Verification of Male Infertility Biomarkers in Seminal Plasma by Multiplex Selected Reaction Monitoring Assay*§

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Seminal plasma is a promising biological fluid to use for noninvasive clinical diagnostics of male reproductive system disorders. To verify a list of prospective male infertility biomarkers, we developed a multiplex selected reaction monitoring assay and measured the relative abundance of 31 proteins in 30 seminal plasma samples from normal, nonobstructive azoospermia and post-vasectomy individuals. Median levels of some proteins were decreased by more than 100-fold in nonobstructive azoospermia or post-vasectomy samples, in comparison with normal samples. To follow up the most promising candidates and measure their concentrations in seminal plasma, heavy isotope-labeled internal standards were synthesized and used to reanalyze 20 proteins in the same set of samples. Concentrations of candidate proteins in normal seminal plasma were found in the range 0.1–1000 µg/ml but were significantly decreased in nonobstructive azoospermia and post-vasectomy. These data allowed us to select, for the first time, biomarkers to discriminate between normal, nonobstructive azoospermia, and post-vasectomy (simulated obstructive azoospermia) seminal plasma samples. Some testis-specific proteins (LDHC, TEX101, and SPAG11B) performed with absolute or nearly absolute specificities and sensitivities. Cell-specific classification of protein expression indicated that Sertoli or germ cell dysfunction, but not Leydig cell dysfunction, was observed in nonobstructive azoospermia seminal plasma. The proposed panel of biomarkers, pending further validation, could lead to a clinical assay that can eliminate the need for testicular biopsy to diagnose the category of male infertility, thus providing significant benefits to patients as well as decreased costs associated with the differential diagnosis of azoospermia. Molecular & Cellular Proteomics 10.1074/mcp.M110.004127, 1–13, 2011.

Human infertility affects ~15% of couples, with the male contributing to the infertility in 50% of all cases (1, 2). One of the most severe forms of male infertility is azoospermia, which is characterized by an absence of sperm in the semen (3). Azoospermia is diagnosed in 20% of subfertile men and has two forms: obstructive azoospermia (OA)¹ and nonobstructive azoospermia (NOA). OA is caused by a physical obstruction in the male reproductive tract. The biological outcome of OA is thus identical to that of vasectomy, which is a surgical severance of the vas deferens. NOA is more complicated infertility syndrome with the azoospermia being secondary to a failure to produce sperm; NOA may be further subclassified as maturation arrest, Sertoli cell-only syndrome, and hypospermatogenesis (4).

For most men with azoospermia, testicular biopsy is the only currently used method to definitively distinguish between OA and NOA (5, 6). Thus, there is an urgent need for an alternative noninvasive approach with better diagnostic potential. The differential diagnosis of normal, NOA, and OA (or post-vasectomy (PV)) men is required for the following reasons: (i) in infertile patients, use of markers capable of differentiating NOA and OA could eliminate the requirement for a diagnostic testicular biopsy and (ii) in healthy individuals who have undergone a vasectomy, markers capable of differentiating normal and PV seminal plasma will reveal whether the vasectomy (and later, its possible reversal) was successful.

Proteins are highly promising biomarkers for clinical diagnostics. However, no biomarkers currently exist for the definitive differential diagnosis of OA and NOA. Blood plasma levels of inhibin B or follicle-stimulating hormone were proposed for NOA diagnosis, but these molecules had poor specificity and sensitivity (7, 8). Protein levels in local fluids, such as seminal plasma, may have a better potential for genitourinary

¹ The abbreviations used are: OA, obstructive azoospermia; PV, post-vasectomy; NOA, nonobstructive azoospermia; SP, seminal plasma; KLK3, kallikrein 3; IS, internal standard; SRM, selected reaction monitoring; iSRM, intelligent SRM; MS/MS, tandem mass spectrometry; Q1, first quadrupole; Q2, second quadrupole; Q3, third quadrupole.
diagnostics (9). For example, PTGDS protein has been recently proposed as a seminal plasma biomarker for the diagnosis of OA (10). However, PTGDS could not distinguish NOA from normal and OA (PV) groups with high confidence.

Recent progress in biological mass spectrometry has facilitated the identification of several thousand proteins in biological fluids (11, 12). Although identification of proteins is now straightforward, routine quantification by mass spectrometry, especially for low abundance proteins in complex mixtures, is still a challenge that requires considerable methodological and instrumental advances. Quantitative selected reaction monitoring (SRM) assays (13, 14) were introduced as a means to supplement antibody-based ELISAs that are widely used in clinical practice. Quantification and verification of biomarkers by SRM assays is an emerging field of proteomics (15–17).

The proteome of seminal plasma is as complex as the proteome of blood serum and contains large amounts of semenogelins, kallikrein 3 (also known as prostate-specific antigen), and other high abundance proteins (18–20). Using two-dimensional LC-MS/MS, we have recently identified between 2000 and 2100 proteins in seminal plasma from normal and PV individuals (20). A group of 79 proteins was found to be underexpressed by more than 1.5-fold in PV samples, based on semi-quantitative spectral counting comparisons (20).

To narrow down the list of proteins in this work, we used the strategy of stepwise elimination of poorly performing candidates. In the final assay, we aimed at analyzing only those proteins the peptides of which would be reproducibly quantified by SRM in the unfraccionated digest of seminal plasma. With the final multiplexed SRM assay, we measured the concentration of 20 proteins in 30 normal, NOA, and PV seminal plasma samples. We included the NOA group based on the hypothesis that like PTGDS protein (10), other proteins absent in PV seminal plasma may also be underexpressed in NOA seminal plasma. For the first time, we propose a panel of biomarkers for the differential diagnosis of azoospermia with absolute or nearly absolute specificities and sensitivities.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials and chemicals were used: sequencing grade modified trypsin (Promega, Madison, WI), iodoacetamide, DTT (Sigma-Aldrich Canada, Oakville, ON), RapiGest surfactant (Waters, Milford, MA). Heavy isotope-labeled peptides were obtained from Thermofisher Scientific Inc., Ulm, Germany.

