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ORIGINAL ARTICLE – PANCREATIC TUMORS

Circulating Neuroendocrine Gene Transcripts (NETest): A Postoperative Strategy for Early Identification of the Efficacy of Radical Surgery for Pancreatic Neuroendocrine Tumors

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ABSTRACT

Background. Surgery remains the only treatment for the cure of pancreatic neuroendocrine tumors (PanNETs). Biomarkers to identify the completeness of resection and predict recurrence are lacking.

Objective. The aims of this study were to evaluate if the blood measurement of neuroendocrine gene transcripts (NETest) was diagnostic of PanNETs, and whether NETest blood levels could identify complete resection. We compared transcript analysis with the biomarker chromogranin A (CgA).

Methods. This was a prospective, longitudinal, single-center study including 30 patients with a postoperative histological confirmation of PanNET. Blood for NETest and CgA was collected preoperatively and on postoperative day (POD) 1, POD5, and POD30. Transcripts were measured by real-time quantitative reverse transcription polymerase chain reaction and multianalyte algorithmic analysis (NETest; normal < 20), and CgA was measured by enzyme-linked immunosorbent assay (ELISA; normal < 109 ng/mL). Data are expressed as mean $\pm$ standard deviation (SD).

Results. Pancreatic surgical resections ($n = 30$) were $R0$, 26; $R1$, 2; and $R2$, 2. Preoperatively, NETest score was elevated in all 30 patients (44.7 ± 27), but postoperatively, NETest scores significantly decreased ($p = 0.006$) to POD30 (24.7 ± 24). The proportion of patients (15/30) with an elevated score significantly decreased by POD30 ($p < 0.0001$). CgA levels were elevated preoperatively (184 ± 360 ng/mL) in only 9/30 patients, but did not decrease significantly postoperatively at POD30 (260 ± 589 ng/mL, $p = 0.398$). The number of patients with elevated CgA levels remained unchanged (9/30).

Conclusions. The NETest is an accurate diagnostic biomarker for PanNETs (100%). A decrease in NETest levels after radical resection suggests this blood test provides early assessment of surgical efficacy. CgA had no clinical utility.

Multiple treatment approaches are currently available for patients with pancreatic neuroendocrine tumors (PanNETs), but surgery is still considered the only treatment associated with cure.[1–3] A key issue however is the need for strategies to define the effectiveness of surgery, since residual or recurrent disease needs to be identified earlier in order to facilitate retreatment. In this respect, imaging and blood biomarkers remain the two best available techniques in the post-surgery follow-up strategy.

Surgery is generally highly effective in localized non-functioning or functioning tumors with a cure rate of approximately 80–85%.[4–6] In contrast, most patients with metastatic disease have poorer outcomes, although surgery may also play a role in the management of metastatic

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disease.[7–9] Despite the overall efficacy of surgery, it should be noted that < 30% of patients with liver metastases are eligible for surgery. In patients with radically resected (*R0*/*R1*) liver metastases, the 5-year overall survival is around 50–60%.[10] The standard management strategy to identify any alteration in the status of the disease and evaluate tumor recurrence includes a combination of radiological and nuclear medicine techniques[1,2]; however, these techniques have significant limitations in the resolution capacity, which is problematic for the accurate and early detection of either residual tumor or tumor recurrence. Furthermore, repeated imaging to identify subtle changes in disease is expensive and requires increased radiation exposure. An alternative strategy would include a reliable blood biomarker for early assessment of the course of the disease. Thus, in the difficult post-operative monitoring phase, when imaging is less effective due to surgical changes, a sensitive blood test would be invaluable to identify disease recurrence at the earliest possible stage.

