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Alterations of Spermatozoa Proteomic Profile in Men with Hodgkin's Disease Prior to Cancer Therapy

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Purpose: Hodgkin's disease (HD) is a type of cancer affecting men in the reproductive age with potential consequences on their fertility status. This study aims to analyze sperm parameters, alterations in proteomic profiles and validate selected protein biomarkers of spermatozoa in men with HD undergoing sperm banking before cancer therapy.

Materials and Methods: Semen analysis was carried out in healthy fertile donors (control, n=42), and patients diagnosed with HD (patients, n=38) before cancer therapy. We compared proteomic profiles of spermatozoa from donors (n=3) and patients (n=3) using LTQ-Orbitrap Elite hybrid MS system.

Results: A total of 1,169 proteins were identified by global proteomic in both groups. The ingenuity pathway analysis revealed that differentially expressed proteins involved in capacitation, acrosome reaction, binding of sperm to the zona pellucida, sperm motility, regulation of sperm DNA damage, and apoptosis were significantly downregulated in HD patients. Validation of proteins implicated in sperm fertility potential by Western Blot demonstrated that peroxiredoxin 2 (PRDX 2) was underexpressed (p=0.015), and transferrin (p=0.045) and SERPIN A5 (p=0.010) protein levels were overexpressed in spermatozoa of men with HD.

Conclusions: Findings of this study indicates that the key proteins involved in sperm fertility potential are significantly altered in men with HD, which provides substantial explanation for the observed low sperm quality in HD subjects prior to cancer therapy. Furthermore, our results suggest PRDX 2, transferrin and SERPIN A5 as possible candidate proteins for assessing sperm quality in HD patients prior to cancer therapy.

Keywords: Hodgkin disease; Infertility; Mass spectrometry; Proteomics; Spermatozoa; Transferrin

INTRODUCTION

Hodgkin's disease (HD) is one of the most prevalent cancers in men in the reproductive age. According to the American Society of Cancer [1], about 4,570 men are estimated to be diagnosed with HD and about 590 men are projected to die from HD. The five years survival rate in people diagnosed with this HD is about 86% [2] and hence, fertility preservation is crucial for men diagnosed with HD. Sperm cryopreservation is recom-

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mended to all male patients before cancer therapy [3,4].

Lower sperm quality, such as sperm concentration or motility has been reported in HD patients even before cancer therapy when compared to healthy individuals [5-7]. Though the reasons for this lower sperm quality is not fully understood, psychological factors or immune-mediated mechanisms affecting the spermatogenesis have been hypothesized to be the possible contributing causes [8,9]. Molecular changes at the subcellular level of spermatozoa that can explain the low sperm quality in men with HD are still unknown.

Proteomic analysis of spermatozoa is a high-throughput technique that elucidates the complex nature of the sperm [10,11]. Advances in mass spectroscopy and analysis of proteomic profile using bioinformatic tools have been used in the discovery of potential biomarkers in tissues and biological fluids [10-15]. Literature review reveals that the proteomic profile of spermatozoa in HD patients has not been investigated until now. Therefore, we sought to investigate the sperm proteomic profiling of HD subjects in comparison to healthy fertile volunteers in order to elucidate the reason(s) for low sperm quality in HD subjects at the time of sperm banking. The objectives of this study are: (1) to analyze and compare semen parameters of men with HD, who banked their specimen before cancer treatment, with fertile donors; (2) to characterize and compare the spermatozoa protein profile among cancer patients and healthy fertile donors; and (3) to validate proteins of interest implicated in fertility that are differently expressed in Hodgkin's cancer patients when compared to controls.

MATERIALS AND METHODS

1. Study subjects and ethics statement

This study was approved by the Institutional Review Board (IRB) of Cleveland Clinic. All the participants signed an informed written consent at the time of sample collection at the Andrology Center, Cleveland Clinic (No. #13-1554). Semen samples were obtained from 42 healthy male donors of proven fertility (control group) who had fathered a child in the past 2 years before enrollment in the study and 38 patients with HD before cancer therapy (cancer group). All the subjects included in the control group had normal semen parameters [16].

Men between the ages of 20 to 45 years diagnosed with HD were included in the study and these patients have not yet started their oncological treatment. We have excluded men with azoospermia and those under the supportive medication, steroids or drugs. In addition, patients with systemic reproductive tract inflammation, smoking, sexually transmitted disease, and leukocytospermic were excluded from the current study.

2. Semen analysis

Semen analysis was performed according to the World Health Organization (WHO) guidelines (2010) [16] in samples from both control (n=38) and cancer (n=33) groups. Semen samples were collected by masturbation after sexual abstinence of at least 48 hours. Samples were allowed to liquefy completely for 20 minutes at 37°C, and semen analysis was performed according to WHO guidelines [16] using the Microcell counting chamber (Vitrolife, San Diego, CA, USA) to evaluate sperm count, motility and round cells. For the samples from the control group, after completion of routine semen analysis, the remainder of the sample was centrifuged for 7 minutes at 1,000×*g*. The clear seminal plasma was aspirated from the samples and spermatozoa were stored at -80°C for proteomic studies. Semen samples from cancer group were cryopreserved with Freezing Medium Test Yolk Buffer (TYB) using an established protocol [4].

3. Preparation of semen samples

Semen samples from the control group were thawed at room temperature and centrifuged at 3,000×*g* for 30 minutes to remove the remnants of seminal plasma and washed with phosphate buffer saline (PBS). Cryopreserved samples from the cancer group were thawed to room temperature and centrifuged at 3,000×*g* for 30 minutes to remove the TYB and the supernatant was discarded. The pellet was then washed with PBS and centrifuged at 3,000×*g* for 30 minutes. This step was repeated 3 times to remove the remains of TYB.

4. Protein extraction and quantification

Proteins were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing the proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA). RIPA was added to the sperm pellet (approximately 100 μL for 100×10^6 sperm) and incubated overnight at 4°C to allow the complete lysis of the spermatozoa. The supernatant was aspirated after centrifugation at 13,000×*g* for 20 minutes to a clean microcentrifuge tube. The protein concentra-

tion was determined using a commercially available kit, bicinchoninic acid kit (Thermo, Rockford, IL, USA), following the manufacturer instructions.

5. Preparation of semen samples for proteomics

Proteomic analysis was performed on randomly selected donor (n=3) and HD (n=3) samples. Further, normalization of the samples was done by pooling the samples to overcome the biological variation [17,18]. The samples were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer and subjected to one-dimensional-PAGE in triplicate runs. After completion of electrophoresis, the gel was cut into 6 pieces and digested using 5 μL trypsin (10 ng/μL), 50 mM ammonium bicarbonate and incubated overnight at room temperature. The samples (cut pieces) were alkylated with iodoacetamine and reduced with dithiothreitol. The peptides from the digested gel were extracted in two aliquots of 30 μL acetonitrile (10%) with formic acid (5%). The two aliquots were pooled together and evaporated to <10 μL and then diluted with 1% acetic acid to make up a final volume to 30 μL.