**Patients and Specimens**—Seminal plasma samples were obtained by masturbation with informed consent and Mount Sinai Hospital institutional review board approval from normal fertile men about to undergo a vasectomy (n = 12), infertile men with proven nonobstructive azoospermia (n = 10), and previously fertile men who had undergone a vasectomy (n = 8).

**Sample Preparation**—Seminal fluid was allowed to liquefy at room temperature for 1 h after collection. Seminal fluid was aliquoted in 1-ml portions and centrifuged at 13,000 × g for 15 min at room temperature three times to separate plasma from cells and cellular debris. The supernatant seminal plasma was then frozen at −80°C until use.

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**Seminal Plasma Digestion for SRM Assays**—Ten microliters of seminal plasma were diluted 10-fold and subjected to trypsin digestion without prior purification or removal of high abundance proteins. Proteins were denatured with 0.1% RapiGest at 60 °C, and the disulfide bonds were reduced with 10 mM dithiothreitol. Following reduction, the samples were alkylated with 20 mM iodoacetamide. The samples were then trypsin-digested overnight at 37 °C. One hundred femtomoles of heavy 13C6, 15N2-L-lysine-labeled peptide LSEPA-ELTDAVK of KLK3 protein or a mixture of 20 heavy isotope-labeled peptide standards was added to each digest. RapiGest was cleaved with 1% trifluoroacetic acid, and a 96-well plate with all samples was centrifuged at 4000 × g for 20 min. The peptides were extracted with 10 μl of OMIX C18 tips (Varian, Lake Forest, CA), eluted with 65% acetonitrile, and diluted to 130 μl to provide three 40-μl injections.

**Peptide Selection for SRM**—Proteotypic peptides were manually chosen in Scaffold using Orbitrap identification data that included proteins identified with a 1.1–1.5% false discovery rate (20). Typical accuracy of peptide identification was 2–4 ppm. SRM candidate peptides that had clear and intense y-ion fragments (especially at proline residue) were selected. Peptides that had modifications and/or cysteine, methionine, and tryptophan amino acids were avoided, if possible. To confirm the choice of peptides, in silico digestions and fragmentations were performed using Pinpoint software (Thermo Fisher Scientific BRIMS, Cambridge, MA), which was also used to generate SRM methods for the triple quadrupole mass spectrometer.

**LC Conditions**—Tryptic peptides were separated on a 2-cm C18 trap column with an inner diameter of 150 μm. The peptides were eluted from the trap column onto a resolving 5-cm analytical C18 column (inner diameter, 75 μm) with a 15-μm tip (New Objective, Woburn, MA). The LC setup was coupled online to a triple-quadrupole mass spectrometer (TSQ Quantum Ultra or TSQ Vantage; Thermo-Fisher Scientific Inc, San Jose, CA) using a nanoelectrospray ionization source (nano-ESI, Proxeon Biosystems, Odense, Denmark). Buffer A contained 0.1% formic acid in water, and buffer B contained 0.1% formic acid in acetonitrile. A three-step gradient was used with an injection volume of 40 μl, which was loaded onto the column via an EASY-nLC pump (Proxeon Biosystems, Odense, Denmark).

**Verification of Peptide Identity by iSRM and SRM-triggered MS/MS**—Each iSRM method included five peptides in scheduled acquisition windows. Each peptide was monitored with two primary transitions that were selected based on LTQ-Orbitrap identification data (20). A threshold of 500 was used to trigger an acquisition of six additional transitions with 0.1-s scan time each. The resulting raw files were uploaded to Pinpoint software, and an overlay of all individual transitions for each peptide was inspected manually. SRM-triggered MS/MS methods had 1-s full MS/MS scan. The MS/MS spectra were analyzed using the Mascot (Matrix Science, London, UK; version 2.2) search engine on the nonredundant International Protein Index human database (version 3.69; February 10, 2010). Unfractionated digest of normal seminal plasma was used in all of the experiments.

**Label-free SRM**—For a label-free SRM assay, 32 peptides and 96 transitions representing 31 proteins were scheduled within 2-min intervals during a 60-min LC gradient (84-min method) and analyzed by TSO Quantum Ultra in the positive-ion mode. The SRM method had the following parameters: predicted collision energy values, 0.002 m/z scan width, 0.1-s scan time, 0.2 Q1, 0.7 Q3, 1.5 mTorr Q2 pressure, tuned tube lens values, 7 V skimmer offset. The three most intense and reproducible transitions for each peptide based on iSRM results were included in the SRM method. Spiked-in heavy isotope-labeled peptide of KLK3 was used as an internal standard to normalize the relative abundances of all peptides and thus account for
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Fig. 1. Stepwise workflow to verify biomarkers for differential diagnosis of azoospermia by SRM.

| 2100 proteins | Select proteins differentially expressed by spectral counting | Select peptides suitable for SRM assay | Measure relative abundance in pools of samples | Measure absolute concentration, select proteins for differential diagnosis | 16 biomarkers |
|---|---|---|---|---|---|
| Proteins: 79 | 54 | 35 | 30 | 30 | 18 |
| Peptides: 590 | 140 | 68 | 30 | 30 | 18 |
| Samples: pools of 5 normal, 5 PV | pools of 5 normal, 5 NOA, 5 PV | | | | |

![Diagram of workflow]

Variations of the sample preparation protocol. Precursor-to-fragment transitions are presented in supplemental Table S1.

