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ProSAAS peptide of the granin protein family in biochemical diagnostics of pheochromocytoma

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Abstract

Introduction: Pheochromocytoma is a hormonally active tumour originating from neuroendocrine cells of the adrenal medulla. Chromogranin A (CgA) and peptide proSAAS belong to the family of granins and are present in neuroendocrine cells of adrenal medulla, from where they are released to circulation, along with catecholamines. The aim of this study was to assess the usability of proSAAS peptide assay in patients with adrenal pheochromocytoma.

Material and methods: 23 patients (13 females and 10 males) with adrenal pheochromocytoma (benign in 18 patients and malignant in 5) confirmed by histopathology examination, and 35 blood donors as a control group. Plasma free metanephrines, CgA, and proSAAS peptide levels were measured in all participants.

Results: CgA and proSAAS levels in the group of pheochromocytoma patients *vs.* the control were: 209 ng/mL and 0.8 ng/mL *vs.* 59 ng/mL and 0.3 ng/mL ($p < 0.001$), respectively. The following sensitivity and specificity indexes were obtained from ROC curves for CgA: 83% and 92%, respectively, and for the proSAAS peptide: 39% and 88%, respectively. The combination of 2 parameters: normetanephrine and proSAAS (96% and 100%) had a high diagnostic value, and the value of all determined parameters together (metanephrine, normetanephrine, CgA, and proSAAS) was 100%.

Conclusion: A single determination of the proSAAS peptide level is associated with a rather low diagnostic value. But collective determination of CgA and proSAAS may be an additional, valuable tool in biochemical diagnostics of pheochromocytoma. **(Endokrynol Pol 2022; 73 (2): 330–335)**

Key words: *pheochromocytoma; proSAAS; chromogranin A; CgA; adrenal tumour*

Introduction

Pheochromocytoma is a rare cancer. It occurs in 0.05–0.2% of patients with arterial hypertension. In patients with adrenal incidentaloma its incidence is 0.6–4.2%. It usually develops at the age of 30–40 years, but it may also occur in young people (family form) and in the elderly [1, 2]. Biochemical diagnostics consists of demonstrating the hormonal activity of the tumour by measuring the tumour-specific hormones or their metabolites in blood and/or urine. The most sensitive specific biomarker for the presence of pheochromocytoma is plasma free metanephrines [3]. The adrenal medulla is made of neuroendocrine cells, which can produce and secrete various proteins (e.g. granins/secretogranins), along with catecholamines, to the blood. The best-known protein from this family is chromogranin A (CgA) [4]. In addition to CgA, neuroendocrine cells are able to produce other proteins, including proSAAS peptide [5]. The aim of this study is to evaluate of the clinical usefulness of this peptide in the biochemical diagnostics of patients with adrenal pheochromocytoma.

Material and methods

Patients

Twenty-three patients (13 women aged 36–87 years, 10 men aged 41–81 years) with pheochromocytoma were recruited. Eighteen cases of benign pheochromocytoma and five cases of malignant form were diagnosed.

The following were obtained from all patients: clinical history, basic laboratory tests and specialist biochemical tests (plasma metanephrine and normetanephrine levels, serum CgA concentration), abdominal imaging [computed tomography (CT) and/or magnetic resonance imaging (MRI)]. Also, in selected patients scintigraphy [^{123}I-metaiodobenzylguanidine scintigraphy (^{123}I-MIBG) and/or somatostatin receptor scintigraphy (SRS)] was also done. After pharmacological preparation, all patients were referred for surgical treatment (laparoscopic or classic surgery). The diagnosis of pheochromocytoma was confirmed by histopathological examination of tumour specimens and by immunohistochemical assays (CgA and synaptophysin).

The control group consisted of 35 healthy volunteers (blood donors): 17 women aged 19–48 years and 18 men aged 20–52 years. In this group, plasma levels of metanephrine and normetanephrine as well as serum CgA levels were determined.

In both groups of subjects (patients with pheochromocytoma and healthy participants) the plasma concentration of the proSAAS peptide was determined.

