The Clinical Utility of a Novel Blood-Based Multi-Transcriptome Assay for the Diagnosis of Neuroendocrine Tumors of the Gastrointestinal Tract

I.M. Modlin, MD, PhD, DSc[1,2], M. Kidd, PhD[2], L. Bodei, MD, PhD[3], I. Drozdov, PhD[2] and H. Aslanian, MD[4]

OBJECTIVES: **Current monoanalyte blood-based biomarkers for the diagnosis and follow-up of neuroendocrine tumors (NETs) do not achieve satisfactory metrics of sensitivity and specificity. We report the sensitivity and selectivity of the PCR-based test, the NETest, to detect tumors with reference to other benign and malignant gastrointestinal diseases.**

METHODS: **A total of 179 cases (gastrointestinal tumors: *n*=81; pancreatic disease: *n*=98) were prospectively collected and assessed using the NETest or chromogranin A (CgA) to determine metrics for detecting small intestinal and pancreatic NETs.**

RESULTS: **For intestinal carcinoids, the accuracy of the NETest was 93% (all NETs positive and 3 (12%) colorectal tumors were positive). CgA was positive in 80%, but 29% (*n*=7) of colorectal cancers were CgA positive. For pancreatic disease, the NETest accuracy was 94% (96% NETs positive, 2 (6%) of intraductal papillary mucinous neoplasms (IPMNs) were positive). The accuracy of CgA was 56% (29% of pancreatic NETs were CgA positive). Overall, the NETest was significantly more sensitive than CgA for the detection of small intestinal (area under the curve 0.98 vs. 0.75 *P*<0.0001) and pancreatic NETs (0.94 vs. 0.52, *P*<0.0001). NETest scores were elevated (*P*<0.05) in extensive disease and were more accurate (76–80%) than CgA levels (20–32%). The metrics of the multianalyte NETest met the performance criteria proposed by the National Institutes of Health for biomarkers, whereas CgA measurement did not.**

CONCLUSIONS: **This study demonstrates that a blood-based multianalyte NET gene transcript measurement of well-differentiated small intestinal and pancreatic neuroendocrine tumor disease is sensitive and specific and outperforms the current monoanalyte diagnostic strategy of plasma CgA measurement.**

Am J Gastroenterol advance online publication, 2 June 2015; doi:10.1038/ajg.2015.160

INTRODUCTION

A fundamental issue in the management of gastroenteropancreatic (GEP) neuroendocrine tumors (NETs) as identified by the National Cancer Institute is the absence of a sensitive and specific set of tumor biomarkers (1). Advances in the treatment of NET disease require the development of markers that are multidimensional and meet the proposed National Institutes of Health (NIH) performance metrics (2). Existing NET biomarkers are of the monoanalyte class and each identifies with various degrees of sensitivity and specificity single biological hallmarks of disease such as secretion and tumor type (3). Currently, there exists no multianalyte strategy that can provide a measure of the underlying mechanisms of tumor development and growth. As the costs of late or ineffective therapy are abundantly evident, biomarkers represent a high-yielding facet of medicine at a scientific, clinical, and economic level as well as contribute to the improvement of survival and quality of life (3). Biomarker deliverables include accurate diagnosis, earlier disease identification, precise determination of residual disease, minimal disease detection, support of imaging, and demonstration of failure/efficacy of therapy. The biomarker compendium therefore ranges from the mechanistic development of more effective

[1]Department of Surgery, Yale University School of Medicine, New Haven, Connecticut, USA; [2]Wren Laboratories, Branford, Connecticut, USA; [3]Division of Nuclear Medicine, European Institute of Oncology (IEO), Milan, Italy; [4]Section of Digestive Diseases, Yale University, New Haven, Connecticut, USA. **Correspondence:** I.M. Modlin, MD, PhD, DSc, Department of Surgery, Yale University School of Medicine, 333 Cedar Street, PO Box 208062, New Haven, Connecticut 06510, USA. E-mail: imodlin@optonline.net

Received 15 January 2015; accepted 16 April 2015

tests such as multianalyte with algorithm analysis assays (MAAAs) to the identification of novel chemicals expressed in complex diseases such as metabolomics and their clinical deployment (4). Although substantial advances have been made in other cancers, the development of novel biomarkers in NETs has been less successful. This reflects two dominant issues. First, the disease is not perceived as a major health threat with the result that pharmacoeconomics has not driven research as vigorously as in other cancer spheres. Second, the tumors comprise a heterogeneous group of cancers with respect to site, secretory product, as well as proliferative control and molecular genetic changes, making the identification of a globally effective set of markers difficult.

Currently, the measurement of secretory products and more recently circulating tumor cells have been considered as optimal approaches (5). Regarding the former, a diverse variety of potential biomarkers have been proposed (6–9). These include the constitutive neurosecretory peptide chromogranin A (CgA) as well as individual cell type–specific secretory products such as gastrin, serotonin, pancreatic polypeptide, neurokinin A, and vasoactive intestinal peptide (VIP), or metabolic degradation products such as urinary 5-hydroxyindoleacetic acid. These have, for the most part, proven to have considerable utility in gastrinomas (gastrin), insulinomas (insulin), VIPomas (VIP), and glucagonomas (glucagon) that represent <1% of all NETs (3) and in serotonin-producing carcinomas (urinary 5-hydroxyindoleacetic acid). Many of the other biomarkers suffer from a lack of sensitivity (e.g., pancreatic polypeptide in pancreatic NETs has an ~50% sensitivity (10) and neuron-specific enolase has an ~30% sensitivity (11)). Furthermore, the technical challenges associated with collection (e.g., 5-hydroxyindoleacetic acid requires a special diet and 24 h urine collection) or problems with drugs that generate misleading values (e.g., acid suppression medication in the case of CgA) are further confounders (3). Detectable circulating tumor cells have been identified in 43% of intestinal and 21% of pancreatic NETs, but numbers are low and no significant relationships were noted with therapeutic response or serum CgA (12). The overall performance metrics for these markers in clinical practice are low (<50% sensitivity and <30% specificity) that fall below the NIH-proposed acceptable metrics (6,13,14).

The NET default biomarker, CgA (15), is variably processed depending on the neuroendocrine cell type (16) and enters the blood stream as a highly heterogeneous antigen composition comprising complete protein or a series of cleavage products (14,17). Although elevated levels are considered to be sensitive and ~60–90% accurate (18), CgA is generally considered an ineffective first-line diagnostic for NETs (19). Measurements are nonspecific (10–35% specificity) as CgA is elevated in other neoplasia (pancreatic, prostate, small cell lung neoplasia) (20) and a variety of cardiac and inflammatory diseases (21) as well as renal failure (22). One of the commonest causes of spuriously elevated CgA levels are proton pump inhibitor (PPI) administration (17).

