Comparison of the Utility of Cocaine- and Amphetamine-Regulated Transcript (CART), Chromogranin A, and Chromogranin B in Neuroendocrine Tumor Diagnosis and Assessment of Disease Progression

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Context: Prognosis in patients with neuroendocrine tumors (NETs) is often poor, frequently reflecting delayed diagnosis. Hence, accurate and practical NET markers are needed. Cocaine- and amphetamine-regulated transcript (CART) peptide is a potential novel NET marker.

Design and Participants: Circulating levels of CART peptide and the established NET markers chromogranin A (CgA) and chromogranin B (CgB) were measured using RIA in 353 patients with NET (normal renal function) and in controls. Clinical data were collected retrospectively.

Main Outcome Measure(s): The comparative and combined utility of CART, CgA, and CgB for diagnosis and assessment of disease progression was measured in different NET subtypes.

Results: CgA and CgB in combination improved diagnostic accuracy in patients with gut NETs, nongastroenteropancreatic NETs, and NETs with an unknown primary origin compared with each biomarker alone. Measuring CART did not further improve diagnosis in these NET subtypes. For pancreatic NETs, CgB was superior to CgA and CART in detecting stable disease ($P < .007$), whereas CgA and CART in combination were most effective in identifying progressive disease. In phaeochromocytomas/paragangliomas (PCC/PGL), CART was the most useful biomarker for identifying stable ($P < .001$) and progressive ($P = .001$) disease. Consistent with this, plasma CART decreased following PCC/PGL tumor resection, remaining low in all patients in remission, but increasing in those with progressive disease.

Conclusions: CART is a useful marker for identifying progressive pancreatic NETs. CART is superior to CgA and CgB in detecting stable and progressive PCC/PGLs, and may have a role as a surveillance marker for PCC/PGL patients. **(*J Clin Endocrinol Metab* 100: 1520–1528, 2015)**

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Abbreviations: AUC, under the curve; CART, cocaine- and amphetamine-regulated transcript; CgA, chromogranin A; CgB, chromogranin B; eGFR, estimated glomerular filtration rate; ENETS, European Neuroendocrine Tumor Society; GEP, nongastroenteropancreatic; IQR, interquartile range; NET, neuroendocrine tumor; NPV, negative predictive value; OR, odds ratio; PCC, phaeochromocytoma; PGL, paraganglioma; PPV, positive predictive value; RECIST, Response Evaluation Criteria in Solid Tumors; ROC, receiver operator characteristic; SAS, Supra-regional Assay Service; SDHB/ SDHD, succinate dehydrogenase complex.

Neuroendocrine tumors (NETs) are heterogeneous tumors arising from cells that exhibit a neuroendocrine phenotype (1). These cells store various bioactive peptides or amines in large, dense-core vesicles and small, synaptic-like vesicles. Although the incidence of NETs has increased significantly over the last 30 years, 5-year survival remains poor (2, 3). The average delay between onset of symptoms and NET diagnosis is 5–7 years, resulting in almost half of patients presenting with distant metastases. Even in well-differentiated tumors, patients with distant metastases have poor survival (4).

The delay in NET diagnosis is partly due to the nonspecific presentation of these tumors, but is compounded by the lack of early diagnostic biomarkers. The chromogranins are the main soluble proteins within the large, dense-core vesicles, and can be used as markers of neuroendocrine cells (5). Although chromogranin A (CgA) is the most widely used circulating NET biomarker, it is relatively insensitive in identifying early disease (6). Specificity can also be compromised because circulating CgA levels can be increased in a number of conditions other than NETs (7–10). Furthermore, extensive post-translational modification makes CgA assay standardization difficult (11). Chromogranin B (CgB) may be used in conjunction with CgA to improve diagnostic accuracy. Circulating levels of CgB are much less affected by renal failure than CgA (12), although the overall diagnostic sensitivity of CgB as a NET marker is considerably poorer than that of CgA (13).

