Engrailed-2 protein as a potential urinary prostate cancer biomarker: a comparison study before and after digital rectal examination

Michał Piotr Marszałł[a,*], Wiktor Sroka[a,*], Marek Adamowski[a], Piotr Słupski[c], Piotr Jarzemski[c], Joanna Siódmiak[b] and Grażyna Odrowąż-Sypniewska[b]

This study was designed to compare and evaluate the presence of engrailed-2 (EN2) protein in urine collected before and after prostate massage as a diagnostic marker for prostate cancer (PCa). We analysed and compared 76 urine samples (38 before and 38 after prostate massage) from the benign group (BPH) and 66 urine samples (33 before and 33 after prostate massage) from patients with PCa confirmed by prostate biopsy. EN2 levels from the PCa and men with BPH (age range 50–82) were related to the tumour stage, Gleason score and prostate-specific antigen. EN2 levels were determined by enzyme-linked immunosorbent assay in urine. The median EN2 levels in urine after prostate massage were significantly different from those determined in urine before prostate massage (1.25 ng/ml in the PCa group and 0.34 ng/ml in the BPH). The mean EN2 levels in PCa patients were 3.76-fold higher than those in non-PCa patients after prostate massage. The distinct influence of prostate massage on EN2 levels was found to be related to the Gleason score and tumour stage. EN2 may be considered a marker of PCa with certain limitations, such as those related to tumour staging. The specificity and sensitivity of the protocol are highly dependent on prostate massage. ***European Journal of Cancer Prevention*** **24:51–56 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

European Journal of Cancer Prevention 2015, **24**:51–56

Keywords: benign prostatic hyperplasia, biomarker, engrailed-2 protein, prostate cancer

Departments of [a]Medicinal Chemistry, [b]Laboratory Medicine, Collegium Medicum in Bydgoszcz and [c]Department of Urology, J. Biziel Hospital, Nicolaus Copernicus University, Bydgoszcz, Poland

Correspondence to Michał Piotr Marszałł, PhD, Department of Medicinal Chemistry, Collegium Medicum, Nicolaus Copernicus University, ul. Jurasza 2, 85-094 Bydgoszcz, Poland
Tel: +48 52 5853540; fax: +48 52 5853804; e-mail: mmars@cm.umk.pl

*Michał Piotr Marszałł and Wiktor Sroka contributed equally to the writing of this article.

Received 20 November 2013 **Accepted** 15 April 2014

Introduction

Metabolomic and proteomic profiling of endogenous low-molecular compounds and proteins, respectively, may be a way to detect early stages in carcinogenesis, predict cancer stage or monitor treatment response. Qualitative and quantitative analysis by metabolomic and proteomic techniques combined with advanced statistical approaches often enables the development of a metabolic or a proteomic profile that characterizes early changes in specific organ function in cancers. In urology, metabolomics has already been used for the interpretation of cancer-related patterns of the analysed profiles in urogenital cancers (Osl *et al.*, 2008; Kim *et al.*, 2009; Struck *et al.*, 2013). The serum prostate-specific antigen (serum PSA) assay and digital rectal examination (DRE) are used widely as screening tests and are the starting point for further workup. However, despite the various procedures utilizing PSA in the diagnosis of prostate cancer (PCa), such as free-to-total PSA ratio, PSA velocity, PSA density and age-specific ranges, all of them have limitations in clinical practice that prompt an ongoing search for new markers (Downes *et al.*, 2006). Although sarcosine was initially proposed as a potential noninvasive diagnostic and prognostic biomarker for PCa, the most common cancer in men and the leading cause of cancer-related deaths in the male population of the USA (Sreekumar *et al.*, 2009; Jiang *et al.*, 2010), further studies provided evidence that it was not a reliable biomarker of PCa invasiveness and aggressiveness (Fenner, 2011; Jentzmik *et al.*, 2011).

