Urinary endogenous peptides as biomarkers for prostate cancer

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Received August 3, 2022; Accepted February 15, 2023

DOI: 10.3892/ol.2023.13759

Abstract. Prostate cancer (PCa) is one of the most prevalent types of cancer in men worldwide; however, the main diagnostic tests available for PCa have limitations and a biopsy is required for histopathological confirmation of the disease. Prostate-specific antigen (PSA) is the main biomarker used for the early detection of PCa, but an elevated serum concentration is not cancer-specific. Therefore, there is a need for the discovery of new non-invasive biomarkers that can accurately diagnose PCa. The present study used trichloroacetic acid-induced protein precipitation and liquid chromatography-mass spectrometry to profile endogenous peptides in urine samples from patients with PCa (n=33), benign prostatic hyperplasia (n=25) and healthy individuals (n=28). Receiver operating characteristic curve analysis was performed to evaluate the diagnostic performance of urinary peptides. In addition, Proteasix tool was used for *in silico* prediction of protease cleavage sites. Five urinary peptides derived from uromodulin were revealed to be significantly altered between the study groups, all of which were less abundant in the PCa group. This peptide panel showed a high potential to discriminate between the study groups, resulting in area under the curve (AUC) values between 0.788 and 0.951. In addition, urinary peptides outperformed PSA in discriminating between malignant and benign prostate conditions (AUC=0.847), showing high sensitivity (81.82%) and specificity (88%). From *in silico* analyses, the proteases HTRA2, KLK3, KLK4, KLK14 and MMP25 were identified as potentially involved in the degradation of uromodulin peptides in the urine of patients with PCa. In conclusion, the present study allowed the identification of urinary peptides with potential for use as non-invasive biomarkers in PCa diagnosis.

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Key words: prostate cancer, biomarkers, endogenous peptides, urine, mass spectrometry, uromodulin

Introduction

Prostate cancer (PCa) is the second most prevalent cancer and the fifth leading cause of cancer-related deaths in the male population worldwide (1). PCa has a favorable prognosis when diagnosed in early stages, as low-grade localized tumors progress slowly and are highly treatable. Currently, PCa diagnosis is mostly based on serum prostate-specific antigen (PSA) testing and digital rectal examination (DRE) followed by confirmation using multi-core prostatic biopsy (2).

Although PSA is the most widely used biomarker for non-invasive PCa detection, its limitations are well known (2). PSA is not cancer specific as many nonmalignant conditions, such as benign prostatic hyperplasia (BPH), prostatitis and urinary tract infections, may affect the PSA serum levels (3). In fact, a negative prostate biopsy was found in 70-80% of men with PSA levels between 4-10 ng/ml (3). On the other hand, up to 15% of men with a PSA level ≤4.0 ng/ml had biopsy-detected PCa (4). In addition, PSA is unable to distinguish between indolent and aggressive PCa. PSA limited sensitivity and specificity results in unnecessary biopsies, overdiagnosis and overtreatment of patients. Thus, PCa is a major global health problem that imposes a significant social and economic burden. Therefore, there is an urgent need for novel non-invasive biomarkers that can accurately detect PCa and improve disease risk stratification in order to appropriately guide patient management.

Urine is an attractive source for PCa biomarker discovery, it is readily available in large quantities and can be sampled non-invasively (liquid biopsy) (5). Urine composition reflects the physiological or pathological state of major urological tissues, including the prostate (6,7). Therefore, prostate-derived molecules found in urine, including DNA, RNA, proteins and

peptides, may represent potential biomarkers for PCa prognostic, diagnostic and monitoring. Increasing evidences have shown that urinary biomarkers are promising tools to improve PCa management (8).

Mass spectrometry (MS) is a powerful technique to detect and monitor biomarkers in human biofluids. MS-based approaches have been successfully used to profile urinary proteins/peptides in the search for PCa biomarkers (8,9). In this work, we used liquid chromatography-mass spectrometry (LC-MS) to identify and quantify naturally occurring peptides in urine samples from patients diagnosed with PCa, BPH, and healthy individuals. We identified 5 urinary peptides derived from uromodulin with potential for use as PCa biomarker, generating AUC values between 0.788 and 0.951. In addition, the identified peptide panel outperformed PSA in differentiating between PCa and BPH. Thus, we hope to contribute to the identification of candidate peptides as biomarkers for the early and accurate detection of PCa.

Materials and methods

Patients and sample collection. The study cohort included men without prior PCa diagnosis who underwent a transrectal ultrasound (TRUS)-guided prostate biopsy from September 2018 to September 2019 in the Urology Service of Hospital Ernesto Dornelles (Porto Alegre, Rio Grande do Sul, Brazil) or in the Hospital Ana Nery (Santa Cruz do Sul, Rio Grande do Sul, Brazil). Positive and negative prostate biopsies were classification criteria for patients with PCa and BPH, respectively. Tumor grading and staging of PCa patients were based on histological analysis. The diagnosis of BPH was based on lower urinary tract symptoms and evidence by palpation or transrectal ultrasound of prostate enlargement. Urine samples were collected just before prostate biopsy without prior DRE or prostatic massage. Control urine samples were obtained from healthy volunteers without any diagnosed prostate condition, no positive DRE, no prostate alteration and PSA levels <4.0 ng/ml. A total of 86 participants were included in the study, with age range from 42 to 88 years. Urine samples were centrifuged at 4,000 g for 10 min at 4˚C and stored at -80˚C until peptide isolation. This study was approved by Institutional Review Boards of Universidade Federal do Rio Grande do Sul (UFRGS), Universidade de Santa Cruz do Sul (UNISC), Hospital Ernesto Dornelles (HED), and Hospital Ana Nery, under the protocol CAAE number 69852617.1.1001.5347. The study was conducted according to the guidelines of the Declaration of Helsinki and written informed consent was obtained from all participants.

