REVIEW

The use of biomarkers in neuroendocrine tumours

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ABSTRACT

The incidence and prevalence of neuroendocrine tumours (NETs) arising from the gastrointestinal tract are increasing. At the time of diagnosis, histological grade, based on Ki-67 proliferation index on a tumour biopsy or specimen, offers prognostication but with often lengthy survival, this may not reflect current tumour biology later in the disease course. Biomarkers, including plasma chromogranin A, urinary 5-hydroxyindole acetic acid and pancreatic specific hormones (insulin, gastrin, vasoactive intestinal peptide), have a role in diagnosis but despite being incorporated into routine clinical practice, there is a lack of robust prospectively collected data investigating their prognostic and predictive value. Given the increasing number of treatment options available for NETs and prolonged survival, there is no agreement on the order of treatment for individual NET patients but the emergence of novel biomarkers and validation of existing ones, in addition to better understanding of the molecular biology, may help solve this clinical problem.

BACKGROUND

Often thought to be rare, the incidence of neuroendocrine tumours (NETs) is increasing with recent data reporting rates of 5.25 per 100 000¹ compared with 2 per 100 000 in the 1980s. This increase probably reflects better detection with more available imaging and endoscopy with a lower threshold to biopsy lesions. NETs are a heterogeneous group of tumours arising from small intestine (or 'midgut', predominantly ileocaecal), pancreas, stomach, lungs and colorectum exhibiting diverse biological behaviour from relatively indolent to highly aggressive cancers. Given heterogeneity in survival, it is not surprising that recent prevalence rates have been reported up to 35 per 100 000, more common than that of most gastrointestinal cancers including

hepatobiliary, oesophageal and pancreatic carcinomas.¹

NETs are malignant transformations of cells of the diffuse neuroendocrine system, the collection of various neuroendocrine cells scattered throughout the body.² Nomenclature and classification systems continue to evolve which can be confusing to the non-specialist. The older term carcinoid, or 'karzinoide' as introduced by Obendorfer in 1907,³ has been replaced by the term 'neuroendocrine tumour' or neuroendocrine neoplasm, which can be applied to any primary site, for example, pancreatic NET, midgut NET and so on, or as an overall term, gastroenteropancreatic (GEP)-NETs. However, midgut NETs which have metastasised to the liver are sometimes still termed 'carcinoid' tumours as some are associated with carcinoid syndrome including symptoms of diarrhoea and flushing as a consequence of secretion of vasoactive substances including serotonin.

Survival rates vary depending on site of primary tumour, grade and stage but pancreatic NET 5-year survival from recent Surveillance, Epidemiology and End Results registry was only 37.6% and 68.1% for midgut NETs.⁴

Although surgical resection according to oncological principles remains the only chance of 'cure' with localised disease, many cases of NETs present with metastases requiring systemic treatment, of which there are numerous options including somatostatin analogues, interferon, chemotherapy, transarterial (chemo) embolisation, radiofrequency ablation, sunitinib, everolimus and radionuclide targeted therapy. In some cases with liver metastases, where the primary is resectable, resection of the liver metastases plus or minus ablation of non-resectable lesions may be considered as a 'curative' approach.

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Systemic management of cases depends on primary site of tumour and tumour subtype, but mainly stage of disease and tumour grade with the aim being to control tumour growth, prolong survival and reduce symptoms from hormone secretion. Current management in Europe is based on guidelines produced by the European Neuroendocrine Tumour Society (ENETS) and UK & Ireland Neuroendocrine Tumour Society.^{5–7}

With the varied survival of NETs and the increasing number of treatment options available, there is a requirement for biomarkers to predict prognosis and outcome with therapy. Currently used biomarkers include histological and circulating markers.

HISTOLOGICAL MARKERS

The expression of chromogranin A (CgA), neurone specific enolase and synaptophysin detected by immunohistochemistry on tumour tissue assists in diagnosis of NETs but is not prognostic. However, poorly differentiated NETs, which have worse prognosis than well-differentiated tumours, often lose expression of these markers.

