Determination of common urine substances as an assay for improving prostate carcinoma diagnostics

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Abstract. Recently, interest in the identification of non-invasive markers for prostate carcinoma detectable in the urine of patients has increased. In this study, we monitored the abundance of potential non-invasive markers of prostate carcinoma such as amino acid sarcosine, involved in the metabolism of amino acids and methylation processes, ongoing during the progression of prostate carcinoma. In addition, other potential prostate tumor markers were studied. The most significant markers, prostate-specific antigen (PSA) and free PSA (fPSA), already used in clinical diagnosis, were analyzed using an immunoenzymometric assay. Whole amino acid profiles were also determined to evaluate the status of amino acids in patient urine samples and to elucidate the possibility of their utilization for prostate carcinoma diagnosis. To obtain the maximum amount of information, the biochemical parameters were determined using various spectrophotometric methods. All results were subjected to statistical processing for revealing different correlations between the studied parameters. We observed alterations in most of the analyzed substances. Based on the results obtained, we concluded that the specificity of prostate carcinoma diagnosis could be improved by determination of common urine metabolites, since we compiled a set of tests, including the analysis of sarcosine, proline, PSA and uric acid in the urine. These metabolites were not observed in the urine obtained from healthy subjects, while their levels were elevated in all patients suffering from prostate carcinoma.

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Introduction

In 2013, cancer of the prostate (CaP) was determine to be the most common type of tumor in males in the United States (1,2) and worldwide (3). Early diagnostis of CaP is important due to the increase in treatment success leading to elimination of metastatic expansion. Currently, there is no complex test available for CaP diagnosis and evaluation of prostate cancer stage (4). The testing process usually used for the diagnosis of CaP includes digital rectal examination, determination of prostate-specific antigen (PSA) (5), transrectal sonography with biopsy of the prostate (6), magnetic resonance imaging (7) and positron emission tomography (8). PSA, first described in 1977 (9), is the most widely used biomarker of CaP to date. It is commonly used to estimate the stage of disease and disease progression. Despite the fact that the sensitivity (49-91%) and the specificity (68-80%) of PSA are high, the prognosis estimate is unreliable, in early stages in particular (10). In this context, new biomarkers of CaP carcinoma are increasingly studied with the prospect to serve as a useful tool for early diagnosis without clinical examinations and/or invasive interventions (11,12). Novel potential biomarkers with the possibility to be determined in urine include α-methylacyl-CoA-racemase (AMACR) (13), PCA3 (prostatic antigen 3) (14,15) and Annexin A3 (13,16). Other reported potential biomarkers detectable in serum include kallikrein 2, fibronectin 1, urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor, pigment epithelium-derived factor (PEDF), interleukin-6 and interleukin-6 receptor (17,18). Nevertheless, the use of these markers in clinical practice and their role in the active surveillance scenario require further investigation. The amino acid sarcosine is currently the most studied biomarker showing the capability to serve as a diagnostic substance for the early stages of prostate carcinoma. Interest in this molecule was increased in 2009 when Sreekumar and colleagues (19) published their study regarding metabolomic profiles of urine obtained from CaP patients. Even though the linkage of sarcosine with prostate carcinoma development was reported (20,21) as well as its

potential in the diagnosis of early-stage tumors (4,22), its use as a marker is still under discussion (23). Hence, it is necessary to study the functions of sarcosine and other amino acids, acting as the intermediate products of metabolism influenced by tumor growth. Therefore, the aim of the present study was to determine the amino acid profile of urine samples obtained from patients suffering from CaP and to compare them with control subjects. Biochemical analyses of samples were also carried out, and the sarcosine content was determined. Further statistical analysis was performed to reveal correlation between the parameters obtained.

Materials and methods

Biological samples. Urine samples from patients suffering from cancer of the prostate (n=32), obtained from the Department of Urology, St. Anne's University Hospital, Brno were used. The average age of the patients was 68.45 years. All cases were diagnosed with different types of acinar adenocarcinoma. Detailed information concerning the patients is documented in Table I. For a control measurement, urine samples from volunteers (n=32) with an average age of 24.69 years were used. Enrollment of patients into the clinical study was approved by the Ethics Committee of the Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Chemicals and pH measurement. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) at ACS-specified purity unless noted otherwise. As a buffer for ion-exchange liquid chromatographic sample preparation sodium dilution buffer composed of 0.10 g of N_3Na, 11.5 g of NaCl, 14 g of citric acid all diluted in 1 liter of water was used. Chemicals used as a part of the kits for urine biochemical parameters were glucose, pyrogallol red, creatinine and uric acid (Medesa s.r.o. Policka, Czech Republic) and urea. 4-Methylumbelliferyl phosphate was obtained from Tosoh Bioscience (Tokyo, Japan). As a derivatization agent used for ion-exchange chromatographic analyses, ninhydrin with methyl Cellosolve (Ingos, Prague, Czech Republic) and $SnCl_2$ as a reduction agent were used. Washing solutions were prepared in Milli-Q water obtained using reverse osmosis equipment Aqual 25 (Aqual s.r.o., Brno, Czech Republic). The deionized water was further purified by using apparatus Direct-Q 3 UV Water Purification system equipped with a UV lamp from Millipore (Billerica, MA, USA). The resistance was established at 18 $M\Omega \cdot cm^{-1}$. The pH was measured using the pH meter WTW inoLab (WTW, Weilheim, Germany).