6. Liquid chromatography-mass spectrometer analysis

Proteomic profiling of spermatozoa was carried out using a Finnigan LTQ linear ion trap mass spectrometer liquid chromatography (LC)-mass spectrometer analysis (MS)/MS system. The peptides were fractionated by injecting 5 μL volumes into high-performance liquid chromatography column (Phenomenex Jupiter C18 reversed-phase capillary chromatography column; Phenomenex, Torrence, CA, USA). Fractions containing the peptides were eluted in acetonitrile/0.1% formic acid at a flow rate of 0.25 μL/min and were introduced into the source of the mass spectrometer on-line. The micro electrospray ion source was operated at 2.5 kV. A full spectral scan was performed by utilizing the data dependent multitask ability of the instrument to determine peptide molecular weights and amino acid sequence of the peptides [19].

7. Database searching and protein identification

Tandem mass spectra generated by LC-MS/MS system were retrieved using Proteome Discoverer ver. 1.4.1.288. Mascot (ver. 2.3.02; Matrix Science, London, UK), Sequest (ver. 1.4.0.288; Thermo Fisher Scientific, San Jose, CA, USA) and X! Tandem (ver. CYCLONE 2010.12.01.1; The GPM, thegpm.org) search was performed on all the MS/MS raw files. The search was limited to the human reference sequences database (http://www.hprd.org/) assuming the digestion enzyme trypsin. The mass tolerance for parent ion was set to 10 parts per million (ppm) and for fragment ion with 1.0 Da. The search results were uploaded into the Scaffold (ver. 4.0.6.1; Proteome Software Inc., Portland, OR, USA) program as previously described [20]. Protein probabilities were assigned by the Protein Prophet (Systems Biology, Seattle, WA, USA) algorithm. Annotation of proteins was performed using Gene Ontology (GO) terms from National Center for Biotechnology Information.

8. Quantitative proteomics

The relative quantification of the proteins was performed by comparing the number of spectra, termed spectral counts in both cancer and control groups. To achieve false detection rate <1%, protein identification criteria was established at >99% probability as explained in our previous study [21]. The abundance of the proteins was determined by matching the spectra (spectral counts or SpCs), and classified as high (H), medium (M), low (L), or very low (VL). To overcome the sample-to-sample variation, normalization of spectral counts was done using the normalized spectral abundance factor (NSAF). In general, longer proteins have more peptide identifications than shorter proteins. NSAF ratio determines the actual expression of the protein in the samples. Proteins with ratio <1 and >1 are considered underexpressed and overexpressed, respectively. Different constraints for fold-change cutoffs and significance tests (p-value) based on the average SpC from 3 replicate runs were applied to obtain the differentially expressed proteins (DEPs) [20]. Appropriate filters were applied used to minimize the errors due to the presence of low abundance proteins. Abundance and the expression of DEPs are based on the following criteria: (i) VL - SpC range, 1.7–7; NSAF ratio (≥2.5 for upregulated and ≤0.4 for downregulated proteins); and p≤0.001, (ii) L - SpC range, 8–19; NSAF ratio (≥2.5 for upregulated and ≤0.4 for downregulated proteins); and p≤0.01, (iii) M - SpC range, 20–79; NSAF ratio (≥2.0 for upregulated and ≤0.5 for downregulated proteins); and p≤0.05, (iv) H - SpC, >80; NSAF ratio (≥1.5 for upregulated and ≤0.67 for downregulated proteins); and p≤0.05.

9. Bioinformatic analysis

DEPs identified in both the study groups were subjected to functional annotation and enrichment analysis using publicly available bioinformatic annotation tools and databases such as GO Term Finder, GO Term Mapper, UniProt, and Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.niaid.nih.gov). Protein-protein interaction was demonstrated using Ingenuity Pathways Analysis (IPA) based on the criteria: experimental evidence, neighborhood, gene fusion, occurrence, co-expression, existing databases, and text mining. Proprietary software package Metacore™ (GeneGo Inc., St. Joseph, MI, USA) was also used to identify the upstream regulators involved in the enriched pathways. Based on their function and role in fertility potential in male six DEPs were chosen for validation by Western blot (WB).

10. Protein validation by Western blotting

The DEPs involved in the reproductive functions and fertilization process, and top canonical pathways were selected for validation. The function of these proteins are well described in the literature. From the identified proteins, six DEPs were selected for validation. The DEPs were validated by WB using n=6 for the control group and n=6 for cancer group. A total of 20 μg of spermatozoa protein was mixed with equal volume of loading buffer (125 mMol Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol). The sample mixture was boiled for 10 minutes and kept on the ice for 5 minutes. 30 μL of each sample was loaded into each well of a 4% to 15% SDS-PAGE and electrophoresed for 2 hours at 90 V along with a set of molecular weight marker (Sigma Chemical Co., St. Louis, MO, USA). The resolved protein bands were then transferred onto polyvinylidene difluoride (PVDF) membranes at 18 V for 30 minutes using a transfer buffer of 25 mMol Tris base, 192 mMol glycine, and 20% methanol. PVDF membranes were blocked with Tris-buffered saline-Tween-20 (TBST) with 5% bovine serum albumin and used for immunodetection of sperm proteins. For each protein analysis, specific primary antibodies were incubated at 4℃ overnight (Table 1). Next, the membranes were washed four times with TBST for 10 minutes and incubated with the secondary antibodies at room temperature for 1 hour (Table 1). Membranes were washed four times again with TBST (10 minutes) and finally treated with enhanced chemiluminescence (ECL) reagent (GE Healthcare, Marlborough, MA, USA) for 5 minutes. ECL reacted blots were exposed to Chemi-Doc (ChemiDoc™ MP Imaging System; Bio-Rad, Hercules, CA, USA) to detect the chemiluminescence signals.

11. Total protein staining

The total amount of protein present in the membranes was identified using a Colloidal Gold Total Protein Stain (Bio-Rad). The protocol was performed according to manufacturer instructions. Briefly, the membranes were washed twice for 10 minutes in the distilled water and stained with total colloidal gold protein stain for 2 hours at room temperature by gentle shaking. Stained membranes were washed twice with distilled water for 10 minutes, and the densitometry image was captured using calorimetric mode on Chemi-Doc (ChemiDoc™ MP Imaging System; Bio-Rad).

12. Statistical analysis

Data analysis was performed using MedCalc Statistical Software (ver. 17.8; MedCalc Software, Ostend, Belgium). Mann–Whitney test was carried out to compare the semen parameters of control and cancer groups,

Table 1. Primary and secondary antibodies used in this study

Primary				Secondary		
Protein	Manufacturer	Source	Dilution	Antibody	Manufacturer	Dilution
ACE	Abcam ab85955	Rabbit	1:1,000	Anti-Rabbit Goat IgG	Abcam ab97051	1:10,000
PRDX 2	Abcam ab71533					
Transferin	Abcam ab82411					
CCT3	Abcam ab225878		1:5,000			
SERPINA5	Abcam ab172060	Mouse	1:500	Anti-Mouse Rabbit IgG	Abcam ab6728	1:10,000

ACE: angiotensin converter enzyme, PRDX2: peroxiredoxin-2, CCT3: T-complex protein 1 subunit gamma, IgG: immunoglobulin G.

and the results were considered significant with $p<0.05$. The same test was used to compare the expression levels of the proteins validated using WB technique in both the groups.