Although CgA has been reported to predict disease progression in advanced pancreatic NETs (14), additional studies of the currently available circulating NET markers for specific NET subtypes are needed. Recommended grading systems for NETs are based on the mitotic rate or percentage of neoplastic cells, which are immunopositive for the proliferation marker Ki67 (Ki67 index) (15, 16). However, such measurements are often based on relatively small biopsy samples and are not always reflective of the intratumoral heterogeneity that may be present in NETs (17, 18).

Hence, the development of robust circulating NET biomarkers for diagnosis, and to predict disease progression, would facilitate early tumor detection and targeted management (19, 20). A preliminary study showed that patients with a NET had higher plasma cocaine- and amphetamine-regulated transcript (CART) peptide levels than healthy controls (21). CART was first identified as an mRNA transcript up-regulated by cocaine and amphetamine in the rat brain (22). Since then, CART peptide has been shown to be widely expressed in neurons and neuroendocrine cells in tissues including the pituitary, adrenal medulla, gut, and pancreas (22). In addition to normal neuroendocrine cells, CART peptide has been shown to be expressed in NETs including pheochromocytomas (PCC) (23), glucagonomas (24) and insulinomas (25, 26). However, the utility of CART as a circulating NET biomarker has not been assessed.

We evaluated the utility of CART, in comparison with CgA and CgB, as a diagnostic marker in NETs, and in specific NET subtypes, and investigated whether CART can differentiate between stable and progressive disease. Our results suggest that CART is a useful marker for the diagnosis and assessment of tumor behavior in progressive pancreatic NETs and pheochromocytomas/paragangliomas (PCC/PGL).

Materials and Methods

Collection of samples

Local ethical approval was obtained and all participants gave informed consent. Control samples (n = 40) were taken from healthy volunteers working at Imperial College Healthcare NHS Trust, all with normal renal function and not taking any medications. A single nonfasting 5-mL blood sample was taken from each participant and collected into K3 EDTA Vacutainer tubes (Becton, Dickinson, NJ). Samples were centrifuged at 1200× *g* for 10 minutes within 15 minutes of collection. Aliquots of plasma were stored at −20°C until they were assayed in batches.

The Imperial College Healthcare NHS Trust Neuroendocrine Tumor Supra-regional Assay Service (SAS) receives samples for routine clinical analysis from two European Neuroendocrine Tumor Society (ENETS) Centers of Excellence in London: Imperial College Healthcare NHS Trust and the Royal Free London NHS Foundation Trust. Following ethical approval, plasma CART, CgA, and CgB were measured in 481 spare aliquots from confirmed NET patient samples received by the SAS from these two centers. All patient samples for NET biomarker analysis are routinely collected and stored under the same conditions outlined above. Aliquots of plasma were stored at −20°C until they were assayed in batches.

Data collection

Clinical data was obtained retrospectively for all 481 patients from patient case notes, and hospital and laboratory information systems. Information gathered included source of primary tumor, evidence of disease remission, presence of metastases, disease progression, Ki-67 index, tumor burden, and renal function including serum creatinine levels and estimated glomerular filtration rate (eGFR). Patients were excluded from further analysis if there was evidence of renal impairment (eGFR $\leq$ 60 mL/min/1.73m^2) (12). Patients were classified as having stable or progressive disease based on radiological assessment, using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (27). Patients were considered to be in remission if there was no clinical, biochemical, or radiological evidence of residual disease.

RIAs

Plasma CgA and CgB were measured using an in-house sensitive and specific RIA used as part of routine clinical service at the Neuroendocrine Tumor SAS laboratory, Imperial College Healthcare NHS Trust (11, 21). Plasma CART was measured using a well-established specific and sensitive in-house RIA (21, 28). Further details of the assays are included in the Supplemental Methods.

Statistics

Plasma CART concentrations in controls and patients with NET were compared using the nonparametric Kruskal-Wallis test. The comparative and combined utility of CART, CgA, and CgB (for NET diagnosis and for distinguishing between stable and progressive disease) in different NET subtypes was assessed using multiple logistic regression analysis. Least-significant variables were omitted from the model using a backwards selection procedure. The odds ratio, defined as the increase in probability of having a NET with one-unit increase in log values, was also calculated. Decision cut-offs for plasma CART, CgA, and CgB to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using receiver operator characteristic (ROC) curve analysis (31). PPV and NPV were adjusted for NET prevalence (32). Plasma CART concentrations in patients with a secretory or nonsecretory PCC/PGL, and in PCC/PGL patients with and without *SDHx* mutations, were compared using the Mann-Whitney *U* test.

