Sona Krizkova[1]
Marketa Ryvolova[1]
Jaromir Gumulec[2]
Michal Masarik[2]
Vojtech Adam[1]
Petr Majzlik[1]
Jaromir Hubalek[3]
Ivo Provaznik[4]
Rene Kizek[1]

[1]Department of Chemistry and Biochemistry, Mendel University in Brno, Brno, Czech Republic
[2]Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3]Department of Microelectronics, Brno University of Technology, Brno, Czech Republic
[4]Department of Biomedical Engineering, Faculty of Electrical Engineering and Communication, Brno University of Technology, Brno, Czech Republic

Received October 6, 2010
Revised November 29, 2010
Accepted December 17, 2010

Research Article

Electrophoretic fingerprint metallothionein analysis as a potential prostate cancer biomarker

Prostate-specific antigen (PSA) is a routinely used marker of prostate cancer; however, the cut-off values for unambiguous positive/negative prostate cancer diagnoses are not defined. Therefore, despite the best effort, certain percentage of misdiagnosed cases is being recorded every year. For this reason, search for more specific diagnostic markers is of great interest. In this study, systematic comparison of PSA and metallothionein (MT) levels in blood serum of 46 prostate cancer-diagnosed patients is presented. It is clearly demonstrated that PSA levels vary significantly and despite normal total PSA values in the range of 0 – 4 ng/mL were obtained in over 36.9% of cases, positive prostate cancer was diagnosed by biopsy. In contrary, MT levels were considerably elevated in all tested samples and no significant variations were observed. These results are indicating the potential of MT as an additional prostate cancer marker reducing, in combination with PSA, the probability of false positive/negative diagnosis. To increase the throughput of the screening, chip-based capillary electrophoresis was suggested as a rapid and effective method for the fingerprinting analysis of prostate cancer from diseased blood sera.

Keywords:
Blood marker / Glutathione / Metallothionein / Prostate cancer / Prostate-specific antigen
DOI 10.1002/elps.201000519

1 Introduction

Prostate cancer (CaP) is the second most frequently diagnosed cancer in developed countries and the third most common cancer causing death in men. Two large trials have been conducted to determine the impact of the preventive screening on the treatment success. The PLCO (prostate, lung, colorectal, and ovarian cancer screening) Trial – USA, 76 693 men, 55–74 y of age – did not observe a reduction in prostate cancer deaths in the group invited for screening; however, the ERSPC (European Randomized Study of Screening for Prostate Cancer) Trial – Europe, 182 000 men aged from 50 to 74 y – observed a statistically significant 20% reduction in prostate cancer deaths. Although the results of trials differ due to the variations in studied population and protocols, it can be generally stated that early detection of CaP significantly increases the treatment success [1, 2]. Prostate biopsy is the most widely accepted method to diagnose CaP. There are several indications for prostate biopsy including an abnormal digital rectal examination (DRE) or serum prostate-specific antigen (PSA) level elevation. PSA is produced by prostate epithelial cells and belongs to the family of human kallikreins; it is a 33-kDa glycoprotein with neutral serine protease activity. Several isoforms of PSA with p*I* in the range 6.8–7.2 are known [3]. It is present especially in acinar lumen and semen, where its concentration is very high (0.2–0.5 mg/mL). To get into the blood stream, PSA must take the prostatic lumen – capillary blood barrier, which consists from prostatic basal membrane, stroma, capillary basal membrane, and capillary endothelial cell. In serum PSA exists in two forms – free (fPSA, 5 – 50% of detectable PSA) and bound to α1-antichymotrypsin and α2-macroglobulin (50 – 90% detectable PSA). Serum PSA levels have been widely used for diagnostic purposes; however, the false-positive and false-negative results can be obtained. Although using PSA in combination with DRE improved detection and CaP diagnosis, still more than 40% of the tumors are detected in advanced local or metastatic stage [4]. Up to now, the generally accepted upper cut-off level of 4.0 ng/mL tPSA was considered to be normal and levels above this value suggested biopsy [5]; however, a number of studies demonstrating the insufficiency of this information

Correspondence: Dr. Rene Kizek, Department of Chemistry and Biochemistry, Mendel University, Czech Republic
E-mail: kizek@sci.muni.cz
Fax: +420-5-4521-2044

Abbreviations: DRE, digital rectal examination; **fPSA,** free PSA; **MT,** metallothionein; **NC,** non-reducing; **CaP,** prostate cancer; **PSA,** prostate-specific antigen; **RC,** reducing; **tPSA,** total PSA

Colour Online: See the articles online to view Figs. 2-4 in colour.

were published [6–10]. In the range from 4 to 10 ng/mL (so-called 'grey zone'), the most false-positive/negative results are obtained. Nearly, 75% of men with tPSA level in this range indicated for biopsy do not suffer from CaP [11]. Increased tPSA level in serum may be caused by various reasons including benign prostatic hyperplasia, prostatitis, and urethral manipulations [12]. Conversely, there is also a significant number of diagnosed prostate carcinomas with a tPSA below 4 ng/mL (estimated at 20–30%) resulting in undiagnosed disease [13]. tPSA has also been found to be linked to body mass index [14, 15] and other factors such as race [16, 17], prostatic volume [18, 19], sexual [20],] and physical activities [21]. One of the most important factors influencing the level of serum tPSA is the age of the patient [22]. In the work of Anderson et al. normal level of tPSA 1.5 ng/mL in the age group 40–49 y, 2.5 ng/mL for the group 50–59 y, 4.5 ng/mL for 60–69 y and in the group 70–79 y up to 7.5 ng/mL was determined [23].

The problem of tPSA test unreliability is being continuously extensively monitored and reviewed [11, 22, 24–27]. Owing to the variables influencing the diagnosis, more precise biomarkers are required to improve the determination. In 2004 Tricoli et al. [28] summarized prostatic cancer markers and 91 molecular compounds that display some level of correlation with prostate cancer presence, disease progression, cancer recurrence, prediction of response to therapy, and/or disease-free survival. Probably, the most commonly used are sarcosine [29], α-methyl-acyl-CoA racemase [30], and metallothionein [31–33]. Metallothioneins (MTs) are low-molecular-mass intracellular proteins rich in cysteine, which are able to bind heavy metals. Based on their high affinity for metal ions, homeostasis of heavy metal ions is probably MT's most important biological function. MT can also serve as ''maintainers'' of the redox pool of a cell. In mammals, MT has been found to be involved in apoptosis, immunomodulation, transcription regulation, cell proliferation, and activation of enzymes via administration of zinc atoms to the proteins. MT is studied as a marker for various cancer types [34].

Prostatic tissue is a very specific in Zn^{2+} metabolism. Its cells accumulate up to tenfold higher quantity of these ions in comparison with other tissues. Prostate cancer is characterized by distinct and not very clear mechanisms, which serve to maintain the level of Zn^{2+}. These unusual metabolizing of Zn^{2+} has two important consequences: the share on pathogenesis of the disease (due to apoptosis) and increased serum levels of thiol proteins, e.g. MTs (with potential use of these proteins as tumor markers of this disease) [35]. During the last decade there have been published several papers on improving Brdicka reaction, its automation and revealing of the mechanism of the reaction [36–39]. Moreover, the method has been successfully employed for detection of MT in samples from patients with various tumor diseases [36, 37, 40–44]. In all studies, the MT concentration in patient serum is increased 5–6 folds [40, 43, 45–47].

