



Protein Fingerprinting of Seminal Plasma Reveals Dysregulation of Exosome-Associated Proteins in Infertile Men with Unilateral Varicocele

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Purpose: Aberrant expression of seminal plasma proteins are associated with altered homeostasis that may affect the fertilizing ability of spermatozoa. However, the precise roles of seminal exosomes on sperm function remain unclear. The objective of this study was to identify the differentially expressed proteins (DEPs) associated with varicocele-mediated infertility by comparing seminal plasma protein profile of unilateral varicocele patients with proven fertile donors.

Materials and Methods: Semen samples were obtained from 10 proven fertile donors with normal semen parameters and 33 infertile patients with unilateral varicocele. For proteomic analysis, 5 samples from each group were pooled and run in triplicate. Key DEPs (ANXA2, TF, CD63, KIF5B, SEMG1) associated with the exosome function were selected by bioinformatic tools and validated using Western blotting.

Results: A total of 47 seminal plasma proteins were differentially expressed in unilateral varicocele patients compared to fertile donors. Validation of exosome-associated DEPs in unilateral varicocele patients (n=7) and fertile donors (n=7) revealed significant upregulation of ANXA2 (p=0.0016) and downregulation of KIF5B (p=0.009). The main upstream regulators of the DEPs in seminal plasma of unilateral varicocele group were androgen receptor, YB1 and NRF2.

Conclusions: This is the first report to identify DEPs in seminal plasma of unilateral varicocele patients compared to fertile donors. Based on the detection of DEPs associated with exosomal function, Western blotting was used to validate the presence of defective exosome machinery in seminal plasma of unilateral varicocele patients. KIF5B and ANXA2 can be utilized as potential biomarkers of infertility in unilateral varicocele patients.

Keywords: Exosomes; Male infertility; Proteomics; Seminal plasma; Varicocele

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INTRODUCTION

Varicocele is recognized as one of the leading causes

of male infertility. It is prevalent in 15% of the male population and in 40% of infertile men [1]. The occurrence of unilateral varicocele is more common

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compared to bilateral varicocele. Approximately 90% of varicocele cases occur unilaterally and on the left side, while 10% occur bilaterally [2]. Varicocele is also encountered in fertile men with normal spermatogenesis and semen parameters [3]. Poor semen quality, increased seminal oxidative stress and sperm DNA fragmentation are most commonly seen in infertile men with varicocele, whereby these factors compromise the normal physiological functions of sperm [4]. However, the exact molecular mechanisms of varicocele-associated infertility still remain unclear [4,5].

Protein profiling in varicocele patients have identified dysregulation of key sperm proteins associated with sperm motility, capacitation, hyperactivation, and zona pellucida binding [6-9]. In addition to sperm proteins, seminal plasma proteome also plays a key role in determining the fertilization capacity of the sperm [10]. Seminal plasma provides a favorable environment for the maturation of spermatozoa and its proteome reflects the functionality of the male reproductive tract. It carries important information regarding testicular function, and about 10% of the proteins found in seminal plasma originate from the testes [11]. Seminal plasma also harbors proteins associated with various fertilization processes; such as hyperactivation, capacitation, acrosome reaction; and sperm-oocyte interaction [12,13]. High throughput proteomic studies have identified seminal plasma proteins associated with nitric oxide synthesis and their altered metabolism in varicocele condition [14,15]. Previous report compared the seminal plasma proteome profile of adolescents without varicocele to those with varicocele and normal or abnormal semen quality [16]. Similarly, seminal plasma proteome of adult men before and after varicocelectomy [14]; adolescents and adults with treated and untreated varicocele [15] were also compared. Furthermore, molecular markers such as proliferative/apoptotic proteins (IGFBP7 and DNASE 1) were predicted in the semen of adolescents with varicocele having normal and abnormal semen parameters when compared with adolescents without varicocele [17]. Nevertheless, none of these reports ever compared the proteome profile of seminal plasma of infertile men with varicocele to that of fertile men without varicocele for finding a marker to predict the cause of infertility in these men.

Seminal plasma contains exosomes that are released from the epididymis, prostate and seminal vesicles. Exosomes have a complex proteome and sperm-exosome

fusion is essential for the transfer of proteins to the transcriptionally and translationally silent spermatozoa [18]. Exosome-associated proteins are involved in energy pathways, protein metabolism, cell growth and maintenance [19,20]. There are no reports on the role of exosomes in varicocele-associated infertility.

Our case-control study was designed to compare the seminal plasma proteome of infertile men with varicocele against that of fertile control without varicocele as an attempt to find a proteomic biomarker to predict the cause for unilateral varicocele-associated infertility. To further delineate the role of exosomes, we have evaluated proteomic pathways associated with exosomal dysfunction to demonstrate the possible underlying cause of infertility in unilateral varicocele patients.

MATERIALS AND METHODS

1. Study subjects and ethics statement

This study was approved by the Institutional Review Board of Cleveland Clinic (IRB No. 17-422). All the subjects who participated in this study had signed a written consent. Semen samples were obtained from 10 healthy male donors of proven fertility, and 33 infertile patients with unilateral varicocele. Clinical diagnosis of varicocele was performed by scrotal palpation and was graded as described in our earlier publication [9].

2. Inclusion and exclusion criteria

Clinical examination, patient history and semen analysis results were considered for sample selection. Patients aged 20 to 40 years (36.3 ± 7.7 years) who attended the clinic for infertility treatment from March 2012 to March 2014 were enrolled in this study. Patients with recurring fever 90 days prior to semen analysis were excluded from the study. Similarly, men with leukocytospermia (Endtz positive), azoospermia, severe and moderate oligozoospermia (<10 million sperm/mL) were excluded from the study as described in our previous study [9]. All fertile men of the age group (40.0 ± 9.8 years) without the presence of clinical varicocele were included in the control group. These fertile men had fathered at least one child in the past 2 years before their enrollment in the study. Both the patients and fertile men were not exposed to any radiations or chemicals or environmental pollutants.

