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# Correlation of focal neuroendocrine differentiation in prostate cancer with the parameters of predictive value

## Korelacija fokalne neuroendokrine diferencijacije u karcinomu prostate sa parametrima od prediktivnog značaja

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**Abstract**

**Background/Aim.** Neuroendocrine (NE) cells are one of the epithelial populations in the prostate. It is well-known that the focal neuroendocrine differentiation (FNED) in prostate cancer (PC) is an aggressive subtype that most commonly evolves from preexisting PC which does not respond to hormone therapy (androgen independed PC). The incidence and clinical importance of FNED in PC is not clearly understood because of conflicting results in the studies, and evaluation of FNED is not routinely performed in clinical practice. The aim of the present study is to determine the importance of FNED presence in the examined prostate changes with special reference to the relationship of FNED degree in PC with some parameters of predictive value [Gleason score, preoperative serum total prostata specific antigen (PSA) value, tumor volume and tumor stage]. **Methods.** The study included the biopsy material from 100 untreated consecutive prostate pathological changes: 70 PC, 20 prostatic intraepithelial neoplasia (PIN) and 10 benign prostatic hyperplasia (BPH). The patients with PIN and BPH were the control groups. A block containing part of the main bulk of pathological change was chosen as representative based on hematoxylin-eosin appearance, and a section of this block was immunohistochemically stained for the tissue PSA (to mark prostatic secretory cells) and chromogranin A, serotonin and synaptophysin (to mark NE cells). **Results.** We found a very pronounced degree of FNED differentiation in 16 (22.9%) PC. Ten (62.5%) of them had Gleason score ≥ 7, the average serum PSA level was 32.62 ± 30.80 ng/mL, average tumor volume was 43.18 ± 31.45 mL and 6 (37.5%) of this PC were detected in D clinical stage with distant hematogenous metastases. The FNED is negatively correlated with the serum PSA level, Gleason score and clinical stage positively correlated with the tumor volume, but without statistically significant differences. **Conclusion.** The FNED has no significant role in the prognosis of PC.

**Key words:**
**prostatic neoplasms; neuroendocrinology; prostate-specific antigen; risk factors; neoplasm staging; prognosis.**

**Apstrakt**

**Uvod/Cilj.** Neuroendokrine (NE) ćelije su deo epitelne populacije prostate. Dobro je poznato da fokalna neuroendokrina diferencijacija (FNED) u karcinomu prostate (KP) predstavlja agresivni subtip, koji obično nastaje iz već postojećeg KP koji nije pokazao nikakav odgovor na hormonsku terapiju (tzv. androgen nezavisni KP). Incidenca i klinički značaj FNED u KP nisu u potpunosti razjašnjeni zbog kontradiktornih rezultata studija i zbog toga što se evaluacija FNED ne primenjuje rutinski u kliničkoj praksi. Cilj ove studije bio je utvrđivanje značaja prisusutva FNED u ispitivanim patološkim promenama u prostati sa posebnim osvrtom na odnos stepena FNED u KP sa nekim parametrima od prediktivnog značaja [Gleason score, preoperativne vrednosti ukupnog serumskog prostata specifičnog antigena (PSA), tumorski volumen i klinički stadijum]. **Metode.** Studija je sprovedena na biopsijskom materijalu 100 uzastopnih patoloških promena prostate: 70 KP, 20 prostatičnih intraepitelnih neoplazija (PIN) i 10 benignih hiperplazija prostate (BHP). Bolesnici sa PIN i BHP činili su kontrolnu grupu. Kalupi sa najreprezentativnijim uzorcima patoloških pro-

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mena dijagnostikovanih na rutinskim hematoksilin-eozin preparatima, imunohistohemijski su obojeni na tkivni PSA (za obeležavanje prostatičnih sekretornih ćelija) i hromogranin A, serotonin i sinaptofizin (za obeležavanje NE ćelija). **Rezultati.** Veoma izražen stepen FNED nađen je kod 16 (22,9%) KP. Među njima, kod 10 (62,5%) je dijagnostikovan Gleason score ≥ 7, prosečna vrednost serumskog PSA bila je 32,62 ± 30,80 ng/mL, prosečni tumorski volumen bio je 43,18±31,45 mL, dok su 6 (37,5%) KP otkrivene u D kliničkom stadijumu sa verifikovanim udaljenim hematogenim metastazama. FNED je bio u negativnoj korelaciji sa vrednostima serumskog PSA, Gleason skorom i kliničkim stadijumom i pozitivno je korelisan sa tumorskim volumenom, ali bez statistički značajne razlike. **Zaključak.** FNED nema značajnu ulogu u prognozi karcinoma prostate.

**Ključne reči:**
**prostata, neoplazme; neuroendokrinologija; prostata, specifični antigen; faktori rizika; neoplazme, određivanje stadijuma; prognoza.**

## Introduction

Prostate cancer (PC) can be manifested in different forms from small slowly growing neoplasia to tumor with an aggressive metastasizing potential [1]. PC is the second most common epithelial malignant tumor in men. It is estimated that 1.1 million men all around the world had a diagnosis of PC in 2012, with almost 70% of cases (759,000) diagnosed in more developed countries. The rates are highest in Australia/New Zealand and Northern America [age-standardised rate (ASR) 111.6 and 97.2 per 100,000] and in the Western and Northern Europe. These data are expected according to the fact that the prostate specific antigen (PSA) testing and prostate biopsy became standard in mentioned regions [2]. Also, worldwide, there is an increasing proportion of men older than 65 years in whom PC is prevalent [3]. The incidence rates are also high in less developed regions such as the Caribbean (79.8), Southern Africa (61.8) and South America (60.1), but still low in Asian men with the estimate rates of 10.5 and 4.5 South-Central and in Eastern Asia. PC is the fifth cancer death cause in men (6.6% of all men deaths). The mortality rates are very high in predominantly black men, very low in Asia and intermediate in the Americas and Oceania [2]. According to the data from the Institute of Public Health of Serbia „Dr Milan Jovanovic – Batut" for 2014, PC is the second leading cause of morbidity and third among the causes of death with 1,748 new cases each year in Ser-bia [4]. Despite its increasingly frequent occurrence, the knowledge of the PC biology is not clear enough. Still, it is very difficult to predict the clinical course and the outcome of advanced PC. It is completely necessary to improve understanding of the PC development as well as the new credibile biomarkers are needed for a therapy planning with a relevant aim to avoid overtreatment, or undertreatment [1].