Optimally, a biomarker should be found uniquely in the malignant tissue of interest and generate a positive signal that can be measured without confounding "noise" from normal tissues or other nonmalignant pathologies. Although gene expression profiling has engendered considerable enthusiasm as a future strategy, it identifies thousands of genes expressed at higher levels in malignant compared with benign tissues, but only limited numbers of transcripts or proteins have been identified that are uniquely elevated in cancer (23). Optimization therefore requires identification of multiple markers and the development of mathematically weighted algorithms to identify abnormal quotients reflective of a specific neoplasia (24).

Using gene microarray-based approaches of both malignant NET tissue and blood, we have developed a robust, reproducible PCR-based 51 marker signature (multigene test, a MAAA) with high sensitivity (85–98%) and specificity (93–97%) for the detection of gut NETs or "carcinoids" in circulating blood (25,26). The test has been considered to have achieved performance metric requirements (27) and has been considered in Delphic Consensus assessments and reviews as likely to supplant current monoanalyte biomarkers (28,29). A prospective validation in a spectrum of clinical situations would optimize its general application (27).

We report the sensitivity and selectivity of the PCR-based test to detect tumors in comparison with CgA with particular reference to other benign and malignant gastrointestinal diseases. For small intestinal NETs, we evaluated the accuracy of the test by prospectively comparing it with a variety of gut adenocarcinomas. For pancreatic NETs, we compared results with prospectively collected patients who were undergoing investigation (endoscopic retrograde cholangiopancreatography or endoscopic ultrasound) for upper gastrointestinal symptoms. Performance metrics were determined for each tumor type under these "real-world" conditions.

METHODS

Sample collection

All samples were collected and analyzed according to a standard institutional review board protocol (Yale University: 17 June 2013) in accordance with the World Medical Association Declaration of Helsinki Principles (25). All individuals from whom blood was obtained were seen (June 2012 to March 2014) at the Yale School of Medicine Smilow Cancer Center outpatient clinics following informed consent. Blood samples (5 ml) were collected in 9 mg K_2EDTA tubes (BD Vacutainer Venous Blood Collection Tubes, BD Diagnostics, Franklin, NJ). Aliquots of whole blood were stored at −80 °C within 2 h of collection (samples immediately stored on ice/4 °C after sampling) per standard molecular diagnostics protocols for PCR-based studies (30). A second aliquot (2 ml) was spun (1,000 r.p.m., 10 min) and the plasma was collected for CgA ELISA as previously described (25,26,31). The investigators were unaware of the clinical diagnosis when the samples were assayed and analyzed.

Intestinal tumors

The small intestinal NET set comprised NETs (n=41) and the gastrointestinal carcinoma set (n=40) included esophageal (n=4), gastric (n=3), small bowel (n=6), colon (n=11), rectal (n=13), and

Table 1. Clinical characteristics of the intestinal tumors

Group 1									
Characteristic					**Small intestinal NETs (*n*=41)**				
Median age (range) (years)					58 (33–78)[a]				
Gender (M/F)					7:34[a]				
Tumor distribution	**No.**	**Grade**			**Stage**		**Treatment**		**Current PPIs**
		G1	**G2**	**G3**	**Locoregional**	**Distant**	**Untreated**	**Current SSAs**	
Small Intestine	41	37	4	0	9	32	10	26	0
Characteristic					**Gastrointestinal adenocarcinomas (*n*=40)[b]**				
Mean age (range) (years)					62 (27–81)[a]				
Gender (M/F)					14:26[a]				
Tumor distribution		Esophageal		Gastric	Small Bowel		Colon[c]	Rectal	Anal[d]
Number		4		3	6		11	13	3

M/F, male/female; NET, neuroendocrine tumor; PPI, proton pump inhibitor; SSA, somatostatin analog.
[a]No significant difference in age (P=0.27, Mann–Whitney) or gender (P=0.08, two tailed: χ^2 test).
[b]Patients currently treated (postoperative: n=12, or chemotherapy: n=28).
[c]Two patients on PPIs (omeprazole 20 mg).
[d]Anal small cell carcinoma.

anal (n=3). The group demographics are included in **Table 1**. The median age for small intestinal NETs was 58 years (33–78) with a sex distribution of 7:34 (male/female). The median age for the gastrointestinal tumor disease set was 62 years (27–81) with a sex distribution of 14:26 (male/female).

Pancreatic tumors

The pancreatic set included pancreatic NETs (n=45) and samples prospectively collected by endoscopic retrograde cholangiopancreatography/endoscopic ultrasound (December 2013 to February 2014: n=53). The group demographics are included in **Table 2**. The median age for pancreatic NETs was 57 years (44–83) with a sex distribution of 17:28 (male/female). The median age for the pancreatic disease set was 66 years (33–78) with a sex distribution of 21:32 (male/female).

MAAA PCR-based test (NETest)

We used a two-step manual technique protocol (RNA isolation with complementary DNA production and quantitative real-time PCR). Transcripts (mRNA) were isolated from 1 ml EDTA-collected whole blood samples using the mini blood kit (Qiagen, Valencia, CA). This approach captures all circulating RNAs irrespective of the source (circulating tumor cells, oncosomes, exosomes, and so on) and provides a "snapshot" of the whole blood concentration. The RNA quantity was 50 µl and the quality was >1.8 ($A_{260:280}$ ratio); analysis of the RNA pattern on electrophoresis (Agilent Technologies, Santa Clara, CA) showed >5.0 RNA integrity number (RIN) (32). The standard Qiagen isolation protocol (heme/genomic DNA contamination not detected) with no modifications was used. Complementary DNA was produced from 50 µl RNA using a High-Capacity Reverse transcriptase kit (Life Technologies, Grand Island, NY: complementary DNA production 2,000–2,500 ng/µl) and stored at −80 °C. Quantitative real-time PCR was performed (384-well plate, HT-7900) with the complementary DNA (200 ng/µl) and 16 µl of reagents/well (Universal Master Mix II with UNG, Life Technologies, triplicate wells; 50 °C for 2 min, 95 °C for 10 min, then 95 °C for 15 s, 60 °C for 60 s for 40 cycles) as previously described (25,26). The overall efficiency of the PCR probes was 1.94±0.11 (ref. 26). The interassay variability for clinical samples was 0.5–1.2% and the intra-assay reproducibility was 0.4–1.0% (ref. 26).

A NET score (0–8) is derived from the PCR data using MATLAB (R2011a, Mathworks, Natick, MA) (33). Briefly, the MATLAB algorithm classifies a blood sample as "control" or "GEP-NET" from the different learning algorithms (support vector machine, linear discrimination analysis, K-Nearest Neighbor, and Naive Bayes) (25). During the iterations, a score of 0 was assigned to predicted "controls" (internally labeled as "N" reflecting normal), whereas a score of 1 was assigned to "GEP-NET" predictions (internally labeled as "T" reflecting tumor). The latter "T" was weighted as either "1" or "2" depending on disease activity. The class of the new sample was decided by counting positive and negative votes from each classifier and selecting a label with the most votes. Each vote had equal weight (the output values of the classifiers were not taken into account). Values ranged from 0 to 8; a value of >2 is a positive tumor score (25,26,33).

