., UK) was used to search the peptide masses. If a protein's Mascot score was

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higher than 56, it was considered correctly identified. A protein score = $-10*\log(P)$, where P is the likelihood that the observed match is a random event, was used to determine the Mascot significance score. A protein score greater than 56 was deemed significant ($p \leq 0.05$). Low-scoring ID proteins were disregarded since they tended to be random matches and insignificant ($p > 0.05$). Due to the low quantity of some proteins and their inability to produce sufficiently powerful mass fingerprints, as well as the fact that some spots were mixed proteins, not all spots of interest could be recognized.[14−18]

2.8. Bioinformatics Analysis. The protein−protein interaction (PPI) network of differentially expressed genes was constructed through the STRING database (https://string-db.org/). Roles of the plasma proteins, their interaction, and related networks that were differentially expressed in the ECD and ECND samples were investigated. Protein lists provided by mass spectroscopy were evaluated by the STRING database to discover their functions and pathways. The detected proteins were categorized in accordance with molecular function and biological processes using the PANTHER (protein analysis through evolutionary relationships http://www.pantherdb.org).

3. RESULTS

3.1. Proteomic Analysis and Identification of Differentially Expressed Proteins. The differential protein expression among two groups (ECD and ECND) (16 samples from 8 gels) were performed through the 2D-DIGE and MALDI-TOF-MS. In Figure 1, the representative fluorescent protein profiles of a 2D-DIGE containing samples labeled with Cy5 for ECND (Figure 1A), labeled with Cy3 for ECD (Figure 1B), labeled with Cy2 for the pooled internal control (Figure 1C), and merged 2D-DIGE gels of samples labeled with Cy2/Cy3/Cy5 (Figure 1D).

In Figure 2, the gel identifies 850 protein spots, and 65 spots were statistically significant between the two groups (ECD and ECND) (ANOVA, $P \leq 0.05$; fold-change ≥1.5). The identified protein spot patterns were reproducible across all 8 gels, leading to alignment and further analysis. Utilizing an internal standard with Cy2-labeling, the entire set of gels was normalized, and quantitative differential analysis of the protein levels was completed. From the preparative gel, the statistically significant spots ($n = 60$) between two groups were excised manually for the identification of protein by MALDI-TOF-MS.

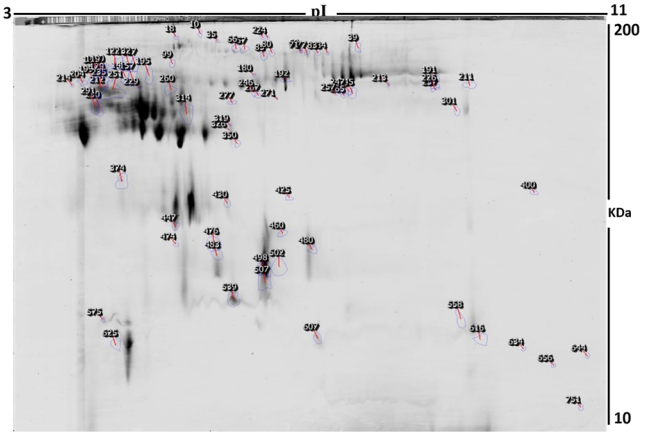

Figure 2. Representative image of protein spots from the plasma of the study groups. The identified differentially expressed proteins were expressed as numbered spots (over 1.5-fold change, $p < 0.05$) and successfully identified with MALDI-TOF-MS.

From the 60 protein spots excised from the preparative gel, PMFs effectively recognized 33 protein spots by MALDI-TOF-MS. Out of these identified 33 proteins, 27 spots were unique protein sequences that were matched to entries in the SWISS-PROT database by Mascot with high confidence scores (Tables 1 and S2). The sequence coverage ranges of the proteins identified by PMFs ranged between 13% and 95% (Table 1 and Figure 2). Among the 33 proteins identified, 17 protein spots were upregulated and 16 were downregulated in ECD patients (Table 1 and Figure 2). α-2-macroglobulin, Ras association domain-containing protein 3, apolipoprotein A-I, α-1B-glycoprotein, zinc-α-2-glycoprotein (ZAG), etc., were significantly identified as upregulated proteins; the complete list is provided in Table 1. The significantly downregulated proteins included haptoglobin apolipoprotein A-IV, hemopexin, α-1-antichymotrypsin, etc.; the full list is provided in Table 1. Among the proteins which were successfully determined, four proteins (haptoglobin, α-1-antichymotrypsin, apolipoprotein A-I, and serotransferrin) were observed on the gels in many locations, which may be a result of post-translational changes, enzyme cleavage, or the existence of various proteins (Table 1).