The age, ethnicity, family history, level of preoperative serum PSA, free/total PSA ratio and outcome of digital rectal examination (DRE) determine the risk of clinically significant PC [5]. PSA is not highly specific, but its combination with DRE is considered as the most commonly used clinical procedures for early detection of PC. The risk assessment of localized PC is related to the plasma PSA level, Gleason score and tumor-node-metastasis (TNM) classification [6]. The prostate cancer antigen 3 (PCA-3) has higher specificity, although its sensitivity is little bit weaker. But, it has an important role in predicting the patients who will benefit from a biopsy of prostate [7]. In addition, one of the standard diagnostic procedures in PC diagnose is also a transrectal ultrasound (TRUS) biopsy of prostate with the minimum of 10–12 cores [6]. An important step in predicting the outcome of invasive prostate carcinoma was the introduction of the Gleason's grading system. It has become a widely accepted pathological method with proven prognostic significance and reproducibility [8]. There was a considerable inter-observer variability in grading prostate cancer in some researches, which imposed the need for the additional prognostic parameters such as neuroendocrine differentiation (NED) [9–11].

The factors with the important role in the development of androgen independent PC, including NED, are still not clear enough, which is the reason of insufficient knowledge of the way to intervene, prevent, or delay the malignancy [3]. Neuroendocrine (NE) cells of the prostate were originally described by Pretl [12] in 1944. They are distributed in the prostate glands of all anatomic zones and consists less than 1% of normal glandular epithelium of prostate and have characteristic lateral dendritic processes spreading. The density of NE cells in peripheral prostatic acini is the highest in the neonatal period and after puberty, and this is possibly under the androgenic hormones influence. The NE cells probably play an important role in endocrine and neuronal regulation of normal prostate. However, their apparent function is not entirely clear [13]. NED in PC can occur in three different forms: focal NED (FNED) in conventional prostate adenocarcinoma, carcinoid tumor (according to the WHO marked as well differentiated neuroendocrine tumor), and small cell neuroendocrine carcinoma (according to the WHO marked as poorly differentiated neuroendocrine tumor) [14]. The histologic features seen in the NED subtype of PC are similar to neuroendocrine tumors of any other organs and consists of differently sized nests as well as the insular or trabecular patterns of mostly round cells with the low grade cytologic features and characteristics "salt and pepper" chromatin distribution [13]. NED is found in 30%–100% of all PC [15] but it is prominent in only 5%–10% of them [16]. In general, the most common histopathological pattern is focal NED in conventional adenocarcinomas of prostate [15]. PC with NED differs from conventional PC histologically by the presence of NE cells which do not express the generic PC markers like the prostate specific antigen (PSA), prostate specific acid phosphatase (PSAP), prostate specific membrane antigen (PSMA), androgen receptor (AR), P501S and the prostate specific androgen regulated homeobox gene protein (NKX3.1), but characteristically expresses the neuroendocrine markers such

as chromogranin A, synaptophysin, CD56 and neuron-specific enolase (NSE) [17, 18]. Today, it is widely accepted that the main product chromogranin A (CgA), is a distinguished marker of NE cell differentiation and is also a general marker of population of NE cell. Other commonly found secretory products include serotonin (5-HT), NSE, bombesin, calcitonin and other members of the calcitonin gene family, such as katacalcin, calcitonin-gene-related peptide, somatostatin, parathyroid hormone-related protein (PTHrP) and thyroid-stimulating-like peptide [3]. A diagnosis of PC with focal NED is mostly made on a needle biopsy or on the metastatic lesions biopsies with the low or negative PSA levels. Probably, it is a subset of PC which is usually related to the androgen receptor resistance and worse prognosis [19]. There are the conflicting data reported in the literature regarding the prognostic significance of NED in PC. Some researchers showed a significant correlation between NED, tumor grade and poor prognosis. In several studies, an increased number of NED tumor cells in the advanced tumor stages, high grade versus low-grade tumors and, especially after the androgen suppression therapy during the tumour progression, was reported [20]. On the other hand, other authors did not find a correlation between the number of NED tumor cells, tumor grade and prognosis. The controversial data of the prognostic significance of NED markers may be explained by the non-standardized patient cohorts, different methods, and other difficulties, such as the limited volume of tissue samples and irregularly distributied NE cells [21–23]. Focal NED is considered to be strongly related as well to poor prognosis in advanced PC as to androgen-indepedent tumors [3]. Some studies considered NED in the tumor, determined either with immunohistochemistry, or with the measuring of the tumor NE cells product concentracion in the peripherial blood, as a significant prognostic parameter associated with survival after the endocrine therapy [24, 25]. This highly agrressive form of PC is increasingly observed in the patients who failed the first- and second-line hormone therapy [26]. The standard therapeutic approaches for PC are ineffective. To date, no specific treatment for PC with focal NED has been found. The antiangiogenetic drugs represent the potential alternatives but are still in a process of clinical research [27].

The aim of the present study is to determine the importance of the focal NED presence in the most important prostate pathological changes with a special reference to the relationship of focal NED degree in PC with some parameters of predictive value (Gleason score, preoperative serum total PSA value, tumor volume and tumor stage).