These negative data have intensified proteomic research to find new prostate markers to overcome the PSA-related limitations. Alpha-methylacyl-coenzyme A racemase (AMACR), basic human urinary arginine amidase (BHUAE), vascular endothelial growth factor (VEGF), annexin A3 (ANXA3) and prostatic inhibin-like peptide (PIP) are examples of new potential biomarkers for which no practical use has been found so far because of their low sensitivity and specificity. Recent years have witnessed a growing interest in engrailed-2 (EN2). Typically, EN2 is a homeodomain-containing transcription factor (Morgan, 2006). Being members of the Hox family, EN proteins are multiple regulatory factors at different stages of development, involving transcriptional and translational regulation (Shah and Sukumar, 2010). All the subgroups of EN proteins (EN1–EN5) play crucial roles in development, regulation of numerous processes including apoptosis, receptor signalling, differentiation, motility and angiogenesis. Also, aberrations in this subgroup's gene expression have been reported in

0959-8278 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CEJ.0000000000000046

abnormal development and malignancy, indicating that altered expression of the *Hox* genes could be meaningful for both oncogenesis and tumour suppression. Recent findings have shown that the *Hox* genes are involved in leukaemia, neuroblastoma and in breast, cervical, lung and ovarian cancers (Shah and Sukumar, 2010). The Hox proteins may also be involved in multiple growth factor pathways that contribute towards hormone-resistant PCa. Economides and Capecchi (2003) have shown that *HOXB13* is essential for the development of rat prostate. Moreover, exogenous overexpression of *HOXB13* in the PCa cell line (lnCaP) can suppress hormone-mediated transactivation of the androgen receptor and consequently prevent the growth of these cells (Jung *et al.*, 2004a, 2004b; Shah and Sukumar, 2010). In contrast, aberrant *Hox* expression may be associated with tumorigenesis (Miller *et al.*, 2003).

Most recently, a potential diagnostic application of EN2 transcription factor, which is subsequently re-expressed in PCa, has been reported (Morgan *et al.*, 2011). A statistically significant relationship between urinary EN2 level and PCa volume has been identified in an ongoing study (Pandha *et al.*, 2012). The aim of our study was to identify and quantify EN2 protein in voided urine and urine samples collected before and after DRE in patients with PCa and benign prostatic hyperplasia (BPH). We showed that EN2 levels determined in urine collected after digital rectal exam could be helpful in differentiating patients with PCa from those with BPH.

Materials and methods

Patients and sample collection

Biological samples were collected from patients qualified for radical prostatectomy (RP) or transurethral resection of the prostate at the Department of Urology, Jan Biziel University Hospital in Bydgoszcz, Poland. The patients were divided into two groups. The cancer group included patients diagnosed with PCa who qualified for RP and the BPH group included patients qualified for transurethral resection of the prostate with no evidence of malignancy, but diagnosed with benign prostate growth. All the diagnoses were confirmed by histopathological examination of the prostate removed from patients with cancer and by histopathological examination of tissues collected during resection in patients with BPH. Because of ethical concerns, concerns about the patients' health and fear of potential complications, the patients in the BPH group were not prostatectomized, which is why histopathological examination of the entire prostate was not performed. Patients with negative digital rectal exam, negative biopsy results and no malignancy in the resected tissue qualified for the BPH group. Prostate volume was determined by the pathologist in the cancer group and by transrectal ultrasound in the BPH group. Tumour volume was determined by visual inspection of the percentage of the specimens affected by cancer. All the histopathological procedures were performed according to the Protocol for the examination of specimens from patients with carcinoma of the prostate gland (Srigley *et al.*, 2009) and Key issues in handling and reporting radical prostatectomy specimens (Srigley, 2006). Because the aim of this study was also to determine whether prostate massage could affect the EN2 concentration, patients who had undergone prostate biopsy or other urological procedures within 1 month before hospital admission were excluded from participation in this study.

Two urine samples were collected from each patient. The first sample was collected in the early morning into a sterile urine container. The second sample was collected after prostate massage performed by a urologist. Prostate massage consisted of three sweeps per lobe, depressing the prostate (0.5–1 cm) in a milking action. Sodium azide solution was added to the samples to a final concentration of 1 mmol/l in urine. Samples were divided into smaller portions and stored immediately after finalizing the preparation protocol at −80°C until further analysis.

The study was approved by the local ethics committee (Collegium Medicum Ethics Committee, #KB66/2011). All patients signed documents for their voluntary participation in this study. The sampling was performed between January 2011 and June 2012.

