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

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KEYWORDS

Prostate specific antigen, Kallikrein-related peptidases, SNP variant, Multiple reaction monitoring, Mass spectrometry, Quantitative proteomics

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Abstract

Prostate specific antigen (PSA) is a well-established tumor marker, which is frequently employed as model biomarker to develop and evaluate emerging quantitative proteomics techniques, partially due to wide access to commercialized immunoassays serving as “gold standard”. We designed a multiple reaction monitoring (MRM) assay to detect PSA proteoforms in clinical samples (n=72), utilizing specificity and sensitivity of the method. We report for the first time, a PSA proteoform, coded by SNP-L132I (rs2003783), observed in 9 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, providing close agreement between each quantitation based on three selected peptides (LSEPAELTDAVK, IVGGWECEK and SVILLGR) and a routinely used commercialized immunoassay. Additionally, we have disclosed that the peptide IVGGWECEK is shared with kallikrein-related peptidase 2 and therefore not unique for PSA. Hence, we propose to use another tryptic sequence (SVILLGR) for accurate MRM-quantification of PSA in clinical samples.

1. Introduction

Moving towards biomarker verification and clinical implementations of novel assays, mass spectrometry based quantitative analyses of biomarkers is at an increasing rate becoming an important route for current proteomics studies. Although, MS instrumentation offers various powerful strategies for biomarker discovery (1), the validation phase of these putative protein candidates primary still relies on immunoreaction based assays, such as ELISA (2). These immunoassays are considered to be effective diagnostic tools routinely used in clinical practice, however often associated with lengthy and expensive development of high quality antibodies, yet displaying significant differences between tests from different vendors. Further on, immunoassays depend on indirect readouts (colorimetric, fluorescent, or radioactive) and may produce false positive results due to nonspecific binding. On the other hand MS nowadays is able to measure analytes with high quantitative accuracy and established MS methods originally developed for quantitation of small molecules, such as multiple reaction monitoring (MRM) (3), have been successfully introduced also for proteins (4-6). As compared with traditional ELISA techniques, MRM assays can be cost-efficiently, quickly developed methods and offer exceptional multiplexing capability (7).

Interestingly, prostate specific antigen (PSA), being a successful biomarker of prostate cancer, has been frequently chosen as 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 liquefaction of seminal coagulum by degrading 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 release of $\approx 5\text{-}50\ \mu\text{mol/L}$ concentrations of mainly catalytic PSA into seminal fluids is regulated by the nuclear androgen receptor, with levels in blood normally being a million-fold lower ($20\ \text{pmol/L}$) wherein PSA is non-catalytic and predominantly lined in a covalent complex with $\alpha\text{-}1\text{-antichymotrypsin}$ (SERPINA3) (27-29). PSA in blood may elevate due to benign conditions including prostatitis or benign prostate hyperplasia (BPH) but modestly elevated PSA in blood at middle age is also strongly associated with metastasis or death from prostate cancer decades later (30, 31) and PSA-screening can reduce cancer-related deaths but may also lead to over-diagnosis and overtreatment (32, 33). Hence, controversy remains regarding the merits of the PSA-test (34, 35), although it remains as mainstay in monitoring of therapeutic intervention, detection of disease recurrence or progression (36).

PSA was chosen as 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 ($^{13}\text{C}_2$ and $^{15}\text{N}_1$ on each Gly residues), was utilized as internal standard (IS), while known amounts of purified PSA was spiked into female serum and a selected reaction monitoring (SRM) transition channel ($y\text{-}7$) was monitored with excellent reproducibility, achieving limit of detection (LOD) of $4.5\ \mu\text{g/mL}$. PSA and 5 other proteins were selected in a multiplexing study that has systematically selected the most useful signature peptides and monitored 3 transitions per peptide (9). The most abundant transitions (IVGGWECEK: $539.3\rightarrow 865.3$ and LSEPAELTDAVK: $636.7\rightarrow 943.4$) were used for quantification on nano-flow LC

