An In-Depth Glycosylation Assay for Urinary Prostate-Specific Antigen

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ABSTRACT: The concentration of prostate-specific antigen (PSA) in serum is used as an early detection method of prostate cancer (PCa); however, it shows low sensitivity, specificity, and a poor predictive value. Initial studies suggested the glycosylation of PSA to be a promising marker for a more specific yet noninvasive PCa diagnosis. Recent studies on the molecular features of PSA glycosylation (such as antenna modification and core fucosylation) were not successful in demonstrating its potential for an improved PCa diagnosis, probably due to the lack of analytical sensitivity and specificity of the applied assays. In this study, we established for the first time a high-performance PSA Glycomics Assay (PGA), allowing differentiation of α2,6- and α2,3-sialylated isoforms, the latter one being suggested to be a hallmark of aggressive types of cancer. After affinity purification from urine and tryptic digestion, PSA samples were analyzed by CE-ESI-MS (capillary electrophoresis–electrospray ionization coupled to mass spectrometry). Based on positive controls, an average interday relative standard deviation of 14% for 41 N-glycopeptides was found. The assay was further verified by analyzing PSA captured from patients’ urine samples. A total of 67 N-glycopeptides were identified from the PSA pooled from the patients. In summary, the first PGA successfully established in this study allows an in-depth relative quantitation of PSA glycoforms from urine. The PGA is a promising tool for the determination of potential glycomic biomarkers for the differentiation between aggressive PCa, indolent PCa, and benign prostate hyperplasia in larger cohort studies.

Since 1994, the protein concentration of prostate-specific antigen (PSA) in serum is a FDA-approved early detection method for prostate cancer (PCa), although its sensitivity is reportedly rather poor.¹ When the PSA value is found to be elevated (above 3 ng/mL or 4 ng/mL), the patient will be advised to undergo more thorough investigations (e.g., digital rectal examination (DRE), multiparametric magnetic resonance imaging (mpMRI), and/or prostate biopsy).¹² Additional examinations are mandatory because the PSA test lacks the specificity that is required for the early diagnosis of PCa, as age, benign prostate hyperplasia (BPH), or prostatitis can also result in elevated levels of PSA.¹ Furthermore, a recent study by Foley et al. showed that the PSA test has an overall low predictive value for PCa and is incapable of discriminating between aggressive and nonaggressive prostate tumors.³⁴ Therefore, the need for more specific and predictive biomarkers is eminent, to effectively discriminate between aggressive and nonaggressive tumors and to avoid unnecessary prostate biopsies.

Literature suggests that the analysis of PSA glycosylation could offer a more efficient PCa diagnosis,⁵−⁷ because the single N-linked glycosylation site (N⁶⁹) of PSA is occupied by a very heterogeneous group of glycans.⁵−⁷ Differences in the linkages of sialic acids, which can be α2,3-, α2,6- or α2,8-linked, have been associated with cancer progression,⁹ with α2,6-sialylated glycans involved in blocking the galectin binding to β-galactoside, and α2,3-sialylated glycans suggested to be involved in (aggressive) types of cancer.¹⁰¹¹ Further investigations into whether a higher abundance of α2,3-sialylated species present on PSA could be correlated to PCa, found an overall a higher specificity and sensitivity when compared with the conventional PSA test.¹² However, this study disregarded the presence of α2,6-sialylated glycans and did not detect other molecular features such as fucosylation or bisecting. Another recent study investigated the glycosylation features of PSA in urinary samples collected after DRE by using an immunopurification step.¹³ Lectins were used to investigate the level of core fucosylation and the presence of α2,6-linked sialic acids.
Table 1. Clinical Patient Information

| # of samples | Non-PCa patients | PCa patients |
|--------------|------------------|-------------|
| Median (Range) | 66 (46-75) | 69 (60-87) |
| Mean ± SD | 65 ± 9 | 70 ± 7 |

| Serum PSA (ng/mL) | Median (Range) | 6.0 (2.9-12.0) | 7.5 (2.6-156.5) |
| Mean ± SD | 6.3 ± 2.9 | 35.4 ± 57.3 |

| Prostate volume (mL) | Median (Range) | 60.8 (29.7-136.0) | 60.0 (31.0-122.0) |
| Mean ± SD | 64.3 ± 29.2 | 61.0 ± 23.9 |

| Ethnicity | Caucasian | 9 | 11 |
| African American | 1 | 1 |
| Unknown | 0 | 1 |

| Clinical tumor staging | T1c | 7 | 8 |
| T2 | 3 |
| T2a | 0 |

| Gleason Score | 3-3 | 6 |
| 3-4 | 5 |
| 4-3 | 1 |
| 4-4 | 0 |
| 4-5 | 1 |

“Two patient samples were excluded from further analysis due to low signal in urinary PGA.