Results

Plasma CART as a diagnostic marker for NETs

Plasma CART was measured in 481 patients with a NET (age range 19–101 y; median age, 60 y; male n = 242; female n = 239). Of these 481 patients with NET, 353 were not in remission and/or had no evidence of renal impairment with eGFR at least 60 ml/min/1.73m^2) (pancreatic, 117; gut, 131; PCC/PGL, 38; nongastroenteropancreatic [GEP], 28; unknown primary, 39). Plasma concentrations of CART, CgA, and CgB in each NET subtype compared with controls (n = 40) are shown in Table 1. All three biomarkers were significantly elevated in NET subtypes compared with controls with the exception of CgA in patients with a PCC/PGL.

The diagnostic utility of measuring a combination of biomarkers was assessed using multiple logistic regression analysis (Table 2). Measuring CgA in combination with CgB significantly improved diagnostic accuracy in patients with gut NETs and NETs with an unknown primary origin. Measuring the peptide CART in addition to CgA and CgB did not further improve diagnosis in these NET subtypes. CgB was the best diagnostic marker for pancreatic NETs, with no diagnostic advantage in also measuring CgA or CART.

CART was the single best diagnostic biomarker for PCC/PGL ($P < .001$), with no additional benefit from measuring either CgA or CgB. Sympathetic PGL, arising from either the paravertebral axis (base of skull to pelvis) or the adrenal medulla (PCC), hypersecrete catecholamines and metanephrines (33). This hypersecretion can be confirmed by measuring urine or plasma concentrations of catecholamines or their metabolites metanephrines. Measurement of methoxytyramine, the O-methylated metabolite of dopamine, has been reported to be particularly useful in the diagnosis of dopamine-producing, extra-adrenal PGL tumors (34, 35). In contrast, parasympathetic PGLs are often nonsecretory, representing a particular diagnostic challenge (36). Further analysis was thus carried out to evaluate the utility of CART as a biomarker in patients with a nonsecretory PCC/PGL. Values for urine/plasma catecholamine/metanephrine levels were available in 33/38 patients. In 26/33 patients, these were elevated above the reference range and these patients were identified as having a secretory PCC/PGL. The remaining seven patients had catecholamine/metanephrine levels within the reference range and were hence identified as having nonsecretory tumors. There was no significant dif-

Table 1. Plasma CART, CgA, and CgB Concentrations in Patients With a NET compared with Healthy Controls

Biomarker	Plasma Concentration, Median (IQR)	*P* Value (Relative to Controls)
Controls (n = 40)		
CART	51 (36–67)	–
CgA	33 (26–41)	–
CgB	70 (63–81)	–
All NETs (n = 353)		
CART	84 (54–225)	<.0001
CgA	65 (42–263)	<.0001
CgB	125 (84–193)	<.0001
Pancreatic NETs (n = 117)		
CART	84 (56–300)	<.0001
CgA	51 (39–111)	<.0001
CgB	110 (71–201)	<.0001
Gut NETs (n = 131)		
CART	77 (51–140)	<.0001
CgA	107 (52–421)	<.0001
CgB	128 (89–191)	<.0001
PCC/PGL (n = 38)		
CART	114 (65–390)	<.0001
CgA	43 (32–77)	NS
CgB	113 (64–161)	<.0001
NonGEP NETs (n = 28)		
CART	124 (54–1000)	<.0001
CgA	48 (37–348)	<.0001
CgB	121 (74–220)	<.0001
NETs with Unknown Primary (n = 39)		
CART	106 (54–224)	<.0001
CgA	199 (54–679)	<.0001
CgB	157 (107–239)	<.0001

NS, nonsignificant.

$P < .05$ was considered significant. All plasma concentrations are in pmol/L. Comparisons between groups were made using the Kruskal-Wallis test.