## Methods

The study included the biopsy material from 100 untreated consecutive prostate pathological changes [70 prostate cancer, 20 prostatic intraepithelial neoplasia (PIN) and 10 benign prostatic hyperplasia (BPH)] diagnosed at the Institute of Pathology, Faculty of Medicine, University of Priština-Kosovska Mitrovica and Institute of Pathology, Clinal Center Kragujevac, Kragujevac, Serbia. Diagnosis of PC was made on the core biopsies in 20 cases, the transurethral resection specimens in 15 cases and the fine needle aspiration biopsies in 35 cases. The diagnosis of PIN and BPH was made on the core biopsies in all cases. The patients with PIN and BPH were the control groups. The tissue samples were fixed in 10% neutral buffered formalin solution. The formalin-fixed, paraffin-embedded sections, 4–5 μm in thickness, were clasically processed and stained with hematoxylin-eosin (HE). The original histological slides were reviewed by the author. The Gleason grading of the prostate carcinomas was carried out according to the official recommendations of the Urological Section of the Swedish Society of Pathology [28]. For each case, a block containing part of the main bulk of pathological change was chosen as representative based on the HE appearance, and a section of this block was immunohistochemically (IHC) stained. The antibodies to the following antigens were used: to mark prostatic secretory cells: anti PSA - DAKO Code No A0562, ER-PR8, dilution 1 : 1000 (as a positive control we used normal prostate tissue); to mark NE cells: anti-chromogranin A - DAKO Code No M0869 DAK-A3, dilution 1 : 800 (as a positive control we used tissue of carcinoid tumor); serotonin - DAKO Code No M0758 5HT-H209, dilution 1 : 20 (as a positive control we used normal gaster tissue); synaptophysin - DAKO Code No M0776 SY38, dilution 1 : 10 (as a positive control we used normal endocrine pancreas tissue). The selected sections of tissue were stained by means of the labeled streptavidin – biotin method (DAKO Cytomation; 1 : 100) [29]. The anti PSA IHC staining of cells was recorded into 4 groups: negative (< 10% of positive cells), weakly positive (+), (10%–40% positive cells), moderate positive (++), (40%–90% positive cells) and very positive (+++), (> 90% positive cells) [30]. At least 500 cells were counted on each slide. NED IHC staining was recorded as NED negative [≤ 10 positive NE cells per 10 high power fields - HPF (×400)] and NED positivite [> 10 positive NE cells per 10 high power fields - HPF (×400)]. Based on the immunoreactivity of one, two or all three antibodies, the FNED degree was classified as: low (1 NED IHC stain positive), moderate (2 NED IHC stains positive) and very pronounced (3 NED IHC stains positive) [16]. All IHC stainings slides were interpreted by three independent researaches and the final interpretation was the mean of their own IHC results. The IHC interpretation was blinded for the clinicopathological data. The PC staging was classifed by Whitmore-Jewel and TNM system. The clinical stage combined DRE, serum PSA levels, TRUS and MRI (magnetic resonance imaging). Determination of extraprostatic extension, surgical margin status, involvement of seminal vesicle and lymph node status were made by the histological examination. The tumor volume was determined by TRUS. PSA was measured by the chemiluminescent immunoassay method in all the patients. Normal levels of the laboratory were 0–4 ng/mL.

The data primarily obtained were analyzed by the descriptive statistical methods (absolute numbers, measures of central tendency – mean value, as well as the measures of variability – standard deviation), the methods for testing statistical hypotheses (the $\chi^2$ test for testing the difference in the frequency among the groups; the Kruskal-Wallis and Mann-

Whitney test for testing the differences in the values of the characteristics among the groups), with the nonparametric correlation analysis – rank correlation and with the ROC (receiver operating characteristic) analysis. The statistical hypotheses were tested at a significance level of 0.05.

**Results**

Of the total number of 100 patients, 70 had PC, 20 had PIN and 10 had BHP. The patients with PIN and BPH were the control groups. The mean age of patients in the PC group was 71.8 ± 5.48, in the PIN group it was 69.8 ± 8.01 and in the BPH group it was 72.6 ± 6.13 (Table 1).

One of primary diagnostic procedures was also the determination of preoperative values of total serum PSA (ng/mL) in the patients with PC, PIN and BPH (Tables 2 and 3). The normal serum PSA levels were ≤ 4 ng/mL. The median PSA value was: in the PC group - 35.82 ng/mL, in the PIN group - 9.15 ng/mL and in the BPH group - 8.68 ng/mL.

The distribution value of serum PSA was statistically significant in the PC group compared to the control groups ($p < 0.0001$). Almost one half of all PC patients (47.1%) had the PSA levels > 40 ng/mL without a difference among the interval subgroups ($t = 0.49$; $p = 0.314$). There was a statistical significance in the interval subgroups in the PIN and BPH patients according to the PSA levels 5–10 and 11–20 ng/mL ($t = 5.96$; $p < 0.001$).

In the PC group with very pronounced focal NED, the average preoperative values of total serum PSA was 32.62 ± 30.80 ng/mL.

Immunostaining for the PSA and NED markers such as chromogranin A, serotonin and synaptophysin was performed on the representative tissue blocks of PC, PIN and BPH (Table 4).

There was a statistical significance for > 90% of the positive cells compared to 40%–90% of the positive cells subgroup on PSA immunostain (Figure 1) in all examined groups ($t = 4.22$; $p < 0.0001$). There was a respectable incidence of 40%–90% of the positive PSA cells at the PC group in almost 23% of cases, but without a statistical significance ($\chi^2=3.804$; $p = 0.149$).

**Table 1**

**Age distribution of the patients with PC, PIN and BPH**

| Diagnosis | Total (n) | Min | Max | Mean | SD |
|---|---|---|---|---|---|
| PC | 70 | 58 | 82 | 71.8 | 5.48 |
| PIN[c] | 20 | 53 | 80 | 69.8 | 8.01 |
| BPH[c] | 10 | 66 | 86 | 72.6 | 6.13 |

**[c] – control group; PC – prostate cancer; PIN – prostatic intraepithelial neoplasia; BPH – benign prostate hyperplasia; SD – standard deviation.**

**Table 2**

**Preoperative values of total serum PSA (ng/ml) in the patients with PC, PIN and BPH**

| PSA (ng/mL) | PC[†] | PIN[c] | BPH[c] |
|---|---|---|---|
| | n (%) | n (%) | n (%) |
| 5–10 | 7 (10.0) | 12 (60.0) | 7 (70.0) |
| 11–20 | 10 (14.3) | 6 (30.0) | 1 (10.0) |
| 21–30 | 12 (17.1) | 2 (10.0) | 1 (10.0) |
| 31–40 | 8 (11.4) | 0 (0.0) | 1 (10.0) |
| > 40 | 33 (47.1) | 0 (0.0) | 0 (0.0) |
| Total | 70 (100.0) | 20 (100.0) | 10 (100.0) |

**PC – prostate cancer; PIN – prostatic intraepitelial neoplasia; BPH – benign prostate hyperplasia; PSA – prostate specific antigen; [c] – control group; [†] – statistical significance compared to the control groups ($p = 0.05$).**

**Table 3**

**Characteristics of preoperative values of serum PSA (ng/mL) in PC, PIN and BPH**

| Diagnosis | Total (n) | Min | Max | Median | Percentile | |
|---|---|---|---|---|---|---|
| | | | | | 10% | 90% |
| PC | 70 | 6.00 | 960.40 | 35.82 | 10.54 | 266.97 |
| PIN[c] | 20 | 3.16 | 27.61 | 9.15 | 5.44 | 20.80 |
| BPH[c] | 10 | 0.80 | 31.20 | 8.68 | 1.05 | 30.90 |

**PC – prostate cancer; PIN – prostatic intraepitelial neoplasia; BPH – benign prostate hyperplasia; PSA – prostate specific antigen; [c] – control group.**