3. Semen analysis

Routine semen analysis was performed as per World Health Organization guidelines [21]. Reactive oxygen species (ROS) levels and sperm DNA fragmentation were measured using chemiluminescence assay and terminal deoxynucleotidyl transferase-mediated fluorescein end labeling assay, respectively [9,22]. Semen samples were centrifuged for 7 minutes at 1,000g, and clear seminal plasma was aspirated and stored at -80°C for proteomic analysis.

4. Preparation of samples for proteomic studies

Seminal plasma samples were thawed at room temperature and centrifuged at 3,000g for 30 minutes to completely remove contaminating spermatozoa and somatic cells. The protein concentration was determined using a bicinchoninic acid kit (Thermo, Rockford, IL, USA). Pooled samples from unilateral varicocele group (n=5) and fertile donor group (n=5) were used for proteomic analysis. The samples were run in triplicate in one dimensional (1D)-polyacrylamide gel electrophoresis (PAGE). A total of 15 µg of protein per sample was loaded into a 12.5% Tris-HCl 1D gel and electrophoresed with constant voltage of 150 V for 35 minutes. After electrophoresis, each gel lane was cut into 6 pieces, digested using 5 µL trypsin (10 ng/µL) and 50 mM ammonium bicarbonate, and incubated overnight at room temperature. 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.

5. Liquid chromatography-tandem mass spectrometry analysis

Proteomic profiling of seminal plasma was carried out using a Finnigan LTQ linear ion trap mass spectrometer liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) system. The peptides were fractionated by injecting 5 µL into high performance liquid chromatography column (Phenomenex Jupiter C18 reversed-phase capillary chromatography column). Fractions containing the peptides were eluted in acetonitrile/0.1% formic acid at a flow rate of 0.25 µL/min and introduced into the source of the mass spectrometer on-line. A full spectral scan was performed by utilizing the data dependent multitask abil-

ity of the instrument to determine peptide molecular weights and amino acid sequence of the peptides [23].

6. Protein identification and quantitative proteomics

Similar pipeline of analysis was followed to identify the proteins using Proteome Discoverer ver. 1.4.1.288. Charge state deconvolution and deisotoping were not performed. 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). The search was limited to the human reference sequences database (33292 entries) and results were uploaded into the Scaffold (ver. 4.0.6.1; Proteome Software Inc., Portland, OR, USA) software as previously described [9]. 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.

Relative quantity of the proteins was performed by comparing the number of spectra, termed spectral counts in both the unilateral varicocele and fertile donor groups. 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) [9].

7. Bioinformatic analysis

Differentially expressed proteins (DEPs) identified in both 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>). ClueGo application on the Cytoscape platform was used to analyze the involvement of DEPs in affected processes, pathways, cellular distribution, and protein-protein interactions [24]. Proprietary software package Metacore™ (GeneGo Inc., Saint Joseph, MI, USA) was also used to identify the upstream regulator proteins involved in the enriched pathways.

8. Protein validation by Western blotting

From the list of DEPs, 5 proteins were selected for validation by Western Blotting (WB) (n=7/group). The selection was based on their role in seminal exosome-related functions: CD63 antigen isoform D precursor (CD63), annexin A2 (ANXA2), transferrin (TF), kinesin-1 heavy chain (KIF5B), and semenogelin 1 (SEMG1). A total of 50 µg of protein per sample was loaded into a 4% to 15% sodium dodecyl sulfate-PAGE for 2 hours at 90 V. The resolved protein bands were then transferred onto polyvinylidene difluoride (PVDF) membranes and for each protein analysis, specific primary antibodies were incubated at 4°C overnight (Table 1). Subsequently, the membranes were incubated with the secondary antibodies at room temperature for 1 hour (Table 1) and finally reacted with enhanced chemiluminescence reagent (GE Healthcare, Marlborough, MA, USA) for 5 minutes. Membranes were exposed to Chemi-Doc (ChemiDoc™ MP Imaging System; Bio-Rad, Hercules, CA, USA) to detect the chemiluminescence signals.

All the PVDF membranes used for protein identification were subjected to total protein staining. The membranes were washed briefly twice for 10 minutes in the distilled water and stained with total colloidal gold protein stain (Bio-Rad) 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 colorimetric mode on Chemi-Doc (ChemiDoc™ MP Imaging System; Bio-Rad).

9. 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 fertile donor group and the unilateral varicocele group, and the results were considered significant for 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 analysis

A majority of the patients (93.9%, 31 of 33 patients)

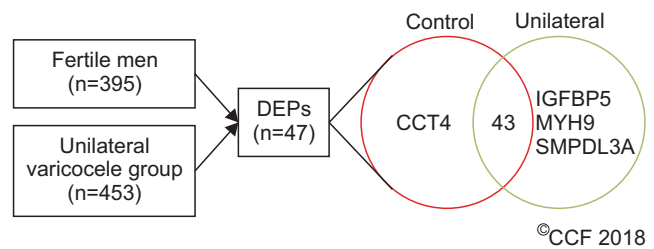


Fig. 1. Differentially expressed proteins (DEPs) in fertile men (control) and unilateral varicocele group (unilateral).

Table 1. List of primary and secondary antibodies

Primary				Secondary		
Protein	Antibody	Manufacturer	Dilution	Antibody	Manufacturer	Dilution
ANXA2	Anti-human mouse IgG	Abcam ab54711	1:1,000	Anti-mouse rabbit IgG	Abcam ab6728	1:10,000
CD63	Anti-human rabbit IgG	Abcam ab118307	1:500	Anti-rabbit goat IgG	Abcam ab97051	1:10,000
KIF5B		Abcam ab167429	1:10,000			1:10,000
TF		Abcam ab82411	1:1,000			1:10,000
SEMG1	Anti-human goat IgG	Santa Cruz sc34719	1:200	Anti-goat donkey IgG	Santa Cruz sc2020	1:10,000

ANXA2: annexin A2, KIF5B: kinesin-1 heavy chain, TF: transferrin, SEMG1: semenogelin 1, IgG: immunoglobulin G.