Sample preparation for the determination of the urine amino acid profile. The urine sample (500 μl) was pipetted into mineralization vials and mixed with 500 μl of 35% HCl and mineralized using the microwave equipment MW 3000 (Anton Paar, Graz, Austria) using parameters: Power 80; Ramp, 15 min; Hold, 90 min; Max, pressure 25 bar, Rotor XF100-6. The mineralized sample (100 μl) was diluted with 900 μl of dilution buffer and centrifuged using Centrifuge 5417R (Eppendorf, Hamburg, Germany) under the following conditions: temperature 4˚C, 25,000 x g for 20 min. Subsequently, 500 μl of the sample was diluted in 500 μl of 0.6 M NaOH prior to analysis by ion-exchange chromatography.

Sample preparation for determination of sarcosine. The urine sample (500 μl) was pipetted into a 96-well evaporation plate (Deepwell plate 96; Eppendorf AG) and evaporated by the nitrogen blow-down evaporator Ultravap 96 with spiral needles (Porvair Sciences Ltd., Leatherhead, UK). After this procedure, the sample was diluted with 500 μl of dilution buffer and was subsequently used for analysis by ion-exchange chromatography.

Ion-exchange liquid chromatography. For determination of sarcosine, an ion-exchange liquid chromatography (Model AAA-400; Ingos) with post column derivatization by ninhydrin and an absorbance detector in visible light range (VIS) was used. A glass column with an inner diameter of 3.7 and length of 350 mm was filled manually with strong cation exchanger (Ostion LG ANB; Ingos) in sodium cycle with ~12 μm particles and 8% porosity. The column was thermostated at 60˚C. Double channel VIS detector with an inner cell of 5 μl was set to two wavelengths: 440 and 570 nm. Prepared solution of ninhydrin was stored under nitrogen atmosphere in the dark at 4˚C. Elution of amino acids was carried out by buffer containing 10.0 g of citric acid, 5.6 g of sodium citrate, and 8.36 g of natrium chloride per liter of solution (pH 3.0). The flow rate was 0.25 $ml \cdot min^{-1}$. The reactor temperature was set to 120˚C.

Spectrophotometric analysis. For determination of all biochemical parameters a BS-400 automated spectrophotometer (Mindray, Shenzhen, China) was used. It is composed of cuvette space tempered to 37˚C, reagent space with a carousel for reagents (tempered to 4˚C), sample space with a carousel for preparation of samples and an optical detector. The cuvette contents are mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples. Contamination is reduced due to its rinsing system. For detection itself, the following range of wavelengths were used: 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm.

Total protein was determined using the SKALAB CBT 600T kit (Skalab, Svitavy, Czech Republic), glucose was determined using a glucose assay (Greiner, Stuttgart, Germany), creatinine using a creatinine kit (Greiner), uric acid using a uric acid kit (Greiner), and urea using the Urea UV 5+1 assay (Greiner) according to the manufacturer's instructions.

Immunoenzymometric assay (IEMA). For analysis of PSA and fPSA in the sample of urine, IEMA was used. Measurement was carried out using the automated analyzer AIA 600 II (Tosoh Bioscience). Seventy microliters of urine sample was pipetted into the testing cup ST AIA-PACK PSAII obtained by Tosoh Bioscience containing lyophilized reagent (magnetic microbeads with murine anti-PSA and mouse anti-PSA conjugated with bovine alkaline phosphatase). Subsequently, the sample was incubated at 37˚C for 10 min. Non-bound antibodies were removed by washing solution (Tosoh Bioscience). Finally fluorogenic substrate (4-methylumbelliferyl phosphate) was added, and the intensity of the fluorescence for determination of the activity of the enzyme was measured.

Statistical analysis. The statistical analysis was carried out using several tests. All values in the present study are expressed

Table I. Overview of the information of the CaP patients, from whom urine samples were obtained.