Urinary peptide isolation. Urine endogenous peptides were isolated by trichloroacetic acid (TCA) precipitation as described by Parker *et al* (10). Briefly, 700 μl of urine samples were concentrated by vacuum centrifugation using SpeedVac. Samples were concentrated to more accurately reproduce the methodology described by Parker *et al* (10), as the protocol was originally standardized for human plasma samples, which contain larger amounts of proteins/peptides than those normally found in urine. After concentration, samples were mixed 1:1 with PBS and urinary proteins were precipitated with 1 volume of 20% TCA for 1 h at 4˚C. Samples were centrifuged at 16,000 x g for 10 min at 4˚C and the peptide-containing supernatants were collected. Purified peptides were desalted using HLB OASIS cartridges (Waters), following manufacturer's instructions. Peptides were quantified using Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Scientific, 23275) and stored at -20˚C until analysis.

LC-MS analysis. The isolated urinary peptides were analyzed by LC-MS using a nanoACQUITY UPLC system coupled to a Xevo G2-XS Q-Tof mass spectrometer (Waters) with a low-flow probe at the source. Peptides were separated by analytical chromatography (Acquity UPLC BEH C18, 1.7 μm, 2.1x50 mm, Waters) at a flow rate of 8 μl/min, using a 7-85% water/ACN 0.1% formic acid linear gradient over 42 min. The MS survey scan was set to 0.5 s and recorded from 50 to 2,000 m/z. MS/MS scans were acquired from 50 to 2,000 m/z, and scan time was set to 1 s. Data were collected in data-independent mode (MS^E). Two independent LC-MS^E runs were performed for each sample and each run contained 25 μg of sample.

LC-MS data analysis. LC-MS^E data were processed and searched using ProteinLynx Global Server (PLGS 3.0.3, Waters Corporation). Database searches were conducted against *Homo sapiens* protein sequences retrieved from UniProtKB/Swiss-Prot database, with the following parameters: oxidation of methionine (M), proline (P) and lysine (K) as variable modifications, without any enzyme specificity and maximal missed cleavage of 0. Peptides and protein tolerances were set as automatic, allowing minimum fragment ion per protein as 2, minimum fragment ion per peptide as 2, minimum peptide matches per proteins as 1 and false discovery rate (FDR) as 4%. Only peptides detected in the two technical replicates were considered for further analysis in order to improve confidence. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE (11) partner repository with the dataset identifier PXD037031.

Raw files containing MS^E spectra and peptide ID files (fragment.csv) generated by PLGS were imported into Skyline software (12) to create a comprehensive spectral library. MS1 precursor ion chromatograms (M, M + 1, and M + 2) were extracted for each peptide and the integrated areas of isotope peaks were used for label-free peptide quantification. Log2-transformed values were submitted to differential expression analysis using the NormalyzerDE tool (13). Peptides with differential abundance between study groups were submitted to receiver operating characteristic (ROC) curve analyses using SPSS Statistics software, version 18 (SPSS Inc., Chicago, III., USA).

In silico protease cleavage sites prediction. The prediction of proteases potentially involved in the generation of the identified urinary peptides was performed using the Proteasix tool (14), which uses the MEROPS peptidase database as a reference. Peptide sequence data were prepared in the required input format and the analysis was conducted using the default settings. In order to retrieve high-confidence cleavage site predictions, we selected a specificity threshold >80%. TCGA expression data of predicted proteases in PCa and normal prostate tissue were retrieved from UALCAN (15).

Table I. Demographic and clinical data of the study cohort.

Parameters	Groups			Comparisons (P-value)		
	PCa (n=33)	BPH (n=25)	Control (n=28)	PCa vs. BPH	PCa vs. Control	BPH vs. Control
Median age, years (95% CI)	69 (64-73)	61 (58-66)[a]	57 (53-60)[b]	0.012[c]	$1.98x10^{-6}$[c]	0.076[c]
PSA median, ng/ml (95% CI)	7.93 (7.00-9.00)[d]	6.65 (5.41-9.31)[e]	0.93 (0.58-1.52)[e]	0.368[f]	$2.56x10^{-10}$[f]	$1.02x10^{-6}$[f]
Gleason score, n						
Gleason 6	13	n.a.	n.a.	n.a.	n.a	n.a
Gleason 3+4/4+3	17	n.a.	n.a.	n.a.	n.a.	n.a.
Gleason 8	3	n.a.	n.a.	n.a.	n.a.	n.a.

[a]From 23 individuals; [b]from 25 individuals; [c]one-way ANOVA with Tukey's post hoc test; [d]from 32 individuals; [e]from 20 individuals; [f]Kruskal-Wallis with Dunn's post hoc test. All statistical significance was set at P<0.05. PCa, prostate cancer; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; CI, confidence interval; n.a., not applicable.

