Blood Chromogranin A Is Not Effective as a Biomarker for Diagnosis or Management of Bronchopulmonary Neuroendocrine Tumors/Neoplasms

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Keywords

Bronchopulmonary · Lung · Chromogranin A · Biomarker · Carcinoid · Diagnosis · Prognosis

Abstract

Background: Identification of circulating tumor markers for clinical management in bronchopulmonary (BP) neuroendocrine tumors/neoplasms (NET/NEN) is of considerable clinical interest. Chromogranin A (CgA), a “universal” NET biomarker, is considered controversial as a circulating biomarker of BPNET. **Aim:** Assess utility of CgA in the diagnosis and management of BPNET in a multicentric study. **Material and Methods:** CgA diagnostic metrics were assessed in lung NET/NENs ($n = 200$) and controls ($n = 140$), randomly assigned to a Training and Test set (100 BPC and 70 controls in each). As-

say specificity was evaluated in neoplastic lung disease ($n = 137$) and nonneoplastic lung disease ($n = 77$). CgA efficacy in predicting clinical status was evaluated in the combined set of 200 NET/NENs. CgA levels in bronchopulmonary neuroendocrine tumor (BPNET) subtypes (atypical [AC] vs. typical [TC]) and grade was examined. The clinical utility of an alteration of CgA levels ($\pm 25\%$) was evaluated in a subset of 49 BPNET over 12 months. CgA measurement was by NEOLISA™ kit (EuroDiagnostica). **Results:** Sensitivity and specificity in the training set were 41/98%, respectively. Test set data were 42/87%. Training set area under receiver operator characteristic analysis differentiated BPC from control area under the curve (AUC) 0.61 ± 0.05 $p = 0.015$. Test set the data were AUC 0.58 ± 0.05 , $p = 0.076$. In the combined set ($n = 200$), 67% BPNET/NEN ($n = 134$) had normal CgA levels. CgA levels did not distinguish histological subtypes (TC vs. AC, AUC $0.56 \pm$

0.04, $p = 0.21$), grade ($p = 0.45$ – 0.72), or progressive from stable disease (AUC 0.53 ± 0.05 $p = 0.47$). There was no correlation of CgA with Ki-67 index (Pearson $r = 0.143$, $p = 0.14$). For nonneoplastic diseases (chronic obstructive pulmonary disorder and idiopathic pulmonary fibrosis), CgA was elevated in 26–37%. For neoplastic disease (NSCLC, squamous cell carcinoma), CgA was elevated in 11–16%. The neuroendocrine SCLC also exhibited elevated CgA (50%). Elevated CgA was not useful for differentiating BPNET/NEN from these other pathologies. Monitoring BPNET/NEN over a 12-month period identified neither CgA levels per se nor changes in CgA were reflective of somatostatin analog treatment outcome/efficacy or the natural history of the disease (progression). **Conclusions:** Blood CgA levels are not clinically useful as a biomarker for lung BPNET/NEN. The low specificity and elevations in both nonneoplastic as well as other common neoplastic lung diseases identified limited clinical utility for this biomarker.

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Introduction

Cancer biomarkers are essential for the identification of disease and as tools to quantify disease progression (PD) and outcome. Numerous types of biomarkers have been proposed and studied [1]. Among these, dysregulated physiological processes such as increased secretory products have been investigated since they represent a process specific to a cell type and are quantifiable, thereby providing a reflection of disease status or a surrogate for treatment efficacy [2]. The acquisition of biological fluids (e.g., urine, saliva, tears, and plasma) is less invasive than tissue biopsy and all possess assessable analytes ranging from nucleic acids to tumor cells [3]. Irrespective of the diverse biomarkers available, a critical issue in assessing clinical utility is the need for rigorous establishment of diagnostic sensitivity and specificity [4]. A further metric of particular clinical relevance is the demonstration that alterations in biomarker levels can be correlated with clinical management of disease. Overall, imaging and blood biomarker measurements remain the 2 main strategies for monitoring disease status. The former, however, requires sophisticated equipment, often includes exposure to radiation and in some relatively indolent diseases, such as neuroendocrine tumors (NET), has limitations in sensitivity [5].

Bronchopulmonary neuroendocrine tumors (BPNET; also known as carcinoids) are neuroendocrine neoplasms (NEN) that range from low-grade typical (TC) to intermediate-grade atypical (AC) tumors [6]. The primary diag-

nosis for BPNET/NEN is by histological analysis of tissue obtained by surgery or biopsy [7]. Identification of a neuroendocrine phenotype relies on the elucidation of tumor architectural features and immunohistochemical markers such as chromogranin A (CgA), synaptophysin, and CD56 [6]. Assessment of the rate of tumor cell proliferation is, to some extent, defined by Ki-67 immunostaining [8]. Differentiation of histological variants, TC from AC, is undertaken by mitotic count: TC <2 mitoses/10 high power field, AC 2–10 mitoses/10 high power field [6, 8]. In addition, the presence of necrotic features in AC tumors compared to TC is considered to be of value in distinguishing the groups. Imaging is of some benefit in defining the disease. Thus, CT, MRI $^{68}\text{Gallium-DOTATE/TOC/NOC}$ PET/CT, and FDG PET/CT have been used with varying degrees of success [9, 10]. Irrespective of grade, surgical resection is the treatment of choice and considered as the definitive curative approach [11]. Postresection disease monitoring is variously undertaken using both blood CgA measurement and imaging [12].