RESULTS

1. Semen parameters in men with Hodgkin's disease

Semen analysis revealed significant decrease ($p<0.05$) in sperm concentration, total count and total motile sperm count in cancer group when compared to control group (Table 2).

2. Proteomic shotgun analysis revealed a total of 1049 different peptides common to both experimental groups

In this study we had used LC-MS/MS, a high throughput technique to obtain the proteome profile of spermatozoa from men diagnosed with HD before cancer therapy and compared this profile with the proteome profile of fertile donors. LC-MS/MS spectrometric analysis identified a total of 1,169 different peptides, out of which a total 1,049 proteins were common to both experimental groups, 101 were unique to the spermatozoa of proven fertile donors, and 19 were unique to the spermatozoa of patients with HD before cancer therapy (Fig. 1A). Relative abundance of the identified proteins revealed that 508 in very low abundance, 280 in low abundance, 286 in medium and 95 in high abundance (Fig. 1B). Among the identified proteins, 134 were detected as DEPs between the two experimental groups (Table 3). The sperm proteome of men with HD before cancer therapy had 35 overexpressed and 80 underexpressed DEPs compared to the sperm proteome of fertile donors (Fig. 1B). In the proteomic analysis of our study, we were also able to identify 16 DEPs unique to fertile donors and 3 DEPs unique to men with HD (Fig. 1C).

3. Ingenuity pathways Analysis revealed cell-to-cell signaling and interaction as top biofunction dysregulated in spermatozoa of men with Hodgkin's disease

IPA analysis of the DEPs revealed that pathways

Table 2. Sperm parameter of men from control and cancer group

Group	Volume (mL)	Concentration ($\times10^6$/mL)	Motility (%)	Total count ($\times10^6$)	Total motile sperm ($\times10^6$)
Control (n=42)	3.63±1.71	70.72±32.22	58.43±12.38	255.17±156.54	147.51±91.04
Patients (n=38)	3.48±1.78	35.98±23.34	53.60±24.51	119.74±83.48	75.49±65.36
p-value	0.62	<0.0001	0.44	<0.0001	0.0004

Values are presented as mean±standard deviation. Mann–Whitney test was carried out to compare the semen parameters of control and cancer groups, and the results were considered significant with $p<0.05$.

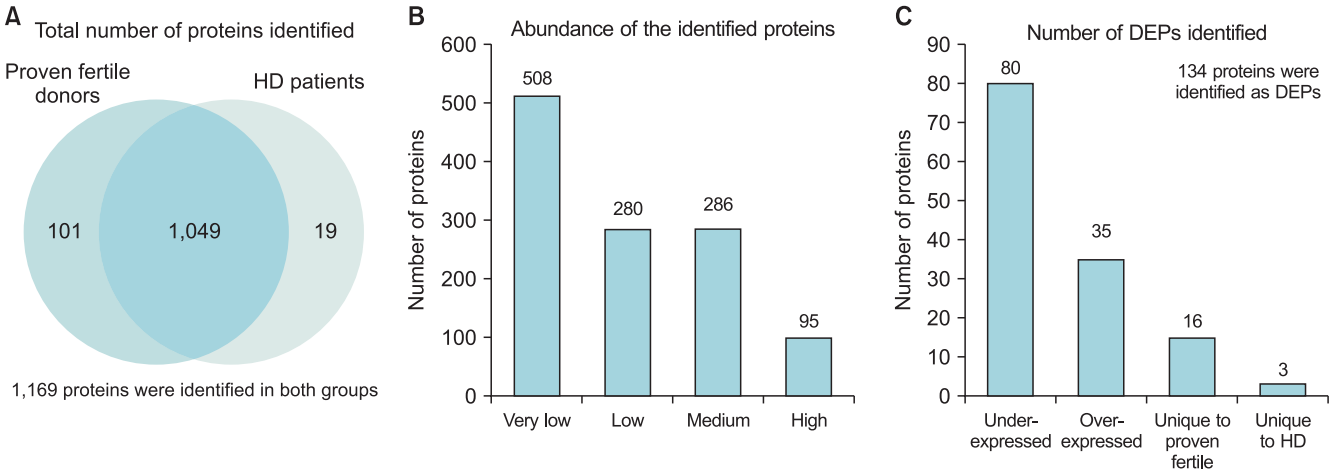

Fig. 1. (A) Total number proteins identified in the spermatozoa of proven fertile donors' group and patients with Hodgkin's disease (HD) before cancer therapy by liquid chromatography-mass spectrometer analysis (MS)/MS spectrometry. (B) Distribution of the identified proteins based on their relative abundance. (C) Differentially expressed proteins (DEPs) of experimental groups.

Table 3. Differently expressed proteins identified in sperm proteome of men with HD compared with donors