A variety of blood markers have previously been proposed but unfortunately none is considered to have adequate sensitivity and specificity, leading to chromogranin A (CgA) becoming the default measurement, despite substantial reservations in respect of its efficacy.[11,12] One study specifically evaluated the utility of CgA measurements in determining surgical efficacy in patients affected by PanNETs, and identified CgA to be < 30% effective.[11] As a result, there is no rigorous method to identify residual disease or assess treatment efficacy. Furthermore, the recent advance of biomarker strategies has demonstrated that monoanalyte biomarkers are far less effective than multianalyte tools to define the numerous biological processes that represent an evolving tumor.[13]The advent of sophisticated molecular biology techniques has led to the identification of more accurate and innovative markers.[14] In this respect, the recent development of a blood test panel of NET marker genes, derived from the transcript profile of NET cells (NETest), has provided novel information.[15–18] This neuroendocrine gene panel (NET molecular signature) has been validated, using a series of independent datasets, by a number of different groups.[19–23] A series of reports has documented that the efficacy of treatment with therapies such as long-acting release (LAR) octreotide, peptide receptor radionuclide therapy (PRRT), or surgery can be demonstrated by a decrease in the blood transcript levels.[19,24–27] In contrast, in patients exhibiting progression on such treatments, a continued elevation or a subsequent rise in transcript levels is evident.[19,25,26] The purpose of this study was to evaluate whether NETest was effective in the diagnosis of PanNETs, and if alterations in circulating NET transcripts could assess the extent and efficacy of pancreatic surgical resections.

METHODS

Study Design

This prospective, longitudinal, single-center study was conducted according to the 'Standards for Reporting Diagnostic accuracy studies' 2015,[28] and was approved by the San Raffaele Hospital Ethical Committee (ClinicalTrials.gov identifier: NCT03012789).

Patients

Between November 2017 and April 2018, patients affected by gastroenteropancreatic (GEP) NET were enrolled if they met the following inclusion criteria: age > 18 years; pathologically confirmed, well-differentiated (G1, G2, G3) GEP-NET candidates for surgical resection; treatment-naïve, as well as those who underwent previous treatments; computed tomography (CT) or magnetic resonance [MR] documentation of disease; and World Health Organization (WHO) status ≤ 2. Exclusion criteria included a known history of HIV seropositivity. All patients signed an informed consent form. For the purpose of the present study, only patients with histological confirmation of PanNETs were included.

Test Methods

NETest represented the index test, whereas CgA was chosen as the reference standard test since at present it is considered the most used and best-described biomarker in the field of neuroendocrine tumors.[1] Regarding NETest, a two-step protocol (RNA isolation, complementary DNA [cDNA] production, and polymerase chain reaction [PCR]) was used. Real-time PCR was performed (384-well plate, HT-7900 machine) with 200 ng/μL of cDNA and 16 μL of reagents per well (Fast Universal PCR master mix, Applied Biosystems®). All primers used were exon spanning and were < 150 bprs. PCR values were normalized to housekeeping genes and expression was quantified against a population control (calibrator sample).[16] Thereafter, multianalyte algorithm analyses (MAAA) were undertaken. Final gene expression results were expressed as an activity index score from 0 to 100%,[29] based on the integration of the majority vote and summated expression of five gene clusters, including the proliferome, epigenome, growth factor signalome, and genes involved in pluripotency.[29] The NETest score was calculated as a percentage, with the upper limit of normal < 20.[21] The clinical assessment of NETest scores has demonstrated that any value ≥ 20 represents neuroendocrine tumor disease. Values between 20 and 40 represent low activity disease; values > 40 represent progressive disease; and values > 80 represent high-

level progressive disease.[19,30] CgA was assayed by the NEOLISA assay (Eurodiagnostica®). The upper limit of normal is 108 ng/mL. All blood samples were de-identified to the laboratory group. Results of the index test (NETest) and reference standard (CgA) test were available to the assessors (Wren Laboratories®), whereas clinical information was only available to the clinical study group and not to the performers and readers of both the index and reference standard tests.