CgA, a member of the granin family, is a protein in the secretory granules of neuroendocrine cells, which is implicated in the synthesis of signaling molecules [13]. There are a number of CgA-derived peptides, such as pancreastatin and catestatin, involved in the secretory regulation of various hormones as well as metabolic regulation and vasoconstriction [14]. The use of CgA as an immunohistochemical marker is widespread and accepted and is utilized in diverse endocrine diseases [15]. As a circulating biomarker, CgA can be measured in serum or plasma and is commonly used in assisting the diagnosis and management of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) [16]. Despite its common usage, the specificity of CgA is <50%, and the sensitivity in GEP-NETs has been variously reported to range from 60 to 90% [5]. Further concerns include the widely differing individual assay metrics and spurious results associated with the use of pharmacological agents (proton pump inhibitors) and diverse pathological conditions, especially renal disease [17, 18].

Although BPNET/NEN share many homologies with GEP-NET, including symptomology and the secretion of bioactive peptides, a recent NET Biomarker Consensus concluded that GEP-NET circulating biomarkers (i.e., CgA) were neither sensitive nor specific for bronchopulmonary neuroendocrine neoplasia [5]. A number of investigations have evaluated circulating CgA in BPNET/NEN with varying stages of disease and found no significant differences corresponding with nodal metastasis and tumor size following TNM classification [19]. No finite

assessment of the clinical utility of CgA in BPNET/NEN has yet been undertaken.

It is generally accepted that circulating CgA exhibits poor diagnostic metrics in NETs [20]. The Consensus on Biomarkers for NETs [5] considers it as a monoanalyte and a class/type “0” according to the NIH biomarker classification. Based upon the concerns relating to the low sensitivity and specificity of CgA as a biomarker in GEP-NETs and the minimal studies conducted with large cohorts of BPNET/NEN, we undertook a more robust evaluation of circulating CgA. The aim of this study was to assess the clinical value of circulating CgA measurement in a comprehensive cohort of healthy controls, BPNET/NEN with varying histology, grade, status and disease extent, nonneuroendocrine lung diseases, and in BPNET/NEN patients monitored over time.

Subjects and Methods

Patients

All patients provided informed consent for plasma biomarker analysis authorized by local Ethics Committees. This multicenter retrospective cohort was collected between June 2013 and February 2018. It included patients and nonaffected family members attending oncology, endocrinology, and pulmonology out-patient clinics. Inclusion criteria included histological confirmation of disease. No exclusion criteria were used. Cohorts included healthy controls, BPNET/NEN of various grade, histology, status, and therapies, as well as nonneuroendocrine lung diseases (Table 1). Whole-blood plasma for CgA measurement was collected in EDTA-plasma preparation tubes.

The clinical utility of CgA was evaluated in 4 areas: histology, PD, efficacy of somatostatin analogs (SSA), and as a prognostic biomarker. First, we examined the relationship between histological subtype, Ki-67, and CgA levels. For this, we compared CgA plasma levels in TC and AC and evaluated whether these correlated with Ki-67 expression in tumor tissue (data from clinical records). Because this was a multicentric study, no central review was undertaken. Histological assessment at each institution was per standard European Neuroendocrine Tumour Society criteria [12]. We then evaluated its use as a clinical monitoring tool in longitudinally followed patients ($n = 49$). Subjects underwent testing at baseline and thereafter every 3 months for a total of 12 months. Second, the relationship between CgA and clinical status was examined. This was assessed by imaging every 3 months and disease stabilization (SD) or PD determined by RECIST 1.0 criteria in the 49-patient cohort. This allowed us to determine whether CgA provided a real-time measurement that accurately correlated with disease status (disease stabilization [SD] or PD). Third, we evaluated the effect of SSAs on CgA levels. A subset of twenty patients (41% of the 49) was treated with Sandostatin LAR (20 or 30 mg), and CgA levels were evaluated in terms of response to therapy. Fourth, we examined whether CgA had use as a type 0 NIH biomarker (a marker of the natural history of disease or prognostic marker, and whether it correlated with clinical outcomes – SD or PD).

Disease Cohorts

We examined circulating CgA levels from BPNET/NEN ($n = 200$) compared to healthy controls ($n = 140$), benign pulmonary disease: idiopathic pulmonary fibrosis (IPF: $n = 50$), chronic obstructive pulmonary disorder (COPD: $n = 27$); neoplastic lung disease: small cell lung cancer (SCLC: $n = 32$), squamous cell carcinoma (SCC: $n = 43$), and adenocarcinomas cell carcinoma (ACC: $n = 62$).