**Table 4**

**Immunohistochemical (IHC) staining in PC, PIN and BPH**

| Diagnosis | Immunohistochemical staining | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PSA (%) | | CgA | | Ser | | Syn | |
| | 40–90%$^{+}$ | > 90%$^{+}$ | ≤ 10$^{+}$/10 HPF | > 10+/10 HPF | ≤ 10+/10 HPF | > 10+/10 HPF | ≤ 10+/10 HPF | > 10+/10 HPF |
| | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) |
| PC | 16 (22.9) | 54$^{\dagger}$(77.1) | 42 (60.0) | 28 (40.0) | 18 (25.7) | 52$^{\dagger}$(74.3) | 31 (44.3) | 39 (55.7) |
| PIN$^{c}$ | 1 (5.0) | 19$^{\dagger}$(95.0) | 11 (55.0) | 9 (45.0) | 7 (35.0) | 13 (65.0) | 13 (65.0) | 7 (35.0) |
| BPH$^{c}$ | 1 (10.0) | 9$^{\dagger}$(90.0) | 10$^{\dagger}$(100.0) | 0 (0.0) | 9$^{\dagger}$ (90.0) | 1 (10.0) | 10$^{\dagger}$ (100.0) | 0 (0.0) |

**PC – prostate cancer; PIN – prostatic intraepitelial neoplasia; BPH – benign prostate hyperplasia; PSA – prostate specific antigen; CgA – chromogranin A; Ser – serotonin; Syn – synaptophysin; $^{+}$ – IHC positive cells; $^{c}$ – control group; $^{\dagger}$ – statistical significance compared to the subgroup within the IHC stain ($p = 0.05$).**
**10HPF – 10 high power field.**

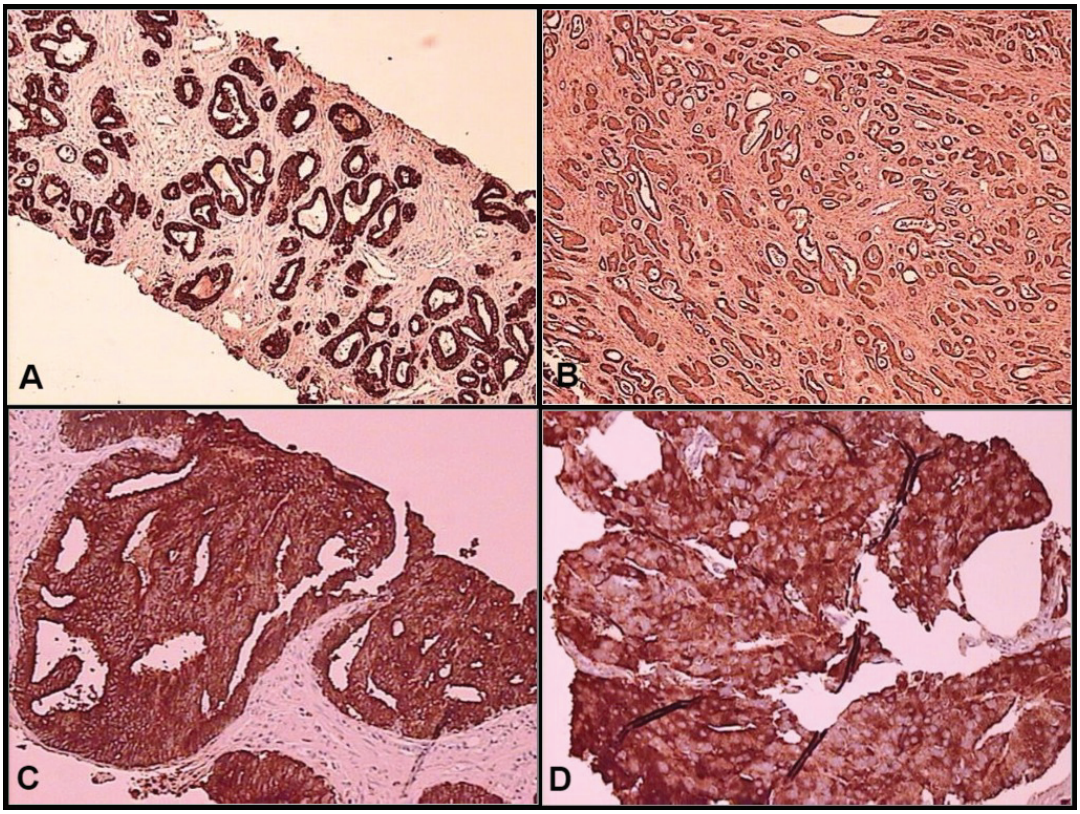


**Fig. 1 – Prostate cancer (strong positive reactivity of > 90% cells, immunostain for prostate specific antigen):**
**A) Gleason grade 2 (×100); B) Gleason grade 3 (×50);**
**C) Gleason grade 4 (×200); D) Gleason grade 5 (×200).**

Immunostain for chromogranin A (Figure 2) showed a statistically significant difference between PC, PIN and BHP ($\chi^2 = 6.625$; $p = 0.035$) concerning the presence of ≤ 10 positive cells per 10HPF in all the BPH cases. There was a respectable incidence of such cells at PC in 60% and PIN in 55% cases, but without a statistical significance (Mann-Whitney U = 78.00; $p = 0.690$). There was no statistical significance in the PC group based on the presence of ≤ 10 positive cells compared to > 10 positive cells per 10HPF ($t = 1.96$; $p = 0.05$), as well as in the PIN group ($t = 0.45$; $p = 0.32$).

Immunostain for serotonin (Figure 3) showed a statistically significant difference between PC, PIN and BHP ($\chi^2 = 15.964$; $p = 0.002$) concerning the presence of ≤ 10 positive cells per 10 HPF in 90% of BPH cases. There was a respectable incidence of such cells at PC in 25.7% and PIN in 35% of cases, but without a statistical significance. There was no statistical significance in the PIN group based on the presence of ≤ 10 positive cells compared to > 10 positive cells per 10 HPF ($t = 1.40$; $p = 0.08$). There was the statistically significant presence of > 10 positive cells compared to ≤ 10 positive cells per 10 HPF in the PC group ($t = 4.65$; $p < 0.001$) and BPH group ($t = 4.22$; $p < 0.0001$). In comparison with chromogranin A, there was a statistically significant representation of cases with 10 positive cells/10 HPF based on immunostain for serotonin suggesting that serotonin had better performances in this study ($t = 3.02$; $p = 0.001$).