Data Collection

Two venous whole blood samples were collected in 5 mL ethylenediaminetetraacetic acid (EDTA) tubes. Two × 5 mL blood samples were obtained at the same time prior to surgery, on postoperative day (POD) 1, POD5, and 1 month after surgery (POD30). After collection of blood samples, one EDTA tube (whole blood sample) was frozen (−80 °C), whereas the second EDTA tube was spun for 10 min to separate plasma, and then frozen (−80 °C). All samples were de-identified and sent by courier on dry ice to Wren Laboratories LLC® for measurement. A prospectively collected database was queried for all clinical and pathological details. The initial diagnostic work-up always included at least one high-quality relevant imaging examination (CT or MR imaging [MRI]) and was always completed with either ^{68}Ga-DOTATOC positron emission tomography (PET)/CT or ultrasound endoscopy (EUS) with fine-needle aspirate (FNA). 18F-fluorodeoxyglucose (^{18}F-FDG) PET/CT was not performed routinely, nor is it recommended that this should be undertaken. All patients included in the present series were treated with proton pump inhibitors (PPI; pantoprazole 40 mg) for at least 1 month after surgery. The preoperative variables considered were sex, age, presence of pain, presence of jaundice, body mass index (BMI), type of diagnosis, tumor function, radiological tumor diameter, and positivity of ^{68}Gallium and ^{18}F-FDG PET/CT. The surgical procedure was planned according to site and dimension of the tumor. Postoperative surgical complications were also recorded and classified according to the Clavien–Dindo classification of surgical complications.[31] Tumor size was defined as the maximum diameter in the pathological specimen, and T and N stages were classified according to current American Joint Committee on Cancer (AJCC)[32] and European Neuroendocrine Tumor Society (ENETS)[1] classifications. Immunostaining routinely included synaptophysin, CgA, and Ki67 proliferative index, assessed by MIB1 antibody staining and expressed as the percentage of cells with positive nuclear staining in 2000 cells counted in the area of highest nuclear labeling. Tumor grade was classified according to the 2017 WHO classification into G1 (Ki67 index $< 3\%$), G2 (Ki67 index 3–20%) and G3 (Ki67 index $> 20\%$).[33] Surgical margins were classified into three categories: *R*0 (no residual tumor), *R*1 (microscopic residual tumor), and *R*2 (macroscopic residual tumor). The presence of microvascular invasion, perineural invasion, and necrosis was assessed.

All patients included in the study were followed regularly after surgery. The follow-up protocol included a 6-month, high-quality imaging examination (MRI), and an outpatient visit on a yearly basis. Last follow-up was updated in June 2019.

Statistical Analysis

Continuous data were reported as mean ± standard deviation (SD). For categorical data, number and proportion (%) were displayed. The comparison between subgroups was carried out using Student's *t* test or Mann–Whitney U test for continuous variables. A paired *t* test was used to compare means of test methods measurements before and after surgical resection. Qualitative data were compared using the Chi square test or Fisher's exact test when necessary. The relationship between continuous variables was evaluated by univariate linear regression analysis. Statistical analyses were performed using SPSS 16.0 for Windows software (SPSS Inc, Chicago, IL, USA). *P*-values were considered significant when ≤ 0.05.

RESULTS

Participants

Overall, 40 patients submitted to surgical resection with a preoperative diagnosis of GEP-NET were enrolled, of whom 30 patients had a postoperative histological confirmation of PanNET. Demographics, clinical details, and pathological findings are summarized in Tables 1 and 2.

Test Results

NETest: The preoperative NETest score was increased in all patients, with a mean value of 44.7 (SD ± 27); nine patients had a high disease activity (NETest score > 40). Pancreatic NET disease was identified in 30/30 patients. The mean preoperative NETest score was significantly higher compared with the NETest score measured on POD1, POD5, and POD30 {preoperative: 44.7 (SD ± 27) vs. POD1: 27.3 (SD ± 23) [$p = 0.001$], vs. POD5: 25.7 (SD ± 19.6) [$p = 0.005$], vs. POD30: 24.7 (SD ± 24) [$p = 0.006$]} (Fig. 1). The decrease in NETest levels remained stable after surgical resection and no statistically significant differences were detected between the three postoperative assessments (POD1, POD5, POD30). The

TABLE 1 Demographics and perioperative characteristics of 30 patients with histologically proven PanNETs