Analytic Strategy

All plasma samples were randomly assigned to a training and test group. Each of these 2 sets comprised 100 carcinoids and 70 controls. Once all samples were measured, an independent correlation of each value with the clinical diagnosis was undertaken. Diagnostic accuracy and metrics (sensitivity, specificity) were calculated for each set. The area under the curve (AUC) and p value were then calculated between various cohorts (non-NE lung diseases, histological variants and grade, and in clinical status). For clinical utility, we correlated changes in CgA levels with disease outcome.

CgA Enzyme-Linked Immunosorbent Assay

CgA was measured using the NEOLISA™ CgA kit (EuroDiagnostica, Malmö, Sweden). The assay was undertaken in a clinical laboratory (Wren Laboratories CL-0704, CLIA 07D2081388). The upper limit of normal for this CgA ELISA assay is ≤ 108 ng/mL [21].

Statistical Analysis

D’Agostino and Pearson normality tests and the Shapiro-Wilk normality test both identified that CgA was not normally distributed ($K2: 59.07$ – 145.1 ; W -value: 0.257 – 0.73 ; both $p < 0.0001$). We therefore used nonparametric statistics for analyses [22]. Nonparametric (Mann-Whitney U test, 2-tailed) comparisons, Fisher’s exact test (2-tailed), and receiver operator characteristic curve analyses were therefore used between cohorts. In one instance, the relationship between 3 categorical variables was tested using a 2-tailed χ^2 test. Metrics calculated included sensitivity, specificity, the z score (values >1.96 are significant), calculation of the Youden J index (performance of a diagnostic), Kaplan-Meier survival analysis, and spider plots. For spider plot analyses, a 2-way ANOVA (using Sidak’s multiple corrections test) was undertaken to evaluate differences between means in each of the 2 categories (treated vs. untreated) at the different time points. Prism 6.0 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and MedCalc Statistical Software version 16.2.1 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2017) were utilized. Values are provided as mean $\pm$ SD and (median [interquartile range]).

Results

A. Diagnostic Utility

A1 Discovery Set: BPNETs/NENs and Controls

The initial study established circulating CgA plasma levels in a Training Set and thereafter in a Test Set. CgA levels in BPNET/NEN were compared with controls and the metrics for each set defined utilizing the NEOLISA™ 108 ng/mL cutoff.

Table 1. Patient demographics – discovery and test cohorts

Variable	Controls (<i>n</i> = 140)	BPNET/NEN (<i>n</i> = 200)	AdenoCa (<i>n</i> = 62)	SCC (<i>n</i> = 43)	COPD (<i>n</i> = 27)	IPF (<i>n</i> = 50)	SCLC (<i>n</i> = 32)
Age, years, mean (range)	53 (18–89)	60 (21–82)	64.5 (36–84)	64.2 (41–82)	62.7 (22–85)	66 (43–87)	63.4 (31–81)
Gender (male:female)	70:70	80:120	36:26	26:17	13:14	50:10	26:6
Tumor type/ grade [§]	N/A	TC: 116 ^{&} (58%) AC: 84 (42%)	T1: 17 (27%) T2: 22 (35%) T3: 5 (8%) T4: 12 (19%) No data: 6 (11%)	T1: 3 (7%) T2: 14 (33%) T3: 12 (28%) T4: 7 (16%) No data: 7 (16%)	N/A	N/A	N/A
Disease extent	N/A	Metastatic: 100 (50%) ^{&&}	Metastatic: 42 (68%)	Metastatic: 36 (84%)	N/A	N/A	Metastatic: 18 (56%)
Disease status (RECIST 1.0)	N/A	SD: 145 (72.5%) PD: 55 (27.5%)	DE: 6 (9%) SD: 19 (31%) PD: 37 (60%)	SD: 22 (51%) PD: 21 (49%)	N/A	N/A	ND
Current treatment	N/A	Everolimus: 1 (0.5%) Pembolizumab: 2 (1%) CapTEM: 2 (1%) Chemotherapy: 6 (3%) PRRT: 2 (1%) Radiotherapy: 1 (0.5%) SSA: 41 (20.5%) Currently not treated: 145 (72.5%)	Targeted therapy: 9 (14%)* Chemotherapy: 16 (26%)** Currently not treated or blood sample collected prior to treatment start: 37 (60%)	Immunotherapy: 5 (12%)* Chemotherapy: 13 (30%)* Radiation therapy: 5 (12%) Currently not treated or blood sample collected prior to treatment start: 20 (46%)	N/A	N/A	Chemotherapy: 8 (25%)*

[&] MEN-1 (*n* = 1).

^{&&} Lymph node metastases (*n* = 54); stage IV disease (including liver metastases) (*n* = 46).

* Crizotinib, Nivolumab; ** Cisplatinum-based (with or without Vinorelbine).

*** Nivolumab.

**** Predominantly Cisplatinum-based (with Vinorelbine).

***** Predominantly Etoposide and cisplatinum.

[§] For adenocarcinoma and squamous cell cancer (SCC), this is the T-classification for lung tumors. N/A, not applicable.

