Differential Proteomic Profiling of Spermatozoal Proteins of Infertile Men With Unilateral or Bilateral Varicocele

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OBJECTIVE
To compare the sperm protein profile between infertile men with unilateral varicocele and infertile men with bilateral varicocele.

METHODS
This prospective study investigated 50 infertile patients with clinical varicocele (33 unilateral and 17 bilateral) seeking fertility workup between March 2012 and April 2014. Routine sperm parameters, reactive oxygen species, total antioxidant capacity, and sperm deoxyribonucleic acid fragmentation were assessed in their semen. Sperm protein profile was characterized only in pooled samples of 5 unilateral and 3 bilateral varicocele samples, respectively, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an Linear Trap Quadrupole-Orbitrap Elite hybrid mass spectrophotometer system. Differences in protein expression were analyzed using gel analysis software, followed by protein identification using mass spectroscopy analysis. Differentially expressed proteins and their abundance were quantified by comparing spectral counts, followed by bioinformatics analysis.

RESULTS
Unique expression of 64 proteins in the bilateral group and 31 proteins in the unilateral group was obtained. Core functions of the top protein interaction networks were post-translational modification (~122 proteins associated with acetylation), protein folding, free-radical scavenging, cell death, and survival. The top molecular and cellular functions were protein degradation, free radical scavenging, and post-translational modifications, whereas the top pathways were protein ubiquitination and mitochondrial dysfunction. Major biological pathways for the 253 differentially expressed proteins were metabolism, apoptosis, and signal transduction.

CONCLUSION
Functional proteomic profiling helps identify the differential processes or pathways that are affected based on the nature of varicocele (bilateral or unilateral) and provide insights into the mechanistic implications of varicocele-associated male infertility. UROLOGY 85: 580–588, 2015. © 2015 Elsevier Inc.

Varicoceles are found in 35%-50% of men with primary infertility and in ~80% of men with secondary infertility, suggesting a progressive decline in fertility over time. Factors that may contribute to the pathogenesis of varicocele include hypoperfusion leading to hypoxia, heat stress, oxidative stress, hormonal imbalances, and exogenous toxicants.1 However, the exact mechanism underlying varicocele-associated testicular dysfunction and infertility remains unclear.2

Proteomics is emerging as a new frontier in male infertility research and is being extensively used to investigate protein alterations in different disease states.3 Proteomic analysis has previously been used to screen for differentially expressed sperm proteins in infertile men afflicted with various etiologies including varicocele.4,7 Two-dimensional electrophoresis and MALDI-TOF/TOF-MS was used to compare the proteomic profile of oligozoospermic, grade 3 varicocele patients with that of healthy fertile men,8 and differentially expressed proteins in grade 2 varicocele patients.9 However, no proteomics-based study has as yet, to our knowledge, looked into the potential alteration in sperm protein expression within the varicocele patient pool, mainly between unilateral and bilateral varicocele patients. Unilateral varicoceles are more commonly present on the left side (35%-40%) compared with bilateral varicoceles (10%-15%). Thus, the aim of the present study was to examine and compare potential differences between the sperm protein profile within a subset of varicocele patients, that is, men with unilateral varicocele with that of bilateral varicocele patients.
**METHODS**

**Patients and Semen Samples**
On study approval by the institutional review board, semen samples were collected from 50 patients with infertility as a primary diagnosis and clinical varicocele (unilateral, n = 33 and bilateral, n = 17) as a secondary diagnosis. All participating patients were examined, and their varicocele(s) were diagnosed.10 Axospermic men and men with a sperm concentration of <10 million sperm/mL were excluded from the present study. After liquefaction, manual semen analysis was performed to determine sperm concentration and motility, and smears were prepared for assessment of sperm morphology according to the World Health Organization criteria.11 Samples with a round cell concentration of >1 × 10⁶/mL were tested for leucocytes or white blood cells/mL and confirmed by the Endtz test.5 Samples positive for leucocytes were excluded from the final proteomic pool. Reactive oxygen species (ROS) formation was measured by chemiluminescence assay using a Berthold luminometer (Autolumat Plus 953, Berthold Technologies, Oakland, TN), as described previously.12 Total antioxidant capacity was measured in the seminal plasma using the antioxidant assay kit.6