Statistical analysis. Demographic and clinical data of study groups were analyzed using one-way ANOVA and Tukey's post hoc test or Kruskal-Wallis and Dunn's post hoc test, depending on the normality of the data. Peptide peak area values (log2-transformed) were used for quantitative analysis using NormalyzerDE tool and differential abundance between the study groups were analyzed by unpaired, moderated t-test (empirical Bayes Limma approach) with Benjamini-Hochberg correction. Comparisons between different age groups were performed using one-way ANOVA or Kruskal-Wallis tests, depending on the normality of the data. Peptide levels in low- and high-grade tumors were compared using a Mann-Whitney non-parametric test. Statistical significance was set at P<0.05. Peptides with differential abundance between the study groups were submitted to ROC curve analyses using SPSS Statistics software. Expression data from predicted proteases in normal and PCa tissues were retrieved from TCGA using UALCAN portal and analyzed by unpaired Welch's t-test.

Results

Clinical data of the study cohort. A total of 86 participants were included in the study: 33 patients with PCa, 25 patients with BPH and 28 healthy controls (Table I). Men in the PCa group were significantly older than men from BPH and control groups (P=0.012 and P<0.0001, respectively). Median PSA levels were significantly lower (P<0.0001) in control group compared to the PCa and BPH groups. In addition, there was no statistically significant difference in mean PSA levels between BPH and PCa groups (P=0.368).

Endogenous urinary peptide profiles. Endogenous peptides were isolated from urine samples by protein precipitation using TCA. Varying amounts of peptides were recovered from each sample, from 105 to 1,260 μg. Twenty-five micrograms of each sample were analyzed by LC-MS^E. The analysis resulted in the identification of 10 peptides derived from uromodulin and 9 peptides derived from alpha-1-antitrypsin (Table SI). Peptides detected in >95% of the analyzed samples were subjected to label-free quantitative analysis based on precursor (MS1) peak area using NormalyzerDE tool. These peptides, highlighted in Table SI, were named UMOD-P1 to UMOD-P7. Comparative analyzes revealed 5 peptides (UMOD-P1 to UMOD-P5) with differential abundance between the study groups, all displaying lower abundance in the PCa group (Table II; Fig. 1). These five peptides showed statistically significant differences between the BPH and PCa groups, with four of them also significantly altered between the control and PCa groups. No peptide showed significant differences between control and BPH groups. Therefore, our data indicate that urine endogenous UMOD peptides have the potential to discriminate between benign and malignant prostate conditions. We subdivided the study groups into distinct age groups to evaluate possible differences in UMOD peptide levels between younger and older individuals. No statistically significant differences were found between age groups in any of the study groups (Table SII). In addition, no significant differences were observed in UMOD peptide levels between low-(Gleason score 6) and high-grade (Gleason score >6) tumors (Table SIII).

Diagnostic performance of urine endogenous peptides. ROC analyses were performed for urinary peptides that showed differential abundance between the study groups. ROC curves were constructed using the log2-transformed precursor peak area values of UMOD-P1, UMOD-P2, UMOD-P3, UMOD-P4 and UMOD-P5 in samples from PCa, BPH and control groups (Fig. 2). UMOD peptides showed similar potential to discriminate between control and PCa groups, displaying AUC values between 0.850-0.873, with sensitivity and specificity ranging from 84.85 to 87.88% and 78.57 to 82.14%, respectively (Fig. 2A and C). In BPH x PCa analysis, UMOD peptides showed AUC values between 0.788 and 0.839, with sensitivity and specificity ranging from 78.79 to 84.85% and 76 to 88%, respectively (Fig. 2B and C). UMOD-P1 showed the best diagnostic performance for differentiating BPH and PCa groups, with sensitivity and specificity levels of 84.85 and 88%, respectively. We then evaluated the ability of combinations of two or more peptides to discriminate between study

Table II. Comparison of peak area values of UMOD peptides between study groups.

		Peptide peak area (log2)									Comparisons			
		Control			BPH			PCa			Control vs. PCa		BPH vs. PCa	
Peptide	Peptide sequence	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	P-value[a]	Adjusted P-value[b]	P-value[a]	Adjusted P-value[b]
UMOD-P1	DQSRVLNLGPITR	16.79-21.71	19.96	1.11	17.18-20.70	19.58	0.76	12.98-20.07	18.53	1.20	$1.89x10^{-6}$	$1.32x10^{-5}$	0.00044	0.00311
UMOD-P2	IDQSRVLNLGPITR	11.74-22.43	20.26	2.37	17.89-21.40	20.26	0.76	13.47-20.79	19.19	1.22	0.01102	0.01928	0.01410	0.02467
UMOD-P3	QSRVLNLGPITR	10.89-18.61	16.77	1.53	14.54-17.47	16.52	0.65	9.75-17.02	15.52	1.19	0.00012	0.00042	0.00248	0.00867
UMOD-P4	SGSVIDQSRVLNLGPITR	-	-	-	23.30-26.17	25.23	0.70	15.80-26.04	24.06	1.76	-	-	0.03157	0.04420
UMOD-P5	SGSVIDQSRVLNLGPITRK	11.38-22.34	19.84	2.26	17.69-21.88	19.69	0.88	15.24-20.47	18.61	1.08	0.00242	0.00565	0.00929	0.02168

[a]Unpaired moderated t-test (empirical Bayes Limma approach). [b]Benjamini-Hochberg adjusted P-value. Statistical significance was set at $P<0.05$. PCa, prostate cancer; BPH, benign prostatic hyperplasia; UMOD-P, uromodulin peptide; SD, standard deviation.