Patient	Age (years)	Histology	Stage	Comorbidities	Smoker
86	80	Low differentiated acinar adenocarcinoma (GS 4+5)	pT2c cN0cM0	Polyneuropathy, hypothyreose, glaucoma	No
87	65	Acinar adenocarcinoma (GS 3+3)	pT2c cN0cM0	HHD, HLP	No
88	71	Acinar adenocarcinoma (GS 3+4)	pT2c cN0cM0	HHD, DM II, AFL	Stop-smoker
89	62	Medium differentiated acinar adenocarcinoma (GS 3+2)	pT2c cN0cM0	HLP, VAS	No
90	73	Medium differentiated acinar adenocarcinoma (GS 3+3)	pT2c cN0cM0	HHD	No
91	61	Medium differentiated acinar adenocarcinoma (GS 3+3)	pT2a cN0cM0	HHD, CMP	No
92	76	Low differentiated acinar adenocarcinoma (GS 3+4)	pT3b cN0cMx	GIST	No
93	64	Medium differentiated acinar adenocarcinoma (GS 2+3)	pT2c cN0cM0	HHD, thyropathy, PAOD	Yes
94	77	Low differentiated acinar adenocarcinoma (GS 5+4)	pT3b cN0cM0	HHD, DM II	Yes
95	61	Low differentiated acinar adenocarcinoma (GS 3+4)	pT3b cN0cM0	-	Yes
96	67	Acinar adenocarcinoma (GS 3+4)	pT2c cN0cM0	-	Yes
97	65	Medium differentiated acinar adenocarcinoma (GS 4+3)	pT3b cN0cM0	HLP, arthritis	No
98	78	Acinar adenocarcinoma (GS 3+3)	pT1c cN0cM0	HHD, COPD	Yes
99	66	Low to medium differentiated acinar adenocarcinoma (GS 3+4)	pT2c cN0cM0	HHD	No
100	65	Medium differentiated acinar adenocarcinoma (GS 3+3)	pT2c cN0cM0	-	No
101	62	Low differentiated acinar adenocarcinoma (GS 3+4)	pT2c cN0cM0	HHD	No
102	66	High grade acinar adenocarcinoma (GS 4+5)	pT3b cN0cM0	DM II, HHD, HLP, hepatopathy	No
103	63	Acinar adenocarcinoma (GS 3+4)	pT2a cN0cM0	HHD, HLP, COPD	No
104	62	Differentiated acinar adenocarcinoma (GS 2+3)	pT2a cN0cM0	HHD, HLP, COPD	Yes
105	60	Medium differentiated acinar adenocarcinoma (GS 3+3)	pT2c cN0cM0	HHD, HLP, AB	No
106	68	Acinar adenocarcinoma (GS 3+4)	pT2c cN0cM0	HHD, A-Fib	No
107	72	Low differentiated acinar adenocarcinoma (GS 5+4)	pT3b cN0cM0	-	Yes
108	72	Medium differentiated microacinar adenocarcinoma (GS 3+3)	pT2c cN0cM0	HHD, ICHS, HLP	No
109	71	Acinar adenocarcinoma (GS 3+3)	pT2c cN0cM0	DM II, depressive disorder	No
110	67	Acinar adenocarcinoma (GS 3+3)	pT3a cN0cM0	HLP	No
111	84	Acinar adenocarcinoma (GS 4+5)	cT3-4 cN0cM1	Hypertension, vertigo	No
112	65	Acinar adenocarcinoma (GS 3+4)	pT3a cN0cM0	IHD, HHD, DM II., HLP	No
113	70	Acinar adenocarcinoma (GS 3+4)	pT3a cN0cM0	Hypertension, vertigo	No
114	84	Acinar adenocarcinoma (GS 5+3)	cT3-4 cN1cM1	CKD - hemodialyzed	No
115	65	Acinar adenocarcinoma (GS 3+3)	pT1c cN0cM0	CKD, HHD, PUD, anemia	Stop-smoker
116	68	High grade acinar adenocarcinoma (GS 5+4)	pT3b cN0cM0	Hypertension, overactive bladder	No
117	72	Acinar adenocarcinoma (GS /+/)	pT3b cN0cM0	Hypertension	No

HHD, hypertensive heart disease; HLP, hyperlipoproteinemia; IHD, ischemic heart disease; DM II, diabetes mellitus type 2; AFL, atrial flutter; VAS, vasculitis; CMP, cardiomyopathy; GIST, gastrointestinal stromal tumor; PAOD, peripheral artery occlusive disease; COPD, chronic obstructive pulmonary disease; AB, asthma bronchiale; A-Fib, atrial fibrillation; CKD, chronic kidney disease; PUD, peptic ulcer disease.

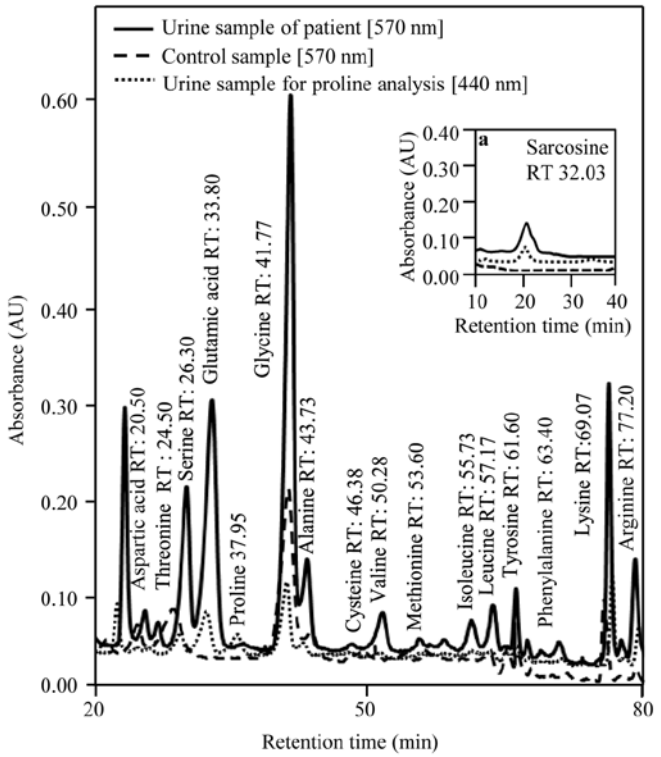

Figure 1. Overlay of chromatograms representing the urine amino acid profiles of — studied patients suffering from prostate cancer, – – control urine samples and ---- chosen patient samples analyzed at different wavelengths necessary for proline analysis. Increases in the amino acid content indicate the possible importance of amino acids in early CaP diagnosis. In inset (a), the results from the sarcosine analysis are indicated showing increased content of sarcosine in patient urine.