Certain histological markers are used to determine grade. The grade of a NET refers to the biological aggressiveness of the tumour with high grade tumours having worse outcomes than low grade NETs. Classifications of low versus high grade tumours have not been uniformly based on consistent pathological characteristics. Some authors have used presence of necrosis, atypia and mitotic count^{8–10} and others focused on Ki-67 proliferation index.^{11 12}

Several authors including ENETS and the WHO have suggested a three-tiered prognostic grading system for GEP-NETs.^{11 12} In these systems, Ki-67 proliferation index, using immunohistochemistry with the MIB-1 antibody, and counting cells undergoing mitosis, the mitotic count, are routinely used to classify NETs as low (G1), intermediate (G2) and high (G3) grade tumours as detailed in table 1.¹³ This grading system has been validated as classifying three different prognostic groups.^{14 15}

Grading often assists management strategy at diagnosis. For example, G3 tumours tend to be aggressive and are felt to represent a different disease process than G2 or G1 NETs. Hence first line therapy in metastatic G3 tumours often involves chemotherapy. Metastatic G1 NETs are often indolent and in some

cases undergo surveillance without treatment or offered somatostatin analogues (if somatostatin receptor (SSTR) scintigraphy is positive), which recently have been shown to have an antitumour effect in addition to symptomatic treatment of 'carcinoid syndrome'.^{16 17} Initial management of metastatic G1 and G2 NETs are similar in published guidelines.¹⁸ However, a change in therapy may be offered early in G2 NETs if there is tumour progression despite initial treatment and chemotherapy is offered first line in metastatic G2 foregut NETs.¹⁹

Grading is based on biopsy material or resected specimen obtained at the time of diagnosis and provides good prognostication at time of diagnosis. Due to prolonged survival, this may be several years prior to a decision for a change in treatment and thus grade may not necessarily reflect current tumour biology. In these cases, the staging of the tumour is evaluated by assessing change in tumour size over time using interval scans based on Response Evaluation Criteria in Solid Tumors.²⁰ If there is progression of disease on imaging, then treatment is escalated. Although guidelines suggest interval imaging every 6–12 months,⁵ there is a lack of evidence for this interval, it may not reflect tumour biology and there is associated radiation exposure. Therefore, dynamic biomarkers, easily obtained through sampling of blood or urine, are required to predict progression of disease, prognosis and response to therapy.

The main existing biomarkers in clinical use are plasma CgA and 24 h urinary 5-hydroxyindole acetic acid (5-HIAA).

CHROMOGRANIN A

The neuroendocrine cells that give rise to NETs secrete a variety of bioactive products that define their role. The best 'general' marker for NETs is plasma CgA, an acidic, hydrophilic 70–85 kDa protein which can aid diagnosis. Alongside other members of the granin family, it is a precursor protein that can be processed at multiple cleavage sites to produce a large number of small bioactive peptides with a wide range of biological activity, although questions on physiological function remain.

There have been numerous studies looking at CgA as a diagnostic biomarker in NETs. Plasma CgA is raised in GEP-NETs, bronchial NETs and pheochromocytomas among other NETs.²¹ Sensitivity and specificity vary depending on the type on NET and burden. In a recent large study, sensitivity was 85.3% and specificity 96% when compared with healthy controls.²²

Although there have been several retrospective studies, there are few prospective studies investigating the prognostic or predictive value of CgA in NETs.

These studies can be categorised into:

1. those correlating CgA to clinicopathological features
2. those investigating the prognostic value of CgA

Table 1 Grading of neuroendocrine tumours as proposed by European Neuroendocrine Tumour Society

Grade	Ki-67 (%)	Mitoses per 10 hpf
G1	≤2	<2
G2	3–20	2–20
G3	>20	>20

Hpf, high power fields.

3. those investigating the value of CgA in monitoring for recurrence following curative resection
4. those using CgA to monitor response to therapy (as a predictive marker).

Several studies have shown plasma CgA correlates with tumour stage or burden in NETs. CgA has been shown to be increased in patients with metastatic disease compared with localised NETs in a number of retrospective studies on heterogeneous populations of midgut and pancreatic primary tumours.^{22–24} Various studies have also shown higher CgA levels in those with a greater volume of liver metastases.^{25 26}

CgA has also been found to inversely correlate with quality of life as determined by standardised questionnaire²⁷ but it does not seem to correlate with symptoms of carcinoid syndrome.²⁸

Plasma CgA, as a prognostic marker, has been adopted into clinical practice but in several retrospective studies, evidence is not consistent in proving its prognostic value. Several studies are summarised in table 2, which have found CgA to be a prognostic marker but evidence is not consistent or robust.