Table 2. Semen parameters in fertile donors and unilateral varicocele patients

Parameter	Healthy fertile men (n=10)	Unilateral varicocele patients (n=33)	p-value
Sperm concentration (10 ⁶ /mL)	69.9±37.7	30.8±28.2	0.006
Sperm motility (%)	57.1±16.0	41.5±17.3	0.021
Normal sperm morphology (%)	8.4±3.7	2.6±1.9	<0.001
ROS levels (RLU/s/10 ⁶ sperm)	142.7 (36.2–337.7)	494.8 (114.2–2,547.8)	0.03
Sperm DNA fragmentation (%)	8.5–18.2	3.3–35.3	0.0159

Values are presented as mean±standard deviation, median (interquartile range), or range only.

ROS: reactive oxygen species, RLU: relative light units.

For all values, p<0.05 indicate a significant difference based on the Mann–Whitney test.

Table 3. Differentially expressed proteins and their abundance in unilateral varicocele group compared with fertile men group

Uniprot No.	Gene name	Protein name	Fertile men group		Unilateral varicocele group		NSAF ratio	Expression
			SC	Abund	SC	Abund		
P50991	CCT4	T-complex protein 1 subunit delta isoform a	2.7	VL	0	-	0.00	Unique to fertile group
P33176	KIF5B	Kinesin-1 heavy chain	12.3	L	2.3	VL	0.10	UE
P10323	ACR	Acrosin precursor	10.7	L	2.7	VL	0.18	UE
Q02383	SEMG2	Semenogelin-2 precursor	1,321.0	H	503.0	H	0.25	UE
P04279	SEMG1	Semenogelin-1 preproprotein	698.0	H	304.3	H	0.27	UE
P15259	PGAM2	Phosphoglycerate mutase 2	7.3	VL	2.3	VL	0.27	UE
D6RI10	SPINK2	Serine protease inhibitor Kazal-type 2 isoform 1 precursor	9.7	L	3.3	VL	0.31	UE
P26641	EEF1G	Elongation factor 1-gamma	10.3	L	4.3	VL	0.33	UE
P07205	PGK2	Phosphoglycerate kinase 2	25.3	M	12.0	L	0.33	UE
P23284	PIIB	Peptidyl-prolyl cis-trans isomerase B precursor	41.7	M	32.3	L	0.46	UE
P02787	TF	Serotransferrin precursor	63.3	M	179.7	H	1.52	OE
P15144	ANPEP	Aminopeptidase N isoform X1	100.3	H	285.0	H	1.64	OE
P63261	ACTG1	Actin, cytoplasmic 2	52.3	M	106.3	H	1.67	OE
P35237	SERPINB6	Serpin B6 isoform a	8.0	L	20.0	M	2.00	OE
P12277	CKB	Creatine kinase B-type	15.7	L	43.0	M	2.07	OE
P19440	GGT1	Gamma-glutamyltranspeptidase 1 precursor	12.0	L	36.3	M	2.08	OE
P08697	SERPINF2	Alpha-2-antiplasmin isoform X1	11.0	L	30.3	M	2.14	OE
Q9Y6R7	FCGBP	IgGfC-binding protein precursor	16.3	L	48.7	M	2.31	OE
P40925	MDH1	Malate dehydrogenase, cytoplasmic isoform 2	8.7	L	24.3	M	2.32	OE
P32119	PRDX2	Peroxiredoxin-2	11.3	L	33.0	M	2.43	OE
O15393	TMPRSS2	Transmembrane protease serine 2 isoform 2	8.0	L	25.3	M	2.47	OE
P05090	APOD	Apolipoprotein D precursor	2.0	VL	7.0	VL	2.56	OE
P27487	DPP4	Dipeptidyl peptidase 4	18.3	L	73.7	M	2.77	OE
Q9HAT2	SIAE	Sialate O-acetyltransferase isoform 1 precursor	6.7	VL	29.3	M	2.78	OE
P04083	ANXA1	Annexin A1	7.7	VL	27.3	M	2.88	OE
P08473	MME	Neprilysin isoform X1	15.0	L	75.3	M	2.93	OE
P49221	TGM4	Protein-glutamine gamma-glutamyltransferase 4	31.3	M	183.0	H	3.17	OE
P29622	SERPINA4	Kallistatin isoform 2 precursor	9.0	L	39.0	M	3.26	OE
P08962	CD63	CD63 antigen isoform D precursor	3.0	VL	11.3	L	3.79	OE
Q9H0B8	CRISPLD2	Cysteine-rich secretory protein LCCL domain-containing 2 precursor	2.0	VL	14.3	L	4.03	OE
P51159	RAB27A	Ras-related protein Rab-27A	3.0	VL	15.0	L	5.04	OE
P07355	ANXA2	Annexin A2 isoform 2	4.7	VL	27.7	M	5.41	OE
Q9UBX7	KLK11	Kallikrein-11 isoform 2	0.7	VL	12.3	L	6.81	OE
Q04760	GLO1	Lactoylglutathione lyase	1.3	VL	11.7	L	7.80	OE
P02763	ORM1	Alpha-1-acid glycoprotein 1 precursor	10.0	L	86.0	H	7.96	OE
P54802	NAGLU	Alpha-N-acetylglucosaminidase precursor	2.3	VL	25.7	M	7.99	OE
P01019	AGT	Angiotensinogen preproprotein	1.7	VL	16.7	L	8.03	OE
P11047	LAMC1	Laminin subunit gamma-1 precursor	2.0	VL	32.3	M	9.87	OE