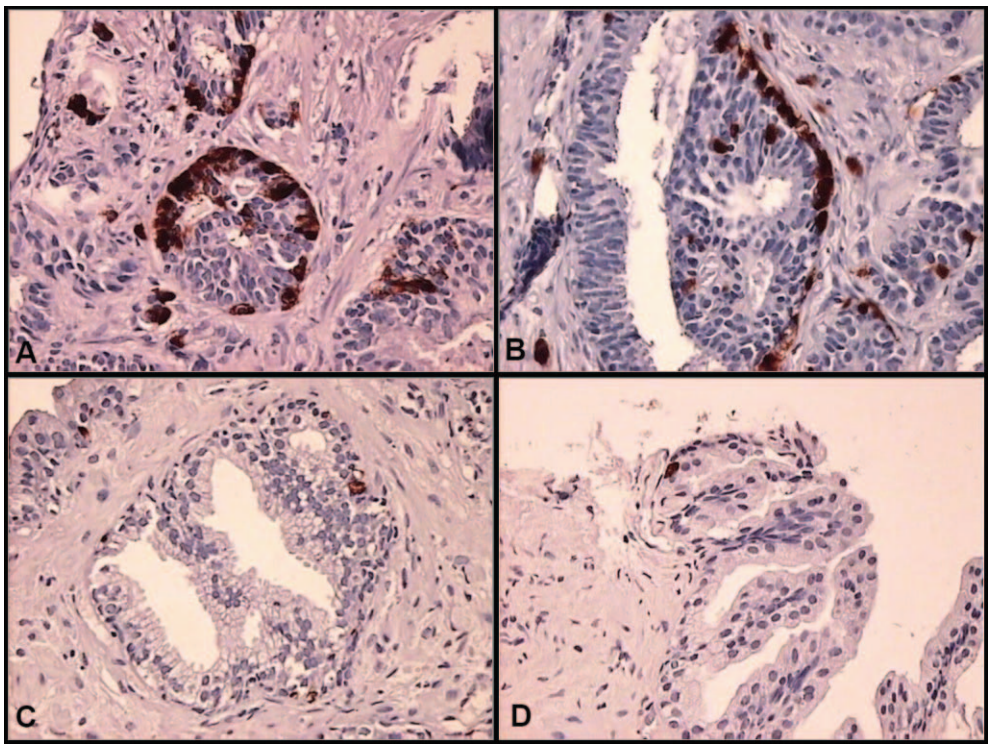


**Fig. 2 – Immunostain for chromogranin A:**
**A) Prostate cancer, Gleason grade 4 (strong positive reactivity of > 10cells/10HPF, ×400);**
**B) Prostate cancer, Gleason grade 4 (≤ 10 positive cells/10HPF, ×400); C) HG prostatic intraepithelial neoplasia (≤ 10 positive cells/10HPF, ×400); D) BPH (≤ 10 positive cells/10HPF, ×400);**
**BPH – benign prostatic hyperplasia; 10HPF – 10 high power fields benign prostatic hyperplasia.**

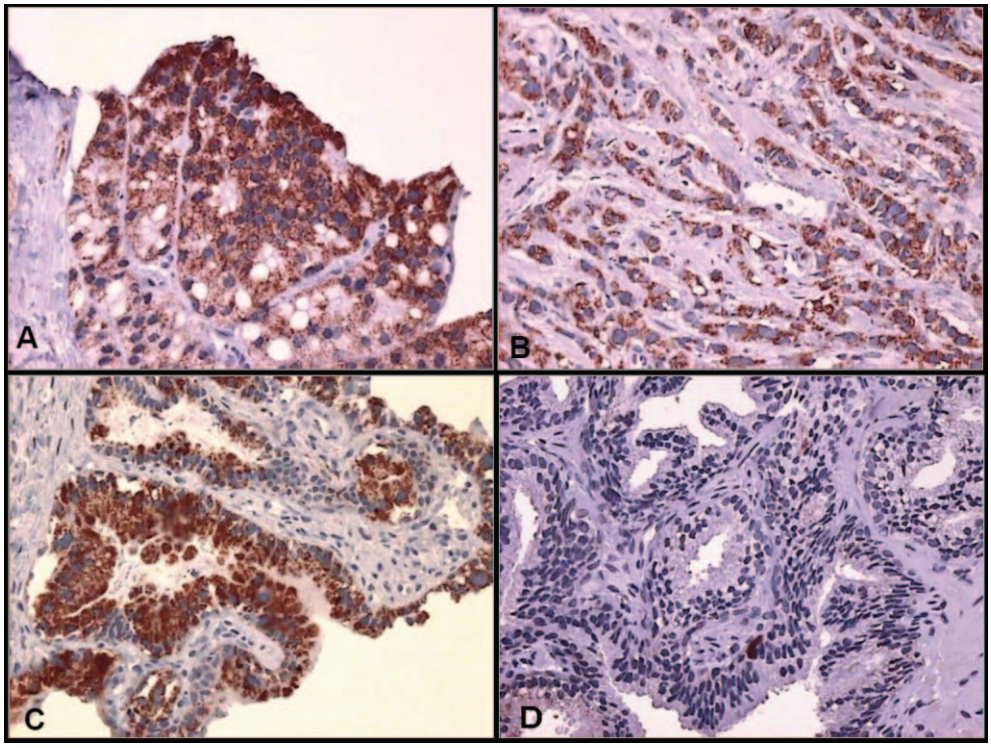


**Fig. 3 – Immunostain for serotonin:**
**A) Prostate cancer, Gleason grade 4 (strong positive reactivity of > 10cells/10HPF, ×400);**
**B) Prostate cancer, Gleason grade 5 (strong positive reactivity of > 10cells/10HPF, ×400);**
**C) HG PIN (strong positive reactivity of >10cells/10HPF, ×400);**
**D) BPH (≤ 10 positive cells/10HPF, ×400).**
**PIN – prostatic intraepithelial neoplasia; BPH – benign prostatic hyperplasia; 10HPF – 10 high power fields.**

Testing the differences in distribution, a degree of immunostain reactivity for synaptophysin (Figure 4) showed that there was a statistically significant difference between PC, PIN and BHP ($\chi^2 = 12.031$; $p = 0.002$) concerning the presence of ≤ 10 positive cells per 10 HPF in all cases of BPH. There was no statistical significance in the presence of > 10 positive cells per 10 HPF in the PC group ($t = 0.96$; $p = 0.17$) and the PIN group ($t = 1.40$; $p = 0.08$) compared to ≤ 10 positive cells per 10 HPF.

In order to improve the comparability of qualitative characteristics of each of the applied marker of neuroendocrine differentiation in the diagnosis of PC and PIN, the comparative qualitative values of these parameters were determined (Table 5).