3.2. Principal Component Analysis. The visualization of each study group and outlier detection was carried out using principal component analysis (PCA). Figure 3 shows the score plots obtained from ECND and ECD groups. The PCA model, demonstrating the ECD and ECND groups clustered in a two-dimensional score plot, indicated that the proteomics profile significantly differed between two groups. The samples were colored according to the color of their group. In Figure 3, the primary source of variance (PC1, explaining 62.61% of the variance) allows separation of the ECD (blue circles) and ECND (red circles) groups.

3.3. PPI Network. We searched for known and predicted interactions for the differentially expressed proteins identified by2D- DIGE proteomics in the STRING PPI database and constructed a PPI network (Figure 4). We identified 9 networks (KEGG and REACTOME), with 36 nodes and 109 edges. The network predicted interactions between albumin, hemoglobin subunit β, haptoglobin-related protein, hemopexin, apolipoprotein A-IV, identified as downregulated in the ECD group, and apolipoprotein A-I, α-2-macroglobulin, serotransferrin, serum amyloid A-1 protein, etc that were upregulated. Biological process pathways identified in STRING for the cholesterol metabolism (false discovery rate 5×10^{-6}), PPAR signaling pathway(false discovery rate 3.9×10^{-3}), binding and uptake of ligands by scavenger receptors(false discovery rate 4×10^{-10}), scavenging of heme from plasma (false discovery rate 3.4×10^{-9}), plasma lipoprotein assembly, remodeling, and clearance(false discovery rate 8×10^{-9}), plasma lipoprotein remodeling(false discovery rate 1.1×10^{-7}), transport of small molecules(false discovery rate 2×10^{-6}), regulation of IGF transport, and uptake by insulin-like growth factor binding proteins (IGFBPs) (false discovery rate 8.2×10^{-5}) are shown in Table S3.

The PANTHER classification system was used to identify the proteins by their molecular functions (Figure 5A), biological processes (Figure 5B), and cellular components (Figure 5C). The functional category showed that most of the differentially expressed proteins identified were enzymes with

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Table 1. Proteins That Were Identified and Their Differences in Abundance between the Plasma Samples from the ECND and ECD Are Shown in Table 1[a]