Determination of EN2

A Human Homeobox protein engrailed-2 ELISA Kit (EIAab Human Homeobox protein engrailed-2 ELISA Kit, Wuhan EIAAB Science Co., LTD, Whuan, China, catalogue number: E1851h) was used for determination of the human EN2 protein in the urine. Ninety-six-well plates of an immunoassay kit were precoated with an antibody specific for the EN2 protein. All urine samples (100 µl) and standards were transferred to the wells with a biotin-conjugated polyclonal antibody specific for the EN2 protein. Avidin conjugated to horseradish peroxidase was added to each well and incubated. Enzyme–substrate reaction was terminated by the addition of sulphuric acid solution. The incubation time and temperature were followed rigorously according to the protocol provided by EIAab. Spectrophotometric measurements were performed using a microplate reader set to 450 nm. Serial dilutions were prepared from a 20 ng/ml stock solution and the standard curve range was from 0.31 to 20 ng/ml.

Statistical analysis

All statistical calculations and graphical presentation were performed using Statistica 10 (StatSoft, Tulsa, Oklahoma, USA) with a medical analysis package. For unpaired comparison of the cancer and the benign groups, the Mann–Whitney *U*-test was used. To test the significance of the differences between the EN2 level, pathological stage (pT2a, pT2b, or pT2c) and the tumour grade (Gleason score = 5, 6, or 7) in the cancer group obtained

before and after prostate massage, the Wilcoxon test was used. The receiver operating characteristic (ROC) curves were generated to determine the diagnostic value of EN2 and PSA.

Results

Overall, 66 urine samples (33 × 2) from cancer patients and 76 urine samples (38 × 2) from the benign group were analysed and compared. The group characteristics including age, serum PSA levels (mean, median), tumour volume and EN2 levels (mean, median) are presented in Table 1. A comparison of age between the two groups showed no relevant difference (mean age 62 and 65, respectively). The mean (3.86 ng/ml) and the median (3.47 ng/ml) values of serum PSA levels were, as expected, lower in the benign group than in the cancer group (7.74 and 6.33, respectively; $P < 0.001$). A total of 22 patients from the benign group had PSA less than 4 ng/ml, the PSA of 15 patients was between 4 and 10 ng/ml and one individual had PSA levels higher than 10 ng/ml. In the cancer group, three patients had PSA levels lower than 4 ng/ml, 26 patients had PSA levels between 4 and 10 ng/ml and four patients had PSA levels higher than 10 ng/ml.

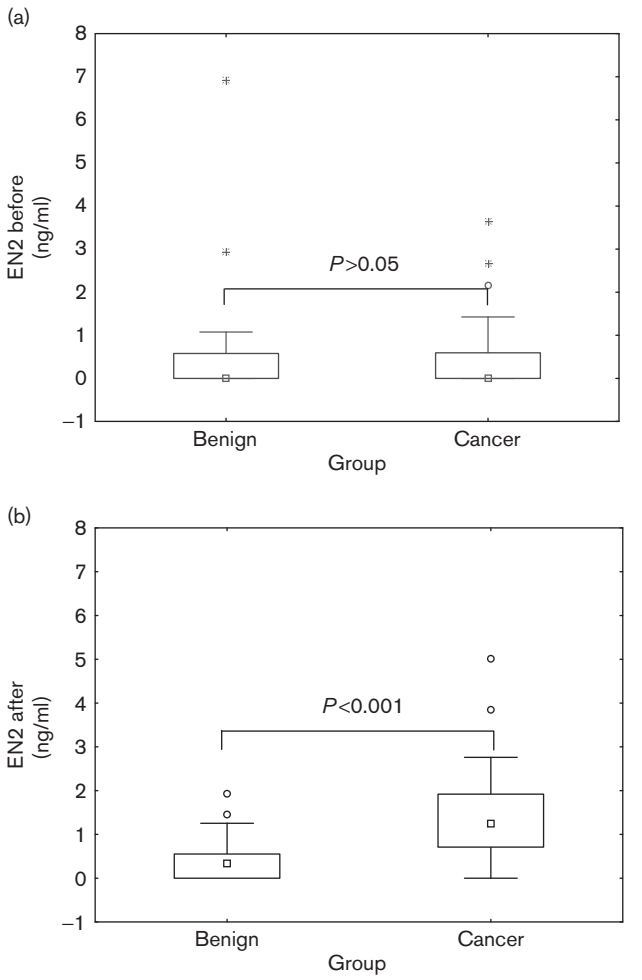
Enzyme-linked immunosorbent assay was used to determine the presence of EN2 in urine samples with a cut-off point of 0.31 ng/ml (specified by the manufacturer). EN2 was detected in 18 of the 33 urine samples of cancer patients and in 18 of the 38 samples from the benign group in the urine collected before prostate massage. However, the difference between the groups did not reach the level of statistical significance ($P > 0.05$). The same threshold was used to determine the presence of EN2 in urine samples collected after prostate massage. EN2 was detected in 30 of the 33 samples from cancer patients and in 18 of the 38 samples from the benign group. Statistical comparison of EN2 levels showed that the patients with PCa had higher levels of EN2, but only in the samples collected after prostate massage ($P < 0.001$) (Fig. 1).