Table II. Overview of the amino acid content in the urine of 32 prostate cancer patients and 32 controls.

	Cancer patients		Healthy controls		
Amino acid	Mean (μmol/mmol)	SD	Mean (μmol/mmol)	SD	P-value
ASP	**3.07**	**2.69**	**0.74**	**2.28**	**0.000**
THR	**1.72**	**2.12**	**0.08**	**2.92**	**0.013**
SER	3.43	0.72	3.28	1.66	0.640
GLU	0.92	3.55	0.50	3.02	0.605
PRO	**4.79**	**1.96**	**0.31**	**0.55**	**0.000**
GLY	4.08	1.54	3.61	1.76	0.260
ALA	2.65	1.15	2.36	1.72	0.433
CYS	1.43	1.81	1.02	1.47	0.324
VAL	1.14	1.61	0.72	1.45	0.280
MET	**0.75**	**1.51**	**0.02**	**1.17**	**0.025**
ILE	**0.85**	**2.27**	**0.23**	**1.73**	**0.005**
LEU	**1.58**	**2.11**	**0.17**	**1.54**	**0.000**
TYR	**24.11**	**34.93**	**9.87**	**15.44**	**0.039**
PHE	9.16	25.50	20.70	83.89	0.459
HIS	68.17	70.99	49.72	68.92	0.296
LYS	32.82	67.23	18.62	34.28	0.291
ARG	**24.80**	**33.82**	**8.17**	**16.01**	**0.015**
SAR	**23.49**	**18.96**	**0.00**	**0.00**	**0.000**

Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Cys, cysteine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe: phenylalanine; His, histidine; Lys, lysine; Arg, arginine; Sar, sarcosine; SD, standard deviation. All values are related to creatinine content.

as means ± SD. Firstly, data were checked for normality using Shapiro-Wilk test. t-tests were used to analyze differences between cases and controls. To outline dependencies between variables, hierarchical clustering on normalized data was used. A P-value <0.05 was considered to indicate a statistically significant result. Statistica Software 10 (StatSoft, Inc., Tulsa, OK USA) was used for analyses.

Results and Discussion

Amino acid determination in the urine samples. The purpose of the first part of the present study was to investigate the amino acid content in the urine of patients suffering from prostate carcinoma and to compare the results with the urine samples of the controls obtained from healthy individuals. Previously, it was reported that metabolism of amino acids is perturbed in tumor cells (24,25), and urine amino acid profiles are consistently altered during tumor development (20,26,27).

Proline. As shown in Fig. 1, the content of most of the analyzed amino acids in the urine obtained from the CaP patients was considerably increased. Proline was absent in the control samples while found in all patient samples at relatively high amounts. Proline is the only secondary amino acid incorporated into protein. It functions with its own distinct metabolic system, responsive to special metabolic requirements (28). POX/PRODH, the first enzyme in proline catabolism, is induced by genotoxic (p53), inflammatory (PPARγ) (29) and nutrient stress (glucose deprivation) (30). Polyak and colleagues (31) used adenoviral-p53 expression with subsequent, extensive analysis of gene expression. They found that 14 out of 7,202 genes were induced more than 7-fold. Proline oxidase was one of them and was marked as p53-induced gene-6 (PIG6). Proline catabolism catalyzed by POX produces proline-dependent radical oxygen species (ROS) specifically superoxides, resulting in proline-dependent apoptosis with potential to serve as a novel mitochondrial tumor suppressor (32,33). In addition, Liu *et al* (34) showed that transcription factor MYC inhibits POX/PRODH expression and, thus, inhibits its function. microRNA miR-23b* an inhibitor of POX is highly expressed in different types of tumors (35,36). Due to the lack of POX, the conversion of proline to pyrroline-5-carboxylate (P5C) is altered (37), and subsequently the amount of proline in urine is increased. Based on these facts and the results showing increased levels of proline compared to controls, proline appears to be a biomolecule with the potential to enlarge the spectrum of diagnostic tools for CaP.

Sarcosine. The role of sarcosine as a potential biomarker of prostate carcinoma was confirmed. Sarcosine was determined in all patient urine samples (Fig. 1a). An elevated amount of sarcosine was probably caused by the overexpression of glycine

Table III. Overview of the biochemical parameters and levels of ions and pH in urine samples of 32 prostate cancer patients and 32 controls.