sl no:	spot no[b]	accession no	protein name	MASCOT ID	*P* value[c] (ANOVA)	ratio[d] ECD/ECND	exp[e]
1	120	P00738	haptoglobin	HPT_HUMAN	0.006	−1.60	DOWN
2	616	P68871	hemoglobin subunit β	HBB_HUMAN	0.016	−2.72	DOWN
3	247	P02768	albumin	ALBU_HUMAN	0.028	−1.73	DOWN
4	35	P01023	α-2-macroglobulin	A2MG_HUMAN	0.05	1.5	UP
5	460	P00738	haptoglobin	HPT_HUMAN	0.041	−1.66	DOWN
6	245	P06727	apolipoprotein A-IV	APOA4_HUMAN	0.028	−1.65	DOWN
7	213	Q86WH2	Ras association domain-containing protein 3	RASF3_HUMAN	0.028	1.57	UP
8	480	P02647	apolipoprotein A-I	APOA1_HUMAN	0.032	1.61	UP
9	474	Q15007	pre-mRNA-splicing regulator WTAP	FL2D_HUMAN	0.035	2.21	UP
10	57	P04217	α-1B-glycoprotein	A1BG_HUMAN	0.05	−1.74	UP
11	319	P25311	zinc-α-2-glycoprotein	ZA2G_HUMAN	0.015	1.5	UP
12	132	P02790	hemopexin	HEMO_HUMAN	0.008	−1.5	DOWN
13	480	Q15819	ubiquitin-conjugating enzyme E2 variant 2	UB2 V2_HUMAN	0.032	1.61	UP
14	147	A5D8V7	coiled-coil domain-containing protein 151/outer dynein arm-docking complex subunit 3	CC151_HUMAN/ ODAD3_HUMAN	0.034	−1.5	DOWN
15	625	Q96HE7	ERO1-like protein α	ERO1A_HUMAN	0.05	1.60	UP
16	191	P02787	serotransferrin	TRFE_HUMAN	0.05	1.5	UP
17	483	P02647	apolipoprotein A-I	APOA1_HUMAN	0.05	1.5	UP
18	226	P02787	serotransferrin	TRFE_HUMAN	0.024	1.5	UP
19	195	P20132	L-serine dehydratase/L-threonine deaminase	SDHL_HUMAN	0.041	−1.5	DOWN
20	634	P0DJI8	aerum amyloid A-1 protein	SAA1_HUMAN	0.004	1.7	UP
21	350	P00738	haptoglobin	HPT_HUMAN	0.004	−2.18	DOWN
22	126	P01011	α-1-antichymotrypsin	AACT_HUMAN	0.019	−1.5	DOWN
23	199	P01011	α-1-antichymotrypsin	AACT_HUMAN	0.020	−1.60	DOWN
24	447	P02647	apolipoprotein A-I	APOA1_HUMAN	0.05	1.5	UP
25	425	O95996	adenomatous polyposis coli protein 2	APCL_HUMAN	0.05	−1.69	UP
26	214	Q96HQ0	zinc finger protein 419	ZN419_HUMAN	0.034	−1.86	DOWN
27	127	P46439	glutathione *S*-transferase Mu 5	GSTM5_HUMAN	0.0008	−1.72	DOWN
28	237	P01817	immunoglobulin heavy variable 2−5	HV205_HUMAN	0.015	1.58	UP
29	267	Q03591	complement factor H-related protein 1	FHR1_HUMAN	0.016	−1.51	DOWN
30	180	P80294	metallothionein-1H	MT1H_HUMAN	0.016	1.5	UP
31	10	P07148	fatty acid-binding protein, liver	FABPL_HUMAN	0.018	−1.50	DOWN
32	476	Q13522	protein phosphatase 1 regulatory subunit 1A	PPR1A_HUMAN	0.021	1.69	UP
33	290	Q96I76	G patch domain-containing protein 3	GPTC3_HUMAN	0.05	−1.5	DOWN

[a]Average ratio between the two groups, together with the fold changes that correspond to those ratios, and a one-way ANOVA with a 2D-DIGE that has a *p*-value less than 0.05. [Analysis type: MALDI-TOF-MS; database: SwissProt; taxonomy: *Homo sapiens*]. [b]Protein accession number for the SWISSPROT Database. [c]*P*-value (ANOVA). [d]Ratio between the groups. [e]Protein expression between the groups.

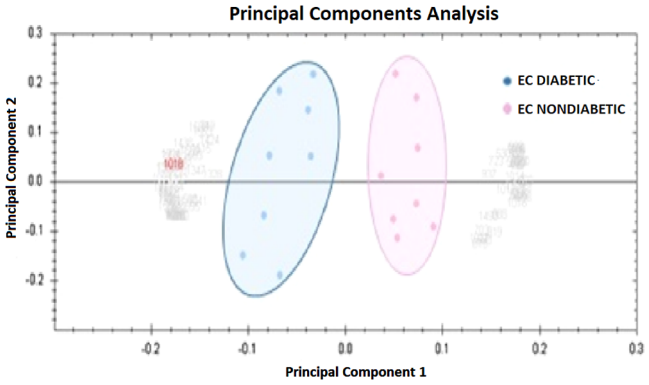

Figure 3. PCA plot of the two principal components. Both together explained 62.61% of the selected spot's variability. Colored dots and numbers are the representation of gels and spots, respectively.

catalytic activity (53%), followed by binding (35%) (Figure 5A). With regard to biological processes, the majority of the identified proteins were involved in cellular processes and biological regulation (38%), followed by biological regulation (14%) (Figure 5B). The majority of the identified proteins were located in the cellular anatomical entity (80%), followed by the protein containing complex (20%) (Figure 5C).

4. DISCUSSION

This study provides an overview of the plasma proteomic differences between ECD patients and ECND patients. We identified 33 differentially regulated proteins, of which 17 were upregulated and 16 were downregulated in the ECD group when compared with the ECND group. Notably, none of these protein alterations were seen in the endometrial tissue proteomics of the same patients we reported previously.[11] Hence, the plasma proteomic differences found in this study are likely to have come from metabolic alterations and the pathophysiological interplay between EC and diabetes. The majority of the altered proteins are involved in acute phase reaction, cholesterol metabolism, scavenging of heme from plasma, and plasma lipoprotein assembly and mobilization.