The aim of this article was to determine MT and PSA (tPSA and fPSA) levels as well as content of other biologically active compound such as glutathione in serum of men diagnosed with CaP. Also, the application of MT as an additional CaP marker increases the reliability of diagnosis without an invasive examination suggested.

2 Materials and methods

2.1 Group characteristic

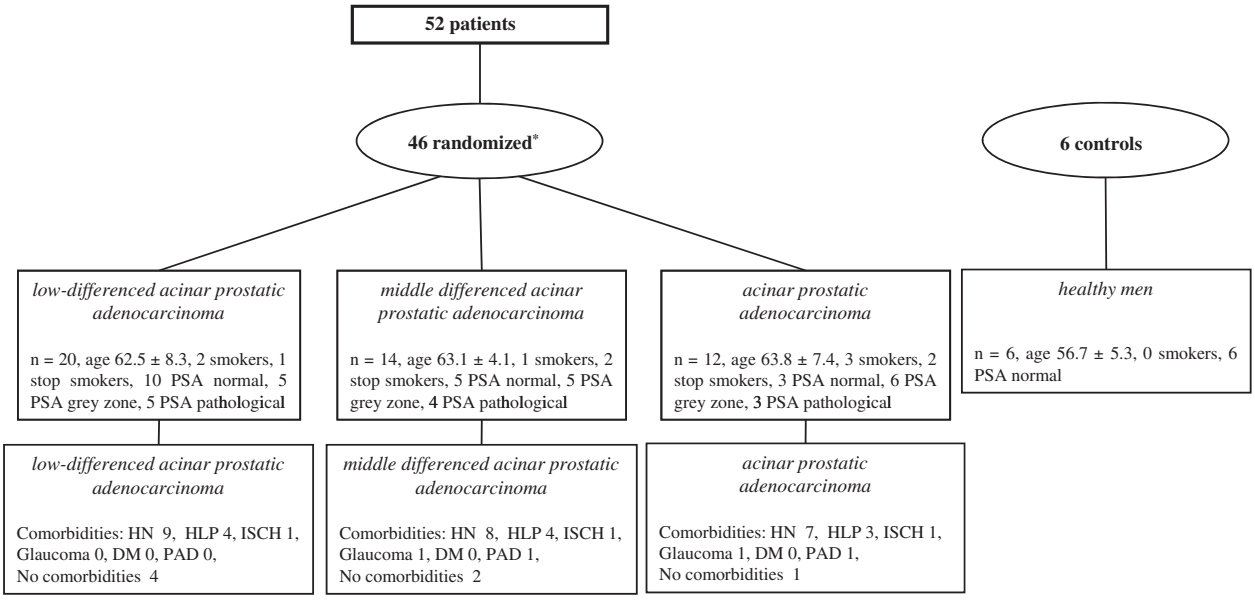
A set of 46 patients and 6 controls has been used for this pilot study. For the structure of the studied set, see Fig. 1. The group of patients underwent (in chronological order) a DRE, serum tPSA determination, and transrectal needle-core biopsy. DRE was positive in cca one-quarter of the patients and therefore indicated this group for further diagnostic procedures. Bioptic and histological examination was performed at the Department of Pathological Physiology, Faculty of Medicine, Masaryk University. Age of patients ranged from 48 to 78 y with an average of 62.7 y. Histologically, all patients had acinar adenocarcinoma of varying degrees of differentiation [48]. None of the patients had metastases in local lymph nodes or in distant lymph nodes, bones, or in another location. Subsequently, two months after the histological confirmation the blood samples were drawn and processed within 2 h and preserved deep-frozen at −80°C. When taking samples, none of the patients suffered from any infectious disease, or acute ongoing inflammatory process. The samples were primarily intended for routine biochemical tests at the Department of Pathological Physiology, Faculty of Medicine, Masaryk University. Serum was separated by centrifugation at $4000 \times g$ for 10 min. For further investigations only sera non-used for routine biochemical tests were used. The samples were stored in −80°C until assayed. Collection of the samples was approved by the Ethical commission of Faculty Hospital Brno.

2.2 Sample preparation for electrochemical analysis

The samples of blood serum were denatured at 99°C in a thermomixer (Eppendorf 5430, Germany) for 15 min with shaking and centrifuged at 4°C, $15\,000 \times g$ for 30 min. (Eppendorf 5402). Heat treatment effectively denatures and removes thermolabile and high-molecular-weight proteins from samples [49]. The prepared samples were used for all MT and GSH analyses.

2.3 Differential pulse voltammetry – Brdicka reaction

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to



*6 patients were excluded due to lack of medicinal information

HLP – Hyperlipidemia
HN - Hypertension
IHD - Ischaemic heart disease
DM - Diabetes mellitus
PUD - Peptic ulcer disease
PAD - Peripheral artery disease
ICHDK - Peripheral artery occlusive disease

Figure 1. Characteristics of entire studied cohort. *The samples were excluded due to lack of information.

746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4°C). A hanging mercury drop electrode was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and glassy carbon electrode was the auxiliary. For data processing GPES 4.9 supplied by EcoChemie was employed. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)+NH_4Cl$, pH = 9.6) was used. The parameters of the measurement were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV, $E_{ads} = 0$ V. All experiments were carried out at temperature 4°C employing thermostat Julabo F25 (Labortechnik GmbH, Germany) [37, 50].

2.4 Determination of low-molecular-mass thiols

High-performance liquid chromatography with electrochemical detection (HPLC-ED) system consisted of two solvent delivery pumps (Model 582 ESA, Chelmsford, MA, USA), Zorbax Eclipse AAA Column (4.6 × 150 mm 3.5-μm particle size; Varian, CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The sample (30 μL) was injected using autosampler (Model 540 Microtiter HPLC, ESA, USA). HPLC-ED experimental conditions were as follows – mobile-phase compositions: A: 80 mmol/L trifluoroacetic acid and B: methanol which were mixed in the gradient from 3% B in 1st min, 10% B in 2nd–6th min and 98% B from the 7th min of the separation; flow of the mobile phase was 0.8 mL/min, temperature of the separation was 40°C; working electrodes potential was 900 mV; detector temperature was 30°C; each measurement was repeated three times. Retention time of the reduced glutathione (GSH) was 5 min. GSH concentration was calculated from a calibration curve (0.5–100 μM). The signal was quantified as a sum of current responses from all working electrodes [51, 52]. In the case of real sample measurements, the shift of the retention time was about ±2%.

2.5 Total protein quantification – Biuret method

Total protein content in the samples was determined using the automated chemical analyzer BS-200 (Mindray, China). Reagent and sample handling was controlled by BS-200 software (Mindray). The commercially available kit from Diagon (Hungary) was used. The standard calibration curve was prepared from BSA (bovine serum albumin) by diluting from stock solution at a concentration of 1 mg/mL within the concentration range from 0.97 to 1000 μg/mL in 0.1 M

phosphate buffer pH 7.0. To the 180 μL of the Biuret kit solution 45 μL of the sample was added. The solution was mixed and incubated for 10 min at 37°C. The absorbance was measured at 510 nm.