hyphenated with a hybrid QTrap mass spectrometer. This work was further explored in an encouraging inter-laboratory study that has compared MRM analytical performances on 7 proteins and 3 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 (PIM) on a linear ion trap instrument (Thermo LTQ) (10). Also in this study, LSEPAELTDAVK was selected for quantification of recombinant PSA spiked into female plasma, because 3 additional PSA peptides (HSQPWQVLVASR, HSLFHPEDTGQVFQVSHSFPHPPLYDMSLLK and FLRPGDDSSHDLMLLR) were noticed to ionize less efficiently. Notably, this methodological study reported for the first time on quantification of PSA in two prostate cancer patient samples (300 and 5000 ng/mL) using MRM-MS. Prostate cancer cell lines were also investigated by an SRM-MS assay in order to correlate PSA levels with clinical tests selecting two signature peptides, LSEPEALTAVK and HSQPWQVLVASR (21).

Although, the progress of methodological developments has accelerated, promising successful clinical implementations in the near future, the number of real samples from patients remained reduced (n=9 with prostate cancer (13) and n=3 with BPH in (12), respectively) using barely LSEPAELTDAVK for quantification. The same group has utilized IVGGWECEK for specific detection of cysteine-containing peptides in plasma using laser-induced photo-dissociation (photo-SRM) for protein quantification (17). Nevertheless, these important studies offered PSA quantification in patient samples at 4-30 ng/mL levels following albumin depletion, tryptic digestion, solid-phase extraction and conventional HPLC separation of 100 μ L serum. For further validation PSA concentrations determined

by MS methods were correlated to a clinical ELISA test with high concordance (13). A novel enrichment strategy employing mass spectrometric immunoassay (MSIA) SRM was applied to access PSA in serum samples measuring SVILLGR as well as an isoform specific tryptic peptide DTIVANP (19). N-linked glycopeptides of PSA were targeted in a study of 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 digital ProteomeChip (Cell Biosciences, Santa Clara, CA) used for selective enrichment of proteotypic peptides with nanoLC-SRM-MS was demonstrated in 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 MRM assay developed for differential quantification of free and total PSA (fPSA and tPSA) in clinical serum samples (n=9) with 0.3-18.9 ng/mL concentrations, determined by an immunoassay (15). Good sensitivity was achieved with LOQs of 2.03 and 0.86 ng/mL for fPSA and tPSA, respectively. The same research group has further improved 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 and high-resolution liquid chromatographic separations without involvement of antibodies (16).

All of these previous reports presented 2 peptides selected for 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 being also in present in human kallikrein-related peptidase 2 (KLK2 or hK2) and that LSEPAELTDAVK is coded on the exon of *KLK3* with a single nucleotide polymorphism (SNP) resulting amino acid exchange of L132I (rs2003783).

Due to 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. Since MRM assay outcomes rely on the detection of a specific peptide of the given protein and tryptic digestion may not always be complete, we screened multiple proteotypic peptides with multiple transitions.

Our study is the first to report on the detection of a proteoform of PSA as the translated gene product of a 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 to quantify PSA in seminal fluid and blood compared to commercialized immunoassays in the largest clinical sample set reported so far (n=72).

2. Material and Methods

2.1. Biological samples

Seminal plasma was prepared from semen obtained from young men undergoing investigation for infertility prior to final diagnosis of disorders (n=30) and healthy volunteers (n=5), following the guidelines of the Helsinki Declaration (<http://www.wma.net/en/20activities/10ethics/10helsinki/>) as described earlier (38). The collection of seminal plasma was approved by the ethical board at Lund University (approval number: LU 532-03) and stored at -20°C until use. Free PSA levels ranging from 0.35 to 1.9 mg/mL were determined by 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 1 µg/µL final PSA concentration.

