Identification of a Novel Proteoform of Prostate Specific Antigen (SNP-L132I) in Clinical Samples by Multiple Reaction Monitoring*

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Prostate specific antigen (PSA) is a well-established tumor marker that is frequently employed as model biomarker in the development and evaluation of emerging quantitative proteomics techniques, partially as a result of wide access to commercialized immunoassays serving as “gold standards.” We designed a multiple reaction monitoring (MRM) assay to detect PSA proteoforms in clinical samples (n = 72), utilizing the specificity and sensitivity of the method. We report, for the first time, a PSA proteoform coded by SNP-L132I (rs2003783) that was observed in nine samples in both heterozygous (n = 7) and homozygous (n = 2) expression profiles. Other isoforms of PSA, derived from protein databases, were not identified by four unique proteotypic tryptic peptides. We have also utilized our MRM assay for precise quantitative analysis of PSA concentrations in both seminal and blood plasma samples. The analytical performance was evaluated, and close agreement was noted between quantitations based on three selected peptides (LSEPAELTDAVK, IVGGWECEK, and SVILLGR) and a routinely used commercialized immunoassay. Additionally, we disclose that the peptide IVGGWECEK is shared with kallikrein-related peptidase 2 and therefore is not unique for PSA. Thus, we propose the use of another tryptic sequence (SVILLGR) for accurate MRM quantification of PSA in clinical samples. Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.028365, 2761–2773, 2013.

With the move toward biomarker verification and the clinical implementation of novel assays, mass-spectrometry-based quantitative analysis of biomarkers is increasingly becoming an important route for current proteomics studies. Although MS instrumentation offers various powerful strategies for biomarker discovery (1), the validation phase for these putative protein candidates still relies primarily on immunoreaction-based assays such as ELISA (2). These immunoassays are considered to be effective diagnostic tools and are routinely used in clinical practice, but they are often associated with the lengthy and expensive development of high-quality antibodies, and sometimes significant differences exist between tests from different vendors. Furthermore, immunoassays depend on indirect readouts (colorimetric, fluorescent, or radioactive) and may produce false positive results as a result of nonspecific binding. Nevertheless, MS nowadays is able to measure analytes with high quantitative accuracy, and established MS methods originally developed for the quantitation of small molecules, such as multiple reaction monitoring (MRM) (3), have been successfully introduced for proteins (4–6). As compared with traditional ELISA techniques, MRM assays can be cost-efficient, utilize quickly developed methods, and offer exceptional multiplexing capability (7).

Interestingly, prostate specific antigen (PSA), a successful biomarker of prostate cancer, has been frequently chosen as a model protein in MRM method development studies (8–21). PSA is a prostatic kallikrein-related serine peptidase (KLK3) with restricted chymotrypsin-like specificity that is mainly responsible for the liquefaction of seminal coagulum via degradation of the major gel-forming proteins SEMG1 and SEMG2 (22–24). Catalytically active PSA is a 237-amino-acid single-chain glycoprotein with a molecular weight close to 28 kDa (25, 26). Abundant prostate-restricted expression of the epithelial cells and the release of mainly catalytic PSA into seminal fluids in concentrations of approximately 5 to 50 μmol/l are regulated by the nuclear androgen receptor, with levels in blood normally being a million-fold lower (20 pmol/l). PSA is non-catalytic and predominantly lined in a covalent complex with α-1-antichymotrypsin (SERPINA3) (27–29). PSA levels in

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1 The abbreviations used are: ACN, acetonitrile; IS, internal standard; MRM, multiple reaction monitoring; PSA, prostate specific antigen; SNP, single nucleotide polymorphism; SRM, selected reaction monitoring.
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blood may become elevated because of benign conditions including prostatitis or benign prostate hyperplasia, but modestly elevated PSA in the blood of a middle-aged patient is also strongly associated with metastasis or death from prostate cancer decades later (30, 31). PSA screening can reduce cancer-related deaths, but it may also lead to overdiagnosis and overtreatment (32, 33). Thus controversy remains regarding the merits of the PSA test (34, 35), although it persists as a mainstay in the monitoring of therapeutic intervention and in the detection of disease recurrence or progression (36).

PSA was chosen as a model protein in the first isotope-dilution MS study that measured protein concentrations directly in serum without using immunoaffinity chromatographic enrichment (8). The heavy-isotope-labeled tryptic peptide of PSA, IVGGWECEK (13C2 and 15N1 on each Gly residue), was utilized as an internal standard (IS) and known amounts of purified PSA were spiked into female serum, and a selected reaction monitoring (SRM) transition channel (y-7) was monitored with excellent reproducibility, achieving a limit of detection of 4.5 μg/ml. PSA and five other proteins were selected in a multiplexing study that systematically selected the most useful signature peptides and monitored three transitions per peptide (9). The most abundant transitions (IVGGWECEK; 539.3 → 865.3 and LSEPAELTDAVK; 636.7 → 943.4) were used for quantification on nano-flow LC combined with a hybrid QTrap mass spectrometer. This work was further explored in an encouraging interlaboratory study that compared MRM analytical performance on seven proteins and three different MS platforms (11) while using differently labeled LSEPAELTDAVK (+8 Da), eliminating the interference in the y-9 transition channel previously reported. Excellent sensitivity was obtained using a combination of immunoextraction and product ion monitoring on a linear ion trap instrument (Thermo LTQ) (10). Also in that study, LSEPAELTDAVK was selected for the quantification of recombinant PSA spiked into female plasma, because three additional PSA peptides (HSQP-WOVLVASR, HSLFHPEDTGGVFQVSHSFHPPLYDMSLLK, and FLRPGDSSHDLMLLR) were noticed to ionize less efficiently. Notably, this methodological study reported for the first time the quantification of PSA in two prostate cancer patient samples (300 and 5000 ng/ml) using MRM-MS. Prostate cancer cell lines were also investigated in an SRM-MS assay in order to correlate PSA levels with clinical tests selecting two signature peptides, LSEPEALTAVK and HSQP-WOVLVASR (21).

