Expression of neuroendocrine differentiation markers in lethal metastatic castration-resistant prostate cancer

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SAINIO MIIKA: EXPRESSION OF NEUROENDOCRINE DIFFERENTIATION MARKERS IN LETHAL METASTATIC CASTRATION-RESISTANT PROSTATE CANCER

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Neuroendokriininen erilaistuminen on yleinen ilmiö eturauhassyövässä, ja joissakin tutkimuksissa sen esiintyminen primaarikasvaimessa on yhdistetty heikompaan ennusteeseen. Neuroendokriinisen erilaistumisen esiintyvyyttä ja merkitystä eturauhassyövän metastaaseissa on tutkittu tähän mennessä niukasti.

Tässä tutkimuksessa tarkastelimme kolmen eniten käytetyn neuroendokriinisen erilaistumisen merkkiaineen (kromograniini A, neuronispesifinen enolaasi ja synaptofysiini) ilmentymistä 89 metastaasissa, jotka kerättiin ruumiinavauksissa 31:ltä kastraatioresistenttiin eturauhassyöpään kuolleelta mieheltä. Vertasimme tuloksia 89 prostatektomianäytteeseen, jotka edustivat hormonisensitiivistä syöpää. Selvitimme myös neuroendokriinisten merkkiaineiden ilmentymisen yhteyttä androgeenireseptorin, ERG-onkogeenin ja Ki-67 -proliferaatiomerkkiaineen ilmentymiseen metastaaseissa.

Yksi 31:stä kuolemaan johtaneesta eturauhassyövästä oli morfologiselta luokitukseltaan pienisolukarsinooma, loput 30 luokiteltiin tyypillisiksi adenokarsinoomiksi. Metastaasit ilmensivät neuronispesifistä enolaasia ja synaptofysiiniä merkittävästi enemmän kuin primaarikasvaimet (6,3 % syöpäsoluista vs. 1,0 %, $p < 0,001$ ja 4,0 % vs. 0,4 %, $p < 0,001$, tässä järjestyksessä). Ainakin hajanaisia syöpäsoluja, jotka ilmensivät yhtä tai useampaa merkkiainetta, havaittiin 78 % metastaaseista, mutta laajempi positivisuus oli harvinaisempaa: 3/89 (kromograniini A), 8/89 (neuronispesifinen enolaasi) ja 5/89 (synaptofysiini) metastaaseista ilmensi merkkiaineita yli 10 % syöpäsoluista. Kromograniini A:n ja synaptofysiinin ilmentymisen välillä metastaaseissa oli merkittävä korrelaatio ($r= 0,64$, $p < 0,001$), mutta neuronispesifisen enolaasin ilmentymisellä ei ollut yhteyttä kahden muun merkkiaineen esiintymiseen. Neuroendokriinisen erilaistumisen laajuus vaihteli huomattavasti myös saman potilaan eri metastaasien välillä. Androgeenireseptorinegatiivisissa metastaaseissa havaittiin merkittävästi useammin laajaa (> 10 % soluista positiivisia) kromograniini A:n ja neuronispesifisen enolaasin ilmentymistä ($p = 0,02$ molemmissa) kuin androgeenireseptoripositiivisissa. Sen sijaan yhdenkään merkkiaineen hajanaisempi positiivisuus ei ollut yhteydessä androgeenireseptorin ilmentymiseen.

Neuroendokriininen erilaistuminen on hyvin monimuotoinen ilmiö kastraatioresistentin eturauhassyövän metastaaseissa, ja se on yhteydessä kasvaimen androgeenireseptorinegatiivisuuteen, joka puolestaan on yhdistetty heikompaan ennusteeseen.

Neuroendokriininen erilaistuminen on yleisempää kastraatioresistentissä syövässä hormonisensitiiviseen verrattuna. Lisää tutkimusta tarvitaan tarkentamaan neuroendokriinisen erilaistumisen merkitystä eturauhassyövän etenemisessä.

ABSTRACT

Neuroendocrine differentiation (NED) is a common phenomenon in prostate cancer, and it has been associated with poor prognosis in some studies of primary prostate cancer. Incidence and patterns of NED in metastatic prostate cancer sites have not been examined widely.

In this study, we studied expression of three commonly used markers of NED (chromogranin A, neuron specific enolase and synaptophysin) in 89 metastases from 31 men that died of castration-resistant prostate cancer and underwent rapid autopsy, and in 89 hormone-naïve primary tumors removed by radical prostatectomy. In addition, we examined NED association with androgen receptor, ERG and Ki-67 expression in metastatic tumor sites.

Morphologically, 1 of 31 cases was classified as small cell carcinoma, and the remaining 30 were classified as usual prostate adenocarcinoma using a recently proposed classification of prostate cancers with NED. Metastases showed more expression of neuron specific enolase and synaptophysin compared to prostatectomies (6.3% of cells vs. 1.0%, $p < 0.001$ and 4.0% vs. 0.4%, $p < 0.001$, respectively). At least focal expression of one of the markers was seen in 78% of metastases. Strong expression was relatively uncommon, seen in 3/89 (chromogranin A), 8/89 (neuron specific enolase), and 5/89 (synaptophysin) metastases. Expression of chromogranin A and synaptophysin correlated with each other ($r = 0.64$, $p < 0.001$), but expression of neuron specific enolase did not correlate with the two other markers. Extent of NED varied significantly between different metastatic sites in individual patients. Absent androgen receptor expression was associated with strong expression of chromogranin A ($p = .02$) and neuron specific enolase ($p = .02$), but not with focal expression of any marker. No clear association was found between expression of NE markers and ERG or Ki-67.

In conclusion, NED is a common and heterogeneous phenomenon in metastatic, castration-resistant prostate cancer. NED is more often present in castration-resistant prostate cancer compared to hormone-naïve disease, and it is associated with androgen receptor negativity. More research is needed to understand significance of NED in the progression of prostate cancer.

ABBREVIATIONS

PCa, prostate cancer; CRPC, castration-resistant prostate cancer; NED, neuroendocrine differentiation; NE, neuroendocrine; CgA, chromogranin A; NSE, neuron specific enolase; TMA, tissue microarray; DNPC, double-negative prostate cancer; AR, androgen receptor; ERG, ETS-related gene

TABLE OF CONTENTS

1 INTRODUCTION

Prostate cancer (PCa) is a highly prevalent cause of cancer death among males worldwide [1]. The majority of PCa deaths are caused by metastatic disease that has progressed to castration resistant prostate cancer (CRPC) after patients have received androgen deprivation treatment. It has been suggested that the presence of neuroendocrine differentiation (NED) in prostate cancer cells could have therapeutic implications for men with CRPC [2].