	Cancer patients		Healthy controls		
Parameter	Mean (μmol/mmol)	SD	Mean (μmol/mmol)	SD	P-value
K^+	**5.73**	**3.84**	**8.03**	**4.08**	**0.042**
Na^+	13.18	8.12	10.62	4.38	0.186
Cl^-	9.12	6.98	9.98	4.14	0.607
Uric acid	**15.44**	**57.95**	**0.26**	**0.10**	**0.026**
Urea	**38.66**	**18.42**	**23.79**	**12.26**	**0.002**
PSA	**4.93**	**7.52**	**0.00**	**0.00**	**0.000**
Glucose	0.05	0.13	0.02	0.02	0.341
Pyrogallol	0.08	0.21	0.00	0.00	0.081
fPSA	**17.46**	**2.12**	**0.00**	**0.00**	**0.000**
pH	6.05	0.75	6.49	0.32	0.058
Creatinine	**15.04**	**4.74**	**9.57**	**18.94**	**0.019**

Levels of K^+, Na^+, Cl^-, uric acid, urea, PSA, glucose, pyrogallol and fPSA were measured in mmol/mmol of creatinine; creatinine was measured in mmol•l^{-1}. Statistical significant differences between patients and controls were observed for levels of K^+ ions, uric acid, urea and creatinine and are indicated in bold print. SD, standard deviation. fPSA, free PSA.

N-methyl transferase (GNMT), cleaving glycine to sarcosine (23). Overexpression of GNMT, encoded by the *GNMT* gene, was previously observed in patients suffering from CaP (38). The expression of *GNMT* induced in this manner leads to elevated synthesis of GNMT that subsequently contributes to the regulation of the levels of S-adenosylmethionine (SAM), subsequently affecting the gene expression by influencing DNA methylation (38). The role of SAM is to transfer the methyl groups and to use them for formation of many essential compounds as creatine or phosphatidylcholine. It has been previously reported that the increased conflux of GNMT results in the elevated formation of sarcosine through increased utilization of SAM (39). The absence of sarcosine in control samples indicates that it is applicable for diagnosis, due to the reduction in false-positive or negative results (23) similar to proline.

Total amino acid content. Furthermore, the basic statistical comparison of amino acid content in the cases and controls was carried out, and the results are summarized in Table II. Values measured were recalculated to urinary creatinine concentration. From these means, standard deviations and P-values were calculated. All amino acids were significantly increased except for phenylalanine amounts.

Statistical significant results were observed for aspartic acid, threonine, methionine, isoleucine, leucine, tyrosine and arginine. Levels of sarcosine and proline in the controls were negligible (mean 0.31 μmol/mmol of creatinine for proline) or at zero (absent or below the limit of detection) for sarcosine. Levels of these biomolecules found in samples obtained from the patients included proline (4.79 μmol/mmol of creatinine) and sarcosine (23.49 μmol/mmol of creatinine) (Table II). These findings support possible utilization of these biomolecules for diagnosis. Relative standard deviation of proline (1.96%) indicated relatively similar values in all cases. In contrast, the relative standard deviations for sarcosine showed higher scatter (18.96%). This was probably caused by the different stages of carcinomas diagnosed in the patients. Levels of other amino acids were altered when compared to the control samples, supporting the general theory concerning the perturbation of tumor cell metabolism (24,25).

Biochemical parameters of the urine samples. Using various spectrophotometric methods, the concentrations of K^+, Na^+, Cl^-, uric acid, urea, PSA, glucose, total proteins (pyrogallol method), fPSA, creatinine and pH were measured simultaneously with the amino acids (Table III). All parameters were related to creatinine content and subjected to basic statistical analysis. Statistically significant differences between patients and controls were observed for levels of K^+ ions, uric acid, urea and creatinine. Serum PSA is currently the most widely used method for CaP diagnosis (40-42). Detecting cancer using low PSA values risks excessive unnecessary biopsies and the detection of clinically insignificant disease. Although PSA has high diagnostic value in the early diagnosis of CaP, there is a considerable overlap of PSA values between various stages of prostate cancer, and decreasing levels cannot be used to evaluate treatment efficacy in all patients (43). Based on this fact, it has been suggested that the ratio of fPSA/tPSA may improve the specificity of PSA for the diagnosis of CaP (44). As shown in Table III, the levels of both PSA and fPSA were below the detection limits in the controls. In patients, the levels were 4.93 μmol/mmol of creatinine and 17.46 μmol/mmol of creatinine for PSA and fPSA, respectively, with relative standard deviations of 7.52% for PSA and 2.12% for fPSA.

Androgen deprivation therapy (ADT) was found to delay disease progression in the management of advanced CaP. Nevertheless, the suppression of testosterone associated with ADT may often lead to hypogonadal conditions with harmful effects on renal function leading to acute kidney