Stable-isotope Dilution: SRM—Heavy isotope-labeled peptide standards were synthesized for 20 proteins (supplemental Table S2). Twenty standard peptides were mixed and diluted to the final concentration of 18 fmol/μl. Ten microliters of internal standard mixture were spiked into a digest of each seminal plasma sample (equivalent of 0.5 μl of original seminal plasma) prior to C18 microextraction. Each seminal plasma sample (one normal, one NOA, and one PV) was digested in triplicate. Each digest was subjected to C18 microextraction and finally analyzed by LC-SRM in duplicate. Forty peptides and 120 transitions representing 20 proteins were scheduled within 1.5-min intervals during a 30-min LC gradient (54-min method) and analyzed by TSQ Vantage in the positive-ion mode. SRM method had the following parameters: optimized collision energy values, 0.010 m/z scan width, 0.015–0.040-s scan time, 0.4 Q1, 0.7 Q3, 1.5 mTorr Q2 pressure, tuned S-lens values, +1 V declustering voltage.

Data Analysis—Raw files recorded for each sample were analyzed using LCquan (version 2.5.6). The peak areas were examined manually for verification and used for quantification. All of the areas were normalized by an internal standard to account for variation of sample preparation and mass spectrometry. For a stable-isotope dilution SRM method, Pinpoint was used to validate the retention times and relative intensities of three transitions of both endogenous tryptic peptides and spiked-in standards. Pinpoint was also used to calculate the light to heavy ratio and coefficients of variation for all peptides.

Statistical Analysis—Three groups of samples were compared by GraphPad software using the nonparametric one-way analysis of variance Kruskal-Wallis test, followed by Dunn’s multiple-comparisons test for differences between groups. A one-way test has been applied because protein concentrations were expected to decrease in PV because of physical obstruction. In all cases, a p value <0.05 was considered significant. Receiver operating characteristic area, sensitivity, and specificity were calculated with GraphPad software.

RESULTS

Multi-step Strategy for Biomarker Verification—Our approach for biomarker discovery included a stepwise selection of candidates from the list of all identified proteins in seminal plasma. The initial list of candidate biomarkers was assembled based on at least 1.5-fold difference in spectral counts between normal and PV pools; some candidates were not detected at all in the PV pool. In total, 79 candidate proteins were selected for SRM assay development (supplemental Table S3).

To verify a list of 79 candidate biomarkers, we designed and followed a multi-step strategy with sequential elimination of poorly performing peptides and proteins (Fig. 1): (i) based on discovery data, choose proteins that have peptides suitable for SRM assay development (54 proteins); (ii) develop preliminary SRM assays (35 proteins); (iii) verify peptide identity (30 proteins); (iv) measure the relative abundance of candidates in pools of five samples; (v) measure the relative abundance in individual samples and select candidates that showed statistically significant differences between groups (18 proteins); and (vi) using spiked-in synthetic heavy isotope-labeled peptide standards, measure concentration of proteins in seminal plasma. Our strategy resulted in 16 biomarkers that will be further validated in hundreds of seminal plasma samples to provide two to four biomarkers for ELISA development and routine use in clinical practice.

Selection of Proteotypic Peptides and SRM Transitions—In a typical SRM assay, a unique peptide is measured, and its concentration is assumed to be equal to the concentration of its parent protein. In our work, proteotypic peptides and SRM transitions were chosen based on LTQ-Orbitrap identification data (20).

The same nanoLC and electrospray sources and conditions used for identification (LTQ-Orbitrap) and quantification (triple quadrupole) assured identical efficiency of peptide ionization. Similar peptide fragmentation patterns in ion traps and quadrupoles (21) facilitated selection of SRM transitions. One to five peptides identified with LTQ-Orbitrap were chosen per protein. Doubly charged tryptic peptides that had 8–15 amino acids and had clear, intense, and unambiguous y-ion fragments (especially at proline residue) were preferentially selected. Peptides that had cysteine (especially at the N terminus) and/or modifications such as partially oxidized methionine, partially deamidated glutamine, or asparagine were avoided, when possible. All of the candidate peptides were searched with Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the uniqueness of each peptide sequence. Five transitions were chosen per peptide based on y-ion fragment intensities. Finally, 140 peptides representing 54 proteins remained in the list.

Observing Peptides with SRM—In total, 140 peptides and 700 transitions were grouped into 46 different LC-SRM methods. Each method was run once, and the results were manually inspected with Pinpoint software. Peptides that had
multiple coeluting peaks for individual SRM transitions were considered as positive hits. A significant number of low abundance proteins failed at this step and were removed. As a result, 68 peptides representing 35 proteins remained.

**Confirmation of Peptide Identities**—Because SRM assays are prone to false positive quantification caused by interfering ions, confirmation of the identity of observed peaks is required. The best means for such confirmation is the SRM analysis of synthetic peptides. Multiplex SRM development, however, requires several hundred synthetic peptides, which is quite costly. Large libraries of unpurified synthetic peptides were proposed as an alternative (22). Even though the latter approach allows for better tuning of instrumental parameters (collision energy and tube lens voltage), it is quite tedious if optimization of hundreds of peptides is required. Ultimately, a digest of a suitable biological fluid represents the most complete library of peptides required for SRM development. The large number of peptides identified with LTQ-Orbitrap in the strong cation exchange chromatography-fractionated digest is typically suitable for SRM quantification with triple quadrupole in the unfractionated digest.