2.6 tPSA and fPSA determination

Total PSA and fPSA was determined by immunochemical analyzer AIA 600 II (Tosoh, Japan). AIA 600 II is specifically designed for measurement of immunochemical parameters in biological fluids using reagents of AIA-PACK series. Analyses were carried out according to the manufacturer's instructions. The instrument was calibrated using AIA-PACK Calibrator set using 6-point calibration (Tosoh). All reactions are performed in the special disposable test cups containing dried and lyophilized reagents. Immunochemical antigen–antibody reaction is employing magnetic particles (1.5 mm). Samples are incubated at 37°C. 4-Methylumbelliferyl phosphate is used as a substrate and fluorescence corresponding to enzymatic activity on magnetic particles is measured.

2.7 Capillary chip electrophoresis

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA) were carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad). A sample (4 μL) was mixed either with 2 μL of non-reducing (NC) sample buffer or 2 μL of reducing (RC) sample buffer (30 μL of NC sample buffer and 1 μL β-mercaptoethanol), and after 4 min boiling, 84 μL of water was added. After the priming of the chip with the gel and gel-staining solution in the diluted priming station sample, the mixture (6 μL) was loaded into the sample wells. The Pro260 Ladder included in the kit was used as a standard. For operation and standard data analysis Experion software v. 3.10 (Bio-Rad) was used.

2.8 SDS-PAGE and Western blotting

The electrophoresis was performed according to Laemmli [53] using a Mini Protean Tetra apparatus with gel dimension of 8.3×7.3 cm (Bio-Rad). First 15% m/V running, then 5% m/V stacking gel was poured. The gels were prepared from 30% m/V acrylamide stock solution with 1% m/V bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 or 30 min, respectively. Prior to analysis the samples were mixed with non-reduction sample buffer in a 2:1 ratio. The samples were boiled for 2 min, and then 4 μL of the sample was loaded onto a gel. For determination of the molecular mass, the protein ladder ''Precision plus protein standards'' from Biorad was used. The electrophoresis was run at 150 V for 1 h (Power Basic, Bio-Rad) in Tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine, and 3.5 mM SDS, pH = 8.3). Silver staining of the gels was performed according to Oakley et al. [54].

After the electrophoretic separation the proteins were transferred onto a PVDF membrane by using Biometra Fastblot apparatus (Biometra, Germany). PVDF membranes were activated by soaking in methanol for 30 s prior to blotting. Further, the membrane was equilibrated for 5 min in blotting buffer (12.5 mM Tris-base, 75 mM glycine, and 15% v/v methanol). The blotting sandwich was composed of three layers of filter paper soaked in blotting buffer, membrane, polyacrylamide gel, and additional three layers of soaked filter paper. The blotting was carried out for 1 h at a constant current of 0.9 mA for 1 cm^2 of the membrane. After the transfer, the membrane was blocked in 1% BSA in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4) for 30 min. The incubation with chicken primary antibody in dilution of 1:500 in PBS with 0.1% of BSA was carried out for 12 h at 4°C. After the three times repeated washing with PBS containing 0.05% v/v Tween-20 (PBS-T) for 5 min the membrane was incubated in the presence of secondary antibody (rabbit anti-chicken labelled with horseradish peroxidase, Sigma-Aldrich, USA in dilution 1:5000) for 1 h at room temperature. Then, the membrane was washed three times with PBS-T for 5 min and incubated with chromogenic substrate. Three chromogenic substrates for horseradish peroxidase were tested (0.4 mg mg/mL AEC – 3-aminoethyl-9-carbazole in 0.5 M acetate buffer with 0.1% H_2O_2, pH 5.5), after the adequate development the reaction was stopped by rinsing with water. The procedure was adopted from work of Krizkova et al. [42].

2.9 Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA), MATLAB and STATISTICA.CZ Version 8.0 (Czech Republic). The results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). Statistical significances of the differences were determined using STATISTICA.CZ. Differences with $p < 0.01$ were considered significant and were determined by using *T*-test, which was applied for means comparison.

The measured data were analyzed using clustering method with Gaussian mixture model. Gaussian mixture models form clusters by representing the probability density function of observed variables as a mixture of multivariate Gaussian densities. Data objects are fit to data using an expectation maximization algorithm, which assigns posterior probabilities to each component density with respect to each observation. Clusters are assigned by selecting the component that maximizes the posterior probability.

Gaussian mixture modeling uses an iterative algorithm that converges to a local optimum.

3 Results and discussion

3.1 Tested cohort characteristics

In our study, sera of 46 patients suffering from CaP were analyzed. Histologically, all patients had acinar adenocarcinoma of varying degrees of differentiation. The Gleason score ranged from 5–9, i.e. moderate to poorly differentiated tumors. Age of patients ranged from 48–78 y with an average of 62.7 y. Staging of the tumor ranged from 1c–4, i.e. bioptically detectable to large fixed or invading tumors. None of the patients had metastases in local lymph nodes or in distant lymph nodes, bones, or in another location. Following comorbidities typical for patient's age were found: hyperlipidemia, hypertension, ischaemic heart disease, diabetes mellitus, and peripheral artery disease. Only 15% of patients did not suffer from any comorbidity. For the structure of the studied set of patients, see Fig. 1.

3.2 Biomarker determination

3.2.1 PSA determination

The entire cohort had serum tPSA value before biopsy, ranging from 0.56 to 19.46 ng/mL with an average of 6.65 ng/mL and median 5.89 ng/mL Part of the patients was indicated for further examinations due to tPSA velocity higher than 0.5 ng/mL/year. Biopsy followed one month after elevated tPSA was detected. tPSA values obtained for the control group ranged from 0.30 to 1.29 ng/mL. Statistically processed results are shown in Fig. 2A. As demonstrated in the literature [55, 56], the ratio of fPSA and tPSA level may significantly improve the diagnosis. Therefore, fPSA was determined in the whole file to complement the levels of tPSA and MT. It was observed that average percentage fPSA (% fPSA) across the whole file was 15.4% fPSA and the value for control group was 35% fPSA.

3.2.2 Metallothionein determination

In the whole cohort MT level ranged from 1.59 to 2.70 μM with average and median of 2.12 and 2.07 μM, respectively. In the control group consisting of 6 men MT levels were in the range from 0.55 to 0.89 μM. This value corresponds to the literature value obtained for a group of healthy individuals (0.51 ± 0.2 μM) [47]. Statistically processed results are shown in Fig. 2B.