Better diagnostic markers of focal NED were serotonin [odds ratio (OR) = 3.30] and synaptophysin (OR = 4.13) for PC, and chromogranin A (OR = 1.52) for PIN. Also, the additional ROC analysis showed that synaptophysin had the best NED diagnostic characteristics for prostate cancer considering the largest area under the ROC (AUC = 0.662; $p = 0.011$) compared to serotonin (AUC = 0.638; $p = 0.029$) and chromogranin A (AUC = 0.550; $p = 0.430$) (Figure 5 and Table 6).

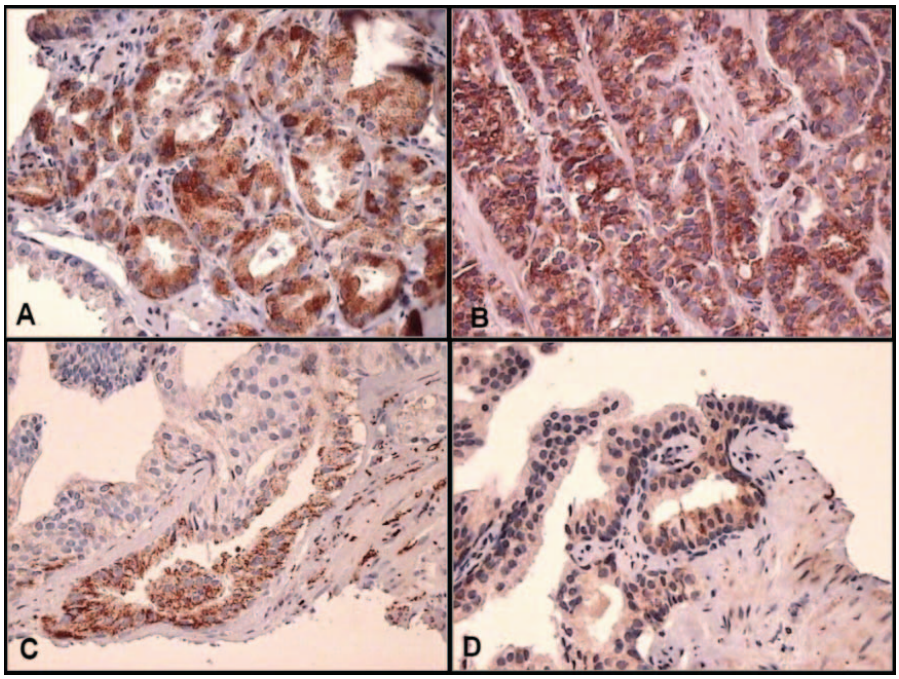


**Fig. 4 – Immunostain for synaptophysin:**
**A) Prostate cancer, Gleason grade 3 (strong positive reactivity of >10cells/10HPF, ×400);**
**B) Prostate cancer, Gleason grade 4 (strong positive reactivity of >10cells/10HPF, ×400);**
**C) HG PIN (≤10 positive cells/10HPF, ×400); D) BPH (≤ 10 positive cells/10HPF, ×400).**
**For abbreviations see under Figure 3.**

**Table 5**

**Performance of markers of neuroendocrine differentiation (NED)**

| Marker of NED | Prostate cancer | | | | | Prostatic intraepithelial neoplasia | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SE | SP | OR | PPV | NPV | SE | SP | OR | PPV | NPV |
| Chomogranin A | 40.0% | 70.0% | 1.55 | 75.7% | 33.3% | 45.0% | 65.0% | 1.52 | 24.3% | 82.5% |
| Serotonin | 74.3% | 53.3% | 3.30 | 78.8% | 53.5% | 65.0% | 33.8% | 0.95 | 19.7% | 79.4% |
| Synaptophysin | 55.7% | 76.7% | 4.13 | 84.8% | 42.6% | 35.0% | 51.2% | 0.57 | 15.2% | 75.9% |

**SE – sensitivity; SP – specificity; OR – odd ratio; PPV – positive predictive value; NPV – negative predictive value.**

**Table 6**

**The performance of neuroendocrine differentiation (NED) markers and focal NED degree in a prostate cancer diagnosis**

| Parameter | AUC | p | SE | SP |
|---|---|---|---|---|
| Chromogranin A | 0.550 | 0.430 | 40.0% | 70.0% |
| Serotonin | 0.638 | 0.029† | 74.3% | 53.3% |
| Synaptophysin | 0.662 | 0.011† | 55.7% | 76.7% |
| Focal NED degree | 0.644 | 0.023† | 62.9% | 66.7% |

**SE – sensitivity; SP – specificity; AUC – area under curve; † – statistically significant ($p < 0.05$).**

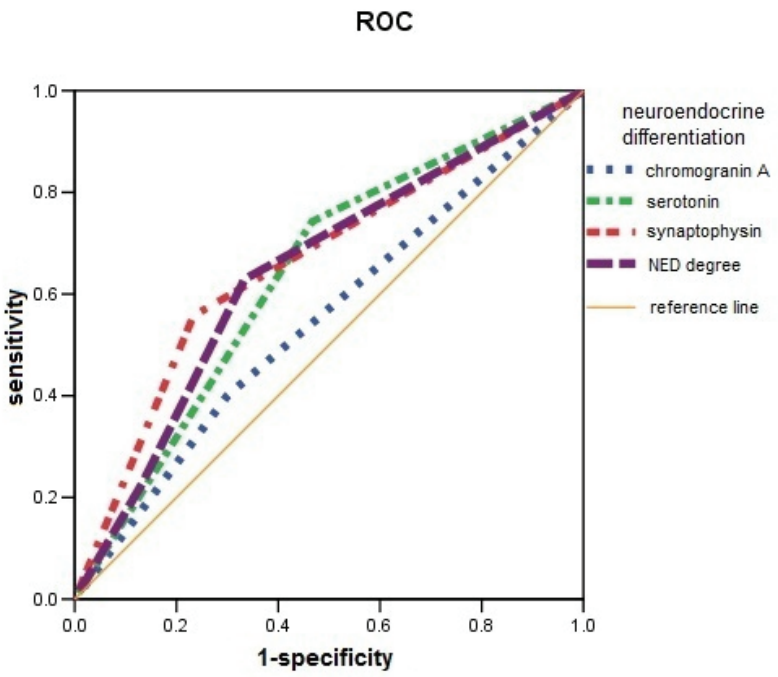


**Fig. 5 – Receiver operating characteristic (ROC) curve parameters for neuroendocrine differentiation (NED) in prostate cancer.**

In order to better understand neuroendocrine differentiation, we determined the degree of focal NED according to the strong reactivity of at least 10 cells per 10HPF. The degree of focal NED divided into three groups was based on immunostain of only one (low), two (moderate), or all three (very pronounced) NED markers (Table 7).