4.1. Proteins with an Increased Abundance in ECD Patients. The results show an increased abundance of α-2

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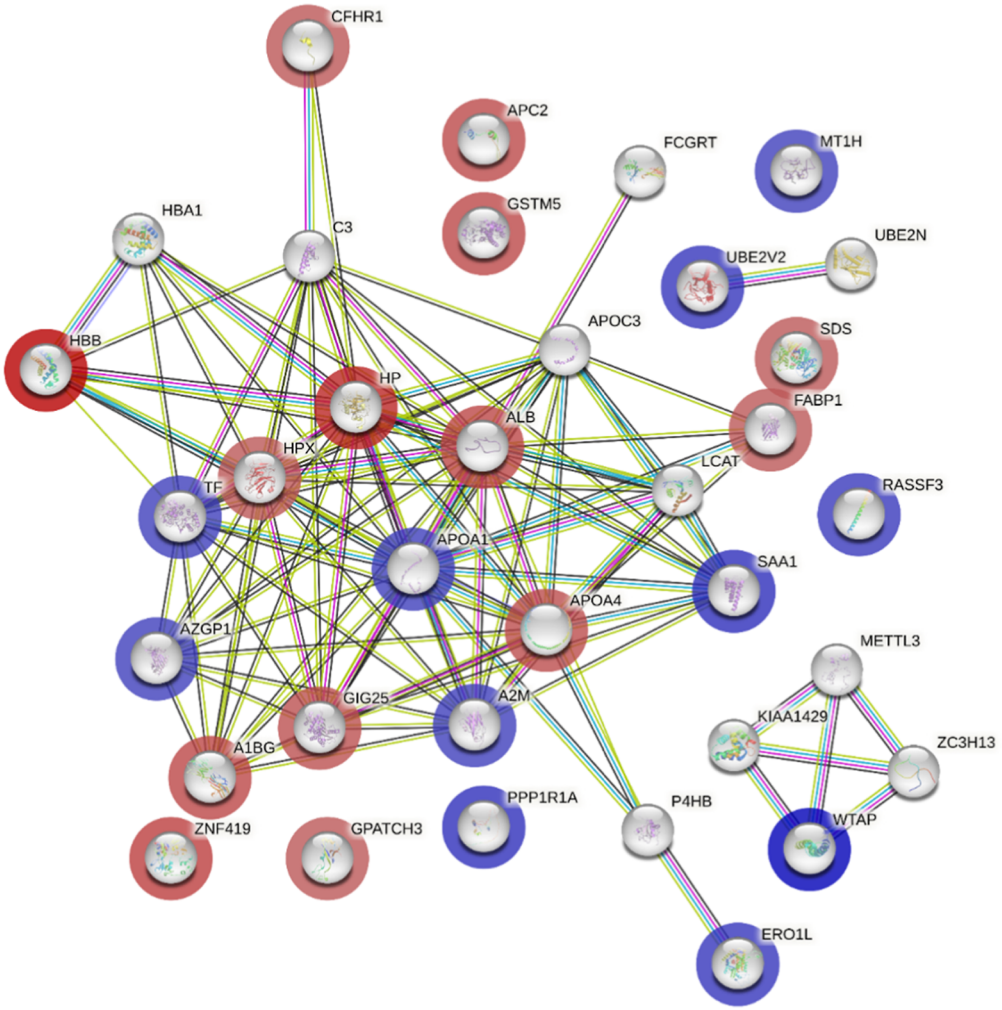

Figure 4. Most enriched interaction network of the differentially expressed proteins in ECD compared with ECND. The nodes with blue halo indicate upregulated; the nodes with red halo indicate downregulated. The proteins without halo are proposed by the STRING database and indicate potential targets that were functionally coordinated with the differentially expressed proteins. Solid black line indicates coexpression; green line indicates gene neighborhood; dark blue line indicates gene co-occurrence; purple line indicates experimentally determined protein interactions.

