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Original article

SERUM CHROMOGRANIN–A LEVELS IN NEUROENDOCRINE NEOPLASMS AS PROGNOSTIC MARKER IN CORRELATION WITH THE CLINICAL COURSE OF THE DISEASE AND THE INFLUENCE OF OCTREOTID THERAPY

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Abstract

Introduction. Neuroendocrine neplasms (NEN) arise from neuroedocrine cells in various tissues and organs, have diverse biological behavior and express neuroendocrine markers synaptophysin and chromogranin A (CgA).

Aim of the study. The aim of this study was to correlate the serum CgA levels before and after surgical and/or oncological treatment with octreotide and to determine the prognostic value of CgA variations during the follow-up.

Material and methods. We used ELISA to analyze 699 serum samples from 410 patients during 9 years, due to carcinoid syndrome, benign neuroendocrine tumor (NET), localized neuroendocrine carcinoma (NEC) and patients with metastatic NEC (MS).
Data from hospital databases were used for follow-up of 60 patients, divided into responders and non-responders, regarding their response to therapy.

Results. The mean serum CgA value in 410 analyzed patients was by 3.47-fold increase compared to the maximal reference values. The highest increase was measured in patients with NEC/MS, with mean 12.94-fold increase, followed by patients with localized NECs, with mean 4.57. During follow-up, CgA values were reduced, with a significant difference between the groups of responders and non-responders.

Conclusions. Reduction of the CgA level for at least 49.5% during the first 12 months after therapy was correlated with stable disease course, and serum CgA elevation or decrease less than 34% during the first 12 months after the therapy was correlated to unfavorable clinical course. Serum CgA levels are useful for the diagnosis of NENs and during the

follow-up for detection of recurrence, disease progression and evaluation of the oncologic therapy response.

Keywords: chromogranin A, ELISA, octreotide therapy

Introduction

Neuroendocrine neplasms (NEN) arise from neuroedocrine cells in various tissues and organs, have diverse biological behavior and share common feature – expression of neuroendocrine markers synaptophysin and chromogranin A (CgA). The incidence of these tumors has been estimated to be in the range between 2.5 to 5 per 100,000 people per year and prevalence of 35 per 100,000[1].

NENs most commonly occur in the gastrointestinal tract, pancreas and lung[2]. Still, neoplasms with neuroendocrine features appear in other organs and systems as well – genitourinary, breast, thyroid gland, skin, thymus, etc. Their morphology varies in different sites of origin, but they all have common macroscopic and microscopic characteristics: most of them are nodular solid tumors, composed of nests, trabeculae or sheets of relatively uniform cells with eosinophilic cytoplasm and typical "salt and pepper" chromatin, tending to form rosettes.

The biological behavior of these neoplasms is mainly estimated through mitotic counts and proliferative index based on Ki67 staining. Since the vast majority of NENs (~70%) arise in the gastroenteropancreatic system[3], the established grading system for these tumors, with some modifications, is also used for grading of NENs in other organic systems. Basically, NENs are divided into two different groups: well-differentiated neuroendocrine tumors (NETs) with low proliferative activity and poorly differentiated neuroendocrine carcinomas and (NECs) with high mitotic and proliferative index[4].

Production of CgA, a member of the family of acidic proteins stored in the secretory granules of the neuroendocrine cells, is common to both groups of NENs. This feature has been widely used by the pathologists for determination of the neuroendocrine nature of the neoplasm by the means of immunohistochemistry, and by the biochemists for determination of the levels of CgA in patients' plasma or serum, both for diagnosis, prognosis and for follow-up. There are different methodologies for determination of CgA with variable sensitivity and specificity[5]. It is also very important to be aware of existence of any causes for false positive results in patients prior to the analyses, such as use of proton pump inhibitors, kidney dysfunction or atrophic gastritis, some other, less common causes, as well as daily CgA variations sometimes up to 20%[6, 7]. These variables must be taken into consideration during interpretation of the results of the CgA tests. Most commonly the serum CgA levels are determined with different ELISA test kits expressing the results either in ng/ml or U/L, with variations in suggested reference values, for example from <108 ng/ml, <94 ng/ml, 2-18 U/L or according to some authors cut-off value of 85.3 ng/ml[8]. Serum CgA changes of more than 40-50% during subsequent measurements are considered clinically significant[9].