Protein	Accession No.	Average SpC		NSAF ratio	p-value	Expression
		Donors	Patients	Patients/ Donors		
Tetratricopeptide repeat protein 25	13899233	12.7	0	0.00	0.00003	Unique to donors
Mannose-1-phosphate guanyltransferase beta isoform 1	11761619	4.7	0	0.00	0.00000	Unique to donors
60S ribosomal protein l7a	4506661	5.3	0	0.00	0.00000	Unique to donors
Vitamin K epoxide reductase complex subunit 1-like protein 1 isoform 1	46309463	3.7	0	0.00	0.00000	Unique to donors
Translocation protein SEC63 homolog	6005872	2.0	0	0.00	0.00001	Unique to donors
Dynein heavy chain 6, axonemal isoform X1	578802753	3.3	0	0.00	0.00001	Unique to donors
Dynein intermediate chain 1, axonemal isoform 2	526479830	7.0	0	0.00	0.00003	Unique to donors
Guanine nucleotide-binding protein subunit beta-2-like 1	5174447	2.0	0	0.00	0.00003	Unique to donors
Mitochondrial import receptor subunit TOM22 homolog	9910382	6.0	0	0.00	0.00009	Unique to donors
Cation channel sperm-associated protein subunit beta precursor	51339295	2.0	0	0.00	0.00009	Unique to donors
Calcyphosin isoform b	18104964	3.3	0	0.00	0.00018	Unique to donors
Radial spoke head protein 6 homolog A	13540559	3.3	0	0.00	0.00018	Unique to donors
Glutamine--trna ligase isoform b	441478305	3.7	0	0.00	0.00029	Unique to donors
LETM1 domain-containing protein 1 isoform X7	578823569	2.3	0	0.00	0.00044	Unique to donors
Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	111160296	7.0	0	0.00	0.00057	Unique to donors
Nucleosome assembly protein 1-like 1	21327708	4.3	0	0.00	0.00059	Unique to donors
Dynein intermediate chain 2, axonemal isoform X4	530412670	15.7	0.3	0.03	0.00012	Underexpressed
Cytoplasmic dynein 1 heavy chain 1	33350932	11.3	0.7	0.04	0.00890	Underexpressed
Mitochondrial fission 1 protein	151108473	8.7	0.3	0.04	0.00162	Underexpressed
V-type proton atpase subunit B, brain isoform	19913428	18.7	0.7	0.05	0.00017	Underexpressed
Alpha-soluble NSF attachment protein	47933379	8.0	0.3	0.05	0.00232	Underexpressed
Arachidonate 15-lipoxygenase B isoform d	85067501	23.7	1.0	0.06	0.00013	Underexpressed
26S proteasome non-atpase regulatory subunit 4	5292161	9.0	0.7	0.09	0.00096	Underexpressed
V-type proton atpase catalytic subunit A	19913424	15.7	1.0	0.09	0.00404	Underexpressed
Dipeptidyl peptidase 3 isoform 1	86792661	12.7	1.0	0.10	0.00073	Underexpressed
Armadillo repeat-containing protein 4 isoform 1	585866234	18.7	1.7	0.12	0.00525	Underexpressed
Thioredoxin domain-containing protein 3	148839372	18.3	2.3	0.13	0.00029	Underexpressed
Long-chain-fatty-acid--coa ligase 3	42794754	9.7	1.0	0.13	0.00137	Underexpressed
Exportin-1 isoform X1	530368070	8.3	2.0	0.13	0.00101	Underexpressed
Protein ERGIC-53 precursor	5031873	10.0	1.0	0.14	0.00368	Underexpressed
Lipid phosphate phosphohydrolase 1 isoform 2	29171738	17.7	3.3	0.18	0.00241	Underexpressed
Probable inactive serine protease 37 isoform 1 precursor	285394164	9.0	1.3	0.18	0.00875	Underexpressed
Surfeit locus protein 4 isoform 1	19557691	9.7	1.7	0.19	0.00200	Underexpressed
Speriolin isoform 1	197276668	6.3	1.0	0.20	0.00041	Underexpressed
Dynein heavy chain 17, axonemal	256542310	88.0	9.0	0.20	0.00004	Underexpressed
EF-hand calcium-binding domain-containing protein 1 isoform a	13375787	11.3	2.0	0.20	0.00517	Underexpressed
Cytosolic non-specific dipeptidase isoform X2	530414265	30.7	4.7	0.20	0.00006	Underexpressed
Spectrin alpha chain, non-erythrocytic 1 isoform X1	578817797	8.0	1.0	0.21	0.00052	Underexpressed
EF-hand calcium-binding domain-containing protein 14	7662160	6.7	1.0	0.22	0.00087	Underexpressed
Dynein heavy chain 8, axonemal isoform X1	578811443	132.3	18.0	0.22	0.00009	Underexpressed
Dynein heavy chain 7, axonemal	151301127	18.0	2.3	0.22	0.00094	Underexpressed
Heat shock protein 75, mitochondrial isoform 1 precursor	155722983	8.3	1.7	0.23	0.00239	Underexpressed
Carnitine O-palmitoyltransferase 1, muscle isoform isoform a	4758050	11.3	3.0	0.23	0.00408	Underexpressed
Actin-like protein 7A	5729720	16.7	3.0	0.23	0.00013	Underexpressed

Table 3. Continued 1

Protein	Accession No.	Average SpC		NSAF ratio	p-value	Expression
		Donors	Patients	Patients/ Donors		
Growth/differentiation factor 15 precursor	153792495	9.0	1.7	0.23	0.00201	Underexpressed
Adenylyl cyclase-associated protein 1	5453595	32.0	6.7	0.24	0.00071	Underexpressed
Glutathione reductase, mitochondrial isoform 2 precursor	305410789	18.7	3.3	0.24	0.00203	Underexpressed
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial isoform 1 precursor	20149568	14.0	2.3	0.25	0.00445	Underexpressed
L-amino-acid oxidase isoform 2 precursor	384381475	76.0	16.0	0.25	0.00002	Underexpressed
Purine nucleoside phosphorylase	157168362	10.7	2.3	0.26	0.00259	Underexpressed
Protein FAM209B isoform X2	578835992	8.0	2.0	0.28	0.00724	Underexpressed
Heat shock protein 105 isoform 1	42544159	8.7	1.7	0.29	0.00781	Underexpressed
Myoferlin isoform X2	530393412	39.3	6.0	0.29	0.00003	Underexpressed
Protein-glutamine gamma-glutamyltransferase 4	156627577	232.3	35.7	0.29	0.00005	Underexpressed
Myosin regulatory light chain 12B	15809016	11.7	3.0	0.30	0.00106	Underexpressed
Probable serine carboxypeptidase CPVL isoform X1	530384848	27.0	5.7	0.30	0.00204	Underexpressed
Uncharacterized protein KIAA1683 isoform X1	530415216	23.3	6.3	0.32	0.02207	Underexpressed
Protein FAM71B	222418633	46.7	13.0	0.34	0.00178	Underexpressed
26S proteasome non-atpase regulatory subunit 6 isoform 2	7661914	18.7	4.7	0.34	0.00962	Underexpressed
26S proteasome non-atpase regulatory subunit 13 isoform 1	157502193	19.7	5.3	0.35	0.00841	Underexpressed
Dnaj homolog subfamily B member 11 precursor	7706495	15.0	3.7	0.35	0.00330	Underexpressed
26S proteasome non-atpase regulatory subunit 3	25777612	35.3	11.0	0.36	0.00033	Underexpressed
Calpain small subunit 1	51599151	9.3	3.0	0.36	0.00997	Underexpressed
Filamin-B isoform 2	105990514	25.7	5.7	0.37	0.00474	Underexpressed
Disintegrin and metalloproteinase domain-containing protein 32 precursor	148664238	6.0	1.7	0.37	0.00091	Underexpressed
Casein kinase II subunit beta isoform 1	23503295	9.3	3.0	0.38	0.00436	Underexpressed
Calcium-binding tyrosine phosphorylation-regulated protein isoform a	24797108	63.3	23.7	0.38	0.00038	Underexpressed
Glutamate carboxypeptidase 2 isoform 1	4758398	69.3	13.7	0.39	0.00100	Underexpressed
Dipeptidyl peptidase 2 isoform X1	530426726	17.7	5.3	0.39	0.00112	Underexpressed
Tissue alpha-L-fucosidase precursor	119360348	19.0	5.3	0.39	0.00523	Underexpressed
Acylamino-acid-releasing enzyme	23510451	27.0	8.0	0.40	0.00434	Underexpressed
T-complex protein 1 subunit zeta-2 isoform X1	578830267	36.7	13.3	0.42	0.00010	Underexpressed
Cathepsin F precursor	6042196	21.0	7.3	0.43	0.00109	Underexpressed
Actin-related protein T2	29893808	45.7	15.7	0.44	0.00119	Underexpressed
Transmembrane and coiled-coil domain-containing protein 2	56847610	23.3	9.0	0.45	0.00067	Underexpressed
Peroxiredoxin-2	32189392	33.3	13.7	0.45	0.00393	Underexpressed
Creatine kinase B-type	21536286	51.0	17.7	0.46	0.00013	Underexpressed
Beta-galactosidase-1-like protein isoform X1	530370954	35.3	12.3	0.46	0.00023	Underexpressed
T-complex protein 1 subunit gamma isoform a	63162572	128.7	50.3	0.46	0.00014	Underexpressed
Retinal dehydrogenase 1	21361176	20.3	7.0	0.47	0.00457	Underexpressed
T-complex protein 1 subunit theta isoform 1	48762932	77.7	28.0	0.48	0.00236	Underexpressed
T-complex protein 1 subunit zeta isoform a	4502643	71.7	28.7	0.49	0.00183	Underexpressed
Glutamine synthetase isoform X1	578800828	23.3	8.3	0.50	0.00915	Underexpressed
Isocitrate dehydrogenase [NADP] cytoplasmic	538917681	59.0	21.7	0.50	0.00110	Underexpressed
Elongation factor 1-delta isoform 1	304555581	32.3	13.0	0.50	0.00303	Underexpressed
Hypoxia up-regulated protein 1 isoform X2	530397761	177.3	63.0	0.50	0.00124	Underexpressed
T-complex protein 1 subunit beta isoform 1	5453603	120.7	50.3	0.50	0.00125	Underexpressed
T-complex protein 1 subunit eta isoform a	5453607	129.7	58.3	0.55	0.00129	Underexpressed
Acrosin precursor	148613878	255.7	136.0	0.58	0.00052	Underexpressed