macroglobulin in the ECD group. It is one of the most abundant serum proteins in humans. α-2 macroglobulin is an antiprotease protein produced mainly in the liver, and the production is associated with the amount of albumin excreted in the urine. When protein is lost significantly in albuminuria, the production of high molecular weight α-2 macroglobulin is increased in the liver as a compensatory mechanism.[19] Its increased serum levels in patients with diabetes have been known for a long time.[20] The higher level of α-2 macroglobulin is therefore expected due to the presence of diabetes in individuals in the ECD group. Apolipoprotein A-I was found upregulated in ECD patients compared with the ECND patients. It is the main protein component of high-density lipoprotein. In a nested case−control study of a large European cohort, HDL-cholesterol had an inverse association with EC risk.[21] A significantly lower level of apolipoprotein A-I in patients with EC has been reported previously.[22] Moreover, it is also inversely associated with diabetes.[23] But, in the present study, the serum level of apolipoprotein A-I is higher in EC patients with diabetes. It appears that the interplay of molecular changes with concomitant diabetes and EC conditions led to the upregulation of apolipoproteins A-I. It needs further investigation to find out the exact mechanisms that led to such a reversal. Moreover, the role of the effect of dyslipidemia treatment on apolipoprotein A-I dysregulation in the ECD group may need to be explored. ZAG, another protein that is usually found lower in patients with T2D is upregulated in ECD patients in our study.[24] It is an adipokine involved in lipid mobilization. Since diabetes treatment was reported to increase the serum levels of ZAG, the upregulation observed in the ECD patients may be attributed to it.[25,26]

Serum transferrin, one of the most abundant proteins in the blood, functions as an iron transporter. High level of serum transferrin is reported in patients with T2D. It is known to be associated with fasting glucose and HbA1c levels.[27] Increased serum transferrin levels were also reported in patients with EC when compared to normal individuals.[22] As both disease conditions were reported to increase serum transferrin levels, the upregulation of serum transferrin in ECD patients is reasonable. ERO1-like protein α (Ero1-α) is an endoplasmic reticulum oxidase involved in oxidative protein folding. Overexpression of Ero1-α is seen in several cancers and is associated with tumor growth and angiogenesis.[28] Expression of Ero1-α in ECs was found higher than normal.[29] In addition, increased expression of Ero1-α was found associated with poor prognosis of cervical cancer, and its knockout resulted in

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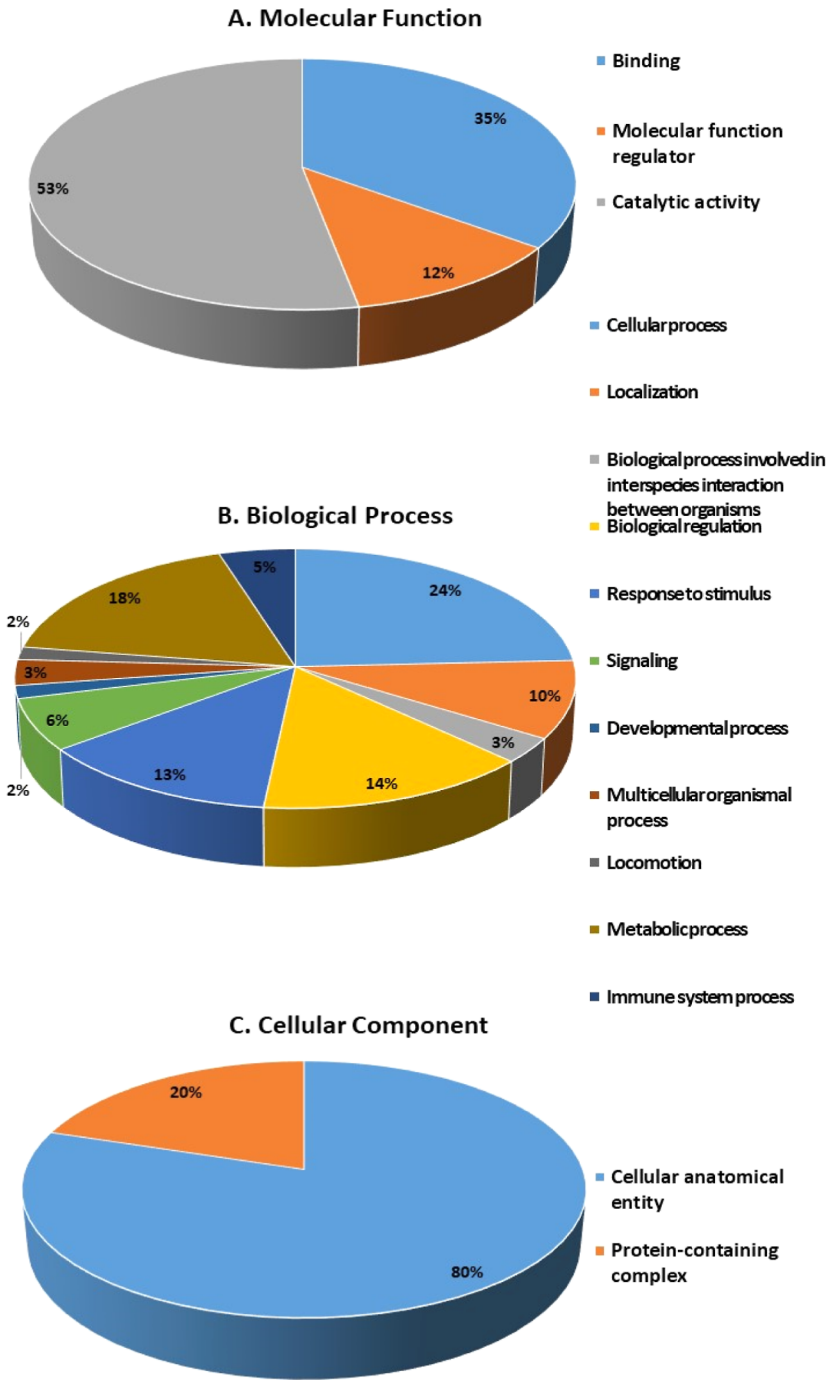