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Laboratory methods

In all patients with suspected chromatoffin tumours, blood was collected simultaneously into 3 test tubes at baseline (before surgery): 1 tube (6 mL) with $EDTA_2K$ for the determination of plasma metanephrine and normetanephrine levels, 1 tube (5 mL, $EDTA_2K$ + aprotinin) for the determination of the proSAAS peptide plasma level, and 1 tube (4 mL) with clot activator for determination of serum CgA concentration. Tubes with $EDTA_2K$ and $EDTA_2K$ + aprotinin were transported to the laboratory on ice and centrifuged for 10 min at 3500 rpm. The tube for the determination of CgA concentration, 30 minutes after clotting, was centrifuged for 10 minutes at 3500 rpm. Plasma and serum were frozen (–80°C) in cryotubes until the assay (< 1 month).

In the group of healthy subjects (blood donors), blood was sampled into 3 test tubes, and the biological material was secured for hormonal tests according to the same scheme. All determinations were performed simultaneously with those done for patients with pheochromocytoma.

Concentrations of metanephrine and normetanephrine were determined by radioimmunoassay (RIA) with LDN kits (Metanephrine Plasma RIA$^{Fast\ Track}$, Normetanephrine Plasma RIA$^{Fast\ Track}$, Labor Diagnostika NORD GmbH & Co. KG, Germany). The analytical sensitivity for metanephrine was 5.8 pg/mL, and 21.4 pg/mL for normetanephrine. The reference range provided by the manufacturer was < 65 pg/mL for metanephrine, and < 196 pg/mL for normetanephrine.

Chromogranin A concentration was determined by immunoradiometric assay (IRMA) with CisBio kits (CGA-RIACT, Cisbio Bioassays, France). The analytical sensitivity of the test was 1.5 ng/mL. The reference range provided by the manufacturer for serum CgA was 19.4–98.1 ng/mL. Intra-assay coefficients of variation (CV) were as follows: 6% for 30 ng/mL, 3.8% for 144 ng/mL, and 2.2% for 996 ng/mL. Inter-assay CV values were as follows: 8.5% for 29 ng/mL, 5.7% for 144 ng/mL, and 5.3% for 996 ng/mL.

Determination of the proSAAS peptide concentration was performed with the enzyme immunoassay (ELISA) method by FineTest (Human ProSAAS / PCSK1N, Wuhan Fine Biotech Co., Ltd. China). Analytical sensitivity of the test was < 0.078 ng/mL. Intra-assay coefficient of variance (CV) was <8%. Inter-assay coefficient of variance (CV) was < 10%.

Statistics

Results were expressed as median and range. Statistical analysis was performed assuming non-parametric distribution, and the Mann-Whitney U test was employed to compare metanephrine, normetanephrine, CgA, and proSAAS levels between patients and the control group. Sensitivity and specificity were calculated using receiver operator characteristic (ROC) curve analysis. $P < 0.05$ was considered to indicate statistically significant differences. All statistical analyses were performed using statistical software (PQStat ver. 1.6.6.202).

Ethics

The study was approved by the Bioethics Committee of the Centre of Postgraduate Medical Education.

Results

In 23 patients with pheochromocytoma, the concentration of specific biomarkers was determined in order to demonstrate the tumour hormonal activity. Plasma concentrations of metanephrine and normetanephrine were 305 pg/mL and 349 pg/mL *vs.* the control group: 6 pg/mL and 48 pg/mL, respectively. Additionally, the concentration of the non-specific biomarker CgA was determined. This concentration was higher in samples from patients with tumours than in the control group (209 ng/mL *vs.* 59 ng/mL; $p < 0.001$). In this group of subjects, the concentration of the proSAAS peptide was determined. It was higher in patients with pheochromocytoma than in the group of healthy subjects (0.8 ng/mL *vs.* 0.3 ng/mL; $p < 0.001$) (Tab. 1, Fig. 1 and 2).