revealing, however, no association with the pathology of the disease (Gleason score). This observation could result from the rather small sample size (18 controls vs 36 PCa cases) and the only partial cover of sialic acid linkages, because only the levels of α2,6-sialic acids were considered. Another study compared the total serum N-glycome of PCa patients and BPH patients, revealing differences with respect to sialylation as well as fucosylation.7 An overall increase of α2,3-sialylated glycans and alterations in fucosylation was shown in PCa patients, which is in agreement with previous studies.14,15 Notably, as these studies analyzed the total plasma or serum N-glycome after glycan release, no information was obtained regarding the protein carriers of the affected glycans. Several other groups explored specifically the level of fucosylation of serum PSA, demonstrating an overall increase in total fucosylation for free PSA16 or total PSA,17 while another study reported decreased N-glycan core-fucosylation and an increase in α2,3-sialylated glycans for PSA in PCa patients.18 In a previous study by CE-ESI-MS (capillary electrophoresis-electrospray ionization coupled to mass spectrometry), on a proteolytically cleaved PSA standard (human seminal plasma), we could identify 75 different glycan species (including isomeric separation of differently linked sialic acids).19 Additionally, a recent in-depth study by liquid chromatography-electrospray ionization coupled to mass spectrometry (LC-ESI-MS(;/MS)) on a similar sample, identified 23 glycan compositions on the single N-glycosylation site of PSA and in addition showed, with a relatively small cohort (61 BPH and 31 PCa cases), that for urinary PSA the fucosylation was decreased in PCa patients and the level of sialylation was increased compared with BPH patients.20 While some of these studies noted differences in the level of PSA sialylation and fucosylation, they often featured limited precision, sensitivity, and/or resolution.5,16–18,14,20

Answering to the need for an analytically more specific and sensitive high-resolution assay to assess PSA glycosylation features of benign, indolent or aggressive tumors, we report here an in-depth high-performance urinary PSA Glycomics Assay (PGA) for patients suspected of PCa, combining the strengths of previously published assays. The assay includes the capturing of PSA from urine, an in-solution digestion, and the analysis of glycopeptides with CE-ESI-MS(/MS), taking advantage of the separation of sialylated isomers in CE. In addition, as restricted quantities of PSA are found in urine (before DRE),15 a sheathless interface (CESI) with a dopant enriched nitrogen gas was used, improving analytical sensitivity and repeatability for glycopeptide identification,21 making this assay suitable to detect rather small differences in the relative abundance of different glycoforms.

- EXPERIMENTAL SECTION

**Materials.** Ammonium bicarbonate (ABC), ethanol (EtOH), sodium bicarbonate (NaHCO₃), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium phosphate dibasic dihydrate (Na₂HPO₄·2H₂O), and monopotassium phosphate (KH₂PO₄) were obtained from Merck (Darmstadt, Germany). Glacial acetic acid, DL-dithiothreitol (DTT), hydrochloric acid (HCl), and iodoacetamide were acquired from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate, formic acid (FA), and water of LC-MS grade water were purchased from Pfluka (Steinheim, Germany). Trypsin from bovine pancreas was obtained from Promega (Madison, WI). Milli-Q water (MQ) was obtained using a Q-Gard 2 system (Millipore, Amsterdam, The Netherlands). HPLC supraGradient acetonitrile (MeCN) was acquired from Biosolve (Valkenswaard, The Netherlands).

PSA standard derived from semen was purchased from Lee Biosolutions (St. Louis, MO). Five times concentrated (5×) PBS consisted of 0.16 M Na₂HPO₄·2H₂O, 0.02 M KH₂PO₄·0.73 M NaCl at pH 7.2. Next, 1× PBS was prepared from the 5× PBS by diluting it with MQ and resulting in a pH of 7.6.

