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Proteomic Analysis of Seminal Plasma from Normal Volunteers and Post-Vasectomy Patients Identifies over 2000 Proteins and Candidate Biomarkers of the Urogenital System

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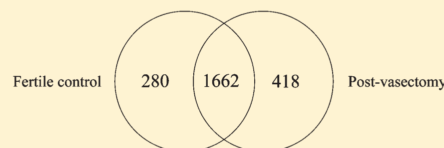
S *Supporting Information*

ABSTRACT: Seminal plasma is a fluid that originates from the testis, epididymis, prostate, and seminal vesicles, and hence, proteomic studies may identify potential markers of infertility and other diseases of the genito-urinary tract. We profiled the proteomes of pooled seminal plasma from fertile Control and post-vasectomy (PV) men. PV seminal plasma samples are void of proteins originating from the testis and the epididymis due to ligation of the vas deferens, and hence, comparative analysis of Control and PV data sets allows for identification of proteins originating from these tissues. Utilizing offline MudPIT and high-resolution mass spectrometry, we were able to identify over 2000 proteins in Control and PV pools each and over 2300 proteins all together. With semiquantitative analysis using spectral counting, we catalogued 32 proteins unique to Control, 49 at lower abundance in PV, 3 unique to PV, and 25 at higher abundance in PV. We believe that proteins unique to Control or at lower abundance in PV have their origin in the testis and the epididymis. Public databases have confirmed that many of these proteins originate from the testis and epididymis and are linked to the reproductive tract. These proteins may serve as candidate biomarkers for future studies of infertility and urogenital diseases.



KEYWORDS: seminal plasma, vasectomy, proteomics, infertility, testis, epididymis

The analysis of complex protein mixtures such as these found in tissues, cultures, and bodily fluids has recently become an attractive area of research due to advances in mass spectrometry and other proteomics-based techniques. Through the use of tandem mass spectrometry, it is now possible to obtain reproducible fragments of peptides that can be used to characterize proteins present in a sample using the so-called "bottom-up" approach. This technology allows for identification of hundreds to thousands of proteins, spanning several orders of magnitude in abundances and in a variety of complex fluids.

When applied to the analysis of seminal plasma, mass spectrometry is capable of identifying proteins present in the ejaculate, making it a valuable tool when searching for potential clinical biomarkers of the urogenital tract. This has relevance in the diagnosis, monitoring, and treatment of diseases of the male reproductive tract, such as infertility, prostatitis, and cancer of the prostate or testis.

Seminal plasma is the liquid surrounding the spermatozoa, providing a protective, nourishing environment.[1] It is composed of fluids secreted from the seminal vesicles (65%), prostate (25%), testes and epididymis (10%), and the periurethral glands (minimal).[2] Using two-dimensional gel electrophoresis as well as mass spectrometry (MS), several high-abundance proteins in human seminal plasma have been identified. These include common matrix and adhesion proteins such as fibronectin, semenogelin I, semenogelin II, and laminin, as well as enzymes such as prostate-specific antigen (PSA), prostatic specific acid phosphatase (PSAP), and creatine kinase.[3,4] Additionally, antimicrobial proteins such as lactoferrin, protease inhibitors such as α-1-antitrypsin, and transport proteins such as albumin are also abundant in the semen.[4,3] More recently, there have been several studies that used mass spectrometry to investigate the proteome of seminal plasma. In MS-based studies of seminal plasma Fung et al.[5] identified over 100 protein and peptide components in seminal fluid; Pilch et al.[1] identified 923 proteins in seminal plasma from one healthy individual of unknown fertility status; Yamakawa et al.[6] identified up to 501 polypeptide spots in seminal plasma from fertile men; and Wang et al.[7] identified 625 proteins in seminal plasma from fertile men.

Determining the specific tissue of origin of individual proteins identified in the ejaculate remains a challenging task. If seminal plasma proteins are to potentially act as markers for diseases of the reproductive tract (such as prostate cancer, testicular cancer, prostatitis, benign prostatic hypertrophy, and infertility), it is

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important to begin to understand the origins of the seminal proteins from within the reproductive tract. Using methods such as ELISA and immunohistochemistry, the tissue of origin of high-abundance proteins such as fibronectin, semenogelin I, semenogelin II, and lactoferrin was determined to be from the seminal vesicles.[8] Such methods are impractical for localizing the potentially thousands of lower-abundance proteins of the seminal plasma.

Our goal was to develop complementary methods that could eventually lead to identification of the tissue of origin of the vast majority of seminal plasma proteins. By using a shotgun proteomic approach, we attempt to compare the proteomes of seminal plasma of normal fertile Controls and men who have undergone a vasectomy. This would allow proteins to be distinguished on the basis of whether they originate proximal or distal to the site of ligation of the vas deferens. Post-vasectomy seminal plasma should be void of proteins originating from the testis and epididymis but will contain proteins derived from the prostate, seminal vesicles, and periurethral glands. Seminal plasma samples of five individuals from each of the two diagnostic groups were pooled together, in an attempt to account for variations between the protein composition of individual samples.

■ MATERIALS AND METHODS

Sample Collection and Processing

Semen from fertile men (Controls) and post-vasectomy men (PV) was collected after a minimum of 3 days of sexual abstinence. Samples were allowed to liquefy for 2−3 h at room temperature and centrifuged at 13,000*g* for 10 min. The supernatant (seminal plasma) was aliquoted into 1.5 mL Eppendorf tubes and stored at −80 °C until further analysis. Total protein concentration was measured using the Biuret assay and ranged between 26 and 55 mg/mL. Five Control and five post-vasectomy (PV) seminal plasma samples were combined to make "Control" and "PV" pools, such that each sample contributed an equivalent amount of protein to the 3 mg of total protein pool. Many aliquots of such pools were prepared to allow for repeated analysis, as necessary (see below).

Trypsin Digestion

Three Control and three PV pools were denatured with 8 M urea (7 M final), reduced with 200 mM dithiothreitol (15 mM final, Sigma) for 30 min at 50 °C, and alkylated with 500 mM iodoacetamide (125 mM final, Sigma) in the dark, on a shaker at room temperature for 60 min. Next, samples were desalted using PD-10 columns (GE Healthcare) in 50 mM ammonium bicarbonate, frozen, and partially lypohilized to reduce sample volume. The samples were left to digest overnight at 37 °C by addition of 400 μL of 50 mM ammonium bicarbonate (pH 8), 60 ug of sequencing grade modified porcine trypsin (1:50 trypsin/protein concentration, Promega), and 200 μL of methanol. To stop the digestion in the morning, the samples were acidified to pH 2 with 20 μL of formic acid.

