Towards Understanding Male Infertility After Spinal Cord Injury Using Quantitative Proteomics*§

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The study of male infertility after spinal cord injury (SCI) has enhanced the understanding of seminal plasma (SP) as an important regulator of spermatozoa function. However, the most important factors leading to the diminished sperm motility and viability observed in semen of men with SCI remained unknown. Thus, to explore SP related molecular mechanisms underlying infertility after SCI, we used mass spectrometry-based quantitative proteomics to compare SP retrieved from SCI patients to normal controls. As a result, we present an in-depth characterization of the human SP proteome, identifying ~2,800 individual proteins, and describe, in detail, the differential proteome observed in SCI. Our analysis demonstrates that a hyper-activation of the immune system may influence some seminal processes, which likely are not triggered by microbial infection. Moreover, we show evidence of an important prostate gland functional failure, i.e. diminished abundance of metabolic enzymes related to ATP turnover and those secreted via prostasomes. Further we identify the main outcome related to this fact and that it is intrinsically linked to the low sperm motility in SCI. Together, our data highlights the molecular pathways hindering fertility in SCI and shed new light on other causes of male infertility. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.052175, 1424–1434, 2016.

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For many years, seminal plasma (SP)¹, the liquid component of semen, was believed to have a single and simple physiological significance as the carrier of spermatozoa through both male and female reproductive tracts. It was around 50 years ago when the compositional complexity of this fluid started to be investigated, demonstrating that SP not only aids in cellular transport but also provides energy and metabolic support to the transiting spermatozoa (1, 2). Today, growing evidence indicates that SP plays a role far beyond what was once envisioned, including acting as an essential regulator of spermatozoa function contributing to (enabling/hindering) the cellular ability of fertilization (3). Composed of secretions derived from the testis, epididymis and male accessory glands (prostate, seminal vesicles and bulbourethral glands), SP is a mixture of sugars, inorganic ions, organic salts, (phospho)lipids and proteins (4). Such a heterogeneous composition emphasizes the complex biochemical cascades triggered within SP during, and immediately after, ejaculation and defines the beneficial and/or detrimental nature of SP in the overall reproductive process (5). From a clinical point of view, studies have confirmed the participation of SP in the etiology of male infertility. By studying semen of men with spinal cord injury (SCI), who become infertile after a traumatic injury and often present with an unusual seminal profile characterized by normal sperm concentration but extremely impaired sperm motility and viability, Brackett et al. demonstrated how SP can impair sperm function leading to infertility (6). Specifically, the authors mixed SP obtained from SCI

¹ The abbreviations used are: SP, Seminal plasma; CASA, Computer-aided semen analysis; CID, Collision induced dissociation; DAVID, Database for Annotation, Visualization and Integrated Discovery; DDA, Data dependent acquisition; DMSO, Dimethyl sulfoxide; DTT, Dithiothreitol; ESI, Electrospray ionization; FDR, Protein false discovery rate; HCD, Higher energy collisional dissociation; HPLC, High-performance liquid chromatography; hSAX, Hydrophilic Strong Anion Exchange; KEEG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LTQ-Orbitrap, Linear trap quadrupole-Orbitrap; PVS, Penile vibratory stimulation; SCI, Spinal cord injury; STR, Straightness; TEAB, Triethylammonium bicarbonate; VAP, Average path velocity; WBC, White blood cell; WHO, World Health Organization.
patients with spermatozoa from normal donors and vice versa. Defining sperm motility as the main evaluation parameter, it was found that, after 5 min, SP from SCI men inhibited motility of spermatozoa from normal controls. Conversely, SP from controls improved cellular motility of spermatozoa from SCI patients, clearly indicating that the SP of these patients present abnormalities that are somehow deleterious to sperm. In order to strengthen these findings, the same authors compared *vas deferens* aspirated to ejaculated spermatozoa in SCI patients and controls (7). Interestingly, sperm motility and viability were significantly higher when spermatozoa were directly aspirated from the *vas deferens* before any contact with the glandular fractions of the ejaculate in SCI patients. Although aspirated cells from patients presented somewhat lower motility and viability compared with controls, implying that epididymal or testicular factors may also be responsible, by far the greatest decrease in the measured sperm parameters was observed after contact with SP.