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

2.2. *In silico* selection of signature peptides

For the identification of PSA isoforms the UniProtKB/TrEMBL database (v.52 2011_11) was used that included both reviewed and non-reviewed sequence variants. All listed sequence variations (10 PSA forms, see **Suppl. Table 1**), including N-terminal signaling

peptides, were used for further processing of *in silico* digestion using trypsin. The theoretical digestion was performed by the PeptideMass tool (available at the ExPASy Proteomics Server web site, <http://expasy.org/sprot/>) using the following settings: iodoacetamide as alkylation agent and no miss-cleavage. The resulted tryptic peptides of all isoforms of PSA were investigated for uniqueness by blast search on the UniProtKB website (<http://www.uniprot.org/uniprot/>). The isoform specificity of the proteotypic peptides was also noticed at this step (**Table 1**). Finally, a list of tryptic peptides was prepared filtering by size (from 7 to 26 AAs) 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, isotope labeled with ^{15}N and ^{13}C in lysine ($\Delta\text{mass}=+8$) and arginine ($\Delta\text{mass}=+10$) of AQUA QuantPro quality (peptide purity higher than 97%, concentration precision equal or better than $\pm 25\%$) (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 internal standard peptide was used to estimate the concentration of PSA in the samples. The list of transitions is presented in **Suppl. Table 2**.

2.3. Preparation of peptide samples

The crude, synthetic peptides were dissolved in 100 μL of 20% 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 (9-26 μL) corresponding to 0.2 mg protein was processed, resulting in different dilution factors used for calculation of PSA levels. The samples were reduced with 10 mM dithiothreitol at 37°C for 60 min, 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 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 addition of 10 μL of 1% formic acid. The resulting protein digests were dried on speed vacuum centrifugation and 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 by speed vacuum centrifugation. Dry protein samples were re-suspended with 100 μL of 6 M urea in 50 mM NH_4HCO_3 solution and the two flow-through fractions were combined and then were 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 time for analysis, both the seminal and blood plasma samples were spiked with heavy surrogate peptides (including the non-unique IVGGWECEK) at 20 fmol/ μ L and 2 fmol/ μ L, respectively and diluted ten times in 5% ACN with 0.1% formic acid.

2.4. MRM assay of PSA

During the method development the software tool of 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, all possible y-ion series that matched the criteria (from $m/z > \text{precursor}-2$ 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 5 most intense transitions were selected for each peptide by manual inspection of the data in Skyline and scheduled transition lists were created for the final assay at both doubly and triply charge states when it was applicable (see **Suppl. Table 2**).

2.5. Mass spectrometric analysis

Tryptic peptide digests were injected (2 μ L) onto and desalted on-line on a trap column (Easy Column™ C18-A1 5 μ m, 2 cm x 100 μ m, Thermo Scientific, Waltham, MA) and separated on a capillary analytical column (15 cm x 75 μ m, packed with ReproSil C18-AQ 3 μ m, 120 Å particles from Dr. Maisch GmbH, Ammerbuch, Germany), using an Easy n-LC II system (Thermo Scientific, Odense, Denmark) at 300 μ L/min flow rate. The mobile phases

were A: 100% LC-MS purity water with 0.1% FA and B: 100% ACN with 0.1% FA. The peptides were eluted with a 45-min linear gradient starting with 10% B to 35% B, followed by a 5 minutes linear gradient to 90% B and a column wash at 90% B for 5 minutes.

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

2.6. 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 internal standard peak areas. Peak integration was automatically performed by the software using Savitzky-Golay smoothing, whereas all imported data were inspected manually to confirm the correct peak detection. Further statistical analysis was done using Microsoft Excel.

3. Results

3.1. Selection of proteotypic peptides of PSA

We have 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 observed microheterogeneity of PSA in clinical samples could only be identified 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 principle on a triple quadrupole mass spectrometer (TSQ Vantage). Our strategy was based on theoretically derived tryptic peptides (*in silico* digestion) of 10 PSA proteoforms found in the UniProtKB database (see **Suppl. Table 1**). Following filtering the initial set of 30 sequences to fit to MRM experimental conditions, 14 proteotypic peptides were recognized, of which 3 were also isoforms specific:

FLRPGDDSSIEPEEFLTPK for Q8NCW4,

MWVPVVFLTLSVTWIGER for Q8WTQ8,

STCSVSHPYSDLEGK for Q8IXI4.

One of the sequences (IVGGWECEK) was recognized to be present in both PSA and hK2 and thus could not be regarded as unique proteotypic peptide (see **Table 2**). In blood and

seminal fluid however, the concentration of PSA is about two orders of magnitude higher than that of hK2, thus IVGGWECEK could also quantify PSA with reasonable approximation. During the MRM assay development 14 synthetic peptides were tested resulting in a list of 6 suitable sequences (FLRPGDDSSHDLMLLR, 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 as well as good peak shape without apparent interference in 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.