Table 3. Continued 2

Protein	Accession No.	Average SpC		NSAF ratio	p-value	Expression
		Donors	Patients	Patients/ Donors		
Ruvb-like 2	5730023	137.3	66.7	0.60	0.00609	Underexpressed
T-complex protein 1 subunit alpha isoform a	57863257	132.0	65.3	0.61	0.00065	Underexpressed
T-complex protein 1 subunit delta isoform a	38455427	108.3	54.3	0.61	0.00479	Underexpressed
Sperm equatorial segment protein 1 precursor	21717832	100.7	53.7	0.62	0.00076	Underexpressed
Ruvb-like 1	4506753	99.7	51.3	0.65	0.00355	Underexpressed
Endoplasmin precursor	4507677	543.7	238.7	0.66	0.00276	Underexpressed
Leucine-rich repeat-containing protein 37A2 precursor	116325993	163.0	78.3	0.67	0.00214	Underexpressed
Myeloperoxidase precursor	4557759	69.3	81.0	1.53	0.01314	Overexpressed
Nuclear pore membrane glycoprotein 210-like isoform 1 precursor	117414168	126.7	120.7	1.56	0.00216	Overexpressed
Nuclear pore complex protein Nup155 isoform 1	24430149	86.0	74.7	1.60	0.01024	Overexpressed
Angiotensin-converting enzyme isoform 1 precursor	4503273	141.3	142.3	1.65	0.00027	Overexpressed
Lactotransferrin isoform 1 precursor	54607120	702.3	829.3	1.67	0.00002	Overexpressed
Clusterin preproprotein	355594753	116.7	147.0	1.79	0.00055	Overexpressed
Protein S100-A8	21614544	15.0	25.0	2.03	0.02068	Overexpressed
Galectin-3-binding protein precursor	5031863	17.3	26.0	2.23	0.01154	Overexpressed
Plasma serine protease inhibitor preproprotein	194018472	40.7	69.7	2.33	0.00003	Overexpressed
Neutrophil gelatinase-associated lipocalin precursor	38455402	9.3	20.0	2.39	0.00320	Overexpressed
Erythrocyte band 7 integral membrane protein isoform a	38016911	9.3	23.3	2.43	0.00067	Overexpressed
Serotransferrin precursor	4557871	11.3	21.3	2.47	0.04142	Overexpressed
Myeloblastin precursor	71361688	6.0	13.7	2.69	0.00081	Overexpressed
Semenogelin-2 precursor	4506885	261.3	606.0	2.83	0.00678	Overexpressed
Sulfhydryl oxidase 1 isoform a precursor	13325075	7.0	15.3	3.05	0.00162	Overexpressed
Extracellular matrix protein 1 isoform 1 precursor	221316614	7.3	16.0	3.06	0.00007	Overexpressed
Epididymal secretory protein E3-alpha precursor	11386189	4.7	12.7	3.25	0.00651	Overexpressed
Alpha-1-antitrypsin precursor	189163532	13.7	34.7	3.43	0.00003	Overexpressed
Beta-2-microglobulin precursor	4757826	4.0	11.7	3.57	0.00238	Overexpressed
Prosaposin isoform a preproprotein	11386147	20.0	42.7	3.71	0.00007	Overexpressed
Prolactin-inducible protein precursor	4505821	238.0	788.3	3.85	0.00122	Overexpressed
Semenogelin-1 preproprotein	4506883	94.0	393.7	5.08	0.00009	Overexpressed
Lipoprotein lipase precursor	4557727	4.3	17.0	5.55	0.00068	Overexpressed
Fibronectin isoform 1 preproprotein	47132557	112.7	505.0	6.44	0.00000	Overexpressed
Zymogen granule protein 16 homolog B precursor	94536866	5.7	29.7	6.65	0.00007	Overexpressed
Integrin beta-2 isoform X1	578836536	2.3	15.0	8.92	0.00004	Overexpressed
Integrin alpha-M isoform 1 precursor	224831239	5.3	33.0	10.26	0.00078	Overexpressed
Mucin-5B precursor	301172750	22.0	214.3	12.40	0.00000	Overexpressed
Mucin-6 precursor	151301154	3.7	36.0	16.60	0.00050	Overexpressed
Laminin subunit beta-2 isoform X1	530372442	1.3	17.3	22.70	0.00054	Overexpressed
BPI fold-containing family B member 1 precursor	40807482	4.7	95.3	27.49	0.00049	Overexpressed
Bactericidal permeability-increasing protein precursor	157276599	0.3	6.7	28.80	0.00085	Overexpressed
Plasma protease C1 inhibitor precursor	73858570	0.3	2.7	34.56	0.00067	Overexpressed
Alpha-1-acid glycoprotein 1 precursor	167857790	0.7	18.0	43.86	0.00001	Overexpressed
Polymeric immunoglobulin receptor isoform X1	530366266	0.3	22.3	97.14	0.00486	Overexpressed
Endothelial lipase precursor	5174497	0.0	2.0	-	0.00000	Unique to patients
Apolipoprotein A-IV precursor	71773110	0.0	5.3	-	0.00022	Unique to patients
Carcinoembryonic antigen-related cell adhesion molecule 8 precursor	21314600	0.0	3.7	-	0.00099	Unique to patients

HD: Hodgkin's disease, SpC: spectral count, NSAF: spectral abundance factor.