	Asp	Thr	Ser	Gly	Pro	Glu	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Sar
Asp	**1.00**	**0.91**	**0.60**	**0.91**	**0.74**	**0.63**	**0.35**	**0.47**	**0.70**	**0.58**	**0.69**	**0.93**	**0.46**	**0.21**	**0.48**	**0.46**	**0.69**	*-0.07*
Thr	**0.91**	**1.00**	**0.75**	**0.85**	**0.53**	**0.67**	**0.43**	**0.51**	**0.69**	**0.67**	**0.73**	**0.86**	**0.50**	**0.34**	**0.55**	**0.51**	**0.59**	*-0.04*
Ser	**0.60**	**0.75**	**1.00**	**0.64**	0.17	**0.55**	**0.36**	**0.42**	**0.67**	**0.42**	**0.45**	**0.54**	0.15	**0.33**	**0.46**	**0.26**	**0.32**	0.03
Gly	**0.91**	**0.85**	**0.64**	**1.00**	**0.48**	**0.62**	**0.31**	**0.49**	**0.72**	**0.49**	**0.58**	**0.83**	**0.41**	**0.29**	**0.44**	**0.42**	**0.61**	*-0.35*
Pro	**0.74**	**0.53**	0.17	**0.48**	**1.00**	**0.42**	**0.21**	0.16	**0.37**	**0.34**	**0.53**	**0.73**	**0.34**	*-0.06*	**0.34**	**0.29**	**0.65**	**0.25**
Glu	**0.63**	**0.67**	**0.55**	**0.62**	**0.42**	**1.00**	0.03	**0.24**	**0.52**	**0.41**	**0.42**	**0.72**	**0.53**	**0.33**	**0.68**	**0.28**	**0.45**	*-0.10*
Ala	**0.35**	**0.43**	**0.36**	**0.31**	**0.21**	0.03	**1.00**	**0.50**	**0.46**	**0.67**	**0.40**	**0.36**	**0.35**	**0.24**	0.06	**0.46**	**0.43**	0.02
Cys	**0.47**	**0.51**	**0.42**	**0.49**	0.16	**0.24**	**0.50**	**1.00**	**0.56**	**0.66**	**0.58**	**0.51**	**0.44**	**0.31**	0.07	**0.54**	**0.42**	*-0.07*
Val	**0.70**	**0.69**	**0.67**	**0.72**	**0.37**	**0.52**	**0.46**	**0.56**	**1.00**	**0.55**	**0.68**	**0.67**	**0.29**	**0.24**	**0.49**	**0.25**	**0.49**	*-0.12*
Met	**0.58**	**0.67**	**0.42**	**0.49**	**0.34**	**0.41**	**0.67**	**0.66**	**0.55**	**1.00**	**0.57**	**0.59**	**0.60**	0.18	**0.29**	**0.58**	**0.50**	0.00
Ile	**0.69**	**0.73**	**0.45**	**0.58**	**0.53**	**0.42**	**0.40**	**0.58**	**0.68**	**0.57**	**1.00**	**0.76**	**0.44**	**0.27**	**0.41**	**0.44**	**0.58**	*-0.01*
Leu	**0.93**	**0.86**	**0.54**	**0.83**	**0.73**	**0.72**	**0.36**	**0.51**	**0.67**	**0.59**	**0.76**	**1.00**	**0.57**	**0.34**	**0.51**	**0.53**	**0.70**	*-0.07*
Tyr	**0.46**	**0.50**	0.15	**0.41**	**0.34**	**0.53**	**0.35**	**0.44**	**0.29**	**0.60**	**0.44**	**0.57**	**1.00**	**0.20**	0.18	**0.43**	**0.59**	*-0.06*
Phe	**0.21**	**0.34**	**0.33**	**0.29**	*-0.06*	**0.33**	**0.24**	**0.31**	**0.24**	0.18	**0.27**	**0.34**	**0.20**	**1.00**	0.11	**0.32**	**0.10**	*-0.10*
His	**0.48**	**0.55**	**0.46**	**0.44**	**0.34**	**0.68**	0.06	0.07	**0.49**	**0.29**	**0.41**	**0.51**	0.18	0.11	**1.00**	**0.32**	**0.34**	0.02
Lys	**0.46**	**0.51**	**0.26**	**0.42**	**0.29**	**0.28**	**0.46**	**0.54**	**0.25**	**0.58**	**0.44**	**0.53**	**0.43**	**0.32**	**0.32**	**1.00**	**0.59**	*-0.02*
Arg	**0.69**	**0.59**	**0.32**	**0.61**	**0.65**	**0.45**	**0.43**	**0.42**	**0.49**	**0.50**	**0.58**	**0.70**	**0.59**	0.10	**0.34**	**0.59**	**1.00**	*-0.04*
Sar	*0.07*	*-0.04*	0.03	*-0.35*	**0.25**	*-0.10*	0.02	*-0.07*	*-0.12*	0.00	*-0.01*	*-0.07*	*-0.06*	*-0.10*	0.02	*-0.02*	*-0.04*	**1.00**

Figure 2. Correlation between amino acid amounts, represented by their correlation coefficients. A positive correlation is indicated in bold print, a negative correlation with italics, insignificant correlation is indicated with normal font. Sarcosine was found to be negatively correlated with most of the amino acids. The most significant dependencies were observed between sarcosine and proline and glutamic acid.

injury (45,46). Deteriorated kidney tissue loses its capability to maintain its naturally functions resulting in disturbances in urine electrolytes (Table III). We found 5.73 μmol/mmol of creatinine of K^+ ions, and their concentrations in the controls were established at 8.03 μmol/mmol of creatinine. The downward trend observed in K^+ ions was also noted in Cl^- ions, but at a much lower level (mean 9.12 μmol/mmol of creatinine for patients compared to 9.98 μmol/mmol of creatinine measured in the controls). An opposite upward trend was observed in Na^+ ions (mean 13.18 μmol/mmol of creatinine in patients and 10.62 μmol/mmol of creatinine in controls). Levels of different ions in the urine of prostate cancer patients are not well understood, mainly due to problems regarding other associated health complications, significantly affecting the urine electrolyte composition, such as inflammation. Hence, these factors prevent their utilization as auxiliary diagnostic markers of CaP.

