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The utility of blood neuroendocrine gene transcript measurement in the diagnosis of bronchopulmonary neuroendocrine tumours and as a tool to evaluate surgical resection and disease progression[†]

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Abstract

OBJECTIVES: The management of bronchopulmonary neuroendocrine tumours (BPNETs) is difficult, since imaging, histology and biomarkers have a limited value in diagnosis, predicting outcome and defining therapeutic efficacy. We evaluated a NET multigene blood test (NETest) to diagnose BPNETs, assess disease status and evaluate surgical resection.

METHODS: (i) Diagnostic cohort: BP carcinoids ($n = 118$)—typical carcinoid, $n = 67$ and atypical carcinoid, $n = 51$; other lung NEN (large-cell neuroendocrine carcinoma and small-cell lung carcinoma, $n = 13$); adenocarcinoma, ($n = 26$); squamous cell carcinoma ($n = 23$); controls ($n = 90$) and chronic obstructive pulmonary disease ($n = 18$). (ii) Surgical cohort, $n = 28$: BP carcinoids ($n = 16$: typical carcinoid 12; atypical carcinoid 4); large-cell neuroendocrine carcinoma, $n = 3$; lung adenocarcinoma, $n = 8$ and squamous cell carcinoma, $n = 1$. Blood sampling was performed presurgery and 30 days post-surgery. Transcript levels measured by quantitative polymerase chain reaction were calculated as activity scores (0–100% scale: normal < 14%) and compared with chromogranin A (enzyme-linked immunosorbent assay; normal < 109 ng/ml).

RESULTS: NETest was significantly elevated in carcinoids ($48.7 \pm 27\%$) versus controls ($6 \pm 6\%$, $P < 0.001$) with metrics: sensitivity 93%, specificity 89%, positive predictive value 92% and negative predictive value 91%. NETest differentiated progressive disease ($73 \pm 22\%$) from stable disease ($36 \pm 19\%$, $P < 0.001$) and R0 resections ($10 \pm 5\%$, $P < 0.001$, area under the curve: 0.98). Levels in chronic obstructive pulmonary disease and lung cancers were 18–24% while elevated in small-cell lung carcinoma/large-cell neuroendocrine carcinoma ($59 \pm 10\%$). In BPNETs on postoperative Day 30, NETest decreased by 60% ($P < 0.001$). Chromogranin A was elevated in only 40% of carcinoids and not altered by surgery.

CONCLUSIONS: Blood NET gene levels accurately identified BPNETs (100%) and differentiated these from controls, benign and malignant lung disease. Progressive disease could be identified and surgical resection verified. Chromogranin A had no clinical utility. Monitoring NET transcript levels in blood will facilitate management by detecting residual tumour and identifying progressive disease.

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INTRODUCTION

Bronchopulmonary neuroendocrine tumours (BPNETs) present with protean pulmonary symptomatology, and diagnosis is frequently long delayed. In many circumstances, it is serendipitous based upon chest imaging and identification of an unsuspected lesion [1]. A minority (3–5%) presents with hormonally related symptoms such as carcinoid syndrome, Cushing's syndrome, acromegaly or inappropriate antidiuretic hormone secretion. Consequently, minimal clinical information is available to facilitate a specific diagnosis [2]. Neuroendocrine lesions of the lung comprise a spectrum of tumours that represent approximately 25% of all lung neoplasia [1]. No effective biomarkers exist and, although diagnosis is facilitated by anatomical imaging modalities, they rarely indicate that a mass lesion is neuroendocrine in origin [2]. Molecular imaging [somatostatin receptor (SSR) scintigraphy or ^{68}Ga -labelled somatostatin analogue (SSA) positron emission tomography (PET)/computed tomography (CT)] that detects SSR expression complements anatomical findings and evaluation of disease extent. In addition, SSR scintigraphy can constitute the basis for indicating radiolabelled SSA treatment (theranostic concept) [2]. Specifically, in BPNETs, molecular imaging may be helpful in the identification of the aetiology of bronchial masses [2]. Since most BPNETs express SSR, especially subtype 2, ^{68}Ga -DOTA-SSA-PET/CT can detect bronchial carcinoids >6 mm in diameter [3]. Simultaneously, it allows a whole-body assessment of the extent of disease, which may have an impact on the subsequent therapeutic decision. In the post-surgical or metastatic setting, ^{68}Ga -DOTA-SSA-PET/CT complements anatomical follow-up imaging with high sensitivity. ^{18}F -Fluorodeoxyglucose-PET/CT in BPNETs shows variable increase in glycolytic metabolism dependent upon the proliferative activity of individual lesions and typical and atypical carcinoids show variable uptake. Large-cell neuroendocrine carcinoma (LCNEC) and small-cell lung carcinoma (SCLC) are usually intensely metabolically active, and ^{18}F -fluorodeoxyglucose is more effective in their monitoring than ^{68}Ga -DOTA-SSA-PET/CT [4]. For practical purposes, bronchoscopy, biopsy and surgery represent the definitive management strategy [5].

Management of a BPNET is based upon surgical resection. Thereafter, the clinical course of BPNETs may be unpredictable since histopathology has limitations in defining biological behaviour, and no effective predictive or prognostic histological or blood biomarkers exist [6]. The accurate delineation of BPNETs is sometimes difficult, despite a histological classification into 4 subgroups—typical carcinoid, atypical carcinoid LCNEC and SCLC. In many instances, it may therefore be challenging to accurately predict the behaviour of an individual tumour [7].