Table 3. Continued

Uniprot No.	Gene name	Protein name	Fertile men group		Unilateral varicocele group		NSAF ratio	Expression
			SC	Abund	SC	Abund		
P19652	ORM2	Alpha-1-acid glycoprotein 2 precursor	1.7	VL	23.0	M	10.75	OE
Q92896	GLG1	Golgi apparatus protein 1 isoform 2 precursor	0.7	VL	8.7	L	11.36	OE
O15230	LAMA5	Laminin subunit alpha-5 isoform X1	1.7	VL	47.0	M	16.65	OE
O60635	TSPAN1	Tetraspanin-1	0.3	VL	9.7	L	18.89	OE
P04908	HIST1H2AB	Histone H2A type 1-B/E	0.3	VL	23.0	M	63.82	OE
P01833	PIGR	Polymeric immunoglobulin receptor isoform X1	0.3	VL	38.3	M	79.11	OE
P24593	IGFBP5	Insulin-like growth factor-binding protein 5 precursor	0.0	-	7.0	VL	0.00	Unique to unilateral varicocele group
P35579	MYH9	Myosin-9	0.0	-	8.0	L	0.00	Unique to unilateral varicocele group
Q92484	SMPDL3A	Acid sphingomyelinase-like phosphodiesterase 3a isoform a precursor	0.0	-	8.3	L	0.00	Unique to unilateral varicocele group

SC: spectral count, Abund: abundance, NSAF: normalized spectral abundance factor, VL: very low, L: low, H: high, M: medium, UE: underexpressed, OE: overexpressed.

presented with a left side varicocele while only 6.1% (2/33) had a right side varicocele. Of these 48.5% (16/33) had grade 1, 21.2% (7/33) grade 2, 12.1% (4/33) grade 1/2, and 3.0% (1/33) grade 2/3. A total of 84.8% (28/33) of the patients had grade 1–2. Only 15.2% (5/33) of the patients presented with grade 3 or higher. A total of 76.7% of the patients (23/30) with unilateral varicocele were identified with primary infertility and 23.3% (7/30) with secondary infertility. Among those with primary infertility, 26.1% (6/23) had infertility of <1 year, 30.4% (7/23) equal to 1 year and 43.5% (10/23) of >1 year.

The hormonal profile was only evaluated for 4.5% of the patients (16/33) with varicocele, from which 31.3% (5/16) had testosterone levels <400 ng/dL and 68.8% (11/16) had levels between 400–800 ng/dL. Unilateral varicocele patients had significantly lower sperm motility, concentration and normal sperm morphology compared to fertile donors (Table 2). ROS and sperm DNA damage levels were significantly higher in the varicocele patient group when compared with fertile men (Table 2).

2. Identification of differentially expressed proteins

Global proteomic analysis identified a total of 395 and 453 proteins in fertile donor group and unilateral varicocele group, respectively. Quantification of pro-

teins based on NSAF ratio revealed that 47 proteins were differentially expressed ($p \leq 0.05$). Three of these DEPs were unique to the unilateral varicocele group and one to the fertile donor group (Fig. 1). Abundance of the DEPs based on their spectral counts differed between the two groups (Table 3). The proportion of very low abundance DEPs was high in fertile donor group (20 DEPs), whereas moderate abundance DEPs were higher in unilateral varicocele group (20 DEPs) (Fig. 2). Expression profiles of the DEPs identified in both groups are shown in Fig. 3.

3. Functional annotation of differentially expressed proteins

ClueGo analysis identified the GO terms associated with the DEPs reported in the seminal plasma. Enriched pathways associated with the exosomal function were selected and the percentage of associated genes are shown in Table 4. Network analysis revealed that the DEPs identified in the seminal plasma are associated with regulation of vesicle fusion and positive regulation of endocytosis related to exosomes (Fig. 4).

4. Identification of transcriptional factors regulated by differentially expressed proteins

Metacore analysis predicted 13 DEPs (APOD [apolipoprotein D precursor], SERPINF2 [alpha-2-antiplasmin

isoform X1], ORM1 [alpha-1-acid glycoprotein 1 precursor], TSPAN1 [tetraspanin-1], CRISPLD2 [cysteine-rich secretory protein LCCL domain-containing 2 precursor], RAB27A [Ras-related protein Rab-27A], ANPEP [aminopeptidase N isoform X1], MME [neprilysin isoform X1], KLK11 [Kallikrein-11 isoform 2], TMPRSS2 [transmembrane protease serine 2 isoform 2], DPP4 [dipeptidyl peptidase 4], TGM4 [protein-glutamine gamma-glutamyltransferase 4], and GLO1 [lactoylglutathione lyase] that were regulated by androgen receptor (Fig. 5A). Nuclease-sensitive element-binding protein 1 (YB-1): associated with fertility (Fig. 5B), and nuclear factor erythroid 2-related factor 2 (NRF2) involved in antioxidant gene expression (Fig. 5C), were identified as main upstream regulating transcription factors. In addition,