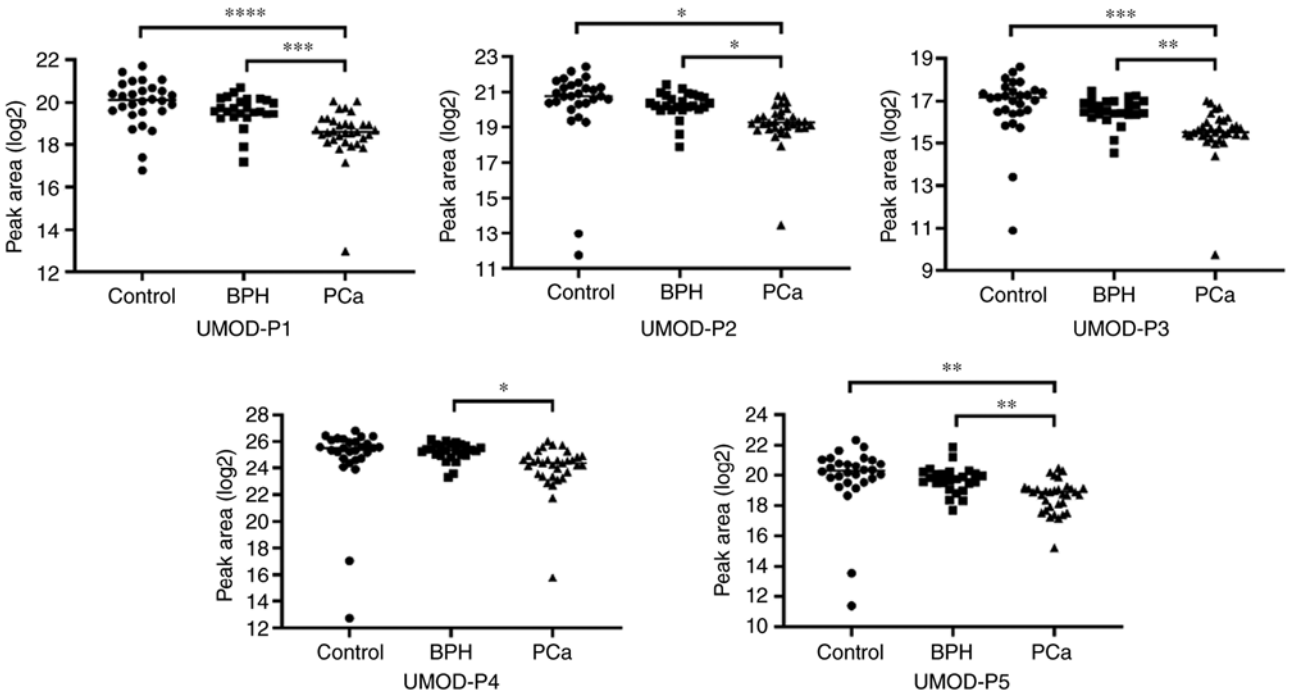
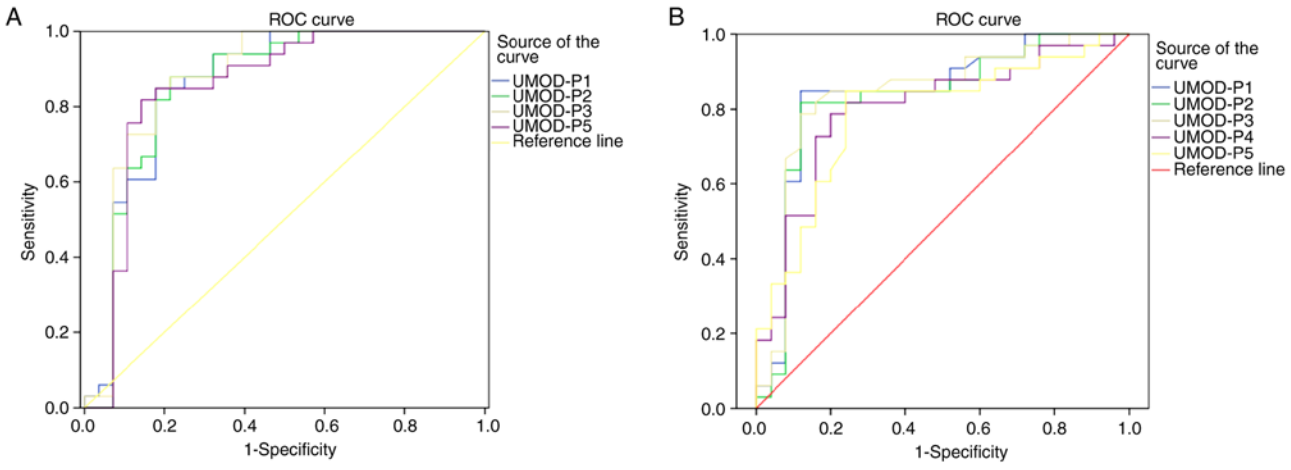


Figure 1. Urinary peptides with differential abundance between the study groups. Peak area values of UMOD peptides significantly altered between BPH and PCa, and control and PCa groups. Differences between groups were analyzed using unpaired moderated t-test (empirical Bayes Limma approach) with Benjamini-Hochberg correction. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. PCa, prostate cancer; BPH, benign prostatic hyperplasia; UMOD-P, uromodulin peptide.