Strong-Cation Exchange Liquid Chromatography

Each of the trypsin-digested Control and PV samples was diluted 2-fold to 2.5 mL with mobile phase A (0.26 M formic acid in 10% acetonitrile) and loaded onto a strong-cation exchange (SCX) PolySULFOETHYL A column (The Nest Group, Inc.) connected to an Agilent 1100 HPLC system. A 60 min method with an increasing mobile phase B (1 M ammonium formate, 0.26 M formic acid in 10% acetonitrile) gradient at a flow of 200 μL/min was used to elute the peptides. The peptide elution profile was monitored with absorbance at 280 nm, and fractions were collected every minute, resulting in 60 fractions. On the basis of the absorbance elution profile, fractions 26−30 as well as 50−54 were pooled together, whereas fractions 31−49 were stored individually, all at −80 °C.

Mass Spectrometry

A total of 21 SCX fractions from each of the three Control and PV pools were desalted and preconcentrated using the Omix C18MB (Varian Inc.) tips and eluted with 5 μL of buffer A (0.1% formic acid and 0.02% trifluoroacetic acid in 65% acetonitrile). To each sample, 80 μL of buffer B (0.1% formic acid and 0.02% trifluoroacetic acid in 5% acetonitrile) was added, of which 40 μL was loaded from a 96-well microplate autosampler onto a C_{18} trap column using the EASY-nLC system (Proxeon Biosystems, Odense, Denmark) and running Buffer C (0.1% formic acid in water). The trap column consisted of IntegraFrit capillary (inner diameter 150 μm, New Objective) cut to 3 cm in length and packed in-house with 5 μm Pursuit C_{18} (Varian Inc.). Peptides were eluted from the trap column with an increasing concentration of Buffer D (0.1% formic acid in acetonitrile) onto a resolving 5 cm long PicoTip Emitter (75 μm inner diameter, 8 μm tip, New Objective) packed in-house with 3 μm Pursuit C_{18} (Varian Inc.). Pooled fractions 26−30 and individual fractions 31−39 were subjected to an 88 min liquid chromatography gradient, whereas fractions 40−49 and pooled 50−54 were analyzed with a 55 min gradient at a flow of 400 nL/min. This liquid chromatography setup was coupled online to LTQ-Orbitrap XL (Thermo Fisher Scientific, San Jose, CA) mass spectrometer using a nanoelectrospray ionization source (Proxeon Biosystems, Odense, Denmark) with capillary temperature set to 160 °C and spray voltage of 2 kV. The full MS^1 scan from 450 to 1450 m/z was acquired in the Orbitrap at a resolution of 60,000 with subsequent MS^2 scans on the top six parent ions in the linear ion trap (LTQ) in data-dependent mode. Dynamic exclusion, monoisotopic precursor selection and charge state screening were enabled. Unassigned charge states as well as charges +1 and ≥+4 were rejected from MS^2 fragmentation.

Data Analysis

XCalibur RAW files were uploaded into Mascot Daemon (v. 2.2), and Mascot Generic Files (MGF) were generated using extract_msn with the following parameters: minimum mass, 300 Da; maximum mass, 4000 Da; automatic precursor charge selection; minimum peaks, 10 per MS/MS scan for acquisition; and minimum scans per group, 1. MGF files were then searched with Mascot (Matrix Science, London, U.K.; version 2.2) and X!Tandem (Global Proteome Machine Manager, version 2006.06.01) against the nonredundant IPI.Human v.3.54 database containing 75,426 forward and 75,426 reverse protein sequences. Data was searched with one missed cleavage allowed, fixed carbamidomethylation of cysteines, and the following variable modifications: oxidation of methionines, deamidation of asparagines and glutamines, cyclization of N-terminal glutamines and glutamic acids (pyro-Glu), and protein N-terminal acetylation. A parent tolerance of 7 ppm and fragment tolerance of 0.4 Da were used for both search engines with trypsin as the digestion enzyme. The resulting Mascot DAT and X!Tandem XML search result files were all loaded into Scaffold (version 2.0, Proteome Software Inc., Portland, Oregon) with "MudPIT" (multidimensional protein identification technology) and "Thinning on" options checked. Scaffold result data was filtered using the X!Tandem LogE filter and Mascot ion-score filters in order to achieve a protein false-positive rate (FPR) of ∼1.1−1.5%. Scaffold protXML reports were exported and uploaded into

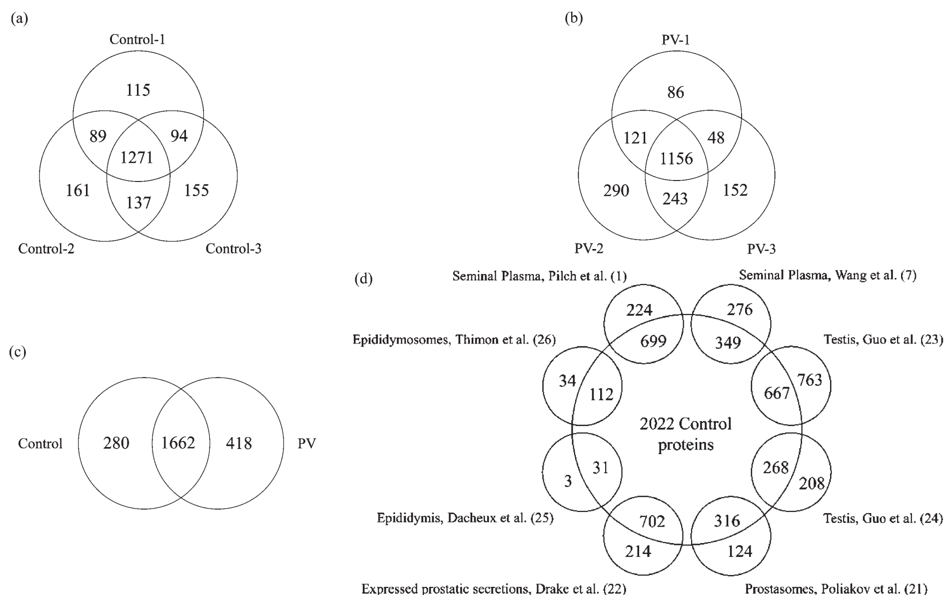

Figure 1. Venn diagram showing overlap of proteins identified among triplicate (a) Control seminal plasma samples, in total 2022 proteins; (b) post-vasectomy (PV) seminal plasma samples, in total 2096 proteins; (c) overlap of proteins between Control and PV pools, in total 2360 proteins. (d) Protein overlap between Control seminal plasma and other studies of fluids and tissues from the reproductive tract.

Protein Center (Proxeon Biosystems, Odense, Denmark) to retrieve Genome Ontology annotations.