Proteins are highly abundant molecules in human SP. The average protein concentration ranges from 35 to 55 mg/ml (8). Proteins also constitute the main level of functional interaction with spermatozoa. Some SP proteins are known to be specific for key cellular processes such as sperm capacitation (9), sperm–zona pellucida interaction, and sperm–oocyte fusion (10, 11). We previously presented an initial qualitative report of the SP proteome from SCI patients and control individuals (12). In that study, a total of 638 individual proteins were identified and 119 proteins showed differential expression. At that time, we observed that prostatic proteins such as prostatic specific acid phosphatase (PSAP) and other enzymes like carboxypeptidases (e.g. CPE) were absent in patients. At the same time, a variety of proteins including apolipoproteins (e.g. APOB) and immunoglobulins (e.g. IGHG2) were found exclusively in samples from SCI patients. These findings suggested a deviation from homeostasis occurring in the SP of SCI patients, presumably altering its function and accounting for the poor seminal quality, which is typical of these individuals (12). Nevertheless, how a SCI post-traumatic scenario influences the actions of SP proteins leading to infertility and which pathways hinder sperm function remained unclear.

In the present study, we used MS based proteomics to qualitatively and quantitatively assess the SP proteome of SCI patients and controls. We investigated SP obtained from SCI patients both on pooled samples as well as individual patients. As a result, we obtained the most extensive list of human SP proteins reported to date (2,820 identified proteins). We also report on the possible molecular mechanisms underlying SCI related infertility. Thus, our results not only describe the SCI related infertility in a molecular level but also improve the general knowledge about the relation between SP and spermatozoa functionality, constituting a valuable data set for future studies.

**EXPERIMENTAL PROCEDURES**

**Subjects, Semen Collection, and Seminal Analysis**—All SCI patients and control volunteers included in the study presented good general health and were participating in the Male Fertility Research Program of the Miami Project to Cure Paralysis, University of Miami Miller School of Medicine. The study was approved by the University of Miami Institutional Review Board, and informed consent was obtained from each of the subjects. In total, seminal samples from 12 SCI patients and 11 controls were collected, analyzed and further prepared for proteomic analysis. The mean age ± standard error of SCI subjects was 38 ± 10 years and the level of injury ranged from C4 to T6. All patients had already passed the period of spinal shock (≥12 months after injury) by the time of semen collection, which was obtained using the standard method of penile vibratory stimulation (PVS) as described elsewhere (13); only antegrade specimens were collected. All control volunteers were noninjured and normospermic men with no known history of infertility. Fertility status and/or proved paternity were not considered for including a donor in the control group. Controls collected semen by masturbation in specific sterile containers after at least 3 days, but not longer than 7 days, of ejaculatory abstinence. After liquefaction at room temperature, semen analysis was performed for all samples. Sperm concentration (millions of sperm/ml ejaculate), total sperm count, sperm motility (% with forward progression) were evaluated by computer-aided semen analysis (CASA) using the IVOS II Clinical system version 12.2 (Hamilton Thorne, MA). The progressive motility settings were 250 μm/s for the path velocity (VAP) and 80% for the straightness (STR). The cut-off parameter for the slow cells was 5.0 μm/s for the VAP and 11.0 μm/s for the progressive velocity (VSL). The seminal white blood cell (WBC) concentration (millions of WBC/ml ejaculate) was measured by unstained wet smear. All reference values were used according to the guidelines from the World Health Organization (WHO) (14). Detailed information about the individual patients are provided in [supplemental Table S1](#).