Uric acid is an important antioxidant and free radical scavenger formed in the body as a product of purine degradation. Several studies have reported that the uric acid level is depleted during tumor development (47,48). In contrast, uric acid may be increased due to cancer therapy, such as by chemotherapy or irradiation during treatment. Kolonel *et al* (50) carried out comprehensive analysis of different types of cancer (prostate, stomach, colorectal, lung, urinary bladder and leukemia). There were no significant associations between the type of cancer and uric acid level except for prostate cancer. In prostate carcinoma a positive association was found. Similarly we observed distinct differences in the content of uric acid (mean 15.44 μmol/mmol of creatinine in patients and 0.26 μmol/mmol of creatinine in controls; Table III). Our results confirmed the implication of uric acid in cancer pathogenesis and indicates good accessibility of uric acid as a possible additional diagnostic marker of CaP. This compound can be measured simply and at low costs, and with the possibility of method automation. In patients suffering from CaP, higher levels of urea are observed. Values of 38.66 μmol/mmol of creatinine for patients compared to controls (mean 23.79 μmol/mmol of creatinine; Table III) again indicate impaired functioning of the kidneys. Higher levels of urea excreted in the urine may potentially serve as a marker of acute kidney injury (AKI), commonly observed in patients undergoing anticancer therapy. Koyner *et al* (51) showed that fractional excretion of urea is not able to be used to detect a difference in AKI course, severity, and outcomes, but on the other hand may serve as an early detection marker of kidney injury, a frequent complication in CaP patients. It clearly follows from the results obtained that patients were affected by kidney function deterioration. For this reason we also noted higher levels of creatinine, a marker of the correct glomerular filtration rate of the kidney (52), ordinarily used for standardization of urine waste substances (mean 15.04 μmol/l in cases compared to 9.57 μmol/l in control samples; Table III).

For glucose content, only minimal differences with no statistical significance were found as well as in total proteins determined using pyrogallol red (Table III). Higher levels of proteins in urine (mean 0.08 μmol/mmol of creatinine compared to 0.00 μmol/mmol of creatinine in control samples) were probably caused by the presence of chronic inflammation accompanying prostate cancer. Inflammation has been proposed as one of the potential carcinogens for CaP. It was shown that inflammation may be found in prostate biopsy tissues, prostatectomy specimens, and chips from transurethral resection of the prostate (53). Minimal differences were observed also in the pH of the urine, but generally urine samples from patients with prostate carcinoma showed

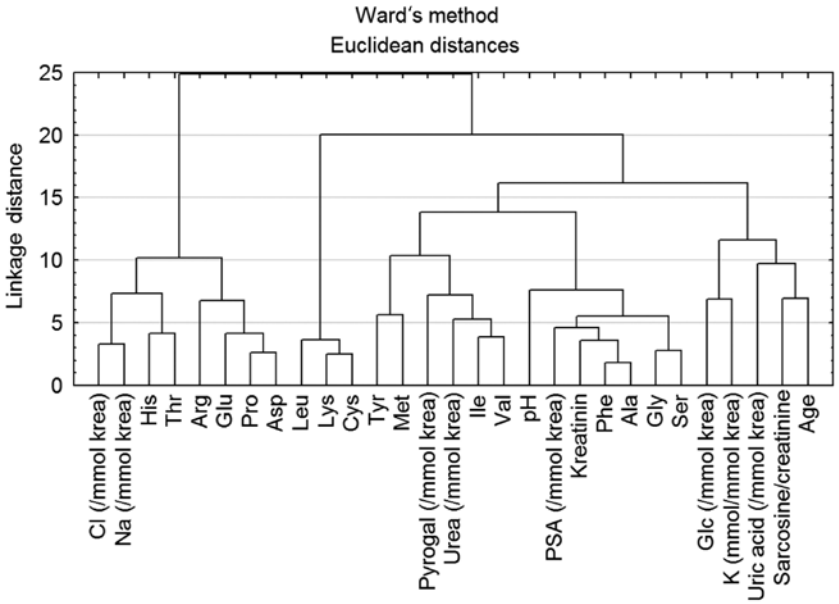

Figure 3. Dendrogram showing the cluster analysis between the different parameters. Note closely clustered sarcosine with age and Na^+ with Cl^-.