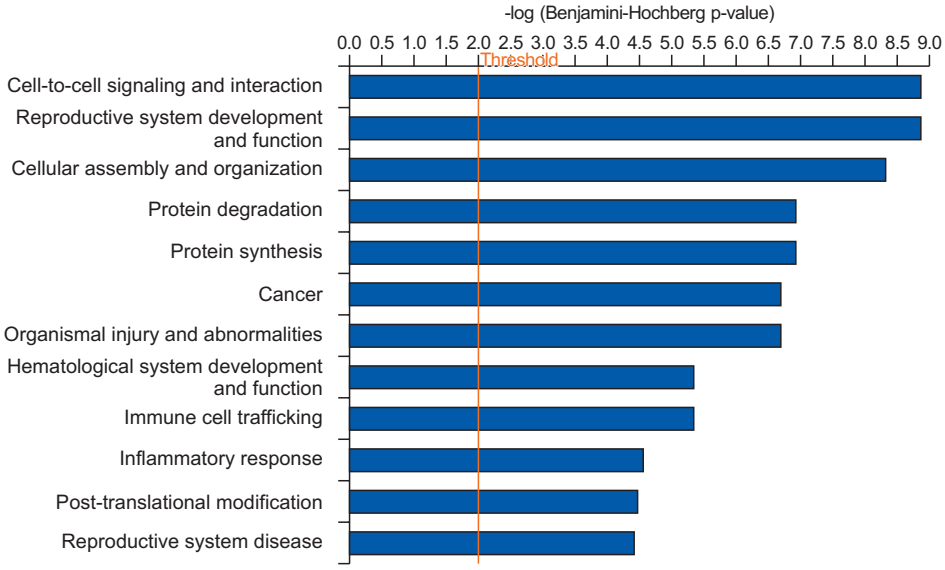

Fig. 2. Diseases and biofunctions identified in the spermatozoa of proven fertile donors' group and patients with Hodgkin's disease before cancer therapy by Ingenuity Pathway Analysis with a cut off=4.44 and p=0.01.

associated with top diseases and biofunctions were dysregulated in HD group with a cut off equal to 4.44 and a p=0.01 (Fig. 2). The top three pathways identified in the diseases and biofunctions are cell-to-cell signaling and interaction, reproductive system development and function, and cellular assembly and organization (Fig. 2). Functional analysis of DEPs identified that majority of the sperm proteins were associated with sperm functions (Fig. 3). Binding of sperm and binding of zona pellucida processes were affected due to the aberrant expression of sperm proteins, such as some subunits of T-complex protein 1 (TCP1, CCT2, CCT3, CCT4, CCT6A, CCT7, and CCT8), acrosin (ACR), probable inactive serine protease 37 (PRSS37) and angiotensin-converting enzyme (ACE). Other DEPs have a connection between the binding to zona pellucida and binding to sperm, (Fig. 3A). Additionally, the DEPs (ACR, TCP1, CCT2, CCT3, CCT4, CCT6A, CCT7, and CCT8) were also involved in the carcinoma functions in spermatozoa of men with HD (Fig. 3B). IPA analysis also showed that the DEPs ACE and ACR were involved in several reproductive functions such as sperm binding, fertility and fertilization process of the spermatozoa (Fig. 3C). Furthermore, IPA analysis also showed that production of reactive oxygen species (ROS) with metabolism of ROS and synthesis of ROS were affected due to altered expression of several DEPs (IL4I1, ITGAM, ITGB2, LTF, MPO, PRDX2, PRTN3, RACK1, S100A8, SERPIN A1, TF, TRAP1, APOA4, FN1, and IDH1) (Fig. 3D).

4. Western Blot validation of spermatozoa proteins in men with Hodgkin's disease

Based on selection criteria and the biological role, five proteins (ACE, PRDX2, CCT3, TF, and SERPINA5) were validated by WB. Our results showed that the expression of ACE was comparable in control and HD groups (Fig. 4A). The expression level of CCT3 was found to be similar in HD and control groups (Fig. 4C). Furthermore, our WB results revealed an underexpression (p=0.015) of PRDX 2 (Fig. 4D), while overexpression of TF (p=0.045) and SERPINA5 (p=0.010) in HD group when compared to fertile donor group.

DISCUSSION

Proteomic analysis using bioinformatic tools offers a comprehensive information regarding the protein distribution, and functional and molecular pathways associated with the peptides identified in spermatozoa [13]. Previous studies in the global proteomics analysis of spermatozoa resulted in the discovery of biomarkers for different pathologies [10,13-15,22]. In this study we used LC-MS/MS, a high throughput technique, to profile the spermatozoa proteins from men diagnosed with HD prior to cancer therapy and compared it with the proteome profile of fertile donors. The semen analysis was performed according to WHO guidelines (2010) [16], and the results indicated that the men from cancer group had lower sperm concentration, total sperm count and total motile count, while there was no significant difference in the motility. The quality