RESULTS

Sample Preparation and LC−MS Analysis

Collected ejaculate samples from healthy Controls and post-vasectomy men were allowed to liquefy. Knowing that seminal plasma has high proteolytic activity resulting in clot liquefaction and postclot protein degradation,[9−11] we decided to work with liquefied seminal plasma without addition of protease inhibitors as it may be impractical and difficult to arrest or control proteolysis in a routine clinical setting. Despite the presence of endo- and exopeptidases, which can cleave proteins in seminal plasma during and post liquefaction, trypsinization of these cleaved proteins would still yield terminal and internal peptides, respectively. Complete protein cleavage at multiple sites beyond which fully tryptic peptides could no longer be obtained is possible, although unlikely, mostly for very small proteins. For this reason, we believe that by working with samples that have been subjected to peptidase activity, we missed very few proteins, if any, and were able to generate a list of proteins that were to a large extent resistant to cleavage. A control protein, KLK3 (PSA), was measured using ELISA in Control and PV pools, as well as individual patient samples. The concentrations were found to be 1.11 and 1.59 mg/mL in Control and PV pools, respectively, and 0.79 mg/mL (mean, n = 12) in Control and 1.48 mg/mL (mean, n = 8) in PV individual samples. This agrees well with a previously published seminal plasma range of 0.4−3.0 mg/mL.[12] Since KLK3 is secreted by the prostate gland, its concentrations should be similar in Control and PV samples. To account for interindividual variability, samples from five patients of the same diagnostic group were pooled together and then divided into three replicates, each containing 3 mg of protein. Following digestion, each replicate was fractionated using strong-cation exchange liquid chromatography to simplify the complexity of the peptide mixture, thereby increasing the depth of peptide identification. Control and PV groups were analyzed in triplicate in order to gauge reproducibility and increase protein identification and prediction confidence. The first 10 fractions were analyzed with an 88 min gradient versus 55 min for the remaining 11 fractions, as the former had greater sample complexity resulting in more peptide identifications.

Data Analysis

Data was searched with Mascot and X!Tandem since use of multiple search engines increases protein sequence coverage and number of protein identifications. Search results from the two search engines were merged using Scaffold 2.0 in MudPit mode for analysis and visualization.[13] Mascot and X!Tandem filter settings were adjusted for individual Control and PV groups to achieve an FPR of 1.1−1.5%. FPR was computed as $2 \times FP/(TP+FP)$, where FP (false positive) is the number of proteins matching the reverse database and TP (true positive) is the number of proteins matching the forward database.[14,15] A triplicate sample data set corresponding to Control and PV clinical diagnostic groups was uploaded into Scaffold and normalized according to the number of spectra in each sample. Cellular component and protein function from Genome Ontology for each clinical category was retrieved using Protein Center. Since one protein may have multiple functions and can be localized in several cellular compartments, many of the proteins have been annotated to more than one classification within the Genome Ontology.

Proteins Identified in the Control and PV Groups

We identified 1657, 1658, and 1569 proteins in each replicate belonging to the Control group, with a total of 2022 proteins (including 15 proteins that matched reverse sequences) at FPR of 1.5% (Figure 1a, Supplementary Table 1). Genome Ontology analysis shows that the majority of proteins have cytoplasmic, followed by membrane, extracellular, and nuclear origin (Figure 2a). The largest percentages of proteins are implicated in binding followed by catalytic function (Figure 3). These annotations agree well with the results of Pilch et al., where the top origin was cytoplasmic and top function was assigned to binding proteins.[1]

In triplicate analysis of the PV group we found a total of 2096 proteins (including 12 proteins that matched reverse sequences)

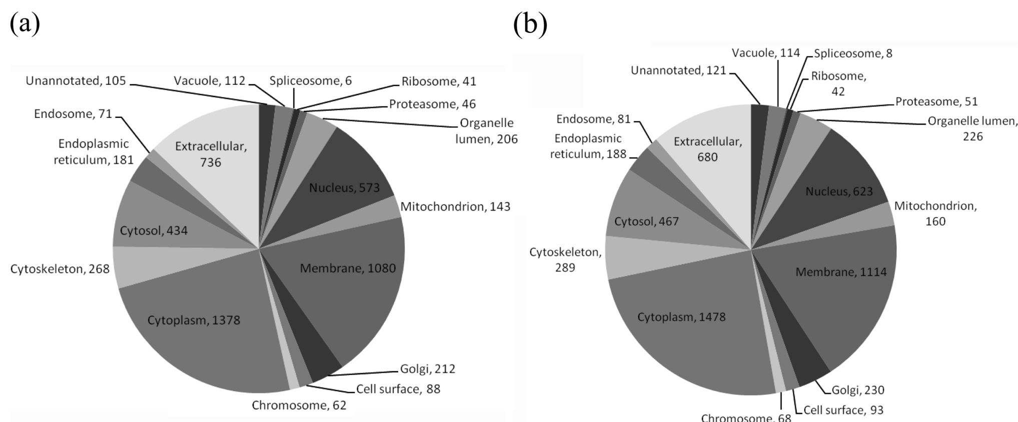

Figure 2. Distribution of cellular component of proteins identified in (a) Control and (b) post-vasectomy (PV) seminal plasma samples.

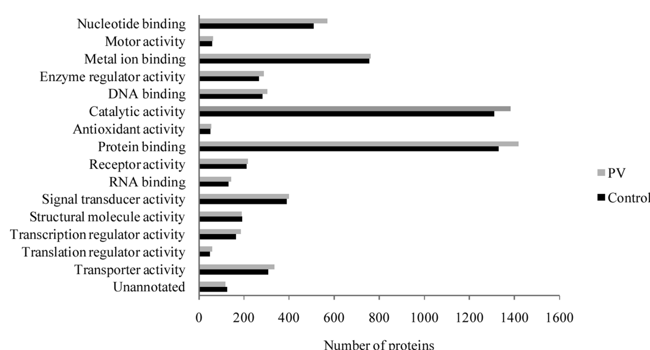

Figure 3. Distribution of molecular function of proteins identified in Control and post-vasectomy (PV) seminal plasma samples.

at FPR of 1.1% with 1411, 1810, and 1599 proteins in each replicate (Figure 1b, Supplementary Table 2). Genome Ontology breakdown of cellular component and function illustrates that these proteins are distributed in the same manner as those in the Control group (Figure 2b, Figure 3).

Detailed information about peptide sequences, scores and modifications for Control, PV, and combined Control-PV data sets can be found in Supplementary Table 5, Supplementary Table 6, and Supplementary Table 7, respectively. To inspect single-peptide protein identifications, supplementary Scaffold files "Control_SP.sfd", "PV_SP.sfd", and "Control-PV_SP.sfd" can be opened using a Scaffold viewer downloaded freely at http://www.proteomesoftware.com/Proteome_software_prod_Scaffold2_download-main.html. Filter settings at the peptide level should be set to "Control-SP", "PV-SP", and "Control-PV-SP" for the respective data sets.