As shown in Fig. 2C, no direct correlation between tPSA and MT level was found; however, statistically significant difference between patients and controls has been observed. The Gaussian mixture model cluster method composed of two compact and well-defined clusters assigned to control and patient data (see Fig. 2C). Both clusters can be clearly distinguished by visual inspection. Formally, a simple manifold (line) can be easily put in 2D space to separate the clusters. Centroid (center of weights) of control data was [MT; tPSA] = [0.66; 0.68] (μM; ng/mL), centroid of patient

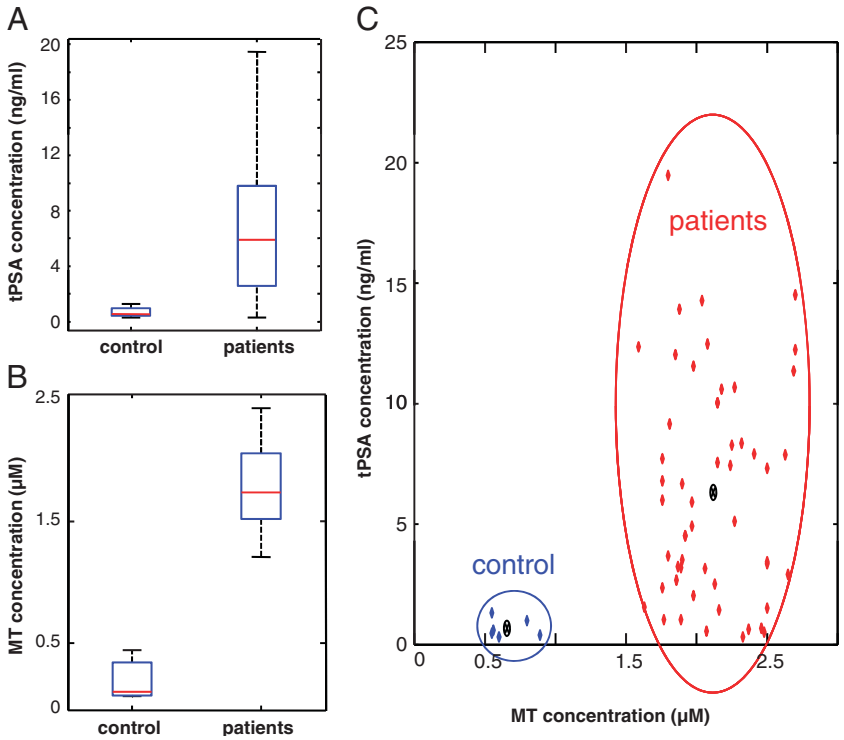


Figure 2. (A) Average value of tPSA for the entire cohort as well as for the control group (including standard deviation), (B) average value of MT for the entire cohort as well as for the control group (including standard deviation) and (C) statistical correlation between tPSA and MT levels.

data was [MT; tPSA] = [2.12; 6.31] (μM; ng/mL). Cluster centroids are marked by ⊗ sign in Fig. 2C. To support the finding, simple statistics was computed on MT and tPSA data. MT increased from 0.66 ± 0.15 μM to 2.12 ± 0.31 μM (see Fig. 2B) when tPSA increased from 0.68 ± 0.39 ng/mL to 6.31 ± 4.62 ng/mL (Fig. 2A). Further, two-tailed *t*-test of the null hypothesis that data in control and patient clusters are independent random samples from Gaussian distributions with equal means and unequal unknown population variances was performed against the alternative that the means are not equal. The hypothesis was rejected at 1% significance level ($p = 0.01$) for MT as well as tPSA.

According to the diagnostic criteria tPSA level in the range 0–4 ng/mL is considered to be physiological, tPSA level in 4–10 is considered to be suspect for CaP and level above 10 ng/mL is considered to be pathological with respect to age. Patients were divided into three main cohorts according to the tPSA level meeting the physiological, suspect and pathological values 0–4 ng/mL ($n = 17$), 4–10 ng/mL ($n = 16$) and >10 ng/mL ($n = 13$). In all groups CaP was diagnosed bioptically in various stages of differentiation (see Fig. 1). The average concentrations of studied analytes for each set are plotted in Fig. 3A. Although normal tPSA values were determined in first group, MT levels were significantly elevated ($p = 0.05$). False-negative result in this group clearly demonstrates the unreliability of tPSA as a CaP marker and the necessity of additional information. Especially in this group examination of MT level as a simple non-invasive method can help to disclose a false-negative diagnosis, which is essential for successful therapy. In grey zone, where the elevated tPSA levels can be attributed to the age, examination of MT level can resolve the diagnosis