There was a statistically significant difference compared BHP to PC and PIN concidering the apperance of low degree of focal NED in all cases ($p = 0.002$). There was no statistical significance between the PC and PIN group (Mann-Whitney U = 618.000; $p = 0.394$), nor in the subgroups inside PC ($t = 1.15$; $p = 0.12$) and PIN ($t = 0.78$; $p = 0.20$).

A Gleason score (GS) was based on primary and secondary on the Gleason pattern. We found GS 7 in almost one half of all 70 PC patients, more precisely, in 33 (47.1%) being statistically significant when compared to other Gleason scores ($t = 3.38$; $p = 0.03$). In the PC group with very pronounced focal NED we found the Gleason score to be ≥ 7 in 10 (62.5%) cases. GS was 3 and 4 in 3% of patients, 5 in 17% an 6 in 10% of patients.

The most common clinical stage in the PC patients was D2 – 31 (44.3%), having no statistical significance inside the groups ($t = 1.89$; $p = 0.058$). In the PC group with very pronounced focal NED, we found D2 in 6 (37.5%) cases. In the stage $B_1$ , there was 1% of patients, in $B_2$ - 6%, in $B_3$ - 20%, in $C_1$ - in 6%, in $C_2$ - 19% and in $C_3$ - 4% of patients.

The average volume of all prostate cancers was 47.3 ± 30.39 mL (max = 183 mL; min = 10 mL). Most prostate cancer (51.4%) had the volume of 21–40 mL ($t = 3.81$; $p < 0.001$). In the PC group with very pronounced focal NED, the average volume was 43.18 ± 31.45 mL.

The results of correlation analysis of the examined parameters are shown in Table 8. The focal neuroendocrine differentiation is negatively correlated with the preoperative serum PSA level, the Gleason score and clinical stage and positively correlated with tumor volume, but without statistically significant differences.

**Table 7**

**The degree of focal neuroendocrine differentiation (NED)**

| Degree of focal neuroendocrine differentiation | PC<br>n (%) | PIN[c]<br>n (%) | BPH[c]<br>n (%) |
|---|---|---|---|
| low | 26 (37.1) | 10 (50.0) | 10[†] (100.0) |
| moderate | 28 (40.0) | 6 (30.0) | 0 (0.0) |
| very pronounced | 16 (22.9) | 4 (20.0) | 0 (0.0) |
| Total | 70 (100.0) | 20 (100.0) | 10 (100.0) |

**PC – prostate cancer; PIN – prostatic intraepitelial neoplasia; BPH – benign prostate hyperplasia; [c] – control group; [†] – statistical significance ($p = 0.05$).**

**Table 8**
**Correlation interdependence matrix of neuroendocrine differentiation (NED) and other histopathological parameters in the prostate cancer patients**

| Parameter | | NED degree | PSA immunostain | Gleason score | PSA serum | Tumor volume | Clinical stage |
|---|---|---|---|---|---|---|---|
| NED degree | cc | 1.000 | 0.126 | -0.181 | -0.197 | 0.083 | -0.058 |
| | p | . | 0.298 | 0.135 | 0.102 | 0.494 | 0.636 |
| PSA immunostain | cc | 0.126 | 1.000 | -0.017 | 0.083 | -0.101 | -0.132 |
| | p | 0.298 | . | 0.889 | 0.493 | 0.405 | 0.275 |
| Gleason score | cc | -0.181 | 0.298 | 1.000 | 0.423** | 0.195 | 0.317** |
| | p | 0.135 | -0.017 | . | 0.000 | 0.105 | 0.007 |
| PSA serum | cc | -0.197 | 0.083 | 0.423** | 1.000 | 0.284* | 0.334 |
| | p | 0.102 | 0.493 | 0.000 | . | 0.017 | 0.002 |
| Tumor volume | cc | 0.083 | -0.101 | 0.195 | 0.284 | 1.000 | 0.152 |
| | p | 0.494 | 0.405 | 0.105 | 0.017 | . | 0.210 |
| Clinical stage | cc | -0.058 | -0.132 | 0.317** | 0.334 | 0.152 | 1.000 |
| | p | 0.636 | 0.275 | 0.007 | 0.002 | 0.210 | . |

**PSA – prostate specific antigen.**
**cc – Spearman's correlation coefficient; *$p < 0.05$, **$p < 0.01$ – degree of significance.**

## Discussion

Neuroendocrine differentiation is found in almost all prostate cancers, but it is expressed in only 5%–10% of them [16]. It can occur either as individualy, or as a group of accumulated tumor cells [31–33]. The apparent function of NE cells is not entirely clear [13]. Although the neurosecretory granules tend to localize close to the plasma membrane of NE cells, their greatest density is within the cytoplasmic dendritic extensions, which is a characteristic of these cells. Neuroendocrine differentiation can be seen in three different forms in prostate cancer: focal NED in conventional prostate adenocarcinoma, carcinoid tumor and small cell neuroendocrine carcinoma [14]. It is now widely accepted that the main product, chromogranin A (CgA), is a distinguished marker of NE cell differentiation and is also the general marker of the population of NE cell. The most PC cells show immunoreactivity with CgA [3, 33], but some cells show synaptophysin and serotonin immunoreactivity. Usually, CgA positivity can be found in 31% and synaptophysin positivity in 8% of prostate cancer [34]. There are conflicting data reported in the literature regarding the prognostic significance of neuroendocrine differentiation in prostate cancer. Some authors believe that it has a negative effect on prognosis [35]. In several researches an increased number of NED tumor cells in the advanced tumor stages, high grade versus low-grade tumors and, especially after suppression by androgen drugs during the tumour progression was reported [20]. However, some researches do not find any link between focal NED and prognosis [21, 36]. In our study, to assess the presence and degree of focal NED in prostate cancer, so as in prostatic intraepithelial neoplasia and benign prostatic hyperplasia, the antibodies to chromogranin A, serotonin and synaptophysin were used. The majority of cases of PC showed positive immunostain of > 10 cells/10HPF on serotonin in 52 (74.3%) PC. There was no statistical significance when chromogranin A and synaptophysin were applied. The obtained results were not in accordance with the data from the literature, which stated that the most cells are positive for chromogranin A [3, 33]. In most cases of PIN, there was a dominant finding of positive immunostain of ≤ 10 cells/10HPF on chromogranin A and synaptophysin. Only serotonin immunostain showed positivity of > 10 cells/10HPF in most cases. In all BHP cases there was a positive immunostain of ≤ 10 cells/10HPF on chromogranin A and synaptophysin, and in 9 (90.0%) of cases when serotonin immunostain was applied. There is a statistically significant difference in immunoreactivity of all the markers of neuroendocrine differentiation between prostate cancer and the control groups: for chromogranin A, for serotonin and for synaptophysin. On the basis of the mentioned above, serotonin proved to be the most sensitive marker of neuroendocrine differentiation.