Somatostatin analogs have become essential components in the therapeutic protocols of these tumors during the past 25 years. Octreotide is a synthetic octapeptide, a somatostatin analog, which has a prolonged half-life compared to native somatostatin for more than 30 times, and a pharmacodynamic action lasting up to 8-12 hours after administration[10]. Its potent anti-secretory effects and multiple mechanisms of action, combined with its established safety profile, make it a reliable treatment option. Furthermore, clinical data support the antiproliferative effect of octreotide in patients with well-differentiated metastatic midgut NETs. Octreotide may also be considered as a treatment option in patients with well-differentiated metastatic non-midgut NECs, regardless of functional status[11].

Aim of the study

The aim of this study was to correlate the serum CgA levels before and after surgical or surgical and oncological treatment with octreotide and to determine the value of serum CgA variations during the follow-up as a prognostic marker.

Patients, material and methods

Patients

We analyzed 699 serum samples from 410 patients taken during a period of 9 years (December 2010 - December, 2019). The indications for testing were: i) clinical suspicion for NEN/carcinoid syndrome, ii) postoperative follow-up after histologically verified NEN and iii) monitoring the effects of octreotide therapy.

The gender and age distribution is shown in Table 1.

Table 1. Gender and age of patients

Gender	Age - Means	Age - N	Age Std. Dev.	Age - Minimum	Age - Maximum
Males	53.76	169	16.0	8.0	81.0
Females	52.86	241	15.3	13.0	83.0
All Groups	53.23	410	15.58	8.0	83.0

Patients were divided into 4 groups: group 1 – tested for clinical suspicion for carcinoid syndrome; group 2 – patients with benign neuroendocrine tumor (NET); group 3 – patients with localized NEC and group 4 – patients with metastatic NEC (MS).

Most of the patients were tested only once (n=301), 57 patients were tested twice, 20 patients three times, and there were 32 patients tested 4 or more times, including 1 patient that was tested 20 times during this period.

Tables 2 and 3 depict the distribution of the patients according to the indication for testing and distribution according to gender.

Table 2. Frequency distribution according to indication for testing

	Count	Cumulative Count	Percentage	Cumulative Percentage
Clinical suspicion / Carcinoid syndrome	195	195	47.56	47.56
Benign NET / Pheochromocytoma/ Endocrine adenoma*	108	303	26.34	73.90
NEC – localized	62	365	15.12	89.02
NEC- MS	45	410	10.97	100.0

Table 3. Gender distribution of the diagnostic groups

Gender	Clinical suspicion/ Carcinoid syndrome	Benign NET/ Pheochromocytoma/ Endocrine adenoma	NEC - localized	NEC- MS	Total
Males	64	49	23	33	169
Females	131	59	39	12	241
Total	195	108	62	45	410

Methods

CgA serum levels

Serum levels of CgA were analyzed with ELISA. Serum from blood samples was separated immediately after collection and stored frozen at -80^{o}C until analysis which was conducted in batches of 8-16 samples per run. ELISA tests were performed with ELISA reader (Humareader HS, Human, Germany). Absorbance was measured at 450 nm with reference wavelength 620 nm.