**Sperm Protein Extraction and In-gel Digestion**
Only samples with sufficient sperm concentration after running the ROS and sperm DNA fragmentation tests could be considered for pooling. We therefore selected samples for pooling from 5 of the 33 unilateral and 3 of the 17 bilateral varicocele patients who met the criteria. After complete digestion, they were extracted from the polyacrylamide gel electrophoresis 1-dimensional gel electrophoresis. Samples were assayed in triplicate for global proteomic analysis and quantified using the label-free spectral counting method. The bands were reduced with dithiothreitol and alkylated with iodoacetamide before the in-gel digestion using trypsin.

**Liquid Chromatography—Mass Spectrometer**
After complete digestion, they were extracted from the polyacrylamide in 2 aliquots of 30-μL 50% acetonitrile with 5% formic acid. The combined extracts were concentrated to <10 μL and resuspended in 1% acetic acid to a final volume of ~30 μL for liquid chromatography—mass spectrometer (LC-MS) analysis. The LC-MS system was a Finnigan Linear Trap Quadropole-Orbitrap Elite hybrid mass spectrometer system (Thermo Fisher Scientific, Waltham, MA). The high performance liquid chromatography column was a Dionex 15 cm × 75 μm internal diameter Acclaim Pepmap C18, 2 μm, 100 A reversed-phase capillary chromatography column. Five microliter of the extract was injected, and the peptides eluted from the column were introduced into the MS. The digest was analyzed to determine peptide molecular weights and tandem mass spectra (MS/MS) to determine amino acid sequence in successive instrument scans.

**Database Searching and Protein Identification Criteria**
Tandem mass spectra were extracted by Proteome Discoverer, version 1.4.1.288 (Thermo Fisher Scientific Inc., Philadelphia, PA). Charge-state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (version 2.3.02; Matrix Science, London, United Kingdom), SEQUEST (version 1.4.0.288; Thermo Fisher Scientific, San Jose, CA) and X! Tandem (version CYCLONE; 2010.12.01.1; The GPM; thegpm.org).

To validate MS/MS–based peptide and protein identifications, Scaffold (version 4.0.6.1; Proteome Software Inc., Portland, OR) was used. Peptide and protein identifications were accepted as previously described.13 Protein probabilities were assigned by the ProteinProphet (Systems Biology, Seattle, WA) algorithm.15 Proteins were annotated with Gene Ontology (GO) terms.16

**Quantitative Proteomics**
For proteomic analysis, the relative quantity of the proteins was determined by comparing the number of spectra used to identify each protein. The total number of mass spectra that identified a particular protein (spectral counts [SpCs]) was used to measure the relative abundance of proteins in the samples being compared. Normalization of SpCs using the normalized spectral abundance factor (NSAF) approach was applied to each group separately before relative protein quantification.17 Differentially expressed proteins (DEP) were obtained by applying different constraints for significance tests and/or fold-change cutoffs based on the average SpCs of the protein from multiple runs.

Appropriate filters were used to identify DEP that were dependent on the overall abundance of the proteins.18 The abundance of the proteins was classified as high, medium, low, or very low based on their average SpCs among the 3 replicate runs. Different constraints for significance tests (P value) and/or fold-change cutoffs (or NSAF ratio) were applied for these 4 abundance categories, as shown in the following:

**Very Low.** SpC range, 1.7-7; P ≤ .001; and NSAF ratio (≥2.5 for upregulated and ≤0.4 for downregulated proteins).

**Low.** SpC range, 8-19; P ≤ .01; and NSAF ratio (≥2.5 for upregulated and ≤0.4 for downregulated proteins).

**Medium.** SpC range, between 20 and 79; P ≤ .05; and NSAF ratio (≥2.0 for upregulated and ≤0.5 for downregulated proteins).