Variable	Patients
Sex	
Male	11 (37)
Female	19 (63)
Age, years [mean (SD)]	54 (± 13.4)
BMI, kg/m^2 [mean (SD)]	25.3 (± 4.3)
Function	
Nonfunctioning	22 (73)
Functioning[a]	8 (27)
Incidental diagnosis	
No	21 (70)
Yes	9 (30)
Pain	
No	24 (80)
Yes	6 (20)
Jaundice	
No	28 (93)
Yes	2 (7)
Radiological tumor diameter, mm [mean (SD)]	25 (± 11.7)
^{68}Gallium PET	
Negative	1 (3)
Positive	20 (67)
Not performed	9 (30)
^{18}F-FDG PET	
Negative	6 (20)
Positive	12 (40)
Not performed	12 (40)
Surgical procedure	
Distal pancreatectomy[b]	17 (57)
Pancreaticoduodenectomy	7 (23)
Enucleation	4 (13)
Middle pancreatectomy	1 (3.5)
Total pancreatectomy[b]	1 (3.5)
Postoperative complications[31]	
None	10 (33)
Clavien–Dindo I–II	16 (53.5)
Clavien–Dindo III–IV	3 (10)
Clavien–Dindo V	1 (3.5)

Data are expressed as *n* (%) unless otherwise specified

PanNETs pancreatic neuroendocrine tumors, *BMI* body mass index, *PET* positron emission tomography, *18F-FDG* 18F-fluorodeoxyglucose, *SD* standard deviation

[a] *n* = 7 insulinoma; *n* = 1 ACTHoma

[b] *n* = 1 distal pancreatectomy + liver wedge resection; *n* = 1 total pancreatectomy + liver wedge resection

TABLE 2 Pathological findings of 30 patients with histologically proven pancreatic neuroendocrine tumors

Variable	Patients [*n* = 30]
Tumor location	
Head	11 (37)
Body	10 (33)
Tail	9 (30)
Tumor grade[33]	
PanNET-G1	12 (40)
PanNET-G2	17 (57)
PanNET-G3	1 (3)
Ki67, % [mean (SD)]	6 (± 7.3)
Tumor diameter, mm [mean (SD)]	23 (± 13.5)
T stage[32]	
*T*1	13 (43)
*T*2	11 (37)
*T*3	6 (20)
N stage[32]	
*N*0	15 (50)
*N*1	12 (40)
*N*x	3 (10)
M stage[32]	
*M*0	26 (87)
*M*1	4 (13)
Stage[32]	
I	11 (37)
II	5 (17)
III	10 (33)
IV	4 (13)
Resection margins	
*R*0	26 (87)
*R*1	2 (6.5)
*R*2	2 (6.5)
Microvascular invasion	
No	15 (50)
Yes	15 (50)
Perineural invasion	
No	23 (77)
Yes	7 (23)
Necrosis	
No	27 (90)
Yes	3 (10)

Data are expressed as *n* (%) unless otherwise specified

PanNETs pancreatic neuroendocrine tumors, *SD* standard deviation

proportion of patients with an elevated NETest decreased significantly (Fisher's exact test: $p < 0.001$) from preoperation (30/30) to POD30 (15/30) [Fig. 2]. The majority (12/15) exhibited modestly elevated scores of 27 at POD30; three patients exhibited scores of ≥ 40, i.e. patient 1 (*R*2), 40; patient 2 (*R*1), 93; and patient 3 (*R*0),

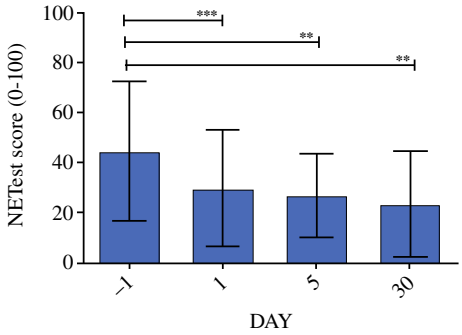

FIG. 1 Relationship of the NETest score to surgical resection. NETest were measured at four different time points: preoperative, POD1, POD5, and POD30. Bars represent the mean (middle line) and standard deviation (top and bottom whiskers). *** represents statistical significance with a p-value ≤ 0.001; ** represents statistical significance with a p value ≤ 0.01. *NETest* neuroendocrine gene transcripts, *POD* postoperative day