Using ROC curves, all tests were compared. Analyses were performed for single parameters (metanephrine, normetanephrine, CgA, proSAAS), for combinations of 2 laboratory parameters (metanephrine/normetanephrine, metanephrine/CgA, normetanephrine/CgA, metanephrine/proSAAS, normetanephrine/proSAAS, CgA/proSAAS), and for all 4 tests. Respective area under the curve (AUC) values for metanephrine and normetanephrine were as follows: 0.82 [95% confidence interval (CI): 0.70–0.94] and 0.96 (95% CI: 0.90–1), while for CgA AUC 0.93 (95% CI: 0.86–0.99) and proSAAS peptide AUC 0.83 (95% CI: 0.71–0.94). The highest discriminant value was found to be the determination of normetanephrine

Table 1. ***Comparison of metanephrine, normetanephrine, chromogranin A (CgA), and proSAAS levels in patients with pheochromocytoma*** *vs.* ***control group***

Test	Pheochromocytoma (n = 23)	Control group (n = 35)	p
Metanephrine [pg/mL]	305 (6–6822)	6 (6–70)	< 0.001
Normetanephrine [pg/mL]	349 (21–6806)	48 (21–92)	< 0.001
Chromogranin A [ng/mL]	209 (61–1234)	58.9 (25.7–102.2)	< 0.001
proSAAS [ng/mL]	0.8 (0.3–20)	0.3 (0.3–1.8)	< 0.001

Results were expressed as median and range.

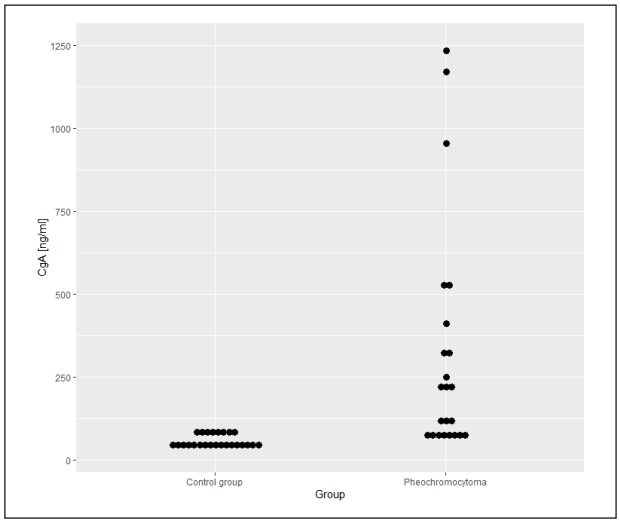

Figure 1. *Comparison of chromogranin A (CgA) levels in pheochromocytoma and the control group*

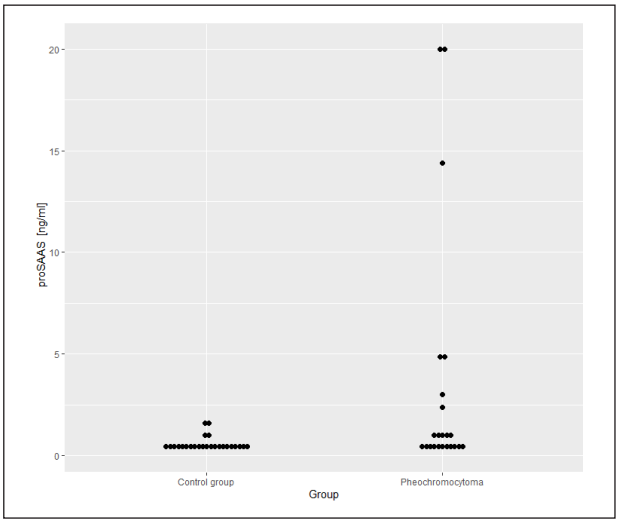

Figure 2. *Comparison of proSAAS levels in the pheochromocytoma group and the control group*

Table 2. ***Area under the curve (AUC) values for various combinations of parameters studied in patients with pheochromocytoma***

Test	AUC (95 CI range)	p
Metanephrine + Normetanephrine	0.97 (0.92–1)	< 0.001
Metanephrine + CgA	0.95 (0.89–1)	< 0.001
Normetanephrine + CgA	0.99 (0.96–1)	< 0.001
Metanephrine + proSAAS	0.89 (0.79–0.98)	< 0.001
Normetanephrine + proSAAS	0.99 (0.99–1)	< 0.001
CgA + proSAAS	0.95 (0.89–1)	< 0.001
Metanephrine + Normetanephrine + CgA + proSAAS	1	< 0.001

CgA — chromogranin A; CI — confidence interval

Table 3. ***Comparison of the diagnostic value of parameters tested in biochemical diagnostics in patients with pheochromocytoma of the adrenal gland***

Test	Sensitivity (%)	Specificity (%)
Metanephrine	56	100
Normetanephrine	91	100
CgA	83	92
proSAAS	39	88

CgA — chromogranin A

in plasma (sensitivity 91%, specificity 100%). Sensitivity and specificity indices for CgA and proSAAS peptides were 83%, 92%, 39%, and 88%, respectively (Tab. 3).