**High.** SpC, >80; P ≤ .05; and NSAF ratio (≥1.5 for upregulated and ≤0.67 for downregulated proteins).

**Bioinformatics Analysis**
Functional annotation and enrichment analysis were performed using GO Term Finder19; GO Term Mapper; UniProt; Software for Researching Annotations of Proteins20; and Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.niaid.nih.gov). Ingenuity pathway analysis (IPA; Ingenuity Systems) was used to obtain consensus-based, comprehensive, functional context for the large list of proteins derived in this study.

**RESULTS**

**Patient Characteristics**
Patient characteristics and results of semen analysis and sperm advanced tests for varicocele patients in this study are shown in Supplementary Table 1. The majority of infertile patients had a left-sided varicocele (94%), mostly of grade 1 or 2 (83.6%). Infertile patients with bilateral varicocele had a higher grade on the left compared with the right (mostly grade 1). Semen parameters in both varicocele groups were
not significantly different, except for a higher sperm DNA fragmentation in the bilateral group. ROS levels in both varicocele groups were higher than the established reference levels of 93 relative light units/s/10^6 sperm. Comparisons between samples from men with varying varicocele grades were not feasible because of insufficient sample size.

**Proteomic Profiling**

For the global proteomic profiling analysis, each pooled sample from the unilateral and bilateral varicocele group was run in triplicate. The total number of proteins identified from these 3 runs, respectively, was 795, 713, and 763 proteins in the unilateral (Supplementary Table 2A-C) and 1024, 999, and 1017 proteins in the bilateral (Supplementary Table 3A-C) varicocele group. Based on the normalized SpCs, some of the more abundant proteins in both varicocele groups were lactotransferrin isoform 1 precursor (LTF), fibronectin isoform 3 preprotein, semenogelin-2 precursor (SEMG2), A-kinase anchor protein 4 isoform 2 (AKAP4), and semenogelin-1 preprotein (SEMG1).
Identification and Classification of Differentially Expressed Proteins
A total of 253 DEP that fit the filtering criteria (high, medium, low, and very low abundance) were identified (Fig. 1A). Thirty-one DEP were unique to the unilateral group (7 medium, 19 low, and 5 very low abundance), whereas 64 DEP were unique to the bilateral group (46 low and 18 very low abundance). One hundred fifty-eight DEP were overlapping proteins, which could be either overexpressed or underexpressed in each varicocele group. One hundred thirteen proteins were overexpressed (19 high, 74 medium, and 20 low abundance), whereas 45 proteins were underexpressed (13 high, 22 medium, and 4 low abundance) in the unilateral compared with the bilateral group (Fig. 1B) and distribution of protein abundance in each varicocele group (Fig. 1C). DEP classification by cellular location, molecular functions, and biological processes (GO Term Mapper) (Fig. 1D1-D3), and the major biological pathways in 151 proteins (from the 253 DEP) that were assigned to the Reactome database are in Figure 2.

DEP Participation in Top Networks and Pathways
The top networks of the DEP identified by IPA and DAVID in our study and their possible roles in various functions and their subcellular localization is shown in Figure 3A,B.

A total of 32 DEP (4 nuclear, 26 cytoplasmic, and 2 subcellular proteins) were involved in the topmost network with core functions of post-translational modification (PTM) and protein folding (Fig. 3A), in which 17 were overexpressed and 15 underexpressed in the unilateral compared with the bilateral group. A total of 35 DEP (2 nuclear, 28 cytoplasmic, 4 plasma membrane, and 1 extracellular protein) were identified in the topmost network with core functions of cell death and survival, free-radical scavenging, and small-molecule biochemistry (Fig. 3B), of which 1 was overexpressed and 32 were underexpressed in the unilateral compared with the bilateral group.