To confirm the identity of selected peptides, we used: (i) correlation of LC retention time between discovery and SRM gradients (Fig. 2A); (ii) iSRM assays (Fig. 2B); (iii) SRM-triggered MS/MS fragmentation assays followed by Mascot database search and comparison with LTQ-Orbitrap MS/MS fragmentation (Fig. 2, C and D). First, correlation of retention times was a prompt method to test peptide identity because peptides typically elute within a narrow and specific range of acetonitrile gradient. Here, retention times of 31 peptides in the SRM LC gradient (60 min) were correlated to the retention times of the identification LC gradient (90 min), and correlation with $R^2 = 0.99$ was found. Identical nanoLC and nanoelectrospray ionization sources facilitated high coefficient of linear correlation. High resolution in the first quadrupole (Q1 0.2 full width at half-maximum) in SRM experiment ensured little or no interference, even in the complex matrix of the unfractionated digest.
Second, all peptides were confirmed with iSRM assays (supplemental Fig. S1). Typically, iSRM assays are used to either reconstruct MS/MS spectra of peptides or to quantify peptides with five to eight transitions (23). In this work, we used iSRM to quickly acquire eight transitions per peptide and used these data to prove the identity of peptides, reconfirm the choice of most intensive transitions, and exclude transitions with significant interferences. Six or more coeluting peaks corresponding to individual transitions ensured the identity of the peptides (18). The three most intense and selective transitions were chosen for the final SRM assay.

Third, 12 peptides representing 12 proteins were confirmed by an SRM-triggered MS/MS fragmentation assay followed by Mascot database search and also comparison with LTQ-Orbitrap MS/MS fragmentation (supplemental Fig. S2). This approach, however, was applicable to high abundance proteins only and was not efficient for medium and low abundance proteins.

Finally, we excluded all ambiguous peptides and selected a single peptide for each protein. Thirty one proteins, including KLK3, were used in the label-free assay (Table I and Fig. 3).

### Table I

**Proteins and peptides selected for a 32-peptide label-free SRM assay**

| Protein | Proteotypic peptide | Pool of five samples, ratio normal/PV | Individual samples, ratio of medians normal/PV |
|---------|---------------------|--------------------------------------|-----------------------------------------------|
| LDHC    | EELFLS1PCVGLGR      | ∞                                   | 64000^a                                        |
| SPAG11B | ICVPFLGPR           | ∞                                   | 11000^a                                        |
| TEX101  | LMSS1LAVPMFVR       | ∞                                   | 14900^a                                        |
| MUC15   | DGI1PM2DDPLR        | 12000^a                            | 14300^a                                        |
| CES7    | DAQAPPVYFYEFPR     | 14100^a                            | 14200^a                                        |
| PTGDS   | AQPGFTETIVLFQPQDK  | 406000^c                           | 66                                              |
| ECM1    | ELLALIQLER         | 6.3                                | 47                                              |
| MGAM    | AYVAFPDFFR         | 8.4                                | 31                                              |
| CEL     | LGILGDSVDFIK       | 6.3                                | 41                                              |
| CAMP    | FALLGDFPR          | 3.1                                | 26                                              |
| ADAM7   | TYYEELLYEYK        | 7.3                                | 19                                              |
| FAM12B  | NAYVVMQNPFLK       | 9.4                                | 15                                              |
| CRISP1  | YCDMTESNFLER       | 3.4                                | 12                                              |
| PATE4   | ENELCSTTAYFR       | 5.3                                | 5.6                                             |
| SPINT3  | DLLPVCAFMKEK     | 10.0                               | 5.3                                             |
| GPR64   | GEINPQYDK          | 21.0                               | 4.8                                             |
| ALDH1A1 | TIPIDGNYTPYTR     | 3.7                                | 4.0                                             |
| CA4     | ASISGQDLAPYQAK     | 18.0                               | 2.5                                             |
| NPC2    | LVIVWQLQDQK        | 3.4                                | 1.9                                             |
| ABP1    | GGFRNYAGLYK        | 3.4                                | 1.5                                             |
| LGALS3BP| SDLAVPSELALLK     | 1.5                                | 1.1                                             |
| DAG1    | VT1PTDLIASSGDIK   | 2.3                                | 1.1                                             |
| GAS6    | LVAEPDFR          | 2.2                                | 1.0                                             |
| LTBP3   | NQCLCPPDPTGR      | 1.9                                | 0.9                                             |
| SERPINA1| SVLQLQSLIK       | 2.5                                | 0.8                                             |
| CD177   | GGGIFSMLR         | 2.5                                | 0.8                                             |
| GSTM3   | LDDLFPNLFLYLDXG  | 1.5                                | 0.7                                             |
| DEF1118 | ACCIPSNEDHR       | ∞                                  | 0.7                                             |
| MXRA5   | FSILSSOWL         | 2.0                                | 0.6                                             |
| KLK3    | LSEPATELTAVK       | 0.6                                | 0.5                                             |
| SERPINA5| TLX1LAD7PPFNFR   | 1.1                                | 0.5                                             |

^a Peptide abundances were normalized to the internal standard (spiked in heavy peptide of KLK3).

^b Based on 12 normal and 8 PV samples.

^c In PV samples, protein was not identified, or its peptide level measured by SRM was below limit of quantification.

In *Silico Assessment of Selectivity of SRM Transitions at High Resolution in Q1*—To inquire whether the higher resolution of the first quadrupole resulted in a lower number of potential interferences, we assessed *in silico* interferences at 0.2 full width at half-maximum in Q1 versus typical resolution of 0.7 full width at half-maximum. Because all peptide forms, all fragments, and all possible post-translational modifications cannot be considered, we assessed only unmodified peptides and b- and y-fragment ions. First, a database of all peptides identified in the seminal plasma digest was assembled; it included 12,073 peptides (20). Using Pinpoint, we evaluated 32 peptides and 273 transitions corresponding to y-ions within the 300–1500 m/z range. All of the transitions were matched against all possible combinations of b- and y-ions for 12,073 peptides with +2, +3, and +4 charges. Surprisingly, at high resolution in the first quadrupole (0.2 Q1), only three interfering transitions were found for peptides of DAG1 (y3), GPR64 (y9), and MUC15 (y12) proteins. For example, transition DPVQEAWAEDVDLR (+2), 821.889 m/z → EDVDLR...
(+2), 373.687 m/z found for PKM2 protein interfered with transition VTIPTDLASSGIK (+2), 821.967 m/z → ILK (+2), and 373.280 m/z for DAG1 protein. These three interfering transitions were outside the typical range of transitions used in our assay (y4−y10). Interestingly, at a higher Q1 value (0.7), there were nine interfering transitions. Such interferences could be discriminated based on peptide hydrophobicity and, if they still interfered in the LC dimension, could be excluded. Thus, higher resolution in Q1 indeed provided higher selectivity of SRM assay.