The two-factor model of the combination of tests was analysed. The highest diagnostic value was found for the combination of plasma normetanephrine and proSAAS peptide levels in patients with pheochromocytoma. Sensitivity and specificity indices for these parameters were 96% and 100%, respectively.

Table 4. ***Comparison of the diagnostic value of various combinations of tested parameters in the biochemical diagnosis of patients with pheochromocytoma***

Test	Sensitivity (%)	Specificity (%)
Metanephrine + Normetanephrine	81	100
Metanephrine + CgA	83	96
Normetanephrine + CgA	91	96
Metanephrine + proSAAS	65	96
Normetanephrine + proSAAS	96	100
CgA + proSAAS	78	92
Metanephrine + Normetanephrine + CgA + proSAAS	100	100

CgA — chromogranin A

The combination of 4 tested parameters: metanephrine, normetanephrine, CgA, and proSAAS, was also analysed. This combination of tests turned out to have the best diagnostic value: 100% sensitivity and specificity (Tab. 2 and 4, Fig. 3).

Discussion

In the human body, granin family proteins are produced by different types of neuroendocrine cells. The role of most of them as potential biomarkers is unknown or little understood. Some of these proteins, such as CgA, currently comprise the most frequently determined tumour marker in the biochemical diagnostics of neuroendocrine tumours, including gastrointestinal tract

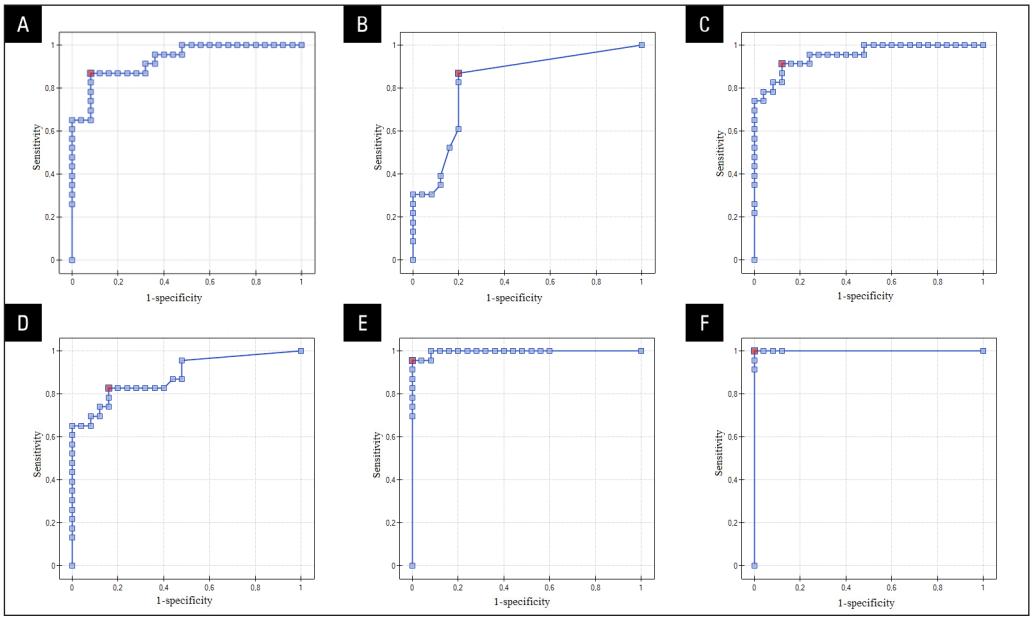

Figure 3. *Receiver operating characteristic curve (ROC) curves for biomarkers.* **A.** *ROC curve for chromogranin A (CgA).* **B.** *ROC curve for proSAAS.* **C.** *ROC curve for CgA + proSAAS.* **D.** *ROC curve for proSAAS+ plasma metanephrine.* **E.** *ROC curve for proSAAS + plasma normetanephrine.* **F.** *ROC curve for proSAAS + CgA + plasma metanephrine and normetanephrine*