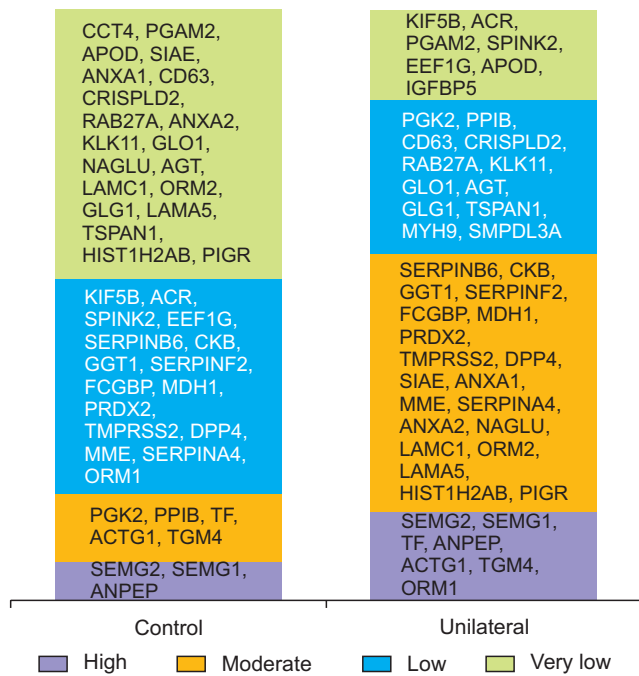


Fig. 2. Distribution of differentially expressed proteins abundance in fertile group (control) and unilateral varicocele group (unilateral).

these DEPs were also involved in sperm function such as binding to zona pellucida, sperm-egg recognition, regulation of ROS, and in sperm maturation processes,

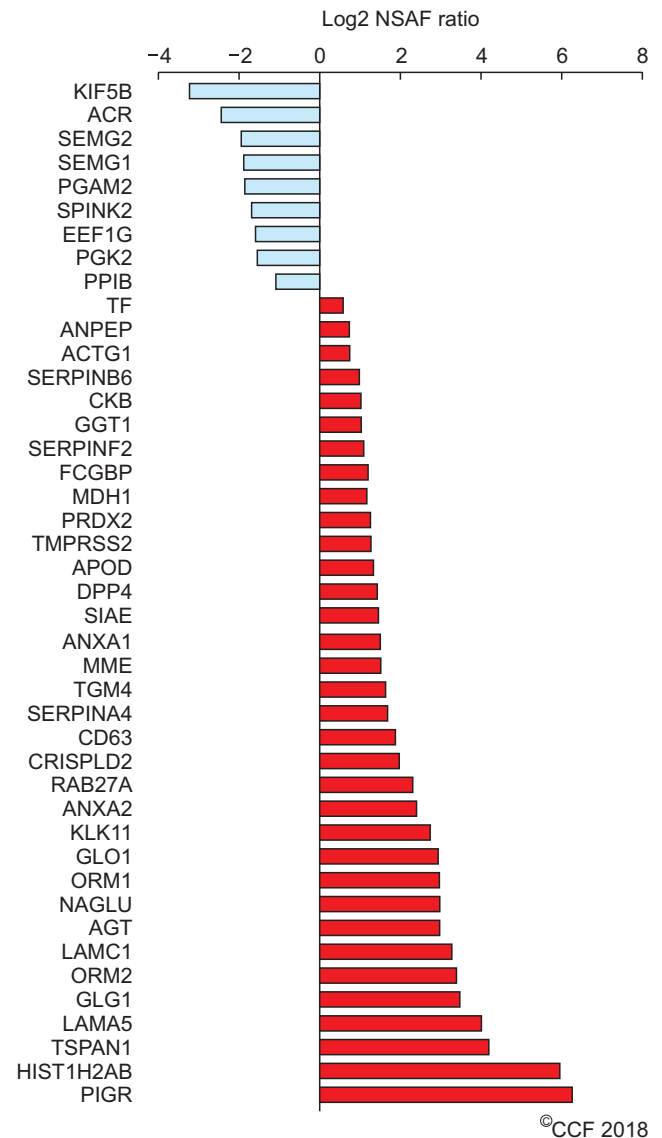


Fig. 3. Overexpressed and underexpressed proteins in seminal plasma of unilateral varicocele patients relative to fertile men. NSAF: normalized spectral abundance factor.

Table 4. Gene ontology (GO)-associated with exosomes present in seminal plasma

No.	GO ID	GO term	Ontology source	% associated genes
1	GO:0031340	Positive regulation of vesicle fusion	Biological process	37.50
2	GO:1902571	Regulation of serine-type peptidase activity	Biological process	21.43
3	GO:1900003	Regulation of serine-type endopeptidase activity	Biological process	21.43
4	GO:0048260	Positive regulation of receptor-mediated endocytosis	Biological process	5.88
5	GO:0004867	Serine-type endopeptidase inhibitor activity	Molecular function	5.00
6	GO:0002020	Protease binding	Molecular function	4.80
7	GO:0031338	Regulation of vesicle fusion	Biological process	4.23
8	GO:0045807	Positive regulation of endocytosis	Biological process	4.03

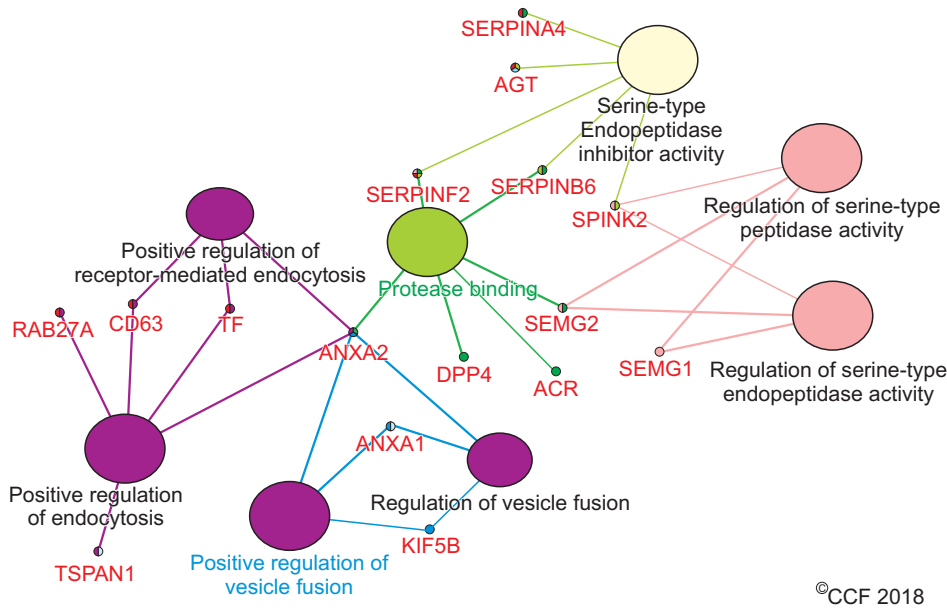


Fig. 4. *In-silico* network analysis of differentially expressed proteins associated with exosomal functions in the seminal plasma of fertile men and unilateral varicocele patients.