One of the landmark prospective randomised control trials, looking at the effect of octreotide long-acting release in midgut NETs (PROMID), did not find elevated CgA to be prognostic or predictive and did not find any significant reduction in CgA, with either placebo or active treatment at 6 months.¹⁶ This was despite a significantly longer time to progression with octreotide. This is in contrast to the RADIANT-1 trial, a phase II study of oral Everolimus (although in advanced pancreatic NETs), where elevated baseline CgA was associated with shorter progression-free survival and overall survival.²⁹

After curative resection of localised NETs, a few studies have focused on the use of biomarkers in monitoring for recurrence. CgA has been shown to rapidly diminish or normalise after radical resection of primary lesion and metastases in a small study³⁰ with progressive increase in CgA levels useful in detecting recurrence after 'curative' resection of midgut NETs with a sensitivity of 85%–91%.^{31 32} A recent small

study has also found CgA levels of 110 U/l sensitive in detecting recurrence after resection.³³

The use of CgA as a predictive marker is more controversial. In a large study of 344 patients, an increase in CgA was associated with tumour progression and shorter survival in a heterogeneous group undergoing surveillance or treatment.²⁶ Others have also found increasing CgA correlates with tumour progression²³ and decreasing levels correlate with radiological response to a number of therapies but definitions of response and cut-off levels have not been consistent.^{24 34}

Outcomes from studies mentioned so far are based on radiological response, usually measured by Response Evaluation Criteria in Solid Tumors. However, no correlation was found between CgA and symptomatic response in a prospective study of octreotide long-acting release in patients with carcinoid syndrome.²⁸ Responses to other treatments have also been studied with varied results. Neither CgA nor CA19-9 was found to be predictive of response to chemotherapy³⁵ but changes in plasma pancreastatin (derivative of CgA) 2 weeks after transarterial chemo-embolisation correlated with radiological response.³⁶

CgA is used in routine practice for diagnosis, prognostication, monitoring response and for surveillance of recurrence. However, evidence is inconsistent from mainly retrospective studies, some only including univariate analysis, and some being small and based on heterogeneous populations undergoing many treatments. Additionally, cut-off levels or definitions of 'response' in CgA vary between studies, which make them clinically difficult to use systematically as a prognostic or predictive marker. The majority of studies have explored the relationship of CgA with overall survival and not progression-free survival.

Furthermore, pitfalls exist with measuring plasma CgA due to false-positive elevation in impaired renal function,⁷ steroid treatment,⁷ chronic atrophic gastritis⁷ and proton-pump inhibitor treatment.⁷ Additionally, there is no recognised international standard for the CgA assay. Stridsberg *et al*³⁷ compared the three commercially available assays and demonstrated

Table 2 Summary of studies evaluating CgA as a prognostic marker of PFS and OS

Author	n	Primary tumour	CgA cut-off	Prognostic of PFS?		Prognostic of OS?	
				Univ	Multiv	Univ	Multiv
Ekeblad <i>et al</i> ⁹	324	Pancreatic	3×ULN	ND	ND	Y	N
Korse <i>et al</i> ²⁷	30	Midgut	1000 µg/l	ND	ND	Y	ND
Janson <i>et al</i> ²⁵	301	Midgut	5000 µg/l	ND	ND	Y	ND
Rinke <i>et al</i> ¹⁶	85	Midgut	ULN	N	ND	N	N
Ahmed <i>et al</i> ⁴³	360	Midgut	'Increasing levels'	ND	ND	Y	N
Oberg <i>et al</i> ⁶¹	207	Pancreatic	2×ULN (73 ng/ml)	N	ND	ND	ND
Turner <i>et al</i> ³⁵	79	Mixed	ULN	Y	Y	ND	Y

CgA, chromogranin A; Multiv, multivariate analysis; N, no, not prognostic; ND, not done; OS, overall survival; PFS, progression-free survival; ULN, upper limit of normal; Univ, univariate analysis; Y, yes, prognostic.

sensitivities varying between 67% and 93% in NET patients. ENETS have suggested that reference laboratories should be preferred for assays, serial measurements should be performed using the same assay and, where possible, proton-pump inhibitor therapy interrupted for at least three half-lives prior to sampling.²¹ Thus, there is a need for robustly designed prospective studies evaluating CgA as a biomarker.

BIOMARKERS IN FUNCTIONING PANCREATIC NETS

Individual amines and peptide hormones, secreted by specific 'functioning' pancreatic or small duodenal NETs, can produce rare clinical syndromes (table 3). However, most pancreatic NETs are 'non-functioning' and do not produce specific symptoms but may secrete pancreatic polypeptide (PP) or human chorionic gonadotrophin without overt physiological consequences.^{38–39} PP has been found to be elevated in gastrointestinal or pancreatic NETs with a sensitivity of 50%–80%⁴⁰ but a combination of CgA and PP has been shown to be useful in non-functioning pancreatic NETs with a sensitivity of almost 95%.⁴¹

Serum gastrin concentration is usually markedly elevated in gastrinomas which present with simple or complicated peptic ulcer disease. Gastrin levels are more than 10 times the upper limit of normal, and are accompanied by a high basal acid output and low stomach pH. A secretin test is often required to establish diagnosis in cases with mild hypergastrinaemia or normal gastric acid secretion. Chronically elevated gastrin levels cause hyperplasia of the neuroendocrine enterochromaffin-like (ECL) cells of the stomach which can lead to 'ECLomas' or type 2 gastric NETs. These are associated with increased CgA levels.