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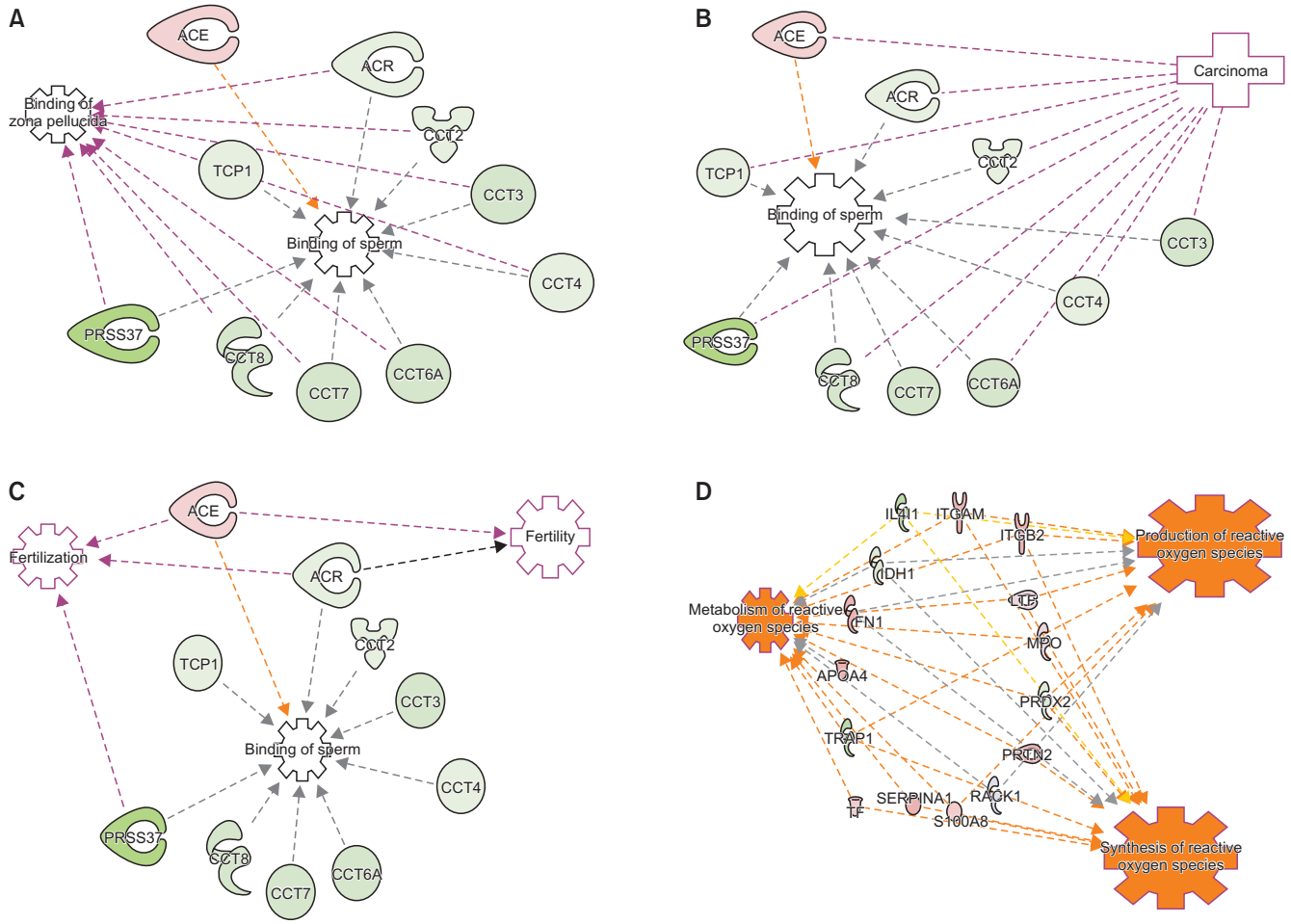

Fig. 3. Ingenuity Pathway Analysis of the differently expressed protein in spermatozoa of proven fertile donors' group and patients with Hodgkin's disease before cancer therapy, with the connecting protein related to (A) binding of zona pellucida and binding of sperm; (B) biding of sperm and carcinoma; (C) binding of sperm, fertility and fertilization; and (D) metabolism, production and synthesis of reactive oxygen species. TCP1: T-complex protein 1 subunit alpha, CCT3: T-complex protein 1 subunit gamma, ACR: acrosin, PRSS37: probable inactive serine protease 37, ACE: angiotensin-converting enzyme, IL4I1: L-amino-acid oxidase, ITGAM: integrin alpha-M; IDH: isocitrate dehydrogenase, ITGB2: integrin subunit beta 2, LTF: lactotransferrin precursor, PRTN2: transcription regulatory protein 2, RACK: receptor for activated C kinase, S100A8: S100 calcium-binding protein A8, SERPIN: serine proteinase inhibitors, TF: transferrin, TRAP: heat shock protein 75 kDa mitochondrial percursor, APOA: apolipoprotein A, FN1: fibronectin, MPO: myeloperoxidase.

of sperm in HD patients was lower when compared to fertile donors, however the sperm parameters values from patients were still within the WHO (2010) [16] reference values. The presence of lower sperm quality in men with cancer, particularly with HD obtained in our study is compliant with previous reports [5-7]. However, semen analysis alone can not predict whether the patients are fertile, subfertile or infertile. Semen parameters are used as a screening test to detect the contribution of male factor, but they fail to provide a full understanding of fertility potential [23,24]. As several factors (hyperactivation, capacitation, acrosome reaction, oxidative stress, sperm DNA fragmentation, etc.) in addition to semen quality contribute to the ability of spermatozoa to fertilize an oocyte, use of high-throughput techniques can allow a better understanding of the spermatozoal fertility potential at molecular level. In the present study, proteomic profiling of spermatozoa showed altered expression of key proteins associated with sperm function, which may contribute to the diminished fertility potential seen in some HD patients.

To minimize the variability existent in the complex nature of the spermatozoa [12], we had pooled the samples and run it in triplicate. LC-MS/MS spectrometry identified a total of 1,169 different peptides in the two experimental groups and about 1,049 were common to both experimental groups. The proteomic profile of spermatozoa is not new and similar studies have been