AC, atypical carcinoid; AdenoCa, adenocarcinoma; BP, bronchopulmonary; CapTEM, capecitabine and temozolomide; COPD, chronic obstructive pulmonary disorder; IPF, idiopathic pulmonary fibrosis; ND, no data; NEN, neuroendocrine neoplasia; NET, neuroendocrine tumor; PD, progressive disease; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer; SD, stable disease; SSA, somatostatin analog; TC, typical carcinoid.

Fig. 1. CgA levels in BPNET/NEN training and test sets. **a** Training set CgA levels: In BPNET/NEN, mean level was elevated versus controls (* $p < 0.01$). In the test set, mean BPNET/NEN level was not different to controls ($p = 0.2$). **b** The AUC for differentiating BPNET/NEN from controls for the training set was 0.61 (95% CI

0.53–0.69, $p = 0.015$) and for the test set was 0.58 (95% CI 0.50–0.66, $p = 0.076$). **c** CgA levels were elevated in 33% ($n = 66$) of the 200 BPNET/NEN. The remaining 134 (67%) samples fell within normal levels (≤ 108 ng/mL). CgA, chromogranin A.

A1.1 Training Set. The mean circulating CgA level in BPNET/NEN from the training set ($n = 100$, $604 \pm 1,961$ ng/mL; [83 (35–273)]) was significantly higher ($p < 0.01$) compared to controls ($n = 70$, 65 ± 38 ng/mL; [55 (41–75)]; Fig. 1a). AUC analysis could differentiate BPNET/NEN from controls (AUC 0.61 ± 0.05 , $p = 0.015$, z score: 2.43, Youden index J: 40%) but although significant, it should be noted that an AUC of 0.6 would be considered a poor biomarker (Fig. 1b). The sensitivity and specificity for the training set were calculated as 41 and 98%, respectively.

A1.2 Test Set. The mean CgA level was not elevated ($p = 0.2$) in BPNET/NEN from the test set ($n = 100$, $534 \pm 2,030$ ng/mL; [64 (41–107)]) compared to the controls ($n = 70$, 60 ± 21 ng/mL; [57 (48–67)]; Fig. 1a). The AUC analysis was not statistically significant (AUC 0.58 ± 0.05 , $p = 0.076$, z score: 1.78, Youden index J: 30%; Fig. 1b). The sensitivity and specificity were 42 and 87% respectively.

A1.3 Combined Set. Mean CgA levels were $569 \pm 1,990$ ng/mL (71 [38–161]) in the combined set of 200 BPNET/NEN. CgA levels were normal in 67% ($n = 134$) and elevated in 33% ($n = 66$) of the cohort (Fig. 1c).

A2 CgA Levels in Comparator Sets: Benign and Malignant Lung Disease

CgA was evaluated in a cohort of benign lung disease and in nonneuroendocrine malignant lung neoplasia to assess the specificity for BPNET/NEN.

In the benign cohort, IPF patients ($n = 50$, 103 ± 114 ng/mL; [70 (42–121)]) had an elevated mean value compared to controls ($n = 140$, 62 ± 31 ng/mL; [57 (46–69)]), $p = 0.038$). Twenty-six percent ($n = 13$) of IPF patients had elevated CgA levels compared to 2.8% ($n = 4$) of controls. This was significant ($p < 0.0001$, Fisher's test). Those with COPD also had an increased mean level ($n = 27$, 144 ± 157 ng/mL; [79 (50–182)]), $p = 0.0037$) compared to controls (Fig. 2a). The proportion of COPD with elevated CgA levels was 37% (10/27); this was significant ($p < 0.0001$, Fisher's test).

In lung ACC, the mean CgA level was not elevated ($n = 62$, 60 ± 30 ng/mL; [56 (15–78)]), $p = 0.55$ versus controls); 7 (11%) exhibited elevated levels. The mean CgA level was marginally elevated in SCC ($n = 43$, 74 ± 87 ng/mL; [44 (36–68)]), $p = 0.045$ versus controls); 7 (16%) were elevated. Both ACC and SCC mean CgA levels were significantly lower than in COPD ($p < 0.005$) but were not different to IPF mean levels ($p = 0.07$ –0.08).

The mean level of CgA in SCLC was significantly elevated ($n = 32$, 118 ± 83 ng/mL; [106 (54–150)]) versus controls (62 ± 31 ng/mL; [57 (46–69)]); $p < 0.0001$) but was not elevated versus COPD (144 ± 157 ng/mL; [79 (50–182)]); $p = 0.76$) or IPF ($p = 0.11$). The proportion of SCLC with elevated CgA levels was 50% (16/32); this was significant ($p < 0.0001$, Fisher's test) versus controls but was not significant versus BPNET/NEN ($p = 0.07$, Fisher's test).