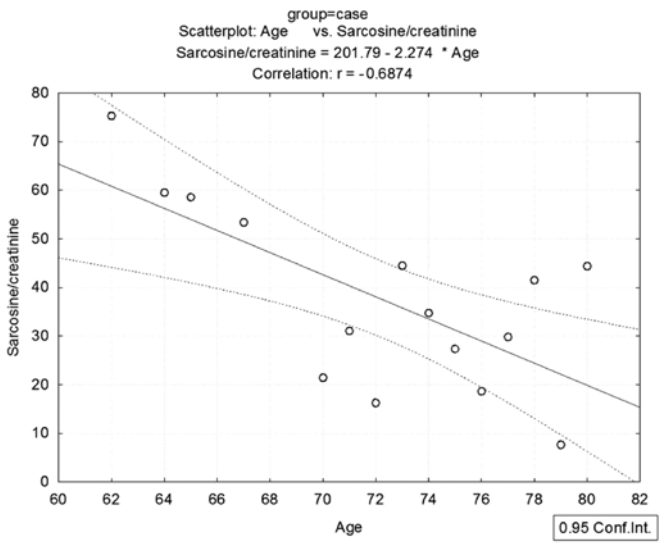

Figure 4. Evidence of the sarcosine dependence on age as indicated from the dendrogram from the cluster analysis. The negative correlation between age and sarcosine is indicated.

lower pH values (mean 6.05) when compared with the control samples (mean 6.49). Slightly acidic pH corresponds with higher levels of proteins and uric acid; nevertheless, this value is still within the physiologic range (54).

Furthermore, we carried out a correlation analysis for each amino acid determined in the urine samples from the CaP patients. From the values obtained from previous measurements, the correlation coefficients were prepared and subsequently compared to each other (Fig. 2). A positive correlation is highlighted in bold print, negative in italics, no correlation in normal font. As shown in Fig. 2, for sarcosine, instead of serine, alanine, methionine and histidine, negative correlations were observed. In contrast, when comparing sarcosine with proline, a positive correlation was noted. Negative correlations indicate an increased sarcosine amount in urine in comparison with other amino acids. The only negative correlation with statistical significance was observed in the case of glycine. On the biochemical basis of sarcosine formation during prostate carcinoma development this phenomenon is caused due to glycine degradation at the expense of sarcosine creation. Montrose *et al* (55) previously confirmed that within tumor tissue, sarcosine dehydrogenase (SARDH), the enzyme which converts sarcosine to glycine, is dysregulated. On the other hand, enzymes generating sarcosine from glycine, glycine *N*-methyl transferase (GNMT) and dimethylglycine dehydrogenase (DMGDH) are elevated in CaP patients (56). This process may explain the increases in sarcosine levels in urine. A high positive correlation of proline was probably random and caused by high increases in proline levels in the patients when compared to sarcosine.

Cluster analysis of all measured urine parameters. For revealing the correlation between all parameters, Ward's method of hierarchical cluster analysis was carried out (Fig. 3). Because of the different units for each parameter, the data were standardized to average zero and standard deviation 1. Due to this fact the dimensionless y-axis - linkage distance was used. From the dendrogram, two main dependencies may be observed. First one is the dependence of Na^+ and Cl^- ions. As mentioned above, the concentration of ions in urine electrolytes is highly influenced by kidney conditions and associated diseases. Due to this fact, the correlation of these two parameters was difficult to evaluate. The dependence of sarcosine on age was more significant (Fig. 3). Although the significant dependence was evident, in order to obtain more detailed insight into this correlation it was necessary to perform a correlation test. Statistical analysis revealed a negative dependence between sarcosine and age (r=-0.068) (Fig. 4). Sarcosine was originally proven to be a mechanistic biomarker of mainly aggressive prostate cancer (19). In accordance with this fact, levels of sarcosine are decreased in elderly patients who have a higher

probability to suffer from non-aggressive prostate carcinoma with a lower ability to produce sarcosine due to dysregulation of the enzymes producing and catabolizing sarcosine (57). The current hypothesis is that most aged men have prostate cancer, and they have cells in their prostates that if observed on a needle biopsy would be diagnosed as prostate cancer (58). This fact is supported by the study of Powell *et al* (59) showing that prostate carcinoma can be found in 50% of males 50 years of age, and 70-80% of men over the age of 70 years, dying from non-prostate cancer-related causes. These lesions are called 'microscopic' or 'latent' foci of prostate cancer, typical of their small size, non-progressiveness, clinically insignificance and rare detection through routine prostate cancer screening. These properties are fundamentally different from aggressive tumors in most cases detected in younger men producing more sarcosine when compared to the non-aggressive forms of the tumors.

In conclusion, in the present study various urine parameters were compared between patients suffering from prostate carcinoma and healthy individuals. Our results indicate that urine sarcosine, proline, uric acid and PSA may serve as a set of non-invasive, rapid, screening panel for CaP examination. Searching for new non-invasive markers of prostate carcinoma is still a great challenge for researchers. PSA achieves relatively excellent results, but specificity could be enhanced. For this reason we searched for correlations between well-known substances with potential for routine analysis in urine samples. We found one profile that may be utilized with relatively high meaningful evaluation from urine samples. This profile includes analysis of the widely studied amino acid sarcosine, amino acid proline, PSA and uric acid. Higher levels of these substances were not found in control samples obtained from health individuals, but were greatly increased in the samples from CaP patients. Inclusion of these analytes to a test panel could increase the specificity of prostate carcinoma diagnosis. Other parameters such as urea, K^+ ions or other amino acids were also altered, but their presence in control samples hindered their utilization for diagnosis. Moreover, their increased levels may rather indicate kidney injury following treatment. Analysis of the above mentioned substances with potential to serve as non-invasive biomarkers can be achieved at a relatively low cost, but the utilization in clinical practice requires examination of a larger cohort of patients.

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