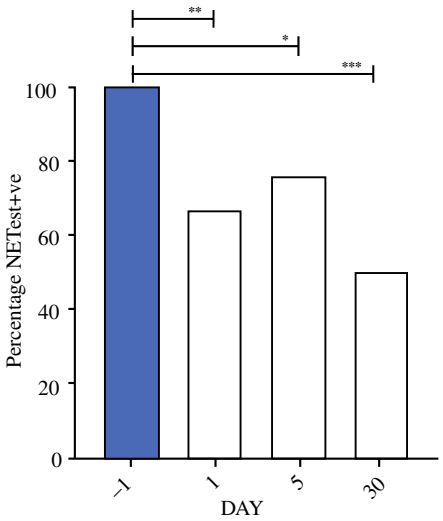

FIG. 2 NETest score changes and surgery. Percentage of patients who were NETest-positive at each of the four different time points: preoperative, POD1, POD5, and POD30. Bars represent the percentage with a positive score. *** represents statistical significance with a p-value ≤ 0.0001; ** represents statistical significance with a p-value ≤ 0.001; * represents statistical significance with a p-value ≤ 0.01. *NETest* neuroendocrine gene transcripts, *POD* postoperative day

80. Patient 3 had a G2 tumor (Ki67 18%) with extensive nodal involvement and the increased postoperative NETest levels were consistent with active residual disease. The highly elevated scores of patients 1 and 2 (40 and 93) were similarly consistent with known residual disease given their *R*1 and *R*2 resections.

A consideration of the group of patients who underwent *R*0 resection provided information of interest. Thus, 13 (50%) patients in this group exhibited a positive NETest score at POD30 [31.2 (SD ± 14.7)]. These blood transcript data are suggestive of residual disease, although surgical and histological assessment confirmed complete resection. This is consistent with previously reported recurrence rates after PanNET surgery.[34]

Regarding preoperative staging, nine patients overall (30%) did not receive ^{68}Gallium PET, six of whom were affected by insulinoma and the remaining three had been diagnosed with ACTHoma ($n = 1$) and nonfunctioning (NF)-PanNETs ($n = 2$). All patients who did not undergo ^{68}Gallium PET ($n = 9$) had an elevated preoperative NETest score [61.5 (SD ± 32)], whereas only four had a mildly elevated NETest score 1 month after surgery [28 (SD ± 3.3)]; all four of these patients were diagnosed as insulinoma. The remaining five patients ($n = 2$ insulinoma, $n = 1$ ACTHoma, $n = 2$ NF-PanNETs) not submitted to preoperative ^{68}Gallium PET had a normal NETest score 1 month after surgery.

CgA: Preoperative CgA levels were elevated in 9 (30%) of the 30 patients. In these patients, CgA plasma levels did not decrease significantly after surgery and were similar between preoperative and postoperative (POD1, POD5, and POD30) measurements {preoperative: 184 ng/mL (SD ± 360 ng/mL) vs. POD1: 93 ng/mL (SD ± 87.5 ng/mL) [$p = 0.054$], vs. POD5: 170 ng/mL (SD ± 335.3 ng/mL) [$p = 0.836$], vs. POD30: 260 ng/mL (SD ± 589 ng/mL) [$p = 0.398$]} (Fig. 3). The proportion of patients with an elevated CgA did not differ ($p = 1.000$) preoperatively to POD30 (9/30) [Fig. 4]. Separately, no differences were noted in CgA decrease between patients submitted to a radical resection (*R*0–*R*1) and those who underwent debulking surgery (*R*2). In the *R*0 group, seven patients (27%) had elevated CgA measurements at POD30 [223 ng/mL (SD ± 117 ng/mL)].