In order to improve the comparability of qualitative characteristics of each applied NED marker, a special attention was paid to the comparative qualitative values of these parameters. According the sensitivity and specificity of NED markers and the odd ratio, synaptophysin had the best performance in diagnosis of prostate cancer with sensitivity of 55.7% and specificity of 76.7% (OR = 4.13) vs. serotonin (OR = 3.30) and chromogranin A (OR = 1.55). Nevertheless, chromogranin A had the best characteristics in the diagnosis of PIN with sensitivity of 45% and specificity of 65% (OR = 1.52) vs. synaptophysin (OR = 0.95) and serotonin (OR = 0.57). For the purpose of better understanding the characteristics of each of the applied neuroendocrine differentiation markers in the diagnosis of PC and PIN, the degree of focal NED was determined according to positive immunostain of > 10 cells/10HPF to only one (low focal NED), two (moderate focal NED), or all three (very pronounced focal NED) NED markers. Focal NED in PC was low in 26 (37.1%) cases, moderate in 28 (40%) cases and very pronounced in 16 (22.9%) cases, with no statistically significant

difference. Focal NED in PIN was low in 10 (50.0%) cases, moderate in 6 (30%) cases and very pronounced in 4 (20.0%) cases, with no statistically significant difference. Focal NED in BPH was low in all cases, which is in accordance with the literature data [33]. There was no statistically significant difference in the NED degree between PC and PIN.

A clinical stage was determined by the clinical and ultrasound examination in all patients with PC, and it was indexed by the alphabetical classification. The most of PC were diagnosed in the stage D – with verified distant hematogenous metastases mainly at the spine and ribs (in over 75% of patients), but without statistically significant differences compared to another clinical stages. The data are very disappointing because the majority of the patients visited the doctor at the time of the existence of distant hematogenous metastases, when there was almost no possibility of a cure. Such cases became rare in the countries with developed screening, where prostate cancer is usually discovered in the stages A or B [37, 38]. In the patients with distant metastasis, death is almost inevitable in about 15% within next 3 years, in 80% within next 5 years, in 90% within following 10 years. During the present study, we obtained the data regarding fatal outcome of 5 (16.13%) patients, among 31 patients with D stage, within the first 12 months from the time of diagnosis.

A histological grade, as a significant indicator of the survival of patients, is administered as a primary factor in almost all existing algorithms [39]. In this study, the most commonly diagnosed PC was that of the Gleason score 7 in almost one half of patients – 33 (47.14%), which is consistent with the findings of other authors [39]. The rarest diagnosed Gleason scores were 3 and 4, in the 2 (2.86%) cases, which is also in line with the data from the literature, according to which it is considered inadvisable to diagnose PC Gleason score 2–4 on prostate needle biopsies [40].

The volume of prostate cancer (mL) was determined by the ultrasound examination. Average PC volume was 47.29 ± 30.39 mL (max = 183 mL; min = 10 mL), which is consistent with findings of other authors [41]. Considering that theoretically lymph-node metastasis can be found only when the primary tumor volume is higher than 4 mL; 0.5 mL cancer would take approximately 12 years to reach 4 ml if its doubling time was 48 months [42]. Further development of the tumor is faster if the tumor at the time of diagnosis has a larger volume. The majority of patients in this study (51.4%) had PC of 21–40 mL, with a statistically significant difference compared to other interval sizes.

The correlation analysis of examined parameters showed there was a statistically significant positive coefficient of correlation between the preoperative serum PSA level on one side and the Gleason score (0.423), tumor volume (0.284) and clinical stage (0.334) on the other, but without a statistical significance. Most authors also find the good positive correlation of serum PSA level with the Gleason score, clinical stage and tumor volume [43]. Some authors consider PSAD (PSA density) [44] and PSAV (PSA velocity) [45] to be better prognostic parameters of serum PSA. The tumor volume and serum PSA were satisfactory positively correlated, meaning that the higher PSA levels correspond to the larger tumors volumes, which is consistent with findings of the other authors [46, 47]. A clinical stage was positively correlated with several parameters. First of all, with the preoperative serum PSA levels < (0.334) and the Gleason score (0.317) with a statistical significance, which is consistent with the data from the literature [39, 43, 48]. The correlation analysis within the parameters of neuroendocrine differentiation showed that the most cells were positive for synaptophysin, with the significant level and high correlation (0.751), which gave it the importance of the most sensitive NED marker in this study. According to the results of the majority of authors, chromogranin A is the best NED marker [34, 49, 50]. Based on the demonstrated sensitivity, chromogranin A and serotonin are the NED markers whith the high and moderate correlation coefficients (0.677 and 0.545, respectively). The results of expression levels findings regarding immunoreactivity to serotonin and synaptophysin showed that they were well- connected with each other, with a statistically significant level, which pointed to the need to combine at least two, but optimally three markers of FNED. All the results of the NED markers expression are negatively correlated with the preoperative serum PSA levels (-0.197), as well as with the Gleason score, however, with statistical significance only for synaptophysin and the Gleason score (-0.280). This means that lower the Gleason score values correspond to a larger number of synaptophysin positive cells. These results are consistent with the data from the literature, according to which the focal NED is in good positive correlation with the Gleason score [16, 51]. The same authors state that focal NED does not correlate with the clinical stage, which was confirmed in this study, but without statistically significant differences (-0.058). There is a positive correlation between NED and tumor volume (0.083). Tumor volume was a significant predictor of biochemical recurrence (BCR) – free survival among the patients who underwent radical prostatectomy [52].

## Conclusion

A large number of PC (77.1%) and almost all cases of PIN (95%) and BPH (90%) show a strong expression of tissue PSA, which is a confirmation of the important role of this marker in the diagnosis of PC and differential diagnosis of metastatic cancer of unknown origin. Prostate cancer and PIN show a significant positive reaction for chromogranin A, synaptophysin, serotonin as markers of focal neuroendocrine differentiation. BPH showed significantly less positive reaction for the same markers. Serotonin and synaptophysin proved to be more sensitive markers than chromogranin A in the diagnosis of FNED of PC. Very pronounced neuroendocrine differentiation was diagnosed in 16% PC and 4% PIN. There was no significant correlation between the degree of focal NED on one side, and the preoperative serum PSA, Gleason score and clinical stage, on the other hand. Based on the above, it can be concluded that the FNED has no significant role in the prognosis of the disease.

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