**Seminal Plasma Preparation and Lys-C/Tryptsin Sequential Protein Digestion**—Immediately after semen analysis, all samples were centrifuged at 1,000 × g for 15 min. The pellet was discarded and the supernatant (i.e. seminal plasma) recovered and stored at −80 °C until use. To perform the proteomics assays, all samples were thawed at room temperature and centrifuged at 1,000 × g for 30 min at 4 °C to eliminate remaining cellular debris or occasional spermatozoa. Supernatants were collected and total protein concentration was assessed for each SCI and control subject by the Bradford assay (15). Initially, samples from 10 SCI patients were pooled together to form a SCI representative pool. Similarly, the 11 control donors were pooled together to form a representative control pool. SCI and control pools were prepared containing 1 mg of total protein. Both groups were further analyzed in triplicate. In a second step, SCI individual samples were prepared to contain 1 mg of protein each. SCI and control pools and the 12 individual SCI samples were resolved on 50 mM triethylammonium bicarbonate (TEAB), 8 mM urea buffer and vortexed thoroughly. The disulfide bonds of SP proteins were reduced with 10 mM dithiothreitol (DTT) at 37 °C for 1 h and then alkylated with 55 mM chloroacetamide at room temperature for 30 min in the dark. A urea concentration of 6 M was adjusted with 50 mM TEAB and 20 μg of Lys-C (Wako, Osaka, Japan) was added for the first digestion step (enzyme to protein ratio 1:50). Samples were then diluted to 2 M urea with 50 mM TEAB and 20 μg of trypsin (modified sequencing grade; Promega, Wisconsin, USA) was added to the samples (enzyme to protein ratio 1:50). For both digestion conditions proteinolysis were carried out at 37 °C for 4 h (Lys-C) and overnight (trypsin).

**Stable Isotope Dimethyl Labeling and Hydrophilic Strong Anion Exchange (hSAX) Chromatography for Peptide Separation**—The digested samples were dried down by vacuum centrifugation (1,500
injected in the system. Peptide separation was achieved with a flow rate of 5 µL/min using a Dionex Ultimate 3000 HPLC system (Dionex Corp., CA) to an Orbitrap Elite (Thermo Scientific, CA) to an Orbitrap XL ETD (Thermo Scientific). The Orbitrap Elite was operated in positive ion mode with a normalized collision energy of 35% and a resolution of 15,000. Tandem mass spectra were generated for up to 12 peptide precursors in the linear ion trap for fragmentation using higher energy collisional dissociation (HCD) at normalized collision energy of 35% after accumulation to a target value of 5,000 for a maximum of 100 ms.

**Protein Identification, Quantification, and Statistical Analysis—**Raw MS spectra were processed by MaxQuant (version 1.5.2.8) for peak detection and quantification. MS/MS spectra were searched against the Uniprot human reference proteome database (70,076 sequences, downloaded on November 6th, 2015) by Andromeda search engine enabled contaminants and the reversed versions of all sequences with the following search parameters: Carbamidomethylation of cysteine residues as fixed modification and Acetyl (Protein N-term), Oxidation (M) as variable modifications. Trypsin/P was specified as the proteolytic enzyme with up to 2 missed cleavages allowed. The mass accuracy of the precursor ion was defined by the time-dependent recalibration algorithm of MaxQuant, fragment ion mass tolerance was set to 0.6 Da. The maximum false discovery rate for proteins and peptides was 0.01 and a minimum peptide length of six amino acids was required. Quantification mode with the dimethyl Lys 0 and N-term 0 as light labels and dimethyl Lys 4 and N-term 4 as heavy labels was selected. All other parameters are the default settings in MaxQuant. Quantitative ratios were calculated by MaxQuant based on two light and medium label partners for each protein and normalized by shifting the median of the total ratio population to 1. Normalized ratios were used for the differential expression analysis and statistical significance was assessed using paired t test on proteins that are quantified all replicates of the pool experiment and at least two samples of the individual experiment. Statistical analyses were performed using the R software (version 3.0.0).

**GO Enrichment/Pathway Analysis/Complex Analysis—**Classification and functional enrichment analysis of the identified and differentially expressed proteins (adj. p < 0.05) were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov), a Bioinformatics Database for the biological process (BP), molecular function (MF), and cellular component (CC). Tissue origin analysis was also performed through the use of DAVID. The Kyoto Encyclopedia of Genes and Genomes (KEEG) (http://www.genome.jp/kegg/) database was used to map the differential expressed proteins to KEGG pathways for biological interpretation. For the creation and investigation of protein-protein interaction maps, Cytoscape 2.8.2 (http://www.cytoscape.org) with Bisogenet 1.41 (http://apps.cytoscape.org/apps/bisogenet) plugin was used. This plugin integrates data from well-known interaction databases including DIP, BIOGRID, HPRD, BIND, MINT, and INTACT and displays the result as an interaction network within Cytoscape. For our purposes only the physical interactions were considered.

