

# Chromogranin A as a biochemical marker for the management of neuroendocrine tumors: a multicenter study developed in Argentina

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## Summary

Elevated circulating levels of chromogranin A (CgA) are found in the neuroendocrine tumors (NETs), but diagnostic usefulness of this marker is still debatable. To assess the role of CgA for the identification and follow up of gastroenteropancreatic neuroendocrine tumors (GEP-NET), a multicenter prospective longitudinal study has been carried out in Argentina. CgA was measured by RIA in 119 histologically proven GEP-NET patients and in 39 healthy controls. A cut-off value of 2.8 nmol/L was established from a receiver-operating characteristic (ROC) curve, as discriminating between controls and patients with active disease (specificity 100% and sensitivity 92.3%). CgA levels were higher in functioning than in non-functioning tumors (median 55 nmol/L vs 5 nmol/L,  $p < 0.05$ ). Metastases were present in 83 patients and their CgA levels were significantly higher than levels in the 36 patients without metastases (median 44 nmol/L vs 6.4 nmol/L,  $p < 0.0001$ ). CgA levels are strongly correlated with tumor metastatic spread. Sensitivity differed between patients with localized disease (median 6 nmol/L), extensive disease (median 22 nmol/L) and very extensive disease (median 44 nmol/L) ( $p < 0.001$ ). In conclusion, due to its high sensitivity and specificity,

CgA is useful in a newly discovered GEP-NET, especially when no abnormal hormone secretion can be demonstrated. CgA levels were significantly higher in functioning tumors than in non-functioning tumors and increased with metastatic spread. If serial evaluation of CgA levels is sufficient for the detection of tumor growth changes remains to be prospectively demonstrated.

**Key words:** chromogranins, neuroendocrine tumors, neuroendocrine marker.

## La Cromogranina A como marcador tumoral en la evaluación de los tumores neuroendócrinos: un estudio multicéntrico desarrollado en la Argentina

### Resumen

En los pacientes con tumores neuroendócrinos (TNE) se detectan en la circulación niveles elevados de cromogranina A (CgA), pese a lo cual su utilidad como marcador tumoral es aún motivo de debate. Con el objetivo de determinar el rol de la CgA en la evaluación y seguimiento de los tumores neuroendócrinos gastroenteropancreáticos (TNE-GEP) llevamos a cabo en la Argentina un estudio multicéntrico, longitudinal y prospectivo. La CgA fue medida con un equipo comercial por RIA en 119 pacientes con TNE-GEP histológicamente confirmado y en 39 sujetos control. Con una curva ROC (re-

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ceiver-operating characteristic) *establecimos un valor de corte de 2,8 nmol/l como capaz de discriminar entre los sujetos control y los pacientes con enfermedad activa (100% de especificidad y 92,3% de sensibilidad). La concentración de CgA era mayor en los tumores funcionantes que en los no funcionantes (mediana 55nmol/l vs 5nmol/l,  $p < 0.05$ ). Ochenta y tres pacientes presentaban metástasis y su nivel de CgA era significativamente mayor que el de los 36 pacientes sin metástasis (mediana 44nmol/l vs 6,4nmol/l,  $p < 0.0001$ ). Los niveles circulantes de CgA se correlacionaron con la extensión de la enfermedad metastásica, con una sensibilidad muy alta para diferenciar entre los pacientes con enfermedad localizada (mediana 6nmol/l), enfermedad extensa (mediana 22nmol/l) y enfermedad muy extensa (mediana 44nmol/l) ( $p < 0.001$ ). En conclusión, la CgA mostró una elevada sensibilidad y especificidad en la evaluación de pacientes con TNE-GEP recientemente diagnosticados, especialmente cuando no se demuestra ninguna secreción hormonal anormal. La concentración plasmática de CgA fue significativamente más elevada en los tumores funcionantes que en los no funcionantes y aumentó con la diseminación metastásica. Restaría demostrar en forma prospectiva si la determinación seriada del nivel de CgA es capaz de detectar precozmente cambios en el crecimiento tumoral.*