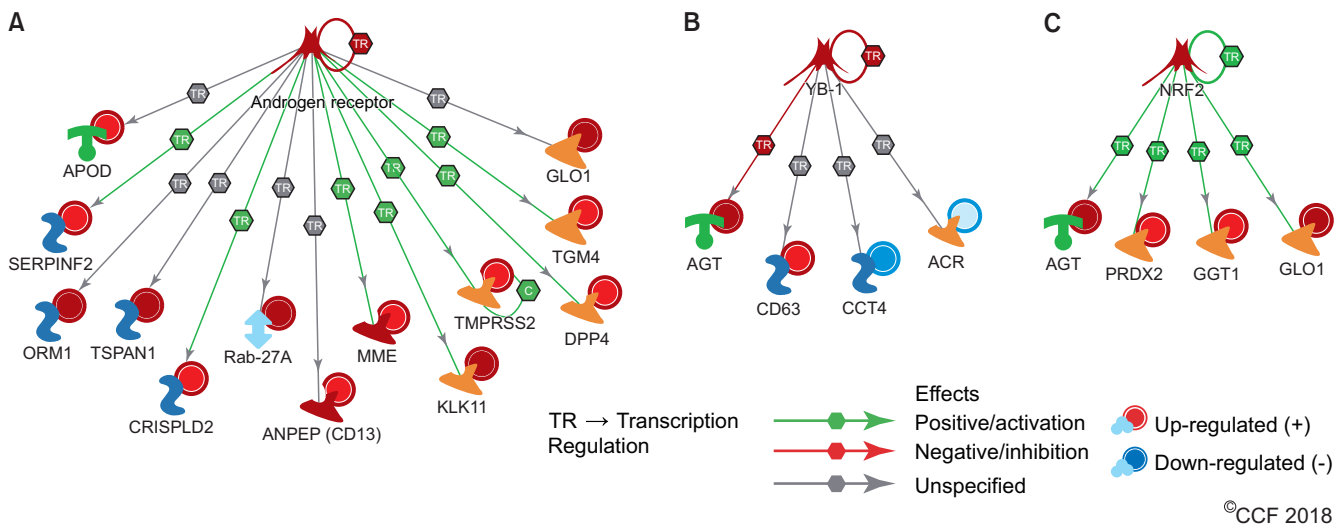


Fig. 5. Upstream transcriptional factors involved in the regulation of differentially expressed proteins identified in the seminal plasma of unilateral varicocele patients relative to fertile men. (A) Androgen receptor; (B) nucleosome-sensitive element-binding protein 1 (YB-1), and (C) nuclear factor erythroid 2-related factor 2 (NRF2).

such as regulated exocytosis, protein processing and protein maturation.

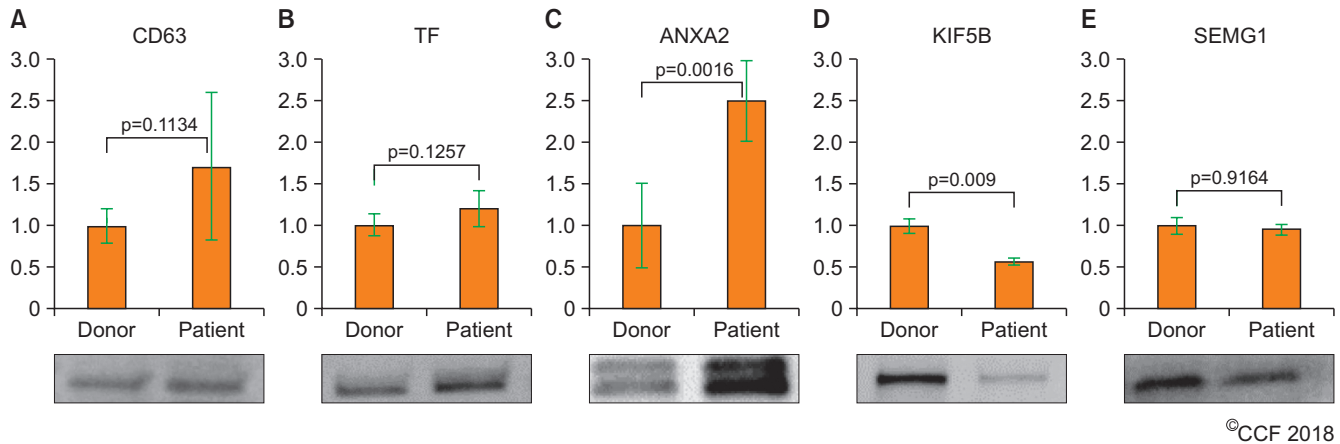
5. Validation of expression profile of proteins by Western blotting

Global proteomics data based on the NSAF ratio of the 5 DEPs showed that CD63, ANXA2 and TF were upregulated, whereas KIF5B and SEMG1 were downregulated in the unilateral varicocele group. We demonstrated the presence of exosomes in the seminal plasma by the expression of CD63 (exosomal marker) using WB (Fig. 6A). ANXA2 was significantly upregu-

lated (2.49 ± 0.44 -fold variation to control; $p=0.0016$) (Fig. 6C), whereas TF expression (1.19 ± 0.22 -fold variation to control) showed an increasing trend in the unilateral varicocele group ($p=0.1257$) (Fig. 6B). KIF5B expression was significantly lower (0.57 ± 0.08 -fold variation to control; $p=0.009$) in varicocele patients (Fig. 6D). SEMG1 did not show any significant decrease (0.95 ± 0.19 -fold variation to control; $p=0.9164$) between groups (Fig. 6E).