Insulinomas present with symptoms of hypoglycaemia and can be difficult to diagnose. The diagnosis can be established using blood glucose levels >2.2 mmol/l, plasma insulin levels >6 µU/l, plasma C-peptide levels >200 pmol/l and absence of sulfonylureas in plasma/urine.

URINARY 5-HIAA

NETs arising from the midgut may result in symptoms due to the secretion of serotonin or other peptides, that is, carcinoid syndrome, characterised by diarrhoea and flushing. The urinary breakdown metabolite of serotonin is 5-HIAA and is usually measured over a 24 h collection. The 24 h urinary 5-HIAA as a marker in NETs has not been investigated to the same extent as CgA but its overall sensitivity and specificity in carcinoid syndrome is 70% and 90%, respectively.⁴² It may be normal in non-metastatic midgut NETs but can be elevated despite a lack of symptoms.

High or increasing urinary 5-HIAA levels have been demonstrated to be associated with worse overall survival in a number of retrospective studies.^{25 43–45} It may also correlate with the extent of disease with elevated levels associated with volume of metastases.⁴⁶

Somatostatin analogues, the mainstay of therapy of GEP-NETs and in carcinoid syndrome, have been demonstrated to reduce levels of urinary 5-HIAA and in some cases this occurs alongside symptomatic improvement.⁴⁷ In an open-label study of prolonged release lanreotide (n=71), urinary 5-HIAA was useful in monitoring response.⁴⁸ Reductions in urinary 5-HIAA were seen, but although CgA correlated with survival and quality of life in patients treated with somatostatin analogues, urinary 5-HIAA did not.²⁷

Considering other treatments, in patients undergoing trans-hepatic arterial embolisation, over a half had a 50% reduction in 5-HIAA levels. A correlation was found with survival and on multivariate analysis, percentage change in 5-HIAA was an independent predictor of survival.^{31 49} One of the largest series (n=111) investigating treatment with interferon (75% midgut NETs) demonstrated >50% reduction of urinary 5-HIAA in 42% of cases (termed a biochemical response).⁵⁰

Approximately 20% of patients with carcinoid syndrome have carcinoid heart disease (CHD). Correlations have been demonstrated between 5-HIAA and severity of CHD⁵¹ and between rising urinary 5-HIAA and progression of CHD.⁵²

Table 3 Syndromes related to functioning pancreatic neuroendocrine tumours

Tumour	Symptoms	Malignancy	Survival
Insulinoma	Confusion, sweating, dizziness, unconsciousness, relief with eating	10% of patients develop metastases	Complete resection cures most patients
Gastrinoma	Zollinger–Ellison syndrome of severe peptic ulceration and diarrhoea	Metastases develop in 60% of patients	Complete resection results in 10-year survival of 90%; less likely if large primary
VIPoma	Werner–Morrison syndrome of profuse watery diarrhoea with marked hypokalaemia	Metastases develop in up to 70% of patients; majority found at presentation	Complete resection with 5-year survival of 80%–90%; with metastases, 30%–60%
Glucagonoma	Necrolytic migratory erythema, weight loss, diabetes mellitus, stomatitis, diarrhoea	Metastases develop in >60% of patients	More favourable with complete resection; prolonged even with liver metastases
Somatostatinoma	Cholelithiasis; weight loss; diarrhoea and steatorrhoea; diabetes mellitus	Metastases likely in about 50% of patients	Complete resection associated with 5-year survival of 95%; with metastases, 60%

Reduced levels of 5-HIAA were seen after radical resection of primary lesion and metastases in a small study.³⁰ However, unlike rising CgA indicating tumour progression during postoperative surveillance, urinary 5-HIAA levels did not increase and are a relatively insensitive method to detect recurrence compared with plasma CgA (18% vs 85%).³¹

In summary, as with CgA, various retrospective and a few prospective studies have investigated 5-HIAA as a prognostic or a predictive marker and the results are inconsistent. Many studies only include univariate analysis while others have studied heterogeneous populations undergoing numerous treatments. Cut-off levels or definitions of 'response' or 'change' in 5-HIAA vary between studies, often arbitrarily chosen.