PCa cells with NED differ from neuroendocrine (NE) cells in normal prostate: PCa cells with NED have proliferative activity, and their protein expression is often different from normal NE cells. The origin of PCa cells expressing neuroendocrine markers is unclear. They might either originate from the same stem cells as normal NE cells, or be differentiated from cancerous prostate epithelia [3].

Cancer cells with NED can be found in 10-100% of all PCas [4]. This broad reported range is likely due to variable tissue imaging and staining techniques, and variable criteria used to evaluate NED in different studies [4]. Traditionally, PCas with NED have been categorized into conventional prostate adenocarcinomas with focal NED, carcinoid tumors and small cell NE carcinomas. However, Epstein et al. have recently proposed a new working classification consisting of usual prostate adenocarcinoma with NED, adenocarcinoma with Paneth cell -like NED, carcinoid tumor, small cell carcinoma, large cell NE carcinoma and mixed tumor with NE carcinoma and adenocarcinoma features [5].

NED is commonly focal in hormone-naïve primary PCa, usually constituting <1% of all tumor cells. Androgen deprivation therapy often leads to increased NED [6,7]. Increased NED has also been associated with poorly differentiated PCa (Gleason Score >6) [8]. NED prognostic significance in hormone-naïve and well-differentiated prostate cancer is controversial, although it has been researched in several studies [2,3,9]. However, NED appears to predict poorer prognosis in patients with high grade PCa both prior and after androgen deprivation therapy: Berruti et al. showed that NED predicts shorter time to PSA progression and shorter survival in patients treated with androgen deprivation therapy but not in patients that have “hormone-naïve” (no exposure to androgen deprivation) disease [10], and Krauss et al. found that primary PCas (Gleason Score ≥ 7) with >1% NE cell populations treated with radiation therapy only are more likely to develop distant metastases, whereas PCas (Gleason Score ≥ 7) with <1% NE cells have outcomes similar to those where NED is absent [11].

The most commonly used immunohistochemical markers for detecting NE phenotype cells are chromogranin A (CgA), neuron specific enolase (NSE) and synaptophysin [12]. Despite being widely used, it has recently been speculated that NSE might not be a sufficiently specific marker

for NED [5]. Of these three markers, CgA appears to be the most specific in detecting NED and predicting the prognosis of PCa [12].

The incidence of NED in primary prostate tumors and its effect on clinical outcome has been examined in several previous studies [2,10,11]. However, although NED in primary PCa is associated with higher incidence of metastatic disease, few articles have focused on NED in metastatic tumor sites.

Mucci et al. described CgA and synaptophysin expression in 2.8% and 2.7% of metastatic CRPC autopsy samples, respectively, using tissue microarray (TMA) technique. However, both CgA and synaptophysin expression was detected five times more often in whole tissue sections of the same samples [13]. Shah et al. and Roudier et al. reported in their autopsy studies of patients that had died of metastatic CRPC, that expression of CgA or synaptophysin was mostly focal, and was not associated with survival of patients [14, 15]. In a recent autopsy study, Bluemn et al. evaluated 10% of metastatic CRPCs as neuroendocrine PCa. In the same study, they observed a significant increase (5% to 21%) in proportion of a “double-negative prostate cancer” (AR negative, NED marker negative, DNPC) in men who died of metastatic CRPC in 1997-2011 (pre-approval of abiraterone and enzalutamide) vs 2012-2016 (post approval of these drugs). In the same study, the proportion of neuroendocrine PCa did not differ among the two groups [16]. In other related work, Aprikian et al. and Quek et al. studied NED in hormone-naïve PCa lymph and bone metastases [17,18]. CgA was expressed in 46% [16] and 12% [18] of lymph node metastases, and in 52% of bone metastases [17]. Quek et al. reported that any CgA expression in lymph metastases predicted worse prognosis [18].

In total, NED in metastatic CRPC tissue has previously been studied in only 140 patients in four different studies [13–16]; Hormone-naïve metastatic tissue has been studied in 202 patients, 54 of whom had received hormonal treatment but reportedly had not developed castration resistance at the time of obtaining study specimens [17,18]. Supplementary Information contains a more detailed review of these six studies focused on NED in metastatic CRPC.

To extend understanding of the role of NED in lethal metastatic PCa, we examined NED in multiple metastatic sites in 31 men. We used three different NE markers, CgA, NSE and synaptophysin, and examined NED association with AR expression, ERG expression, and proliferation marker Ki-67.

2 MATERIALS AND METHODS

The use of clinical material was approved by the ethical committee of the Tampere University Hospital (TAUH) and the National Authority for Medicolegal Affairs and the Johns Hopkins Medicine Institutional Review Board (autopsy samples).

Our study material consists of 136 metastases obtained in rapid autopsies from 32 men who died of metastatic CRPC between 1995 and 2005. These men consented to participate in an integrated clinical-molecular study of lethal metastatic prostate cancer (PELICAN study), and received care both in community practice and tertiary oncology centers as previously reported [19]. The average age at diagnosis was 63 years (range 40-79), and average years between diagnosis and death was 6 years (range 1-15 years). Among the 32 men, ancestry was as follows: White, Hispanic (1), African-American, Non-Hispanic (4), and White, Non-Hispanic (27). Definitive pelvic radiation therapy was received by 16 of these men, and radical prostatectomy was performed in 8 men. After metastatic disease became clinically evident, all 32 men received androgen deprivation therapy of various kinds, including orchiectomy in 10, and various drug therapies in 29. Clinical data for the autopsy cases are tabulated in Supplementary Table 1.

In addition, we studied 116 primary PCas in men undergoing radical prostatectomy in Tampere University Hospital between 1995 and 1997. These patients had not been treated with androgen deprivation or other prostate cancer therapy prior to radical prostatectomy. The average age at diagnosis was 63 years (range 51-77 years).

