

A Comparison of Three Chromogranin A Assays in Patients with Neuroendocrine Tumours

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ABSTRACT

Background & Aims: Chromogranin A (CgA) is the most important general tumour marker used in the diagnosis and follow-up of patients with neuroendocrine tumours (NET). Chromogranin A assays may have different sensitivities, which is of importance for the clinical diagnosis and handling of NET patients. The aim of this study was to compare the clinical sensitivities of three different CgA assays in NET patients.

Methods: We measured CgA level in 42 NET patients (male/female: 23/19, median age: 63 years, range 29-85 years). Twenty-five patients had liver metastases, eight had local disease, and nine were disease free after surgery. We studied an in-house RIA: RH RIA assay (Rigshospitalet, Copenhagen, Denmark); NEOLISA™ (Euro Diagnostica, Malmö, Sweden) and EURIA CgA RIA (Euro Diagnostica, Malmö, Sweden).

Results: The RH RIA assay showed a clinical sensitivity of 97%, while the NEOLISA and EURIA assays both showed similar clinical sensitivities of 79%. Patients with liver metastases had significantly higher CgA levels compared to disease free patients by all three assays ($P < 0.001$), but only the RH RIA assay was able to discriminate between patients with liver metastases and with regional disease ($P < 0.01$).

Conclusion: Chromogranin A measurements are significantly assay-dependent and caution should be applied in the interpretation of CgA measurement for assessment of NET status. The in-house RH RIA assay was better at predicting NET status than the NEOLISA and EURIA assays.

Key words: chromogranin A – neuroendocrine tumor – assay – enzyme-linked immunosorbent assay – radioimmunoassay.

List of abbreviations: CgA: chromogranin A; EURIA: Eurodiagnostica, radioimmunoassay; GFR: glomerular filtration rate; NEOLISA: Eurodiagnostica, enzyme-linked immunosorbent assay; NET: neuroendocrine tumour; PET-CT: positron emission tomography; PPI: proton pump inhibitor; RH RIA: Rigshospitalet, radioimmunoassay; SPECT-CT: single photon emission computed tomography.

INTRODUCTION

Neuroendocrine tumours (NET) develop from the diffuse neuroendocrine system and are most often located in the gastrointestinal tract, including the pancreas (60-70%) and in the lungs (25-30%) [1]. The tumours are rare but are increasing in incidence according to the North American Surveillance, Epidemiology and End Results Programme [1, 2]. The NET is a highly heterogeneous disease with different organ locations, staging and grading. As a

consequence of the heterogeneous nature of NET, treatment of the disease varies from surgery to different pharmacological treatments and radionuclide therapy [3].

Neuroendocrine tumors' diagnosis and disease status evaluation during follow-up are based on clinical symptoms, imaging methods and circulating tumour markers. In particular, the tumour marker chromogranin A (CgA) has been shown to be a universal marker for NET, and CgA is currently the single most important tumour marker used in the diagnosis and follow-up of NET patients [4]. Chromogranin A levels are elevated in variable degrees in most patients with NET [5, 6].

Chromogranin A is an acidic glycoprotein consisting of 439 amino acids with a molecular mass of 48 kDa. It is stored in the dense secretory granules of the cytoplasm of neuroendocrine and endocrine cells and is co-secreted upon stimulation of these cells together with peptide hormones and neuropeptides [7]. Chromogranin A from both non-pathological and

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pathological neuroendocrine cells is post-translationally processed into different fragments. This post-translational processing may be different in different tissues and also in different tumours, and this may have an impact on the CgA assessment by different assays [8].

For both diagnosis and follow-up of NET patients it is important to have a CgA assay with high sensitivity and specificity. At diagnosis, elevated levels of CgA will result in further diagnostic investigations to establish the NET diagnosis and staging, which is required for initiation of specific NET treatment. During follow-up, increases in CgA levels raise suspicion of NET recurrence or disease progression with the need for further investigations and a possible change in treatment modality [9]. Opposite, false positive CgA levels may lead to a number of unnecessary investigations with increased costs for the individual patient and the health care system.

Chromogranin A levels can be elevated for other reasons than the presence of NET disease. The most common causes are proton pump inhibitor (PPI) treatment, chronic atrophic gastritis and renal insufficiency. Other well known causes include inflammatory bowel disease and liver failure [10].