Table 1 Patient characteristics: number of patients, age, serum PSA levels, tumour volume, EN2 levels (ng/ml) in urine before and after digital rectal examination

	Benign	Cancer
Number of patients	38	33
Mean age (range)	65 (50–82)	62 (51–79)
Median serum PSA (ng/ml)	3.47	6.33
Mean serum PSA (ng/ml) (range)	3.86 (0.05–10.46)	7.74 (1.09–42.56)
Tumour volume	–	28.17 (2.50–90.00)
Mean EN2 (ng/ml) level in urine collected before DRE (range)	0.50 (0.00–6.92)	0.51 (0.00–3.63)
Median EN2 (ng/ml) level in urine collected before DRE	0.00	0.00
Mean EN2 (ng/ml) levels in urine collected after DRE (range)	0.41 (0.00–1.93)	1.54 (0.00–7.25)
Median EN2 (ng/ml) level in urine collected before DRE	0.34	1.25

DRE, digital rectal examination; EN2, engrailed-2; PSA, prostate-specific antigen.

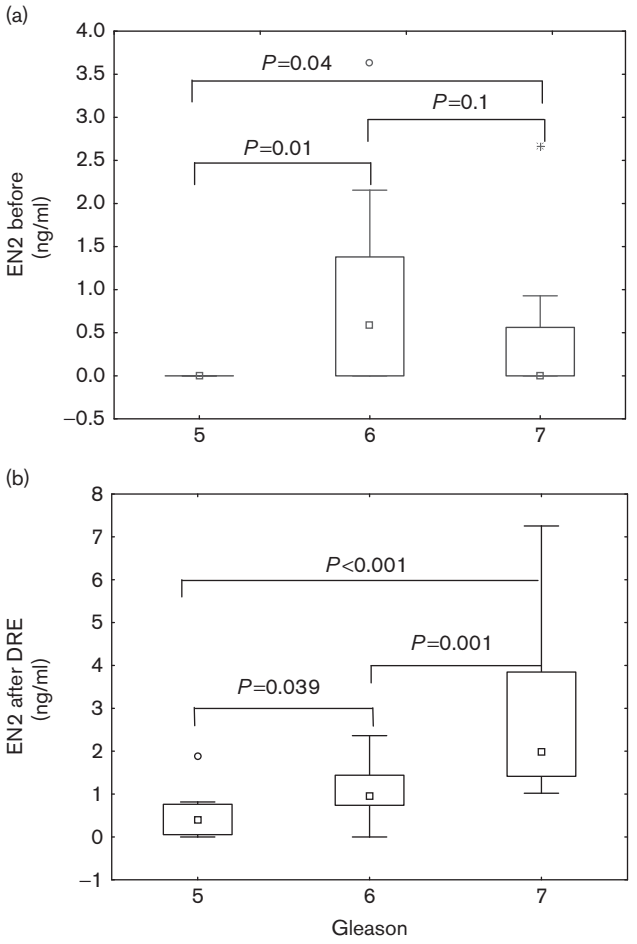
Fig. 1



Group comparison of EN2 concentrations in urine before (a) and after (b) digital rectal examination (DRE). EN2, engrailed-2. Asterisks, extreme values; open circles, outliers; open squares, median values.