(GEP-NEN gastroenteropancreatic neuroendocrine neoplasms) or tumour pheochromocytoma. Proteins from the granin family are currently recognized as non-specific markers of neuroendocrine tumours. and their clinical roles are important in the diagnosis, and monitoring of hormonally inactive tumours. The aim of this study was to evaluate a diagnostic value of one of the proteins from the granin family: the proSAAS peptide. The peptide's role as a biomarker in the biochemical diagnosis of pheochromocytoma has not been explored so far. The most sensitive biochemical indicators in the diagnosis of pheochromocytoma are free metanephrines assayed in plasma. This is because their concentrations are usually elevated, regardless of the hormonal activity of a tumour (hypertension attacks) [6]. Currently, the most sensitive methods are chromatographic techniques, especially the technique of mass spectrometry (LC-MS/MS, liquid chromatography with tandem mass spectrometry). The sensitivity and specificity of this method in the determination of plasma free metanephrines is 94% and 100%, respectively [7]. In our work, we used the isotope method (RIA, radioimmunoassay) to determine plasma concentrations of metanephrine and normetanephrine. The manufacturer of this kit compared this method with the LC-MS/MS technique, obtaining the correlation coefficients $r^2 = 0.97$ for metanephrine and $r^2 = 0.98$ for normetanephrine. Determining plasma concentrations of both metanephrines in patients with pheochromocytoma, we achieved 100% specificity, while sensitivity was higher for normetanephrine (91%) compared to metanephrine (56%). Similar results were obtained by Pussard et al. [8] using the RIA technique for determination of free metanephrines in plasma. In the group of patients with 46 pheochromocytoma and 13 paraganglioma tumours, the specificity of the assay for both metanephrines was 95%, while the sensitivity for metanephrine and normetanephrine was 61% and 97%, respectively. Regardless of the biochemical method used to demonstrate hormonal activity of the adrenal medulla, which may suggest existence of a tumour, its presence has to be confirmed by histopathological examinations. This type of neoplasm was confirmed in all 23 patients who had undergone adrenalectomy. Additionally, in some patients immunohistochemical tests for the presence of specific markers (chromogranin A and synaptophysin) were done in the tumour tissue.

The adrenal medulla is made of neuroendocrine cells, capable of synthesizing, storing, and secreting into blood various proteins, peptides, and neuropeptides, along with catecholamines. This group of compounds includes secretory proteins belonging to the granin family (secretogranin). Granins are proteins found in secretory granules of endocrine, neuroendocrine, and nerve cells, etc. Among secretogranins the following are distinguished: CgA, chromogranin B (CgB), secretogranin II (CgC), secretogranin III (SgIII or 1B1075), secretogranin IV (SgIV or HISL-9), secretogranin V (SgV or 7B2), secretogranin VI (SgVI or NESP55), secretogranin VII (SgVII or VGF), and proSAAS [9, 10].

The best-known granin family protein is CgA. It is a glycoprotein made up of 439 amino acids. For the