**Palabras claves:** cromograninas, tumores neuroendócrinos, marcador neuroendócrino.

Chromogranin A (CgA) is a 49 kilodalton acidic glycoprotein that is produced exclusively by endocrine and neuroendocrine cells<sup>1</sup> and is a ubiquitous component of secretory dense core granules, secreted with coresident peptides and biogenic amines.<sup>2</sup> CgA detection in tissue slices by immunohistochemistry confirms the neuroendocrine character of a tumor. Physiologically, the adrenal gland is the main organ source of CgA. It is released by exocytosis and can be detected in blood. When a neuroendocrine tumor develops, it becomes the main source of circulating CgA.<sup>3</sup> It has also been suggested that CgA may be a precious tool to disclose the endocrine nature of a newly discovered gastroenteropancreatic tumor (GEP-NET), for signaling recurrences and thus helpful in the follow up.<sup>4,6</sup> However, previous studies reported different ranges of sensitivity and specificity for circulating CgA, according to the histological characteristics of the tumor,

the disease spread, the method used for CgA determination and the threshold considered as pathologic.<sup>7</sup> In order to clarify this issue, a multicenter observational study has been performed in Argentina in a large series of GEP-NET and healthy controls to establish the best cut-off value for the evaluation of patients with diagnosis of these tumors and to assess the accuracy of CgA for their follow up

## Patients and methods

### Patients

As members of the Group for the Study of Gastroenteropancreatic Neuroendocrine Tumors in Argentina (Argentum), 6 different centers participated in this multicenter prospective longitudinal study. Serum samples were obtained between August 2006 and December 2007 from 119 subjects [53 males and 66 females, aged  $53 \pm 15$  years old (range 13-78 years)] with GEP-NETs diagnosed from 1 to 240 months earlier (median 45 months). All patients had a pathologically proven tumor by histological and immunohistochemical diagnosis after surgery or biopsy of primary tumor or metastases. The primary GEP-NET tumor in the 119 patients arose from yeyuno ileum in 42, pancreas in 26, colon in 9, stomach in 7, appendix in 7, rectum in 7, esophagus in 2 and biliary tract in 1. Eighteen tumors whose primary location could not be identified were classified as GEP-NETs because of the presence of enterochromaffin cells in the biopsy of metastases. In 27 patients specific peptidic hormones (glucagon, insulin, gastrin) or biogenic amines metabolites (5-hydroxyindoleacetic acid) were available.

Exclusion criteria included kidney insufficiency (creatinine  $> 120 \mu\text{M/L}$ ), liver failure, Parkinson's disease, treatment with proton pump inhibitors and pregnancy.

Conventional imaging (abdominal and thoracic CT and/or MRI) as well as somatostatin receptor scintigraphy were used for staging. Eighty-three patients (70%) presented with metastases. The status of the disease was defined as localized, locally advanced (regional lymph nodes involvement), extensive disease ( $< 3$  metastases affecting either liver, bone or lung) and very extensive disease (multiple metastases in the liver or metastases affecting at least two organs).

Moreover, according to the status of the disease at the enrollment, patients were divided into three groups:

- 1) New diagnosis when a patient was diagnosed at a centre for the first time with evidence of disease at study entry.
- 2) Stable disease when a patient medically treated had stable disease for at least 6 months.
- 3) Remission when a patient previously treated (surgically or medically) had no evidence of disease.

### Control grup

They were strictly selected from patients visiting our outpatient clinics because of problems not related to gastrointestinal diseases. All the healthy controls had normal renal function based on the creatinine concentration and none of them was treated with proton pump inhibitors at the time of the study. The control group was composed of 39 healthy subjects [20 males and 19 females, mean age 32 years old (range 16-75 years)].

### Ethics

All patients gave written informed consent and the study protocol was approved by the institutional ethics committee of each participating center. The study was carried out according to the Helsinki Declaration of human studies.