All tissue samples examined were formalin-fixed and paraffin-embedded. Representative areas of tissue blocks were chosen, and TMA cores combined in TMA blocks as described previously [20]. CgA, NSE and synaptophysin immunostains were performed at Fimlab laboratories (Tampere, Finland) using a Leica Bond III automated immunostain system (Leica Biosystems Newcastle Ltd). Deparaffinization and epitope retrieval were performed onboard using Leica Biosystems reagents (Bond Dewax AR9222 and Bond Epitope retrieval 2, AR9640). All antibodies were diluted in Bond antibody diluent (AR9352) and antibody incubation time and temperature for each antibody was 30 min at room temperature. The clones and dilutions for each antibody were as follows: CgA (Dako A430, Clone polyclonal) 1:2000 dilution; NSE (Dako M0873, Clone BBS/NC/VI-H14) 1:5000 dilution; and synaptophysin (Leica Novocastra PA02999, Clone 27G12) as prediluted by supplier. All immunostains were detected and visualized using Bond Polymer Refined Detection kit DS9800 (Leica Biosystems Newcastle Ltd).

Adjacent 4 micron sections from the PELICAN and Tampere TMAs were immunostained for CgA, NSE, and synaptophysin. Within each TMA spot a representative region of 100 contiguous tumor cells was visually selected, and the number of cells expressing the NE marker were counted. All

cells were counted if there were under 100 tumor cells in the sample. In addition, 100 representative normal glandular cells were counted in the prostatectomy samples if found, and scored similarly. For some analyses we classified the metastases into three groups according to the fraction of cells expressing each NE marker. Over 10% of cells showing positivity to the marker

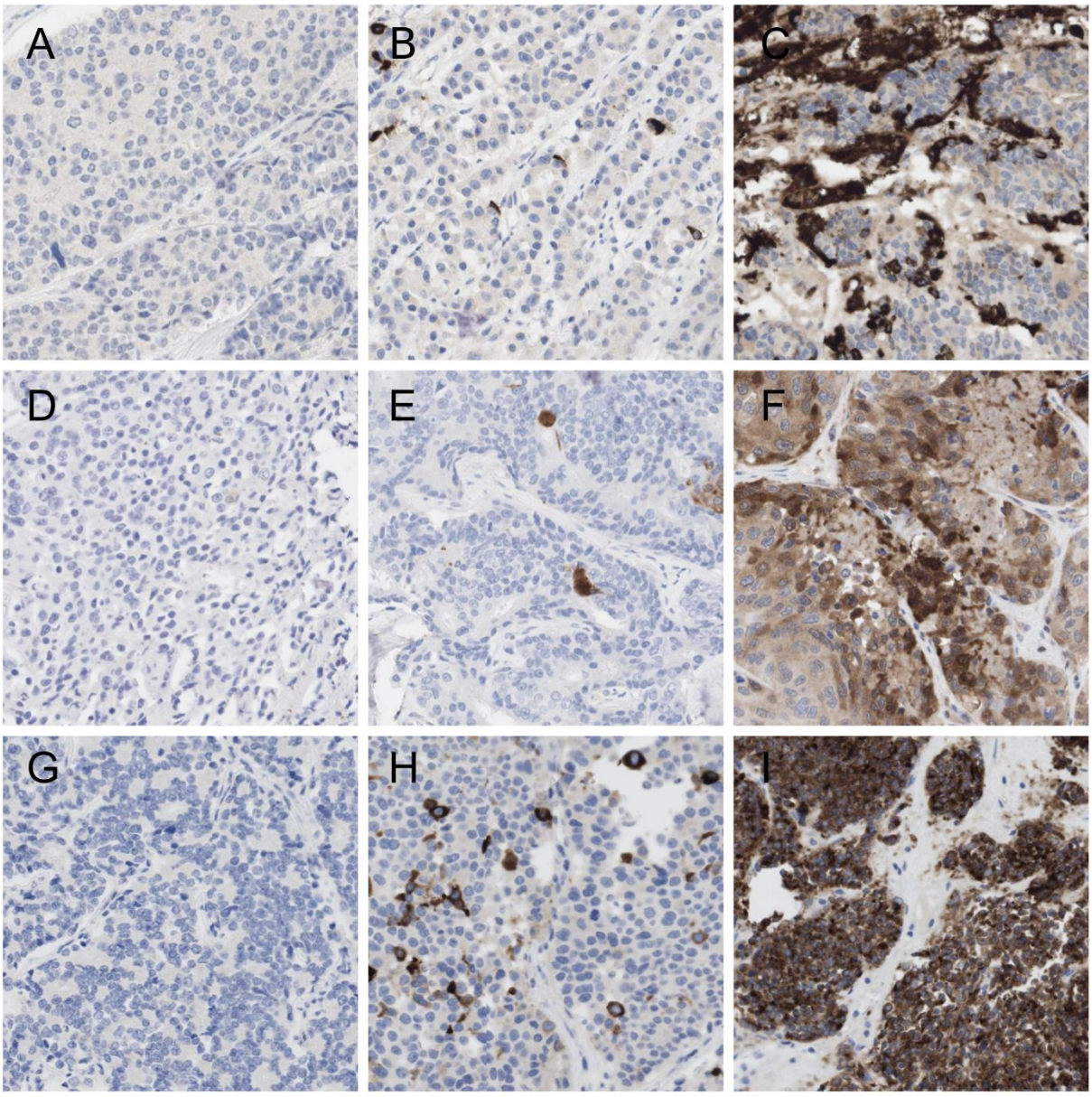

***Figure 1**. Immunohistochemistry: chromogranin A (CgA), neuron specific enolase (NSE) and synaptophysin (Syn) expression in metastases. 20x magnification. (A) A21 rib metastasis with no CgA expression. (B) A16 pericardial metastasis with focal CgA expression. (C) A31 adrenal metastasis with strong CgA expression. (D) A32 subclavicular lymph node metastasis with no NSE expression. (E) A25 femur metastasis with focal NSE expression. (F) A30 liver metastasis with strong NSE expression. (G) A22 periaortic lymph node metastasis with no Syn expression. (H) A10 periportal lymph node metastasis with focal Syn expression. (I) A23 periaortic soft tissue metastasis with strong Syn expression.*

was classified as strong expression, 1-10% positivity as focal expression and 0% as no expression. Figure 1 shows representative images from the immunostained metastasis TMA slides illustrating no expression, focal expression and strong expression of each NE marker, and representative whole TMA spot images for all immunostains used in the study are shown in Supplementary Figure 1. Examples of stains in the Tampere prostatectomy samples are shown in Figure 2. Scoring was performed first by a bachelor of medicine, and after that verified by a surgical pathologist.