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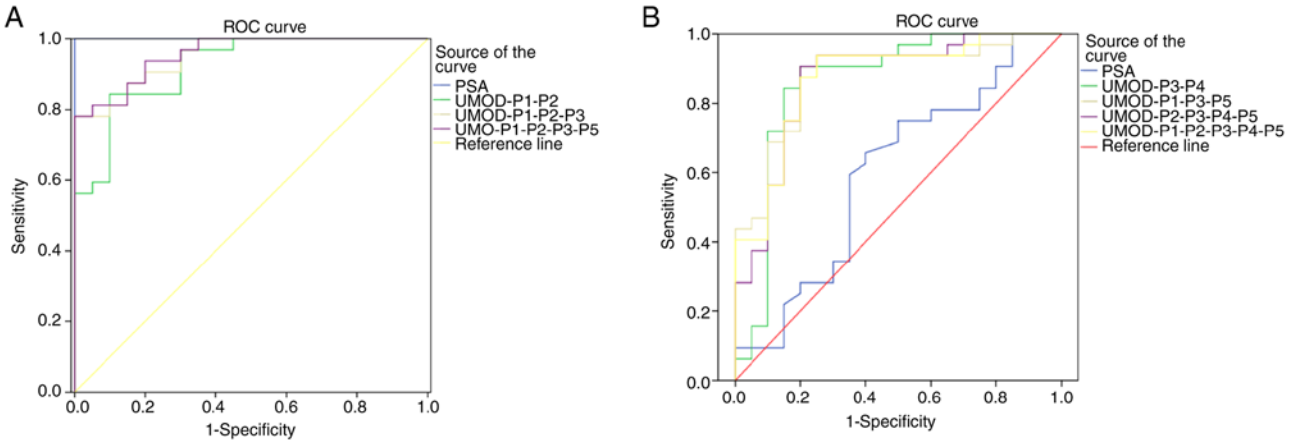
	Control versus PCa groups				BPH versus PCa groups				
	UMOD-P1	UMOD-P2	UMOD-P3	UMOD-P5	UMOD-P1	UMOD-P2	UMOD-P3	UMOD-P4	UMOD-P5
Sample size (n)	61	61	61	61	58	58	58	58	58
Control/PCa group (n)	28/33	28/33	28/33	28/33	n.a.	n.a.	n.a.	n a.	n.a.
BPH/PCa group (n)	n.a.	n.a.	n.a.	n.a.	25/33	25/33	25/33	25/33	25/33
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Area under curve (AUC)	0.859	0.855	0.873	0.85	0.837	0.827	0.839	0.8	0.788
95% CI	0.746–0.935	0.741–0.932	0.763–0.945	0.736–0.929	0.717–0.921	0.705–0.913	0.719–0.923	0.674–0.894	0.661–0.884
Specificity (%)	82.14	78.57	82.14	82.14	88	88	88	80	76
Sensitivity (%)	84.85	87.88	84.85	84.85	84.85	81.82	78.79	78.79	84.85

Figure 2. Performance of urinary peptides as biomarkers for PCa detection. ROC analyses were performed using log2-transformed precursor peak area values of UMOD peptides in (A) control vs. PCa, and (B) BPH vs. PCa groups. (C) ROC curve data for the different comparisons between the study groups. ROC, receiver operating characteristic; PCa, prostate cancer; BPH, benign prostatic hyperplasia; UMOD-P, uromodulin peptide; CI, confidence interval; n.a., not applicable.

groups (Fig. 3). Peptide combinations resulted in AUC values between 0.922 and 0.951 for control x PCa analysis, with sensitivity and specificity levels ranging from 75.76 to 81.82% and 89.29 to 100%, respectively (Fig. 3A and C).



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	Control versus PCa groups			
	UMOD-P1-P2	UMOD-P1-P2-P3	UMOD-P1-P2-P3-P5	PSA
Sample size (n)	61	61	61	52
Control/PCa group (n)	28/33	28/33	28/33	20/32
P-value	<0.0001	<0.0001	<0.0001	<0.0001
Area under curve (AUC)	0.922	0.943	0.951	1
95% CI	0.824–0.975	0.852–0.986	0.864–0.990	0.932–1
Specificity (%)	89.29	96.43	100	100
Sensitivity (%)	81.82	75.76	75.76	100

D

	BPH versus PCa groups				
	UMOD-P3-P4	UMOD-P1-P3-P5	UMOD-P2-P3-P4-P5	UMOD-P1-P2-P3-P4-P5	PSA
Sample size (n)	58	58	58	58	52
BPH/PCa group (n)	25/33	25/33	25/33	25/33	20/32
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.2482
Area under curve (AUC)	0.847	0.851	0.856	0.856	0.598
95% CI	0.729–0.928	0.733–0.931	0.739–0.934	0.739–0.934	0.453–0.732
Specificity (%)	88	72	80	84	60
Sensitivity (%)	81.82	87.88	87.88	78.79	65.62