first time isolated from chromaffin cells of the adrenal medulla [11]. From the clinical point of view, determination of CgA concentration has found an application in biochemical diagnostics of various neuroendocrine tumours, including hormonally active and inactive GEP-NENs. Because CgA is produced and secreted into the circulation by various neuroendocrine cells, it has been defined as a non-specific marker, a so-called circulating tumour marker, which can be determined by various immunochemical methods [enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA), radioimmunoassay (RIA), chemiluminescence immunoassay (CLIA)] in blood (serum, plasma) [12]. The CgA molecule is a precursor of various peptides (e.g. WE-14, catestatin, pancreastatin, and others). Considering the lack of standardization of CgA determinations, currently used immunochemical tests allow for determination of various CgA fragments. Consequently, comparison of results obtained by different laboratories poses a major clinical problem. Antibodies used in the IRMA method, which we used to determine the concentration of CgA, made it possible to bind a stable region (without the influence of proteolysis) of the $CgA_{145-245}$ molecule. The method of CgA determination used by us enables the determination of the entire (intact) chromogranin A molecule. In addition to neuroendocrine tumours of the gastrointestinal tract, CgA can be used in biochemical diagnosis of pheochromocytoma [13]. In particular, its determination may be useful in patients with negative (normal) results of plasma metanephrines, e.g. in patients with a malignant tumour [14]. In a group of 15 out of 23 patients with phaeochromocytoma, its concentration was above the upper reference range (URL) and ranged from 110 to 1234 ng/mL (URL > 98.1). The sensitivity was 83% and specificity was 92%. In other research reports, authors obtained similar test scores in this group of patients (sensitivity 80–98%). Parisien-La Salle et al. obtained the sensitivity of CgA determination of 97% (34 patients with pheochromocytoma) [15]. In another study, Grossrubatscher et al., obtained a sensitivity of 91% in 22 patients with pheochromocytoma [16], while Bilek et al. examined 46 patients with phaeochromocytoma, obtaining 96% sensitivity [17]. A combined determination of plasma metanephrine or normetanephrine and serum CgA concentration in our study showed similar sensitivity (> 80%), but a higher specificity (> 95%).

$ProSAAS_{1-227}$ is a precursor to several peptides of the granin family (big SAAS, little SAAS, PEN, big LEN, little LEN), 2 of which have been identified in rat adrenal medullary chromaffin cells ($SAAS_{1-26}$ and $LEN_{212-227}$). ProSAAS-derived peptides play a role in several physiologically important vital functions, such as circadian rhythm, food intake, energy balance, and foetal neuropeptide processing. Many cells, including endocrine and neuroendocrine cells, have the ability to store and release proSAAS from internal vesicles into circulating blood. ProSAAS-derived peptides are processed in the regulated secretory pathway of neuroendocrine cells [18–22]. In the group of 23 patients with pheochromocytoma, sensitivity of proSAAS peptide determination was 39%, with a specificity of 88%. Simultaneous determination of both granins (CgA + proSAAS) resulted in obtaining much better test evaluation parameters (sensitivity 78%, specificity 92%). Comparing determination of the non-specific proSAAS marker with free metanephrines, the best evaluation was obtained by determination of normetanephrine with proSAAS (sensitivity 96%, specificity 100%). When determining all the parameters together (metanephrine + normetanephrine + CgA + proSAAS), both sensitivity and specificity were 100% in the studied group of patients with pheochromocytoma. This is the first study to describe the clinical importance of proSAAS peptide determination in patients with pheochromocytoma. Comparing the clinical usefulness of the tested peptide with another protein from the granin family, CgA, it can be concluded that a single determination of the level of these proteins shows lower sensitivity and specificity compared to specific biomarkers such as plasma free metanephrines. Hence, in future, CgA, and possibly other secretory proteins from the secretogranin family, may prove to be complementary tests in the diagnosis of catecholamine-secreting tumours, especially in patients whose results of plasma metanephrines are inconclusive in relation to the clinical presentation, or in patients with malignant tumours. In this case, research should be continued in order to clinically evaluate this peptide in this tumour form.

This work has several limitations that should become subjects of further research. The study presents material from 23 patients with confirmed phaeochromocytoma. The authors of this publication state that it would be advisable to conduct a study on a larger group of patients. Additionally, comparative studies in other groups of patients would be worth performing, i.e. patients with other tumours capable of producing catecholamines and their metabolites (paraganglioma tumours), patients with adrenocortical adenomas (hormonally active and inactive), and patients with arterial hypertension (primary and secondary, not causing the presence of pheochromocytoma).

Conclusions

Determination of non-specific markers in biochemical diagnostics of adrenal pheochromocytoma offers a valuable addition to the diagnostics, particularly in

cases when results obtained for specific markers are inconclusive. A single determination of the proSAAS peptide level is associated with a rather low diagnostic value. However, collective determination of CgA and proSAAS may be an additional, valuable tool in biochemical diagnostics of pheochromocytoma.

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