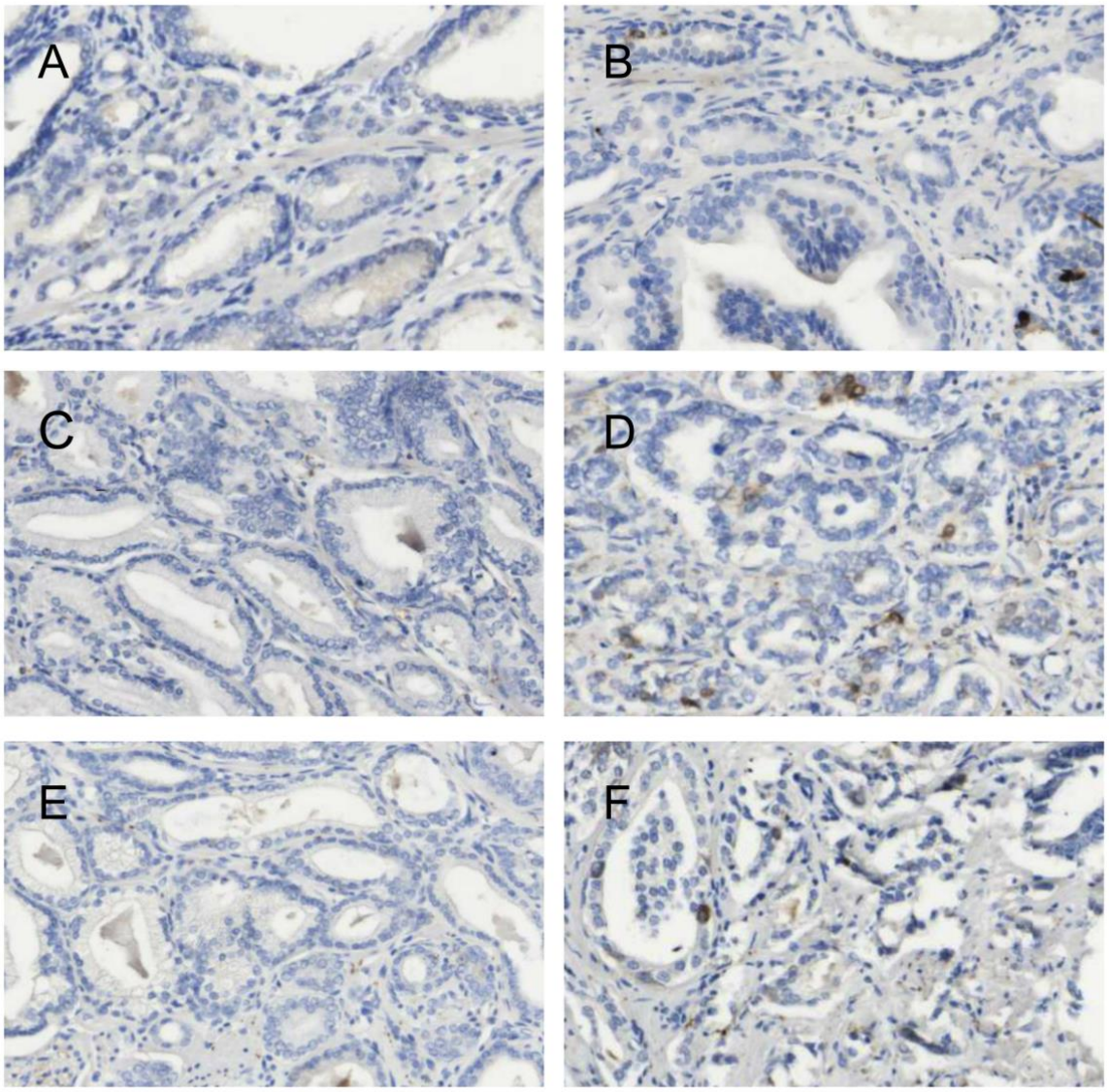

***Figure 2**. Immunohistochemistry: chromogranin A (CgA), neuron specific enolase (NSE) and synaptophysin (Syn) expression in prostatectomies. 15x magnification. (A), (C) and (E) show no expression of neuroendocrine markers. (B), (D) and (F) show single cells expressing CgA, NSE and Syn, respectively. Benign prostate tissue can also be seen along with cancer.*

We combined CgA, NSE, and synaptophysin results with previously performed stains of the same array for androgen receptor (AR), ETS-related gene (ERG) and proliferation marker Ki-67, where antibodies used in immunohistochemistry were: 318 (Novocastra Laboratories Ltd.) against AR, EPR3864 (Epitomics Inc.) against ERG and 138G6 (Cell Signaling Technology) against Ki-67 [20]. For AR and ERG, TMA spots were scored positive/negative, and for Ki-67 the percentage of cells expressing the protein was determined as described [20]. ERG-staining was used as an internal control of successful staining for metastases. ERG is always expressed in normal vascular endothelial cells [21]. We excluded samples that did not show ERG positivity in normal endothelium from analysis due to possible autopsy-associated tissue degradation.

Of 136 metastases addressed in the study, 89 samples stained for CgA, NSE and synaptophysin were available for analyses from 31 men. 47 samples were excluded for the following reasons: missing spot (26), no cancer in spot (6), failed staining (4), and absent ERG staining in endothelial cell nuclei (11). From previous staining of slides from the same TMA, we had 89 metastatic samples scored for AR, 88 for ERG and 88 for Ki-67.

Of 116 Tampere prostatectomies, 89, 87 and 87 samples contained PCa that could be scored for CgA, NSE and synaptophysin, respectively. 15 samples were excluded for the following reasons: missing spot (9), no prostate epithelium (1), and failed staining for all three stains (5). In addition, in 2 spots NSE staining failed, and in 2 other spots synaptophysin staining failed and these spots were excluded. 12 spots contained only benign prostate tissue, so these samples were used only in analyses of staining in noncancerous prostate epithelium. Normal glandular prostate cells could be scored for CgA in 70 prostatectomy samples, for NSE in 65 samples and for synaptophysin in 67 samples. Other prostatectomy samples contained only tumor cells.

Follow-up data was available in 110 of 116 patients, of which CgA, NSE and synaptophysin expression in tumor cells could be scored in 84, 82 and 83 patients, respectively. The average follow-up time was 7.7 years (range 1 month - 20 years). Progression was defined as elevated serum PSA levels (>0.4 ng/ml) in two consecutive samples. Where two such measurements occur, the date of the first measurement is used as the date of progression.