During this 9-year-period four different ELISA kits for serum CgA levels were used with three different reference values:

1. CHROMOGRANIN A ELISA KIT (DAKO) (ref. 2-18 U/ml)
2. NEOLISA CHROMOGRANIN A (EURO DIAGNOSTICA) (ref. <108 ng/ml)
3. NEOLISA CHROMOGRANIN A (SVAR) (ref. <108 ng/ml)
4. CGA-ELISA (Cisbio Bioassays) (ref. <94 ng/ml)

Three of the kits (2-4) had identical or comparable reference values (<108 ng/ml and <94 ng/ml), and the first kit expressed the CgA serum levels in different measurement units (U/L). All 4 kits used identical cubic spline plots, derived from the calibrators included in the kits, for determining the CgA levels in patients' samples, as well as positive and negative controls to validate the measurements. Having in mind that many of the patients were followed through the years, during which different kits were available in the laboratory, in order to overcome this inconsistency and make results comparable, all results were semiquantitatively expressed as quotients,where measurements within the reference range were indexed as 1, and those above the reference range were expressed as a quotient (obtained value/maximal reference value) showing the measured CgA value relative to the maximal reference value of the kit.

Clinical data and follow-up

From the databases of the Institute of Pathology and University Clinic for Radiotherapy and Oncology, we extracted data that enabled relevant follow-up of 61 patients. The mean time of the follow-up was 20,3 months (min. 1; max. 100; SD 22,9). During the follow-up, additional 1-20 measurements of serum CgA per patient were performed, either for evaluation of postoperative CgA levels or for evaluation of the response to the octreotide therapy. For the purpose of this study, the lowest CgA values obtained during the first 12 months after surgery and/or initiation of specific oncologic therapy were recorded and expressed as reduction (in percents) from the highest values obtained during measurement before therapy.

These patients were further subdivided into 2 groups (Responders and Non-responders) regarding their response to therapy (surgical, specific oncologic or combined), and according to the clinical course of the disease, imaging results and biochemical

parameters other than CgA. Patients with stable clinical course, absence of radiological progression or clinical and radiological signs of remission were placed in the first group (responders), while those with progressive disease course during therapy based on their clinical, imaging and biochemical parameters comprised the second group of non-responders.

Statistical analysis

For description of the obtained results, methods from the descriptive statistics were used and the null hypothesis was tested using parametric and non-parametric tests by means of commercial software Statistica for Windows v.10 [StatSoft, Inc. 2011. STATISTICA (data analysis software system), version 10. www.statsoft.com.]. Normality of distribution was tested with the Kolmogorov-Smirnov D test, Lillefors test and Shapiro-Wilk's W test. For testing the differences of serum CgA levels between the patients' groups regarding the therapy response, as well as the significance of differences between patients' subgroups, nonparametric tests for comparison of multiple independent samples (Kruskal-Wallis ANOVA by ranks and Median chi-square test) were done. Correlations between parametric variables were tested with Pearson Product Moment Correlation (r), and for the non-parametric variables the Spearman's Rank Order Correlation (R) was used.

Results

Initial Serum CgA analyses and values

The mean serum CgA value in 410 analyzed patients was by 3.47-fold increase compared to the maximal reference values of the corresponding kits (Table 4).

Table 4. Serum CgA increase patients expressed as relative increase compared to the maximal reference value

	Valid N	Mean	Minimum	Maximum	Std. Dev.
CgA (fold-increase)*	410	3.47	1.0 *	36.11	5.68