3.2. Proteoforms of PSA in seminal and blood plasma

Screening through the seminal plasma samples (n=35), in most cases the LSEPAELTDAVK peptide ($m/z=636.84$ [M+2H]⁺⁺) was observed as single peak (n=28) as shown on the left panel in **Figure 1**. However, in some cases it was detected also as double peaks (n=6) within the scheduled 4-min analytical window as shown on the middle panel in **Figure 1**. Interestingly, the additional peak with shorter retention time ($\Delta t=-0.6$ min) was noticed with identical transitions to the annotated second peak, which was 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 shorter retention time that did not match with the peak of the surrogate LSEPAELTDAVK peptide (see the right panel in **Figure 1**).

Since the transitions of both chromatographic peaks were identical, suggesting isobaric peptides with a slight difference in their hydrophobicity, we have tested a similar sequence replacing the second Leu with a Ile residue (LSEPAEITDAVK) and proved that the first peak indeed represent the common PSA variant: SNP-L132I. This mutation has a frequency about 10% in the population and hence needs to be monitored when quantifying PSA. Following completion of the blood plasma analysis, 2 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 quantification of PSA in samples with heterozygous expression.

3.3. PSA levels in seminal and blood plasma

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 correlated to the known concentration of the heavy peptides, which were spiked into the samples. The endogenous levels of PSA in whole seminal and blood plasma were calculated from the data obtained with four tryptic peptides (LSEPAELTDAVK, LSEPAEITDAVK, IVGGWECEK and SVILLGR as shown in **Table 2**) by adjusting for the dilution at sample preparation. The calculations were made for the four

different peptides individually in seminal and blood plasma as presented in **Tables 3** and **4**, respectively.

Comparing the determination of the PSA concentration in seminal plasma by using four peptides (LSEPAELTDAVK, LSEPAEITDAVK, IVGGWECEK and SVILLGR) has revealed that the SVILLGR peptide had generally the highest levels with the exception for one sample, where it showed the lowest value. The determination with the peptide IVGGWECEK resulted in generally the lowest levels except for the same sample (see **Table 3** and **Suppl. Figure 1**).

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 about 60% of the levels of SVILLGR (see **Figure 2A-C** and **Suppl. Figure 1**). However, the linear regression coefficients between the determinations made by the three different peptides were excellent with R^2 -values ranging between 0.97-0.99 (see **Figure 2A-C**).

Comparison of the determined concentrations of PSA peptides in blood plasma has revealed that the peptides SVILLGR and the combined LSEPAELTDAVK and LSEPAEITDAVK resulted in very similar values (see **Table 4**). As in seminal plasma, the levels determined by peptide IVGGWECEK were the lowest, about 70% of the levels found for the other two peptides (see **Figure 2D-E** and **Suppl. Figure 2**). The linear regression coefficients between the determinations calculated by the PSA peptides were excellent with R^2 values higher than 0.99 (see **Figure 2D-E**). From this result we could conclude that the digestion was effective and tryptic PSA peptides were sufficiently released from complexes with α -1-antichymotrypsin and α -2-macroglobulin predominant in blood. From

this point of view the MRM assay was independent of sample source, as PSA in both free and complexed forms could be determined in seminal and blood plasma, respectively. Comparison of the concentrations of PSA obtained by the standard clinical test (DELFI[®], Perkin Elmer) and the MRM assay has shown that PSA levels were steadily lower than the immunoassay. Judged by these measured levels in blood plasma indicated that the depletion of 7 most abundant proteins has not removed a significant amount of bound PSA. The concentrations obtained with the peptide SVILLGR is about 60% of the fPSA levels determined by DELFI[®], whereas only 50% and 34% for the peptides LSEPAELTDAVK + LSEPAEITDAVK and IVGGWECEK, 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 of higher than 0.99) samples, respectively (see **Figure 3**).