Mann-Whitney, Chi-square and Fisher's exact tests were used as measures of statistical significance. Relationships between different markers were tested by Spearman correlation. Kaplan-Meier analysis was used for comparison of PSA progression among staining groups. P-values below 0.05 were considered statistically significant. Statistical computations were performed by Graphpad Prism 7.01 software.

3 RESULTS

CgA was expressed in 23 of 89 CRPC metastases. 20 of these showed focal positivity (1-10% of tumor cells expressed the marker), and in 3 metastases more than 10% of cells were stained positively for CgA. NSE expression was observed in 57 metastases. In 49 metastases ≤10% of cells were positive, 8 showed NSE positivity more widely. 32 metastases showed positivity for synaptophysin. Focal expression was seen in 27 metastases, 5 expressed the marker strongly. Only 20 metastases were completely negative for all three markers; 78% of all metastatic samples showed at least some NED marker expression.

All 31 men from whom metastatic CRPC tissue was available for study typical prostate adenocarcinoma in initial biopsy and/or radical prostatectomy material according to their biopsy report and surgical pathology report in the 8 men who underwent radical prostatectomy as part of clinical treatment. Morphologically, one of these 31 men (A23) was noted to have small cell features in his CRPC material studied at autopsy. Otherwise, all CRPC metastases were morphologically typical adenocarcinomas with or without NE marker expression. No Paneth cell - like NED, carcinoid tumors, large cell NE carcinomas or mixed NE carcinoma-acinar adenocarcinomas were found [5].

Tampere prostatectomy samples showed CgA expression in 29/89, NSE in 38/87 and synaptophysin in 11/87 samples. Positivity was focal in almost all samples. However, one prostatectomy sample stained strongly for all three markers. Non-tumor cells expressed CgA in 16/70, NSE in 10/65 and synaptophysin in only 5/67 samples. All prostatectomy samples were typical prostatic adenocarcinomas. Table 1 shows the distributions of strong, focal and negative NE marker expression in metastases and prostatectomies.

Table 1. *Distribution of strong, focal and no expression of chromogranin A (CgA), neuron specific enolase (NSE) and synaptophysin in metastases and prostatectomies. Percentage of spots categorized for each staining level for each marker is shown in parentheses.*

	CgA expression			NSE expression			Synaptophysin expression		
	<1%	**1-10%**	**>10%**	**<1%**	**1-10%**	**>10%**	**<1%**	**1-10%**	**>10%**
Metastases, n (%)	66 (74.2)	20 (22.5)	3 (3.4)	32 (36.0)	49 (55.1)	8 (9.0)	57 (64.0)	27 (30.3)	5 (5.6)
AR positive, n (%)	60 (74.1)	20 (24.7)	1 (1.2)	31 (38.3)	45 (55.5)	5 (6.2)	53 (65.4)	25 (30.9)	3 (3.7)
AR negative, n (%)	6 (75.0)	0 (0)	2 (25.0)	1 (12.5)	4 (50.0)	3 (37.5)	4 (50.0)	2 (25.0)	2 (25.0)
ERG positive, n (%)	31 (72.1)	11 (25.6)	1 (2.3)	13 (30.2)	29 (67.4)	1 (2.3)	25 (58.1)	17 (39.5)	1 (2.3)
ERG negative, n (%)	34 (75.6)	9 (20.0)	2 (4.4)	18 (40.0)	20 (44.4)	7 (15.6)	31 (68.9)	10 (22.2)	4 (8.9)
Prostatectomies, tumor, n (%)	60 (67.4)	28 (31.5)	1 (1.1)	49 (56.3)	37 (42.5)	1 (1.1)	76 (87.4)	10 (11.5)	1 (1.1)
Prostatectomies, normal, n (%)	54 (77.1)	16 (22.9)	0 (0)	55 (84.6)	10 (15.4)	0 (0)	62 (92.5)	5 (7.5)	0 (0)

Elevated PSA levels were seen in 63 of 110 Tampere patients during follow-up. Expression of CgA, NSE or synaptophysin in ≥1% of tumor cells in prostatectomy sample was not associated with higher risk to PSA progression compared to patients with absent expression of the NE markers. Supplementary Figure 2 shows Kaplan-Meier PSA progression-free survival curves comparing patients with 0% and ≥1% NE marker expression; A trend of higher progression in the ≥1% CgA group is seen, but is not statistically significant. The NSE and synaptophysin plots are nearly identical in the two groups.

Metastatic tumor samples featured significantly more NSE and synaptophysin expression than prostatectomies. Average NSE expression was 6.3% in metastases and 1.0% in prostatectomies ($p<0.001$). As for synaptophysin, on average 4.0% of cells in metastases and 0.4% of cells in prostatectomies showed expression ($p<0.001$). Normal glandular prostate cells found in prostatectomy samples expressed CgA, NSE or synaptophysin only in 0.4%, 0.2% and 0.1% of cells on average, respectively. All mean expressions of the three markers in metastases and prostatectomies (both tumor cells and normal cells) are shown in Table 2.

AR negative metastases showed strong (>10% of cells positive) CgA and NSE expression significantly more often than AR positive cases. 25% of AR negative metastases expressed CgA strongly, whereas only 1% of AR positive samples showed strong expression ($p=0.02$). 38% of AR negative and 6% of AR positive samples showed strong NSE expression ($p=0.02$). For synaptophysin, 25% of AR negative and 4% of AR positive metastases expressed synaptophysin in over 10% of their cells ($p=0.06$). Table 3 shows the frequencies of strong (>10%) and focal/non-existent (0-10%) expression of the three markers in AR negative and positive metastases.

Table 2. *Mean expressions (% of cells expressing the marker) of chromogranin A (CgA), neuron specific enolase (NSE) and synaptophysin (Syn) in metastases and prostatectomies.*

	Metastases (%)	Prostatectomies, tumor cells (%)	P-value	Prostatectomies, normal cells (%)	Metastases, AR positive (%)	Metastases, AR negative (%)	Metastases, ERG positive (%)	Metastases, ERG negative (%)
CgA	1.9	1.0	0.57	0.4	0.8	12.6	0.9	2.9
NSE	6.3	1.0	<0.001	0.2	3.5	34.3	3.7	8.9
Syn	4.0	0.4	<0.001	0.1	3.4	10.9	2.7	5.4

Metastases expressing NSE strongly (>10%) were ERG negative more frequently than metastases showing focal (1-10%) NSE expression ($p=0.02$). However, CgA and synaptophysin had no significant association with ERG status of metastases.