Fig. 2. CgA levels in benign and malignant lung disease. **a** Mean CgA level in COPD ($n = 27$) was elevated ($p = 0.0037$) compared to controls ($n = 140$). Mean level in ACC ($n = 62$) was not elevated ($p = 0.55$), but the mean level was increased ($p = 0.045$) in SCC ($n = 43$). ACC and SCC CgA levels were significantly less than COPD ($p < 0.005$). IPF ($n = 50$) had elevated mean level compared to controls ($p = 0.038$). SCLC ($n = 32$) also exhibited elevated levels compared to controls ($p < 0.0001$). **b** AUC analysis could not dif-

ferentiate COPD or IPF from BPNET/NEN (AUC 0.52–0.54, $p = 0.48$ – 0.64), and the AUC between ACC and SCC and BPNET/NEN was not statistically significant (AUC 0.61–0.63, $p = 0.06$). SCLC likewise could not be differentiated from BPNET/NEN (AUC 0.56, $p = 0.23$). CgA, chromogranin A; ACC, adenocarcinoma cell carcinoma; COPD, chronic obstructive pulmonary disorder; IPF, idiopathic pulmonary fibrosis; SCC, squamous cell carcinoma; BPNET, bronchopulmonary neuroendocrine tumor.

AUC analysis could not differentiate between either COPD (AUC 0.54 ± 0.05 , $p = 0.48$) or IPF and BPNET/NEN (AUC 0.52 ± 0.04 , $p = 0.64$). For ACC (AUC 0.61 ± 0.04 , $p = 0.06$), SCC (AUC 0.63 ± 0.06 , $p = 0.06$), and SCLC (AUC 0.56 ± 0.05 , $p = 0.23$), the metrics were not significant compared to BPNET/NEN (Fig. 2b).

B. Clinical Utility

B1 CgA Evaluation in BPNET/NEN (TC and AC) and Ki-67

We examined whether CgA levels were associated with histological subtype or correlated with the tissue Ki-67 proliferation index.

B1.1 Histology. CgA levels were evaluated between TC ($n = 116$) and AC ($n = 84$). The mean CgA level was not significantly increased in TC ($415 \pm 1,772$ ng/mL; [69 (34–128)]), $p = 0.11$) compared to the controls ($n = 140$; 62 ± 31 ng/mL; [57 (46–69)]), but was elevated in AC (672 ± 2011 ; [72 (47–274)]), $p = 0.0041$; Fig. 3a). The proportion of TC with elevated CgA was 35 (30%); this was significant compared to controls (4/140; $p < 0.0001$, Fisher's test). The proportion of AC with elevated CgA was 30 (36%); this was significant compared to controls ($p < 0.0001$, Fisher's test). No significant differences in those

with an elevated CgA was noted between TC and AC ($p = 0.45$). An AUC analysis (TC vs. AC), likewise, was not statistically significant and could not differentiate between the 2 histological variants (0.56 ± 0.04 , $p = 0.21$; Fig. 3b).

B1.2 Ki-67. Ki-67 proliferation index data were available in 110 (55%) of the 200 BPNET/NEN. Grade 1 BPNET/NEN ($n = 67$, Ki-67: $\leq 4\%$) had CgA levels averaging $508 \pm 1,906$ ng/mL (78 [32–178]) and in Grade 2 BPNET/NEN ($n = 39$, Ki-67: 5–24%) the levels averaged $943 \pm 2,827$ ng/mL (80 [49–353]). Grade 3 numbers were limited ($n = 4$, Ki-67: $\geq 25\%$) and had CgA levels of 188 ± 157 ng/mL (162 [56–348]). All p values were insignificant ($p = 0.45$ – 0.72) between the 3 grades (Fig. 3c). No significant relationship could be identified between CgA levels and Ki-67 values (Pearson $r = 0.143$, $p = 0.136$; Fig. 3d). CgA measurement could not consistently define histological variants or the different grades of BPNET/NEN. CgA did not correlate with the Ki-67 index for BPNET/NEN.

B2 CgA and Disease Status

CgA was assessed in the context of stage and clinical status (based on RECIST 1.0 criteria) to determine if lev-

Fig. 3. CgA measurement in histological variants and grades of BPNEN/NEN. **a** Mean CgA level was significantly elevated (* $p < 0.005$) for AC ($n = 84$) but not TC ($n = 116$), compared with controls ($n = 140$). Both TC and AC had a similar proportion (30 and 36%, respectively) of elevated levels. **b** AUC analysis could not differentiate CgA levels between TC and AC (AUC 0.56, 95% CI 0.47–0.64, $p = 0.21$). **c** CgA levels between Grade 1 (Ki67 <4%; $n = 67$)

and Grade 2 (Ki67: 5–24%; $n = 39$) BPC were not statistically significant ($p = 0.45$). Grade 3 (Ki67 >24%; $n = 4$) was lower but this was not statistically different ($p = 0.56$ –0.72) compared to Grade 1 and 2. **d** The relationship between Ki-67 grading and CgA levels was not significant (Pearson $r = 0.143$, $p = 0.136$). CgA, chromogranin A; TC, typical; AC, atypical; AUC, area under the curve.

els correlated with PD. Patients with liver metastatic disease (Stage IV) comprised 46 (23%). These patients exhibited an elevated mean CgA level ($2,019 \pm 708$ ng/mL; [109 (63–1,723)]) compared to those with no metastasis ($n = 100$; 194 ± 483 ng/mL; [65 (40–106)], $p = 0.01$) but not versus those with lymph node disease ($n = 54$; 236 ± 466 ng/mL; [64 (32–209)], $p = 0.13$). The proportion of patients with elevated CgA was 43% (Stage IV); 33% (lymph node metastases); and 28% (no metastases). This was not significantly different (χ^2 : $p = 0.18$).