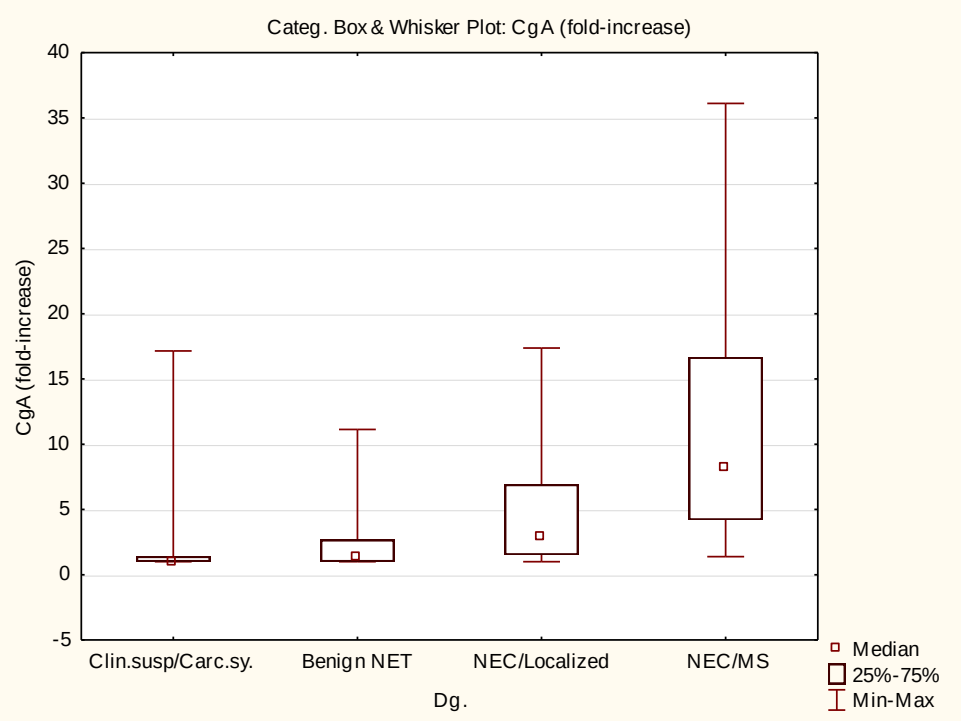
**1 corresponds to serum CgA within reference range*

The highest serum CgA increase was measured in the group of patients with metastatic disease (NEC/MS), with mean 12.94-fold increase (median 8,24; SD 11,7), followed by the group with localized NECs, with mean 4.57 (median 2,84; SD 4,23) (Table 5).

The lowest serum CgA increases were measured in the group with benign NETs including pheochromocytoma and endocrine adenomas, where the mean value was 2.34 (median 1.4; SD 2.12) and in the group of patients tested because of clinical suspicion for carcinoid syndrome, with the mean value of 1.57 (Median 1; SD 1,83) (Figure 1).

Table 5. Mean serum CgA increase in patients according to indication for testing

Diagnosis	CgA (fold-increase) - Means	CgA (fold-increase) - No. of patients	CgA (fold-increase) - Std.Dev.
Clinical suspicion / Carcinoid syndrome	1.57	195	1.83
Benign NET /Pheochromocytoma/ Endocrine adenoma	2.34	108	2.12
NEC – localized	4.57	62	4.23
NEC – MS	12.94	45	11.70
All Groups	3.47	410	5.68



Tukey HSD for unequal N (Spjotvoll/Stoline) test; p<0.05

Fig. 1. Median serum CgA increases in diagnostic groups

We found statistically significant intergroup differences (p<0.05) between all the groups in different combinations, except for the first and the second group (Clinical suspicion/Carcinoid sy. *vs.* Benign NETs/Pheochromocytoma/Endocrine adenomas), where difference was not found.

Serum CgA variations during follow-up

For 61 patients we obtained clinical follow-up data. The mean time of the follow-up of these patients was 20.3 months (min. 1; max. 100; SD 22,9). The patient that enlisted the shortest follow-up had 2 CgA tests, with indication for the first test being suspicion for carcinoid syndrome, but on the re-test 1 month later he showed 83% decrease of the serum CgA in comparison to the initial value. It appeared that for the initial test he had been on therapy with proton-pump inhibitors. This patient was excluded from further statistical analysis.

Table 6. Follow-up interval (months) of patients according to their response to therapy