**Data Availability**—The original mass spectrometric raw data files along with the MaxQuant search result files are available on proteinXchange (18), http://www.proteomexchange.org, under the accession number PXD002145.

**RESULTS AND DISCUSSION**

**Qualitative Map of the Human Seminal Plasma Proteome—**There is a growing body of evidence pointing to SP as a key regulator of spermatozoa homeostasis and as an important effector of male infertility. It has been described that the success of the fertilization process is intrinsically related to the complex protein content present in SP (19), which serves functions in the different steps such as sperm capacitation (9), immune response inside the uterus, formation of the tubal
sperm reservoir, sperm-zona pellucida interaction, and sperm-oocyte fusion (20–22). Additionally, Brackett et al. demonstrated that SP is capable of impairing and/or restoring sperm motility (6). In the present study, we used state-of-the-art proteomics to extend the coverage of the human SP proteome and to perform an in depth analysis (as described in Fig. 1) of proteins present in the SP of SCI patients.

As a result, we have obtained the most extensive proteomic analysis of SP to date, extending coverage by about three times over the most cited study on the human SP proteome (8) (Fig. 2A). In total 2,550 proteins were identified in the pool experiment (Fig. 2B, supplemental Table S2 and S3) at a protein false discovery rate (FDR) of 1%. On average, 2,279 proteins were identified per technical replicate (supplemental Fig. S1A) and the overlap among the experiments are shown in supplemental Figs. S1B and S1C. For the individual experiment (i.e. proteome measurements of individual patients), a total of 1,534 proteins were identified (Fig. 1C, supplemental Table S4 and S5, protein FDR 1%) and ~1,000 proteins were identified per individual patient (supplemental Fig. S1C). When combining both data sets, a total of 2,820 nonredundant proteins were identified (Fig. 1D, supplemental Table S6).

Our data set ranges from high abundance proteins such as the semenogelins, kallikreins, the main serine proteases in SP, lactotransferrin and fibronectin to very low abundance enzymes such as phosphatases and dehydrogenases.

**Fig. 1. Experimental Design.** MS-based quantitative proteomic strategy was used to compare the SP proteome in SCI patients versus controls. SP was obtained from 12 SCI patients and from 11 healthy control donors. All control samples were grouped together forming a representative protein pool whereas SCI samples were either grouped together (10 samples) or analyzed individually (12 samples). Major differences between groups were assessed through the comparison of pooled samples (“pool experiment”) whereas particular variations among individuals were investigated through the analysis of single samples (“individual experiment”). Samples from the “pool experiment” and from the “individual experiment” were subjected to a Lys-C/Trypsin combinatorial protein digestion. The resulting peptides were dimethyl labeled, “light” for peptides from SCI samples (pool and individuals) and “medium” for peptides from control sample (pool). Light and medium labeled peptides were mixed 1:1 and the final mixture submitted to an off-line fractionation on a hSAX column. A total of 48 fractions were collected for the “pool experiment” and 36 fractions for the “individual experiment.” The hSAX fractions were finally analyzed by LC-MS/MS on an LTQ-Orbitrap Elite (“pool experiment”) or on a LTQ-Orbitrap XL (“individual experiment”) mass spectrometer. Differential quantification was assessed using the software package MaxQuant version 1.5.2.8 and the mass difference of the dimethyl labels were used to compare the peptide abundance in different samples, making it possible to calculate the corresponding control/patient protein expression ratios.
over, GO enrichment analysis showed that the majority of proteins is composed of extracellular and intracellular proteins (Fig. 2E). Interestingly, we identified proteins from almost all cellular compartments including the endoplasmic reticulum, mitochondria, nucleus, membrane, cytosol, and cytoplasm. These proteins likely originate from shedded epithelial cells and/or from secretory cells. Furthermore, the identification of high amounts of certain proteins like acrosin (ACR), a serine protease with trypsin-like specificity stored specifically in the acrosome of mature sperm cells (23), shows that cellular disruption or leakages of spermatozoa also contribute to the contamination of SP with cellular proteins. In addition, as previously described, SP bears large amounts of immune system proteins, many of which may be produced and released by leukocytes.