DISCUSSION

In the present study, we compared the protein sig-



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Fig. 6. Protein expression levels of the differentially expressed proteins selected for validation by Western blot in unilateral varicocele group relative to fertile men. (A) CD63 antigen isoform D precursor (CD63), (B) transferrin (TF), (C) annexin A2 (ANXA2), (D) kinesin-1 heavy chain (KIF5B), and (E) semenogelin 1 (SEMG1). Results are expressed as mean±standard error of mean and in fold variation to control.

natures in the seminal plasma of infertile unilateral varicocele patients with fertile men. Earlier studies on seminal plasma proteomics in varicocele patients did not include fertile men as the control group or failed to prove the fertility status of the comparison group [14-16,25,26]. Use of the fertile group in this study contribute to the accurate identification of the candidate proteins. This is essential for the development of potential biomarkers to diagnose infertile men with unilateral varicocele.

In our previous studies, we demonstrated key sperm proteins such as SPAM1, TCP11, TEKT3, and AK7 that were differentially expressed in varicocele patients and were known to be critical for fertility [9,27-29]. However, these proteins are not the sole reason for the pathology associated with spermatozoa. Seminal plasma contains microvesicles or exosomes which are involved in the transfer of proteins essential for sperm maturation and fertilization [18,30]. The results of this study identified DEPs associated with exosomes, and hence our goal was to investigate the role of exosome-associated proteins in determining the infertility status of varicocele patients. First, we established the presence of seminal exosomes by validating the expression of CD63 (universal exosomal marker) [31] by WB in seminal plasma of both fertile donor group and unilateral varicocele patients (Fig. 6A). From the comparative proteomic data, altered expression of the nodal proteins (RAB27A, TF, TSPAN1, ANXA1, ANXA2, and KIF5B) of the predominant network suggest dysfunction in the exosome machinery (Fig. 4). Evaluation of the key proteins (ANXA2, TF, CD63, KIF5B, and SEMG1) by

WB confirmed the significant change in the expression of ANXA2 and KIF5B. These two proteins may play an important role in the regulation of vesicle fusion and positive regulation of endocytosis associated with the exosomes in unilateral varicocele group.

In exosomes, KIF5B plays a major role in the transport of synaptosomal-associated protein 25 (SNAP-25) and assembly of SNARE proteins, which are essential for the release and proper functioning of the exosomes [32-34]. KIF5B was found to be significantly downregulated in the seminal plasma of unilateral varicocele patients (Fig. 6D), leading to the release of the dysfunctional exosomes. Decreased expression of KIF5B in seminal plasma is an indicator of the presence of defective exosomes. Existence of such conditions may affect the transfer of the exosomal elements into the peri-acrosomal region of the spermatozoa during their epididymal transit. Spermatozoa lacking the exosomal elements in their peri-acrosomal space exhibit increased fluidity of the sperm membrane and this results in premature acrosome reaction [18].

ANXA2 is a calcium-regulated binding protein, which is highly enriched in the exosomes, and is associated with membrane trafficking and fusion [35,36]. Our WB results showed a significant increase in ANXA2 (Fig. 6C). Sperm maturation takes place predominantly when spermatozoa transit through the epididymis and undergo changes in the sperm proteome [37,38]. Aberrant expression of ANXA2 may lead to the failure of the exosome fusion machinery, impairing the transfer of exosomal elements to the spermatozoa and resulting in the production of immature spermatozoa [39].

Seminal plasma serves as a rich source of biomarkers that can help in understanding the pre-ejaculation events from spermatogenesis to maturation. Using Metacore analysis, we were able to predict the upstream regulators involved in the transcription and translation of the DEPs detected in this study by LC-MS/MS; namely, androgen receptor (process of sperm production and maturation), YB-1 (fertilization), and NRF2 (antioxidant enzyme expression). Among these, the androgen receptor is the topmost upstream regulator of the DEPs. Testosterone action is dependent on the androgen receptors, which is essential for spermatogenesis and maintains accessory sex organs including prostate. In varicocele patients, decrease in testosterone levels is mainly due to the malfunction of androgen receptors [40]. In our dataset, we identified 13 overexpressed proteins that were under the regulation of androgen receptor, thus affecting the spermatogenesis process (Fig. 5A) in these patients. This was reflected by a significant decrease of sperm concentration in the unilateral varicocele group compared with fertile men (Table 2). One of the principal types of seminal plasma exosomes is prostasomes produced by the prostate [41,42]. Therefore, it is hypothesized that dysregulation of androgen receptor will lead to anomalous testosterone action resulting in decreased spermatogenesis and prostates-mediated sperm maturation. Varicocele is reported to induce hypogonadism and affect testosterone level [40]. Prostasomes from androgen-insensitive and hormone-responsive prostate cancer cell lines have alteration in glycosidase and protease activities compared to physiologically normal prostasomes [43]. Dysregulation of androgen receptor may lead to anomalous testosterone action resulting in decreased spermatogenesis and prostate function. Anomalous prostate function in turn may be responsible for defects in prostates-mediated sperm maturation. Furthermore, differential proteomics followed by immunocytochemistry and WB revealed that acquisition of MMSDH: methylmalonate-semialdehyde dehydrogenase (an androgen-dependent and developmentally regulated epididymal sperm protein) by spermatozoa is mediated *via* epididymosomes [44]. Therefore, the difference in the seminal plasma proteins of exosomal origin in unilateral varicocele patients may be due to alteration in androgen level.