CgA ELISA-based test

CgA was measured using the DAKO ELISA kit (K0025, DAKO North America, Carpinteria, CA) (25,26,31). A cut-off of 19 U/l (DAKO) was used as the upper limit of normal (25).

Table 2. Clinical characteristics of the pancreatic tumors

Group 2									
Characteristic							**Pancreatic NETs (*n*=45)**		
Mean age (range) (years)							57 (44–83)		
Gender (M/F)							17:28		
Tumor distribution	**No.**	**Grade**			**Stage**		**Treatment**		**Current PPIs[a]**
		G1	**G2**	**G3**	**Locoregional**	**Distant**	**Untreated**	**Current SSAs**	
Pancreas[b]	45	17	17	1	19	25	3	0	11
Characteristic							**Pancreatic disease (*n*=53)[c]**		
Mean age (range) (years)							66 (33–78)		
Gender (M/F)							21:32		
Disease			Pancreatitis			Cysts		NETs	Adenocarcinoma
Number			4 (1 on PPI[a])			31 (9 PPI[a])		4 (1 PPI[a])	14 (9 PPI[a])

M/F, male/female; NET, neuroendocrine tumor; PPI, proton pump inhibitor; SSA, somatostatin analog.
[a]Treatment includes lansoprazole (60 mg, *n*=3), omeprazole (5 mg, *n*=3; 20 mg, *n*=6; 40 mg, *n*=9), pantoprazole (40 mg, *n*=7; 60 mg, *n*=1), and rabeprazole (20 mg, *n*=2).
[b]Pathology data were only available for 35 of 45 pancreas NETs.
[c]Significant difference in age ($P<0.01$, Mann–Whitney) but not gender ($P=0.54$, two tailed: χ^2 test).

Statistical analyses

Sensitivity comparisons using respectively χ^2, nonparametric measurements, and receiver operating characteristic (ROC) analysis were made between the MAAA-PCR test and single-analyte plasma ELISAs for NET detection. Both Prism 6.0 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com) and MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) were utilized. The accuracy of each of the single analyte assays was compared with the NETest using ROC curve analyses (continuous variables) and the sensitivity, specificity, and the area under the curve (AUC) were calculated (MedCalc) (34). AUCs were compared and the *Z*-statistic derived (35) (MedCalc).

RESULTS

Intestinal tumors

All (100%, 41/41) small intestinal NETs exhibited a PCR score of ≥2 (**Figure 1a**) compared with 23 (57%) with an elevated CgA ($\chi^2=20.6$, $P<7\times10^{-7}$).

In the prospectively collected gastrointestinal carcinoma series, 3 (7.5%) of 40 exhibited a PCR score of >2 (positive test), whereas 8 (20%) had elevated CgA levels (**Figure 1b**). One (9%) of the 11 colon cancer patients and 2 (15%) of the 13 rectal adenocarcinoma patients exhibited positive scores (4, 3, and 3); each also had elevated CgA levels (**Figure 1c**). None of the esophageal (0/4), gastric (0/3), small bowel (0/6), or anal cancers (0/3) exhibited elevated PCR scores or elevated circulating CgA levels. Six colon cancers (54%) exhibited elevated CgA levels; two patients were receiving PPI therapy. Of the four nontreated patients, one patient exhibited an elevated PCR score; the other three had scores of 0.

Pancreatic disease

A total of 93% (42/45) of pancreatic NETs exhibited a PCR score of >2 (**Figure 2a**) compared with 15 (34%) with an elevated CgA ($\chi^2=35.3$, $P<1\times10^{-9}$). The three patients with ("normal") scores of 2 had been clinically assessed as stable disease following previous resection (pancreas resection (<1 cm insulinomas: *n*=2); Whipple: *n*=1) and were currently receiving somatostatin analog treatment.

Of the 53 patients assessed for potential pancreatic disease, 47 (89%) exhibited a PCR score of ≤2 (negative test) (**Figure 2b**). Four of the six patients with positive scores were subsequently determined (histopathological assessment) to have a pancreatic NET (**Figure 2b**). The other two positive scores were both subsequently confirmed to be cysts with intraductal papillary mucinous neoplastic (IPMN) features. Overall, none of the pancreatitis (0/4) or pancreatic cancer (0/14) cases exhibited elevated scores. All NETs were correctly identified (4/4), whereas (2/31) 6% of pancreatic cysts (IPMNs) exhibited a positive score. Elevated CgA levels were noted in 20 (37%) patients (**Figure 2c**). Only 1 of the 4 (25%) NETs exhibited elevated levels and 1 of 4 (25%) pancreatitis, 12 of 31 (39%) cysts, and 6 (43%) of pancreatic adenocarcinomas had elevated CgA.

Metrics

Analysis of all gastrointestinal tumors (NETs and gastrointestinal cancers) identified a positive NETest in 44 (54%) of the 81 samples, of which 41 of the 44 (93%) were NETs. CgA was positive in 31 cases (38%); 23 (75%) were NETs; the remainder were predominantly colon cancers. An audit of the colon cancer patients identified that two were on PPI therapy, suggesting a possible iatrogenic etiology. The NETest test had significantly better performance metrics than CgA ($P<0.0001$). The differences in performance

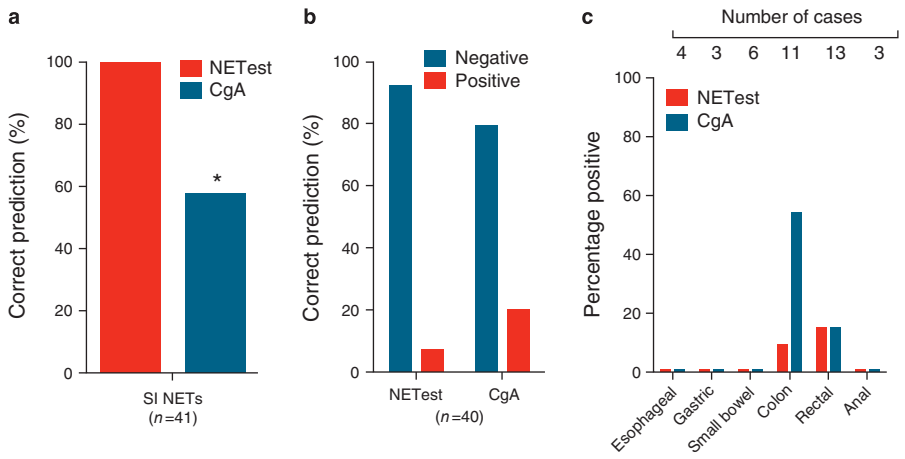

Figure 1. Accuracy of the NET multigene test compared with CgA for identifying SI NETs (group 1). (**a**) PCR analysis identified NETs (n=41) in 100% compared with 57% using CgA. Multigene transcript analysis was significantly more accurate than CgA (*Overall: χ^2=20.6, $P<7\times10^{-7}$). (**b**) Results for both the NETest and CgA in the non-NET gastrointestinal tumor group (n=40). The NETest was positive in 3 (7%), whereas CgA was elevated in 20% (χ^2=1.7, P=NS). (**c**) The NETest and CgA were negative in esophageal, gastric, small bowel, and anal cancers. One colon cancer and two rectal cancers were positive by the NETest, whereas 56% of colon cancers and 15% of rectal cancers exhibited elevated CgA levels (colon cancer: P=0.06, rectal cancer: P=NS). CgA, chromogranin A; NET, neuroendocrine tumor; NS, not significant; SI NET, small intestinal NET.