**Clinical Samples.** Urine samples from 10 healthy female volunteers were collected at the Leiden University Medical Center. As a negative control, a female urine pool (FUP) was made by pooling 10 urine portions. One urine sample from a healthy male volunteer was collected at the Academic Medical Center (Amsterdam, The Netherlands) as well 25 urine samples from patients suspected of PCa. All patients had a serum PSA level of >3 ng/mL and donated their urine prior to prostate biopsy and before DRE. Clinical information and urine specimens were collected with the approval of the medical ethical committee of the AMC (W16_010#16.020). Table 1
illustrates the clinical information on the patients. Urine was collected (8–72 mL) and cooled to room temperature (RT) before it was stored at −80 °C.

**Anti-PSA Beads.** Custom-made anti-PSA specific nanobodies based on sequence N7 from Saerens et al.22 (antigen-binding portion of the heavy chain from cameldil, 0.49 mg/mL PBS) were obtained from QVQ (Utrecht, The Netherlands) and coupled to NHS activated Sepharose 4 Fast Flow-beads (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer’s protocol. Briefly, 7 mL of 0.49 mg/mL anti-PSA in PBS was added to 14 mL of drained beads (1:1200 extra vacuum was applied) during the washing with 600 μL of 1× PBS (pH 7.6) followed by two times with 600 μL of 50 mM ABC. After this step, PSA was eluted by adding 200 μL of 100 mM FA that was collected in a V-bottom microtitrater plate; the sample was evaporated at 45 °C in a vacuum centrifuge (Eppendorf Concentrator 5301, Eppendorf) and reconstituted in 5 μL of 25 mM sodium bicarbonate.

**SDS-PAGE and In-Gel Tryptic Digestion.** For all SDS-PAGE experiments, samples were separated by NuPAGE 4–12% gradient Bis-Tris gel (Thermo Fisher). For reduction, 2.5% of 2-mercaptoethanol (Sigma-Aldrich) was added to the sample prior to SDS-PAGE. The gel was stained with colloid blue (Novex). For in-gel digestions the PSA bands (~32 kDa) were excised and cut into pieces that were washed with 100 μL 25 mM ABC with an incubation time of 5 min at RT. Supernatant was removed and the gel pieces were dehydrated by the addition of 100 μL 30% MeCN with an incubation time of 30 min at RT, after which the supernatant was removed. A final volume of 100 μL 10% MeCN was added with an incubation time of 10 min at RT prior to reduction with 75 μL of 0.9 mM DTT for 30 min at 56 °C. After reduction the supernatant was removed and 100% MeCN was added for 10 min at RT. After removal of the supernatant, 75 μL of 5 mM iodoacetamide was added and kept in the dark for 20 min at RT. The supernatant was removed after the alkylation and 100 μL of 100% MeCN was added for 5 min at RT. After removal, 25 mM ABC was added and the gel pieces were incubated for 10 min at RT. The supernatant was removed and an additional 100% MeCN was added and incubated at RT for 5 min. After removal of the supernatant, the gel pieces were dried in a vacuum centrifuge for 30 min. Samples were reconstituted in a mixture containing the proteolytic enzyme (30 μL of 5 ng/μL trypsin in 25 mM ABC). Digestion was performed overnight at 37 °C. The capturing procedure was performed using software package Gelanalyzer 2010. The software was used to quantify the capturing efficiency by comparing the intensities of the PSA protein bands in SDS-PAGE gel (png file) before and after capturing.

**In-Solution Tryptic Digestion.** The 5 μL reconstituted eluates, described in section PSA Capturing, were reduced and alkylated by adding 1 μL of 12 mM DTT with an incubation time of 30 min at 60 °C followed by the addition of 1 μL 42 mM iodoacetamide with an incubation of 30 min performed in the dark at RT. To ensure that the sulfi de alkylation was stopped, 1 μL of 48 mM DTT was added and incubated for 20 min in full light at RT. After addition of 1 μL of 0.15 mg/mL trypsin in 25 mM ABC, the digestion was performed overnight at 37 °C.