The aim of the present study was to compare the clinical sensitivity of three different CgA assays in patients with known NET using the cut-off values recommended by the manufacturers of the respective assays. The assays compared were an in-house RIA assay, RH RIA assay (Rigshospitalet, Copenhagen, Denmark) and two commercial assays: NEOLISA™ (Euro Diagnostica, Malmö, Sweden) and EURIA CgA RIA (Euro Diagnostica, Malmö, Sweden). The in-house assay is used for CgA measurements at ENETS Centres of Excellence in Denmark, while the EURIA CgA RIA assays are used at several NET centres in Europe.

METHODS

Patients

A total of 42 patients histologically diagnosed with NET were included from the Department of Medicine V (Hepatology and Gastroenterology), Aarhus University Hospital, Denmark, in the period December 2010 to April 2011. Disease status was evaluated using computed tomography (CT, $n = 38$), magnetic resonance imaging (MRI, $n = 7$), somatostatin receptor imaging by computed tomography (CT) in combination with positron emission tomography (Ga-Dotanoc PET-CT, $n = 11$) and/or single photon emission computed tomography (SPECT-CT, $n = 18$). The latter two scans were combined with diagnostic CT scans, all performed as 3-phase CT scans with i.v. contrast enhancement. Follow-up with CT-scans on radically resected NET patients was performed up to 12 months after inclusion in the study.

The study was approved by the local Ethics Committee, Region Midtjylland (133/2012).

Assays

The RH RIA assay is an in-house assay developed at Rigshospitalet (Copenhagen, Denmark) in 1999 from a library of RIAs specific for different epitopes, including the NH₂ and COOH termini and three sequences adjacent to dibasic sites in the remaining part of CgA [10]. The polyclonal antibodies

in the present RIA are raised in rabbits and bind different epitopes between amino acid residue 340 of the CgA molecule and amino acid residues in the C-terminal direction, including e.g. amino acid residue 340-348 of the CgA molecule [10]. The CgA measurement has been improved in 2007 by treating samples with trypsin in a processing-independent analysis (PIA) followed by measurement of CgA by the RIA [11]. For determination of cut-off (set to <130 pmol/L), 88 healthy controls, 50% males and 50% females, median age for all 41 years (IQR 30-51) and 27 patients with gastrointestinal NET, 44% women, median age for all 60 years (IQR 57-70) were studied [11, 12].

The NEOLISA is an enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies raised in mice. The catching antibodies bind an epitope starting at amino acid residue 236 and ending at amino acid residue 251. The detector antibodies bind an epitope from amino acid residue 264 to 279. The cut-off value for the NEOLISA was <3 nmol/L defined by the manufacturer.

The EURIA assay is a competitive RIA using polyclonal antibodies raised in rabbits directed towards epitopes between amino acid residue 116 to amino acid residue 439 in the CgA peptide. The cut-off value for the EURIA defined by the manufacturer was <6 nmol/L.

Plasma samples

For plasma collection we used K2-EDTA tubes (Terumo, Herlev, Denmark). Samples were kept on ice and centrifuged for nine minutes (1850 g, 4°C) within one hour after blood collection. After centrifugation samples were immediately frozen and stored at -20°C until analysis. Analysis and calibration were carried out according to the recommendations of the manufacturers.

Statistical analysis

Sensitivities were calculated using SPSS (version 18.0). Comparisons of the individual assays between groups were performed by the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test. Correlations between assays were performed by Spearman's rho. Bland Altman plots and Box plots are provided for the three assays (Figs. 1, 2).

RESULTS

Patients' characteristics

The 42 patients included in the study were 23 males and 19 females, with a median age of 63 years (range 29-85 years). Thirty-three patients had clinically manifest NET disease at the time of blood sampling verified by imaging techniques. Of these, 25 patients had liver metastases and 8 had regional disease classified as the presence of the primary tumour and/or metastases to other sites than the liver. The remaining 9 patients were radically operated with removal of their primary tumour and no evidence of metastatic disease. Table I shows the primary tumour locations for the 33 patients with present NET disease and the 9 radically operated patients.

Among the 25 NET patients with liver metastases, 20 patients (9 with pancreatic NET, 7 with small bowel NET, 1 with lung NET, 1 with cecal NET and 2 with unknown primary

Table I. Primary tumour location in patients with present NET and patients free from tumour after radical surgery.

Primary tumour	Patients with present NET disease (n = 33)	Patients free from tumour after surgery (n = 9)
Small intestine	12	3
Pancreas	12	1
Lung	3	3
Appendix (goblet)	1	0
Cecum	1	0
Rectum	0	2
Unknown	4	0

NET) had elevated CgA levels measured by all three assays, and 1 patient (small bowel NET) had a normal CgA level measured by all three assays. The remaining 4 patients (3 small bowel NET and 1 unknown primary NET) had elevated levels of CgA using the RH RIA assay while normal CgA levels using the NEOLISA and EURIA assays.