3.4. 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 7 steps into a pooled sample of 7 blood plasma. Analysis was performed in 5 replicates. The peak area of each IS peptide peak was then plotted against the theoretical concentrations (**Figure 4**). Linear regression fitting was performed resulting in R^2 -values were higher than 0.99 within the investigated concentration range (0.03-30 fmol/ μ L). The integrated peak areas of the corresponding endogenous peptides in the sample were constant (except for LSEPAEITDAVK, which was absent). The LOQ of these peptides in blood plasma was estimated the lowest concentration measured with CV <20%

and was found to be 0.1 fmol/ μ L for IVGGWECEK and SVILLGR, whereas it appeared to be somewhat below the lowest measured value (0.03 fmol/ μ L) for LSEPAELTDAVK and LSEPAEITDAVK. This LOQ value 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 have investigated some key parameters. The retention time of the heavy isotope labeled IS peptides were monitored and summarized in **Table 5**, showing a variation of less than 2%.

Technical variations were determined in 6 randomly selected seminal plasma samples analyzed in triplicates (see **Table 6**). The concentrations of endogenous PSA peptides were determined by using Skyline algorithm for integration of the peak area (weighted average of all transitions) and calculating the mean values, SD and the coefficient of variance. The CV ranged between 0.3% and 4.5% (77.7% of all CV was 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 5 separate batches following digestion and spiked with a mixture of heavy IS peptides at 2 fmol/ μ L. The overall variations of the blood plasma workflow was less than 9.4%, judged by the measured concentrations of the given endogenous PSA peptide (see **Table 7**).

4. Discussion

PSA quantification by MRM assay has a scientific history of almost ten years (8), driven by the fact that PSA is available as purified protein product and routinely analyzed in clinical samples by 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. 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 was observed by others (9-14), largely due to the intensive signal generated by the y-9 transition channel. The other frequently used tryptic peptide of PSA, IVGGWECEK (8, 9, 11), was found to be not unique as this N-terminal sequence is present in both PSA and hK2.

Consequently, it is not recommended to be used for quantitation without considerations accounting for mutual contributions of these proteins to the detected endogenous levels.

Furthermore, the experimentally determined concentrations using IVGGWECEK was 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 internal standard. On the other hand, we could classify another unique PSA peptide (SVILLGR) that could be used for quantification displaying excellent analytical properties (see **Figures 2, 3 and 4**). Despite the fact that the SVILLGR is located in vicinity to the glycosylation site of PSA, no difficulties were observed in quantification of PSA using this peptide. It might be explained by the general observation that digestion was efficient even in blood plasma where PSA predominantly is present in complex with other proteins. The comparison of PSA levels determined by three signature peptides indicated that SVILLGR could provide PSA concentrations similar to 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 non-synonymous mutation: L132I (rs2003783), which is located within the LSEPAE(L/I)TDAVK peptide. Due to the isobaric precursor and fragment ions identical transitions were produced and observed in 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 having a shorter retention time. Since both of the two isoforms can be present in the same sample (heterozygous expression profile), the areas of both peaks have to be combined when quantifying the total amount of PSA.

Population based frequency of the allele A in exon 3 of the *KLK3* gene (dbSNP code: rs2003783) showed 10% worldwide, 8% in Asia and Europe, 14% in Africa and 11% in

America as reported in 1000Genomes database (<http://browser.1000genomes.org>). 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 risk of prostate cancer based on a large case-control cohort from Sweden (CAPS) (40). Further on, SNP prediction tools (SIFT and SNPS3D), reporting on 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 investigated rs2003783, mentioning no associations with disease link (41, 42).

Transcript databases registered evidence of 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 similar physicochemical properties to 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).

The fact that this is the first observation of this SNP variant of PSA at expression level is likely to be the result of screening through a large number of individual samples. In accordance with the ever-growing activities 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 to identify 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 to find 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 but 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 the two other, previously reported sequences and also with immunoassay values. The systematic deviation between the concentrations determined by three different peptides (see section 3.3) is most likely due to the different amounts of heavy labeled internal standard peptides spiked into the samples. The absolute amount of the synthetic peptides was not determined and thus is a source of $\pm 25\%$ variation, which covers well the 30-40% difference between IVGGWECEK and SVILLGR determinations. In order to build a clinical assay used in central hospital laboratories, the next step of our developments would be to define the levels of the internal standards.