### Methods: serum CgA determination

All samples were collected after an overnight fasting and centrifuged at 3,000 g within 30 min. Serum was frozen in aliquots and kept at -20°C before the analysis in different assays. CgA was measured with a commercial RIA kit (BioSource Europe, Belgium). This kit is a competitive radioimmunoassay using polyclonal antibodies raised in rabbits. The antibodies are raised against a purified fragment containing amino acid sequence 116-349 in the human CgA molecule. The source of calibrator is human material. The working calibrators are prepared by 10 nmol/L dilutions of the CgA calibrator with assay buffer. Seven standards of 0, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00 nmol/L, two controls of 0.44 nmol/L and 1.97 nmol/L, and patients' samples are performed in duplicate. This assay displays an analytical sensitivity of 1 nmol/L and the cut-off value recommended by the manufacturer is 10 nmol/L. The within and between assay coefficients of variation are 3 % and 8%, respectively, for a CgA level of 1,97nmol/L.

### Statistical analysis

CgA levels are reported as the mean  $\pm$ S.D, the

median and the range. Group comparisons of values from independent groups were performed using the nonparametric Wilcoxon test. A  $p < 0.05$  was considered significant in all tests.

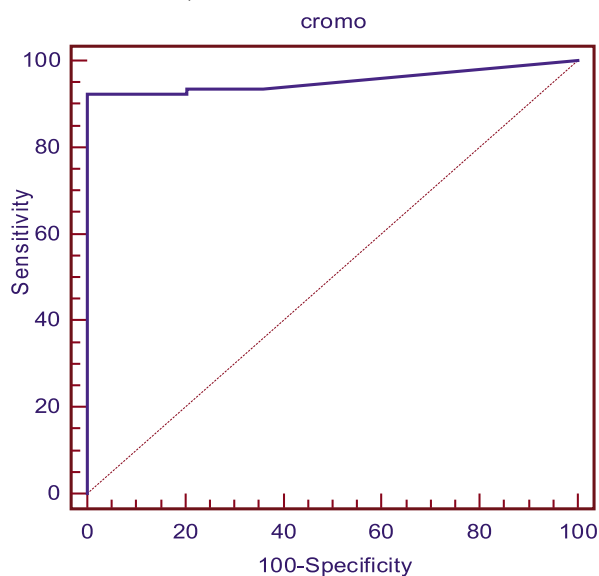
In order to identify a cut-off value that could distinguish between healthy subjects and affected patients, a receiver-operating characteristic (ROC) analysis was performed using a statistical software package. CgA levels of the 39 controls and the 119 patients with GEP-NET were used to construct the curve. The area under the ROC curve (AUC) indicated the marker capability to discriminate between patients and controls. Sensitivity and specificity were calculated using the standard formulae.

### Results

CgA in healthy controls showed a narrow range, between 1 and 2.8nmol/L. Although we used a small control group, we validated the transference of reference interval reported by the manufacturer because any of these subjects fell outside the original limit of 4nmol/L.<sup>8</sup> As shown by the ROC curve (Figure 1), a cut-off value of 2.8nmol/L for the diagnosis was identified, providing the best compromise between a 100% specificity and a 92.3% sensitivity. This value was chosen for further analysis.

Cg A level was in the normal range in all healthy

**Figure 1.** Receiver-operating characteristic (ROC) curve obtained with 39 controls and 119 patients with GEP-NET. The area under the curve was 0.953 (95% CI 0.915-0.978).



controls, 1/32 patients newly diagnosed, 5/29 patients with endocrine tumor in remission and 1/58 patients with stable disease. There was a wide dispersion in CgA levels in patients with GEP-NET, ranging from 1 to 8,000 nmol/L, and it was not normally distributed. CgA levels in GEP-NET patients distributed according to their clinical status at recruitment are shown in Table 1.

**Table 1.** Basal Cg A levels in 119 patients with GEP-NET according to the recruitment group.

Cg A (nmol/L)	New diagnosis (n=32)	Remission (n=29)	Stable (n=58)
Mean ± SD	78.57 ± 98.36	6.88 ± 4.82	240.89 ± 1072
Median	32	6	25
Range	3 - 375	1 - 25	1 - 8000

CgA levels were statistically different in all conditions (p<0.001) except between patients with new diagnosis and stable disease.