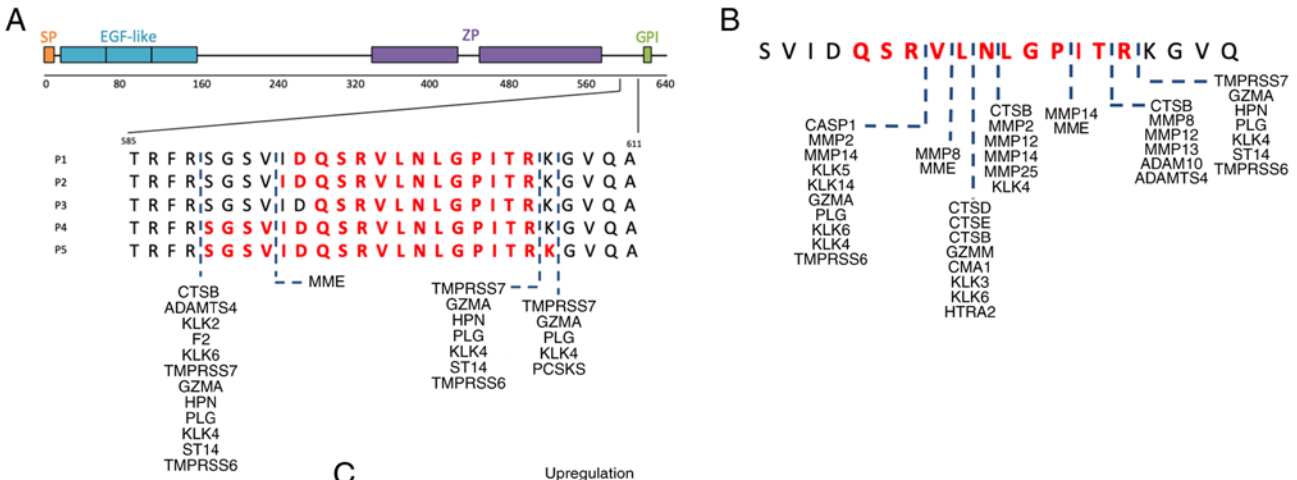
Figure 3. ROC curves for urinary peptide panels and PSA levels. Comparative ROC analysis using peptide combinations or PSA levels for discriminating (A) control vs. PCa, and (B) BPH vs. PCa groups. Data for the respective ROC curves are shown in (C) and (D), respectively. ROC, receiver operating characteristic; PCa, prostate cancer; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; UMOD-P, uromodulin peptide; CI, confidence interval.

The combination of 4 peptides (UMOD-P1, UMOD-P2, UMOD-P3 and UMOD-P5) showed the best performance for discriminating control and PCa groups, with sensitivity and specificity levels of 75.76 and 100%, respectively. AUC values between 0.847 and 0.856 were obtained for BPH x PCa analysis using peptide combinations, with sensitivity and specificity levels ranging from 78.79 to 87.88% and 72 to 88%, respectively (Fig. 3B and D). The combination of UMOD-P3 and UMOD-P4 showed high sensitivity and specificity (81.82 and 88%, respectively) in discriminating between benign and malignant prostate conditions.

We also performed a direct comparison of our biomarker panel with PSA levels (Fig. 3). Peptide panel resulted in specificity levels similar to those seen for PSA (100%) in a control vs. PCa analysis (Fig. 3A and C), but with lower sensitivity. It is important to consider that PSA levels <4.0 ng/ml was an inclusion criterion for the control group, which may be influencing the high levels of sensitivity and specificity observed for PSA. On the other hand, our urinary peptide panel significantly outperformed the PSA in BPH x PCa comparison (Fig. 3B and D), showing significantly higher AUC values and sensitivity/specificity levels (P<0.0001).

Prediction of proteases potentially involved in the generation of UMOD urinary peptides. In silico analyses using Proteasix indicated that different proteolytic events could be involved in generating UMOD-P1 to UMOD-P5. A total of 104 combinations of predicted proteases and cleavage sites were identified using a >80% specificity threshold (Table SIV). The predicted proteases are summarized in Fig. 4A. Among predicted proteases, 13 (~93%) predominantly cleave at the carboxyl side of arginine or lysine residues. No N-terminal cleavage site was identified for UMOD-P1 and UMOD-P3 peptides with the filtering criteria used.

Since UMOD-P1 to UMOD-P5 showed significantly reduced abundance in PCa samples, we speculate that these peptides are generated under normal physiological conditions and undergo additional proteolytic events under malignant conditions of the prostate. Therefore, we searched *in silico* for proteases able to hydrolyze the smallest identified UMOD peptide (UMOD-P3). Proteins potentially involved in the proteolysis of UMOD-P3 were summarized in Fig. 4B and Table SV. The expression profile of the identified proteases in PCa and normal tissue were retrieved from UALCAN portal (Fig. 4C). Proteases KLK3, KLK4, KLK14, HTRA2 and MMP25 were reported to be overexpressed in PCa tumor and could be potentially involved in the degradation of UMOD peptides in individuals with PCa. These additional cleavage events may explain the reduced abundance of UMOD peptides in the urine of individuals with PCa compared to BPH and control groups.



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Protease	Upregulation (PCa versus normal tissue)	Statistical significance
ADAM10	No difference	2.95×10^{-1}
ADAMTS4	No difference	3.16×10^{-1}
CASP1	Normal tissue	1.8×10^{-6}
CMA1	Normal tissue	6.94×10^{-3}
CTSB	Normal tissue	9.64×10^{-13}
CTSD	No difference	8.37×10^{-1}
CTSE	Normal tissue	4.66×10^{-3}
F2	PCa	1.38×10^{-3}
GZMA	No difference	8.89×10^{-1}
GZMM	No difference	5.01×10^{-1}
HPN	PCa	$<1\times10^{-12}$
HTRA2	PCa	1.36×10^{-10}
KLK14	PCa	9.04×10^{-3}
KLK2	PCa	1.95×10^{-10}
KLK3	PCa	9.74×10^{-5}
KLK4	PCa	2.53×10^{-7}
KLK5	No difference	1.93×10^{-1}
KLK6	Normal tissue	5.06×10^{-3}
MME	Normal tissue	6×10^{-5}
MMP12	No difference	5.27×10^{-1}
MMP13	No difference	2.37×10^{-1}
MMP14	Normal tissue	1.76×10^{-9}
MMP2	Normal tissue	2.16×10^{-4}
MMP25	PCa	2.12×10^{-3}
MMP8	No difference	3.53×10^{-1}
PCSK5	Normal tissue	1.42×10^{-3}
PLG	Normal tissue	3.84×10^{-2}
ST14	PCa	1.18×10^{-14}
TMPRSS6	No difference	2.25×10^{-1}
TMPRSS7	Normal tissue	4.94×10^{-7}