Quantification of Proteins Between SCI Patients and Control Subjects—Quantitative ratios of proteins, between SCI and control samples, were obtained by dimethyl labeling and calculated using the software package MaxQuant (24) and requiring a minimum of two pairs of labeled peptides per protein. For the pool experiment, three technical replicates were analyzed and the good reproducibility among replicates (supplemental Figs. S2A and S2B) enabled statistical analysis within and across groups. Similarly, the 12 individual analyses showed good reproducibility, enabling statistical analysis to consider each single patient as one biological replicate in order to assess differences within the SCI group. The data was normalized by shifting the median of all protein ratios to 1 (i.e. no change between SCI and control, supplemental Figs. S3A and S3B). For the pool experiment, about 68% of the identified proteins were also quantified (1,730 proteins). Moreover, about 19% of the identified proteins were found exclusively in one group or the other (344 proteins in SCI and 146 proteins in control). For the individual experiment, around 80% (1,230 proteins) of the identified proteins were also quantified and 14% (212 proteins) were exclusively identified in one group or the other (110 proteins in SCI and 102 proteins in control). For subsequent data and bioinformatics analysis, we only considered proteins that could be either identified or quantified in all replicates from the pool experiment and/or in at least two patients from the individual experiment; p values were calculated and adjusted for multiple testing and proteins showing significant regulation (adjusted \( p < 0.05 \)), either elevated or decreased in the SP of SCI patients, are shown in Figs. 3A and 3B.

Functional interpretation of the regulated proteome was aided by GO enrichment analysis. The majority of elevated proteins in SCI have an extracellular localization followed by membrane-bounded vesicle, and vesicle lumen (Fig. 3C). Proteins showing decreased expression in SCI also mostly represent the extracellular space, vesicles and membrane-bounded vesicles (Fig. 3D). Moreover, proteins diminished in

![Fig. 2. Overview of human SP proteome. A, Venn diagram showing the overlap between the proteins identified in our study and the previously reported proteome of SP from a healthy man (8). B, Venn diagram showing the overlap between the proteins identified in the SCI and control groups in the pool experiment. In total 2,550 proteins were identified and an overlap of 2,060 proteins was observed between the two groups. C, Venn diagram showing the overlap between groups in the individual experiment. A total of 1,534 proteins were identified with an overlap of 1,322 proteins between groups. D, Venn diagram showing the overlap between the two experiments performed in our study. A total of 2,820 unique proteins were identified. E, GO analysis of all identified proteins indicates the subcellular localization of SP proteins.](image)
SCI are more related to typical cellular processes such as protein transport, regulation of apoptosis, carbohydrate metabolic process and small GTPase mediated signal transmission (Fig. 3D) whereas elevated proteins in SCI are mainly related to responses to external stimuli, defense response, immune response and acute inflammatory response (Fig. 3C).