In the 8 NET patients with regional disease, 6 patients (3 pancreatic NET, 1 small bowel NET, one appendix goblet NET and 1 unknown primary NET) had elevated CgA levels measured by all three assays. Two patients (lung NET) had elevated CgA levels measured by the RH RIA assay while normal CgA levels measured by the NEOLISA and EURIA assays.

In the 9 patients free from NET after surgery, 3 (2 small bowel NET, 1 lung NET) had elevated CgA levels measured by all assays, while 2 patients (1 rectal NET, 1 lung NET) had normal CgA levels measured by all assays. The remaining 4 NET patients (1 for each of the following locations: small bowel, lung, rectal and pancreatic) had elevated CgA levels in plasma using the RH RIA assay, but normal levels using the NEOLISA and EURIA assay.

Assay sensitivity and comparison

We evaluated the clinical sensitivity of the RH RIA, NEOLISA and EURIA assays in patients with known NET using the cut-off values recommended by the manufacturers. Table II illustrates that the RH RIA assay showed plasma CgA levels elevated in 32 and normal in 1 of the patients with present NET disease. The clinical sensitivity of the RH RIA assay was 97%. In the 9 patients free from tumour after radical surgery, CgA levels were elevated in 7 and normal in 2.

Using the NEOLISA and EURIA assays, we found that plasma CgA levels were similarly elevated in 26 of the 33 patients with present NET and normal in the remaining 7 patients. In the 9 patients without present NET, CgA levels were elevated in 3 and normal in 6. Accordingly, the clinical sensitivity of the NEOLISA and EURIA assays was of 79%.

A strong correlation was found between CgA measurements by the NEOLISA and EURIA assays ($r = 0.97$, $P < 0.0001$), while the correlation with the RH RIA assay was slightly weaker ($r = 0.86$ for NEOLISA, and $r = 0.80$ for EURIA; for both $P < 0.000$). For the NEOLISA and EURIA assays there were no differences regarding which patients had normal and elevated levels of CgA measured when compared according to the assay cut-off values. However, when comparing the RH RIA assay with the

Table II. Number above and below the chromogranin A (CgA) cut-off value in relation to disease status of the 42 patients using the RH RIA and NEOLISA/EURIA assays.

		Present tumour	No present tumour
RH RIA assay (cut-off <130 pmol/L)	Elevated CgA	32	7
	Normal CgA	1	2
NEOLISA/EURIA (cut-off <3 and <6 nmol/L, respectively)	Elevated CgA	26	3
	Normal CgA	7	6

Table III. Evaluation of chromogranin A (CgA) levels measured by the RH RIA assay and by the NEOLISA and EURIA assays in the 42 patients.

		NEOLISA/EURIA Assay	
		Elevated CgA (≥ 3 or ≥ 6 nmol/L)	Normal CgA (< 3 or < 6 nmol/L)
RH RIA Assay	Elevated CgA (≥ 130 pmol/L)	29	10
	Normal CgA (< 130 pmol/L)	0	3

NEOLISA/EURIA assays we found incongruencies among patients who were considered as having normal or elevated CgA levels (Table III).

Using Bland Altman plots we showed that the RH RIA assay performed differently compared to the NEOLISA and EURIA, which showed more comparable values (Fig. 1). Values above 50 nmol/L for the NEOLISA and EURIA assay are not included in the Bland Altman plots as they were defined as values > 50 nmol/L (7 patient samples).

There was a significant difference in CgA levels dependent of disease stage (Fig. 2), with a significant difference between patients with liver metastases compared to disease free patients for all three assays ($P < 0.001$). There was no difference in CgA levels between disease free patients and patients with regional disease. Only the RH RIA assay demonstrated a significant difference between patients with regional disease and liver metastases ($P < 0.01$).

During follow-up of radically resected NET patients, one patient with lung NET developed radiologically confirmed recurrence at three months after blood sampling. This patient had elevated CgA levels measured by all three assays. For one patient no scans were performed in the follow-up period. For the remaining seven patients, the scans performed during the 12 month follow-up revealed no signs of recurrence.

Kidney function and proton pump inhibitor treatment

Eight of the 42 patients had renal insufficiency defined as elevated plasma creatinine (reference intervals: 60-105 $\mu\text{mol/L}$ for men and 45-90 $\mu\text{mol/L}$ for women) and diminished glomerular filtration rate (GFR) (reference interval > 60 mL/min). Five patients with liver metastases and two with regional disease had elevated levels of CgA. The last patient (83 years old female with hypertension) was free from NET after radical surgery; she had elevated levels of CgA by all three assays, a creatinine level of 152 $\mu\text{mol/L}$ and GFR 28 mL/min.