Expression of CgA and synaptophysin had a correlation coefficient of 0.64 ($p<0.001$) in metastases. Otherwise correlations between different NE markers were relatively weak and statistically nonsignificant. All correlations between NE markers are shown in Table 4. Proliferation marker Ki-67 had no significant association with any of the three NE markers.

Table 3. *Expression of neuroendocrine markers in androgen receptor (AR) positive and negative metastases.*

	CgA 0-10%	CgA >10%	Total	p-value
AR positive	80	1	81	
AR negative	6	2	8	
Total	86	3	89	0.02

	NSE 0-10%	NSE >10%	Total	p-value
AR positive	76	5	81	
AR negative	5	3	8	
Total	81	8	89	0.02

	Syn 0-10%	Syn >10%	Total	p-value
AR positive	78	3	81	
AR negative	6	2	8	
Total	84	5	89	0.06

Of the 89 metastatic samples studied, 46 were located in lymph nodes, 11 in bone and the remaining 32 were classified as visceral metastases (7 subdural, 6 adrenal, 5 liver, 3 seminal vesicle, 2 lung, 2 urinary bladder, 2 pericardial and 1 spleen, diaphragmatic, periadrenal, periaortic soft tissue and retroperitoneal mass metastasis). No significant differences were found in expression of CgA, NSE, synaptophysin, AR, ERG or Ki-67 between lymph, bone and visceral metastases. Anatomic locations of metastases are shown in Supplementary Table 1.

Table 4. *Correlations between expression of chromogranin A, neuron specific enolase and synaptophysin in metastases.*

	CgA		NSE	
	r	p-value	r	p-value
CgA				
NSE	0.06	0.60		
Syn	0.64	<0.001	0.07	0.50

The degree of NED, estimated by expression of CgA, NSE and synaptophysin, varied noticeably among metastases of individual patients as shown in Figure 3. Only 3 of the 31 patients (A14, A23

and A31) showed strong, >10% CgA expression, and all three showed this in only one metastasis, while the rest of their metastases were either completely negative or expressed CgA focally. Interestingly, one metastasis of A14 stained strongly for CgA and was AR negative, whereas the remaining two were negative for CgA and were AR positive. However, all five metastases of A31 were AR positive, including the one showing strong CgA expression. As for A23, the small cell carcinoma case, only one metastasis was analyzable, and was both AR negative and showed strong CgA expression (Supplementary Figure 1).

Similar heterogeneous NE marker expression within individual patients could be observed also for NSE. 7 patients had >10% NSE expression in some of their metastases. 2/4 metastases of A5 expressed NSE strongly. A4, A14, A19, A23, A30 and A31 had one metastasis stained strongly for NSE, in addition. A5 and A23 had remarkably strong (>70%) NSE expression in their above-mentioned metastases, and all metastases of those patients (also the 2 metastases of A5 showing only focal expression) were AR negative. As for A4, A19, A30 and A31, all their metastases were AR positive. Some metastases of A14 expressed AR and others did not, but a pattern between AR and strong NSE expression could not be found.

>10% synaptophysin expression was found in 5 patients: A2, A14, A16, A18 and A23. All five patients showed strong synaptophysin expression only in one of their metastases. A14 had one AR negative and two AR positive metastases as mentioned above. The AR negative metastasis showed strong synaptophysin expression, while the two AR positive metastases were completely negative for synaptophysin and also for chromogranin A. The rest of the patients did not show any clear association between their AR and synaptophysin status.

4 DISCUSSION

In this study we analyzed expression of three commonly used NE markers in 89 PCa metastases in a cohort of 31 men who died of metastatic CRPC. Six prior studies have evaluated NED in PCa metastases and the results vary in these reported studies, resulting from different definitions of NED and different study materials: i.e. both standard slide and TMA techniques have been used, some studies included hormone-naïve PCas in their study population while others studied only CRPC, different markers have been used to evaluate NED and some studies have limited their analyses to only lymph node or bone metastases [13–18]. In our study we tried to establish a reproducible standard method for scoring based on evaluating individual cell expression within representative areas in TMA-based samples. This allowed us to set distinct percent categories for strong and focal expression. To our knowledge, it is the first reported study to analyze expression of all three commonly used markers of NED in metastatic CRPC. The research material we used