Figure 5. Comparative depiction (%) of identified proteins categorized into groups according to their molecular function (A), biological process (B), and cellular components (C).

decreased tumorigenesis and migration of cervical cancer cells in vitro.[30] It appears that the presence of diabetes in ECD patients augmented the expression of Ero1-α, suggesting a poor prognosis for EC patients who have diabetes. Serum amyloid A-1 protein (SAA1) is an acute phase protein found to be overexpressed in ECD patients in the present study. It is a precursor of amyloid A protein, which is a major component of the inflammatory amyloid deposit. Elevated SAA1 levels are known to be associated with T2D.[31] It is overexpressed in ECs and has been reported to be a biomarker for ECs.[32] Most of the cancer cells that produce SAA1 protein are thought to overcome the host immune response by stimulating immunosuppressive IL-10.[33] In addition, the overexpression of SAA1 in cancers was found to be associated with a poor prognosis and decreased survival.[34] Since SAA1 is increased in both diabetes and EC, the presence of diabetes might potentiate the pathways that are linked with the worsening of the EC. Furthermore, SAA1 also acts as an apolipoprotein with HDL.[35] Together with a higher apolipoprotein A-I level, upregulation of SAA1 in ECD patients shows that the coexistence of diabetes and EC led to the upregulation of pathways involved with the HDL. This needs further study as diabetes and EC are known to be inversely associated with HDL.

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Protein phosphatase 1 regulatory subunit 1A (PPP1R1A), an inhibitor of protein-phosphatase 1 is found overexpressed in the ECD group when compared to the ECND. It is reported to be highly expressed in Ewing Sarcoma and it promotes tumor growth and metastasis.[36] PPP1R1A has not been reported previously in ECs. It is also a marker of pancreatic β-cell injury and is released into the extracellular space following injury to the β-cells.[37] The higher abundance of PPP1R1A in ECD patients in our study may be linked to the presence of both EC and diabetes. Ras association domain-containing proteins have been reported in many cancers.[38] Overexpression of Ras association domain-containing protein 3 (RASSF3) inhibits cell proliferation and induces apoptosis.[39] RASSF3 is found to be overexpressed in patients with ECD in the current study. The common antidiabetic drug metformin exhibits antiproliferative activity by inducing mislocalization of K-Ras in the cytoplasm. It inhibited the tumor growth in K-Ras mutated cells in mouse models of EC.[40] RASSF3 complexes with the activated K-Ras.[39] Since most of the patients with diabetes were on metformin treatment in the ECD group, the metformin interference with the K-Ras might have led to an overexpression of RASSF3 in those patients. This could be possibly a new mechanism of the anticancer activity of metformin, which needs further investigation.