Eight of 42 patients received PPI treatment at the time of CgA measurement. All had present NET disease and all had

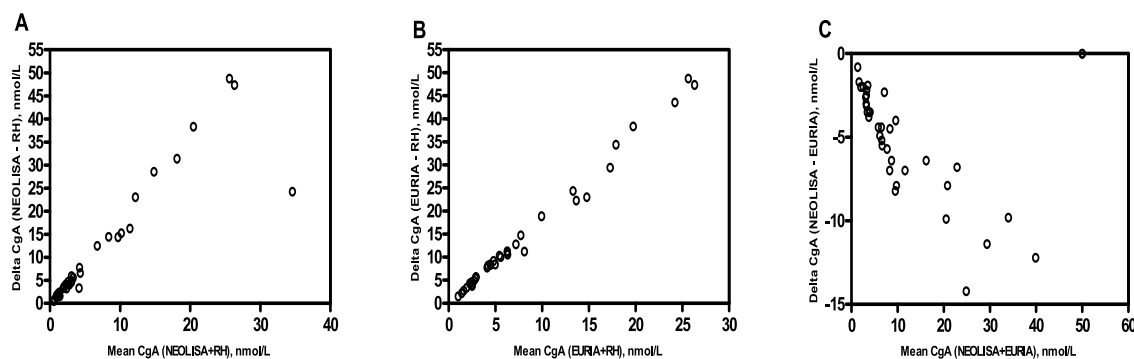


Fig. 1. Bland Altman plots showing differences between the RH RIA and NEOLISA (Panel A), RH RIA and EURIA (Panel B) and NEOLISA and EURIA assay (Panel C).

elevated CgA levels by RH RIA assay; seven had elevated CgA levels also by the NEOLISA and EURIA assays.

Two of the 42 patients had been diagnosed with ulcerative colitis, and both were radically operated for their NET without residual disease at the time of blood sampling. They were treated with Mesalazin (5-ASA). In both cases, CgA levels were elevated using the RH RIA assay but normal using the NEOLISA and EURIA assays.

DISCUSSION

Chromogranin A is the most important general tumour marker used in the diagnosis and follow-up of patients with NET. The main finding in the present study was the marked difference in the clinical sensitivity of the three assays. The in-house RIA assay from Rigshospitalet, Copenhagen had a higher clinical sensitivity than the two commercially available assays from Euro Diagnostica.

We confirmed that the increase in CgA levels to some extent depends on tumour status [13, 14]; however, the present study also demonstrated a significant difference between patients with regional disease and patients with liver metastases when

using the RH RIA assay. This may suggest that the RH RIA assay better predicts disease status compared to the commercially available assays investigated.

Our finding that the clinical sensitivities of CgA assays differ is in line with findings from other studies. Stridsberg et al compared three different commercial CgA assays [CGA-RIA CT (CIS bio international, Gif-Sur-Yvette, Cedex, France); DAKO Chromogranin A ELISA kit (DAKO A/S, Glostrup, Denmark) and CgA (Euro Diagnostica, Malmö, Sweden)] with sensitivities of 67%, 85% and 93%, respectively [8]. Zatelli et al compared the CGA-RIA CT from CIS bio international and the DAKO Chromogranin A ELISA assay from DAKO A/S and found sensitivities of 78% and 84%, respectively [14]. Ferrari et al also compared the CGA-RIA CT from CIS Bio international and the DAKO Chromogranin A ELISA assay from DAKO A/S and found a sensitivity of 79% for both assays [15]. Comparison of the sensitivities found in these studies is, however, difficult as different cut-off values are used in the studies. For the CGA-RIA CT assay the cut-off value was < 99 ng/mL in the study by Stridsberg et al, <70 ng/mL in the study by Ferrari et al and < 53 ng/mL in the study by Zatelli et al. Similarly, the cut-off values used in the evaluation of the DAKO Chromogranin A ELISA assay