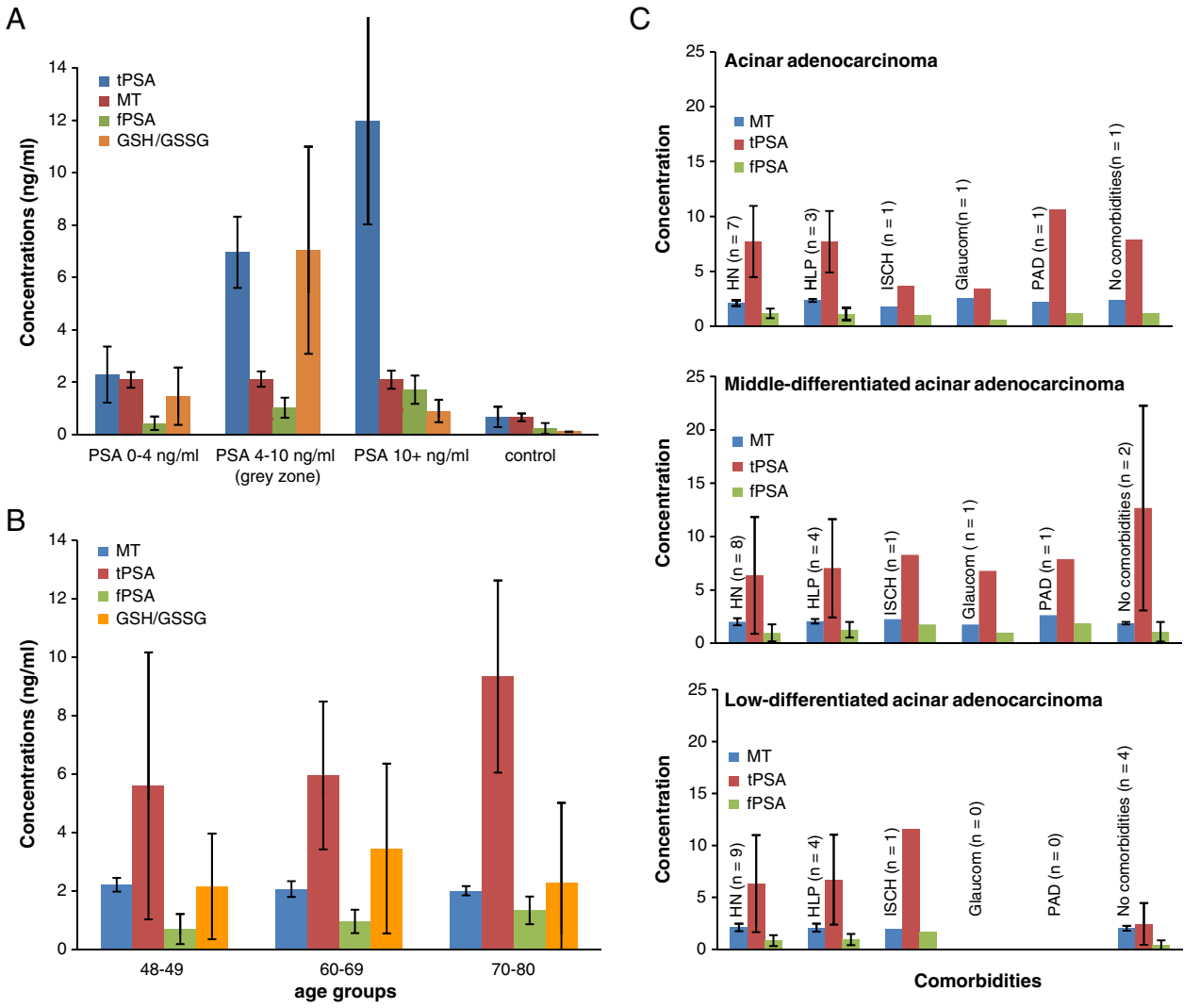


Figure 3. (A) Average concentrations of tPSA, MT, fPSA and GSH/GSSG ratio for the file divided into groups 0–4 ng/mL tPSA, 4–10 ng/mL tPSA and 10+ng/mL tPSA, (B) average values of tPSA, MT,fPSA and GSH/GSSG ratio grouped according to the age of patients, (C) relationship between concentration of tPSA, MT and fPSA and comorbidities in groups – acinar adenocarcinoma, low-differentiated acinar adenocarcinoma and middle-differentiated acinar adenocarcinoma. The error bars indicate variability of the obtained levels among the patients.

without the need for additional invasive examination. The significant difference between MT level of tested patients and control cohort leads to the conclusion that elevated MT value indicates certain health problem and in combination with tPSA as well as fPSA can considerably improve the diagnosis. As expected, tPSA and fPSA values are increasing with age (see Fig. 3B); however, the MT levels are age independent.

3.3 Influence of comorbidities

As mentioned earlier, number of comorbidities such as hyperlipidemia, hypertension, ischaemic heart disease, and peripheral artery disease were found in the tested group of patients. Only 15% of patients did not suffer from any comorbidity. In Fig. 3C patients are grouped according to the tumor stage – acinar adenocarcinoma, low-differentiated acinar adenocarcinoma and middle-differentiated acinar adenocarcinoma. In these groups levels of tested biomarkers for selected comorbidities are plotted. The significant variations of tPSA levels across the whole file complicate the diagnosis; however, it does not exhibit any systematic trend in dependence on age, smoking, comorbidities, and tumor differentiation. It is apparent that no definite conclusion can be made on the relationship between tPSA level and abovementioned factors due to the size of the studied file. On the other hand, MT levels were significantly elevated in the whole file, with no considerable variations, indicating its independency on the tested factors. No correlations with age, comorbidities, and surprisingly even with smoking were found.

3.4 Glutathione and other thiol compounds

Low-molecular-mass thiol compounds including glutathione (GSH) are maintaining the redox pool in the cell. Particularly, GSH plays a key role in cell homeostasis and it is considered as a marker of the oxidative damage. GSH serves as free-radical scavenger and helps regenerate other antioxidants such as ascorbic acid and vitamin E [57]. Augmented GSSG levels and GSSG/GSH ratio are often suggestive of a state of oxidative stress [58]. Low level of GSH, high level of GSSG, and a lower GSH/GSSG ratio have been found in blood from patients with various pathologies such as diabetes, HIV, and cancer [59]. It is known that cancer cells exhibit different levels of antioxidants and have more intensive metabolism, so shifted GSH and GSSG ratio can be expected [60–64].

Normal serum ratio GSH/GSSG is 9:1, lower value indicates oxidative stress [65–67]. In the tested group, significant oxidative stress was observed because 85% of patients had GSH/GSSG ratio even lower than 4.5:1. This observation is consistent with previously published articles [60–64]. The emergence of the oxidative stress and relations

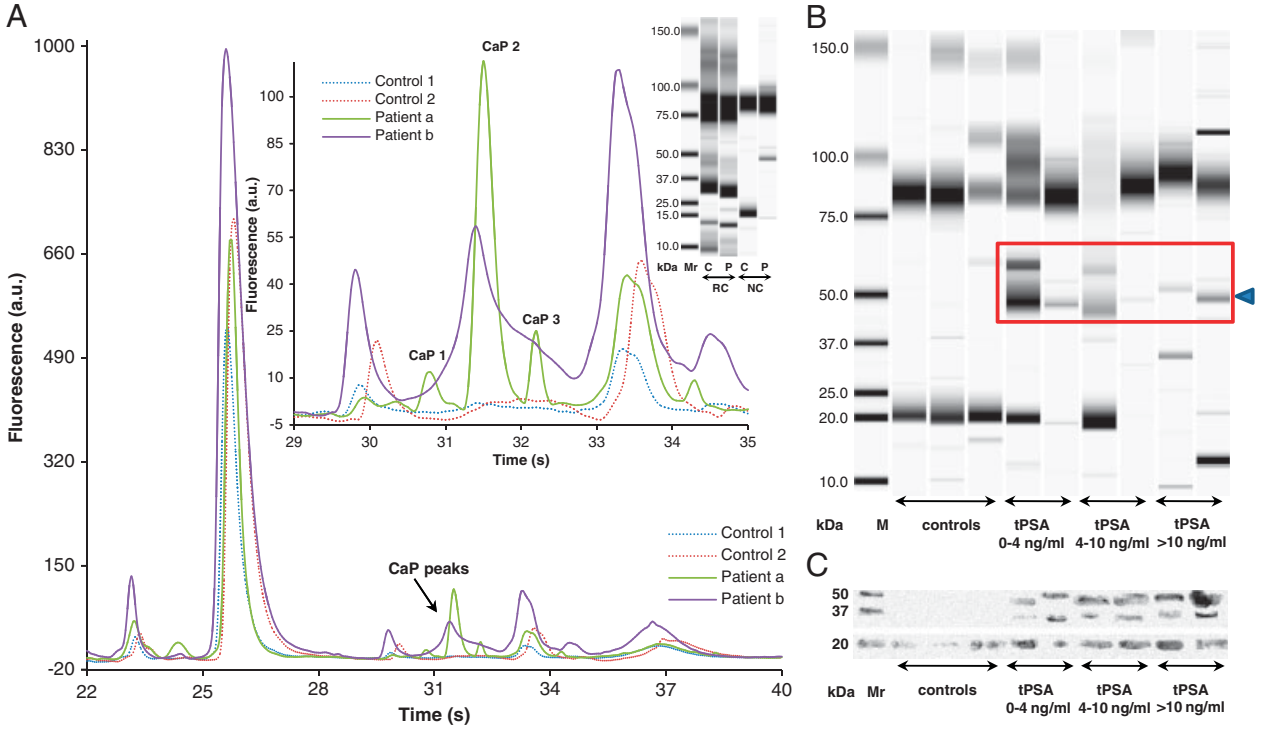


Figure 4. (A) Capillary chip electropherograms of blood sera from patients and control individuals, the arrow indicates the observed differences; arrow indicates position of CaP peaks, in inset: comparison of the samples under RC and NC conditions, detail of the RC electropherogram with significant differences indicated as CaP 1, 2, 3. (B) Virtual gel output from chip capillary electrophoretic system. Differences between patients and control samples are indicated with arrow. (C) Western blot of the analyzed sera.