Although the progress of methodological developments has accelerated, promising successful clinical implementation in the near future, the number of real samples from patients remains low (n = 9 with prostate cancer (13) and n = 3 with benign prostate hyperplasia (12)) with LSEPAELTDAVK used for quantification. The same group has utilized IVGGWECEK for the specific detection of cysteine-containing peptides in plasma using laser-induced photo dissociation (photo-SRM) for protein quantification (17). These important studies offered PSA quantification in patient samples at levels of 4 to 30 ng/ml following albumin depletion, tryptic digestion, solid-phase extraction, and conventional HPLC separation of 100 μl serum. For further validation, PSA concentrations determined via MS methods were correlated to a clinical ELISA test with high concordance (13). A novel enrichment strategy employing mass spectrometric immunoassay SRM was applied to access PSA in serum samples measuring SVILLGR as well as the isoform specific tryptic peptide DTIVANP (19). N-linked glycopeptides of PSA were targeted in a study by the same group selectively capturing and quantifying NKSVILLGR in female serum spiked with known amounts of PSA (18).

PSA was also included in a protein panel developed for monitoring primary urothelial cell carcinomas of bladder (14). A larger number of patient samples (n = 14 control and n = 17 cancer patients) were systematically screened by the nano-LC-MRM assay intended to detect and quantify a few endogenous proteins in urine. Advanced technology integrating isoelectric focusing on a digital ProteomeChip (Cell Biosciences, Santa Clara, CA) used for the selective enrichment of proteotypic peptides with nano-LC-SRM-MS was demonstrated in the quantification of PSA spiked into female serum and in prostate cancer patients using both LSEPAELTDAVK and IVGGWECEK (20). Recently, a study has been published reporting on an MRM assay developed for the differential quantification of free and total PSA (fPSA and tPSA, respectively) in clinical serum samples (n = 9) with concentrations of 0.3 to 18.9 ng/ml, determined by an immunoassay (15). Good sensitivity was achieved, with limits of quantification of 2.03 and 0.86 ng/ml for fPSA and tPSA, respectively. The same research group has further improved the sensitivity of the assay, reaching PSA quantification in spiked female serum at sub-ng/ml levels, and also in a low number of clinical samples, utilizing advanced high-pressure, high-resolution liquid chromatographic separations without the involvement of antibodies (16).

All of these previous reports presented two peptides selected for the quantification of PSA in spiked serum/plasma and in a limited number of clinical samples. However, none of the publications mentioned the fact that IVGGWECEK is not unique for PSA and is also present in human kallikrein-related peptidase 2 (KLK2 or hK2), or that LSEPAELTDAVK is coded on the exon of KLK3 with a single nucleotide polymorphism (SNP), resulting in the amino acid exchange of L132I (rs2003783).

Because of its inherent high selectivity and sensitivity, we have chosen MRM to identify and monitor proteoforms (37) of PSA in clinical samples. For this purpose we developed an MRM assay based on theoretically derived tryptic peptides of 10 PSA isoforms. Because MRM assay outcomes rely on the detection of a specific peptide of the given protein and tryptic digestion might not always be complete, we screened multiple proteotypic peptides with multiple transitions.
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Our study is the first to report on the detection of a proteoform of PSA as the translated gene product of an SNP variant of the KLK3 gene (L132I; rs2003783). It is our conclusion that based on its frequency (ca. 10% worldwide), this allele should also be monitored in order to quantify PSA appropriately, using the signature peptide LSEPA(L/I)TDAVK, in samples with homogeneous and heterogeneous allele expressions.

Additionally, we used three different signature peptides to present data about the analytical performance of our nano-flow LC-MS/MS approach for quantifying PSA in seminal fluid and blood relative to commercialized immunoassays in the largest clinical sample set reported so far (n/H11005 72).

### MATERIALS AND METHODS

**Biological Samples**—Seminal plasma was prepared from semen obtained from young men undergoing investigation for infertility prior to a final diagnosis of disorders (n = 30) and from healthy volunteers (n = 5), following the guidelines of the Helsinki Declaration as described elsewhere (38). The collection of seminal plasma was approved by the ethical board at Lund University (approval number: LU 532-03), and plasma was stored at −20 °C until use. Free PSA levels ranging from 0.35 to 1.9 mg/ml were determined via a time-resolved fluorescent immunoassay (Prostatus Free/Total PSA DELFIA®, Perkin Elmer, Turku, Finland) routinely used at the clinics (39). Prior to analysis, the samples were thawed on ice and diluted in 50 mM ammonium bicarbonate to a final PSA concentration of 1 µg/µl.

Blood plasma samples were obtained from patients diagnosed with advanced stages of prostate cancer, and total PSA levels greater than 100 ng/ml (n = 37, ranging from 120 to 4400 ng/ml) were determined via the DELFIA® assay.