There was no statistical difference between serum concentration of CgA in gastrointestinal tumors and pancreatic tumors (mean 135.03 ± 824.03 nmol/L, median 18.5 nmol/L, range 1-2,000 nmol/L vs mean 152.90 ± 401.80 nmol/L, median 10 nmol/L, range 1-8000 nmol/L) (NS).

Twenty two patients with elevation of peptidic hormones in serum (gastrin, glucagon, insulin) or 5-hydroxyindolacetic acid in urine were named as functioning tumors and 5 patients without any elevation, were considered as having non functioning tumors.

CgA serum concentration was significantly higher in patients with functioning tumors than in patients with non functioning tumors (mean 170.11 ± 420.73 nmol/L, median 55 nmol/L, range 3-2000 nmol/L vs mean 9.74 ± 12.58 nmol/L, median 5 nmol/L, range 1.9-32 nmol/L) (p<0.05).

CgA levels were significantly higher in the 83 patients with metastatic tumors than in the 36 patients with non metastatic tumors (mean 218.15 ± 956.15 nmol/L, median 44 nmol/L, range 1-8000 nmol/L vs mean 10.35 ± 17 nmol/L, median 6.4 nmol/L, range 1-110 nmol/L) (p<0.0001).

To evaluate the influence of the secretory condition of the tumor and the metastatic status, CgA levels in localized and metastatic patients were further divided according to functional status: functioning

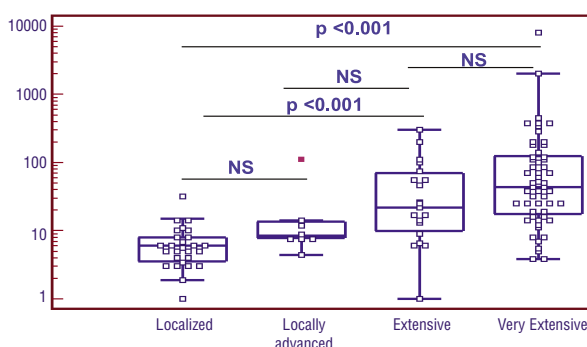
vs non functioning. Table 2 shows the respective influence of the secretory and metastatic status. CgA levels were significantly higher in metastatic patients whatever the secretory status was (p<0.005).

**Table 2.** CgA levels according to the metastatic status: localized (L) and metastatic (M) in functioning (F) and non functioning (NF) tumors, respectively. Kruskal-Wallis test shows a heterogeneity between the subgroups L and M (p<0.005).

CgA (nmol/L)	NF / L	F / L	NF / M	F / M
Mean ± SD	10.72 ± 14.24	7.3 ± 4.97	580 ± 948	99.97 ± 111.89
Median	4.5	5.4	140	75
Range	1.9-32	3-14	40-2000	3.8-375

According to the extent of metastatic spread, 27 patients had localized disease, 8 patients with lymph node involvement were considered as locally advanced disease, 24 patients with less than 3 metastases as extensive disease and 60 patients with 3 or more metastases as very extensive disease. A highly significant positive relationship was demonstrated between the tumor load and CgA levels in patients with localized disease, extensive disease and very extensive disease (p<0.001) (Figure 2).

**Figure 2.** CgA levels in 119 GEP-NET patients according to the extent of metastatic spread: 27 patients had localized disease, 8 locally advanced disease with lymph node metastases, 24 extensive disease with less than 3 metastases and 60 very extensive disease with three or more metastases. Individual levels are presented as dots and median as lines.