Table 1. Results of the chip CE analysis compared with other analyses – clinical samples

Patient No.	Histology	Cap 1	Cap 2	Cap 3	CaP	tPSA	% fPSA	MT
23	D	0	1	1	d	+	+	+
25	D	1	0	1	d	–	+	+
10	D	1	1	0	d	?	+	+
20	D	1	1	0	d	–	–	+
49	D	1	1	0	d	+	+	+
1	D	1	1	1	d	?	+	+
2	D	1	1	1	d	?	+	+
8	D	1	1	1	d	–	+	+
9	D	1	1	1	d	?	+	+
11	D	1	1	1	d	+	+	+
40	D	1	1	1	d	+	+	+
51	D	1	1	1	d	?	+	+
38*	M	0	1	0	ND	?	–	+
27	M	0	1	1	d	–	+	+
36	M	0	1	1	d	?	+	+
47	M	0	1	1	d	–	+	+
21	M	1	0	1	d	–	+	+
52	M	1	0	1	d	?	+	+
12	M	1	1	0	d	?	+	+
16	M	1	1	0	d	+	+	+
34	M	1	1	0	d	+	+	+
45	M	1	1	0	d	+	+	+
15	M	1	1	1	d	?	+	+
29	M	1	1	1	d	?	+	+
37	M	1	1	1	d	–	+	+
48	M	1	1	1	d	+	+	+
19	L	0	0	1	ND	+	+	+
13*	L	0	1	0	ND	–	+	+
50	L	0	1	0	ND	?	+	+
7	L	0	1	1	d	?	+	+
22	L	0	1	1	d	+	+	+
28	L	0	1	1	d	–	+	+
31	L	0	1	1	d	–	+	+
33	L	0	1	1	d	–	+	+
3	L	1	0	1	d	–	+	+
14	L	1	0	1	d	–	+	+
18	L	1	0	1	d	+	+	+
35	L	1	0	1	d	?	+	+
30	L	1	1	0	d	–	+	+
32	L	1	1	0	d	–	+	+
39	L	1	1	0	d	–	+	+
46	L	1	1	0	d	–	–	+
24	L	1	1	1	d	+	+	+
26	L	1	1	1	d	?	+	+
42	L	1	1	1	d	–	+	+
44	L	1	1	1	d	?	+	+

Histology: D – differentiated CaP, M – middle differentiated CaP, L – low differentiated CaP, CaP peaks: 1 – peak present. 0 – peak missing. CaP: d – detected, ND – not detected. tPSA: – tPSA level below 4 ng/mL, ? – tPSA level between 4 and 10 ng/mL,+ – tPSA level above 10 ng/mL. fPSA:+ – % fPSA below 25, – – % fPSA above 25. MT:+ – MT level above 1 μM, – – MT level below 1 μM, * – low sample quality.

Table 2. Results of the chip CE analysis compared to other analyses – control samples

Subject No.	Cap 1	Cap 2	Cap 3	CaP	PSA	% fPSA	MT
1	0	1	0	ND	–	–	–
2	0	1	0	ND	–	–	–
3	0	1	0	ND	?	–	–
4	1	0	0	ND	–	–	–
5	0	1	0	ND	?	–	–
6	1	0	0	ND	–	–	–

CaP peaks: 1 – peak present. 0 – peak missing. CaP: d – detected, ND – not detected. tPSA: – – tPSA level below 4 ng/mL, ? – tPSA level between 4 and 10 ng/mL,+ – tPSA level above 10 ng/mL. fPSA:+ – % fPSA below 25, – – % fPSA above 25. MT:+ – MT level above 1 μM, – – MT level below 1 μM.

to the tumor diseases are not completely clarified and therefore additional research is required.

3.5 Fingerprinting analysis by chip-based CE

In the recent work, the aggregation of MT has been observed and the association with the tumor diseases is under investigation [68, 69]. Owing to the high level of oxidative stress, it can be assumed that MT aggregation could be promoted. This hypothesis was verified by capillary gel electrophoresis.

Capillary electrophoresis is due to the high separation efficiency, an effective tool for analysis of complex biological samples. Short time of analysis is beneficial for high-throughput analyses especially in clinical laboratories. Extremely low sample consumption is advantageous especially when limited sample volume is available. Furthermore, CE is exceptionally suitable for miniaturization and in chip-based arrangement the time of analysis is decreased even further to tens of seconds.

Identification of individual signals in complex samples including blood serum requires time-consuming sample preparation. In contrary, fingerprint analysis is an efficient method providing the essential information. Identification of particular peaks is not necessary, only differences in positive and control sample are needed to indicate the patient for the biopsy. Commercial Chip-CE instrument utilizing optimized capillary gel electrophoresis technique for determination of proteins was employed to analyze the blood sera of patients.

RC and NC conditions were tested. Figure 4A shows selected electropherograms of blood sera of patients with different PSA levels compared with the control blood sera. Under NC condition differences between control samples and patients were observable. The peaks in time range from 30.5 to 33 s were observed in all analyzed samples, corresponding to molecular masses from 45 to 58 kDa. The number of peaks in the samples ranged from 1 to 3 (CaP 1, CaP 2, and CaP 3 peaks), see inset in Fig. 4A. In controls, no peaks were present in this time range. Figure 4B provides

this information expressed as gel electrophoresis. In Fig. 4C Western blotting of selected samples were performed using chicken yolk antibodies and visualization with chromogenic substrate [42]. It is confirmed that observed signals in the range of molecular masses are reactive with the MT antibodies suggesting the structure of MT adduct or MT oligomers (MT di-, tri-, and pentamers). The presence of the observed peaks in analyzed samples in summarized in Tables 1 and 2. The data collected from patients ($n = 46$) were statistically compared with control group ($n = 6$) in three parameters represented by peaks at 42.9, 46.8, and 50.6 kDa. Two-tailed Fisher's exact test was used to consider statistical significance of the hypothesis that both groups differ in peak appearance. Fisher's exact test confirmed that patient and control group significantly differ in Cap 3 (50.6 kDa) peak appearance ($p < 0.05$). The test also confirmed that patient and control groups significantly differ in Cap 1 (42.9 kDa) peak appearance ($p < 0.1$). Cap 2 (46.8 kDa) peaks were not found to be a significant marker of any difference between groups. The data were further analyzed for concurrent presence of two arbitrary peaks to develop a simple marker. It has been found that two concurrent peaks were not present in any subject of the control group, while present in 91.3% of patients.

Although chip-CE analysis is not an unambiguous determination of CaP it may serve as a rapid and cost-effective indication of clinical changes in the organism and may lead to the further examination.