**In Silico Selection of Signature Peptides**—For the identification of PSA isoforms, we used the UniProtKB/TrEMBL database (v.52 2011_11), which includes both reviewed and nonreviewed sequence variants. All listed sequence variations (10 PSA forms; see supplemental Table S1), including N-terminal signaling peptides, were used for further processing of in silico digestion using trypsin. The resulting tryp tic peptides of all isoforms of PSA were investigated for uniqueness via Blast search on the UniProtKB website. The isoform specificity of the proteotypic peptides was also noticed at this step (Table I). Finally, a list of tryptic peptides was prepared, filtering by size (from 7 to 26 amino acids) for synthesis at low purity with and without heavy isotope labeling and carbamidomethylation at cysteine residues (JPT Peptide Technologies GmbH, Berlin, Germany).

**For quantification, four heavy peptides of AQUA QuantPro quality (peptide purity higher than 97%, concentration precision equal to or better than ±25%), isotope-labeled with 15N and 13C in lysine (mass = +8) and arginine (mass = +10) (Thermo Scientific, Ulm, Germany), were used.** These heavy-isotope-labeled peptides were spiked into the biological samples at known concentrations, and the ratio between endogenous (light) and IS peptide was used to estimate...
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the concentration of PSA in the samples. The list of transitions is presented in supplemental Table S2.

Preparation of Peptide Samples—The crude, synthetic peptides were dissolved in 100 μl of 20% acetonitrile (ACN) in order to obtain improved reconstruction of hydrophobic peptides. In experiments of MRM assay development, the crude light and heavy peptides of PSA were separately mixed with equal volumes (50 μl), resulting in 415–454 fmol/μl and 153 fmol/μl final concentrations, respectively.

The protein content of the seminal plasma samples was determined with Bradford reagent (Sigma, Steinheim, Germany). A volume (9 to 26 μl) corresponding to 0.2 mg protein was processed, resulting in different dilution factors used for the calculation of PSA levels. The samples were reduced with 10 mM dithiothreitol at 37 °C for 60 min and alkylated with 50 mM iodoacetamide at room temperature for 30 min in the dark. Tryptic digestion was performed by adding sequencing-grade trypsin (Promega, Madison, WI) at a 1:100 calculated weight ratio and incubating at 37 °C overnight on a block heater with shaking at 900 rpm. The reaction was stopped by the addition of 10 μl of 1% formic acid. The resulting protein digests were dried via speed vacuum centrifugation, restored in 50 μl of 5% ACN with 0.1% formic acid, and stored at −20 °C until analysis.

Seven of the most abundant plasma proteins were depleted from the blood plasma samples (10 μl of each) using a MARS Hu-7 spin column following the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). We collected the flow-through fractions, which were dried via speed vacuum centrifugation. Dry protein samples were re-suspended with 100 μl of 6 M urea in 50 mM NH₄HCO₃ solution, and the two flow-through fractions were combined and then reduced, alkylated, and digested with trypsin under the same conditions as for the seminal plasma samples. The processed blood plasma was restored in 50-μl volumes in 5% ACN with 0.1% formic acid (dilution factor of 5) and stored at −20 °C until analysis.

At the time of analysis, both the seminal and blood plasma samples were spiked with heavy surrogate peptides (including the non-unique IVGGWECF) at 20 fmol/μl and 2 fmol/μl, respectively, and diluted 10 times in 5% ACN with 0.1% formic acid.

MRM assay of PSA—During the method development, the software tool Skyline v1.2 (MacCoss Lab Software, Seattle, WA) was used exclusively. Peptide sequence lists were prepared manually based on the selected proteotypic tryptic sequences. Primarily, high numbers of transitions and all possible y-ion series that matched the criteria (from m/z > precursor-2 to last ion-2, precursor m/z exclusion window: 20 Th) were selected for each peptide at both 2+ and 3+ precursor charge states. Finally, the five most intense transitions were selected for each peptide via manual inspection of the data in Skyline, and scheduled transition lists were created for the final assay at both doubly and triply charged states when applicable (see supplemental Table S2).

Mass Spectrometric Analysis—Tryptic peptide digests were injected (2 μl) onto and desalted online on a trap column (Easy Column™ C18-A1 5 μm, 2 cm × 100 μm, Thermo Scientific, Waltham, MA) and separated on a capillary analytical column (15 cm × 75 μm, packed with ReproSil C18-AQ 3 μm, 120-Å particles, from Dr. Maisch GmbH, Ammerbuch, Germany) using an Easy n-LC II system (Thermo Scientific, Odense, Denmark) at a 300-μl/min flow rate. The mobile phases were (A) 100% LC-MS purity water with 0.1% formic acid and (B) 100% ACN with 0.1% formic acid. The peptides were eluted with a 45-min linear gradient starting with 10% B to 35% B, followed by a 5-min linear gradient to 90% B and a column wash at 90% B for 5 min.

A TSQ Vantage triple quadrupole instrument (Thermo Scientific, San Jose, CA) was used with the Flex ESI-interface and working in SRM mode in positive polarity. The MS analysis was conducted with the spray voltage and declustering potential set at 1750 V and 0 V, respectively. The transfer capillary temperature was set at 270 °C, and a tuned S-lens value was used. MRM transitions were acquired in Q1 and Q3 operated at unit resolution (0.7 full width at half-maximum), and the collision gas pressure in Q2 was set to 1.2 mTorr. The cycle time was 2.5 s in the nonscheduled methods and 1.5 s in the scheduled methods.