**Immune System Related Proteins in the SP of SCI Patients**—Although hypotheses that inflammatory processes may be associated with the poor semen quality observed in SCI patients have been challenged (25, 26), our in-depth study of the SP proteome enlivens this debate. The quantitative assessment of the SP proteome from SCI patients confirmed what we have previously speculated (12), showing that the majority of the elevated proteins in SCI are connected to the immune system. Components of the adaptive immune system (different isotypes of immunoglobulins) as well as of the innate immune system (acute-phase enzymes and complement system proteins) were among the most highly abundant proteins in the SP of SCI patients. The presence of several of these proteins indicates a strong activation of leukocytes in the reproductive tissues of SCI men. Myeloblastin (PRTN3) and neutrophil elastase (ELANE), for example, were exclusively identified in SCI samples but with very high quantities (judged from the MS intensity) (Fig 3A). Both are serine proteases expressed mainly by neutrophils during inflammatory processes (27, 28). Similarly, neutrophil defensin1 (DEFA1), a member of the defensin family of proteins involved in host defense and found specifically in the microbicidal granules of neutrophils (29), was also exclusively identified in the SCI group (Fig 3A). Several studies have demonstrated the importance of protease inhibitors in regulating the activity of proteases released by leukocytes during inflammation (30–32). The in situ balance between released proteases and protease inhibitors determines the overall proteolytic activity in SP and the extent of potential proteolytic damage in the tissue of the surrounding area (33). Different types of protease inhibitors, mainly serine protease inhibitors, were identified in our seminal samples, corroborating the findings of Pilch and Mann (8). Surprisingly, the majority of these protease inhibitors were elevated in SCI. Alpha-2-macroglobulin (A2M) and pregnancy zone protein (PZP) are two homologous glycoprotein protease inhibitors elevated in SCI. Both proteins exhibit...
A broad spectrum, capable of suppressing all classes of proteases by a specific trapping mechanism (34, 35). PZP, exclusively identified in the SCI group, is one of the major pregnancy-associated plasma proteins and closely resembles A2M, which, in turn, is synthetized in reproductive tissues by Sertoli cells under FSH control (36). Although the main function of A2M is to control fibrinolysis by inhibiting plasmin and serum kallikrein and to regulate the coagulation cascade by the inhibition of thrombin (37), some studies have associated A2M with sperm motility (38). We observed that A2M is, on average, threefold more abundant in the SP of SCI patients but no direct correlation between motility and A2M abundance was observed (data not shown). In addition to macroglobulins, about 41% of all characterized protease inhibitors elevated in SCI are members of the largest and most diverse family of protease inhibitors, the serpin superfamily. To date, 36 human proteins have been described to constitute the serpin family, which controls proteolytic pathways that require tight regulation, such as blood coagulation, inflammation, fibrinolysis, and SP liquefaction (39). In total 12 serpins were quantified in our study and we observed, for example, that SERPINB9 and SERPINB13 were exclusively identified in the SP of SCI patients. Thyroxine-binding globulin (SERPINA7) was significantly elevated only in the pool experiment (1.7-fold). Similarly, α1-antichymotrypsin (SERPINA3), kallistatin (SERPINA4), monocyte neutrophil elastase inhibitor (SERPINB1), plasminogen activator inhibitor 1 (SERPIND1), and pigment epithelium derived factor (SERPINF1) were all significantly elevated in the individual experiment. However, α-1 antitrypsin (SERPINA1), protein C inhibitor (SERPINA5), Antithrombin-III (SERPINC1), and plasma protease C1 inhibitor (SERPING1) presented the most pronounced differences between groups in both experiments. SERPINA5, the main serpin in human SP (seminal concentrations ranging between 150–200 μg/ml) (35–37) is, for example, threefold more abundant in SCI (Fig. 4A). Regulation of enzymatic activity in the liquefaction cascade by inhibition of kallikrein activity is the main role of SERPINA5 (40). Although the seminal liquefaction process is not fully understood (41), it is known that kallikreins are secreted from the epithelium of the glandular prostate into SP to ensure the proteolysis of semenogelins (SEMG1 and SEG2) secreted by seminal vesicles. Semenogelins completely arrest the movement of spermatozoa by associating with a cell surface component localized on the flagellum of each cell. Thus, the proteolytic degradation of SEMG1 and SEMG2 leads to a decrease in their inhibitory effect on sperm motility. As a regulatory mechanism, SERPINA5 is trapped by SEMG1 and SEMG2 in seminal vesicles and is released from the coagulum immediately after ejaculation as part of the kallikrein 3 (KLK3) catalyzed liquefaction progresses. SERPINA5, in turn, complexes with KLK3 and inhibits its serine protease activity, controlling the degradation of SEMG1 and SEMG2 and consequently the liquefaction cascade (41, 42).
Using bioinformatics tools we queried the differential SCI proteome to reveal which molecular pathways may be affected by a higher abundance of protease inhibitors. Interestingly, it was observed that the seminal liquefaction cascade seems to be the most harmed seminal process (Fig. 4B). Both PZP and A2M can interfere in this process via a physical interaction with KLK3. Furthermore, an interaction between SERPINA1 and SERPINA3 with KLK3 as well as an interaction between SERPINA3, SERPINA4, and SERPINC1 with KLK2 was observed. Clinical correlates of this fact are intriguing because semen of SCI men typically shows delays in liquefaction (compared with normal men) but still liquefies within 30 min, and would thus be considered normal by World Health Organization (WHO) standards (43). However, so far, we only estimated protein quantities judged from MS intensities and did not perform any protein activity assay for the protease inhibitors described. In any case, our analysis strongly suggests that, in SCI patients, the liquefaction cascade can potentially be delayed because of inhibition of kallikrein activities by different protease inhibitors. Therefore, after ejaculation, sperm cells can remain immotile, entrapped in the gel clot formed by semenogelins. Furthermore, new evidences indicate that some biochemical properties of semenogelins, mainly related to post-translational modifications (unpublished data) also contribute to the poor motility observed in SCI patients. Despite the fact that our findings were made in the context of the SCI proteome, the results offer insight and direction into investigating infertility in the general male population because it is likely that infertility because of hyper viscosity (44) resembles the molecular mechanisms described here.