Comparison of Control and PV Protein Lists

One of our objectives was to identify the tissue of origin of proteins within the semen. Those proteins found exclusively or more abundantly in the Control group compared to the post-vasectomy group are likely to have originated from the testis and/or the epididymis. Comparing Control and PV proteomes, there are 1662 proteins in common, 280 found only in Control, and 418 found only in PV (Figure 1c, Supplementary Table 3). In total, we identified 2360 proteins (including 15 proteins that matched reverse sequences) at FRP of 1.3% in the combined Control and PV groups. The common or unique proteins to each group include proteins that were identified by one peptide and/or in one replicate, rendering the lists not very useful for confident protein selection. Normalized spectral counts were used to identify proteins that were unique to one group or under-expressed in one group in comparison to the other. The following arbitrary criteria were used to select a high-confidence list of potential proteins originating from the testis and/or the epididymis or proteins with higher concentration in one of the groups: (a) proteins that showed an average of at least 3 spectral counts in one group and zero in the other, (b) fold changes ≥ 2, (c) fold changes ≥ 2 but <3 needed to have an average spectral count of at least 10 in one clinical group, (d) fold changes ≥ 3 needed to have an average spectral count of at least 5 in one clinical group, (e) spectral counts in each replicate (belonging to the same clinical group) for criteria a−d needed to be consistent. Selected proteins that had their spectral counts significantly elevated due to shared peptides with other proteins were removed from analysis. Applying the above-mentioned selection criteria we found the following: 32 proteins uniquely present in the Controls, 49 present at lower abundance in PV, 3 uniquely present in the PV, and 25 present at higher abundance in PV (Table 1, Table 2, Table 3, Supplementary Table 3, Supplementary Table 4). In the case of proteins at higher or lower concentration, the majority of the candidates have fold changes much greater than the cutoff of 2. Since the fold changes were calculated from averaged spectral counts, they may be overestimated, especially in cases where only one out of three replicates contains spectral counts.

The 109 proteins that are found uniquely or more abundantly in either of the two clinical groups were searched against UniProtKB, UniGene, BioGPS (formerly Novartis Gene Expression Atlas), and Human Protein Atlas databases for tissue specificity. Only proteins that showed restricted, dominant, or above average expression in the testis, epididymis, seminal vesicle, or prostate were annotated as expressed in these tissues. In order for proteins to be selected as being specific to the above-mentioned tissues, in UniGene expression needed to be "restricted" to the tissue of interest or the tissue had a dominant/major contribution, in BioGPS expression needed to be "exclusive" to the tissue of interest or have at least 3× the average expression, and in Human Protein Atlas expression needed to be "strong". Because not all databases contained epididymis and seminal vesicle in their tissue list and because epididymis is proximal to the site of ligation in post-vasectomy patients and would not contribute to the protein pool in PV samples, proteins expressed in the epididymis were grouped with testis as testis-specific. There are 14 proteins that show testicular/epididymal origin in three out of

Table 1. Proteins Found Uniquely in Control Seminal Plasma (Absent in Post-vasectomy (PV) Seminal Plasma) According to Spectral Counting[a]

	average Spectral Count			
gene name	Control	PV[b]	protein origin[c]	references[d]
LDHC	53.7	0	testis	1,2,3
DPEP3	16.3	0	testis	
TEX101	17.0	0	testis	4,5,6
CEL	15.0	0		
ADAM7	9.7	0	testis	7
LIPI	8.7	0		8
BSPH1	13.7	0		9
OVCH2	10.0	0		
MFGE8	8.7	0		10
PGK2	16.7	0	testis	11,12
ACRBP	7.7	0	testis	13
HIST1H2BA	8.7	0	testis	14,15
PGAM2	7.3	0		
CDH2	6.3	0		16
MFAP4	4.0	0		
SLC2A14	4.3	0	testis	17
REG3G	6.0	0		18
PTPRG	4.3	0		19
HSPA4L	3.7	0		20,21,22
THBS2	4.3	0		23
RNASE13	4.0	0		
VASN	2.7	0		
LRRC37A3	2.7	0		
SI	7.0	0		
SPACA3	3.3	0	testis	24
GAPDHS	3.0	0	testis	25,26
SLC1A1	3.3	0	testis	
C16orf89	2.7	0		
DEFB121	2.7	0		
BSG	2.7	0		
ZPBP	2.7	0	testis	27
AKAP4	2.7	0	testis	28

[a] Refer to Supplementary Table 4 for all information. [b] Post-vasectomy. [c] For protein origin to be annotated, the corresponding gene name needed to be found in testis, epididymis, or prostate in at least two out of four databases. [d] Linked to urogenital tract.

four databases; 7 are in the list of proteins unique to Control and 7 are in the list of proteins at lower abundance in PV (Table 4). When only two out of four databases are considered, then in addition to the 14 proteins above, there are 8 other proteins found in the testis/epididymis: 6 in the list of proteins unique to Control and 2 in the list of proteins at lower abundance in PV (Table 1, Table 2, Supplementary Table 4). There are also 2 proteins found in two out of four databases with expression in the prostate. We were able to identify 19 proteins with "restricted" or "exclusive" expression in the testis, 15 according to UniGene and 16 according to BioGPS. These proteins are marked with an asterisk in Supplementary Table 4. Upon comparison of the 109 proteins to proteomic analysis of three prostate cancer cell lines by Sardana et al., 29 proteins out of 109 among all three cell lines are in common: 15 found in 22Rv1, 17 found in LNCaP, and 20 in PC3 (Supplementary Table 4).[16]

DISCUSSION

We identified 2022 proteins in pooled seminal plasma from 5 fertile (Control) men and 2096 proteins in men who had a vasectomy (PV). Despite PV seminal plasma being void of proteins originating from testis and epididymis, we did not expect to find differences in the number of proteins identified between Control and PV samples. Since testis and epididymis contribute approximately only 10% to the overall proteome of seminal plasma, proteins originating from these tissues would be less abundant and more difficult to detect. In addition, MS based analysis using Data Dependent Acquisition (DDA) further adds variability to protein identification as not all peptides in the survey scan, especially those of lower abundance, are subjected to fragmentation. Due to these reasons, identification of more proteins in PV rather than in Control seminal plasma is plausible.

Despite identification of many low-abundance proteins in seminal plasma, the high-abundance proteins were catalogued with hundreds to thousands of spectra. For example, the semenogelin proteins, SEMG1 and SEMG2, are predominant proteins in human seminal plasma. These are matrix proteins secreted by the seminal vesicles that form a gel-like matrix entrapping spermatozoa and are responsible for the viscous state of semen.[17,8] Another high abundance adhesion molecule is fibronectin, FN1. KLK3, otherwise known as PSA or prostate-specific antigen, is a serine-protease secreted by the prostate gland. It is involved in proteolysis of semenogelins, thereby leading to liquefaction of gelatinous semen and increased motility of spermatozoa.[10] PSA is being used as a serum biomarker of prostate cancer for nearly 20 years.[18] KLK3 is one of several proteases found in seminal plasma, as is SERPINA1, α-1-antitrypsin, a protease inhibitor. ACPP, often referred to as PAP or prostastic acid phosphotase was also used in the past as marker of metastatic prostate cancer but was found to be inferior to PSA and is no longer used.[19,20] LTF, lactoferrin or lactotransferrin, is part of the immune system as it has antimicrobial properties, and albumin, ALB, is a common transport protein. These are some of the high abundance proteins identified in seminal plasma. A complete list of proteins identified in Control and PV seminal plasma can be found in Supplementary Table 1, Supplementary Table 2, and Supplementary Table 3.