The measurement of 5-HIAA is also subject to confounding factors including compliance with 24 h urine collection. Patients are required to adhere to a strict diet for 3 days prior to collection but this is controversial.⁷ This is possibly due to tryptophan-rich foods including plums, pineapples, bananas, aubergines, tomatoes, avocados and walnuts increasing urinary levels. Additionally, certain drugs increase levels, for example, acetanilide, phenacetin, glyceryl guaiacolate (found in cough syrups), cisplatin, fluorouracil (used in NET treatment) and melphalan. Other medications may reduce levels including chlorpromazine, heparin, tricyclic antidepressants, levodopa, monoamine oxidase inhibitors and promethazine. False negative results occur in renal impairment and dialysis. Falsely raised levels occur in malabsorption of coeliac disease, tropical sprue, Whipple's disease and cystic fibrosis in which increased tryptophan metabolites are encountered in urine.

This has led to the ENETS guidelines stating that urinary 5-HIAA is not a consistently reliable prognostic factor in this disease.²¹

N-TERMINAL PRO-BRAIN NATRIURETIC PEPTIDE

Screening of CHD in patients with midgut NETs has been revolutionised by the use of circulating N-terminal pro-brain natriuretic peptide (NT-pro-BNP).⁵³ Using NT-pro-BNP as a screening marker compared with regular echocardiography, the number of patients needed to diagnose one case of CHD reduced from 5.1 to 1.4 with elevated levels in those with CHD.

NUCLEAR MEDICINE IMAGING AND OTHER MARKERS

Expression of NET specific markers is used in nuclear medicine imaging. Most NETs express SSTRs of which there are five SSTR subtypes (SSTR 1–5) with SSTR-2 and SSTR-5 expressed in at least 80% and 77% of gastrointestinal NETs, respectively.⁵⁴ With the exception of insulinomas (only 50% express SSTR2), SSTR imaging, for example, Octreoscan or ⁶⁸Gallium-DOTA-Octreotate Positron Emission Tomography (PET), is the mainstay of staging and may assist in

localising primary lesions in GEP-NETs.⁵⁵ They may also be useful in monitoring for recurrence after radical surgery with a study demonstrating 50% of 'cured' patients with detectable lesions with SSTR scintigraphy.⁵⁶

Unlike adenocarcinomas, PET with 18-fluoro deoxy-glucose⁵⁷ is often negative in low or intermediate grade NETs. However, PET radionuclides such as ⁶⁸Gallium can be conjugated to somatostatin analogues for SSTR imaging. ⁶⁸Gallium-DOTA-Octreotate and ⁶⁸Ga-DOTA-Octreotide and more recently ⁶⁸Ga-DOTANOC have been found to be sensitive for NETs due to detection of more SSTR subtypes and enhanced affinity compared with Octreoscan.⁵⁸ These imaging modalities can help characterise metastases, assess extent of disease and locate primary lesions. However, studies have suggested their role in monitoring response to therapy is limited.⁵⁹

Others prognostic biomarkers studied in NETs include α -fetoprotein, human chorionic gonadotrophin,⁶⁰ neurone specific enolase⁶¹ and angiopoietin-2⁶² but are not used in routine clinical practice. High levels of Neurokinin A has also been associated with poor prognosis.⁴⁶

Promising new biomarkers include circulating nucleic acids⁶³ and circulating tumour cells. Circulating mRNA transcripts specific to NETs have been detected in higher levels in metastatic compared with localised NETs or healthy controls.⁶³ Circulating tumour cells have been demonstrated in a number of carcinomas but using a semiautomated immunomagnetic system, metastasising cancer cells have recently been detected in NETs and found to be valuable as prognostic markers.⁶⁴ Levels are associated with progressive disease and are the subject of ongoing studies. If emerging biomarkers such as these provide a means to assess response at an earlier time point than with radiology, they may provide a more rapid means to assess new therapies or sequence of conventional therapies.

SUMMARY

The incidence and prevalence of NETs are increasing. Given the increasing number of treatment options available and prolonged survival, the algorithm of therapeutic management is unclear. Grade according to Ki-67, but not mitotic count, certainly offers some prognostic information at the time of diagnosis. Plasma CgA, urinary 5-HIAA and pancreatic specific hormones can aid in NET diagnosis and are repeatable during the disease course. However, despite plasma CgA and urinary 5-HIAA being accepted as established biomarkers, there is a need for prospective studies systematically investigating their prognostic and predictive value.

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