**Capillary Electrophoresis—Electrospray Ionization—Mass Spectrometry.** All experiments were performed under optimized conditions previously established.21 Briefly, a 90 cm long bare-fused capillary (30 μm internal diameter and 150 μm outer diameter) was used on a CESI 8000 system (Sciex, Framingham, MA). The system had a temperature controlled sampling tray, and for all experiments a voltage of 20 kV was varied with incubation of the sample on a tube roller. For the capturing of PSA from patient material, the following final parameters were used: 60 μL of 50% anti-PSA bead suspension, 20 mL of plastic Falcon tube, urine adjusted to pH 7, no blocking agent, and an overnight incubation at 4 °C. After the sample was captured, it was centrifuged at 100g for 1 min at RT. After removal of the supernatant, the remaining anti-PSA bead suspension (roughly 500 μL) was transferred to a 96-well polypropylene filter plate (2 mL) containing a 10 μm pore size polyethylene frit (Orochem, Naperville, IL). The liquid was further removed by using a vacuum manifold (MerkMillipore, Darmstadt, Germany). Finally, it was tested whether the elution was hampered if the beads were completely dried (1 min of extra vacuum was applied) during the washing with 600 μL of 1× PBS (pH 7.6) followed by two times with 600 μL of 50 mM ABC.

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applied. Prior to each analysis, the separation capillary was thoroughly rinsed with 0.1 M NaOH (2.5 min), LC-MS grade water (3 min), 0.1 M HCl (2.5 min), water (3 min), and 3 min with the background electrolyte (BGE) of 10% acetic acid (v/v, 1.74 M, pH 2.3). The conductive liquid line was supplemented with BGE, by rinsing with BGE for 3 min. An online preconcentration step was applied, transient isophoresis, by adding 1.0 μL of the leading electrolyte (LE, 250 mM ammonium acetate at pH 4.0) to 1.5 μL of the sample. All samples were injected hydro-dynamically by applying 1 psi pressure for 60 s, corresponding to 1.4% of the total capillary volume (9 nL). After each sample injection, a BGE postplug was injected by applying 0.5 psi for 25 s (0.3% of the capillary volume).

The CE system was coupled to a UHR-QqTOF maXis Impact HD MS (Bruker Daltonics, Bremen, Germany) via a sheathless CE-ESI-MS interface (SCIEX). The complete housing of the nozzle was a customized platform from SCIEX, which allowed the optimal alignment of the position of the capillary spray tip in front of the nanospray shield (Bruker Daltonics). A stable electrospray was obtained by applying a glass capillary voltage between −1100 and −1300 V. All experiments were performed in positive ionization mode. The flow rate and temperature of the drying gas (nitrogen) were adjusted to 1.2 L/min and 150 °C, respectively. MS data were acquired between m/z 200 and 2200 with a 1 Hz spectral acquisition frequency. An internal polymer cone was attached onto the porous tip housing to enable the usage of a dopant (ca. 4% mole percent).21

**Data Analysis.** CE-ESI-MS data was analyzed with Data Analysis 4.2 (Build 395, Bruker Daltonics). Calibration of the MS spectra was performed prior to data analysis with sodium adducts that were present at the beginning of the electropherogram. The data was manually screened for glycopeptides based on the exact mass, migration order and relative intensities (Supporting Information, Table S-1). Fragmentation spectra were acquired for 30% of the identified compositional glycopeptide variants of PSA (Supporting Information, Figures S-1A–S-1T), an additional 16% was identified on the basis of previous research.22 Extracted ion electropherograms (EIEs, smoothed with a Gaussian fit) were acquired with the first three isotopes of the double, triple, and quadruparny charged analytes using a width of ± m/z 0.05 unit.

## RESULTS AND DISCUSSION

A PSA Glycomics Assay (PGA) for the glycosylation analysis of PSA from urine of patients suspected of PCa was established and verified (N = 25). For this, PSA was captured from typically 20 mL of urine, subjected to tryptic treatment, and the obtained glycopeptides were analyzed by CE-ESI-MS. For data processing, glycopeptide signals were integrated, and ratios of the glycopeptide signal intensities were determined. The PGA was further verified by examining factors such as capturing procedure, repeatability, intermediate precision, and biological variation between patients.