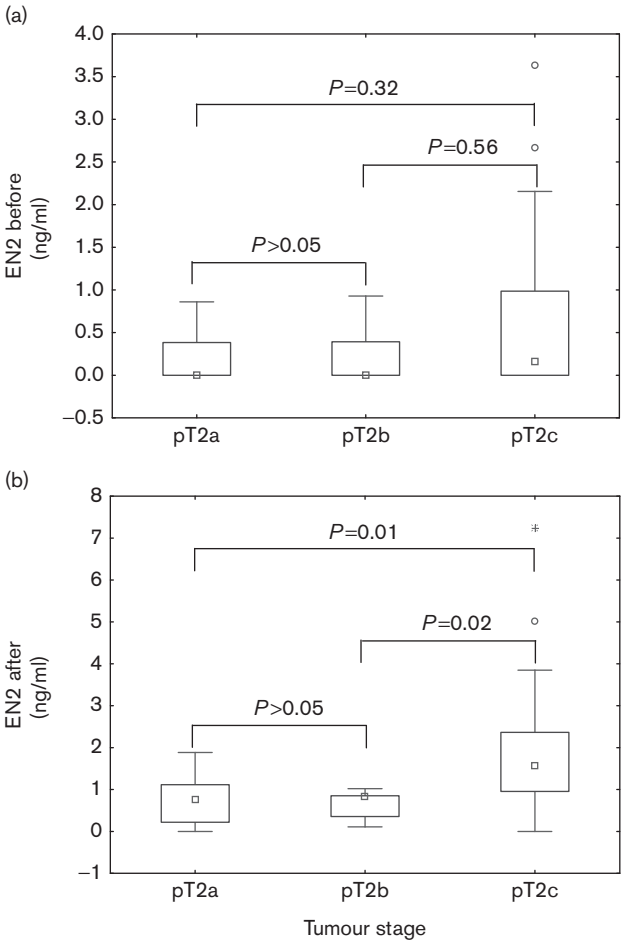
A distinct influence of prostate massage on EN2 levels was observed in the urine when the cancer patients were subdivided into those with Gleason scores of 5 ($n = 8$), 6 ($n = 13$) and 7 ($n = 12$) (Fig. 2). A significant correlation was observed between EN2 levels and the tumour grade. Higher levels of EN2 (median EN2 concentration was 1.98) were identified in the urine from patients with a Gleason score of 7. Lower median EN2 values in urine (0.40 and 0.95) were identified in patients with Gleason scores of 5 and 6, respectively. There was no significant correlation between the EN2 level and the Gleason score in urine samples collected before prostate massage. Also, a similar comparison was performed for the relationship between urinary EN2 levels and pathological stage (Fig. 3). The proportion of men with different pathological stages was 24% for pT2a, 15% for pT2b and 61% for pT2c. The increasing EN2 level was evidently associated with tumour stage in urine sample after prostate massage.

Fig. 2



Comparison of the EN2 level in urine collected before (a) and after (b) digital rectal examination (DRE) in cancer patients subdivided into those with a Gleason score of 5–7. EN2, engrailed-2. Asterisks, extreme values; open circles, outliers; open squares, median values.

Fig. 3



Relationship between urinary EN2 level before (a) and after (b) digital rectal examination (DRE) and tumour stage. EN2, engrailed-2. Asterisks, extreme values; open circles, outliers; open squares, median values.

However, neither the EN2 level in the urine collected before prostate massage nor that after prostate massage was statistically significant. A significant difference ($P=0.02$) was observed only in the more advanced pathological stages (pT2b and pT2c).