DEP Relevant to Spermatogenesis
Proteins annotated with distinct functional categories related to male reproduction and/or spermatogenesis including spermatogenesis, spermatid development and differentiation, male gamete generation, binding of sperm to zona pellucida, sperm-egg recognition, reproductive cellular processes, and sexual reproduction were also identified. Table 1 shows the 21 DEP that may play a role in varicocele or male infertility—related functions and the UniProt number, gene name, protein name, and spermatogenic/reproductive function of these proteins as obtained from UniProt database and Software for Researching Annotations of Proteins annotation tool. Based on the 21 DEP, we further narrowed down 7 proteins that exhibited >40% peptide coverage in at least 2 of the 3 runs, as these are likely candidate proteins of interest. The 7 genes encoding these proteins are GSTM3,
Enriched functional categories of the differentially expressed proteins (DEP). The top pathways and/or molecular and cellular functions or networks analysis using ingenuity pathway analysis revealed the enriched functional categories for the DEP were (but were not limited to) — the post-translational modification, protein folding, protein ubiquitination, free-radical scavenging, lipid and nucleic acid metabolism, small molecule biochemistry, mitochondrial dysfunction, tricarboxylic acid cycle, and acetyl CoA biosynthesis. Top disease and function networks and involvement of DEP are shown in (A) post-translational modification and protein folding, and in (B) cell death and survival, free-radical scavenging, and small molecule biochemistry. In each network, underexpressed DEP are shown in green, whereas overexpressed DEP are shown in red in unilateral compared with the bilateral varicocele group. The gradation of color reflects their intensity or abundance of expression (eg, brighter the red, the larger the protein expression). The abundance of a DEP is based on the normalized spectral count obtained from the protein profiling, whereas the overexpression or underexpression of proteins is based on gene ontology analysis. (Color version is available online.)
<table>
<thead>
<tr>
<th>No.</th>
<th>Uniprot Number</th>
<th>Gene Name</th>
<th>Protein Name</th>
<th>Expression</th>
<th>Unilateral Varicocele Group Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Bilateral Varicocele Group Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Function</th>
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<tr>
<td>1</td>
<td>P21266</td>
<td>GSTM3</td>
<td>Glutathione S-transferase Mu 3</td>
<td>OE</td>
<td>15/58 15/55 17/57</td>
<td>16/58 19/76 18/69</td>
<td>Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. May govern uptake and detoxification of both endogenous compounds and xenobiotics at the testis- and brain-blood barriers.</td>
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<td>3</td>
<td>Q6BCY4</td>
<td>CYB5R2</td>
<td>NADH-cytochrome b5 reductase 2</td>
<td>OE</td>
<td>10/47 7/37 9/44 6/31 8/46 6/30</td>
<td>Involved in desaturation and elongation of fatty acids, cholesterol biosynthesis, drug metabolism, and in erythrocyte, methemoglobin reduction.</td>
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<td>5</td>
<td>Q8IZP9</td>
<td>GPR64</td>
<td>G-protein coupled receptor 64</td>
<td>OE</td>
<td>12/14 11/13 10/12 7/9.4 7/10 7/10</td>
<td>Could be involved in a signal transduction pathway controlling epididymal function and male fertility.</td>
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<td>6</td>
<td>O14967</td>
<td>CLGN</td>
<td>Calmegin</td>
<td>OE</td>
<td>8/22 6/15 5/9.3 2/3.4 3/6.1 2/6.2</td>
<td>Functions during spermatogenesis as a chaperone for a range of client proteins that are important for sperm adhesion onto the egg zona pellucida and for subsequent penetration of the zona pellucida.</td>
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<td>7</td>
<td>Q99497</td>
<td>PARK7</td>
<td>Protein DJ-1</td>
<td>OE</td>
<td>7/44 7/43 5/39 5/22 10/65 11/74</td>
<td>Protects cells against oxidative stress and cell death.</td>
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<td>8</td>
<td>Q8TAA3</td>
<td>PSMA8</td>
<td>Proteasome subunit alpha type-7–like</td>
<td>OE</td>
<td>13/65 12/57 11/53 7/41 7/43 8/43</td>
<td>Component of the spermatoproteasome, a form of the proteasome specifically found in the testis that promotes degradation of histones, thereby participating actively to the exchange of histones during spermatogenesis.</td>
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<td>10</td>
<td>P09622</td>
<td>DLD</td>
<td>Dihydrolipoyl dehydrogenase, mitochondrial</td>
<td>OE</td>
<td>26/60 25/60 28/65 20/55 16/45 16/47</td>