Regarding the immune system related proteins, one plausible explanation for their strong elevation may be offered by urinary tract infections (UTIs) or to male accessory gland infections (MAGIs), caused by the need of frequent catheterization of SCI patients, both of which lead to inflammatory processes of reproductive tissues. The proteomic data showed no contamination whatsoever of SCI seminal plasma with proteins of bacterial or fungal origin (data not shown). Further, the use of antibiotics to treat UTIs resulted in little or no change to the semen parameters of SCI patients (45), suggesting that noninfectious causes of an inflammatory response in the semen may be of more importance than previously thought. Although most men with SCI have leukocytospermia (46, 47), our clinical observation of thousands of semen analyses in SCI patients indicates the lack of a relationship between sperm motility and the concentration of leukocytes in the semen of these men (unpublished data). The origin of the abundant numbers of leukocytes in the semen of SCI men is unclear. Randall et al. investigated the prostate gland as the source but could find no strong evidence to support prostate tissue as the origin of the leukocytes (48).

Prostate Dysfunction Plays a Key Role in SCI Related Infertility—The main protease in SP, KLK3, was 5.9-fold less abundant in patient pools versus control plasma (Fig. 5A). Reduced
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KLK3 was observed in all individual SCI patients with reduction factors ranging from 1.4-fold to 39.8-fold (Fig. 5B). The same was observed for two other kallikreins; kallikrein 2 (KLK2) was 6.4-fold less abundant in SCI pools (Fig. 5C) and KLK11 was 2.5-fold less abundant in the same group (Fig. 5E). The respective figures for individual patients ranged from 1.7 fold to 43.3-fold for KLK2 (Fig. 5D) and from 0.7-fold to 28.6-fold for KLK11 (Fig. 5F).

In total, 15 human kallikreins have been described and, as previously discussed, are the main proteases fostering sperm motility by acting directly or indirectly on the liquefaction cascade after ejaculation. Our study identified five different kallikreins (KLK1, KLK2, KLK3, KLK6, and KLK11) in SP samples and 3 of them (KLK2, KLK3, KLK11) were quantified. It has been reported that KLK3, which is contained in SP at high concentrations under physiological conditions (0.5 to 0.3 mg/ml) (49), is decreased in SP of SCI patients. In agreement with that, our study showed that this protein is sixfold more abundant in control samples and that all the 12 individual SCI patients exhibited a significant decrease in its abundance. Similarly, the same is observed for two other kallikreins, KLK2 and KLK11. Rather than an isolated fact, the decrease of kallikreins seems to be related to an overall failure of the prostate gland because many other prostatic proteins were also decreased in the SP of SCI patients.