Linear Regression Analyses: By univariate linear regression analysis, the only factor significantly correlated with NETest was the presence of a functioning tumor

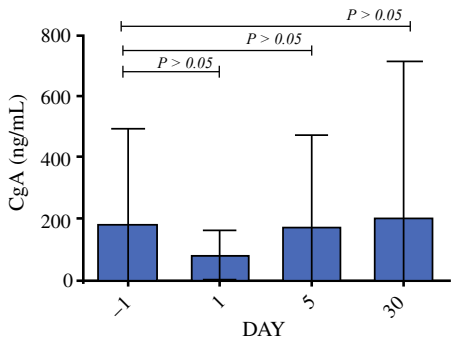

FIG. 3 Relationship of CgA to surgical resection. CgA was measured at four different time points: preoperative, POD1, POD5, and POD30. Bars represent the mean (middle line) and standard deviation (top and bottom whiskers). *** represents the statistical significance with a p-value ≤ 0.001; ** represents the statistical significance with a p-value ≤ 0.01. *CgA* chromogranin A, *POD* postoperative day

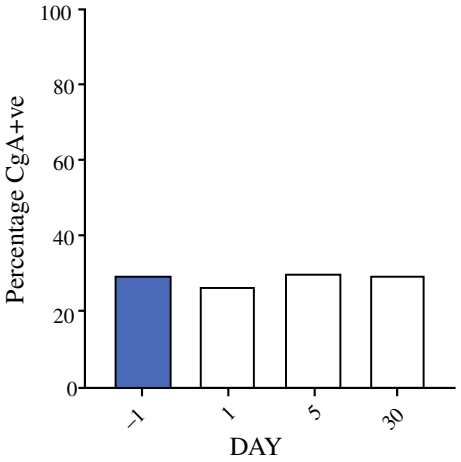

FIG. 4 CgA changes and surgery. Percentage of patients who were CgA-positive (i.e. elevated/abnormal level) at each of the four different time points: preoperative, POD1, POD5, and POD30. Bars represent the percentage with a positive score. No significance was identified. *CgA* chromogranin A, *POD* postoperative

(B = 27.727, 95% confidence interval [CI] 6.289–49.165, p = 0.013) [Table 3]. Study population only included four patients with M1 tumors, making it unlikely that a correlation would be identified. The NETest was however significantly higher in the presence of a functioning tumor {65-fold (SD ± 31) vs. 37-fold (SD ± 23) [p = 0.022]}.

Using univariate linear regression analysis, the only factor significantly correlated with NETest change (preoperative–1 month after surgery) was tumor function (B = 37.460, 95% CI 3.488–71.433, p = 0.032) as assessed by analysis of the molecular secretome levels. CgA plasma levels were similar between patients who had either functioning [70 ng/mL (SD ± 55 ng/mL)] or non-functioning [225 ng/mL (SD ± 351 ng/mL)] neoplasms.

TABLE 3 Univariate linear regression analysis on the effect of demographic, clinical, and pathological variables on preoperative NETest

Variable	Univariate		
	B-value	95% CI	p-Value
Sex			
Male	1	–	0.495
Female	7.368	−14.447 to 29.183	
Age	−0.093	−0.895 to 0.710	0.815
Function			
No	1	–	**0.013**
Yes	27.727	6.289–49.165	
PanNET largest diameter	−0.321	−1.112 to 0.470	0.412
Ki67	−0.510	−1.922 to 0.901	0.458
N stage[32]			
*N*0	1	–	0.639
*N*1	5.111	−17.061 to 27.283	
M stage[32]			
*M*0	1	–	0.466
*M*1	−11.154	−42.042 to 19.734	
Perineural invasion			
No	1	–	0.486
Yes	−8.571	−33.418 to 16.275	
Microvascular invasion			
No	1	–	0.416
Yes	−8.444	−29.395 to 12.506	
Necrosis			
No	1	–	0.144
Yes	−24.938	−58.934 to 9.058	

PanNET pancreatic neuroendocrine tumor, *NETest* neuroendocrine gene transcripts, *CI* confidence interval

DISCUSSION

It is evident that resection of PanNETs is the only potentially curative treatment for the disease. Recurrence of disease post-resection is, like with many other cancers, a key issue determining outcome.[35] Strategies for the early postoperative detection of residual or recurrent disease are confined to its identification by imaging or altered levels of biomarkers specific for disease.[1,2] Anatomical imaging has a limited discriminant index as a result of postoperative healing and fibrosis. Similarly, functional imaging (somatostatin scintigraphy) is ineffective after surgery due to the cells of the inflammatory process expressing somatostatin receptors.[36] When specific blood biomarkers are available (gastrin, insulin, glucagon, or vasoactive intestinal peptide), a rise or fall in their blood values is highly effective in defining cure or residual disease.[1] However, such NETs represent < 5% of PanNETs and therefore there is a need for a universal NET blood biomarker to identify the presence of such tumors.