Recently, a useful database was built on the platform of the Global Proteome Machine at mrm.thegpm.org/thegpm-cgi/peak_search.pl. This database allows predicting isobaric interferences based on peptide m/z, hydrophobicity, and intensity of fragments in different biological matrices.

Sample Preparation—Sample preparation protocols in quantitative proteomics typically include a set of physicochemical procedures (protein denaturation and C18 microextraction), chemical reactions (DTT reduction and iodoacetamide alkylation), and enzymatic reactions (trypsin digestion). Each procedure has less than 100% yield, which can vary from day to day. In our experience, even the most optimal sample preparation protocol may have variability as high as 20%. If the reproducibility of each step of the protocol is slightly compromised, the whole quantification may be compromised (coefficient of variation ≥ 20%). To facilitate high reproducibility of analysis and accurate comparison of protein abundances in individual samples, all of the sample preparation steps in this work were performed on a single 96-well plate, and no additional fractionation of seminal plasma was done prior to LC-SRM.

We evaluated the efficiency of some critical steps of the sample preparation protocol, such as trypsin digestion, alkylation, and C18 microextraction. Three seminal plasma samples were trypsin-digested in duplicate, a known amount of a heavy peptide of KLK3 was spiked into each sample, each digest was subjected to C18 microextraction, and each duplicate was analyzed three times with the SRM assay. Although area values of light KLK3 peptide varied by ~20%, the coefficients of variation for the light-to-heavy ratio were 2.8, 3.0, and 1.8%. Thus, normalization of the area with an internal standard significantly reduced technical variability. To estimate the efficiency of iodoacetamide alkylation, a ratio of alkylated over nonalkylated peptide ELGIC’PDAAVIPIK for a high abundance protein (PIP) was measured by SRM. The yield of alkylated peptide was 99.8%.

Comparison of Normal, PV, and NOA Pools of Samples—A multiplex scheduled SRM assay was used to analyze 31 proteins, including KLK3, in pools of five normal, NOA, and PV samples (supplemental Fig. S3). Significant differences in abundances were found for the majority of proteins. Four proteins (LDHC, TEX101, MUC15, and SPAG11B) were not detected in PV samples, so their abundances were estimated using the level of background signal. The estimated relative abundance of some proteins between samples exceeded 2 or 3 orders of magnitude.

Label-free Analysis of 31 Proteins in 30 Seminal Plasma Samples—Individual seminal plasma samples (12 normal, 10 NOA, and 8 PV) were digested once and analyzed in triplicate. Technical coefficients of variation based on three LC-SRM injections for each of 30 proteins were less than 12, 26, and 28% in normal, NOA, and PV samples, respectively. The concentration of KLK3 was measured with accuracy of ~3%. KLK3 concentration in normal seminal plasma was within the previously published range of 0.4–3 mg/ml (24). The relative abundances for all proteins in individual samples (n = 30) were subjected to nonparametric one-way analysis of variance Kruskal-Wallis test. Eighteen proteins showed a statistically significant difference (p < 0.05) in at least one of three groups (supplemental Fig. S4 and supplemental Table S4).

Validation of SRM Assay with Heavy Isotope-labeled Peptide Standards—To validate our SRM assay and to measure concentrations of proteins in seminal plasma samples, 20 heavy isotope-labeled peptide standards were synthesized:
Fig. 4. Concentration of 20 proteins measured with a stable-isotope dilution SRM assay in normal (n = 12), NOA (n = 10), and PV (n = 8, Post vas.) seminal plasma samples. For more discussion see text.
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18 peptides to quantify candidate biomarkers that showed statistically significant difference in PV samples in comparison with normal samples (supplemental Table S4) and two peptides to quantify prostate-specific proteins (KLK3 and CD177). One representative seminal plasma sample from each group (normal, NOA, and PV) was digested in triplicate, and each digest was analyzed in triplicate. As a result, retention times of all heavy isotope-labeled internal standards were found to be identical to those of the endogenous tryptic peptides (supplemental Fig. S5). Relative intensities of three transitions per peptide were the same for both endogenous tryptic peptides and spiked-in standards. Variabilities of digestion of a normal seminal plasma sample were found less than 9% for each of the 20 proteins, with an average value of 5%. Technical variability of three LC-SRM injections was less than 9% for each of the 20 proteins, with an average value of 2%. Taking into account adequate levels of variability of trypsin digestion and LC-SRM injections, we used a single digestion and two LC-SRM injections for the subsequent analysis of 30 seminal plasma samples. Finally, to demonstrate that label-free quantification using a single internal standard for normalization can be used to shorten the initial list of candidates, we compared the ratios of proteins in the same normal and PV samples measured with either a single or 20 internal standards. Ratios calculated with both methods were in good agreement (supplemental Table S5). This suggested that heavy isotope-labeled peptides could be synthesized and used at later steps of assay development and only for those proteins that performed well in the preliminary studies. To conclude, the use of heavy isotope-labeled internal standards unambiguously proved that our multi-step SRM development approach was valid, rigorous, and fairly reproducible and could be used for SRM-based biomarker verification.