4.2. Proteins with a Decreased Abundance in ECD Patients. Haptoglobin is a known marker of several cancers, with higher serum levels predicting tumor progression and poor prognosis.[41] Higher levels of serum haptoglobin were used to distinguish the ECs from endometrial hyperplasia.[42] In addition, EC tissues overexpress haptoglobin mRNA.[43] Haptoglobin binds with hemoglobin in circulation and protects the tissues. It is an acute-phase protein with antioxidant and anti-inflammatory properties. In diabetes, haptoglobin is known to be positively correlated with HbA1c.[44] However, the presence of both diabetes and EC led to the downregulation of haptoglobin in ECD patients. This might be an outcome of the molecular alterations due to the concomitant conditions of diabetes and EC. Hemopexin, a plasma glycoprotein involved in the removal of free heme from serum, was downregulated in the ECD patients. Its circulating levels are associated with the levels of haptoglobin and low haptoglobin levels lead to lower hemopexin levels as well.[45] This is in agreement with our results as ECD patients showed a decreased abundance of hemopexin compared to that of ECND patients. The hemoglobin β subunit protein performs a variety of physiological functions in addition to transporting oxygen. It is a regulator of innate immunity and was shown to have anticancer activity.[46] The hemoglobin β subunit was found to be downregulated in diabetic patients with complications.[47] Moreover, it was also downregulated in EC tissues when compared to normal tissues and was associated with poor outcomes.[48] Our study found that the serum abundance of hemoglobin β subunit was significantly lower in patients in the ECD group. Interestingly, the proteins involved in the heme transport and removal, such as haptoglobin, hemopexin, and hemoglobin β subunit protein, were downregulated in the ECD patients. A lower abundance of these proteins is likely to increase the circulating free heme and hemoglobin, which will lead to oxidative stress.

Downregulation of apolipoprotein A-IV was seen in patients with EC in many studies.[42,49] It is a lipid-binding protein produced by the small intestine and aids in fat absorption. Apolipoprotein A-IV deficiency is also known to be associated with diabetes and atherosclerosis.[50] Our results are in agreement with this, as the patients in the ECD group had a significantly lower abundance of apolipoprotein A-IV compared with the patients in the ECND group. α-1B-glycoprotein is another protein that was found to be downregulated in the ECD patients in our study. Interestingly, it was reported to be overexpressed in the serum of EC patients and downregulated in patients with diabetes and its complications.[47,51] It looks like the influence of diabetes has dominated the molecular changes underlying the downregulation of α-1B-glycoprotein. α-1-antichymotrypsin, a glycoprotein and acute-phase protein, is upregulated in EC tissues.[52] It is found to increase in most other cancers. Though, the role of α-1-antichymotrypsin in cancers whether it counteracts tumorigenesis or promotes invasiveness is not clearly understood.[53] However, it is found to be downregulated in ECD patients in our study. This reversal of expression might be an outcome of concomitant diabetes in ECD patients. Adenomatous polyposis coli protein 2 (APC) is a tumor suppressor protein that plays a role in cell migration, cell adhesion, proliferation, and differentiation[54] It suppresses the Wnt/β-catenin pathway which is essential for epithelial−mesenchymal transition in many tumor cells.[54] In human ovarian cells, downregulation of APC2 by miR-939 is required for cell proliferation.[55] There have been no previous reports of dysregulation of APC2 in EC cells. The downregulation of APC2 in patients with EC and diabetes could be an indication of a poor prognosis of EC in the presence of diabetes.

Zinc finger protein 419 (ZNF419) is a ferroptosis-related protein associated with certain cancers. Its expression and significance vary widely. In some cancers, high expression was associated with a good prognosis, while in others, downregulation was linked to poor outcomes. There is no evidence of the altered expression of ZNF419 in ECs so far. It is thought to be involved in the ferroptosis of cancer cells, and suppression of ferroptosis plays an important role in the development of EC.[56] Therefore, it is possible that the downregulation of ZNF419 in our study might have been associated with a poor prognosis in ECD patients. Glutathione *S*-transferase Mu 5 (GSTM5) belongs to the family of detoxification enzymes that catalyze the conjugation of glutathione to toxins or xenobiotics. Mutation and suppression of these enzymes are associated with several cancers. GSTM5 was found to be downregulated in bladder cancer tissues and its overexpression suppressed the proliferation and migration of bladder cancer cells.[57] Likewise, GSTM5 expression was found to be suppressed in EC tissues compared to normal tissues.[58] We also found that the abundance of circulating GSTM5 in ECD patients is decreased compared to that in ECND patients. It is another protein alteration, indicating the poor prognosis of EC in the presence of diabetes in our study participants. Fatty acid binding proteins (FABPs) are soluble intracellular proteins that transport fatty acids and lipophilic substances. Many of the FABP members are involved in the cancer pathophysiology. The adipose-secreted FABP-4 suppresses the proliferation and migration of EC cells and was found to be downregulated in the endometrial tissue samples.[59] While the other members, such as FABP3 and FABP5, were associated with poor prognosis of EC.[60,61] However, in the current study, we found FABP-1 (FABPL) downregulated in patients with EC and diabetes compared to those in patients with EC. FABP-1 known as liver FABP is also associated with several cancers. However, its effect has not