Table 2. Comparison of CART, CgA, and CgB as Diagnostic Markers in Patients With a NET

Biomarker	OR (95% CI)	*P* Value	AUC (95% CI)
Controls (n = 40) vs all NETs (n = 353)[a]			
CART	1.58 (0.64–3.94)	.32	0.83 (0.79–0.88)
CgA	12.2 (2.73–54.3)	.001	
CgB	10.6 (1.46–77.8)	.02	
Reduced model using the most significant markers[b]			
CgA	12.1 (2.72–53.9)	.001	0.83 (0.79–0.88)
CgB	15.6 (2.46–98.3)	.004	
Controls (n = 40) vs Pancreatic NETs (n = 117)[a]			
CART	1.61 (0.97–2.68)	.07	0.78 (0.71–0.85)
CgA	1.81 (0.79–4.14)	.16	
CgB	2.47 (1.28–4.75)	.007	
Reduced model using the most significant markers[b]			
CgB	61.3 (5.86–641)	.001	0.77 (0.70–0.84)
CART	3.39 (1.16–9.93)	.03	
Controls (n = 40) vs Gut NETs (n = 131)[a]			
CART	0.22 (0.05–1.08)	.07	0.90 (0.85–0.95)
CgA	271 (15.9–4617)	<.001	
CgB	6.76 (2.26–20.2)	.001	
Reduced model using the most significant markers[b]			
CgA	250 (15.2–4127)	<.001	0.90 (0.85–0.95)
CgB	19.2 (2.85–129)	.002	
Controls (n = 40) vs Non-GEP NETs (n = 28)[a]			
CART	0.96 (0.08–12.2)	.98	0.81 (0.70–0.93)
CgA	12.1 (0.69–213)	.09	
CgB	108 (0.44–26 354)	.10	
Reduced model using the most significant markers[b]			
CgA	12.0 (0.78–182)	.07	0.82 (0.70–0.93)
CgB	104 (0.75–14 412)	.07	
Controls (n = 40) vs PCC/PGL (n = 38)[a]			
CART	26.7 (2.64–270)	.005	0.71 (0.58–0.84)
CgA	1.34 (0.07–25.1)	.84	
CgB	3.51 (0.10–120)	.49	
Reduced model using the most significant markers[b]			
CART	340 (43.2–2682)	<.001	0.83 (0.75–0.90)
Controls (n = 40) vs NETs with unknown primary (n = 39)[a]			
CART	531 (4.47–63 004)	.10	0.96 (0.91–1.00)
CgA	52.5 (3.88–710)	.01	
CgB	17.5 (0.56–551)	.003	
Reduced model using the most significant markers[b]			
CgA	263 (3.40–20 384)	.01	0.94 (0.88–1.00)
CgB	40.8 (3.82–435)	.002	

CI, confidence interval; non-GEP, nongastroenteropancreatic.

Multiple regression analysis including all three biomarkers was carried out first, followed by a backward selection procedure omitting the least significant biomarker from the model, before repeating the regression analysis. $P < .05$ was considered significant. OR is the increase in odds of having a NET with a one log-scale unit increase in concentration of the biomarker.

[a] The combined area under ROC curve using all three biomarkers.

[b] AUC when most significant biomarkers are included.

ference between plasma CART concentrations in patients with secretory tumors compared with nonsecretory PCC/PGL; plasma CART median interquartile range (IQR) was 130 [63–325] pmol/L vs 108 [56–404] pmol/L for secretetory vs nonsecretory. The sensitivity of each biomarker compared with the current reference standard of elevated catecholamines/metanephrines for diagnosis of a secretory PCC/PGL tumor was: 73.1% (CART), 53.8% (CgA), and 53.8% (CgB).