The agreement between the MRM assay and DELFIA® results are 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 and tPSA: 0.05-250 ng/mL) and thus compromised with larger error.

5. Conclusions

Nano-LC-MS/MS technology has matured sufficiently as judged by the high reproducibility reported in our experiments as well as others (14-16). This 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 matrix. Advanced chromatographic systems can already provide high-resolution when combined with intelligent selection of fragments containing target molecules (16) as well as immunoreaction enrichment of biomarkers of sub-ng/mL levels. We believe that this development holds a potential of becoming an optional platform for clinical analyses in the future (49).

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

6. References

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8. Legends to the Figures

Figure 1. Detection of three possible combinations of allele expressions in examples of analyses in clinical samples. Endogenous signals of LSEPAELTDAVK and LSEPAEITDAVK are shown in red, whereas their corresponding heavy labeled IS signals are in blue.

Figure 2. Correlations between the measured concentrations of PSA peptides in (A-C) seminal and (D-F) blood plasma.

Figure 3. Correlation between the PSA levels determined by MRM and DELFIA® assays in (A-C) seminal and (D-F) blood plasma.

Figure 4. Linearity of the MRM assay determined by using heavy labeled IS peptides spiked into a pooled blood plasma sample at various concentrations (0.03-30 fmol/ μ L). The measured levels of the corresponding endogenous peptides are shown as blue diamonds, whereas the LOQ (CV<20%) is indicated with arrow.

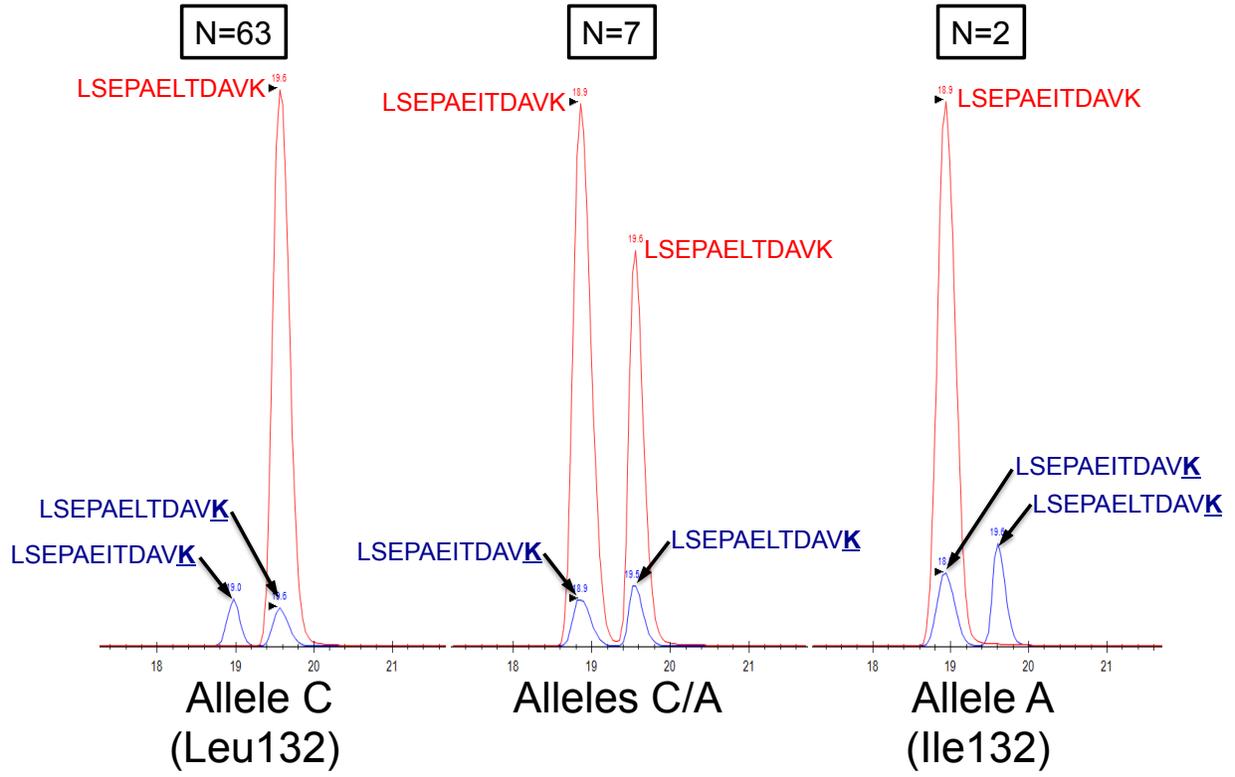


Figure 1.