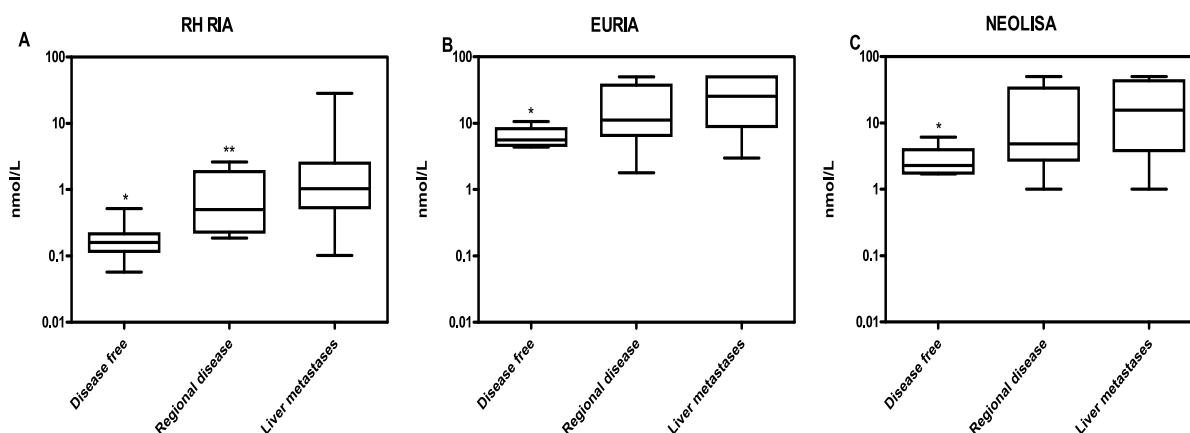


Fig. 2. Chromogranin A measurements in the three disease categories: disease free (n=9), regional disease (n=8), liver disease (n=25) by using the three different assays. Box plots showing the median and range, with the bottom and top of the box illustrating the 25th and 75th percentile for RH RIA (<130 pmol/L), EURIA (<6 nmol/L) and NEOLISA (<3 nmol/L).

* P < 0.001 Disease free patients compared to patients with liver metastases

** P < 0.01 Patients with regional disease compared to patients with liver metastases

was <19 U/L, <34 U/L and <16 U/L, respectively. Therefore, cut-off values for the respective CgA assays should be internationally standardised to compare results from different studies.

The cut-off value for an assay is important for the sensitivity of the assay and we investigated whether the cut-off value could explain the different sensitivities of the assays evaluated. In cases with present NET and elevated CgA by the RH RIA assay but normal CgA levels by the NEOLISA assay, we found that the CgA values measured by NEOLISA were relatively far from the cut-off value of < 3 nmol/L recommended by the manufacturer. This means that it is not desirable to change the cut-off value of this assay in order to reach a higher sensitivity. Similarly, for the EURIA assay, the normal CgA levels measured in patients with present NET disease and elevated CgA levels using the RH RIA assay were relatively far from the cut-off value of < 6 nmol/L recommended by Eurodiagnostica.

Comparison between studies is also difficult due to differences in the study populations. Campana et al demonstrated lower CgA levels in patients with lung NET compared to patients with gastroenteropancreatic NET [16]. Therefore, studies comprising a relatively large number of lung NET patients could be assumed to find lower sensitivities of CgA assays compared to studies with a higher proportion of gastroenteropancreatic NET patients. This should be considered in the interpretation of the studies evaluating CgA as a tumour marker.

We followed patients free from NET after surgery to investigate if they later developed recurrence. One patient with pulmonary NET had recurrence. The remaining seven patients followed-up for 12 months had no signs of recurrence. We investigated if the elevated CgA levels observed in patients free from NET after surgery could be explained by other causes. In three patients, co-morbidity might have contributed to the elevated CgA levels: renal insufficiency (1) or ulcerative colitis (2). None of the patients received PPIs, and none had chronic atrophic gastritis or liver failure.

The present study has some strengths and limitations. We investigated the clinical sensitivity of the three CgA assays in 42 patients with NET, all well characterised by relevant imaging methods and pathology reports. We did not include healthy controls and are therefore not able to draw conclusions regarding the ability of the assays to differentiate between patients with NET and healthy individuals. Future studies should include a larger number of NET patients and also a group of age and gender matched controls.

CONCLUSION

The clinical sensitivity of CgA measurements is assay dependent. The in-house RH RIA assay was better at predicting NET status than the two commercial NEOLISA and EURIA assays. Thus, caution should be applied in the interpretation of CgA measurements for the assessment of NET status.

Conflicts of interest: The authors declare that they have no conflicts of interest that may have influenced this work.

Authors' contribution: L.B.H.: data acquisition, analysis, and interpretation; manuscript preparation. T.P.: data acquisition, analysis and interpretation; critical revision of the manuscript for important intellectual content. C.S.K.: data acquisition, analysis and interpretation; critical revision of the manuscript for important intellectual content. H.G.: design and conception, data analysis and interpretation; critical revision of the manuscript for important intellectual content.

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