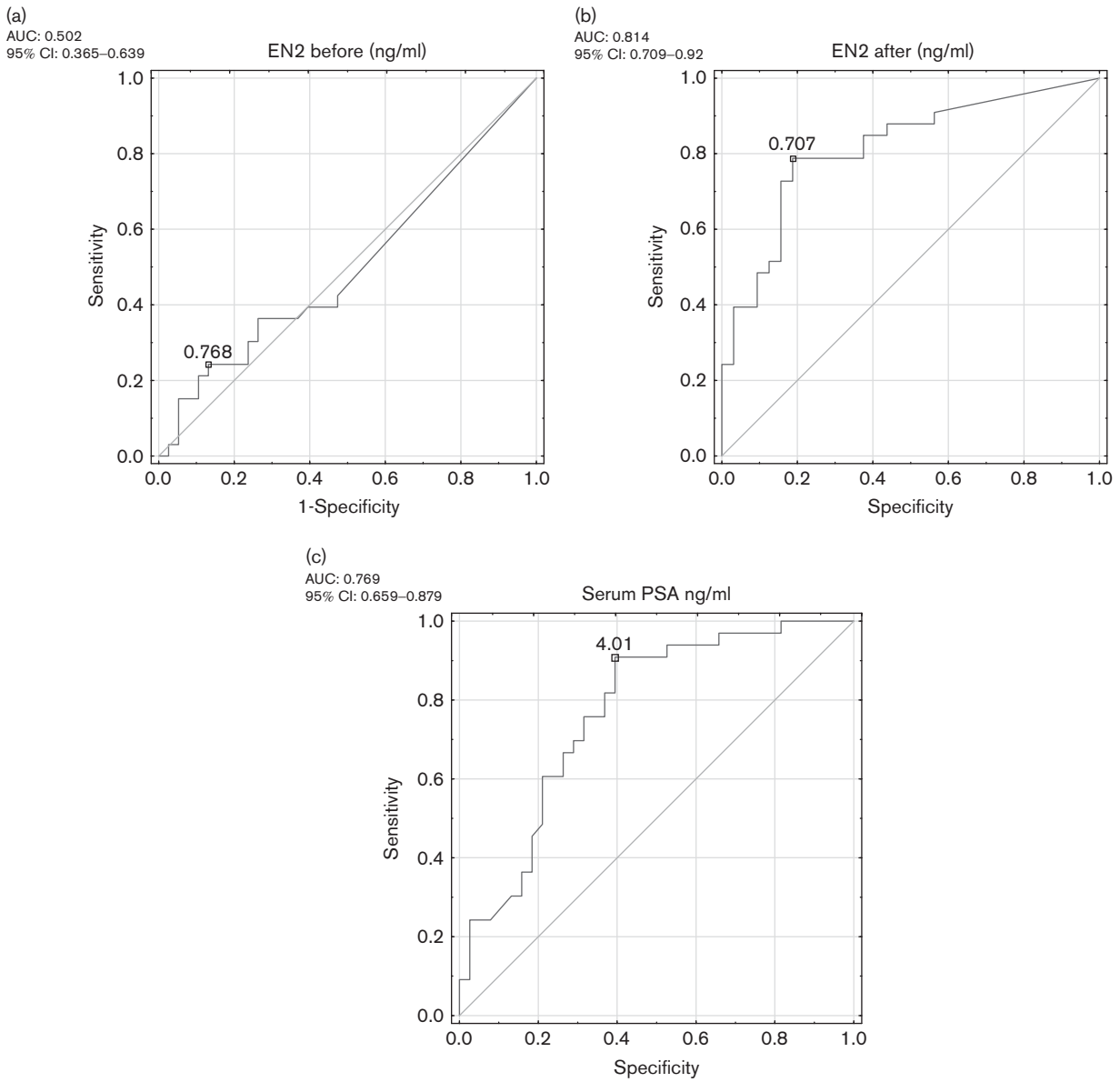
An ROC analysis of urine EN2 concentrations after prostate massage showed a high diagnostic potential of EN2 with the area under curve of 0.814, which was larger than for PSA (0.769) (Fig. 4a and c). Using a cut-off value of 0.70 ng/ml, EN2 protein is able to predict cancer with a true positive rate of 79% and a false-positive rate of 19%. PSA with a cut-off value of 4.01 ng/ml could diagnose patients with a true positive rate of 90% and a false-positive rate of 40%. No acceptable statistical parameters were obtained for the ROC analysis of urine before prostate massage (Fig. 4b). There was no correlation between PSA and the biomarker studied ($r=0.0937$, $P=0.4367$) (Fig. 5).