Data Evaluation and Quantification of PSA—The raw files generated on the triple quadrupole mass spectrometer were imported to Skyline for data analysis. Quantification was based on the calculation of ratios between the corresponding endogenous and IS peak areas. The peak integration was automatically performed by the software using Savitzky-Golay smoothing, and all imported data were inspected manually to confirm the correct peak detection. Further statistical analysis was done using Microsoft Excel.

RESULTS

Selection of Proteotypic Peptides of PSA—We previously found that PSA exists in several molecular forms in seminal plasma (38), which may be commonly regarded as proteoforms, a term recently introduced for a general category of closely related proteins that includes isoforms, splicing variants, and their post-translationally modified forms (37). However, this microheterogeneity of PSA in clinical samples could be observed only by repeatedly detecting the same tryptic peptides of PSA in electrophoretically separated bands. Therefore, we have designed a highly specific and more sensitive approach utilizing MRM principles on a triple quadrupole mass spectrometer (TSQ Vantage). Our strategy is based on theoretically derived tryptic peptides (in silico digestion) of 10 PSA proteoforms found in the UniProtKB database (see supplemental Table S1). Following filtering of the initial set of 30 sequences to fit MRM experimental conditions, 14 proteotypic peptides were recognized, of which 3 were also isoform specific:

| Peptide sequence | m/z [M+H]+ (heavy) | Protein | neXtProt AC |
|------------------|---------------------|---------|-------------|
| IVGGWECF         | 1085.5176           | PSA/hk2 | NX_P07288/NX_P20151 |
| SVILLGR          | 767.5013            | PSA     | NX_07288    |
| LSEPAELTDVK      | 1280.8448           | PSA     | NX_07288    |
| LSEPAETDVK (SNP-L132I)| 1280.8448 | PSA | NX_07288 |

One of the sequences (IVGGWECF) was recognized as present in both PSA and hK2 and thus could not be regarded as a unique proteotypic peptide (see Table II). In blood and seminal fluid, however, the concentration of PSA is about 2 orders of magnitude greater than that of hK2, and thus IVGGWECF could also quantify PSA with reasonable approximation.

During the MRM assay development, 14 synthetic peptides were tested, resulting in a list of six suitable sequences...
(FLRPGDSSHDLMLLR, HSQPWQVLVASR, LSEPAELTDAVK, IVGGWECEK, FMLCAGR, and SVILLGR) that were employed for testing in seminal plasma samples with PSA levels ranging from 0.35 to 1.9 mg/ml. All these sequences could provide acceptable analytical characteristics, including stable and repeatable signal responses and good peak shape without apparent interference in the matrix. In this series of experiments, these six tryptic peptides were systematically observed with good signal intensities and at least acceptable peak shapes. However, none of the isoform specific peptides were detected in the clinical samples investigated in this study.

Proteoforms of PSA in Seminal and Blood Plasma—In screening of the seminal plasma samples (n = 35), in most cases the LSEPAELTDAVK peptide (m/z = 636.84 [M+2H]2+) was observed as a single peak (n = 28), as shown in the left-hand panel in Fig. 1. However, in some cases it also was detected as double peaks (n = 6) within the scheduled 4-min analytical window, as shown in the middle panel in Fig. 1. Interestingly, the additional peak with a shorter retention time (Δt = −0.6 min) was noticed with transitions identical to those of the annotated second peak, as identified by the corresponding heavy-isotope-labeled IS peptide with similar signal intensities (ratio 1:1). Furthermore, one of the seminal plasma samples showed only the more hydrophilic peptide peak with a shorter retention time that did not match the peak of the surrogate LSEPAELTDAVK peptide (see the right-hand panel in Fig. 1).

Because the transitions of both chromatographic peaks were identical, suggesting isobaric peptides with a slight difference in their hydrophobicity, we tested a similar sequence in which the second Leu was replaced with an Ile residue (LSEPAEITDAVK) and proved that the first peak indeed represented the common PSA variant SNP-L132I. This mutation has a frequency of about 10% in the population and thus needs to be monitored when quantifying PSA. Following completion of the blood plasma analysis, two additional samples showed either heterozygous (n = 1) or homozygous (n = 1) expressions of the mutant PSA gene, providing a total rate of 9.72% heterozygotes (n = 7) and 2.78% homozygotes (n = 2) in our sample cohort. The peak areas of both LSEPAELTDAVK and LSEPAEITDAVK peptides were combined for the quantification of PSA in samples with heterozygous expression.

PSA Levels in Seminal and Blood Plasma Samples—The endogenous levels of PSA peptides in the seminal and blood plasma samples were calculated by taking the ratio between the peak areas of the endogenous (light) and IS peptides (heavy) and correlating it to the known concentration of the heavy peptides that were spiked into the samples. The endogenous levels of PSA in whole seminal and blood plasma samples were calculated from the data obtained with four tryptic peptides (LSEPAELTDAVK, LSEPAEITDAVK, IVGGWECEK, and SVILLGR, as shown in Table II) by adjusting for the dilution at sample preparation. The calculations were made for the four different peptides individually in seminal and blood plasma samples as presented in Tables III and IV, respectively.