Measurement of Concentrations of Proteins in 30 Seminal Plasma Samples—Using spiked-in heavy isotope-labeled internal standards, 30 seminal plasma samples were reanalyzed (Fig. 4), and concentrations of 20 proteins in 12 normal, 10 NOA, and 8 PV seminal plasma samples were calculated (Table II). Concentrations of proteins were found in the range 0.1–1000 μg/ml, which corresponded to the range of medium abundance proteins and was near the limit of protein quantification by mass spectrometry. These findings were in good agreement with our previous results and demonstrated that the lowest level of quantification of proteins with SRM assays in the unfractinated digest of seminal plasma was ~0.1 μg/ml. Because several proteins (TEX101, LDHC, etc.) were not detected in PV or NOA samples, the limits of quantification of these proteins were determined by serial dilution analysis of heavy peptides in the seminal plasma digest.

Selection of Biomarkers for Differential Diagnosis of Azoospermia—Concentrations of all proteins in individual samples (n = 30) were subjected to nonparametric one-way analysis of variance Kruskal-Wallis test with a statistical cut-off of p < 0.05. Three groups of proteins were selected: (i) a group of 16 proteins to differentiate normal and PV seminal plasma; (ii) 3 proteins, normal and NOA; and (iii) 11 proteins, NOA and PV (Table III). Some proteins discriminated groups with absolute or nearly absolute specificities and sensitivities and the areas under the receiver operating characteristic curves of 0.96–1.0. To distinguish between three groups of patients, the

| Protein | Molecular mass | Concentration in seminal plasma, median (IQR) | Median coefficient of variation of SRM assay |
|---------|---------------|-----------------------------------------------|--------------------------------------------|
|         | kDa           | μg/ml                                        | %                                          |
| KLK3    | 28.7          | 330 (240–480)                                | 0.7                                        |
| CAMP    | 19.3          | 37 (17–62)                                   | 0.9                                        |
| ECM1    | 60.7          | 34 (19–58)                                   | 0.7                                        |
| CRISP1  | 28.5          | 30 (15–52)                                   | 1.3                                        |
| PTGDS   | 21.0          | 9.0 (5.5–15)                                 | 1.2                                        |
| MGAM    | 210.0         | 8.1 (4.8–17)                                 | 1.3                                        |
| SPINT3  | 10.3          | 7.0 (3.0–8.2)                                | 0.9                                        |
| GPR64   | 112.0         | 5.2 (2.6–6.5)                                | 1.5                                        |
| NPC2    | 22.0          | 4.2 (3.4–6.2)                                | 0.9                                        |
| CD177   | 46.4          | 3.8 (1.7–20)                                 | 7.9                                        |
| FAM12B  | 17.6          | 2.3 (1.6–5.9)                                | 1.1                                        |
| LDHC    | 36.3          | 1.8 (1.2–5.3)                                | 1.1                                        |
| CEL     | 79.3          | 1.5 (0.9–2.1)                                | 1.7                                        |
| ADAM7   | 85.7          | 1.4 (0.8–2.7)                                | 2.9                                        |
| CES7    | 63.9          | 1.3 (0.8–3.1)                                | 2.8                                        |
| ALDH1A1 | 54.9          | 1.2 (1.0–1.9)                                | 2.8                                        |
| TEX101  | 26.7          | 1.1 (0.8–1.9)                                | 1.1                                        |
| MUC15   | 36.3          | 0.93 (0.53–1.1)                              | 1.4                                        |
| CA4     | 35.0          | 0.80 (0.50–1.4)                              | 1.2                                        |
| SPAG11B | 11.4          | 0.23 (0.10–0.38)                             | 1.3                                        |

TABLE II
Concentration of 20 proteins in 30 seminal plasma samples measured with a 40-peptide stable-isotope dilution SRM assay

* IQR, interquartile range.
* Technical variability based on single digestion and duplicate injection of normal samples.
* Limit of quantification of protein calculated based on the limit of quantification of a synthetic peptide in the seminal plasma digest.

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A combination of two markers from groups (ii) and (iii) is required (Fig. 5).

**Tissue Specificity Analysis**—We performed tissue specificity analysis using microarray mRNA expression profiles available at BioGPS (biogps.gnf.org), a “centralized gene portal for aggregating distributed gene annotation resources” (supplemental Fig. S6). Tissue specificity was a key parameter in predicting PV (OA) markers, because testis-specific proteins were absent in PV seminal plasma because of surgical severance of the vas deferens. All of the top candidates were highly specific to the testis. Cell specificity analysis of the top six candidates revealed that Leydig cell-specific proteins were only slightly decreased, whereas Sertoli cell-specific proteins and germ cell-specific proteins were significantly underexpressed in NOA (Fig. 6).

**DISCUSSION**

**Development of SRM Assays**—The challenges of development of SRM assays include: (i) selection of proteotypic peptides, (ii) selection of transitions, (iii) optimization of MS and LC parameters, and (iv) multiplexing of dozens of peptides in a single assay. Selection of proteotypic peptides for SRM is a critical step of assay development. It is hardly possible to predict prior to the experiment which peptide will be fairly separated in LC and efficiently ionized in electrospray, thus providing stable and intense signal. Proteotypic peptides can be selected based on identification data or by searching publicly available databases, if the peptide of interest has previously been identified. In recent years, a large volume of identification data became available in public databases such as GPM Proteomics Database (mrm.thegpm.org) (25) and Peptide Atlas (www.peptideatlas.org) (26). These databases, however, still lack high quality data for many low and medium abundance proteins. Once proteotypic peptides are selected, optimization of SRM transitions and LC-MS parameters can be accomplished with SRM software such as Pinpoint (Thermo Fisher Scientific BRIMS, Cambridge, MA), MRMaids (www.mrmaid.info), or MRM Pilot (Applied Biosystems Inc., Foster City, CA).