A recent study has described a specific role for CgA in the diagnosis of PCC/PGL in patients with a mutation in genes encoding subunits B and D of the succinate dehydrogenase complex (*SDHB/SDHD*) (37). Patients with an *SDHB/SDHD* mutation and a secretory PCC/PGL often secretes catecholamines at lower rates compared with patients with a sporadic PCC/PGL (38). Hence, this relative biochemical inactivity may delay diagnosis and can make surveillance difficult. In the current study, 26 patients had a confirmed secretory PCC/PGL and an *SDHx* mutation: *SDHB* mutation n = 8, *SDHD* mutation n = 3. In this small subset of patients, CART levels were similar in patients with secretory PCC/PGL tumors with and without *SDHB/SDHD* mutations (plasma CART: 114 ± 79–828 [*SDHx* mutation–positive] (n = 11) vs 81 ± 53–192 pmol/L [*SDHx* mutation–negative] (n = 7). Further details regarding catecholamine/metanephrine secretion in secretory tumors and mutation analysis are provided in Supplemental Results.

Supplemental Table 1 shows specificity, sensitivity, PPV, and NPV for CART, CgA, and CgB in NET diagnosis, adjusting for NET prevalence (32).

Plasma CART as a marker for NET disease progression

Regression analysis was used to investigate circulating CART, CgA, and CgB as markers of disease progression (Table 3). The three biomarkers in combination produced the greatest area under the curve (AUC) for identifying progressive disease in patients with pancreatic NETs, a NET of unknown origin, and a PCC/PGL. In the latter two groups, plasma CART was the single best biomarker, with no additional benefit from measuring CgA or CgB. In contrast, measuring a combination of CgA and CART improved the identification of patients with progressive pancreatic NETs. Similar to biomarker performance in NET diagnosis, sensitivity, specificity, PPV, and NPV were comparable for all three biomarkers in distinguishing stable and progressive disease (Supplemental Table 2).

The Ki67 proliferative index is an accepted marker for staging of NETs and can be used to predict NET prognosis (15, 39). Ki67 index was available in 162 patients with NETs (median, interquartile range: 5%, 2–15%). In these patients, circulating CART and CgB levels were significantly correlated with the Ki67 index (CgB $P = .02$; CART $P < .05$), but circulating CgA levels were not.

Table 3. Comparison of CART, CgA, and CgB in Identifying Stable and Progressive Disease in Patients With a NET

Biomarker	OR (95% CI)	*P* Value	AUC (95% CI)
All NETs			
(n = 349, stable n = 239, progressive n = 110)[a]			
CART	5.21 (2.82–9.60)	<.001	0.78 (0.72–0.84)
CgA	1.78 (1.03–3.08)	.04	
CgB	2.85 (0.78–10.5)	.11	
Reduced model using the most significant markers[b]			
CgA	2.16 (1.32–3.55)	.002	0.78 (0.72–0.83)
CART	6.22 (3.19–11.1)	<.001	
Pancreatic NETs			
(n = 116, stable n = 80, progressive n = 36)[a]			
CART	3.30 (1.80–6.08)	<.001	0.86 (0.79–0.94)
CgA	1.89 (1.07–3.35)	.03	
CgB	1.20 (0.69–2.06)	.52	
Reduced model using the most significant markers[b]			
CgA	3.90 (1.40–10.9)	.009	0.86 (0.79–0.94)
CART	11.7 (3.81–35.8)	<.001	
Gut NETs			
(n = 131, stable n = 101, progressive n = 27)[a]			
CART	1.08 (0.56–2.05)	.82	0.69 (0.57–0.81)
CgA	1.10 (0.65–1.87)	.71	
CgB	2.04 (0.97–4.29)	.06	
Reduced model using the most significant markers[b]			
CgB	1.63 (1.17–2.27)	.004	0.69 (0.57–0.81)
Non-GEP NETs			
(n = 28, stable n = 14, progressive n = 14)[a]			
CART	0.85 (0.03–21.1)	.92	0.69 (0.47–0.91)
CgA	2.37 (0.16–34.5)	.53	
CgB	5.63 (0.04–731)	.49	
Reduced model using the most significant markers[b]			
CgA	3.74 (0.95–14.6)	.06	0.71 (0.51–0.92)
PCC/PGL			
(n = 38, stable n = 25, progressive n = 13)[a]			
CART	3.24 (0.60–17.3)	.17	0.89 (0.78–1.00)
CgA	4.63 (0.47–46.0)	.19	
CgB	1.02 (0.32–3.22)	.97	
Reduced model using the most significant markers[b]			
CART	35.3 (4.04–308)	.001	0.86 (0.75–0.98)
NETs with unknown primary			
(n = 39, stable n = 19, progressive n = 20)[a]			
CART	3.61 (1.22–10.7)	.02	0.82 (0.68–0.96)
CgA	2.08 (0.72–6.08)	.18	
CgB	0.82 (0.25–2.72)	.75	
Reduced model using the most significant markers[b]			
CART	12.6 (1.94–81.9)	.008	0.74 (0.58–0.90)