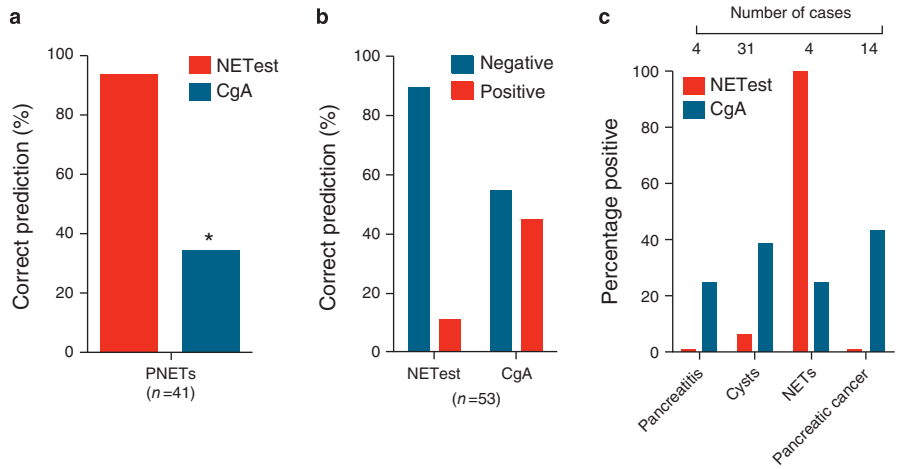

Figure 2. Accuracy of the multigene test compared with CgA for detecting pancreatic NETs (group 2). (**a**) PCR analysis identified NETs (n=45) in 93% compared with 34% with CgA. This was significantly better than CgA (*χ^2=35.3, $P<1\times10^{-9}$). (**b**) Results for both the NETest and CgA in the pancreatic diseases group (n=53). The NETest was positive in 6 that included 4 NETs. CgA was elevated in 20 patients (including 1 NET) (χ^2=8.6, P<0.003). (**c**) The NETest was negative in all pancreatitis and all pancreatic cancer cases. The NETest was positive in two cysts and all NETs (n=4). Elevated CgAs were identified in all four groups in 20–40% of the patients. CgA, chromogranin A, NET, neuroendocrine tumor; PNET, pancreatic NET.

metrics for differentiating a NET using the multigene test vs. the CgA in the 81 samples are included in **Figure 3a**. For the NETest, the sensitivity was 100%, specificity 93%, positive predictive value (PPV) 93%, and negative predictive value (NPV) 100%. For CgA, sensitivity was 56%, specificity 80%, PPV 74%, and NPV 64%. A formal comparison using ROC analysis identified the AUC for the NETest was 0.98±0.013, whereas it was 0.75±0.06 for CgA (**Figure 3b**). Comparison of the AUCs (35) demonstrated the NETest was significantly more accurate than CgA (difference between areas: 0.23, Z-statistic=4.37, P<0.0001, **Table 3**).

The majority (32 of 41, 78%) of small intestinal NETs had extensive metastatic disease (hepatic, bone, peritoneal deposits). Of these, 27 (66%) had only liver metastases. A comparison between hepatic metastases and those with limited, locoregional disease (stage IIIB, 9/41, 22%) confirmed the NETest was significantly elevated in those with liver involvement (5.3±0.2 vs. 4.1±0.2, P<0.02). The AUC was 0.75 (P=0.03; **Figure 4** and **Table 4**). Although CgA levels were increased in patients with hepatic metastases (81±55 U/l), this was not significant vs. limited disease (25±2 U/l) and ROC analysis identified an AUC of

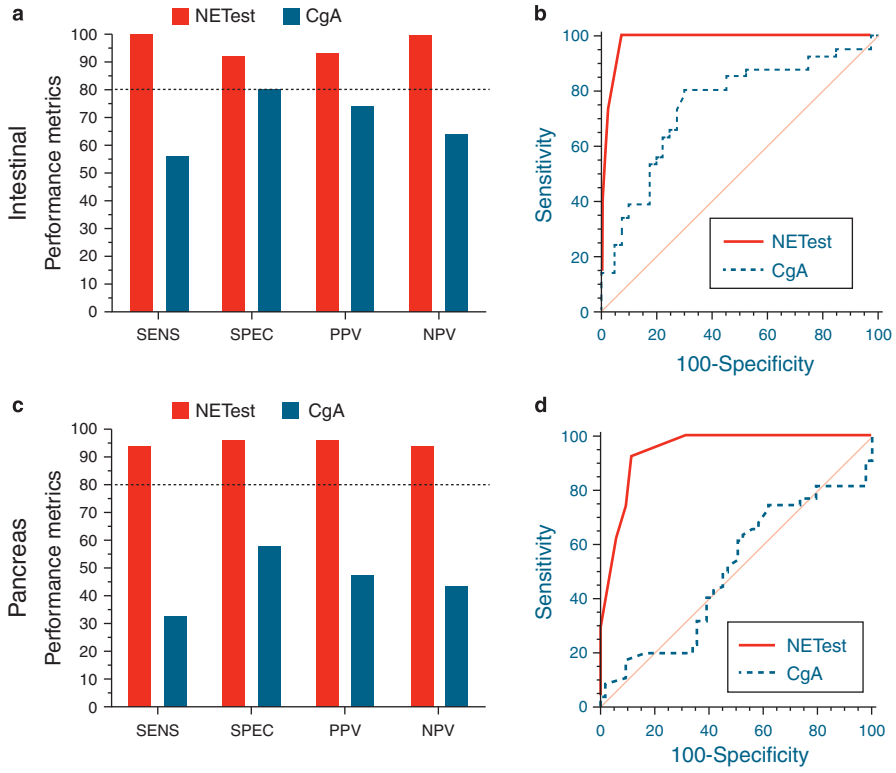

Figure 3. Performance metrics for the multigene test vs. CgA in intestinal and pancreatic disease. The dotted line (**a** and **c**) represents 80% (standard cutoff level for biomarkers). The dotted line (**b** and **d**) represents 50% for the AUC. (**a**) The sensitivity, specificity, PPV, and NPV for the multigene test in gastrointestinal neoplasia were all >80%. The metrics for CgA ranged from 56 to 80%. (**b**) Receiver operating characteristic (ROC) curves for PCR gene analysis compared with CgA. The AUC for PCR gene analysis was 0.98 and for CgA was 0.75. This difference was highly significant (*Z*-statistic: 4.4, *P*<0.0001). (**c**) The sensitivity, specificity, PPV, and NPV for the multigene test in pancreatic diseases were all >80%. The metrics for CgA ranged from 33 to 58%. (**d**) The AUC for PCR gene analysis was 0.94 and for CgA was 0.52. This difference was highly significant (*Z*-statistic: 6.7, *P*<0.0001). AUC, area under the curve; CgA, chromogranin A; NPV, negative predictive value; PCR, multigene test; PPV, positive predictive value; SENS, sensitivity; SPEC, specificity.