Comparing the determination of the PSA concentration in seminal plasma using four peptides (LSEPAELTDAVK, LSEPAEITDAVK, IVGGWECEK, and SVILLGR) revealed that the SVILLGR peptide generally had the highest levels, with the exception of one sample for which it showed the lowest value. Determination with the peptide IVGGWECEK generally resulted in the lowest levels, except for that same sample (see Table III and supplemental Fig. S1).

Taking the difference between the determinations, the combination of LSEPAELTDAVK and LSEPAEITDAVK indicated levels that were about 85% of the levels of SVILLGR, and...
IVGGWECEK levels were about 60% of the levels of SVILLGR (see Figs. 2A–2C and supplemental Fig. S1). However, the linear regression coefficients between the determinations made by the three different peptides were excellent, with $R^2$ values ranging between 0.97 and 0.99 (see Figs. 2A–2C). Comparison of the determined concentrations of PSA peptides in blood plasma revealed that the peptide SVILLGR and the combination of LSEPAELTDAVK and LSEPAEITDAVK resulted in very similar values (see Table IV). As in seminal plasma, the levels determined by the peptide IVGGWECEK were the lowest—about 70% of the levels found for the other two peptides (see Figs. 2D and 2E and supplemental Fig. S2). The linear regression coefficients between the determinations calculated using the PSA peptides were excellent, with $R^2$ values greater than 0.99 (see Figs. 2D and 2E). From this result we could conclude that the digestion was effective and tryptic PSA peptides were sufficiently released from complexes with $\alpha$-1-antichymotrypsin and $\alpha$-2-macroglobulin predominant in blood. From this point of view, the MRM assay was independent of the sample source, as PSA levels in both free and complexed forms could be determined in seminal and blood plasma, respectively.

Comparison of the concentrations of PSA obtained via the standard clinical test (DELFIA®, PerkinElmer Life Sciences) and the MRM assay showed that PSA levels were consistently lower than the immunoassay. These measured levels in blood plasma indicated that the depletion of the seven most abundant proteins did not remove a significant amount of bound PSA. The concentration obtained with the peptide SVILLGR was about 60% of the fPSA level determined using DELFIA®, whereas it was only 50% and 34% of that level for the peptides LSEPAELTDAVK and LSEPAEITDAVK, respectively. However, the correlation coefficients between the immunoassay and MRM assay determinations for the PSA concentrations were excellent in seminal ($R^2$ values of 0.82–0.85) and exceptional in blood plasma ($R^2$ values greater than 0.99) samples, respectively (see Fig. 3).

Reproducibility and Precision of MRM Assay—The linearity of the MRM assay was determined by spiking a mixture of heavy-labeled IS peptides diluted in seven steps into a pooled...
sample of seven individual blood plasma samples. Analysis was performed in five replicates. The peak area of each IS peptide peak was then plotted against the theoretical concentrations (Fig. 4). Linear regression fitting was performed, resulting in $R^2$ values greater than 0.99 within the investigated concentration range (0.03–30 fmol/μl). The integrated peak areas of the corresponding endogenous peptides in the sample were constant (except for LSEPAEITDAVK, which was absent). The limit of quantification of these peptides in blood plasma was estimated at the lowest concentration measured with $\text{cv} < 20\%$ and was found to be 0.1 fmol/μl for IVGGWECEK and SVILLGR, whereas it appeared to be somewhat below the lowest measured value (0.03 fmol/μl) for LSEPAELTDAVK and LSEPAEITDAVK. This limit of quantification corresponds to a PSA concentration of 0.86 ng/ml.

In order to evaluate the analytical performance of the experimental workflows, including tryptic digestion only (seminal plasma samples) or depletion combined with digestion (blood plasma samples), we investigated some key parameters. The retention times of the heavy-isotope-labeled IS peptides were monitored and are summarized in Table V, showing a variation of less than 2%.

Technological variations were determined in six randomly selected seminal plasma samples analyzed in triplicate (see Table VI). The concentrations of endogenous PSA peptides were determined by using the Skyline algorithm for integration of the peak area (weighted average of all transitions) and calculating the mean value, S.D., and $\text{cv}$. The $\text{cv}$ ranged between 0.3% and 4.5% (77.7% of all $\text{cv}$ values were below 3%). Notably, the least variation in these samples was observed with the LSEPAELTDAVK peptide, and the most with the SVILLGR peptide.

Biological replicates were also generated by depleting a blood plasma sample in five separate batches following digestion and spiking with a mixture of heavy IS peptides at 2 fmol/μl. The overall variation in the blood plasma workflow was less than 9.4%, as judged by the measured concentrations of the given endogenous PSA peptides (see Table VII).

**DISCUSSION**

PSA quantification via MRM assay has a scientific history of almost 10 years (8), driven by the fact that PSA is available as a purified protein product and routinely analyzed in clinical samples by means of specific immunoassays in hospitals. Based on sequence MS/MS data and observation frequency, there are a number of valuable proteotypic tryptic peptides that quantification methods can employ efficiently. Considering the high specificity and sensitivity of MRM transitions in triple quadrupole mass spectrometers, the approach appears to be suitable for targeted protein identification as well. By deriving isoform specific unique peptides of PSA, we were able to develop such an MRM assay focused on the identification of three additional isoforms of PSA based on three tryptic peptides. Additionally, all other tryptic peptides of PSA were monitored simultaneously in order to evaluate our analytical strategy and identify further signature sequences suitable for quantification in clinical samples.