**Discussion**

This multicenter observational study deals with the practical value of CgA as a biochemical marker for the evaluation and follow-up of GEP-

NET tumors. CgA is a glycoprotein released together with hormones upon stimulation of normal neuroendocrine cells but is also released from neuroendocrine tumors. In normal and tumoral neuroendocrine cells, CgA exists in its native form coexisting with several CgA related peptides. Different neuroendocrine cells can also process CgA differently. Tumors can also release different molecular forms of CgA. Therefore, CgA released from different neuroendocrine tumors can be expected to exist in different molecular forms. CgA circulates as an intact molecule together with cleavage breakdown products and the clinical value of CgA measurement relies on the detection of most of its circulating forms.<sup>9</sup>

We used a commercial competitive radioimmunoassay with a polyclonal antibody directed against several epitopes of the CgA amino acid sequence 116-439, that measure intact and fragmented CgA.<sup>10</sup> In the present study, the specificity was 100% when a cut-off value of 2.8 nmol/L calculated from a ROC curve analysis was used. This high specificity may be related to the setting up of a control group including only strictly selected healthy subjects. Healthy subjects showed a narrow range of CgA levels and any of them had high baseline CgA as was shown by others.<sup>11,12</sup> This high specificity indicates that CgA has a good diagnostic value in the evaluation of a newly discovered neuroendocrine digestive tumor, specially when no abnormal hormone secretion can be demonstrated.<sup>6</sup> The method also showed a high sensitivity (92.3%), probably because this competitive assay using polyclonal antibodies can detect more antibodies binding epitopes than a non-competitive assay, like some IRMA and ELISA assays using monoclonal antibodies. Our results agree with a recent study comparing commercial kits that showed for RIA assay a sensitivity of 93% using polyclonal antibodies and 67% using monoclonal antibodies, and a specificity of 88% and 96%, respectively.<sup>10</sup> The performance of the RIA kit using polyclonal antibodies was better than previous reports with a modest sensitivity (77.8%, 84% and 85%) and specificity (71.3%, 85.3% and 85%) for both IRMA and ELISA assays.<sup>10,12</sup>

In the GEP-NET group, CgA was in the normal range only in a patient newly diagnosed with an insulinoma not detected by conventional images. A possible explanation is that CgA levels are strongly related with the tumor burden<sup>4</sup> and insulinomas are

usually detected at an early stage of their oncological evolution because of the hypoglycaemia.

As regard to the functional status, CgA levels were significantly higher in functioning tumors than in non functioning tumors.<sup>7</sup> In disagreement with previous reports,<sup>4,7,13</sup> no difference in CgA levels between functioning pancreatic and gastrointestinal tumors was found in the present study. Taking into account the metastatic status, it has been suggested that CgA is a reflection of tumor spread with the highest level when liver metastases had developed.<sup>13,14</sup> In keeping with previous reports<sup>6,25</sup> our results confirmed higher CgA levels in metastatic patients when compared with those without metastases. Only one patient with liver metastases had normal levels of CgA. He had a poorly differentiated neuroendocrine carcinoma with a high proliferative index (54%) that usually does not or scarcely express CgA. When the secretory activity and the spread of the disease were analyzed, CgA level was related to the presence of metastases independently of the functional status.

In this prospective study among the patients in follow up for a long time, only 5 of 58 patients with endocrine tumor in remission and 1 of 29 patients with stable disease by other clinical criteria showed normal levels of CgA. This might suggest that CgA remains indicative of the patient status and that changes in CgA levels accurately correlate with positive tumor response.<sup>16</sup> However it remains to be established if the sensitivity of CgA as tumor marker is sufficient to detect small tumoral changes. In the guidelines for the management of gastroenteropancreatic tumors from ENETS<sup>17</sup> and UKNET work<sup>18</sup> CgA was considered as the minimally required biochemical test for diagnosis and for follow-up.

In agreement with previous reports,<sup>4-6,19,20</sup> our study demonstrated a statistically significant difference among groups with increasing metastatic spread. In disagreement, a recent Italian multicenter study<sup>12</sup> demonstrate that CgA level was lower in patients with very extensive metastatic spread than in those with metastases limited to the liver.

In conclusion, due to its high specificity, CgA is a useful biochemical marker for the evaluation of patients with NET-GEP. CgA levels were significantly higher in functioning tumors than in non functioning tumors and increased with metastatic spread. The capability of CgA levels to detect tumor growth changes should be further investigated.

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