Emerging precision medicine strategies have drawn attention to the utilization of molecular tools such as noninvasive liquid biopsies (circulating biomarkers) to facilitate and optimize cancer management.[37] Current biomarkers for NET disease are monoanalyte amines (serotonin, histamine) or peptides (gastrin, insulin, CgA) measuring secretory activity only. Recently, a molecular NET transcriptomic analysis (NETest) has been proposed as an NET liquid biopsy.[16] The NETest is a multianalyte transcript-based biomarker providing a signature whose individual 'omic' clusters (proliferome, metabolome, signalome, etc.) reflect the diverse molecular biological aspects of the tumor that defines clinical disease. Thus, the NETest, as opposed to CgA or other monoanalyte peptides/

hormones, is a multianalyte molecular signature representing biological information pertinent to clinical neuroendocrine disease course. It has numerous documented applications, including diagnosis, identification of residual disease post-surgery, disease status identification, and assessment of treatment efficacy.[38] The NETest has been shown to correlate with disease positivity on imaging.[18] In addition, a positive NETest result can also precede the standard imaging detection of the disease by 1–2 years.[30] Independent validation of this NET liquid biopsy in the assessment of pancreatic surgical resection is required. In previous studies in small bowel and lung NET, the NETest has been reported to be 97% accurate, 99% sensitive, and 95% specific in NET diagnosis. These metrics meet and exceed the National Institutes of Health (NIH) proposed criteria of an optimal diagnostic biomarker.[13] We therefore undertook to validate the NETest as a diagnostic and disease status identification marker in PanNETs.

The current default biomarker, CgA, has been considered to have limited, if any, clinical value in the assessment of the efficacy of surgical resection.[13] In this study, CgA did not correlate with disease status (positive in less than 1/3 patients). After resection, CgA values did not differ significantly and the biomarker provided no information that was clinically relevant in assessing the efficacy of the surgical resection undertaken. In contrast, the NETest correlated with disease status (positive in 100%) and after resection significantly decreased, which is consistent with the primary tumor as the source of the gene transcripts.

A previous study regarding the use of the NETest in GEP-NET in individuals undergoing small bowel, hepatic, and pancreatic NET surgery demonstrated that surgical resection decreased NETest levels in blood.[26] It also demonstrated that the failure of NETest blood levels to return to normal was associated with imaging evidence of residual/recurrent disease. The elevations of the NETest were evident prior to imaging evidence of disease, becoming apparent over time periods that ranged from 3 months to 2 years.[26] In some instances, evidence of NET recurrence was evident on histological needle biopsy when repeated imaging (all modalities) failed to identify disease, despite repeated elevations of the NETest.[39]

In our study, the resection of PanNETs was associated with a significant decrease in the NETest in the majority (27/30) of patients. In 15 patients, NETest levels returned to normal. Among the remaining 15 patients, 12 exhibited a mean NETest level of 27 after resection (POD30), which is consistent with the presence of residual disease. However, three patients exhibited levels of > 40 at POD30. Of these three patients, one had a PanNET with both nodal and liver metastases and an *R*2 resection was performed. This patient was then submitted to an adjuvant treatment with PRRT. The second individual had previously been treated with neoadjuvant PRRT and then underwent an *R*1 resection. The staging of this patient was NET G1 T1N0 at final histology. The third patient had a G2 tumor (Ki67 18%) with extensive nodal involvement. It is very likely that these patients either have residual disease or will develop recurrent disease with the passage of time. Since two of the three were *R*1 and *R*2 resections, respectively, it can be assumed that the NETest has correctly identified known residual disease.