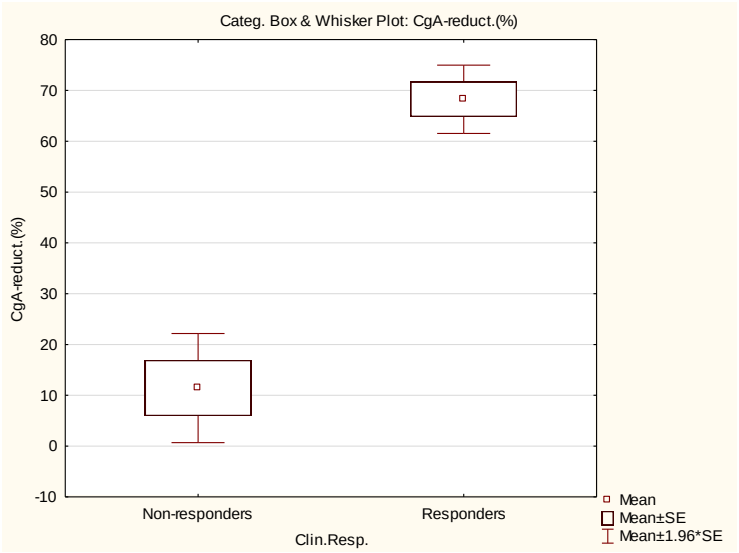
Clinical Response	Follow-up (m) - Means	Follow-up (m) - N	Follow-up (m) - Std. Dev.	Follow-up (m) - Minimum	Follow-up (m) - Maximum
Non-responders	11.2	5	12.19	2.0	32.0
Responders	43.43	14	29.45	10.0	100.0
All Groups	34.95	19	29.53	10.0	100.0

The group of responders (stable disease course) consisted of 38 patients and in the second group (non-responders) there were 22 patients. The mean follow-up intervals for the two groups were 43.4 months and 11.2 months, respectively (Table 6).

There was no statistically significant difference in the distribution of the patients in these 2 groups according to their gender ($p>0.05$).

Subsequent serum CgA measurements revealed reduction in CgA values, with a significant difference between the two groups (responders *vs*. non-responders) ($p<0.001$). The mean serum CgA reduction in the group of responders during the first 12 months after initiation of the therapy (surgical, octreotid or combined) was 68.25% (SD 18.78), while in the group of non-responders the mean serum CgA reduction was 11.42% (SD 22.6) (Figure 2).

These data suggest that cases where the initial serum CgA was elevated by 2.3 fold (the mean increase in the patients of the second diagnostic group – Benign NET/ Pheochromocytoma/Endocrine adenoma) or more, compared to the maximal reference range, subsequent reduction of the CgA level for at least 49.5% (mean CgA reduction in responders 68.3% - SD 18.8) during the first 12 months after surgery, initiation of specific oncologic therapy or both, was correlated with a stable disease course. Opposite to this, serum CgA elevation or decrease less than 34% (mean CgA reduction 11.4 + SD 22.6 in non-responders) during the first 12 months after surgery, initiation of specific oncologic therapy or both, were correlated to unfavorable clinical course and progressive disease.



T-Test: value -9.25; df=45; p<0.001

Fig. 2. Serum CgA reduction in the group of Non-responders *vs*. Responders

Discussion

Our study showed significantly higher serum CgA in patients with NECs, especially cases with metastatic disease in comparison to patients tested because of clinical suspicion for carcinoid syndrome. The highest increase was detected in cases with metastatic disease (mean 12.9-fold increase over the maximal reference range), followed by cases with localized NEC (mean 4.57-fold increase over the maximal reference range). This difference is probably attributable to the higher tumor burden in patients with metastatic disease, as found in several other studies[12-14]. Because of the linear relationship of CgA to tumor burden, its measurement also provides prognostic information.

Different guidelines from 2010 to 2020 recommend 1-4 measurements of CgA or 5-HIAA in follow-up of midgut carcinoids per year, while patients with foregut tumors can also be monitored with CgA or 5-HIAA measurements, if they were positive for these markers at initial diagnosis. As hindgut tumors usually do not secrete serotonin, consequently only CgA monitoring is recommended[15-19].The follow-up for midgut NETs, except for small well differentiated tumors of appendix, should last for at least 7 years in different clinical settings for various tumors, but the upper limit was not established. According to ClinLab Navigator and UK guidelines, 2-3 serum CgA measurements per year should be performed during follow-up[9, 20].