Figure 4. Predicted proteases involved in the generation of UMOD peptides. UMOD urinary peptides originate from a region near to C-terminal of the precursor uromodulin protein. (A) Peptides identified in our study are in bold red and the predicted cleavage sites and proteases are indicated by dashed lines. (B) Additional cleavage sites predicted for UMOD-P3. (C) Expression profile of predicted proteases in PCa and normal tissue. Data were retrieved from TCGA using UALCAN portal and correspond to normal and PCa tissues from different individuals. Statistical analysis was performed using unpaired Welch's t-test. PCa, prostate cancer; SP, signal peptide; EGF, epidermal growth factor; ZP, zona pellucida; GPI, glycosylphosphatidylinositol.

Discussion

Most prostate tumors grow slowly and are confined to the prostate gland, with a good prognosis if diagnosed early. However, the PSA-based tests currently used for PCa diagnosis, including age-adjusted PSA ranges, PSA velocity, PSA density and percentage of free PSA, have important limitations (4,16). PSA has low specificity for discriminating between malignant and benign prostatic conditions, as well as to differentiate between indolent and aggressive disease, leading to overdiagnosis and overtreatment (4). Thus, new biomarkers have been proposed to improve PCa diagnosis, including the urinary RNAs PCA3 and TMPRSS2-ERG (17). The clinical utility of these biomarkers for PCa diagnosis is still conflicting across different studies, which describe AUC values ranging from 0.660 to 0.770 (8,9,17). Assays based on these molecules, such as Progensa and Mi-Prostate Score (MiPS), have been used mainly to assist in the decision to perform a new biopsy in the case of an inconclusive first biopsy (8,9).

Reliable biomarkers able to provide an early and accurate diagnosis may improve patient management, reducing biopsies and overtreatment. In this work, we applied simple and low-cost protocols for peptide biomarker discovery in urine for PCa diagnosis. Using this approach, we identified a panel of urinary peptides with high specificity in differentiating between PCa and control groups (AUC=0.951). Most importantly, these urinary peptides outperformed PSA in discriminating between malignant and benign prostate conditions (AUC=0.847), showing high sensitivity (81.82%) and specificity (88%). Overall, urinary peptides resulted in AUC values comparable to or greater than those observed for other urinary PCa biomarkers based on RNA, DNA or proteins (8,17).

Our sample preparation protocol is simplest, cheapest and fastest when compared to experimental approaches traditionally used in the isolation of urinary protein/peptide biomarkers for LC-MS analysis (6,18-23). We analyzed naturally occurring urinary peptides and, therefore, our protocol did not include an enzymatic protein digestion step (e.g., trypsin digestion). Furthermore, we used TCA protein precipitation to isolate endogenous urinary peptides, eliminating expensive devices and time-consuming centrifugation steps typical of ultrafiltration-based protocols (10). Overall, the standardized methodology is compatible with LC-MS-based assays for routine clinical applications, which could facilitate its future translation for medicine and patient care.

Numerous studies have revealed the high intertumoral and intratumoral heterogeneity in PCa (24). Therefore, a single biomarker is unlikely to provide the sensitivity and specificity needed to accurately diagnose and stratify PCa, as every single biomarker has its own performance limits. Thus, more recent studies have focused on the identification and assessment of multiple biomarkers to improve diagnostic accuracy and disease risk stratification (25,26). Here, we found that combining peptides in a biomarker panel improved diagnostic performance compared to individual biomarkers, showing higher AUC values in discriminating PCa from control and BPH groups.

The invasive techniques for cancer diagnosis and monitoring are slowly being replaced by liquid biopsies. Liquid biopsies allow for easy and minimally invasive sample collection for biomarker detection and quantification (7,27). In this scenario, urine has been recognized as a good source of biomarkers for urological tumors, including PCa (28). However, many studies use prostate massage prior sample collection for biomarker discovery with the aim of increasing the amount of prostate-derived molecules released in the urine (29,30). Furthermore, it is important to consider that a significant portion of men still refuse DRE on account of discomfort or embarrassment. Thus, our study was based on the analysis of biomarkers present in urine without previous prostate massage in order to increase patient acceptance and adherence in a future clinical application.

About 70% of urinary proteins are derived from exosomes, secretory/excretory products and cells shed from urogenital tract, including prostate gland. As urine is stored in the bladder for hours, digestion of urinary proteins occurs prior to voiding by the action of endogenous proteases (31). Therefore, the presence/abundance of urinary peptides is altered according to the physiological/pathological state of main urogenital tissues. Cancer cells secret proteases that can act on proteins present in the urine, ultimately leading to a differential abundance of urinary peptides in individuals with cancer (18,32). Previous studies have shown that endogenous urinary peptide signatures have diagnostic value for PCa, especially in discriminating between PCa and BPH, resulting in high sensitivity (67.4 to 91.7%) and specificity (71.2 to 90.5%) (33-35). Our urinary peptide panel outperformed PSA in discriminating between PCa and BPH, showing high levels of sensitivity (87.88%) and specificity (88%).