The issue of an elevated NETest in the presence of image-negative disease needs to be considered. It has been previously reported that the NETest identifies disease 12–18 months before image-positive evidence of disease is present.[30] A separate study conducted in 111 patients identified that elevated NETest levels exhibited a 96% concordance with imaging.[22] A consideration of the sensitivity of the blood transcript analysis compared with imaging suggests that elevated levels of NET transcripts post-surgery at POD30 most likely represent identification of early disease that is too small to be identified by current imaging techniques. This information is supported by the reports of histologic-positive but image-negative disease in 50% of a cohort of patients ($n = 11$) with hepatic metastases evaluated by imaging and histopathology.[40]

The molecular genomic data in this investigation should be considered as consistent with clinical reports that note an approximately 50% recurrence rate after radical pancreatic surgery.[1,34,41] Overall, our work indicates that measurements of NET transcript levels in blood identify and will facilitate early detection of residual disease.[26] We believe that the window of opportunity to manage and, if necessary, treat low burden disease can be substantially optimized by early identification after surgery of molecular evidence of microscopic residual disease. Consideration should be given to rigorous follow-up of this 'at-risk' group with appropriate diagnostic resources. Using this strategy, it will be possible to assess whether initiating intervention at an early stage prior to the recognition of image-positive disease may be of benefit. While NETs may require less aggressive therapeutic intercession, and a watch-and-wait program with the NETest may be clinically effective, it is well-recognized that pancreatic NETs exhibit the highest malignancy, and similar early intervention strategies have been employed advantageously in the treatment of breast, lung, and colon cancer.[42–44]

Overall, data provide evidence that a blood-based multigene biomarker is accurate and provides information that is concordant with the operative assessment of the efficacy of surgical resection. However, a prospective large study is needed to precisely define how effective the

NETest alone is in identifying recurrence or progression after pancreatic surgery and how much earlier it can detect alteration in disease status.

A point of interest is that the alterations in the levels of the NETest do not necessarily correlate with the extent of disease resected when examined as an aggregate. This reflects the fact that the test measures a number of different 'omic indices' in each tumor. Specific tumors have different levels of 'omic clusters', as would be predicted from the unique nature of an individual tumor. Thus, in this series, it was noted that functional tumors overall had a higher NETest level than nonfunctional lesions. Further analysis of the genes involved in regulating secretion (the 'secretome') in this group of tumors demonstrated unequivocally that the expression levels of these genes were significantly elevated in the functional group [summated expression: 175-fold (SD $\pm$ 64) versus non-NET blood] compared with the nonfunctional group [47-fold (SD $\pm$ 23), $p = 0.012$]. Such molecular information is consistent with the known discrepancy between the size of a tumor and its individual unique malignant propensity or biological behavior. The latter are specific characteristics of the functional molecular biology of each tumor rather than its size.[45] Size, for the most part, simply represents the time point at which a physician encounters a particular tumor. Thus, at some time point in the evolution of any tumor, it must have been < 1 cm, suggesting that the omic characteristics that define tumor biology are probably more relevant than tumor size.

The study only examined the 30-day period after surgery and requires extension to define at what point elevated NETest levels will be identified by imaging as representing disease recurrence. Nevertheless, the data clearly indicate that resection of PanNET disease is followed by a decrease in NETest blood levels. This demonstrates that elevated blood values are consistent with the presence of NET disease. A similar outcome has been reported in the evaluation of bronchopulmonary NET disease (BPNET).[27] In this study, resection of a tumor was demonstrated to clearly correlate with a decrease in NETest blood levels. Failure to undertake a complete resection or disease recurrence was identifiable by an increase in blood NETest levels.[27]

CONCLUSIONS

Our study demonstrates that the NETest is independently validated as an accurate diagnostic biomarker for PanNETs. These results are concordant with effective surgical resection and provide a good assessment of initial postoperative disease status. It seems likely that early detection of residual disease or recurrence will facilitate the early introduction of effective therapy and thereby increase the likelihood of effectively treating disease when it is at a lower burden. A multianalyte gene blood test and imaging may therefore be used as clinical tools that can provide adjunctive information able to facilitate the management of PanNET disease during postoperative monitoring. Given the concordance of blood transcript levels and disease presence, it seems reasonable to consider that in the post-surgery follow-up, where imaging is often difficult to interpret, a blood-based assessment of disease may be easier, more accurate, and more effective in the early identification of residual disease.

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