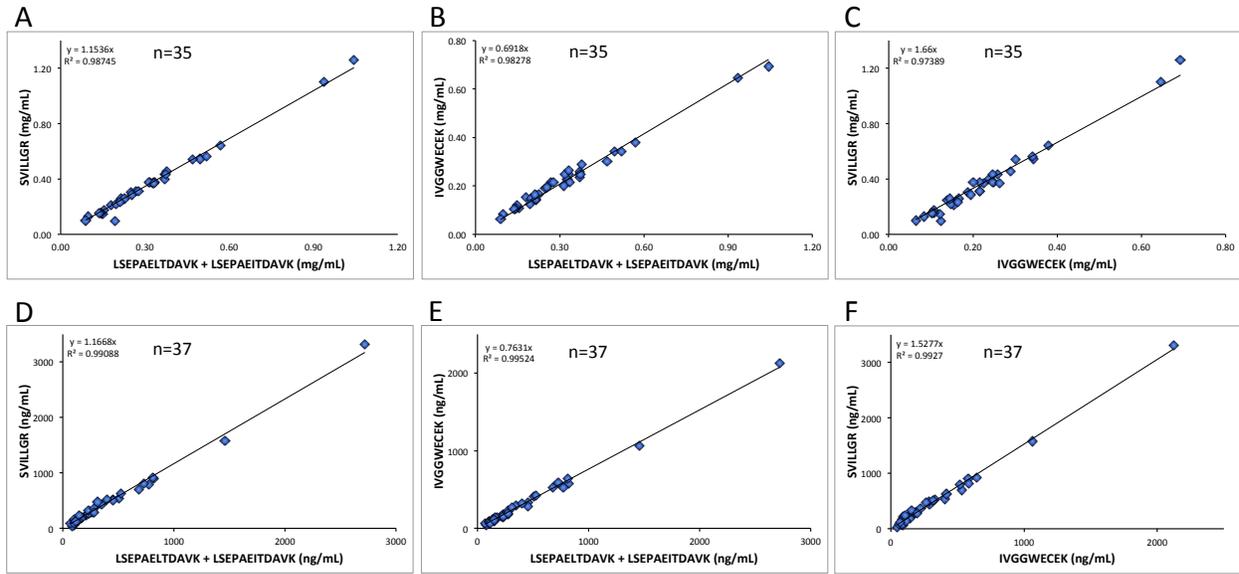


Figure 2.