**Capturing Procedure.** Several parameters of the capturing procedure were optimized and are summarized in Supporting Information, Table S-2. Briefly, a volume of 60 μL of anti-PSA beads was found to give the highest capturing efficiency (N = 2, Supporting Information, Figure S-2). The use of bare NHS Sepharose beads did not result in nonspecific binding of PSA as the PSA was observed only in the flow-through and not in the eluate of the bare NHS Sepharose beads, while anti-PSA beads did capture PSA and no PSA was observed in the flow through (Supporting Information, Figure S-3). PSA recoveries were largely comparable when using plastic vs glass retainers, and plastic retainers were chosen for their convenience for further experiments (N = 1, Supporting Information, Figure S-4). Furthermore, the pH of the FUP (N = 2), was investigated, and a similar recovery was found for all conditions (pH 6, 7, and 8) (Supporting Information, Figure S-5). For subsequent experiments, pH 7 was chosen. It was tested whether the addition of a blocking agent prevented nonspecific binding of PSA to the walls of the retainer and could improve the capturing efficiency (Supporting Information, Figure S-6). Notably, the use of bovine serum albumin (BSA) and casein as a blocking agent contaminated the affinity-purified sample but did not result in increased PSA yields. Therefore, for further experiments, no blocking agent was added to the FUP. Incubation at room temperature showed a wide variation in capturing efficiency, with 4 °C being the most preferable temperature (Supporting Information, Figure S-7). The incubation time did not appear to influence the PSA recovery, as a recovery between 72% and 78% was observed across the range of incubation times (2, 4, 6, 8 h and overnight, N = 1). Due to practical considerations, further experiments were performed with an overnight incubation. As a vacuum system was used with a 96-well format for the washing and elution of the PSA from the anti-PSA beads, it was tested whether PSA yields changed when the 96-well plate was run dry during the washing procedure (an extra minute of vacuum was applied, Supporting Information, Figure S-8). A similar capturing efficiency was observed when the well was completely dried compared to wells that were kept wet during the washing procedure (80% vs 73%, N = 2). FA (100 mM) proved to be a suitable elution reagent, as no PSA was detected postelution on the beads after the beads were incubated with the loading buffer and analyzed on a NuPAGE SDS-PAGE gel (data not shown). Furthermore, PSA quantitation by SDS-PAGE with Coomassie staining (N = 3, Supporting Information, Figure S-9) revealed that the capturing procedure recovered approximately 58% of the PSA spiked into a female urine pool (FUP). To reveal any potential glycosylation bias of the affinity capturing procedure, glycosylation profiles of PSA before and after capturing were compared, applying in-gel tryptic cleavage of PSA followed by CE-ESI-MS. We chose for a gel-based approach in order to remove any possible sample matrix confounders. A total of 41 N-glycopeptides were detected, and comparable profiles were obtained before and after affinity purification (N = 3, Supporting Information, Figure S-10). The relative abundances of the 10 most abundant N-glycopeptides are compared in Figure 1 and show no bias of the glycosylation profile induced by the affinity purification procedure, with an average relative standard deviation (RSD) of 6.5% (noncaptured) and 3.0% (captured) for the 10 most abundant glycopeptides (normalized to the sum of all identified glycoforms). In addition, an average RSD of 14.8% (noncaptured) and 8.3% (captured) for all glycopeptides was observed. However, it has to be noted that the in-gel digestion was performed on the ~32 kDa band and possible discrimination of potential nicked forms of PSA that may migrate at different heights is, therefore, not taken into account.

**PSA Glycopeptide Detection by CE-ESI-MS.** The analysis of a tryptic digest of the captured PSA standard identified 41 N-glycopeptides with the CE-ESI-MS setup. All

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glycans were attached to the dipeptide N69K as listed in Supporting Information, Table S-1. The base peak electropherogram of a typical PSA tryptic digest analysis (Figure 2) shows three distinctive glycopeptide clusters separated on the basis of the level of sialylation (Figure 2B,C). Furthermore, isomer separation was achieved for α2,3- and α2,6-sialylated species, in accordance with our previous work.19 Figure 2B,C demonstrate that α2,3- and α2,6-linked isomers are baseline resolved, confirming the ability of CE-ESI-MS to discriminate between the different linkages. However, compared with our previous work,19 nonsialylated glycoforms were not detected in this sample. The difference is most likely due to the use of different PSA batches in the two studies. Notably, the peaks that are observed in the base peak electropherogram in the nonsialylated region (area marked by an asterisk in Figure 2A) correspond to other analytes in the sample such as tryptic unglycosylated peptides.