Currently, this is the most exhaustive list of proteins to be found in seminal plasma. Of the 923 seminal plasma proteins identified by Pilch et al.,[1] 699 are in common with our list (Figure 1d, Supplementary Table 1). There were also 349 proteins in common between our list and that of Wang et al.,[7] where 625 proteins were identified in fertile patients (Figure 1d, Supplementary Table 1). An indication of the tissues of origin of some of these proteins could be determined by comparing our protein lists with proteomic studies from other reproductive tract fluids and tissues. Poliakov et al. characterized the protein composition of membrane-bound vesicles (prostasomes) secreted by the prostate gland. Of the 440 proteins identified by Poliakov et al.,[21] 316 are found in our Control list (Figure 1d, Supplementary Table 1). Examination of expressed prostatic secretions (EPS) from preprostatectomy patients reported 916 proteins,[22] 702 of these are also found in our Control samples (Figure 1d, Supplementary Table 1). Two separate studies of testicular biopsy specimens from men by Guo group identified 1430[23] and 476[24] proteins. Comparative analysis of 1430 testicular proteins shows 667 proteins shared with our Control and 671 with our PV (Figure 1d, Supplementary Tables 1 and Supplementary Table 2).

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Table 2. Proteins Found at Lower Abundance in Post-vasectomy (PV) Seminal Plasma Relative to Control Seminal Plasma According to Spectral Counting[a]

	average spectral count				
gene name	Control	PV[b]	fold change[c] Control/PV	protein origin[d]	references[e]
MUC5B	29.7	0.3	89.0		29
PTGDS	52.3	1.3	39.3	testis, epididymis, prostate	30,31,32
CPVL	9.7	0.3	29.0		
ELSPBP1	22.0	1.0	22.0	testis	33,34
GPR64	13.7	0.7	20.5	testis	35,36
C20orf114	6.7	0.3	20.0		
CA4	6.0	0.3	18.0		37,38
STOM	5.7	0.3	17.0		
BGN	9.3	0.7	14.0		
CES7	23.0	1.7	13.8		39
PFKP	4.3	0.3	13.0		
LOC642103	29.0	2.7	10.9		
SPINT3	20.7	2.0	10.3	testis	
MGAM	28.0	3.3	8.4		
COL18A1	15.3	2.0	7.7		40
NID1	12.3	1.7	7.4		
CRISP2	7.3	1.0	7.3	testis	41,42,43
FAM12B	19.3	2.7	7.3	testis, epididymis	44
BCAN	15.3	2.3	6.6		
ECM1	114.0	18.0	6.3		
A2M	46.7	7.7	6.1		45,46,47
PATE4	5.3	1.0	5.3	prostate	
SERPINA6	5.0	1.0	5.0		
APCS	10.0	2.3	4.3		
EEF1G	9.7	2.3	4.1		
AK1	10.0	2.7	3.8		48
ALDH1A1	17.3	4.7	3.7		49
PDGFA	4.7	1.3	3.5		50
NPC2	234.0	68.3	3.4	testis, epididymis	51,52
CRISP1	59.0	17.3	3.4	testis	41,53,54,55
ABP1	71.3	21.0	3.4		47
CFI	9.0	2.7	3.4		
CAMP	11.3	3.7	3.1		
CALR	14.0	4.7	3.0		
CPAMD8	12.7	4.7	2.7		
PPA1	9.7	3.7	2.6		
CD177	82.0	32.3	2.5		47
SERPINA1	155.3	61.7	2.5		56
BPIL1	17.3	7.0	2.5		
FBLN2	17.0	7.3	2.3		57
DAG1	12.3	5.3	2.3		
FAM3C	10.0	4.3	2.3		
PGLYRP2	10.7	4.7	2.3		
CHGB	12.0	5.3	2.3		
GAS6	34.0	15.3	2.2		58
NP	16.0	7.3	2.2		
RNASE1	58.3	28.0	2.1	testis	
CD14	32.7	16.0	2.0		
MXRA5	56.0	28.3	2.0		47

[a] Refer to Supplementary Table 4 for all information. [b] Post-vasectomy. [c] Fold changes may be overestimated especially in cases where only one out of three replicates contains spectral counts (refer to Supplementary Table 4 for all information). [d] For protein origin to be annotated, the protein needed to be found in testis, epididymis, or prostate in at least two out of four databases. [e] Linked to urogenital tract.

Table 3. Proteins Found at Higher Abundance in Post-vasectomy (PV) Seminal Plasma Relative to Control Seminal Plasma According to Spectral Counting[a]

	average spectral count			
gene name	Control	PV[b]	fold change[c] Control/PV	references[d]
ELA2	1.0	15.0	−15.0	
AZU1	1.0	6.0	−6.0	
HIST1H2BL	3.3	18.3	−5.5	
PRELP	0.7	5.3	−5.5	
FGB	5.0	26.3	−5.3	
MPO	8.7	36.7	−4.2	
AGL	1.7	6.7	−4.0	
HIST1H4H	4.0	14.3	−3.6	
GSTT2	3.0	10.0	−3.3	
COL6A2	2.7	8.3	−3.1	
MYO1C	3.0	9.0	−3.0	
FMOD	5.3	15.7	−2.9	
SYTL1	4.7	11.3	−2.4	
PAEP	56.7	134.3	−2.4	59,60
FLJ58816	4.3	10.0	−2.3	
ALDH7A1	4.3	10.0	−2.3	
MYH9	28.0	63.3	−2.3	
C11orf54	5.3	11.7	−2.2	
ORM2	111.3	241.3	−2.2	
STAT3	7.7	16.0	−2.1	61
ORM1	224.7	466.7	−2.1	
PYGB	16.3	33.7	−2.1	
APOA1	17.7	36.3	−2.1	
ACAT2	7.7	15.7	−2.0	
C3	35.7	70.0	−2.0	

[a] Refer to Supplementary Table 4 for all information. [b] Post-vasectomy. [c] Fold changes may be overestimated especially in cases where only one out of three replicates contains spectral counts (refer to Supplementary Table 4 for all information). [d] Linked to urogenital tract.