<td>Lipoamide dehydrogenase is a component of the glycine cleavage system as well as of the alpha-ketoacid dehydrogenase complexes. Involved in the hyperactivation of spermatozoa during capacitation and in the spermatozoal acrosome reaction.</td>
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<td>No.</td>
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<td>Expression</td>
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<td>Bilateral Varicocele Group</td>
<td>Function</td>
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<tr>
<td>11</td>
<td>Q8TC29</td>
<td>ENKUR</td>
<td>Enkurin</td>
<td>UE</td>
<td>2/12 N/A N/A</td>
<td>7/38 8/48 6/38</td>
<td>Adapter that functions to localize a calcium-sensitive signal transduction machinery in sperm to a calcium-permeable ion channel.</td>
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<td>13</td>
<td>P49327</td>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>UE</td>
<td>46/16 41/16 45/13</td>
<td>75/28 57/26 72/26</td>
<td>Catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH.</td>
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<td>14</td>
<td>O75952</td>
<td>CABYR</td>
<td>Calcium-binding tyrosine phosphorylation-regulated protein</td>
<td>UE</td>
<td>4/22 6/9.8 10/26</td>
<td>20/62 23/66 23/69</td>
<td>Functions as a regulator of both sperm motility and sperm head-associated functions such as capacitation and the acrosome reaction.</td>
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<td>15</td>
<td>P12277</td>
<td>CKB</td>
<td>Creatine kinase B-type</td>
<td>UE</td>
<td>15/39 14/39 15/45</td>
<td>19/68 18/68 21/62</td>
<td>Reversibly catalyzes the transfer of phosphate between ATP and various phosphagens.</td>
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<td>16</td>
<td>P04279</td>
<td>SEMG1</td>
<td>Semenogelin-1</td>
<td>UE</td>
<td>41/62 36/64 40/66</td>
<td>70/77 64/73 73/72</td>
<td>Contributes to the activation of progressive sperm movements as the gel-forming proteins are fragmented by KLK3/PSA.</td>
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<td>17</td>
<td>Q02383</td>
<td>SEMG2</td>
<td>Semenogelin-2</td>
<td>UE</td>
<td>23/47 22/49 26/51</td>
<td>51/65 46/70 50/74</td>
<td>Participates in the formation of a gel matrix (sperm coagulum) entrapping the accessory gland secretions and ejaculated spermatzoa.</td>
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<td>18</td>
<td>P16562</td>
<td>CRISP2</td>
<td>Cysteine-rich secretory protein 2</td>
<td>Unilateral only</td>
<td>4/16 5/21 7/38</td>
<td>N/A N/A N/A</td>
<td>Regulates some ion channels’ activity and calcium fluxes during sperm capacitation.</td>
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<td>19</td>
<td>P78540</td>
<td>ARG2</td>
<td>Arginase-2, mitochondrial</td>
<td>Unilateral only</td>
<td>7/28 7/27 7/28</td>
<td>N/A N/A N/A</td>
<td>Plays a role in the regulation of extracellular arginine metabolism and also in the downregulation of nitric oxide synthesis.</td>
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<tr>
<td>20</td>
<td>P46926</td>
<td>GNPDA1</td>
<td>Glucosamine-6-phosphate isomerase 1</td>
<td>Unilateral only</td>
<td>10/44 5/22 5/22</td>
<td>N/A N/A N/A</td>
<td>May trigger calcium oscillations in mammalian eggs. These oscillations serve as the essential trigger for egg activation and the early development of the embryo.</td>
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<tr>
<td>21</td>
<td>Q9NQ60</td>
<td>EQTN</td>
<td>Equatorin</td>
<td>Bilateral only</td>
<td>N/A N/A N/A</td>
<td>N/A N/A N/A</td>
<td>Acrosomal membrane-anchored protein involved in the process of fertilization and in acrosome biogenesis.</td>
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ATP, adenosine triphosphate; CoA, coenzyme A; KLK3/PSA, kallikrein 3/prostate-specific antigen; N/A, not available; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); OE, overexpressed; UE, underexpressed.
In the present study, we found no significant differences in semen parameters between unilateral and bilateral varicocele patients. This emphasizes the well-established fact that routine semen parameters are poor diagnostic markers of infertile men, particularly in those with varicoceles. Elevated ROS levels in both groups indicate increased oxidative stress in men with varicocele. ROS levels were higher in the bilateral compared with the unilateral group, although not significant. Increased DNA fragmentation levels in the bilateral compared with the unilateral group suggest a progressive decline in DNA integrity of bilateral varicocele patients. Increased oxidative stress is associated with high DNA damage both in men with normal and abnormal semen parameters.