We could confirm that the most sensitive and reliable unique peptide was LSEPAELTDAVK, as has been observed by others (9–14), largely because of the intensive signal generated by the $y$-9 transition channel. The other frequently used tryptic peptide of PSA, IVGGWECEK (8, 9, 11), is not unique, as this N-terminal sequence is present in both PSA and hK2. Consequently, it is not recommended for use in quantitation.
without accounting for the mutual contributions of these proteins to the detected endogenous levels. Furthermore, the concentrations experimentally determined using IVGGWECEK were found to be the lowest, although they should be a combination of PSA and hK2 (1000:1 molar ratio in both seminal and blood plasma). Considering that the amount of heavy IVGGWECEK spiked into the plasma samples was unknown, the consistently lower levels of PSA determined by this peptide reflect the lower absolute amount of IS.

We were able to classify another unique PSA peptide (SVILLGR) that could be used for quantification and displayed excellent analytical properties (see Figs. 2, 3, and 4). Despite the fact that SVILLGR is located in the vicinity of the glycosylation site of PSA, no difficulties were observed in the quantification of PSA using this peptide. This might be explained by the general observation that digestion was efficient even in blood plasma, in which PSA is present predominantly in complex with other proteins. The comparison of PSA levels determined by three signature peptides indicated that SVILLGR could provide PSA concentrations similar to those determined with the other two sequences in most individual samples. The possible correlation between the degree of PSA glycosylation and the efficiency of proteolytic release of SVILLGR may be further investigated.

The most important outcome of our study was the discovery of an SNP variant of PSA in 9 out of 72 clinical samples carrying the nonsynonymous mutation L132I (rs2003783), which is located within the LSEPAELTDAVK peptide. Because of the isobaric precursor and fragment ions, identical transitions were produced and observed in the analysis of those specific samples. The peaks of LSEPAELTDAVK and LSEPAEITDAVK were baseline separated in the reversed-phase gradient used, clearly indicating that the LSEPAEITDAVK sequence is more hydrophilic and has a shorter retention time. Because both of the isoforms can be present in the

| Sample | PSA | LSEPAELTDAVK | LSEPAEITDAVK | LSEPAELTDAVK+LSEPAEITDAVK | SVILLGR | IVGGWECEK |
|--------|-----|--------------|--------------|-----------------------------|---------|-----------|
| ID     | ng/ml^a | ng/ml^b | ng/ml^b | ng/ml^b | ng/ml^b | ratio | ng/ml^b | ratio |
| BP1    | >100 | 832 | n.d. | 832 | n.a. | 833 | n.a. | 571 | n.a. |
| BP2    | >100 | 354 | n.d. | 354 | n.a. | 425 | n.a. | 286 | n.a. |
| BP3    | >100 | 203 | n.d. | 203 | n.a. | 229 | n.a. | 151 | n.a. |
| BP4    | >100 | 471 | n.d. | 471 | n.a. | 517 | n.a. | 324 | n.a. |
| BP5    | n.a. | 278 | n.d. | 278 | n.a. | 285 | n.a. | 101 | n.a. |
| BP6    | n.a. | 809 | n.d. | 809 | n.a. | 902 | n.a. | 635 | n.a. |
| BP7    | 2200 | 1429 | n.d. | 1429 | 0.65 | 1557 | 0.71 | 1047 | 0.48 |
| BP8    | 383 | 237 | n.d. | 237 | 0.62 | 266 | 0.69 | 174 | 0.45 |
| BP9    | 228 | 161 | n.d. | 161 | 0.71 | 178 | 0.78 | 124 | 0.54 |
| BP10   | n.a. | 259 | n.d. | 259 | n.a. | 288 | n.a. | 187 | n.a. |
| BP11   | 318 | 234 | n.d. | 234 | 0.74 | 261 | 0.82 | 144 | 0.45 |
| BP12   | 270 | 166 | n.d. | 166 | 0.61 | 188 | 0.70 | 124 | 0.46 |
| BP13   | 1100 | 682 | n.d. | 682 | 0.62 | 683 | 0.62 | 521 | 0.47 |
| BP14   | n.a. | 768 | n.d. | 768 | n.a. | 773 | n.a. | 510 | n.a. |
| BP15   | 223 | 179 | n.d. | 179 | 0.80 | 196 | 0.88 | 134 | 0.60 |
| BP16   | 201 | 147 | n.d. | 147 | 0.73 | 139 | 0.69 | 107 | 0.53 |
| BP17   | 870 | 497 | n.d. | 497 | 0.57 | 526 | 0.60 | 360 | 0.41 |
| BP18   | 225 | 148 | n.d. | 148 | 0.66 | 211 | 0.94 | 96 | 0.43 |
| BP19   | 183 | 106 | n.d. | 106 | 0.58 | 60 | 0.33 | 79 | 0.43 |
| BP20   | 1224 | 83 | n.d. | 83 | 0.68 | 35 | 0.29 | 46 | 0.38 |
| BP21   | 106 | 79 | n.d. | 76 | 0.66 | 87 | 0.82 | 65 | 0.61 |
| BP22   | 157 | 106 | n.d. | 106 | 0.68 | 162 | 1.03 | 88 | 0.56 |
| BP23   | 203 | 120 | n.d. | 120 | 0.59 | 119 | 0.59 | 79 | 0.39 |
| BP24   | 1619 | 126 | n.d. | 125 | 0.78 | 86 | 0.53 | 80 | 0.49 |
| BP25   | 587 | 443 | n.d. | 443 | 0.76 | 497 | 0.85 | 298 | 0.51 |
| BP26   | 454 | 251 | n.d. | 251 | 0.55 | 330 | 0.73 | 156 | 0.34 |
| BP27   | 451 | 288 | n.d. | 288 | 0.64 | 365 | 0.81 | 227 | 0.50 |
| BP28   | 2563 | 162 | n.d. | 162 | 0.63 | 183 | 0.71 | 149 | 0.58 |
| BP29   | 600 | 403 | n.d. | 403 | 0.67 | 314 | 0.86 | 221 | 0.54 |
| BP30   | 389 | 280 | n.d. | 280 | 0.72 | 289 | 0.74 | 202 | 0.52 |
| BP31   | 325 | 136 | n.d. | 136 | 0.42 | 145 | 0.45 | 121 | 0.37 |
| BP32   | 1653 | 534 | n.d. | 534 | 0.51 | 631 | 0.60 | 439 | 0.42 |
| BP33   | 499 | 520 | n.d. | 520 | 0.64 | 491 | 0.96 | 285 | 0.57 |
| BP34   | 168 | 123 | n.d. | 123 | 0.73 | 121 | 0.72 | 57 | 0.34 |
| BP35   | 4449 | 2743 | n.d. | 2743 | 0.62 | 3319 | 0.75 | 2280 | 0.51 |
| BP36   | 2632 | 156 | n.d. | 156 | 0.59 | 238 | 0.90 | 99 | 0.38 |
| BP37   | 1077 | 377 | n.d. | 362 | 0.69 | 812 | 0.75 | 623 | 0.50 |