**Figure 1.** Relative abundance of the 10 most abundant glycopeptides of prostate-specific antigen after either capturing (from spiked female urine pool) or no capturing (PSA standard, 1.5 μg) procedure. All samples were loaded on to a SDS-PAGE gel, the approximately ∼32 kDa band was excised from the gel and underwent a trypic in-gel digestion prior to CE-ESI-MS analysis. Error bars represent the standard deviation (N = 3). PEP illustrates the tryptic peptide sequence N69K.

**Figure 2.** CE-ESI-MS analysis of tryptic (glyco)peptides from PSA standard. (A) Representative base peak electropherogram observed for a tryptic digest of PSA. The migration window assigned with the * symbol typically contains the nonsialylated N-glycopeptides of PSA, which were not detected in this sample. Two distinct clusters (B,C) were observed, both containing N-glycopeptides. Extracted ion electropherograms for monosialylated N-glycopeptides (B) and disialylated N-glycopeptides (C) of PSA are illustrated, and each color represents an individual glycopeptide in its isomeric forms (multiple peaks). In total, 41 different N-glycopeptides were identified (not all data shown, a complete overview can be found in Table S-1, Supporting Information). PEP illustrates the tryptic peptide sequence N69K.
and interday RSD were below 3% and 7%, respectively, for the 10 most abundant glycopeptides (normalized to the sum of all identified glycoforms). Results for the remaining 31 identified N-glycopeptides (relative abundance of low abundant glycopeptides) are shown in Supporting Information, Figure S-11.

The RSDs for all glycopeptides showed an average intraday variation of 5% and an average interday variation of 14%.

Analysis of PSA Glycopeptides Captured from Patient Urine. The biological variation in PSA glycosylation was examined using the developed PGA applied to urine samples of 25 patients. Before sample treatment, the patient urine samples were split into three batches, and each of them processed on one of three consecutive days. Each batch included a negative (FUP) and a positive control (FUP spiked with 1.5 μg of standard PSA). Furthermore, after tryptic digest, a small portion (0.5 μL) of all the patient samples was pooled. This pool was used for the identification of the glycopeptides and structural elucidation by tandem MS (N = 3). A total of 67 N-glycopeptides could be identified in the pooled patient sample. All glycans were attached to the dipeptide N69 K, in agreement with the previous result, and a complete overview of the glycopeptides is given in Supporting Information, Table S-1.

After data treatment, 23 of the 25 patient samples passed the quality criteria (>20 glycopeptides identified with S/N > 9, ppm error <10 ppm), resulting in 13 PCa patients and 10 non-PCa patients. The whole assay, including sample preparation and CE-ESI-MS measurement, was completed per batch (11 samples) within 60 h, of which 8 h was dedicated to hands-on time; the remaining hours consisted of all incubation steps as well as the analysis of the samples on the analytical platform.

In order to assess the intermediate variation of the PGA, one positive control (spiked FUP, N = 3) was included each day with the sample preparation of the patient urines (Supporting Information, Figures S-12 and S-13). The complete PGA showed an average RSD of 6% for the 10 most abundant glycopeptides of the positive controls. The CE-ESI-MS detection method showed a low technical variation, with an average RSD of 2% for the 10 most abundant glycopeptides as determined using the sample of the pooled PSA digests. A large variation with an average RSD of 50.1% (non-PCa patients), 47.2% (PCa patients) and 48.6% (all patients) was observed between the patients’ PSA glycosylation profiles, which are believed to largely reflect biological variation (Supporting Information, Figures S-12, S-13 and Table S-3). The much higher biological variation as compared with the technical variation highlights the potential use of the established PGA for biomarker discovery. The biological variation within the clinical

Figure 3. Relative abundance observed for the most abundant glycopeptides of prostate-specific antigen. PSA was spiked into a female urine pool, digestion was performed in-solution and was used to test the repeatability (intraday, N = 3) and intermediate precision (interday, N = 3) of the PGA with CE-ESI-MS. PEP illustrates the tryptic peptide sequence N69 K.