CI, confidence interval; PCC/PGL, pheochromocytomas/paragangliomas; non-GEP, nongastroenteropancreatic.

Multiple regression analysis including all three biomarkers was carried out first, followed by a backwards selection procedure omitting the least significant biomarker from the model, before repeating the regression analysis. $P < .05$ was considered significant. OR: increase in odds of having a NET with a one log-scale unit increase in circulating concentrations of the biomarkers.

[a] The combined area under ROC curve using all three biomarkers.

[b] AUC when most significant biomarkers are used.

Plasma CART as a surveillance marker for NETs

Most patients with a NET remain under long-term surveillance, even those who have undergone complete resection of their tumor, as recurrence is common. We compared the utility of CART, CgA, and CgB in differentiating patients with a NET in remission from those with active disease. All three biomarkers were significantly lower in patients in remission (n = 56) compared with those with active disease (n = 353) (ROC AUC, SE [95% CI] for CART: 0.74, 0.04 (0.70–0.79) ($P < .0001$); CgA: 0.72, 0.04 (0.67–0.76) ($P < .0001$); and CgB: 0.79, 0.03 (0.74–0.83) ($P < .0001$). There was no significant difference between the AUCs for the three biomarkers.

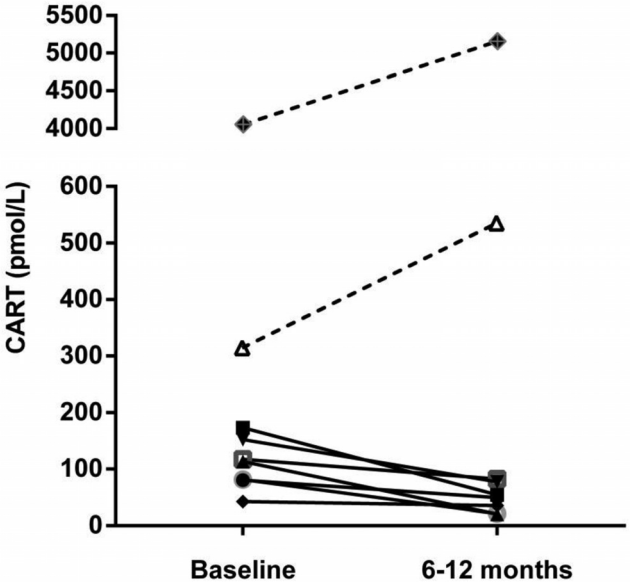

Figure 1. Longitudinal study of plasma CART concentrations in patients with stable and progressive PCC/PGL. Plasma CART was measured at baseline and at 6–12 months in PCC/PGL patients with stable (solid lines) and progressive (dashed lines) disease.

Plasma CART was the most useful marker for identifying stable or progressive PCC/PGL. Hence, we measured plasma CART concentrations preoperatively and for up to 12 months postoperatively in seven PCC/PGL patients who had undergone surgical resection and were in clinical remission, and in two patients with progressive disease (Figure 1). Plasma CART levels decreased in all patients who entered clinical remission following surgery, and remained low. In contrast, plasma CART concentrations increased in the two patients with progressive disease. This suggests that CART may be a useful surveillance marker in patients with a PCC/PGL, although further work is needed to investigate this as the sample size in this subgroup analysis was small.