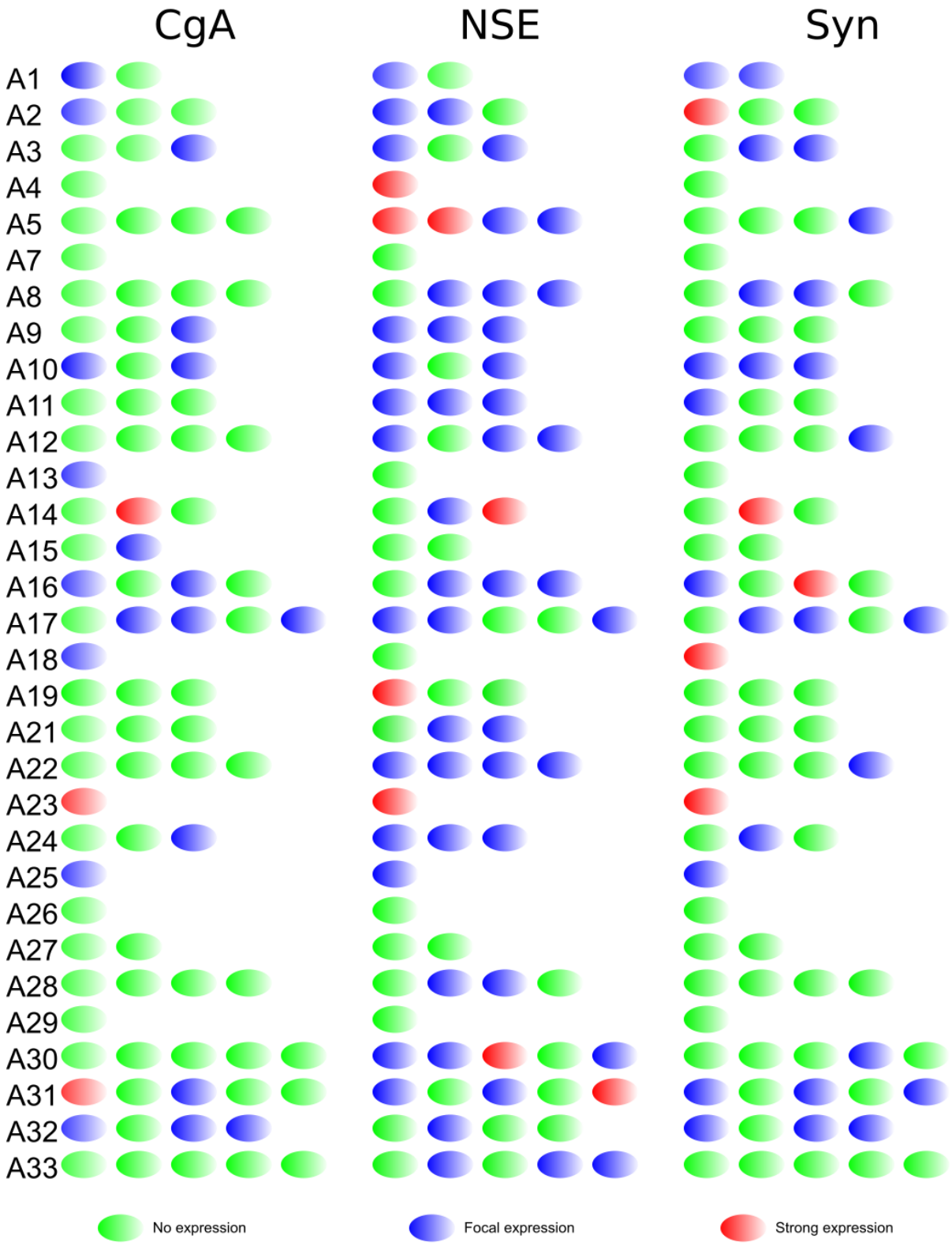

***Figure 3**. Expression of neuroendocrine markers in metastases. The results illustrate intra and inter-patient heterogeneity in expression of all three NED markers (CgA, NSE, Syn).*

(in one group all patients had lethal metastatic CRPC and in the other group patients had not received any treatment prior to prostatectomy) made it possible to compare expression of CgA, NSE, synaptophysin, AR, ERG and Ki-67 in detail. A limit of our study was the small number of cases available, limiting subgroup analysis. For example, the number of AR negative metastases, only 8, was a restriction for more detailed examination, although we managed to observe significant association between AR negativity and strong expression of CgA and NSE.

AR negativity in primary PCa has been associated with NED marker expression and poor prognosis [22,23]. The work reported here is the first to show that NED, evaluated by expression of NE markers, associates with AR negativity also in metastatic CRPC tissue. Strong, >10% expression of CgA and NSE was more common in AR negative metastases. In contrast, focal expression (1-10% of cells) of NED markers had no detectable association with the AR status of metastases.

Bluemn et al. described a recent increase in “double-negative” CRPC (both AR and NE marker negative) [16]. In our material, all 8 AR negative metastases showed NED to some extent, however, 4 of these metastases expressed NE markers only focally, and would likely have been considered NE negative and thus would likely be designated DNPC tumors using the scoring system described in Bluemn et al. [16]. If these 4/89 (4.5%) of metastases are considered DNPC, this rate of DNPC is similar to the results of the Bluemn et al. study, where 5% of all CRPC metastases collected between 1998 and 2011 were DNPC. Cases collected after introduction of enzalutamide and abiraterone in 2011 showed a DNPC rate of 21% in Bluemn et al. The autopsy CRPC material studied here was collected between 1995 and 2005, before approval of enzalutamide and abiraterone, so our results are consistent with those of Bluemn et al.

It is a commonly thought that PCa cells with NED do not express AR [24,25], however AR positive NE cell populations in PCa have also been described [26]. In our material, 81/89 metastases were classified as AR positive, and many of these showed NED to some extent. Most AR positive metastases had scattered AR negative subpopulations of cells. Because dual AR and NE marker staining was not performed in our study, we cannot determine if these small AR negative subpopulations express NED markers.

However, in one case, in TMA spots separated by 8 microns, we found evidence of two distinct coexisting subpopulations of bone metastatic PCa cells, small islands of AR-NED+ cells among a mass of AR+NED- cells (Supplementary Figure 3). Such subpopulations have been described in primary prostate cancer [24,25], but to our knowledge this phenomenon has not been detected

previously in metastatic PCa to bone or other organs. Additional studies are needed to determine if this is a common phenomenon in metastatic PCa, and determine the origin of this biphasic phenotype. This could be evidence of tissue organization-based plasticity [27], and could have implications for further examination of the tumor dormancy [28]. In similarly adjacent sections, there is also evidence that some PCa metastases are populated largely by AR+NED+ cells (Supplementary Figure 3). To our knowledge, this is the first time this has been reported.

In our material CgA was expressed in 26%, NSE in 64% and synaptophysin in 36% of the metastases. CgA and synaptophysin expression was more common in our material compared to previous studies by Mucci et al. [13], Shah [14], Bluemn [16] and Quek et al. [18]. On the other hand, Aprikian et al. observed focal CgA expression in 46% of bone and 52% of lymph node metastases in their mostly hormone-naïve cohort [17]. However, direct comparison between the studies is difficult and probably not especially useful due to different scoring systems and research material.

The expression of NE markers in Tampere prostatectomy samples that had not received androgen withdrawal therapy was weak, focal and clearly less common compared to metastases. As it has been shown earlier, androgen deprivation is associated with increased NED, so this confirms these prior findings [6,7]. Normal glandular cells found in prostatectomy samples expressed NE markers in 0.1-0.4% of cells, which is in line with previous studies reporting that <1% of normal prostate cells are neuroendocrine [2]. Expression of the three NE markers differed clearly between normal NE cells and neuroendocrine differentiated cancer cells in our material: in normal NE cells CgA was the most expressed marker and synaptophysin the least, whereas NSE was most expressed among cancer cells and CgA the least. Different protein expression patterns among normal NE cells of the prostate, and prostate cancer cells displaying NED has been suggested to indicate that the cells arise from different lineages [3].

We also studied association between primary PCa CgA, NSE and synaptophysin expression and PSA progression in the Tampere patients. Prognostic significance of NED in hormone-naïve primary PCa is controversial [2]. Neither NSE nor synaptophysin expression has been associated with poorer outcomes in any study that we are aware of [2]. In the 84 Tampere PCa patients studied here with average follow-up of 7.7 years, by Kaplan-Meier analysis, a higher rate of progression in the ≥1% CgA group is seen, but is not statistically significant. The NSE and synaptophysin plots are nearly identical in the two groups (Supplementary Figure 2).