### Table III

| Protein | Statistical significance of difference of groups | ROC area | Sensitivity at ≥95% specificity (%) | Exclusive tissue specificity | Testis cell specificity |
|---------|-----------------------------------------------|----------|-------------------------------------|------------------------------|------------------------|
| LDHC    | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig, Germ  |
| SPAG11B | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig        |
| TEX101  | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig, Germ  |
| MUC15   | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig        |
| PTGDS   | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig, Germ  |
| ECM1    | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig        |
| CEL     | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig        |
| FAM12B  | <0.001                                        | 1.00     | 100                                 |                              | Sertoli, Leydig, Germ  |
| CAMP    | <0.01                                         | 0.97     | 88                                  |                              | Germ                   |
| SPINT3  | <0.01                                         | 0.96     | 75                                  |                              | Germ                   |
| CES7    | <0.01                                         | 0.95     | 75                                  |                              | Germ                   |
| MGAM    | <0.01                                         | 0.94     | 88                                  |                              | Germ                   |
| ADAM7   | <0.01                                         | 0.92     | 50                                  |                              | Leydig                 |
| CRISP1  | <0.05                                         | 0.91     | 75                                  |                              | Leydig, Germ           |
| GP6R64  | <0.05                                         | 0.89     | 63                                  |                              | Leydig                 |
| CA4     | <0.05                                         | 0.89     | 63                                  |                              | Leydig                 |

**Normal (n = 12) versus PV (n = 8)**

- LDHC: <0.001, 1.00, 100, Testis Sertoli, Leydig, Germ
- SPAG11B: <0.001, 1.00, 100, Testis Sertoli, Leydig, Germ
- TEX101: <0.001, 1.00, 100, Testis Sertoli, Leydig, Germ
- MUC15: <0.001, 1.00, 100, Testis Leydig
- PTGDS: <0.001, 1.00, 100, Sertoli, Leydig, Germ
- ECM1: <0.001, 1.00, 100, Sertoli, Leydig
- CEL: <0.001, 1.00, 100, Sertoli, Leydig
- FAM12B: <0.001, 1.00, 100, Sertoli, Leydig, Germ
- CAMP: <0.01, 0.97, 88, Germ
- SPINT3: <0.01, 0.96, 75, Testis Germ
- CES7: <0.01, 0.95, 75, Testis Germ
- MGAM: <0.01, 0.94, 88, Testis Germ
- ADAM7: <0.01, 0.92, 50, Leydig
- CRISP1: <0.05, 0.91, 75, Leydig
- GP6R64: <0.05, 0.89, 63, Leydig
- CA4: <0.05, 0.89, 63, Leydig

**Normal (n = 12) versus NOA (n = 10)**

- TEX101: <0.01, 0.99, 90, Testis Sertoli, Leydig, Germ
- LDHC: <0.01, 0.93, 90, Testis Sertoli, Leydig, Germ
- CEL: <0.05, 0.85, 50, Testis Sertoli, Leydig, Germ

**NOA (n = 10) versus PV (n = 8)**

- ECM1: <0.01, 0.96, 75, Testis Leydig
- CAMP: <0.01, 0.96, 88, Testis Leydig
- MGAM: <0.01, 0.95, 88, Testis Leydig
- MUC15: <0.05, 0.94, 63, Testis Sertoli, Leydig
- CES7: <0.01, 0.94, 75, Testis Sertoli, Leydig
- SPINT3: <0.01, 0.93, 50, Testis Germ
- FAM12B: <0.01, 0.91, 13, Testis Leydig, Germ
- CRISP1: <0.01, 0.90, 75, Testis Leydig, Germ
- SPAG11B: <0.01, 0.90, 75, Testis Leydig, Germ
- ADAM7: <0.05, 0.89, 63, Testis Leydig
- GP6R64: <0.05, 0.83, 10, Testis Leydig

### Notes

1. Nonparametric one-way ANOVA Kruskal-Wallis test followed by Dunn’s multiple-comparisons test for differences between three groups.
2. Area under receiver operating characteristic (ROC) curve.
It should be noted that the sensitivity of SRM assays is still not sufficient to compete with ELISA. In our experience, proteins can be quantified with SRM assays in unfractionated digests of biological fluids if their lowest concentration is near 0.1 μg/ml (27). Furthermore, these levels are even higher for iSRM and SRM-triggered MS/MS assays. Such assays work well for high and medium abundance proteins but do not provide conclusive information for low abundance proteins because of the increased level of interferences and distorted peak shapes for low intensity transitions. Even though iSRM and SRM-triggered MS/MS assays can be valuable confirmatory tools, their use is limited to proteins with concentrations in seminal plasma higher than 1–10 μg/ml. The use of synthetic peptide standards may be the most straightforward way to validate the identity of lower abundance peptides.

Relative Abundance of Proteins in Pools and Individual Samples—The same pools of normal and PV seminal plasma samples were used for protein identification (20) and SRM quantification, and relative abundances of proteins were compared. Interestingly, the relative abundances of proteins that were observed with more than 20–30 spectral counts in the PV pool correlated fairly well to the relative SRM abundances. Proteins with less than 20 spectral counts showed poor correlation between spectral counting and SRM data. Spectral counting, however, was a fair guide to filter over 2000 proteins and choose the initial list of 79 candidate biomarkers (supplemental Table S3).

A relatively wide distribution of abundances in individual seminal plasma samples was found for some proteins (supplemental Fig. S4). This finding might question the significance of 1.5-fold change used as a cut-off in this and other biomarker studies. The majority of proteins with such relatively small differences between groups would likely underperform in the verification phase. A striking example in the present work was PATE4 protein, which showed high promise in the PV pool but failed when individual samples were tested because of its extremely wide (more than 100-fold) distribution. Such an outcome could be explained by possible intermittent secretion of PATE4 (prostate and testis expressed 4 protein) by both prostate and testis.