There are 78 proteins out of 667 that are in common only with Control, but not PV, and 81 out of 671 that are in common only with PV, but not Control. Out of these 78 proteins, LDHC, DPEP3, TEX101, PGK2, CDH2, MFAP4, HSPA4L, GAPDHS belong to the 32 proteins selected to be unique in Control (Table 1, Supplementary Table 4). FGG, from the list of 81 proteins, has also been selected as uniquely found in PV (Supplementary Table 4). In a subsequent study cataloguing 476 testicular proteins, 268 were found to be in common with Control and PV lists (Figure 1d, Supplementary Table 1 and Supplementary Table 2). Out of 268 shared proteins, there were 20 that were in common only with Control and another 20 in common only with PV. HSPA4L is one protein that was found to be in common only with the Control and belongs to the list of 32 proteins identified as unique in Control (Table 1, Supplementary Table 4). The proteins identified in the testicular biopsy samples would have intracellular and extracellular origin. Since in Control seminal plasma the protein contribution from testis and epididymis is estimated to be only 10% and since proteins from these tissues would be mostly secreted or shed, it is not surprising that we did not identify all 1430 and 476 testicular proteins in our study. Interestingly, we expected to identify a fewer number of testicular proteins in our PV samples compared to Control, however, it is also possible that these testicular proteins are not solely expressed in testis, but also elsewhere in the urogenital system. Proteomic mapping of different regions of the epididymis from four men identified 34 proteins.[25] Proteins TPO, A1M and PBP were not identified in Control seminal plasma, whereas in PV samples in addition to those absent from Control, ELSPBP1 also was not identified (Figure 1d, Supplementary Table 1 and Supplementary Table 2). In a study of membraneous vesicles secreted in the intraluminal compartment of the epididymis (epididymosomes), Thimon et al. identified 146 proteins.[26] Epididymosomes are believed to be involved in the transfer of proteins to spermatozoa as they pass the epididymis, having an important role in sperm maturation.[27] We were able to identify 112 of these epididymosomal proteins in our Control list and 100 in our PV list (Figure 1d, Supplementary Table 1 and Supplementary Table 2). One epididymosomal protein, ARF5, was found in the PV list, but not in Control, whereas the 13 proteins LDHC, TEX101, HSPA2, ADAM7, PGK2, PGAM2, GAPDHS, ZPBP, AKAP4, RUVBL1, HK1, NRAS, and SDCBP2 were not identified in the PV group. Since LDHC, TEX101, ADAM7, PGK2, PGAM2, GAPDHS are in the list of 32 proteins unique to Control, it is suggestive of their epididymal origin. All proteins other than PGAM2 were identified by two or more databases as being expressed in the testis. As vasectomy obstructs the passage of epididymal proteins into seminal plasma, we expected to find a lower number of epididymosomal proteins in PV seminal plasma than in Control. This suggests that proteins that make up epididymosomes are not strictly found in the epididymis but also in other regions of the reproductive tract.

Genome Ontology analysis shows that the majority of the proteins in Control and PV groups have cytoplasmic, followed by membrane, extracellular, and nuclear origin. The possible explanation for the large portion of proteins being cytoplasmic (and intracellular) is due to the presence of prostasomes and epididymosomes in seminal plasma as well as due to protein leakage from surrounding tissue cells.[28] Prostasomes, arising from the prostate, are exosome-like vesicles that form into vesicular bodies from intracellular membranes, taking up cytoplasmic and intracellular proteins in the process. Similarly, epididymosomes originate in the epididymis and are secreted into seminal plasma in an apocrine manner.[26] Since prostasomes and epididymosomes have not been removed from seminal plasma in our study, many of these intracellular proteins would find their way into seminal plasma.[21]

Functionally, a majority of the proteins have been assigned a binding function, followed by catalytic activity. The binding category consists of many different subcategories of which protein binding is the largest. This is a very general protein designation category as protein binding functionality could be implicated in another function. A large proportion of seminal plasma proteins have been assigned a catalytic activity, consistent with many of the proteins being involved in semen coagulum liquefaction and sperm maturation.

Proteins Unique to Control

Many of the 280 proteins found to be unique to the Control group have been identified with one peptide and not in all three replicates. Therefore, in order to improve the confidence in recognition of proteins strictly found in the Control group, selection criteria described earlier were used to narrow the list of 280 proteins down to 32 (Table 1, Supplementary Table 4). Proteins

Table 4. Proteins at Different Abundances in Seminal Plasma from Control and Post-vasectomy (PV) Men According to Spectral Counting That Are Expressed in the Testis/Epididymis or the Prostate in at Least Three out of Four Databases[a]

	average spectral count			
gene name	Control	PV[b]	fold change[c] Control/PV	protein origin
		Unique to Control		
TEX101	17.0	0	PV ≪ Control	testis
PGK2	16.7	0	PV ≪ Control	testis
HIST1H2BA	8.7	0	PV ≪ Control	testis
SLC2A14	4.3	0	PV ≪ Control	testis
SPACA3	3.3	0	PV ≪ Control	testis
GAPDHS	3.0	0	PV ≪ Control	testis
AKAP4	2.7	0	PV ≪ Control	testis
		↓ in PV		
PTGDS	52.3	1.3	39.3	testis
ELSPBP1	22.0	1.0	22.0	testis
SPINT3	20.7	2.0	10.3	testis
CRISP2	7.3	1.0	7.3	testis
FAM12B	19.3	2.7	7.3	testis
PATE4	5.3	1.0	5.3	prostate
NPC2	234.0	68.3	3.4	testis
CRISP1	59.0	17.3	3.4	testis

[a] Refer to Supplementary Table 4 for all information. [b] Post-vasectomy. [c] Fold changes may be overestimated especially in cases where only one out of three replicates contains spectral counts (refer to Supplementary Table 4 for all information).

TEX101, PGK2, HIST1H2BA, SLC2A14, SPACA3, GAPDHS, and AKAP4 are exclusively or mainly transcribed in the testis/epididymis in at least three out of four searched databases (Table 4, Supplementary Table 4), whereas LDHC, DPEP3, ADAM7, ACRBP, SLC1A1, and ZPBP are found in two out of four databases (Table 1, Supplementary Table 4). Note that proteins expressed in the epididymis were grouped as testis-specific since only two databases contained epididymis in their tissue list. The 19 remaining proteins, CEL, LIPI, BSPH1, OVCH2, MFGE8, PGAM2, CDH2, MFAP4, REG3G, PTPRG, HSPA4L, THBS2, RNASE13, VASN, LRRC37A3, SI, C16orf89, DEFB121, and BSG, have not been identified as testis- or epididymis-specific by at least 2 databases. According to UniGene, proteins LDHC, DPEP3, ADAM7, PGK2, ACRBP2, SLC2A14, RNASE13, SPACA3, GAPDHS, ZBPB, and AKAP4 are found to have "restricted" expression in the testis. Similarly, in BioGPS, LDHC, DPEP3, TEX101, ADAM7, PGK2, HSPA4L, SPACA3, GAPDHS, ZPBP, and AKAP4 are "exclusive" to the testis. Note that some of these proteins may have been identified as testis- or epididymis-specific by at least one database (Supplementary Table 4). However, literature searches on these proteins show that several are expressed in the reproductive tract and play a significant role in male fertility. The 32 proteins unique to the Control are mostly extracellular, followed by membrane and cytoplasmic or a combination thereof. Interestingly, whereas in the Control group the extracellular component was the third largest, among these Control-unique proteins the extracellular component dominates. In addition to proteins annotated as expressed in testis/epididymis, the remaining proteins on this list may potentially have their origin in the testis or the epididymis and may represent candidate markers of infertility.