The urinary peptides found in our study were derived from uromodulin (UMOD), also known as Tamm-Horsfall glycoprotein. UMOD is the most abundant protein found in human urine under physiological conditions (36). Previous studies have identified UMOD peptides in urine under physiological conditions, as part of urinary peptidome of healthy individuals (19). Thus, alterations in the presence and/or abundance of urinary UMOD peptides may be associated with the individual's pathological state. Urinary UMOD peptides identified across multiple studies are derived from the C-terminal region of the protein, around 589-607 residues, which suggest that this region is more sensitive or accessible to the action of endogenous proteases (20-22). We identified five UMOD-derived urinary peptides with reduced abundance in PCa samples. M'Koma *et al* (35) also identified urinary UMOD peptides with reduced levels in patients with PCa. These results suggest that PCa-related proteases may be acting on the further degradation of UMOD peptides normally found in urine. From *in silico* analyses of cleavage sites and expression data in PCa x normal tissue, we identified proteases HTRA2, KLK3, KLK4, KLK14, and MMP25 as potentially acting on degradation of the identified UMOD peptides.

Kallikrein-related peptidases (KLKs) are serine proteases that are upregulated in PCa (37), including KLK2, KLK3 (also known as PSA), KLK4, KLK11 and KLK14-15. These proteases have therefore been proposed for use as PCa biomarkers (37,38). KLKs are secreted by prostate cancer cells and many of them have already been detected in human biofluids, including urine (23,37). MMP25 (also known as MT6-MMP) was found upregulated in malignant prostate tissue compared to benign prostate tissue (39). This metalloproteinase displays intrinsic proteolytic activity towards extracellular matrix components and therefore could play a direct role in prostate tumor invasion. MMP25 is a GPI-anchored protein and its presence in urinary exosomes has already been reported (40).

Although our results indicated the potential of urinary peptides as biomarkers for PCa, they should be interpreted considering the limitations of the study, including a relatively small and age-biased cohort. Despite these limitations, the cohort was appropriate to assess the feasibility of profiling endogenous urine peptides and to estimate the potential of these peptides as diagnostic biomarkers for PCa. The present study therefore represents the first phase of the biomarker development pipeline (the discovery phase), in which a small number of individual samples are analyzed to identify biomarker candidates (10). Next, it will be necessary to further evaluate the specificity of the identified peptides for use in PCa diagnosis, since urinary peptides derived from UMOD have already been described as altered in other pathophysiological conditions (21-22,41). It is noteworthy that, although the urine proteome is altered under conditions of urinary tract infection or inflammation, no changes were found in urinary levels of UMOD or enzymes potentially involved in its proteolysis in samples from cases of urinary tract infection or from animal models of prostatic inflammation (42,43). Lastly, our findings require confirmatory studies using larger age-matched cohorts to validate the pathophysiological relevance of the identified urinary peptides and their potential use as PCa biomarkers.

In conclusion, the profiling of urine by LC-MS allowed the identification of endogenous peptides with potential for use as PCa biomarkers. In addition, our peptide panel was able to discriminate between individuals with PCa or BPH with high sensitivity and specificity, overcoming an important

limitation of currently available biomarkers. We also identified disease-associated proteases potentially involved in the degradation of uromodulin peptides detected at low levels in the urine of patients with PCa.

Acknowledgements

The authors would like to thank Dr Ricardo Kaufmann (Urology Service, Ernesto Dornelles Hospital, Porto Alegre, RS, Brazil) for his assistance with the collection of urine and biopsy specimens.

Funding

This work was supported by Programa de Pesquisa para o SUS: Gestão Compartilhada em Saúde-PPSUS (grant no. 17/2551-0001 412-0), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Departamento de Ciência e Tecnologia da Secretaria de Ciência, Tecnologia e Insumos Estratégicos do Ministério da Saúde (DECIT/SCTIE/MS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Secretaria da Saúde do Estado do Rio Grande do Sul (SES/RS). CSD and DRK were supported by CNPq scholarship. MH was supported by FAPERGS scholarship. DCS and RMP were supported by Universidade Federal do Rio Grande do Sul (BIC/UFRGS) scholarship.

Availability of data and materials

The mass spectrometry datasets generated during the current study are available in the PRIDE repository with the dataset identifier PXD037031 (https://www.ebi.ac.uk/pride/archive/projects/PXD037031). Other datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CDSD and KMM participated in the conception and design of the study, and reviewed the literature. CDSD drafted the manuscript. AZ, GB, DRK, DJB, HBF, KMM, LGP, RCD and MFB made substantial contributions to the acquisition of data. CDSD, DDCS, KMM, MH, RMP and NRM analyzed the data. HBF, AZ, KMM and LGP critically revised the intellectual content of the manuscript prior to submission. CDSD and KMM confirm the authenticity of all the raw data. Each author participated sufficiently in the work to take public responsibility for appropriate portions of the content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Review Boards of Universidade Federal do Rio Grande do Sul (UFRGS), Universidade de Santa Cruz do Sul (UNISC), Hospital Ernesto Dornelles (HED) and Hospital Ana Nery under the protocol CAAE number 69852617.1.1001.5347. All subjects provided written informed consent and the study was performed following the guidelines of The Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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