Discussion

The diverse, often nonspecific presentations and the varied clinical course of NETs make their diagnosis and management challenging. Although CgA is the most widely

used NET biomarker, its utility is limited. There is an urgent need for robust, highly specific diagnostic and prognostic circulating NET biomarkers to enable earlier diagnosis and to plan targeted therapy. The peptide CART is widely distributed throughout the neuroendocrine system and preliminary studies suggest a role for CART as a NET marker (12). We have shown that CART may have utility as a circulating NET biomarker to identify progressive disease, particularly for pancreatic NETs and PCC/PGL tumors.

Circulating levels of all three biomarkers, CgA, CgB, and CART, were significantly higher in patients with a NET compared with healthy controls. Although none of the markers were sufficiently specific to be used as definitive diagnostic markers for NETs, the ease of sampling and analysis makes them a useful first-line investigation for delineating the neuroendocrine etiology of these tumors. Although CgA remained the most useful diagnostic marker for all NETs, the diagnostic accuracy of each biomarker varied with NET subtype. Plasma CART was the only useful diagnostic biomarker in patients with PCC/PGL and CgB was the most useful biomarker in patients with pancreatic NETs. This variation highlights the limitations of using a single biomarker in NET diagnosis and supports the proposal that biomarkers in combination can optimize NET diagnosis (13).

We have focused on comparing biomarker performance for NET diagnosis using the odds ratio (OR), rather than paired indicators. There are several benefits to using this approach (41). Comparing accuracy using sensitivity and specificity can be difficult unless one biomarker outperforms the other in both sensitivity and specificity. In addition, sensitivity, specificity, PPV, and NPV vary with the decision cut-off chosen. Furthermore, PPV and NPV are dependent on the prevalence of the disease in the population in whom the test will be used. PPV will increase when there is high disease prevalence in the study population. The prevalence of NETs in the general population is 20 in 100 000 (32). However, measurement of these biomarkers is not performed for general population screening. Therefore, the prevalence of NETs in the population who will be offered measurement of these biomarkers due to clinical suspicion of a NET will be higher than that of the general population, but still significantly lower than our study population. Without knowing the prevalence in the clinical population that will have these biomarkers measured, an accurate prediction of PPV and NPV is not possible. The calculated PPV for each biomarker for NET diagnosis is very low consistent with the low NET prevalence in the study population.

CgA and CgB are increased in a number of nonneuroendocrine disorders, including common conditions such as heart failure and hypertension (42, 43). Additional studies are now required to investigate the specificity of CART as a NET biomarker and to test its utility in the patient population in which it would be used, for example, patients with suspected, but not confirmed, NETs. There are several diagnostic CgA assays, each of which use different antisera, but which do not significantly differ in their ability to diagnose NETs (11). All previous reports of CART as a NET marker have used the same CART RIA (12, 21). Using this RIA, CART does not display a diurnal variation or alter with food intake (21), and seems to be less affected by renal impairment than CgA (12). Similar to CgA (11), false-positive elevations in CART have been reported in nonneuroendocrine malignancy (21), which requires further investigation. A limitation of the current study is its retrospective design, particularly in terms of delineating the effects of all these potential confounders on plasma CART. Hence, there is a need for a future prospective study to establish the effects of proton pump inhibitor therapy or H2 receptor antagonist therapy on circulating CART levels, given that the effects of these agents on CgA and CgB concentrations are well characterized (42). Similarly, the effects of various therapeutic interventions can influence plasma CgA (44, 45) and hence, potentially plasma CART. Again, due to the retrospective design of this study, these effects cannot be identified using the current data. A future prospective study designed to focus on changes in circulating CART in response to specific NET treatments such as somatostatin analogs would be particularly valuable.