According to the literature, many proteins that have testicular or epididymal tissue specificity have been shown to have a strong link to fertility. LDHC (L-lactate dehydrogenase C chain) is a testis-specific enzyme secreted into testicular or epididymal fluid from exfoliated postmeiotic germ cells[29] that affects male fertility through reduced sperm motility.[30] Absence of LDHC inhibits glycolysis, which results in lower levels of ATP (adenosine triphosphate) required for movement of sperm flagellum.[30] The LDHC/sperm ratio is indicative of the status of the seminiferous epithelium.[29] Measurement of LDH-C4 (homotetramer of LDHC) in semen samples of fertile and infertile men showed complete absence of LDH-C4 activity in all azoospermic samples except one and all samples from men who underwent a vasectomy compared to fertile Controls.[31] In mouse, TEX101 is a germ cell marker glycoprotein during gametogenesis.[32−34] It is later found on spermatocytes, spermatids, and testicular sperm after the onset of puberty and is shed as sperm passes the caput epididymis.[33,35] In mouse studies, ADAM7 expressed in the epididymis is transferred to the sperm surface, and during acrosome reaction it is redistributed to the posterior of the sperm head.[36] It is possible that ADAM7 may play a critical role in sperm maturation. PGK2 is involved in glycolysis of spermatozoa as it activates phosphoglycerate-kinase, important for development of spermatozoa.[37,38] PGK2 gene, along with LDHC and GAPDHS, are germ-cell specific genes.[37] GAPDHS, being a glycolytic enzyme found in the fibrous sheath of the sperm tail, is involved in glycolysis, a process that produces ATP required for movement of sperm flagellum.[39,40] GAPDHS knockout mice contain sperm that are immotile, suggesting importance of GAPDHS in male fertility. AKAP4 is another fibrous sheath protein that is involved in sperm motility. In AKAP4 null male mice, sperm numbers were not reduced, but sperm motility was diminished, resulting in infertility.[41] In these mice, sperm had morphological differences such as reduced sperm diameter, shortened flagellum, and improper formation of the fibrous sheath. Mutant mice also showed lower GAPDHS expression levels, suggesting that AKAP4 is necessary for binding of GAPDHS to the fibrous sheath.[41] This finding implies that in AKAP4 free sperm, glycolysis is inhibited. HIST1H2BA gene is expressed exclusively in the testis and also in sperm.[42] The protein is found in the basal area of sperm nuclei,[42] and a variant of this protein is involved in the telomere-binding complex, suggesting its role in spermiogenesis and fertilization.[43] SLC2A14 gene is selectively expressed in the testis, and the protein is involved in glucose transport.[44] SPACA3 gene encodes for a lysozyme protein localized on the acrosome of human spermatozoa and hence may serve as a receptor for egg membrane saccharide *N*-acetylglucosamine.[45] It may therefore be involved in sperm-egg interaction during fertilization. ZPBP is located on the zona pellucida (ZP), the outer region of the egg responsible for sperm binding and initiation of acrosome reaction that is required for sperm penetration.[46] Studies on ZPBP have shown that male mice lacking this gene are sterile as they have reduced sperm motility due abnormal sperm morphology. Furthermore, these mice also suffer from improper acrosome biogenesis resulting in poor Sertoli-spermatid junctions. ACRBP is another acrosomal protein involved in binding of polysulfate groups on ZP glycoproteins in a stereodependent fashion.[47] ACRBP therefore mediates prolonged and proper interaction of sperm with ZP, thereby providing ample time for the sperm head to penetrate through the ZP.

Literature searches on proteins that show expression in only one database or have not been shown to have expression in the testis or epididymis indicate that many of these are also important in the well-being of the reproductive system. MFGE8, being expressed on sperm, is involved in sperm-egg adhesion, and MFGE8 null mice are subfertile.[48] CDH2 is involved in rat blood-testis-barrier function.[49] Studies of HSPA4L protein in mice show high expression in the testis and in spermatogenic cells.[50] In HSPA4L null male mice spermatogenesis is not disrupted; however, they are infertile and are more prone to osmotic stress. The disruption of fertility in mice may be due to an observed higher number of apoptotic spermatocytes and decreased sperm motility.[50] In a study conducted on gene expression patterns in infertile men and rodent models, HSPA4L gene was downregulated in the infertile male mouse compared to wild-type, but not in human males.[51] According to Nonoguchi et al., HSPA4L is expressed during and after the spermatocyte stage in germ cells and also in sperm.[52] THBS2 was also detected in interstitial Leidig cells in mouse testis.[53] BSG is a highly glycosylated protein belonging to the Ig superfamily that is required for reproduction in both males and females.[54] BSG is initially localized on the principal piece of testicular spermatozoa, then in the middle region during epididymal maturation, and finally on the head of the sperm after capacitation, suggesting its importance in sperm-ZP interaction.[55] During the capacitation process, BSG undergoes deglycosylation which may be required for sperm-egg fusion.

From literature searches it is clear that many of the above-mentioned proteins have their origin in the testis and/or epididymis and show a strong link to the male reproductive tract, despite not being annotated as epididymal or testicular by all four databases.

Proteins at Lower Abundance in PV Patients

There are 49 proteins that according to spectral counting are at lower abundance in the PV group relative to the Control. These proteins may potentially be expressed in regions of the reproductive tract other than the testis or the epididymis; hence they are also found in the PV samples but at lower concentration levels. Proteins PTGDS, ELSPBP1, SPINT3, CRISP2, FAM12B, NPC2, and CRISP1 are expressed in the testis/epididymis in at least three out of four tissue databases, whereas GPR64 and RNASE1 are found in two out of four databases (Table 2, Table 4, Supplementary Table 4). PATE4 and PTGDS were also identified as having strong expression in the prostate in three out of four and two out of four databases, respectively (Table 2, Table 4, Supplementary Table 4). Note that proteins expressed in the epididymis were grouped as testis-specific since only two databases contained epididymis in their tissue list. PTGDS, FAM12B, and NPC2 are also shown to be expressed in the epididymis by two out of four databases (Table 2, Supplementary Table 4). According to UniGene, proteins SPINT3, CRISP2, FAM12B, and CRISP1 are found to have "restricted" expression in the testis (Supplementary Table 4). Similarly, according to BioGPS, ELSPBP1, GPR64, SPINT3, CRISP2, FAM12B, and CRISP1 are "exclusive" to the testis (Supplementary Table 4). PATE4 also has "restricted" and "exclusive" expression in the prostate according to UniGene and BioGPS, respectively (Supplementary Table 4). Genome Ontology identifies most of the proteins in this group as extracellular, membrane or cytoplasmic.