Table 3. Performance metrics and ROC comparisons

NETs	AUC (35)	s.e.	95% CI[a]	Difference between areas	*Z*-statistic (36)	*P* value
Intestinal (n=*81*)						
NETest	0.982	0.0129	0.924–0.999	0.234±0.0534	4.373	*P*<0.0001
CgA	0.749	0.0557	0.640–0.839			
Pancreatic (n=*87*)						
NETest	0.939	0.024	0.872–0.978	0.421±0.0637	6.692	*P*<0.0001
CgA	0.518	0.063	0.298–0.545			

AUC, area under the curve; CgA, chromogranin A; CI, confidence interval; NET, neuroendocrine tumor; NETest, multigene test; ROC, receiver operating characteristic.
[a]Binomial exact.

0.52 (*P*=0.9). A comparison between limited, locoregional disease (local lymph node metastasis) and extensive disease (*n*=32) identified that the NETest was higher in the latter (5.3±0.2, *P*=0.006) and the resultant AUC was 0.76 (*P*=0.01). Similar to the hepatic group, CgA levels were elevated but these were not significantly different (88±47, *P*=0.5 vs. limited disease) and the AUC was noninformative (AUC=0.59, *P*=0.6). Regarding metrics, all small intestinal NETs exhibited a positive NETest score compared with 44–61% with elevated CgA levels. The NETest score exhibited an overall accuracy of 76%, whereas it was 32% for CgA. This was highly significantly different (χ^2=14.2, *P*=0.001).

For pancreatic tumors, a combination of set 1 (pancreatic NETs) and set 2 (prospectively collected samples by endoscopic

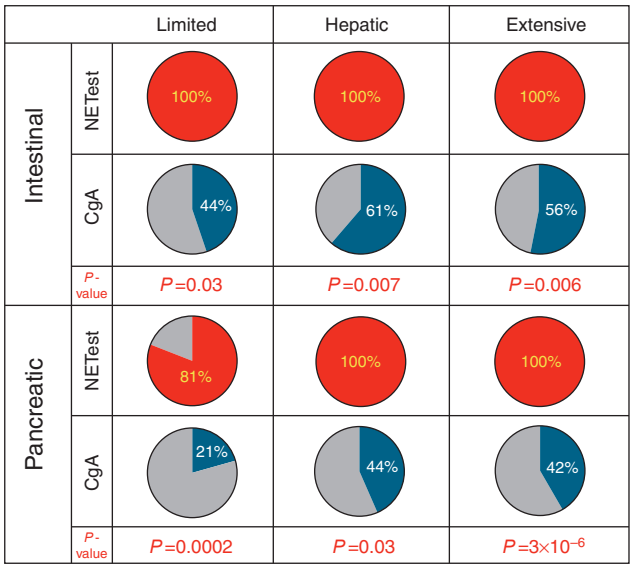

Figure 4. Multigene test scores and CgA levels in intestinal and pancreatic NETs (limited, hepatic, and extensive). The NETest and CgA-positive score percentages are provided for each group. *Small intestinal NETs*: NETest scores were 100% positive in all small intestinal NETs with limited disease. CgA was elevated in 44%. This was significantly different (P=0.03, χ^2 test). In the hepatic metastases group, 100% were PCR positive compared with 61% with elevated CgAs (P=0.007). In the extensive disease group, 100% were PCR positive compared with 56% with elevated CgAs (P=0.006). *Pancreatic NETs*: in locoregional disease, PCR positives were 81% compared with elevated CgAs in 21% (P=0.0002, χ^2 test). In hepatic metastases, 100% were PCR positive compared with 44% with elevated CgAs (P=0.03). In extensive disease, 100% were PCR positive compared with 44% with elevated CgAs (P=3×10^{-6}). CgA, chromogranin A, NET, neuroendocrine tumor.

Table 4. Multigene test scores (NETest) and CgA levels in intestinal and pancreatic NETs

NETs	Biomarker	Limited	Hepatic	Extensive
Intestinal (n=41)	NETest	4.1±0.2	5.1±0.2[a]	5.3±0.2[b]
	CgA	25±2 U/l	81±55 U/l[c]	88±47 U/l[c]
Pancreatic (n=45)	NETest	3.9±0.4	4.7±0.4[a]	5.6±0.3[b]
	CgA	145±84 U/l	682±436 U/l[c]	772±487 U/l[c]

CgA, chromogranin A; NET, neuroendocrine tumor; NETest, multigene test.
[a]P<0.05 compared with limited disease (two-tailed, Mann–Whitney test).
[b]P<0.01 vs. limited disease (two-tailed, Mann–Whitney test).
[c]P=not significant (NS; CgA levels compared with limited disease).

retrograde cholangiopancreatography/endoscopic ultrasound) identified a total of 49 patients with NETs and 49 with other pancreatic pathology. A total of 46 (94%) of NETs were positive by the NETest compared with 13 (27%) by CgA. An audit of these 13 identified that 6 (46%) were on concomitant PPI therapy. Two (4%) of non-NETs (both IPMN) were positive by the NETest, whereas 22 (45%) exhibited elevated CgA levels. An audit of these 22 patients identified that 13 (59%) were also on concomitant PPI therapy. The remainder, however, had no demonstrable medical reason for elevated CgA. A false positive rate (unknown causes) of 16% (8 of 49) was therefore evident for CgA measurement. In the overall group, the NETest test had significantly better (P<0.0001) performance metrics than CgA. The differences in performance metrics for differentiating a NET using the multigene test vs. the CgA in the 98 samples (49 NETs and 49 pancreatic disease) are included in **Figure 3c**. For the NETest, the sensitivity was 94%, specificity 96%, PPV 96%, and NPV 94%. For CgA, sensitivity was 33%, specificity 58%, PPV 47%, and NPV 43%. ROC analysis identified the AUC for the NETest was 0.94±0.02, whereas it was 0.52±0.06 for CgA (**Figure 3d**). Comparison of AUCs (35) identified the NETest to be significantly more efficient than CgA (difference between areas: 0.42, Z-statistic=6.7, P<0.0001, **Table 3**).