The fact that varicoceles are associated with male subfertility is widely accepted, but the exact mechanism remains unclear. Sheehan et al. proposed that molecular and genetic differences could exist among varicocele patients, and these differences may determine whether or not varicocele is associated with male infertility. Although distinct differences have been reported in protein expression in infertile men with and without varicocele, the protein profile in infertile men with unilateral or bilateral varicocele is lacking. Our laboratory has recently examined and compared semen parameters, global protein profiles, and DEP in men of proven fertility and infertile men with varicoceles (unpublished). Thus, to better comprehend the underlying pathology and severity of disease, we compared sperm protein alterations in unilateral and bilateral varicocele patients in this study. We used SDS-PAGE for protein fractionation and high resolution LC–MS/MS analysis on an Linear Trap Quadrupole-Orbitrap Elite instrument, designed to deliver a high resolution and mass accuracy.

Using genome-wide protein profiling analysis, we have identified ~750 proteins in the unilateral and >1000 proteins in the bilateral varicocele group. Of these proteins, 253 were differentially expressed (overexpressed or underexpressed or uniquely expressed) in either group, which suggests their possible involvement in the etiology of varicocele. Identification of a lower number of proteins in the unilateral compared with bilateral group may be due to the presence of low-abundance proteins, which are associated with increased variability due to the complex nature of samples and run-to-run variability of the LC–MS/MS experiment.

In our study, the localization and functional analyses of majority of DEP identified many intracellular proteins (Fig. 1D1), which verifies their origin from spermatozoa and not seminal plasma. DEP were depicted as participating in various biological processes, like small–molecule metabolic process, cellular nitrogen compound metabolic processes, response to stress, biosynthetic process, and signal transduction processes (Fig. 1D2). We found that the DEP’s major molecular functions consisted of ion binding, oxidoreductase and peptidase activity, and enzyme binding and regulator activity (Fig. 1D3). We can therefore hypothesize that alterations in expression levels of these enzymatically involved proteins could affect enzyme kinetics. It could also be presumed that altered proteins in varicocele patients, responsible for ion binding and oxidoreductase activity, will cause changes in cell defense against oxidative or heat stress.