4 Concluding remarks

Blood serum samples of 46 patients with CaP diagnosed and confirmed by biopsy were tested. In this study it is clearly demonstrated that tPSA level vary significantly and despite normal tPSA values in the range of 0 – 4 ng/mL were obtained in over 36.9% of cases, positive prostate cancer was diagnosed by biopsy. In contrary, MT levels were considerably elevated in all tested samples comparing with control samples and no significant variations were observed. Obtained results demonstrate the potential of MT as an additional prostate cancer marker increasing reliability of determination and subsequently reducing the probability of false-positive/negative diagnosis. To increase the throughput of the screening, chip-based capillary electrophoresis was suggested as a rapid and effective method for the fingerprinting analysis of prostate cancer from diseased blood sera. Commercially available platform was successfully used.

The financial support from the grants GACR 301/09/P436, IGA MZ 10200-3, NANOSEMED GAAV KAN208130801, GACR 102/07/1473, GACR 102/09/H083, and MSM00216-30513 is highly acknowledged.

The authors have declared no conflict of interest.

5 References

[1] Smith, R. A., Cokkinides, V., Brooks, D., Saslow, D., Brawley, O. W., *CA – Cancer J. Clin.* 2010, *60*, 99–119.

[2] Terrell, J. D., Roehrborn, C. G., Wians, F. H., Karakiewicz, P. I., Shariat, S. F., *Lab Med.* 2008, *39*, 613–621.

[3] You, J., Cozzi, P., Walsh, B., Willcox, M., Kearsley, J., Russell, P., Li, Y., *Crit. Rev. Oncol./Hematol.* 2010, *73*, 10–22.

[4] Pound, C. R., Partin, A. W., Epstein, J. I., Walsh, P. C., *Urol. Clin. North Am.* 1997, *24*, 395–406.

[5] Canto, E. I., Slawin, K. M., *Ann. Rev. Med.* 2002, *53*, 355–368.

[6] Smith, D. S., Humphrey, P. A., Catalona, W. J., *Cancer* 1997, *80*, 1852–1856.

[7] Yan, Y., *Cancer* 2001, *92*, 776–780.

[8] Collette, L., Burzykowski, T., Schroder, F. H., *Eur. J. Cancer* 2006, *42*, 1344–1350.

[9] Thompson, I. M., Pauler, D. K., Goodman, P. J., Tangen, C. M., Lucia, M. S., Parnes, H. L., Minasian, L. M., Ford, L. G., Lippman, S. M., Crawford, E. D., Crowley, J. J., Coltman, C. A., *New Engl. J. Med.* 2004, *350*, 2239–2246.

[10] Loeb, S., Roehl, K. A., Antenor, J. A. V., Catalona, W. J., Suarez, B. K., Nadler, R. B., *Urology* 2006, *67*, 316–320.

[11] Jamaspishvili, T., Kral, M., Khomeriki, I., Student, V., Kolar, V., Bouchal, J., *Prostate Cancer Prostatic Dis.* 2010, *13*, 12–19.

[12] Thompson, I. M., Ankerst, D. P., Chi, C., Lucia, M. S., Lucia, M. S., Goodman, P. J., Crowley, J. J., Parnes, H. L., Coltman, C. A., *JAMA – J. Am. Med. Assoc.* 2005, *294*, 66–70.

[13] Schroder, F. H., van der Cruijsen-Koeter, I., de Koning, H. J., Vis, A. N.et al.,*J. Urol.* 2000, *163*, 806–811.

[14] Ohwaki, K., Endo, F., Muraishi, O., Hiramatsu, S., Yano, E., *Urology* 2010, *75*, 648–652.

[15] Rundle, A., Neugut, A. I., *Prostate* 2008, *68*, 373–380.

[16] Abdalla, I., Ray, P., Ray, V., Vaida, F., Vijayakumar, S., *Urology* 1998, *51*, 300–305.

[17] Oesterling, J. E., Kumamoto, Y., Tsukamoto, T., Girman, C. J., Guess, H. A., Masumori, N., Jacobsen, S. J., Lieber, M. M., *Br. J. Urol.* 1995, *75*, 347–353.

[18] Fowler, J. E., Bigler, S. S., Kilambi, N. K., Land, S. A., *Urology* 1999, *53*, 1175–1178.

[19] Partin, A. W., Catalona, W. J., Southwick, P. C., Subong, E. N. P., Gasior, G. H., Chan, D. W., *Urology* 1996, *48*, 55–61.

[20] Tchetgen, M. B., Song, J. T., Strawderman, M., Jacobsen, S. J., Oesterling, J. E., *Urology* 1996, *47*, 511–516.

[21] Oremek, G. M., Seiffert, U. B., *Clin. Chem.* 1996, *42*, 691–695.

[22] You, J. J., Cozzi, P., Walsh, B., Willcox, M., Kearsley, J., Russell, P., Li, Y., *Crit. Rev. Oncol. Hematol.* 2010, *73*, 10–22.

[23] Anderson, J. R., Strickland, D., Corbin, D., Byrnes, J. A., Zweiback, E., *Urology* 1995, *46*, 54–57.

[24] Makarov, D. V., Loeb, S., Getzenberg, R. H., Partin, A. W., *Ann. Rev. Med.* 2009, *60*, 139–151.

[25] Nogueira, L., Corradi, R., Eastham, J. A., *BJU Int.* 2010, *105*, 166–169.

[26] Punnen, S., Nam, R. K., *Surg. Oncol. – Oxford* 2009, *18*, 192–199.

[27] Sardana, G., Dowell, B., Diamandis, E. P., *Clin. Chem.* 2008, *54*, 1951–1960.

[28] Tricoli, J. V., Schoenfeldt, M., Conley, B. A., *Clin. Cancer Res.* 2004, *10*, 3943–3953.

[29] Sreekumar, A., Poisson, L. M., Rajendiran, T. M., Khan, A. P., Cao, Q., Yu, J. D., Laxman, B., Mehra, R., Lonigro, R. J., Li, Y., Nyati, M. K., Ahsan, A., Kalyana-Sundaram, S., Han, B., Cao, X. H., Byun, J., Omenn, G. S., Ghosh, D., Pennathur, S., Alexander, D. C., Berger, A., Shuster, J. R., Wei, J. T., Varambally, S., Beecher, C., Chinnaiyan, A. M., *Nature* 2009, *457*, 910–914.

[30] Rubin, M. A., Zhou, M., Dhanasekaran, S. M., Varambally, S., Barrette, T. R., Sanda, M. G., Pienta, K. J., Ghosh, D., Chinnaiyan, A. M., *JAMA – J. Am. Med. Assoc.* 2002, *287*, 1662–1670.

[31] Moussa, M., Kloth, D., Peers, G., Cherian, M. G., Frei, J. V., Chin, J. L., *Clin. Invest. Med. – Med. Clin. Et Exp.* 1997, *20*, 371–380.

[32] Zhang, X. H., Jin, L., Sakamoto, H., Takenaka, I., *J. Urol.* 1996, *156*, 1679–1681.

[33] Pedersen, M. O., Larsen, A., Stoltenberg, M., Penkowa, M., *Prog. Histochem. Cytochem.* 2009, *44*, 29–64.