Nineteen patients (42%) exhibited limited locoregional disease (4 stage I and 15 stage IIIB), the rest (58%) exhibited metastatic lesions, 9 (20%) with hepatic and 17 (38%) with extensive disseminated. A comparison between individuals with hepatic metastases and those with limited, locoregional disease confirmed the NETest was significantly elevated in those with liver involvement (4.7±0.4 vs. 3.9±0.4, P<0.04; **Figure 4** and **Table 4**). Analyzing these data using a ROC analysis confirmed that the NETest score could significantly differentiate these two groups (AUC=0.74, P<0.05). CgA levels were increased in patients with hepatic metastases 682±436 U/l but this was not significant (P=0.72) and neither was the AUC (0.7, P=0.32). A comparison between limited, locoregional disease and extensive disease (n=36, including hepatic metastasis) identified that the NETest was higher in the latter (5.6±0.3 vs. 3.9±0.4, P=0.0005). The AUC was 0.80 (P=0.0009). CgA levels were elevated in the distant group but these were not significantly different to the limited, locoregional group (772±487 U/l, P=0.4). Furthermore, the AUC did not demonstrate a significant difference (AUC=0.56, P=0.69). Regarding metrics, 94% of patients had a positive NETest compared with ~20–40% with CgA. The overall accuracy for NETest scores was 80%, whereas it was 20% for CgA (χ^2=30, P=2×10^{-8}).

DISCUSSION

This study assesses the clinical utility of the NETest for well-differentiated small intestinal and pancreatic NETs under "real-world" conditions, namely, in comparison with CgA, vs. gastrointestinal adenocarcinomas and pancreatic disease, respectively. As the majority (99%) of tumors were G1 and G2, the metrics and utility reflect its use in these well-differentiated lesions. This study demonstrates that the NETest accurately and specifically detects G1 and G2 small intestinal NETs with sensitivities and specificities of 100 and 93%, respectively, and outperformed CgA (Z-statistic >4, P<0.0001). The NETest scores were more often positive and were also higher in distant disease (liver metastases, other sites such as bone) compared with limited disease. NETest levels accurately and sensitively differentiated localized disease from distant disease with better metrics (76%) than CgA alone (32%). Well-

differentiated pancreatic NETs were also accurately differentiated from other pancreatic diseases such as pancreatic cancers (sensitivity=94% and specificity 96%). As a biomarker, the NETest confirmed to be significantly more informative than CgA (*Z*-statistic ~7, $P<0.0001$). As for intestinal NETs, NETest scores were more often positive and were elevated in distant than in locoregional pancreatic disease. In addition, the accuracy of the NETest was significantly superior ($\chi^2=30$, $P<10^{-8}$) particularly because a significant proportion (>60%) of pancreatic NETs failed to exhibit elevated levels. In this study, we tested the efficacy of the NETest in a "real-world" situation comparing NETs with unknown GEP diseases. Our results demonstrate that the blood transcript analysis outperforms the current diagnostic standard (CgA) and meet the optimal NIH recommended assay performance metrics (25,36,37).

The strengths of this study included the large numbers in each group ($n\geq40$, total of 179 cases), the prospective nature, and efficacy of centralized analysis. The weaknesses of the study include the limitations of age/sex matching of samples (difficult in the prospective setting, the pancreatic disease group was significantly older than the pancreatic NET group), but this also reflects the rarity of NET disease. The former is unlikely to be an important factor as age and gender have previously been reported to play no role in NETest results (26). A comparison with other monoanalyte markers including CgA derivatives like pancreastatin and neuron-specific enolase could have provided added information, but it has been previously reported that the transcript analysis significantly outperforms these and hence only CgA was compared (25,36,37).

Neuroendocrine differentiation and immunohistochemical detection of neuroendocrine cells in gastrointestinal adenocarcinomas is a well-described feature in a subset of lesions. CgA-positive cells have been reported in up to 15% of 91 colorectal cancers (38); elevated circulating levels of CgA were detected in 38% of the patients. Elevated CgA is considered a potential marker of neuroendocrine differentiation in gastric adenocarcinomas (39), although the roles of PPI administration (17) and other factors such as hypertension and inflammatory diseases are well-known confounders. In this study, three colon and rectal adenocarcinoma patients exhibited both elevated CgAs and PCR scores. Although the detection of three (3.7%) samples can be considered as false positives, the detection of both circulating NET transcripts and CgA is more consistent with a neuroendocrine phenotype. In this regard, of the five other colon cancers with elevated CgAs, two patients were treated with PPIs. The other three all had circulating NET scores of 0, consistent with no neuroendocrine tumor disease. Given the sensitivity of the NETest as compared with CgA, it may be possible to identify colonic or prostatic adenocarcinomas that exhibit neuroendocrine features. A separate, prospective study would however be necessary to confirm such a hypothesis. This may be of clinical relevance as specific treatment strategies for "neuroendocrine phenotype neoplasia" (prostate) have been reported to be efficacious (40). The potential clinical utility of this observation is further supported by reports that adenocarcinomas with a neuroendocrine phenotype are associated with a poorer prognosis (20,41). Thus, identifying and defining such a group is advantageous.

Gastrointestinal cancers are typically diagnosed at imaging, e.g., endoscopy or colonoscopy, or at follow-up for symptoms indicative of disease, e.g., melena. Tumors localized in the terminal ileum, however, are more difficult to image and access for biopsy (42). CgA in this study was elevated in 57% of NETs and in 0% of small bowel tumors. The NETest was uniformly positive (100%) in the NETs and was negative in all small bowel cancers (0/6). Although the numbers are limited (the disease is rare), it is evident that the NETest demonstrates the appropriate performance metrics to serve as an effective tool for differentiating small bowel lesions and would provide an effective marker for tumor management.

The diagnosis of pancreatic disease is accomplished through imaging tests and pathologic diagnosis. Whereas pancreatic cancers constitute up to 95% of exocrine malignancies, cystic neoplasms are reported to account for <1% of pancreatic cancers. Up to 90% of pancreatic cysts are inflammatory pseudocysts arising from acute or chronic pancreatitis. When a solid mass is detected at imaging, the differential diagnosis between pancreatic NET, particularly the "non-functional/non-syndromic" variant and adenocarcinoma, is problematic and neither the clinical features, such as loss of appetite, abdominal pain, and jaundice, nor the appearance at multidetector computed tomography or magnetic resonance imaging is clearly distinctive of either entity (43). Moreover, no biomarkers can currently differentiate these two tumor types.