YB-1 has pleiotropic functions that include transcriptional regulation and translational silencing [45]. Dif-

ferential expression in the proteins (CCT4 [T-complex protein 1 subunit delta isoform a], ACR [acrosin precursor], and CD63) regulated by YB-1 has a major impact on the binding of sperm to zona pellucida and sperm-egg recognition process (Fig. 5B). This may be the cause for infertility seen in varicocele patients. Besides, YB-1 also regulates conversion of angiotensin I from its precursor protein angiotensinogen (AGT) and subsequently to angiotensin II. These are essential for the motility of spermatozoa [46,47]. In the present study, overexpression of precursor protein indicates the limited conversion of AGT to its active forms, causing decreased sperm motility in infertile men with unilateral varicocele. YB-1 also acts as a transcriptional regulator to prevent DNA damage and promote cell survival [48]. This was in accordance with the results of the DNA fragmentation assay showing high degree of sperm DNA fragmentation in unilateral varicocele patients (Table 2). Therefore, it is logical to draw the conclusion that impaired YB-1 activity leads to accumulation of AGT, which in turn is responsible for declined sperm motility and augmented DNA fragmentation in spermatozoa of infertile men with unilateral varicocele.

NRF2 plays a critical role in the prevention of oxidative damage to the spermatozoa [49]. It is involved in the regulation of enzymes responsible for the protection against oxidative stress: peroxiredoxin-2 (PRDX2), glutamyltranspeptidase 1 precursor (GGT1), and GLO1. Immature spermatozoa tend to generate high levels of ROS resulting in oxidative stress in varicocele patients and affecting the physiological function of normal spermatozoa. In fact, a significant elevated ROS levels was observed in the unilateral varicocele group (Table 2). Therefore, it may be envisaged that the body invokes an adaptive response against ROS *via* NRF2, which in turn results in the overexpression of proteins involved in the antioxidant system (PRDX2, GGT1, and GLO1) (Fig. 5C). However, the trigger is not strong enough to revert back to basal level (comparable to fertile control).

Apart from the exosomal proteins, the other seminal plasma proteins insulin-like growth factor (IGF)-binding protein 5 (IGFBP5), myosin-9 (MYH9) and acid sphingomyelinase-like phosphodiesterase 3a (SMPDL3A) were uniquely expressed in the infertile patients with unilateral varicocele. IGF-binding proteins (IGFBP) including IGFBP5 are extracellular proteins that prolong the half-life of the IGFs and have been

shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors. IGF-1 plays an important role in the regulation of spermatogenesis in the testes. Therefore, in fertile group the IGFBP5 is properly utilized by binding with the basal levels of IGF while it remained unutilized in case of unilateral varicocele where the IGF expression may be low [50,51]. In addition unique expression of MYH9 in unilateral varicocele patients may be attributed to the presence of dead/apoptotic fragmented spermatozoa [52]. The expression of extracellular secretory protein SMPDL3A is associated with the fertility status [53]. Therefore, the unique expression of the IGFBP5, MYH9, and SMPDL3A proteins in infertile men with unilateral varicocele may be due to defect in the spermatogenesis process.

In the present study, we demonstrated the expression of proteins associated with exosomal function to be altered in the seminal plasma of the unilateral varicocele patients. Dysregulation of exosomal function may be mainly due to the improper packaging and release of the exosomes into the seminal plasma. This further hampers the delivery of fertility-modulating proteins to the surface of spermatozoa [20]. This could also be the reason for the significant decrease of sperm motility in varicocele patients compared to healthy fertile men (Table 2). Moreover, the presence of defective exosomes in the seminal plasma results in the early capacitation and spontaneous acrosome reaction, thereby compromising the fertility status of men [54].

One of the limitations of our study was the non-inclusion of fertile men with unilateral varicocele. This would have been an ideal group to compare the alterations seen in the infertile group. However, enrolling this group is very challenging as only infertile men seek medical assistance and most of these infertile men are diagnosed with varicocele (subclinical or clinical) during their fertility evaluation. Furthermore, it is very difficult to obtain semen samples from fertile patients with unilateral varicocele as access to such patients is very limited in a clinical setting. Therefore, to understand which proteins are responsible for maintenance of fertility, we compared the DEPs in the seminal plasma of infertile men with varicocele with that of proven fertile men. Another limitation was that we did not isolate and purify the seminal plasma exosomes for proteomic analysis. As this was the first

report on this topic, we could not predict the DEPs and the networks in which they are involved in prior to conducting this study. However, the validation of exosome-associated proteins and differential expression of key proteins of the network by WB in current study validates our hypothesis. The study findings can be further validated in isolated exosomes in future.

CONCLUSIONS

This is the first report on the DEPs in the seminal plasma of infertile unilateral varicocele patients with respect to fertile healthy men without varicocele. We confirmed the presence of defective exosomal machinery in the seminal plasma of unilateral varicocele patients based on the altered expression of exosome-associated proteins (ANXA2, TF, KIF5B, and SEMG1). It is proposed that KIF5B and ANXA2, can serve as potential protein biomarkers of exosomal dysfunction, while accumulation of AGT could be predictive of enhanced sperm DNA fragmentation and impaired motility of the spermatozoa in infertile men with varicocele.

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Conflict of Interest

The authors have nothing to disclose.

Author Contribution

Conceptualization: AA. Data curation: MKPS, LS. Formal

analysis: AA, MKPS, RS, LS. Investigation: AA. Methodology: MKPS, SG. Project administration: AA. Software: MKPS, LS. Supervision: AA. Validation: MKPS, TRD, ADM. Writing—original draft: MKPS. Writing—review & editing: all authors. Approval of the final manuscript: all authors.

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