As a tumor marker used in follow-up, a 40% to 50% change in serum CgA concentrations should be considered clinically significant in the absence of confounding factors. Much smaller changes in CgA concentrations might be considered significant only if they occur over several serial measurements[18, 19]. Serum CgA results obtained with different kits should not be compared directly, but taking into consideration that changes of 40-50% or more between two consecutive controls are considered clinically relevant, we found it useful

to express the serum CgA levels in a semi-quantitative manner as a relative increase (–fold increase) over the maximal reference value of the respective kits, as it is referred in other studies also[6, 9]. This methodology clearly lowers the resolution level of the measurements, but enables longer follow-up of patients tested with different kits and at higher serum CgA levels (several times the maximal reference value), which is frequently found in symptomatic and advanced NET patients[5-7, 9]. This approach has no influence on the perception of the serum CgA trend during follow-up.

Gut *et al*. found the serum CgA not to be a specific marker for diagnosis of NENs mainly because its levels in blood are influenced by multitude of factors including gastric disorders and use of proton pump inhibitors, kidney dysfunction, inflammatory bowel disease, some other less common causes, as well as daily CgA variations, sometimes up to 20%[6]. Provided that all precautions during interpretation of serum CgA are taken, all reasons for false positive and false negative results are excluded and in a setting of already diagnosed NEN, especially in cases with metastatic disease, serum CgA is a valuable tool for disease monitoring.

Welin *et al*. found that serum CgA elevation is the first indication of recurrence in radically operated midgut carcinoid tumors[21]. They suggested that patients should be monitored 2 times per year for chromogranin, including yearly ultrasonography until CgA levels increase or clinical symptoms appear. After that point, all measures should be undertaken for detection of the recurrent tumor lesions using different imaging methods including computerized tomography, somatostatin receptor scintigraphy and/or positron emission tomography with 5-hydroxytryptophan.

Furthermore, our results suggest that the degree of serum CgA decrease during the first 12 months of the treatment or after operation was correlated with the later clinical course of the disease and efficacy of the octreotid therapy. We found that the mean serum CgA reduction in the group of responders during the first 12 months after initiation of the therapy was 68.25% (SD 18,78), while in the group of non-responders the mean serum CgA reduction was 11.42% (SD 22,6) ($p<0.001$). This is basically in concordance with the principles of octreotid test which is used for selection of candidates for therapy with somatostatin analogs. Similar results were published by Massironi *et al*.[22] who found that patients who responded to subcutaneous injection of 200 mcg octreotide with 50% or more decrease of the CgA level had better outcome. Successful objective response occurred in 68% of patients, successful symptomatic response was found in 72% of patients and biochemical response was detected in 81% of these patients. Opposite to this, patients who had less than 30% CgA decrement after the octreotest, had worse outcome. Eighty-six percent of the non-responders died of the disease with a median survival of 6 months (6-30 months) in comparison to the median survival of the responders which was 48 months (6-138 months) ($p<0.0005$).

Conclusions

Serum CgA levels are useful for the diagnosis of NENs and are also valuable during the follow-up for detection of recurrence, disease progression and for evaluation of the oncologic therapy response.

Serum CgA fluctuations must be interpreted with caution and in context of the disease. Special care must be taken to exclude reasons for false positive results (i.e. proton-pump inhibitors). Serum CgA variations more than 40-50% on two consecutive tests, as well

as smaller but consistent changes in the same direction during several measurements should be considered clinically significant. Depending on the diagnostic group (clinical suspicion for carcinoid syndrome *vs*. benign NET *vs*. localized NEC *vs*. advanced NEC), and in accordance with the guidelines for specific localizations of NENs, regular serum CgA measurements should be done, optimally 1-3 times per year.

Reduction of the CgA level for at least 49.5% during the first 12 months after surgery, initiation of specific oncologic therapy or both, is correlated with a stable disease course. Opposite to this, serum CgA elevation or decrease less than 34% during the first 12 months after surgery, initiation of specific oncologic therapy, or both, would be correlated to unfavorable clinical course and progressive disease.

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