Several of the proteins at lower levels in PV pools have been previously investigated by looking at their concentration levels in semen from men with various reproductive abnormalities. These include PTGDS, A2M, and NPC2. PTGDS (prostaglandin-H2 D-isomerase) is a secretory protein expressed by the Sertoli and Leydig cells as well as the epithelial cells of the prostate.[56,57] In our first study of PTGDS in seminal plasma from men belonging to different fertility groups, median PTGDS levels in normal men were higher 100-fold compared to vasectomized men.[56] In our more recent study of PTGDS in seminal plasma with better defined cases of infertility, median PTGDS levels in normal men was 800 μg/L (n = 10), compared to 9.0 μg/L (n = 20) in men who have had a vasectomy, 11.0 μg/L (n = 9) in men with obstructive azoospermia, and 18.5 μg/L (n = 14) in men with nonobstructive azoospermia.[58] Clearly, PTGDS levels can potentially be used in differentiation of obstructive from nonobstructive azoospermia patients. These PTGDS values by ELISA corroborate quite well with our semiquantitative results. A2M (α-2-macroglobulin) is a protease inhibitor produced in Sertoli cells that is modified and complexed by PSA, allowing it to bind with a receptor on spermatozoa.[59] A2M is believed to have important implications in sperm motility. In a study of seminal plasma A2M concentrations, mean A2M levels have been reduced from 9.15 μg/L in normal to 0.74 μg/L in vasectomised men.[60] NPC2 (epididymal secretory protein E1), an epididymal protein believed to be involved in transporting cholesterol during sperm maturation, is decreased by 40% in seminal plasma from vasectomised men compared to normal men.[61] Following vasectomy reversal, NPC2 protein levels increased to those of normal men. In a proteomic analysis of seminal plasma samples from obstructive and nonobstructive azoospermia patients, NPC2 was not identified in the latter group.[6] This finding suggests the possibility of using NPC2 in differentiating these two causes of male infertility. The above-mentioned quantitative studies confirm our findings.

Many of the remaining proteins that have their origins in the reproductive tract are associated with fertility. ELSPBP1 is present in caudal, corpus, and caput regions of bull epididymis and binds to transiting spermatozoa.[62] In human and porcine sperm, it was found to be bound to the midpiece region of the sperm.[63] GPR64 gene is found to be expressed in all regions of the epididymis with highest expression in the caput portion.[64] Inhibition of GPR64 gene in mice models leads to dysregulation of fluid reabsorption within the efferent ductules, which leads to flow stagnation of spermatozoa in efferent ducts, resulting in infertility.[65] CRISP1 and CRISP2 belong to the CRISP family, which shows strong expression in the male reproductive tract.[66] CRISP1 is a secreted epididymal protein that binds the postacrosomal region of spermatozoa. CRISP1 gene knockout mouse sperm showed reduced ability to fertilize ZP-intact and ZP-free eggs,[67] suggesting the importance of CRISP1 in the sperm-egg fusion processes.[68,69] CRISP2 is believed to have a role in male fertility, as it is an acrosomal and tail protein potentially involved in sperm motility and acrosomal reaction.[70] It is also implicated in adhesion between spermatids and Sertoli cells and gamete interaction.[71] In a proteomic analysis of seminal plasma from healthy and asthenoazoospermic (AS) men, Wang et al. identified 100 proteins that were up- or down-regulated.[7] In that study, A2M, ABP1 and MXRA5 were up-regulated in healthy men, and these same proteins were also at higher concentration in our Control samples. CD177 was up-regulated in AS but was found to be at lower concentration in our Control. Identification of the same proteins further strengthens the hypothesis that they are associated with fertility.

Literature searches on the remaining proteins on our list show that many of these belong to the reproductive tract and are believed to be involved in fertility (Table 2, Supplementary Table 4). The finding that proteins previously annotated as testicular or epididymal are found in the PV samples suggests that they may not strictly be localized to these tissues but could also be expressed in other regions of the reproductive tract. Contribution of proteins from testicular or epididymal tissue to Control seminal plasma samples and absence of such contribution to PV seminal plasma renders these proteins potential indicators of fertility status. Future validation studies are highly warranted.

Proteins Unique to PV and at Higher Abundance in PV Patients

Three proteins, MMP9, FGG, and SAA2, were found to be unique to the PV list (Supplementary Table 4). Furthermore, there are 25 proteins that are at higher abundance in PV relative to Control (Table 3, Supplementary Table 4). According to literature, PAEP and STAT3 seem to be implicated in fertility (Table 3, Supplementary Table 4). Six of the proteins, GSR, PYGB, ORM1, ORM2, MYH9, and MPO, were previously found to be differentially expressed in seminal plasma from healthy and AS men.[7] ORM1, ORM2, and MPO were also found to be up-regulated in AS samples. It has been previously shown that vasectomy alters the expression of epididymal proteins,[72] and hence it is also possible that proteins in other parts of the reproductive tract may also be prone to up- or down-regulation.

Clinical Relevance

Contributions to the seminal proteins arise from the different cellular components of the testis, epididymis, vas deferens, seminal vesicles, prostate, and accessory glands. With this study we have begun to understand the complexity of the seminal proteome and the tissue of origin of specific seminal proteins found in the semen. We believe that seminal plasma is an excellent fluid for clinical research purposes as it can be readily obtained and contains secreted and shed proteins from the above-mentioned tissues. Because a number of proteins within seminal plasma are tissue- and cell-specific, this has the potential for diagnosis and monitoring of specific diseases of the male reproductive system such as prostate and testicular cancer, prostatitis, and male infertility. As an example, a number of these seminal plasma proteins arise specifically from the cells of the testis and epididymis. These proteins are potentially important markers for obstruction of the reproductive tract causing infertility and could be used for the noninvasive diagnosis of the etiology of male infertility, avoiding testicular biopsy.

CONCLUSION

Seminal plasma is a highly complex fluid, with seminal proteins arising from all parts of the male genital tract. We have identified a list of over 2000 proteins in seminal plasma; thus far this is the largest list of proteins identified in this fluid. Upon spectral counting analysis of Control and PV protein lists, we were able to catalogue 81 proteins at higher abundance and 28 at lower abundance in Control relative to PV. We believe that the proteins unique to Control and those at significantly lower abundance in PV have their reproductive tract origin solely or principally in the testis or epididymis. Twenty-two of these proteins have been shown to be testis- or epididymis-specific by at least two out of four gene or protein databases; others are linked to reproductive biology, whereas others have not been annotated to be part of the urogenital system. The proteins identified only in Control and at higher abundance in Control, especially those that originate in testis/epididymis, can serve as candidates for future studies on testicular and epididymal function in men with infertility, testicular cancer, and other urogenital disorders.

ASSOCIATED CONTENT

Supporting Information

Supplementary Tables 1, 2, 3, 4, 5, 6, 7 and supplementary Scaffold files "Control_SP.sfd", "PV_SP.sfd", and "Control-PV_SP.sfd". This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

PV, post-vasectomy; MS, mass-spectrometry; SCX, strong-cation exchange; LC, liquid chromatography; ATP, adenosine triphosphate; SRM, single-reaction monitoring; MudPIT, multiple-dimension protein identification technology; FPR, false-positive rate; ZP, zona pellucida; AS, asthenoazoospermia

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