Discussion

Despite the numerous potential prostatic biomarkers, so far most of them have not been found to be useful in clinical practice (Makarov *et al.*, 2009). As first reported, the EN2 transcription factor is expressed by PC cell lines and prostate tumours, but not in the normal prostate tissue (Morgan *et al.*, 2011). In addition, other studies have shown that HOXC4-6 and HOXC8 are not normally expressed in normal prostatic tissue but are upregulated in biopsy cancer samples (Shah and Sukumar, 2010). Hence, the aim of our study was to compare the diagnostic potential of EN2 as a marker related to its higher expression and secretion by PCa with commonly used PSA as a non-PCa-specific marker.

Determination of EN2 in 100 µl of voided urine by enzyme-linked immunosorbent assay showed that only urine collected after prostate massage can be helpful in

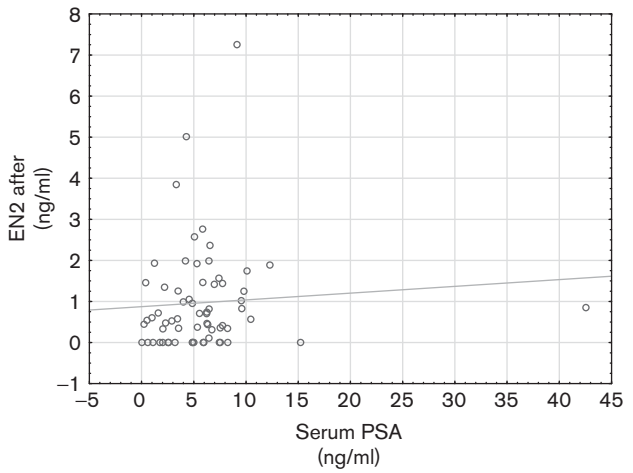
Fig. 4



Comparison of the diagnostic potential of EN2 level before (a) and after (b) digital rectal examination (DRE) and PSA (c) using an ROC curve. AUC, area under the curve; CI, confidence interval; EN2, engrailed-2; PSA, prostate-specific antigen; ROC, receiver operating characteristic.

the differentiation of PCa and BPH. On the basis of our results and other results published in different papers, we can state that there is a connection between PCa and EN2 levels. We found that EN2 levels were higher in urine samples collected after prostate massage in the cancer group and patients with higher concentrations of EN2 were classified higher according to the Gleason score. It needs to be noted that some patients diagnosed with benign growth might have had undiagnosed PCa. All patients from the BPH group presented with negative biopsy results for PCa, negative digital rectal exam results and confirmed BPH on the basis of the histopathological examination of resected prostate tissue samples. Still, these methods lack accuracy and PCa may have been missed. This may explain why some of the patients presented with high EN2 levels. Serum PSA levels had a higher true positive rate (PSA 90% compared with EN2 79%), but EN2 had lower false-positive rate (more false-positive diagnosis using serum PSA – 40%, compared with 19% false-positive diagnosis using EN2). Area under the curve showed more promising results when EN2 concentrations were used in differentiating cancer and benign disease (0.814 EN2 compared with 0.769). This is why we believe that the use of EN2 as a PCa biomarker can be a better solution.

Fig. 5



Relationship between EN2 in urine after a digital rectal examination (DRE) and serum PSA level. EN2, engrailed-2; PSA, prostate-specific antigen.

However, there are significant limitations to the use of EN2 as a biomarker for PCa. First, using EN2 would not eliminate DRE or prostate massage. On the basis of our result, only urine collected after prostate massage can be helpful in differentiation of PCa and BPH. Furthermore, the presently used procedures dependent on the serum PSA levels have a better true positive rate compared with EN2 without uncomfortable procedures. There is a need to carry out a large study that includes cancer, benign hyperplasia and healthy participants to define EN2 as a marker for PCa and not only a differentiation tool between PCa and BPH. Also we believe that combined prognostic values of both EN2 and serum PSA may provide more helpful diagnostic information, but this should be examined more closely on a large scale.

Conclusion

Patients with PCa presented with a higher concentration of EN2 only in urine collected after prostate massage. This is a promising result and should be studied more closely in a larger group of patients that would also include healthy volunteers.