a Total PSA levels determined via the routinely used clinical test based on immunoreaction (DELFIA®).

b Concentration of PSA calculated from the endogenous peptide levels (MW = 28,741 g/mol).

c The ratios between the SRM values and the DELFIA® values are calculated.

d n.d., not detected.
same sample (heterozygous expression profile), the areas of both peaks have to be combined when quantifying the total amount of PSA.

The population-based frequency of allele A in exon 3 of the KLK3 gene (dbSNP code: rs2003783) is 10% worldwide, 8% in Asia and Europe, 14% in Africa, and 11% in America as...
reported in the 1000 Genomes database. A similar frequency rate (12%) was observed in Swedish study cohorts used for re-sequencing and genotyping of all KLK genes (40). It is worth mentioning that the KLK3 gene has 51 SNP sites registered, but only 3 can trigger residue change. The SNP-L132I variant of PSA (Ensembl protein summary: ENSP00000314151.1) was not significantly associated with the risk of prostate cancer based on a large case-control cohort from Sweden (CAPS) (40). Furthermore, SNP prediction tools (SIFT and SNPS3D), used in studies of the possible effects of amino acid substitutions on proteins functions, recognized this SNP as tolerated, and only PolyPhen2 indicated an association with benign disorders in tumors, conserved across multiple species. This controversy was not further supported by the studies investigating rs2003783, which mentioned no associations with disease (41, 42). Transcript databases registered evidence of the existence of transcript variant 3 mRNA (NM_001030047.1), resulting in the entry of PSA isoform 3 in protein databases. The subtle alteration the Leu-Ile exchange caused in the loop it localized has intermediate solvent accessibility (16%) and is predicted to have physicochemical properties similar to those of the wild type, as both residues are medium-sized and hydrophobic (Leu/Ile) (see UniProtKB/Swiss-Prot variant pages: VAR021942). The three-dimensional structure of PSA with Ile132 is available at RCSB (PDB code: 2zch).

### Table V

Summary of the chromatographic retention times (in minutes) for four tryptic PSA peptides (LSEPAELTDAVK, LSEPAEITDAVK, SVILLGR, and IVGGWECEK). The registered values are for the heavy peptides spiked into the biological samples.

| Sample ID | LSEPAELTDAVK | LSEPAEITDAVK | SVILLGR | IVGGWECEK |
|-----------|--------------|--------------|---------|------------|
|           | Average SD %CV Average SD %CV Average SD %CV Average SD %CV |
| SP (n=35) | 19.92 0.21 1.9 19.19 0.22 1.2 19.34 0.33 1.7 14.58 0.33 1.7 |
| BP (n=27) | 20.12 0.19 1.0 19.41 0.22 1.1 19.69 0.20 1.0 15.31 0.24 1.5 |

### Table VI

Quantification of PSA in seminal plasma by the four tryptic PSA peptides LSEPAELTDAVK + LSEPAEITDAVK, SVILLGR, and IVGGWECEK, using all the transitions for one peptide summed together and using each transition on its own. The values are given in fmol/μl for seminal plasma (n = 3) and in amol/μl for blood plasma samples (BP11 and BP20 → n = 3 and BPpooled → n = 5).