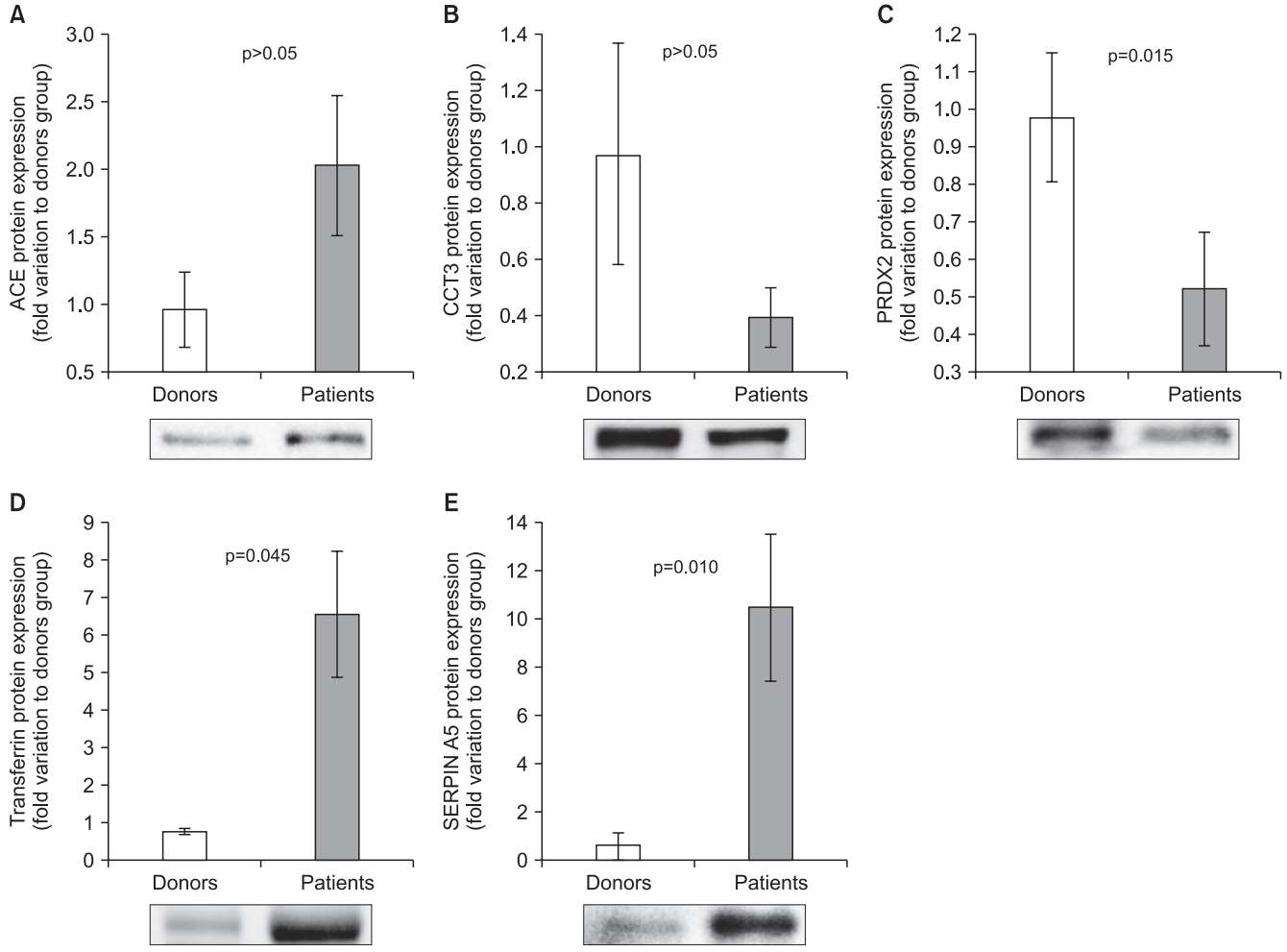

Fig. 4. Protein expression levels of the differentially expressed proteins selected for validation by Western blot in spermatozoa of proven fertile donors' group and patients with Hodgkin Disease before cancer therapy. (A) Angiotensin converting enzyme (ACE); (B) T-complex protein 1 subunit gamma (CCT3); (C) Peroxiredoxin-2 (PRDX2); (D) Transferrin; (E) Plasma serine protease inhibitor (SERPIN A5). Results are expressed as mean±standard error of mean and in fold variation to donors' group.

performed in mature and immature ejaculated spermatozoa from fertile men [14], infertile men [11], and in spermatozoa of infertile men with bilateral varicocele [22]. However, this is the first report on the sperm proteins in men with HD before cancer therapy. To find an explanation for the lower sperm quality in these patients, we had chosen five proteins for validation. These proteins were selected based on their involvement in reproductive functions and fertilization process. Cell to cell signaling and interactions are crucial for the fertilization process [25,26], and the binding of sperm to zona pellucida is in fact crucial for the oocyte fertilization. Our analysis revealed that binding of zona pellucida and binding of sperm were dysregulated in spermatozoa of men with HD. ACE is a protein involved in fertility and our IPA analysis relates this protein to binding of sperm and zona pellucida, and interrelates the binding of sperm with fertilization process and fertility. ACE has been reported to be altered in infertile patients [13,27,28]. In our experiment, the proteomic analysis showed an overexpression in ACE, although the WB validation did not show any significant increase in expression. This protein has been described as a functional protein in sperm motility and capacitation [26,29]. Due to the localization of ACE in the peri-acrosomal section of the sperm head, and an agonist-induced function in acrosomal exocytosis, this protein consequently has a significant role in the fertility process [30]. Studies have reported a correlation between decrease in ACE with an increase in ROS and possible impairment of acrosome reaction [28,29]. PRDXs are central antioxidant enzymes and have a

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role in sperm function and male fertility [31]. PRDX2 is mostly found in the cytosol, eliminating ROS formed as a by-product of metabolism [32]. This protein was found to be underexpressed by the proteomic analysis and validated by WB. Since PRDX2 is an antioxidant enzyme, the underexpression of this protein can be related to low levels of oxidative stress. Transferrin which has been found to be overexpressed by proteomic analysis and validated by WB is also involved in sperm protection [33]. This protein decreases oxidative stress by reducing the availability of free iron [34].

The acrosome is crucial for the fertilization and it contains numerous hydrolytic enzymes, including ACR [35]. ACR is synthesized and stored in the sperm acrosome matrix in the form of proacrosin, an inactive form of ACR. During acrosome reaction, proacrosin transforms to intermediate and mature forms through autoactivation [36]. Previous studies have reported the importance of ACR in fertility and sperm ACR activity is significantly reduced in men with anti-sperm antibodies [37]. Another study reported that the ACR activity is a reflection of male fertility status [38]. ACR is a proteolytic enzyme capable of hydrolyzing the zona pellucida of oocyte, playing an important role in fertilization [39]. This is in line with our IPA analysis results which revealed that ACR was related to binding of sperm, zona pellucida and fertilization process. Our proteomics analysis demonstrated an under expression of ACR. The low expression of ACR in patients can be related with other protein identified by LC-MS/MS, the probable inactive serine protease 37 (PRSS37). PRSS37 is involved in acrosin activation [36], and abnormal activation of proacrosin/acrosin system has been reported in sperm with low levels PRSS37 [36]. Interestingly, our proteomic results showed an underexpression of PRSS37 (Table 3). SERPIN A5 was found to be overexpressed in spermatozoa of men with HD compared with proven fertile donors by LC-MS/MS spectroscopy with concordant results from validation. SERPIN A5 is localized in external plasma membrane and has many functions including inhibition of several serine proteases related to male reproductive tract [40-42]. This protein has a role in motility [40,41], fertilization process [42], and indirectly inhibits the degradation of semenogelin1 and 2 [43]. Our proteomic study results demonstrated the overexpression of semenogelin1 and 2 (Table 3), which is concordant with the overexpression of SERPIN A5 that inhibits the degradation of these proteins. Besides these functions, SERPIN A5 also inhibits acrosin and indirectly protects the male genital tract from the damaging effects of excess acrosin [42].

CCT3 is a subunit of T-complex protein 1, and a chaperone responsible for protein folding in the cells that requires the assistance of enzymes [44]. Low levels of this protein are related to low cell proliferation in cell counts, and induced cell apoptosis [45,46]. This fact can be indirectly responsible for the low sperm count in the HD group. Another fact to consider is the association between high levels of CCT3 and its negative correlation with survival in cancer patients [45,46]. The expression levels of CCT3 are low in sperm of HD patients, which is in compliance with the high survival rate for HD described by the American Cancer Society, [1].

CONCLUSIONS

This study aimed to explain reasons for poor semen quality seen in men diagnosed with HD prior to cancer therapy, as understanding the cellular and molecular mechanisms of cancer-associated poor semen quality may guide future research in fertility preservation. Our proteomic data showed an altered proteomic profile in spermatozoa of men with HD and our WB results validated the vital proteins involved in the fertility process. Validated proteins can serve as potential biomarkers to determine the sperm quality and fertility status in HD patients prior to cancer therapy.

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Conflict of Interest

The authors have nothing to disclose.

Author Contribution

Conceptualization: AA. Data curation: ADM, PNP. Formal analysis: ADM, MKPS, SB. Investigation: AA. Methodology: ADM, PNP. Project administration: AA. Resources: ADM, MKPS. Software: PNP. Supervision: AA. Validation: ADM. Writing–original draft: ADM. Writing–review & editing: all authors.

Data Sharing Statement

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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