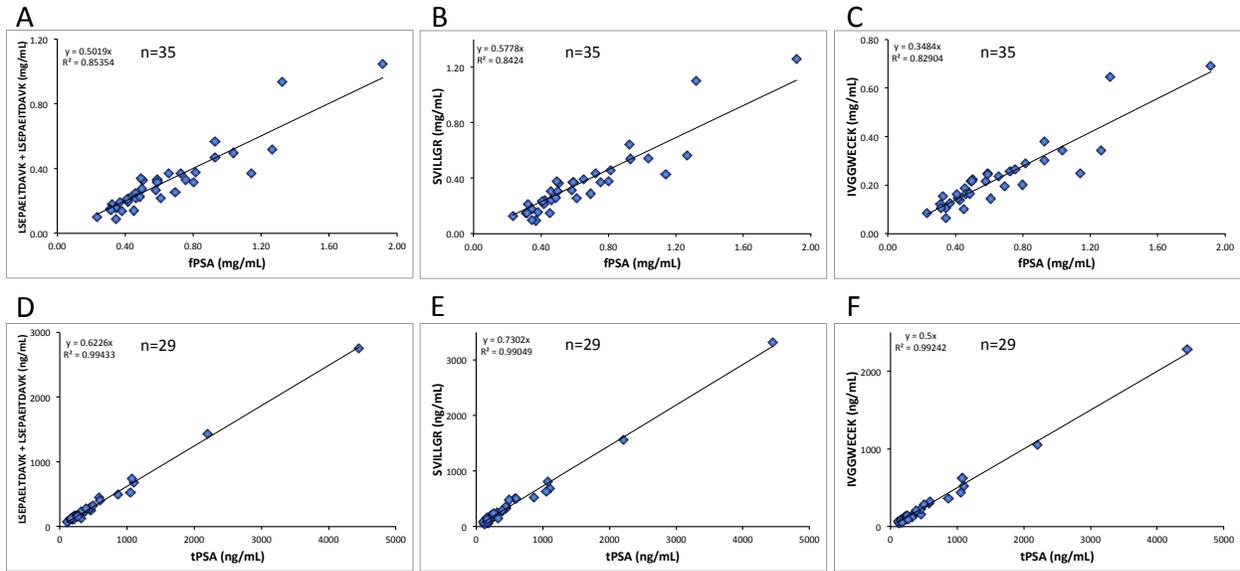


Figure 3.

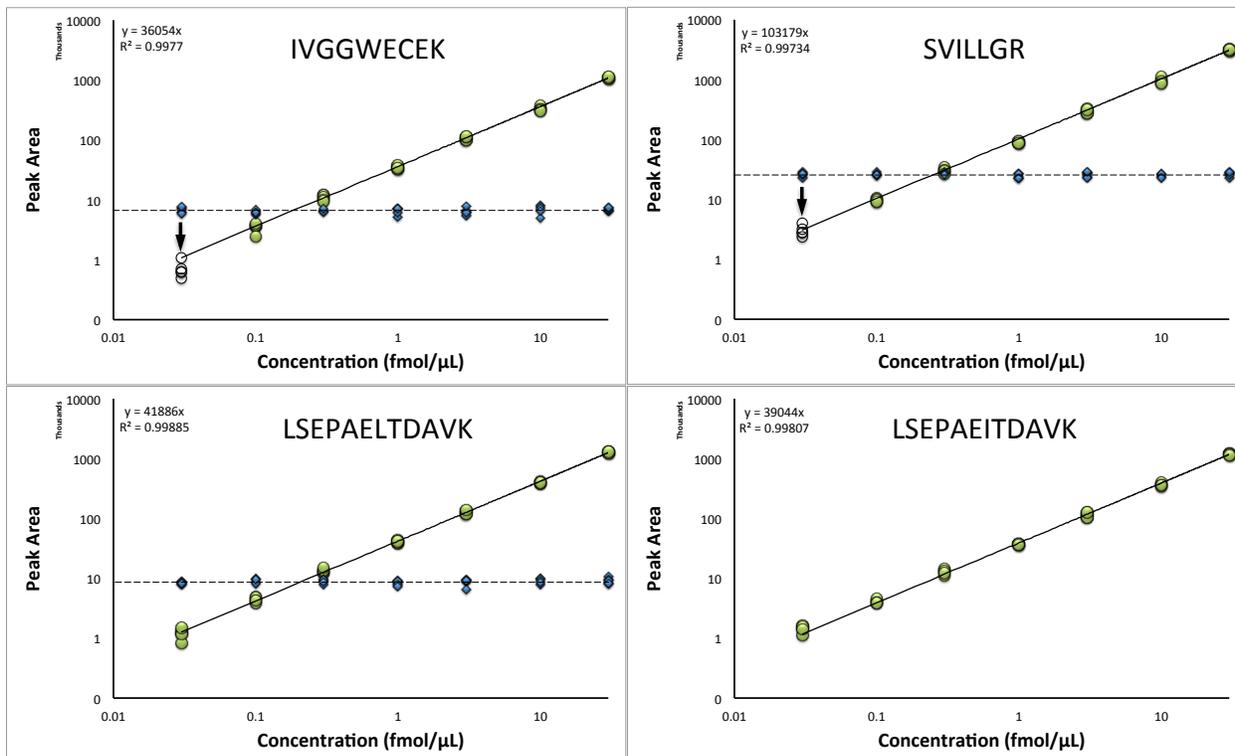


Figure 4.