Patients with PD comprised 28% ($n = 55$) of the 200 BPNEN/NEN cohort, while patients with no image-evidence of disease or stable disease (SD) comprised the remaining cohort ($n = 145$). The PD group did not have a significantly elevated mean CgA level (401 ± 769 ng/mL; [70 (46–157)]) compared to SD ($576 \pm 2,184$ ng/mL; [70 (35–152)], $p = 0.47$). However, mean levels in both PD and SD were significantly higher than the mean level in the controls ($p < 0.05$; Fig. 4a). The proportion of SD with elevated CgA was 47 (32%); this was significant compared

Fig. 4. Assessment of CgA in stable and PD. **a** Mean CgA level between SD ($n = 145$) and PD ($n = 55$) was not significant ($p = 0.47$). Mean levels in SD and PD were increased ($*p = 0.018$ – 0.048) compared with controls ($n = 140$). Both SD and PD had a similar pro-

portion (32 and 31%, respectively) of elevated levels. **b** AUC analysis for differentiating SD from PD was not statistically significant (AUC 0.53, 95% CI 0.44–0.62, $p = 0.47$). CgA, chromogranin A; PD, progressive disease; AUC, area under the curve.

to controls (4/140; $p < 0.0001$, Fisher's test). The proportion of PD with elevated CgA was 17 (31%); this was significant compared to controls ($p < 0.0001$, Fisher's test). No significant differences in those with an elevated CgA was, however, noted between SD and PD ($p = 1.0$). In addition, AUC analysis could not differentiate progressive disease from stable disease (0.53 ± 0.05 $p = 0.47$; Fig. 4b).

B3 Clinical Utility of CgA Levels

Of the 200 BPNET/NEN, only 49 could be studied for clinical utility. Evaluations (clinical, radiological, and CgA) occurred every 3–6 months over a 12-month period (Fig. 5). At baseline, 22 (45%) were clinically characterized as progressive disease, while the remaining 27 (55%) were identified as clinically stable.

Eight of the 22 patients with progressive disease exhibited elevated CgA levels at baseline. Six patients (AC, $n = 2$; TC, $n = 4$) did not undergo any treatment over the 12-month follow-up. CgA levels were elevated in 1 patient at baseline. Levels remained elevated for this patient (AC tumor) but remained normal in the other 5 (Fig. 5a).

Sixteen of the 22 PD patients (AC, $n = 8$; TC, $n = 8$) either started ($n = 7$) SSA therapy (Sandostatin LAR 20–30 mg) or continued ($n = 9$) with therapy. At baseline, 7 had elevated levels. SSA was associated with a decrease in CgA in only 1 patient (6%, AC tumor). No changes were noted in 9. Elevation was evident in 6 (Fig. 5a, b). Spider plot analysis (Fig. 5b) identified that SSA was associated

with a significant increase in CgA ($p = 0.0021$; $n = 16$) compared to those not treated ($n = 6$). This suggests the ineffectiveness of SSA in this cohort of PD patients.

Twenty-seven patients were considered stable at baseline. Eleven exhibited elevated CgA levels. Twenty-three patients (AC, $n = 5$; TC, $n = 18$) were not treated over the 12-month follow-up. In 6 patients, CgA decreased despite no treatment; was unchanged in 12 (was always normal $n = 8$ or remained elevated $n = 4$) and increased in 5 (22%; Fig. 5c). In the nine with elevated levels, 2 were PD. Four patients (AC, $n = 1$; TC, $n = 3$) treated with SSAs (Sandostatin LAR 20 mg) all remained clinically stable. Spider plot analysis (Fig. 5d) identified that SSA was not associated with a significant change in CgA ($p = 0.84$; $n = 4$) compared to those not treated ($n = 23$). This suggests low effectiveness of SSA in this cohort of SD patients.

Discussion

When CgA was initially described as a useful NET biomarker, there was considerable enthusiasm [23]. More recent assessments have expressed concerns [24–26]. CgA is currently rated by NCCN guidelines as a category 3 test which is defined: “Based upon any level of evidence, there is major NCCN disagreement that the test is appropriate” [27], while European Neuroendocrine Tumour Society has specifically identified that there is “little or no

Fig. 5. Clinical utility of CgA and BPNET/NEN monitoring. **a** CgA levels of 22 patients with progressive disease either treated with SSA ($n = 16$) or not treated ($n = 6$) over a 12-month period. **b** Spider plot analysis identified a significant difference (increase, $p = 0.0021$) of SSA treatment on CgA. **c** CgA levels of 27 patients with

SD either treated with SSA ($n = 4$) or not treated ($n = 23$) over a 12-month period. **d** Spider plot analysis was unable to differentiate an impact of therapy ($p = 0.84$) over the evaluation period. CgA, chromogranin A.

systematic empirical evidence” to use CgA for lung [12]. The paucity of BPNET/NEN biomarker studies [5], as well as the current CgA controversy, provided the impetus for a formal assessment of this biomarker in BPNET/NEN disease. We evaluated the efficacy of CgA as a circulating biomarker in BPNET/NEN and compared it to controls, nonneuroendocrine lung diseases, BPNET/NEN of different histological variants and grade, and during disease monitoring.