| Sample ID | LSEPAELTDAVK + LSEPAEITDAVK | SVILLGR | IVGGWECEK |
|-----------|-----------------------------|---------|------------|
|           | All transitions y2 y8       | All transitions y2 y8 | All transitions y2 y8 |
|           | Average SD SD %CV Average SD SD %CV Average SD SD %CV |
| SP7       | 365 366 357 377 371 426 430 426 387 251 251 253 261 249 242 |
| SD        | 3.9 3.6 19 6.1 4.6 19 20 21 36 2.7 2.5 25 9.0 10 |
| BP11      | 102.8 124.06 77.31 66.67 143.16 168.11 161.22 190.77 152.34 113.18 93.87 134.33 361.33 80.06 17.21 |
| SD        | 12.0 0.6 40 6.0 8.9 13.3 1.3 2.5 18 2.4 1.7 1.8 3.9 3.8 2.0 6.1 |
| BP20      | 185.13 241.56 144.82 151.99 262.16 574.34 601.39 563.58 558.15 340.6 410.26 269.57 955.68 232.04 282.73 |
| SD        | 11.35 7.94 23.55 18.97 34.90 17.72 26.17 55.30 58.23 48.54 47.39 125.94 94.28 65.36 169.25 |
| BPpooled1 | 365.61 451.20 263.79 318.46 428.98 533.66 589.65 579.29 432.05 341.91 351.74 291.28 639.77 241.01 185.75 |
| SD        | 60.93 26.63 0.56 105.19 174.07 21.88 41.25 76.38 36.22 130.38 37.22 52.80 259.38 100.67 112.81 |
| BPpooled2 | 16.67 5.90 31.30 32.40 40.59 4.10 6.99 13.19 8.39 38.22 9.30 120.12 40.54 41.77 60.73 |
| BPpooled   | 19.90 24.17 35.76 67.21 77.42 3.65 8.97 13.07 16.26 24.66 8.94 28.18 69.11 57.27 25.63 |

### Table VII

Variability measured over the whole work-up process of a blood plasma sample (BP1), including depletion, digestion, and analysis (n = 5). PSA levels were determined with three tryptic PSA peptides (LSEPAELTDAVK, SVILLGR, and IVGGWECEK). No endogenous levels of the isoform LSEPAEITDAVK were observed in this sample. The concentrations were calculated from known spiked levels of heavy peptide.

| Sample ID | LSEPAELTDAVK | SVILLGR | IVGGWECEK |
|-----------|--------------|---------|------------|
|           | LSEPAELTDAVK | SVILLGR | IVGGWECEK |
|           | Average S.D. % CV | Average S.D. % CV | Average S.D. % CV |
|           | 4.6 7.0 3.0 | 0.43 0.52 0.28 | 9.4 7.4 9.2 |
| Average concentration of PSA (fmol/μl) | 4.6 7.0 3.0 | 0.43 0.52 0.28 | 9.4 7.4 9.2 |
The fact that this is the first observation of this SNP variant of PSA at expression levels is likely to be the result of screening through a large number of individual samples. In accordance with the ever-increasing activity in proteomics research, such findings may pave a path to a new domain of proteoforms, making it possible to detect and screen for mutated proteins. Previous studies have demonstrated the efficiency of MS in identifying post-translationally modified proteins and highly abundant abnormal proteins, such as those responsible for amyloidosis (43–47). This field of proteomics is currently under exploration, indicating a strong disease link with some mutations (48).

Selected reaction monitoring is not optimal in complex matrices, as the likelihood of finding another peptide sharing the same transition is relatively high even within a narrow time window (9). Therefore, multiple transitions of the most suitable proteotypic peptide were selected for quantification. Additionally, the choice of signature peptides is not limited to the experimentally detected peptides; theoretically derived sequences can also be considered (in silico digestion). Comparing PSA quantifications in clinical samples performed with the three different peptides proved that a newly proposed peptide (SVILLGR) was applicable with good concordance with two previously reported sequences, as well as with immunooassay values. The systematic deviation among the concentrations determined by the three different peptides (see the section “PSA Levels in Seminal and Blood Plasma Samples”) is most likely due to the different amounts of heavy-labeled IS peptides spiked into the samples. The absolute amount of the synthetic peptides was not determined and thus is a source of ±25% variation, which covers well the 30% to 40% difference between IVGGWECEK and SVILLGR determinations. In order to build a clinical assay for use in central hospital laboratories, the next step of our development would be to define the levels of the internal standards.

The agreement between the MRM assay and the DELFIA® results is remarkable, particularly in blood plasma samples. The somewhat poorer correlation in seminal plasma samples may be explained by the relevant dynamic range of DELFIA®, which is below the endogenous levels of PSA in seminal plasma (fPSA: 0.04–250 ng/ml; tPSA: 0.05–250 ng/ml) and thus is compromised with larger error.

CONCLUSIONS

Nano-LC-MS/MS technology has matured sufficiently as judged by the high reproducibility reported in our experiments and others (14–16). This has made it possible to process smaller sample volumes, provided that the target proteins are present at low ng/ml levels. Arguably, immunodepletion is still required in order to reach this sensitivity in blood plasma samples, and consequently a portion of target molecules may not be analyzed upon complex formation in the matrix. Advanced chromatographic systems can already provide high resolution when combined with the intelligent selection of fragments containing target molecules (16) and immunoreaction enrichment of biomarkers at sub-ng/ml levels. We believe that this development holds the potential to become an optional platform for clinical analyses in the future (49).

Our goal of identifying specific proteoforms of PSA based on detecting unique tryptic peptides resulted in the important observation that a new PSA isoform could be identified by the altered amino acid sequence within a frequently used tryptic peptide (LSEPAELTDVK → LSEPAEITDVK). This allele of the KLK3 gene coding for the SNP-L132I variant is present in the human population at a significant level (ca. 10%) and consequently has to be considered when screening clinical samples.

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