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been consistent across different types of cancers.[61] There are no previous reports of the dysregulation of FABP-1 in ECs. FABP-1 interacts with PPARα and activates downstream transcriptional targets that are associated with an anti-inflammatory response, cellular differentiation, and apoptosis.[62,63] Considering the other protein alterations in this study, the downregulation of FABP-1 in patients with EC and diabetes is likely to be a sign of severe disease and a poor prognosis.

This study has revealed proteomic differences between patients who had EC and diabetes and those who had only EC. Proteins that are linked with tumorigenesis, progression, and metastasis were found to be significantly altered in patients with EC and diabetes. The results indicate that the presence of diabetes in these patients could lead to severe disease and poor outcomes. We found different protein alterations in this study that include proteins that are associated with diabetes (α-2 macroglobulin, ZAG, and protein phosphatase 1 regulatory subunit 1A), proteins that are associated with EC (glutathione *S*-transferase Mu 5 and Ero1-α), proteins that are associated with both the conditions (serum transferrin, serum amyloid A-1 protein, hemoglobin β subunit, and apolipoprotein A-IV), and proteins that were not found to associate with both the conditions so far (RASSF3, APC2, ZNF419, and FABP-1). The abundance of some proteins reversed in the presence of EC and diabetes. Proteins α-1B-glycoprotein and α-1-antichymotrypsin usually overexpressed in ECs were found to be downregulated in patients with EC and diabetes. Apolipoprotein A-I, typically found downregulated in both ECs and diabetes, was upregulated in patients who had both conditions together in our study. Similarly, haptoglobin reported to be upregulated both in EC and diabetes was downregulated in patients with diabetes and EC. The interaction network analysis showed that apolipoprotein A1, albumin, haptoglobin, and hemopexin are highly enriched indicating the dysregulation of cholesterol metabolism and heme scavenging pathways. Other highly interactive nodes were the hemoglobin β subunit, α-1-antichymotrypsin, apolipoprotein A-IV, SAA1, transferrin, α-2-macroglobulin, α-1B-glycoprotein, and ZAG. These proteins extensively interact with cholesterol metabolism and heme scavenging pathways. Strength of this study includes the use of EC control subjects. It helped us to identify the altered protein abundance due to the presence of diabetes in patients with EC. Limitations of this study is that we did not include a diabetes control to compare any proteomic difference that occurred due to the presence of EC in patients with diabetes. We used 2D-DIGE and MALDI-TOF-MS techniques to detect the altered plasma abundance of proteins; this requires confirmation by using immunoblotting techniques.

5. CONCLUSIONS

The present study revealed the blood proteomic signature of patients with EC and diabetes in comparison with patients who had EC alone. The differential expression of proteins found in patients who had EC and diabetes indicated severe disease and poor prognosis. The protein interaction analysis showed dysregulation of cholesterol metabolism and heme scavenging pathways in these patients. The abundance of some proteins was found to be reversed in the presence of diabetes in patients with EC. It needs further investigation to find out the exact mechanisms of the molecular interplay between diabetes and EC.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07992.

Experimental design; mass spectrometry list of significant differentially abundant proteins; and different canonical pathways obtained from STRING database analysis (PDF)

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Notes

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■ ABBREVIATIONS

EC, endometrial cancer; 2D-DIGE, two-dimensional differential gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; T2D, type 2 diabetes; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LNV, log-normalized volume; PMF, peptide mass fingerprint; PPI, protein−protein interaction; PANTHER, protein analysis through evolutionary relationships; PCA, principal component analysis; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding proteins; ZAG, zinc-α-2-glycoprotein; SAA1, serum amyloid A-1; PPP1R1A, protein phosphatase 1 regulatory subunit 1A; RASSF3, Ras association domain-containing protein 3; APC, adenomatous polyposis coli protein 2; ZNF419, zinc finger protein 419; GSTM5, glutathione *S*-transferase Mu 5

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