We identified that circulating chromogranin levels were clinically uninformative in the majority (67%) of BPNET/NEN including those with extensive disease (only elevated in 43%). Levels were also elevated in benign and other malignant lung disease. AUC analyses could not differentiate BPNET/NEN from other lung diseases or controls, and the overall accuracy metrics are significantly lower than those considered necessary for an

effective biomarker [5]. Of more relevance to NETs, CgA measurement was incapable of differentiating histological variants (TC vs. AC) and exhibited no correlation with Ki-67. Similarly, both stable disease and progressive disease exhibited elevations in a similar proportion of patients (31–32%). Monitoring BPNET/NEN over a 12-month period identified neither CgA levels *per se* nor changes in CgA were reflective of the natural history of the disease (progression).

“False”-positive levels were a feature of this study. These were identified in benign and malignant lung disease. In benign disease, this likely reflects increased numbers of pulmonary neuroendocrine cells compared to healthy controls [28] or the high prevalence of gastroesophageal reflux and the concomitant use of acid suppressive therapy [29, 30]. A localized increase of neuroendocrine cells is well recognized to increase circulating

chromogranin as noted in gastric carcinoids and ECL hyperplasia of the gastric fundus [31], while proton pump inhibitor treatment will induce an elevation in this biomarker regardless of short- or long-term use [32]. It is also possible that the concomitant underlying inflammatory processes in the lung that occur with COPD and IPF [33] may involve the diffuse neuroendocrine system and accentuate release of cytokines and neuropeptides [34].

More than 10% of ACC and SCC (16%) also exhibited high levels. We suspect that there may be some degree of neuroendocrine differentiation in those with elevated levels [35] as has been previously noted [6], or inflammation may play a factor. Small cell lung cancers also exhibited elevated levels that are well-described [36] and likely reflect the histological observation that SCLC is of neuroendocrine origin, although poorly histologically differentiated and clinically distinct from carcinoids. SCLC is an aggressive disease with rapid progression and a marker that is unable to discern between SCLC and BPNET/NEN has obvious limitations given the significantly different clinical courses and management of the respective diseases, especially given the low survival rate of SCLC (5-year survival rate: <7%) [37]. Overall, these data demonstrate that CgA is a poor diagnostic in lung neoplasia due to lack of specificity and also identify that elevated CgA levels are not exclusive to BPNET/NEN.

BPNET/NEN are classified into 2 histological variants (AC and TC) based upon biopsy, mitotic count, and areas of necrosis. The significance of correct identification of histology is heightened by the frequent metastases in AC (>50%) as opposed to TC (25%) [12]. The distinction between the AC and TC phenotype is crucial due to their substantially different clinical outcomes with 5-year survival rates for AC being ~50% compared to ~88% in TC [38]. Additionally, there is controversy regarding the role of the Ki-67 proliferation index in differentiating AC (20%) from TC 5% [39]. CgA levels alone cannot distinguish the 2 histological variants. Of note, similar proportions of TC (30%) and AC (36%) exhibited an elevated CgA. AUC analysis alone could not differentiate between the 2 histological variants. We also could not identify any relationship between CgA levels and Ki-67 (grade) per se. This is consistent with 1 parameter, CgA, functioning as a measure of secretion, and a second, Ki-67, as a measure of proliferation. These 2 parameters do not appear to therefore be linked.

Staging also was not associated with clinically useful alterations in CgA. While CgA is known to be higher in those with extensive, for example, stage IV GEP-NET disease [40] and we did identify Stage IV BPNET/NEN to

have the highest levels, only 43% were elevated. Twenty-eight percent of those with no metastases also exhibited elevated levels, while 33% of those with lymph node metastases were abnormal. This was not significantly different identifying that CgA may not be useful for staging BPNET/NEN.

We then, using RECIST 1.0 criteria, evaluated whether CgA levels could be a predictor of the patient's clinical status. Both progressive disease and stable disease had significantly higher CgA levels than controls. Levels were, however, not significantly elevated in PD compared with SD nor were the proportion of patients with elevated levels (31–32%). It is not therefore surprising that the AUC analysis failed to differentiate the 2 cohorts. The detection of progressive disease in BPNET/NEN is of considerable clinical relevance in terms of management and outcome. The decision to utilize certain therapies and treatments, such as surgical resection or chemotherapeutic drugs, is dictated by the identification of progressive versus stable disease [12]. Our data indicate that CgA levels are a poor determinant of PD and therefore cannot effectively facilitate therapeutic options.