The limitation of using CgA as a biomarker in pancreatic NETs is highlighted by observations that this is elevated in only 34%, whereas NET transcript are elevated in 93%, with a 7% false negative rate. This included three patients with clinical stable disease receiving somatostatin analogs following surgery. The false positive rate was 2%. These included two patients with cysts with IPMN features. One of the two patients also exhibited an elevated CgA. Whereas neuroendocrine features are uncommon in IPMNs (44), these precancerous lesions can coexist with NETs in ~3–5% of cases (45–47), not dissimilar to the current series (2/31 IPMNs exhibited an elevated NETest). The latter is an interesting "false positive" as previous histopathological studies have indicated that IPMNs and NETs can occur in adjacent areas of the pancreas (48,49). Such lesions are rarely functional and have a relatively small size (~1.5 cm), raising the possibility that additive information from a blood biomarker test may be of some clinical utility. It is not clear whether either of these two cysts in this study were associated with NETs, but the combination of a positive PCR score as well as an elevated CgA suggests that this possibility should be considered. The NETest may therefore also have utility in excluding a NET when questionable anatomic imaging is noted.

It should be noted that elevated CgA levels were noted in 20 of the pancreatic disease patients. In all, 39% of cysts and 43% of pancreatic adenocarcinomas had elevated CgA. These data largely parallel PPI use in this cohort; 29% of patients with cysts were receiving PPIs, whereas 64% with pancreatic adenocarcinomas were being treated. The AUC for CgA was low (0.50) and the high PPI usage levels in these patients (~40%) made measurements largely non-informative. The widespread use of PPIs with consequent false positive (80–100% (37,50)) CgA (and its fragments) augments the clinical problem of diagnosis and management based upon CgA.

As opposed to monoanalytes, considerable clinical advantage has been derived from the use of MAAAs such as Mammaprint (51) for breast neoplasia. For NETs, the development of biomathematically weighted scoring systems will facilitate the introduction of a risk probability (52) analysis and allow for the improvement of prognostic and predictive information, thereby aiding clinical decision making (29,53). An alternative possibility is that interface of blood-based transcriptional information relating to the proliferative regulation of tumor growth metastasis and metabolism will likely provide information that is synergistic with neuroendocrine cell function (29). This could facilitate more precise and earlier detection of disease progression or recurrence after surgery. A key opportunity is the use of blood levels of NET transcript levels to provide added value in the prediction of therapeutic efficacy, particularly when other monoanalyte biomarkers such as CgA have proven to be of limited value in defining targeted drug efficacy (26,29,33).

CONFLICT OF INTEREST

Guarantor of the article: I.M. Modlin, MD, PhD, DSc.

Specific author contributions: The authors' relationships with the NETest are as follows: I.M. Modlin: Medical/Scientific Consultant on behalf of Keewaydin Consulting to Wren Laboratories; M. Kidd: Laboratory Director of Wren Laboratories; I. Drozdov: Bering, Statistical Consultant to Wren Laboratories; L. Bodei: Deputy Director of Nuclear Medicine at IEO, Milan, Principal Investigator, LuGenium Consortium for Independent Research.

Financial support: This work was in part supported by Clifton Life Sciences.

Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ The default biomarker for the diagnosis and follow-up of neuroendocrine tumors is chromogranin A (CgA).
- ✓ CgA does not achieve the standardized metrics proposed for biomarker sensitivity and specificity.
- ✓ A multianalyte test based on circulating RNA, the NETest, has attained the performance criteria as an effective clinical test.

WHAT IS NEW HERE

- ✓ A prospective study in a large cohort (~180 cases) of small bowel and pancreatic neuroendocrine tumors (NETs) demonstrates that multigene analysis in blood is more sensitive and specific than CgA and has performance metrics that meet the National Institutes of Health (NIH) criteria for biomarker measurement.
- ✓ Levels are significantly more specific and selective than CgA, which is the current default monoanalyte marker, for differentiating limited, locoregional disease from distant intestinal and pancreatic NET disease.
- ✓ Use of a multianalyte gene transcript test in blood is feasible and provides added value for well-differentiated neuroendocrine tumor disease detection in the clinical setting.

REFERENCES

1. Modlin IM, Moss SF, Chung DC *et al.* Priorities for improving the management of gastroenteropancreatic neuroendocrine tumors. J Natl Cancer Inst 2008;100:1282–9.
2. Palmer C, Duan X, Hawley S *et al.* Systematic evaluation of candidate blood markers for detecting ovarian cancer. PLoS One 2008;3:e2633.
3. Modlin I, Kidd M, Taylor A *et al.* Neuroendocrine tumor biomarkers: current status and perspectives. Neuroendocrinology 2014;100:265–77.
4. McDermott U, Downing JR, Stratton MR. Genomics and the continuum of cancer care. N Engl J Med 2011;364:340–50.
5. Giandomenico V, Modlin IM, Ponten F *et al.* Improving the diagnosis and management of neuroendocrine tumors: utilizing new advances in biomarker and molecular imaging science. Neuroendocrinology 2013;28:28.
6. Modlin IM, Latich I, Zikusoka M *et al.* Gastrointestinal carcinoids: the evolution of diagnostic strategies. J Clin Gastroenterol 2006;40:572–82.
7. Turner GB, Johnston BT, McCance DR *et al.* Circulating markers of prognosis and response to treatment in patients with midgut carcinoid tumours. Gut 2006;55:1586–91.
8. Ardill JE, Erikkson B. The importance of the measurement of circulating markers in patients with neuroendocrine tumours of the pancreas and gut. Endocr Relat Cancer 2003;10:459–62.
9. Meijer WG, Kema IP, Volmer M *et al.* Discriminating capacity of indole markers in the diagnosis of carcinoid tumors. Clin Chem 2000;46:1588–96.
10. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. Gastroenterology 2008;135:1469–92.
11. Bajetta E, Ferrari L, Martinetti A *et al.* Chromogranin A, neuron specific enolase, carcinoembryonic antigen, and hydroxyindole acetic acid evaluation in patients with neuroendocrine tumors. Cancer 1999;86:858–65.
12. Khan MS, Tsigani T, Rashid M *et al.* Circulating tumor cells and EpCAM expression in neuroendocrine tumors. Clin Cancer Res 2011;17:337–45.
13. Modlin IM, Oberg K, Chung DC *et al.* The current status of gastroenteropancreatic neuroendocrine tumors. Lancet Oncol 2008;9:61–72.
14. Kanakis G, Kaltsas G. Biochemical markers for gastroenteropancreatic neuroendocrine tumours (GEP-NETs). Best Pract Res Clin Gastroenterol 2012;26:791–802.
15. Stridsberg M, Oberg K, Li Q *et al.* Measurement of chromogranin A, chromogranin B (secretogranin I), chromogranin C (secretogranin II) and pancreastatin in plasma and urine from patients with carcinoid tumours and endocrine pancreatic tumours. J Endocrinol 1995;144:49–59.
16. Portela-Gomes GM, Stridsberg M. Selective processing of chromogranin A in the different islet cells in human pancreas. J Histochem Cytochem 2001;49:483–90.
17. Lawrence B, Gustafsson BI, Kidd M *et al.* The clinical relevance of chromogranin A as a biomarker for gastroenteropancreatic neuroendocrine tumors. Endocrinol Metab Clin North Am. 2011;40:111–34.
18. Modlin IM, Gustafsson BI, Moss SF *et al.* Chromogranin A--biological function and clinical utility in neuro endocrine tumor disease. Ann Surg Oncol 2010;17:2427–43.
19. Marotta V, Nuzzo V, Ferrara T *et al.* Limitations of chromogranin A in clinical practice. Biomarkers 2012;17:186–91.
20. Wu JT, Erickson AJ, Tsao KC *et al.* Elevated serum chromogranin A is detectable in patients with carcinomas at advanced disease stages. Ann Clin Lab Sci 2000;30:175–8.
21. Sciarra A, Monti S, Gentile V *et al.* Chromogranin A expression in familial versus sporadic prostate cancer. Urology 2005;66:1010–4.
22. Hsiao RJ, Mezger MS, O'Connor DT. Chromogranin A in uremia: progressive retention of immunoreactive fragments. Kidney Int 1990;37:955–64.
23. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
24. Reichard KK, Hanson CA. Hematological diseases: prototypical conditions requiring the diagnostic and prognostic use of molecular data. Semin Diagn Pathol 2013;30:382–92.
25. Modlin I, Drozdov I, Kidd M. The identification of gut neuroendocrine tumor disease by multiple synchronous transcript analysis in blood. PLoS One 2013, e63364.
26. Modlin I, Drozdov I, Kidd M. Gut neuroendocrine tumor blood qPCR fingerprint assay: characteristics and reproducibility. Clin Chem 2014;52:419–29.
27. Halperin DM, Kulke MH, Yao JC. A tale of two tumors: treating pancreatic and extrapancreatic neuroendocrine tumors. Annu Rev Med 2014;17:17.
28. Lewis MA, Yao JC. Molecular pathology and genetics of gastrointestinal neuroendocrine tumours. Curr Opin Endocrinol Diabetes Obes 2013;4:4.