Biological Function of Promising Candidates—We reviewed the previously published literature on the biological function of

Fig. 5. Combination of two proteins, SPAG11B and TEX101, can be used for differential diagnosis of azoosperma. In normal seminal plasma, both SPAG11B and TEX101 are abundant; in NOA, SPAG11B is abundant, whereas TEX101 is significantly decreased; in PV (OA), levels of both SPAG11B and TEX101 are significantly decreased (below limit of quantification of present SRM assay). For quantitative data, see also Table II.
the most promising candidates. TEX101 is a very specific marker for both male and female germ cells during gonadal development (28). TEX101 is a membrane glycoprotein expressed on the cell surface of germ cells during spermatogenesis but shed to the seminal plasma at the late stages of post-testicular sperm maturation (29). The biological function of TEX101 still remains unknown, although it may play a crucial role in the acrosome reaction (30). LDHC is a testis-specific isozyme discovered in male germ cells and is critical for fertilization (31, 32). PTGDS stands for lipocalin-type prostaglandin D synthase, which is expressed in Sertoli cells of the testis and in epithelial cells of the prostate and ductus epididymis (33). However, it is also significantly expressed in the central nervous system and found in cerebrospinal fluid (34) and in the heart (35). PTGDS has been previously studied as a biomarker of azoospermia (10, 36). MUC15 is cell membrane-associated mucin. MUC15 overexpression in colorectal carcinoma cells enhances cell proliferation, cell extracellular matrix adhesion, colony-forming ability, and invasion (37). SPAG11B is a cationic secretory anti-microbial peptide expressed in human epididymis (38, 39). FAM12B is an epididymal secretory protein (40, 41) that has been found up-regulated in epididymides of nonobstructive azoospermic men (42). ADAM7 is a membrane protein expressed specifically in the epididymis and localized to the sperm surface during epididymal transit (43, 44). PATE4 is a hypothetical prostate and testis-expressed secreted protein that has never been previously studied. Its amino acid sequence was predicted based on the sequence of its mRNA transcript (45–47).

**Tissue-specific Proteins Perform Well as Biomarkers**—Previously published work aiming to identify azoospermia-specific biomarkers did not provide conclusive results (19). Using two-dimensional gel electrophoresis, four proteins (STAB2, CEP135, GNRP, and PIP) were proposed as candidate NOA biomarkers. No verification was undertaken, so the potential of the proposed proteins for NOA diagnosis was not clear. In the same work, NPC2, a protein with moderate testis specificity, was proposed as OA biomarker. Our results, however, demonstrated that NPC2 was a mediocre OA biomarker.

In general, proteins highly specific to testis should be absent in PV (OA) seminal plasma because of the physical obstruction and thus should perform as PV (OA) biomarkers with outstanding sensitivity and specificity. On the contrary, nonspecific proteins may still be present in seminal plasma if produced by prostate gland or seminal vesicles. The sensitivity of such markers (NPC2, CA4, and ALDH1A1) is typically low (Table III).

Recently, expression profiles of many proteins (rather than mRNA) became available at the Human Protein Atlas (www.proteinatlas.org) (48). For example, two different antibodies were used for immunohistochemistry analysis of TEX101 protein. Both antibodies confirmed exclusive specificity of TEX101 to the cells in seminiferous ducts (Sertoli and germ cells) but not to Leydig cells (supplemental Fig. S7).

**Cell-specific Proteins May Reveal Aspects of the Molecular Basis of NOA**—Impaired maturation of sperm cells in NOA may originate from the failure of certain types of testis cells (Leydig, Sertoli, or germ). Our analysis shows that testis-specific proteins can be slightly or significantly affected in NOA. Cell-spe-
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cific proteins differentially expressed in NOA may also shed some light on protein networks involved in NOA pathogenesis.

Using the BioGPS database of gene expression profiles (biogps.gnf.org), the abundance of testis-specific proteins was correlated to the specificity of mRNA expression by three types of testis cells. As a result, in NOA conditions, the expression of Leydig cell-specific proteins (ADAM7, SPAG11B, MUC15, and FAM12B) was slightly affected, whereas expression of germ cell and Sertoli cell (e.g. seminiferous tubule) proteins (TEX101 and LDHC) decreased significantly (Table III). Thus, Sertoli or germ cell dysfunction, but not Leydig cell dysfunction, may be associated with NOA. Such a hypothesis was previously investigated using expression of inhibin B (Sertoli cell-specific protein), but the results were not clear-cut (10). Our results support this hypothesis. The large number of cell-specific proteins differentially expressed in normal, NOA, and PV (OA) seminal plasma will facilitate identification of molecular pathways impaired in NOA.

Conclusions—A stepwise workflow to verify biomarkers for differential diagnosis of azoospermia by SRM was presented. A multiplex label-free SRM assay was used to measure the relative abundance of 31 proteins in the unfraccionated digest of seminal plasma, verify 30 candidate proteins in 30 samples, and identify 18 promising biomarkers. To follow up on these candidates, heavy isotope-labeled peptides were used to re-measure concentrations of 20 proteins in the same cohort of samples. Concentrations of promising biomarkers were found in the range 0.1–1000 μg/ml and thus corresponded to medium abundance proteins in seminal plasma. For the first time, we propose a working panel of 16, 3, and 11 azoospermia biomarkers capable of differentiating three pairs of three biological conditions: normal, NOA, and PV (OA). The present panel of identified biomarkers has the potential to eliminate the need for testicular biopsy, providing significant benefits to patients at decreased costs. It is possible that an expanded panel (under development) may be capable of further classifying the three subgroups of the NOA syndrome. Our current and expanded panels could also be examined for the diagnosis of other pathologies of the male reproductive tract such as prostatitis and prostate cancer.

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The on-line version of this article (available at http://mcponline.org) contains supplemental Tables S1–S4 and Figs. S1–S7.

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