We also examined the clinical utility of CgA measurement by monitoring levels in 49 patients over a 12-month period. Of the 49 patients, 22 were defined as progressive disease and 27 were stable at baseline measurements. No significant changes in CgA levels were detected irrespective of the patient's clinical status (SD vs. PD) during treatment, or their response to SSA treatment. Of the 27 patients with stable disease at baseline, 6 patients developed progressive disease during follow-up. Only 2 out of the 6 patients that progressed showed an increase in CgA levels. Changes in CgA levels measured during follow-up were not related to PD, or disease stabilization but were increased in a proportion of PD undergoing treatment. Although these data are limited because they were derived from small subgroups, they do suggest that CgA levels do not consistently reflect either responses to SSA therapy or function as an accurate Type 0 biomarker in BPNET/NEN. Clearly, larger prospective studies are required.

As in any study, there are some limitations. These include that it was multicentric and therefore no central review of histology was undertaken, it was retrospective and some of the cohorts are small. It should be noted that multicentricity may be a positive feature since data from a number of institutions are representative of real-world information. The group sizes studied are also substantial for a relatively uncommon disease. The cohorts of stable and progressive disease evaluated over time are limited and therefore no definitive conclusions can be derived.

However, the data support the general consensus that measurement of a single complex secretory protein cannot capture the complex biological behavior of a proliferative neoplasia [5].

There is a critical need for an effective circulating biomarker since currently only imaging can provide information regarding disease status. Imaging an indolent disease such as BPNET/NEN, however, has significant limitations in terms of costs, radiation exposure, and interpretation [41]. Somatostatin receptors (SSTR1, SSTR2A, SSTR5) expression has been investigated in paraffin-embedded BPNET samples and reported to be of use as a diagnostic, prognostic, and therapeutic marker. Tissue expression levels measured by qRT-PCR may be capable of differentiating BPNET/NEN variants [42]. However, only limited investigation of these markers as circulating targets has been reported (e.g., SSTR2 and 5 detectable on CTCs) [43]. Other strategies reported include ELISA measurement of serum levels of VEGFR-1, which identified that VEGFR-1 levels were elevated and closely correlated with tumor size and metastasis [19]. These important results require further assessment in larger cohorts and an evaluation of whether discrimination between histological variants is feasible. Several miRNAs have been investigated. One study identified a set of 5 miRNAs (miR-129-5p, miR-409-3p, miR-409-5p, miR-185, miR497) that were differentially upregulated in TC versus AC BPCs [44]. A separate study reported that Hsa-miR-222-3p's putative target, *CDKN1B*, was overexpressed in TC compared to AC [45]. The critical limitation of these data is, however, that they are tissue assays and are not currently available as blood tests.

A recent multigene analysis study using whole-blood mRNA has evaluated a 51-NET transcript panel in 25 BPNET/NEN and 25 controls [46]. A multianalyte algorithm analysis technique reported the ability to differentiate BPNET/NEN from controls as well as stable from progressive disease with greater than 90% accuracy [46]. A subsequent follow-up study using the same technology demonstrated the assay had excellent diagnostic metrics (93% sensitivity, 89% specificity) and could accurately differentiate progressive from stable disease (AUC 0.91) [47]. A separate independent study [48], in a large group of patients, confirmed the diagnostic utility of the multigene assay in BPNET/NEN and demonstrated its utility as a marker of progression. The clinical utility of this multi gene analysis (NETest) in the surgical management of BPNET/NEN noted the accuracy of the NETest to identify complete resection and detect residual disease or recurrence [47].

Our study demonstrates that circulating CgA levels are not clinically useful in the diagnosis of BPNET/NEN and have no clinical utility in monitoring disease management. This reflects growing awareness that measurement of monoanalyte biomarkers do not provide an accurate multidimensional view of the complex processes inherent in neoplasia. Although CgA levels were elevated compared to controls, two thirds of all BPNET/NEN patients had normal CgA levels. Assessment of the one-third that were abnormal demonstrated that CgA was incapable of distinguishing progressive from stable disease, failed to define histological variants, exhibited no relationship to grade, and could not be used to predict disease outcome. Moreover, an elevated CgA was commonly identified in benign lung diseases while other neoplasia, for example, NSCLC, also expressed high circulating levels.

CgA is currently considered a controversial biomarker, for GEP-NETs [24, 27] and in particular for BPNET/NEN [12]. We are reminded of the insightful comments of Ovid in AD 1 – “*Tempora mutantur, nos et mutamur in illis*” (Times are changed; we, too, are changed within them) – these immemorial lines penned 2 thousand years ago confirm that previously valid observations may need to be altered with the passage of time. The current study confirms the clearly apparent lack of utility of CgA, a once useful general biomarker, whose efficacy has been supplanted by novel multianalyte genomic biomarkers. We respectfully suggest that further investigations should be undertaken in the evaluation of other markers such as molecular-based assays or alternative proliferative or metabolic regulators of bronchopulmonary NET disease.

Disclosure Statement

M.K. and S.M. are employees of Wren Laboratories. I.A.D. and I.M.M. have consulted for Wren Laboratories LLC. Wren Laboratories is involved in the development of the NETest and is licensed to use it. L.B. has received support from Advanced Accelerator Applications.

Author Contributions

All authors contributed equally to the manuscript.

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