Figure 4. PSA Glycomics Assay derived sialylation traits of all 23 patient urine samples, a “trait” is the relative abundance of all glycopeptides observed within a patient complying with specific characteristics (no sialic acid and one, two, or three sialic acids present on the glycan portion of the glycopeptide). (A) Disialylation was the most abundant sialylation trait observed in the patient group, followed by monosialylation, no sialylation, and trisialylation. Monosialylation and disialylation show a clear inverse correlation. The sialic acid linkage in the monosialylated glycopeptides (B) and disialylated glycopeptides (C) could be further explored, revealing that the α2,6-linked sialic acid was the most abundant species; however, no direct correlation could be found between the level of α2,3 and α2,6-linked sialic acids. Letters in the legend indicate each patient individually (patient characteristics can be found in Supporting Information, Table S-3).
samples and the two different patients groups (non-PCa vs PCa patients) was systematically examined in relation to specific glycosylation traits, as illustrated in Supporting Information, Figure S-14. A clear urinary PSA glycosylation profile could be obtained for a healthy volunteer, with low levels of α2,3-sialylated glycans when compared with the patient profiles, which is in agreement with the literature.12 The most abundant glycans was found to be the complex type (ranging from 85% up to 94% of the relative abundance for the non-PCa patients while a similar profile was found for the PCa patients ranging from 87% up to 95%), followed by minor amounts of the hybrid type (4.9%–8.6% (non-PCa patients) and 3.8%–7.4% (PCa patients)), and high-mannose type glycans (0.7%–7.9% (non-PCa patients) and 1.5%–6.2% (PCa patients)). Furthermore, by grouping the identified glycoforms according to their degree of fucosylation (Supporting Information, Figure S-15), the most abundant group corresponded to the monofucosylated species (all core-fucosylated, 55%–82% (non-PCa patients) and 55%–89% (PCa patients)), followed by nonfucosylated (17%–45% (non-PCa patients) and 11%–45% (PCa patients)), and a small group of difucosylated species (0.2%–2.1% (non-PCa patients) and 0.3%–1.3% (PCa patients)). With respect to sialylation (Figure 4A), disialylation (two sialic acids present on the glycan) was most prevalent (70%–84% (non-PCa patients) and 69%–88% (PCa patients)) followed by monosialylation (14%–15% (non-PCa patients) and 9%–25% (PCa patients)) and nonsialylated glycan species (1.3%–9.0% (non-PCa patients) and 1.9%–7.9% (PCa patients)), with a minor part being trisialylated (0.2%–1.0% (non-PCa patients) and 0.0%–0.5% (PCa patients)). A large variation of sialic acid linkages was observed between patient PSA samples (Figure 4B,C). As indicated in the introduction, sialic acid linkages may be indicative of the disease state of the patient; however, with the current sample size, no significant differences could be observed between non-PCa patients and PCa patients. Moreover, the relationship between the observed sialic acid linkage and the measured PSA serum concentration as well as the prostate volume were studied (Supporting Information, Figure S-16). No direct correlation could be observed and it is therefore recommended to perform additional studies using the present PGA or related high-resolution platforms on medium- to large-size cohorts of patient materials (urine, serum) to further evaluate these glycosylation traits as a biomarker candidate. Even though the preliminary results of this study did not reveal any significant differences, most likely due to the small sample size, several studies have already shown that the fucosylation and sialylation traits of PSA seem to be of relevance for potential markers able to discriminate between PCa and BPH.13,16,17,20