Reactome software analysis dissected the function of about 60% (151 of 253) of DEP. Metabolism, disease, immune system, gene expression, signal transduction, and apoptosis were the major pathways associated with the unilateral varicocele group (Fig. 2). IPA and DAVID functional annotation analysis showed that PTM and protein folding were severely compromised. Top molecular and cellular functions identified by IPA were protein degradation and synthesis, free-radical scavenging, PTM, and protein folding. The top pathways identified were protein ubiquitination, aldosterone signaling, and mitochondrial function. Proteins in molecular interaction networks (Fig. 3A,B) show that majority of DEP were intracellular (Fig. 1D1).

There seems to be very little overlap in proteins reported in studies comparing men with and without varicocele and those reported in the present study. Hosseinifar et al. reported that PARK7 and SEMG2 are underexpressed in the varicocele group compared with healthy controls. In our study, PARK7 was overexpressed and SEMG2 was underexpressed in the unilateral compared with the bilateral group. This may reflect the severity of bilateral varicoceles. The study by Camargo et al. compared the protein profile of varicocele patients before and after varicocelectomy. PARK7 was expressed postvaricocelectomy, whereas SEMG1 and SEMG2 levels decreased postvaricocelectomy. Our study shows that PARK7 is overexpressed and SEMG1 and SEMG2 are underexpressed in the unilateral compared with the bilateral group. This may again be indicative of the aggressive nature of bilateral varicoceles and its detrimental effects on sperm quality and in the pathogenesis of varicocele. Chan et al. had found increased expression of heat shock proteins HSP70 and HSP90 in varicocele patients compared with controls, whereas Hosseinifar et al. subsequently studied the effect of varicocelectomy on SOD1, ATP5D, and HSPA5 expression. In our study, none of these proteins were differentially expressed.

Studies describing the differential expression of proteins exclusively in the unilateral or bilateral varicocele are lacking. For the first time, our study provides an important platform that highlights the distinct differences seen in expression of proteins between the unilateral and the bilateral groups. Of the 253 DEP, 21 proteins were involved in reproductive functions such as motility, capacitation, hyperactivation, acrosome reaction, and zona pellucida binding (Table 1). DEP that are overexpressed in the unilateral or underexpressed in the bilateral group—GSTM3, SPANX1, CYB5R2, CALGN, or PARK7—reflect the severity of
varicocele and its detrimental effect on the sperm parameters (Table 1). Similarly, underexpressed proteins in the unilateral or overexpressed proteins in the bilateral group—ENKUR, SEMG1, SEMG2, SPAM1, or CABYR—are suggested of the compromised semen quality in men with bilateral varicocele (Table 1). These findings have important clinical relevance for the clinicians in further evaluating the pathogenesis of varicocele and in understanding that bilateral varicocele has more damaging effects that may require both unilateral as well as bilateral varicocelectomy to help revert the expression of these proteins. One study limitation was that the semen samples used in our study were not subjected to swim up during sperm preparation and thus may have contained a small amount of round cells. Another study limitation was the absence of protein validation of DEP as candidate markers, by Western blot, enzyme-linked immunosorbant assay, or immunochemistry. Validation of major functional proteins present exclusively in the unilateral or bilateral group will help in establishing their role as potential biomarkers in determining disease severity. Although we did not include a control group without varicocele in our study, it would be very interesting to investigate how expression of these proteins is affected after unilateral or bilateral varicocelectomy, as this would significantly increase the protein validity as potential biomarkers.

CONCLUSION
In this study, for the first time, we compared the global protein profiles of unilateral varicocele with bilateral varicocele and identified 21 DEP participating in reproductive function. All these proteins are likely candidates for potential biomarkers and need to be further validated. The list of shortlisted proteins for validation would be the 8 genes that encode the proteins GSTM3, SPANXB1, PARK7, PSMA8, DLD, SEMG1, and SEMG2. Another good starting point would be genes encoding proteins exclusive to the unilateral or the bilateral group. Candidate proteins of interest will serve as potential markers of unilateral varicocele-related infertility and aid urologists in providing effective solutions and a strategic rationale in the selection of patients who would most benefit from varicocelectomy.

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References

APPENDIX
Supplementary Data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.urology.2014.11.030.