Heterogeneity of NED in CRPC metastases was remarkable in our study. Although CgA, NSE, and synaptophysin are all widely used and accepted markers of NED, their expression did not correlate strongly with each other, correlation between CgA and synaptophysin being the only one to reach

statistical significance. Only one metastasis showed strong expression of all three markers, and this was from case A23, which showed small cell features at autopsy. 78% of metastases expressed at least focally one of the markers, indicating that low degrees of NED can be found in most of the PCas progressed to CRPC. Expression of single NE markers varied considerably also between metastases of one patient. 12/31 patients had both CgA positive and negative metastases, 16/31 both NSE positive and negative metastases, and 14/31 had similar mixed synaptophysin expression. Previous genomic studies in a subset of the 31 rapid autopsy cases studied here showed that metastases in individual men derive from a common clonal ancestor [19,29] but also show strikingly variable degrees of subclonal divergence. The source of NED heterogeneity observed among metastases in the current could be due to subclonal genomic variation, or could be due to differences in local microenvironment or other factors. However we did not observe any association between expression of NE markers and locations of metastases. Similar NED heterogeneity was described previously among bone metastases in individual patients with CRPC in rapid autopsy study by Roudier et al. using CgA staining [15]. Interestingly, expression of PSA and AR varied in a similar way between metastases of single patients in the autopsy study by Shah et al. However, they did not examine heterogeneity of NED in their study material [14].

A possible caveat of our study, also relevant to previous autopsy studies that have used immunohistochemistry, is that stability of different proteins at different tissue sites might vary after death. Degradation rates of different proteins in different tissue sites after death have not been studied very widely, and most papers involve proteins of brain tissue, as autopsy studies have been a major method in research of diseases like Alzheimer's. Stan et al. studied stability of several proteins with different molecular weights in brain tissue, and established that most proteins preserve their stability >40h after death [30]. Of the proteins used in our study, synaptophysin immunoreactivity has been found to begin decreasing significantly 36h post mortem [31]. Intensity of AR immunoreactivity has been shown to decrease already 6h after death in rat brains, but on the other hand, the same study stated that AR intensity was still moderately strong 24h after death [32]. However, it is unknown whether results of protein stability studies performed in animals or normal human tissues can be generalized to PCa, and furthermore, stability of some proteins we examined have not been examined at all in any tissue.

In addition to the time interval between death and autopsy, size of the samples, storage temperature and quality and quantity of used fixative can have impact on protein stability through resulting variations in proteolytic enzyme activity. The time elapsed between obtaining samples and stabilizing it by formalin fixation is also critical to protein stability [33]. In our study, rapid autopsies were performed on average 4h 10min after death (range 2h 30min to 9h 30min), and snap-frozen rapidly in liquid nitrogen. Delay of <10h indicates that probably postmortem

degradation of proteins does not have a prominent effect on our results, but possible bias cannot be ruled out before protein stability has been researched in prostatic cancer tissue.

Whether ERG expression affects development of NE phenotype of PCa has not been widely researched. A recent study found an association between TMPRSS2:ERG-fusion and CgA expression in prostatectomies of CRPC patients [34]. In contrast, another recent study using cell lines reported that ERG positivity could predict reduced NED, associating ERG negativity with NED [35]. In our material of CRPC metastases ERG negativity was associated with strong expression of NSE ($p=0.02$) , confirming the findings of Mounir et al [35]. However, we found no association between ERG status and two other NE markers, CgA and synaptophysin, so this result should be interpreted with caution. Also, it has been shown that AR negative prostate cancer cells do not express ERG even if TMPRSS2: ERG-fusion is present [36], which might have affected our results. In our material, none of 8 AR negative metastases expressed ERG, as expected. Research with larger cohort sizes are needed.

Expression of Ki-67 is an independent predictor of aggressive disease and poor prognosis in PCa [37]. Association between Ki-67 and NE markers in PCa has not been researched previously, and as both Ki-67 and NED predict aggressive PCa, we decided to investigate if any correlation could be found. None of the three NE markers had significant association with Ki-67 expression in our study. Prostatic small cell carcinomas typically express Ki-67 in most tumor cells [5], but we were unable to confirm this in single small cell cancer case as Ki-67 staining showed unexpected strong cytoplasmic positivity (Supplementary Figure 1, case A23) making nuclear Ki-67 difficult to distinguish. It is not clear whether this represents false positive cytoplasmic activity due to endogenous peroxidase activity, or whether it represents actual Ki-67 positivity in the cytoplasm.

5 CONCLUSIONS

NED marker expression is a common and heterogeneous phenomenon in metastatic castration resistant prostate cancer. Expression of chromogranin A, synaptophysin and neuron specific enolase do not correlate strongly with each other, although all three are widely used markers of neuroendocrine differentiation. Metastatic prostate cancer cells expressing both neuroendocrine markers and androgen receptor, as well as cells that express neuroendocrine markers but not androgen receptor, were observed. Our study confirms that strong expression of chromogranin A and neuron specific enolase associates with absent androgen receptor expression in metastatic CRPC consistent with earlier studies in hormone-naïve primary tumors. In contrast, focal expression of neuroendocrine markers did not predict the expression of androgen receptor.

Neuroendocrine marker expression in primary prostate cancer removed by radical prostatectomy was not associated with significantly worse rate of progression in our study.

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7 SUPPLEMENTARY DATA

Supplementary files can be found and opened here: https://doi.org/10.1016/j.prp.2018.04.015.

Supplementary Information contains a more complete version of a review of published studies of NED in prostate cancer.

Supplementary Table 1, metastases file contains clinical data and tissue microarray spot readings in metastatic prostate cancer autopsy patients.

Supplementary Table 2, prostatectomies file contains all tissue microarray reading data from prostatectomy samples.

Supplementary Figure 1 contains representative TMA spot images from H&E, PSA, CgA, NSE, Syn, Ki-67, AR and ERG stains of one of the metastases studied in cases A5, A8, A11, A16, A21, A23, and A31.

Supplementary Figure 2 shows PSA progression curves in patients with and without CgA, NSE and synaptophysin expression in their hormone-naïve prostatectomy sample.

Supplementary Figure 3 contains TMA spot images from AR, CgA, NSE, and synaptophysin stains from case A16 pericardial metastasis, and A25 bone metastasis.