Tissue origin analysis of the SCI regulated proteome was performed using the Database for Annotation, Visualization and Integrated Discover (DAVID). The analysis showed that proteins decreased in SCI primarily originate from the prostate rather than other accessory glands. In fact, 46% of all proteins decreased in SCI are produced by secretory cells of the prostate gland. Moreover, no proteins assigned to the epididymis or testis were indicated in the analysis. The decreased proteins of prostate gland origin varied from kallikreins, members of the Rab family of proteins, to metabolic proteases. Just to name a few, the annexin proteins, ANXA4, ANXA5, and ANXA7, a group of protease inhibitors, which may participate in lipid metabolism by the inhibitory activity on phospholipases A1 and A2 (50) were quantified and show decreased abundance in SCI. Moreover, ACPP, a tyrosine phosphatase that dephosphorylates large numbers of substrates and which has been widely used a biomarker for prostate cancer (51), was observed to be around sevenfold more abundant in the SP of controls. Thus, our proteomic results strongly support what other studies have only hypothesized (12, 52), i.e. that a prostatic dysfunction could somehow be involved in SCI related infertility. However, the possible causes underlying the disruption of prostatic functionality after SCI remain unknown. It is possible that a dysfunctional status of the prostate is because of severe inflammation and infection (prostatitis) culminating in loss of tissue functionality or a block of the glandular secretory ducts. It is also possible that the lack of neurologic control generated by the SCI leads to glandular atrophy and a decrease in functionality as reported in animal models (53). The neurogenic stimulation of the male accessory sexual glands including the prostate arises from sympathetic nerves from the thoracolumbar spinal cord (T11-L2) and from parasympathetic nerves from the sacral spinal cord (S1-S4) (54). Brasso et al. reported that SP concentrations of KLK3 were significantly lower in SCI patients with lesions below T7 compared with patients with lesions at or above this level (52). Studies have shown that the prostate gland is smaller in men with SCI compared with healthy, noninjured men, and that this effect may be because of a prolonged central (rather than peripheral) deinnervation of the prostate gland (55). However, further pathophysiologically studies of the prostate gland are necessary to investigate how a neurological trauma influences glandular function. As observed in our study, a hypofunctional prostate leads to an altered proteome, which, in turn, may lead to a multitude of metabolic consequences. To begin to address this, we analyzed the lipid content of SP samples by MALDI-TOF MS (supplemental Fig. S4). However, no differences in the lipid profiles of SP of SCI patients and control groups were observed. Still, we cannot rule out that the lipid composition of sperm cells may be altered, an aspect that may be further investigated in the future.

As prostate-derived proteins are mainly secreted into SP via prostasomes, we compared our results with the detailed proteomic analysis of prostasomes reported by Poliakov et al. who identified 440 proteins (56). Prostasomes are exosome-like microvesicles resulting from a fusion process between the membrane surrounding the storage vesicle and the plasma membrane of the prostate epithelial cell responsible for exocytosis (56). Out of the 285 proteins decreased in SCI, not less...
than 114 (40%) were found to originate from prostasomes (Fig. 6).

In addition, at least ten key metabolic enzymes involved in ATP turnover are found in both studies, notably 6-phosphogluconolactonase (PGLS), alcohol dehydrogenase [NAPD([+] (AKR1A1), L-xylulose reductase (DCXR), fructose-1,6-bisphosphatase 1 (FBP1), L-lactate dehydrogenase B chain (LDHB), malate dehydrogenase, cytoplasmic (MDH1), phosphoglucomutase-1 (PGM1), phosphoglycerate mutase 1 (PGAM1), glycogen phosphorylase (PYGB), and sorbitol dehydrogenase (SORD). Further proteins either related to oxidoreductase processes, such as aldo-keto reductases and peroxiredoxins, or to GTPase activity and protein transport were also among the characterized prostasome proteins (decreased in SCI) further strengthening the connection between SCI induced infertility and the prostate gland. This appears to be a key mechanism leading to the diminished cellular motility and consequent infertility observed in SCI. The physiological function of these enzymes with capacity to form ATP is not known but Ronquist et al. (57) have demonstrated their operational activity for ATP formation when supplied with substrates. Moreover, these authors have suggested that glycolytic enzymes may be transferred from prostasomes to sperm cells to ensure sperm motility and fertilization, constituting a pool of proteins from which enzymes/proteins insufficiently represented in or lost from sperm cells can be replaced (57). These hypotheses can be corroborated by our results because our data greatly suggest that the absence of proteins derived from prostasomes are associated with decreased sperm motility, as observed in SCI patients.

In conclusion, this study described SCI related infertility on a proteomic level, providing strong molecular evidence for the factors leading to impaired sperm functionality, notably sperm motility. Our data shows that SCI patients show strong actuation of the immune system, which does not seem to be triggered by microbial infection. We also found a striking induction of protease inhibitors and simultaneous loss of proteases explaining the low motility of sperm cells in the SP of SCI men. Last but not least, the data provides strong evidence that a loss of prostate function is a contributing factor in the observed molecular and macroscopic phenotype of SCI infertility.

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