Previous reports of the utility of circulating CgA in the diagnosis of PCC/PGL are conflicting (37, 46–50). Our data does not support the use of CgA in the diagnosis of these tumors. Future collaborative studies to further investigate CgA in PCC/PGL diagnosis could involve CgA antisera from those centers where a positive role for CgA has been described (37, 49, 50). Adding plasma CART to the laboratory biochemical work-up for PCC/PGL diagnosis may be particularly valuable. The current study suggests that plasma CART may be elevated in patients with nonsecretory PCC/PGL tumors, although patient numbers are small and a further, larger study is required to confirm this. If proven to be useful, plasma CART measurement would be invaluable in facilitating earlier detection of these tumors, particularly in the screening of high-risk individuals with *SDHx* mutations, which currently relies heavily on expensive and time-consuming imaging studies (51). Although plasma CART was not higher in patients with secretory PCC/PGL tumors and a mutation in *SDHx*, the conclusions from this data are limited, again by small patient numbers but also by the limited genetic analysis results available for the PCC/PGL patients re-

cruited to this study. Future studies of the role of CART in PCC/PGL diagnosis should include comparison with the O-methylated dopamine metabolite methoxytyramine in addition to plasma and urine metanephrines.

CgB provided the greatest diagnostic accuracy for pancreatic NETs. An early, small study of the role of CgB in NET diagnosis also showed high CgB levels in pancreatic patients with NET (52). Our data suggest that CgA is less useful in pancreatic NET diagnosis. Previous reports of the role of CgA in pancreatic NET diagnosis have been conflicting (21, 53–55). This variability emphasizes the need for a focused panel of NET biomarkers rather than using a single marker for NET diagnosis, particularly in pancreatic NETs.

Recent reports of alternative accurate methods of diagnosing NETs may provide interesting opportunities for future studies to further elucidate the role of CART in NET diagnosis and in identifying progressive disease. Duque et al (56) describe a novel biomarker, a multitranscript molecular signature measured by a serum RT-PCR, which was superior to CgA in its ability to identify GEP NETS (57). Previously, neuron-specific enolase, present in neuronal and neuroendocrine tissue, has been reported as useful in predicting disease progression and response to everolimus in patients with advanced pancreatic NETs (14). The recent CLARINET trial describes CgA as a predictor of response to the lanreotide in grade 1 or 2 pancreatic NETs (45). Pancreastatin, recognized by the CgA assay used in the current study (11) may have a role in identifying carcinoid tumors (58), hepatic metastases from a NET primary (59) and in monitoring response to hepatic artery chemoembolization (60). It would be particularly interesting to compare CART with these biomarkers in exploring response to treatment.

In addition to being useful diagnostic NET markers, our results suggest that CgA, CgB, and CART can be used as markers to identify progressive NET disease, with their utility again depending on the primary NET subtype. Plasma CART was the single best marker to differentiate stable from progressive disease in patients with a PCC/PGL and in those with an unknown primary NET. The combination of CART and CgA was useful in identifying patients with a progressive pancreatic NET. This finding is consistent with previous work suggesting CART acts as a NET marker (21). It is unclear whether the observed increase in plasma CART concentrations in patients with progressive NETs reflects increased secretion from the tumor itself or increased extratumoral secretion in response to the tumor. The Ki67 index grades the malignant potential of a NET based on its mitotic activity (61). Consistent with a role for CART in predicting NET behavior, we have shown a strong correlation between circulating CART and tumor Ki67 index.

In a small, longitudinal cohort of patients with a PCC/PGL, plasma CART remained low in patients with stable disease following tumor resection, but increased in patients with progressive disease. This suggests that plasma CART may also be a useful surveillance marker in patients with PCC/PGL. However, larger, long-term longitudinal studies are needed to assess the utility of CART, alongside CgA and CgB, in patients with and without *SDH*x mutations and also in predicting PCC/PGL progression and outcome.

Another limitation of the current study concerns the wider relevance of the findings made using our in-house CART RIA. Comparing the performance of our CART RIA with that of a commercial CART Enzyme Immunoassay confirmed good assay consistency and suggests that our findings are likely to be applicable to other CART measurement platforms. However, future studies are required to formally establish the accuracy and utility of other CART assays in NET diagnosis and assessment of disease progression.

In summary, our data suggest that circulating CART is a useful marker for diagnosis and identifying progressive disease in patients with a NET. The use of CART in combination with established NET biomarkers may facilitate the monitoring and management of patients with NETs, particularly those with PCC/PGL and pancreatic NETs. Long-term longitudinal studies are now needed to fully establish the role of CART as a marker for NET progression and prognosis.

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