Besides fucosylation and sialylation, a small portion of the glycans species appeared to be bisected within the patient group (<0.3%). Furthermore, one of the high mannose glycans (H6N2) appeared to be present in two forms, either phosphorylated or nonphosphorylated, of which the phosphorylated form appeared to be more abundant in both groups (Supporting Information, Figure S-17). In addition, a phosphorylated species (H6N3) was observed which most likely represents a Man6 N-glycan with a GlcNAc-capped mannose-6-phosphate residue generated by GlcNAc-1-phosphotransferase, although an alternative structure such as a phosphorylated hybrid-type species cannot be ruled out. Next to phosphorylation, sialylation was also observed on two complex species containing an N-acetylglactosamine with either no or one sialic acid attached (Supporting Information, Figure S-18). Sulfation or phosphorylation on the glycan species that did not contain mannoses nor N-acetylgalactosamines was not observed, suggesting that this modification can only appear on these monosaccharides and should definitely be added to other observed glycosylation features (sialylation including linkage information, fucosylation, bisection) when searching for possible disease-specific markers.

### CONSIDERATIONS

During this study, urine samples of 23 of the 25 patients were investigated, and a high biological variation was observed between the patients. As a result of the small sample size, one should be cautious when drawing conclusions regarding the clinical and diagnostic potential of PSA glycosylation features. Further research is essential to assess whether PSA glycosylation of urine and blood samples can be of diagnostic value. Nonetheless, this study showed that the established PGA is capable of characterizing PSA glycosylation, after affinity capture from urinary samples of either healthy volunteers or patients, thoroughly and in a repeatable manner.

Minor questions were prompted by the current study and are worthy of consideration in upcoming investigations such as the chemical stability of PSA in urine matrix, the role played by PSA subforms, and the concentration of urinary PSA. First, it is known that for various major plasma glycoproteins chemical degradation is minimal.23 Little is known about the stability of glycoproteins such as PSA in the urine matrix, neither under healthy nor under disease conditions, and this should be investigated in the future. In addition, while comparing the obtained results of the in-gel (Figure 2) and in-solution digestion (Figure 3), small discrepancies could be found in the overall glycosylation profile. These small differences could be caused by the presence of nicknamed PSA forms, which were not taken into account in the in-gel digestion (after reduction) as only the main PSA form (~32 kDa) was excised for the digestion.24–26 It is uncertain if the PSA antibody captures these nicknamed forms of PSA; however, a study by Sarrats et al.,27 noticed slight differences in glycosylation profiles between different PSA subforms (subforms which we think could be ascribed to nicknamed PSA forms). It is, therefore, of great interest to study the differences between the main PSA form and its nicked forms, especially if these differences could be correlated with different stages of PCa. However, as each PSA form would result in a glycopeptide with a dipeptide Nε6K, a different approach would be needed, such as intact protein analysis or the analysis of different gel bands after reduction and in-gel digestion. Furthermore, the concentration of PSA in urine should be monitored in relation to the volume of urine produced in a given time frame, as it is currently unknown whether larger urine volumes will provide a higher amount of PSA or will result in a more diluted PSA sample. It is unclear whether morning collected urine would result in a more concentrated PSA sample. Another possibility for higher PSA concentration in urine would be to collect the patients’ urine after DRE (as routinely done for other urine marker tests, like PCA3), giving the possibility to lower the current amount of urine (20 mL) that is needed for the PGA assay.13,15,28 We envision expanding the current PGA assay toward PSA quantitation by including relevant internal peptide standards facilitating absolute quantitation by MS of proteolytically generated PSA peptides.
CONCLUSIONS AND PERSPECTIVES

The established high-performance PSA Glycomics Assay reveals multiple glycoforms of urinary PSA, including sialic acid linkage isomers as well as the level of (core-)fucosylation. We could envision a similar assay for analyzing the glycosylation of serum PSA, which, however, poses major challenges with regards to sample purification and analytical sensitivity of the assay. Using the high-performance PGA, we plan to evaluate the PSA glycosylation for early detection of PCa as well as for potential differentiation between aggressive and nonaggressive tumors. We could envision a similar assay for analyzing the glycosylation of serum PSA, which, however, poses major challenges with regards to sample purification and analytical sensitivity of the assay. Using the high-performance PGA, we plan to evaluate the PSA glycosylation for early detection of PCa as well as for potential differentiation between aggressive and nonaggressive tumors. For this, we plan to evaluate the prognostic value of the PGA on the basis of a longitudinal study.

ASSOCIATED CONTENT

Supporting Information

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Additional information is available as stated in the text (PDF)

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Notes

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