[34] Eckschlager, T., Adam, V., Hrabeta, J., Figova, K., Kizek, R., *Curr. Protein Pept. Sci.* 2009, *10*, 360–375.

[35] Eckschlager, T., Adam, V., Hrabeta, J., Figova, K., Kizek, R., *Curr. Protein Pept. Sci.* 2009, *10*, 360–375.

[36] Adam, V., Fabrik, I., Eckschlager, T., Stiborova, M., Trnkova, I., Kizek, R., *TRAC – Trends Anal. Chem.* 2010, *29*, 409–418.

[37] Petrlova, J., Potesil, D., Mikelova, R., Blastik, O., Adam, V., Trnkova, L., Jelen, F., Prusa, R., Kukacka, J., Kizek, R., *Electrochim. Acta* 2006, *51*, 5112–5119.

[38] Raspor, B., *J. Electroanal. Chem.* 2001, *503*, 159–162.

[39] Krizkova, S., Blahova, P., Nakielna, J., Fabrik, I., Adam, V., Eckschlager, T., Beklova, M., Svobodova, Z., Horak, V., Kizek, R., *Electroanalysis* 2009, *21*, 2575–2583.

[40] Fabrik, I., Krizkova, S., Huska, D., Adam, V., Hubalek, J., Trnkova, L., Eckschlager, T., Kukacka, J., Prusa, R., Kizek, R., *Electroanalysis* 2008, *20*, 1521–1532.

[41] Kizek, R., Trnkova, L., Palecek, E., *Anal. Chem.* 2001, *73*, 4801–4807.

[42] Krizkova, S., Adam, V., Eckschlager, T., Kizek, R., *Electrophoresis* 2009, *30*, 3726–3735.

[43] Krizkova, S., Fabrik, I., Adam, V., Kukacka, J., Prusa, R., Chavis, G. J., Trnkova, L., Strnadel, J., Horak, V., Kizek, R., *Sensors* 2008, *8*, 3106–3122.

[44] Adam, V., Baloun, J., Fabrik, I., Trnkova, L., Kizek, R., *Sensors* 2008, *8*, 2293–2305.

[45] Adam, V., Blastik, O., Krizkova, S., Lubal, P., Kukacka, J., Prusa, R., Kizek, R., *Chemicke Listy* 2008, *102*, 51–58.

[46] Vajtr, D., Fabrik, I., Adam, V., Kukacka, J., Prusa, R., Kizek, R., *Tumor Biol.* 2008, *29*, 60–60.

[47] Krizkova, S. Masarik, M., Majzlik, P., Kukacka, J., Kruseova, J., Adam, V., Prusa, R., Eckschlager, T., Stiborova, M., Kizek, R., *Acta Biochim. Polon.* 2010, *57*, 561–566.

[48] Partin, A. W., Kattan, M. W., Subong, E. N. P., Walsh, P. C.et al.,*JAMA – J. Am. Med. Assoc.* 1997, *277*, 1445–1451.

[49] Erk, M., Ivankovic, D., Raspor, B., Pavicic, J., *Talanta* 2002, *57*, 1211–1218.

[50] Fabrik, I., Ruferova, Z., Hilscherova, K., Adam, V., Trnkova, L., Kizek, R., *Sensors* 2008, *8*, 4081–4094.

[51] Potesil, D., Petrlova, J., Adam, V., Vacek, J., Klejdus, B., Zehnalek, J., Trnkova, L., Havel, L., Kizek, R., *J. Chromatogr. A* 2005, *1084*, 134–144.

[52] Petrlova, J., Mikelova, R., Stejskal, K., Kleckerova, A., Zitka, O., Petrek, J., Havel, L., Zehnalek, J., Adam, V., Trnkova, L., Kizek, R., *J. Sep. Sci.* 2006, *29*, 1166–1173.

[53] Laemmli, U. K., *Nature* 1970, *227*, 680–685.

[54] Oakley, B. R., Kirsch, D. R., Morris, N. R., *Anal. Biochem.* 1980, *105*, 361–363.

[55] Jung, K., Brux, B., Lein, M., Rudolph, B., Kristiansen, G., Hauptmann, S., Schnorr, D., Loening, S. A., Sinha, P., *Clin. Chem.* 2000, *46*, 47–54.

[56] Naya, Y., Fritsche, H. A., Cheli, C. D., Stamey, T. A., Bartsch, G., Brawer, M. K., Childs, S., Taneja, S. S., Lepor, H., Partin, A. W., Sokoll, L. J., Chan, D. W., Babaian, R. J., *Urology* 2003, *62*, 1058–1062.

[57] Camera, E., Picardo, M., *J. Chromatogr. B – Anal. Technol. Biomed. Life Sci.* 2002, *781*, 181–206.

[58] Brigelius-Flohe, R., *Free Rad. Biol. Med.* 1999, *27*, 951–965.

[59] Rossi, R., Milzani, A., Dalle-Donne, I., Giustarini, D., Lusini, L., Colombo, R., Di Simplicio, P., *Clin. Chem.* 2002, *48*, 742–753.

[60] Carretero, J., Obrador, E., Anasagasti, M. J., Martin, J. J., Vidal-Vanaclocha, F., Estrela, J. M., *Clin. Exp. Metastasis* 1999, *17*, 567–574.

[61] Huang, Z. S., Komninou, D., Kleinman, W., Pinto, J. T., Gilhooly, E. M., Calcagnotto, A., Richie, J. P., *Int. J. Cancer* 2007, *120*, 1396–1401.

[62] Levy, R. D., Oosthuizen, M. M. J., Degiannis, E., Greyling, D., Hatzitheofilou, C., *Br. J. Cancer* 1999, *80*, 32–37.

[63] Lusini, L., Tripodi, S. A., Rossi, R., Giannerini, F., Giustarini, D., del Vecchio, M. T., Barbanti, G., Cintorino, M., Tosi, P., Di Simplicio, P., *Int. J. Cancer* 2001, *91*, 55–59.

[64] Saydam, N., Kirb, A., Demir, O., Hazan, E., Oto, O., Saydam, O., Guner, G., *Cancer Lett.* 1997, *119*, 13–19.

[65] Kobal, A. B., Prezelj, M., Horvat, M., Krsnik, M., Gibicar, D., Osredkar, J., *Environ. Res.* 2008, *107*, 115–123.

[66] Gougoura, S., Nikolaidis, M. G., Kostaropoulos, I. A., Jamurtas, A. Z., Koukoulis, G., Kouretas, D., *Eur. J. Appl. Physiol.* 2007, *100*, 235–239.

[67] Paolisso, G., Dimaro, G., Pizza, G., Damore, A., Sgambato, S., Tesauro, P., Varricchio, M., Donofrio, F., *Am. J. Physiol.* 1992, *263*, E435–E440.

[68] Krizkova, S., Masarik, M., Eckschlager, T., Adam, V., Kizek, R., *J. Chromatogr. A* 2010, *1217*, 7966–7971.

[69] Krizkova, S., Adam, V., Kizek, R., *Electrophoresis* 2009, *30*, 4029–4033.