29. Oberg K, Modlin I, DeHerder W *et al.* Biomarkers for neuroendocrine tumor disease: a Delphic consensus assessment of multianalytes, genomics, circulating cells and monoanalytes. Lancet Oncol 2015, in press.
30. Raza A, Ali Z, Irfan J *et al.* Analytical variables influencing the HCV RNA determination by TaqMan real-time PCR in routine clinical laboratory practice. Mol Biol Rep 2012;39:7421–7.
31. Modlin IM, Gustafsson BI, Drozdov I *et al.* Principal component analysis, hierarchical clustering, and decision tree assessment of plasma mRNA and hormone levels as an early detection strategy for small intestinal neuroendocrine (carcinoid) tumors. Ann Surg Oncol 2009;16:487–98.
32. Fleige S, Walf V, Huch S *et al.* Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnol Lett 2006;28:1601–13.
33. Modlin I, Drozdov I, Kidd M. A multitranscript blood neuroendocrine tumor molecular signature to identify treatment efficacy and disease progress. J Clin Oncol 2013;31:A4137.
34. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982;143:29–36.
35. Hanley JA, McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. Radiology 1983;148:839–43.
36. Modlin I, Drozdov I, Alaimo D *et al.* A multianalyte PCR blood test outperforms single analyte ELISAs for neuroendocrine tumor detection. Endocr Relat Cancer 2014;21:615–28.
37. Modlin IM, Aslanian H, Bodei L *et al.* A PCR blood test outperforms chromogranin A in carcinoid detection and is unaffected by PPIs. Endocr Connect 2014;14:14–0100.
38. Syversen U, Halvorsen T, Marvik R *et al.* Neuroendocrine differentiation in colorectal carcinomas. Eur J Gastroenterol Hepatol 1995;7:667–74.
39. Syversen U, Ramstad H, Gamme K *et al.* Clinical significance of elevated serum chromogranin A levels. Scand J Gastroenterol 2004;39:969–73.
40. Fisher KW, Montironi R, Lopez Beltran A *et al.* Molecular foundations for personalized therapy in prostate cancer. Curr Drug Targets 2015;16:103–14.
41. Wang J, Wei B, Albarracin CT *et al.* Invasive neuroendocrine carcinoma of the breast: a population-based study from the surveillance, epidemiology and end results (SEER) database. BMC Cancer 2014;14:147.
42. Raghav K, Overman MJ. Small bowel adenocarcinomas--existing evidence and evolving paradigms. Nat Rev Clin Oncol 2013;10:534–44.
43. Manfredi R, Bonatti M, Mantovani W *et al.* Non-hyperfunctioning neuroendocrine tumours of the pancreas: MR imaging appearance and correlation with their biological behaviour. Eur Radiol 2013;23:3029–39.
44. Kloppel G, Basturk O, Schlitter AM *et al.* Intraductal neoplasms of the pancreas. Semin Diagn Pathol 2014;31:452–66.
45. Gill KR, Scimeca D, Stauffer J *et al.* Pancreatic neuroendocrine tumors among patients with intraductal papillary mucinous neoplasms: real association or just a coincidence? JOP 2009;10:515–7.
46. Goh BK, Ooi LL, Kumarasinghe MP *et al.* Clinicopathological features of patients with concomitant intraductal papillary mucinous neoplasm of the pancreas and pancreatic endocrine neoplasm. Pancreatology 2006;6:520–6.
47. Marrache F, Cazals-Hatem D, Kianmanesh R *et al.* Endocrine tumor and intraductal papillary mucinous neoplasm of the pancreas: a fortuitous association? Pancreas 2005;31:79–83.
48. Ishida M, Shiomi H, Naka S *et al.* Concomitant intraductal papillary mucinous neoplasm and neuroendocrine tumor of the pancreas. Oncol Lett 2013;5:63–7.
49. Larghi A, Stobinski M, Galasso D *et al.* Concomitant intraductal papillary mucinous neoplasm and pancreatic endocrine tumour: report of two cases and review of the literature. Dig Liver Dis 2009;41:759–61.
50. Stridsberg M, Eriksson B, Oberg K *et al.* A comparison between three commercial kits for chromogranin A measurements. J Endocrinol 2003;177:337–41.
51. Faltin B, Zengerle R, von Stetten F. Current methods for fluorescence-based universal sequence-dependent detection of nucleic acids in homogenous assays and clinical applications. Clin Chem 2013;59:1567–82.
52. Eastman PS, Manning WC, Qureshi F *et al.* Characterization of a multiplex, 12-biomarker test for rheumatoid arthritis. J Pharm Biomed Anal 2012;70:415–24.
53. Cornejo KM, Kandil D, Khan A *et al.* Theranostic and molecular classification of breast cancer. Arch Pathol Lab Med 2014;138:44–56.