Acknowledgements

The project was supported by a Polish Ministry of Science research grant for young scientists – Iuventus Plus IP 201103397.

Conflicts of interest

There are no conflicts of interest.

References

Downes MR, Byrne JC, Pennington SR, Dunn MJ, Fitzpatrick JM, Watson RW (2007). Urinary markers for prostate cancer. *BJU Int* **99**:263–268.

Economides KD, Capecchi MR (2003). Hoxb13 is required for normal differentiation and secretory function of the ventral prostate. *Development* **130**:2061–2069.

Fenner A (2011). Sarcosine: the saga continues…. *Nat Rev Urol* **8**:175.

Jentzmik F, Stephan C, Lein M, Miller K, Kamlage B, Bethan B, *et al.* (2010). Sarcosine in prostate cancer is not a differential metabolite for prostate cancer aggressiveness and biochemical progression. *J Urol* **185**:706–711.

Jiang Y, Cheng X, Wang C, Ma Y (2010). Quantitative determination of sarcosine and related compounds in urinary samples by liquid chromatography with tandem mass spectrometry. *Anal Chem* **82**:9022–9027.

Jung C, Kim RS, Lee SJ, Wang C, Jeng MH, Jung C, *et al.* (2004a). HOXB13 homeodomain protein suppresses the growth of prostate cancer cells by the negative regulation of T-cell factor 4. *Cancer Res* **64**:3046–3051.

Jung C, Kim RS, Zhang HJ, Lee SJ, Jeng MH (2004b). HOXB13 induces growth suppression of prostate cancer cells as a repressor of hormone activated androgen receptor signaling. *Cancer Res* **64**:9185–9192.

Kim K, Aronov P, Zakharkin SO, Anderson D, Perroud B, Thompson IM, Weiss RH (2009). Urine metabolomics analysis for kidney cancer detection and biomarker discovery. *Mol Cell Proteomics* **8**:558–570.

Makarov DV, Loeb S, Getzenberg RH, Partin AW (2009). Biomarkers for prostate cancer. *Annu Rev Med* **60**:139–151.

Miller GJ, Miller HL, van Bokhoven A, Lambert JR, Werahera PN, Schirripa O, *et al.* (2003). Aberrant HOXC expression accompanies the malignant phenotype in human prostate. *Cancer Res* **63**:5879–5888.

Morgan R (2006). Engrailed: complexity and economy of a multi-functional transcription factor. *FEBS Lett* **580**:2531–2533.

Morgan R, Boxall A, Bhatt A, Bailey M, Hindley R, Langley S, *et al.* (2011). Engrailed-2 (EN2): a tumor specific urinary biomarker for the early diagnosis of prostate cancer. *Clin Cancer Res* **17**:1090–1098.

Osl M, Dreiseitl S, Pfeifer B, Weinberger K, Klocker H, Bartsch G, *et al.* (2008). A new rule-based algorithm for identifying metabolic markers in prostate cancer using tandem mass spectrometry. *Bioinformatics* **24**:2908–2914.

Pandha H, Sorensen KD, Orntoft TF, Langley S, Hoyer S, Borre M (2012). Urinary engrailed-2 (EN2) levels predict tumour volume in men undergoing radical prostatectomy for prostate cancer. *BJU Int* **110 (Pt B)**:E287–E292.

Shah N, Sukumar S (2010). The Hox genes and their roles in oncogenesis. *Nat Rev Cancer* **10**:361–371.

Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, *et al.* (2009). Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* **457**:910–914.

Srigley JR (2006). Key issues in handling and reporting radical prostatectomy specimens. *Arch Pathol Lab Med* **130**:303–317.

Srigley JR, Humphrey PA, Amin MB, Chang SS, Egevad L, Epstein JI, *et al.* (2009). Protocol for the examination of specimens from patients with carcinoma of the prostate gland. *Arch Pathol Lab Med* **133**:1568–1576.

Struck W, Siluk D, Yumba-Mpanga A, Markuszewski M, Kaliszan R, Markuszewski MJ (2013). Liquid chromatography tandem mass spectrometry study of urinary nucleosides as potential cancer markers. *J Chromatogr A* **1283**:122–131.