To date, circulating biomarkers to diagnose and monitor BPNETS have been characterized by their paucity and dearth of clinical utility. A NET Biomarker Consensus Conference concluded that the currently used gastroenteropancreatic (GEP) NET biomarkers, e.g. chromogranin A (CgA), were neither sensitive nor specific for BPNETS and that imaging, histology and biochemistry were of limited value in identifying disease progression and, in some cases, in defining BPNET malignancy or differentiating BPNETS from other lung neoplasia [6]. These is a critical unmet need for the development of a non-invasive tool that can

accurately identify BPNETS, delineate the effectiveness of surgical intervention and subsequently predict disease progression.

The evolution of strategies to evaluate circulating molecular information emanating from neoplasia has advanced to the point that blood sampling can provide considerable oncological information [8, 9]. Such strategies or 'liquid biopsies' have proved to be effective in lung neoplasia, e.g. for monitoring treatment responses to EGFR inhibitors through the identification of mutation T790M in circulating tumour DNA [10]. In this respect, a circulating neoplastic molecular signature is clinically useful in avoiding invasive biopsies, defining therapeutic targets and providing a real-time monitoring tool to evaluate disease status [11].

A multianalyte molecular assay (51 transcripts) to identify NET disease in blood has been reported to have clinical utility in the management of gastrointestinal NET disease [12, 13]. Clinical studies have demonstrated the efficacy of NET-specific transcripts to accurately (~95%) diagnose pancreatic and gastrointestinal tract NETs and that transcript levels can identify post-surgery residual disease, define disease progression and predict treatment efficacy [SSA and peptide receptor radionuclide therapy (PRRT)] [14, 15]. More recently, the NET-specific genes have been detected in BPNET transcriptomes and have been identified in neuroendocrine lung cell lines. Furthermore, tissue levels and matched blood samples exhibited good correlation [16].

In this study, we examined the diagnostic utility of a NET-specific gene assay for BP neuroendocrine neoplasia (NEN) in blood (Fig. 1). We address whether blood levels correlate with clinical status (disease aggression) and whether the signature differs from other lung pathologies. Finally, we evaluate whether the completeness of surgical resection correlates with a decrease in blood transcript values.

SUBJECTS AND METHODS

Patients

All patients provided informed consent for the blood translational analysis authorized by local ethics committees. Whole blood [10 ml; messenger RNA (mRNA)] and plasma (CgA) were collected. For the prospective surgical group, samples were collected at baseline and on Postoperative Day 30 (POD30). Structural imaging (CT/magnetic resonance imaging) evaluated disease status (RECIST 1.0).

Diagnostic cohort (n = 288). This multicentre retrospective cohort was recruited between June 2013 and March 2017. It included patients and non-affected family members visiting oncology, endocrinology and pulmonology outpatient clinics. The inclusion criterion was the histological confirmation of disease. No exclusion criteria were used. The group constituted healthy controls (n = 90); chronic obstructive pulmonary disease (COPD n = 18); and neoplastic lung disease, neuroendocrine neoplasia and other cancers. The neuroendocrine neoplasia cohort included 118 'carcinoids' and 13 other neuroendocrine neoplasms (LCNEC: n = 9; SCLC: n = 4). Other lung cancers included squamous cell carcinoma (SCC: n = 23) and adenocarcinoma (AdenoCa: n = 26) (Table 1). The majority of carcinoids were

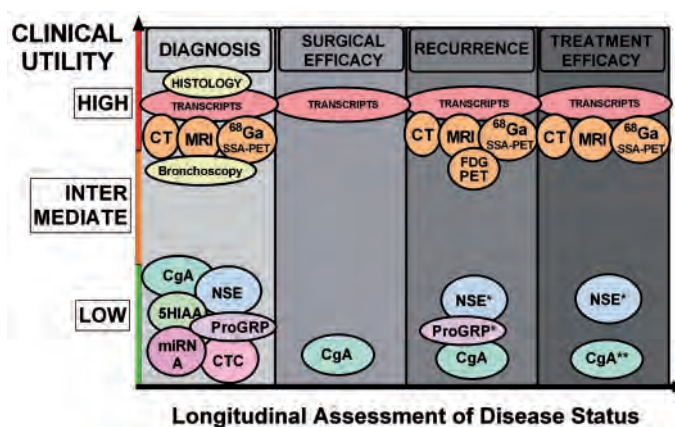


Figure 1: The utility of current clinical tools in the assessment of bronchopulmonary neuroendocrine tumour disease status. Current biomarkers used in bronchopulmonary neuroendocrine tumours management are single analytes and have a low utility, e.g. CgA or NSE. Histology and imaging have a high clinical utility. Imaging is especially useful in assessing disease evolution over time. Monoanalytes have minimal utility in assessing surgical residual disease or recurrence and limited benefit, except NSE in small-cell lung carcinoma. Neuroendocrine circulating transcripts measurement is effective in all 4 stages of disease management. *Only in small-cell lung carcinoma; **No prospective studies. 5HIAA: 5-hydroxyindole acetic acid; CgA: chromogranin A; CT: computed tomography; CTC: circulating tumor cell; FDG: fluorodeoxyglucose; MRI: magnetic resonance imaging; NSE: neuron-specific enolase; ProGRP: pro-gastrin releasing peptide; PET: positron emission tomography; SSA: somatostatin analogue.

Table 1: Demographics of Set I: diagnostic cohort

	Mean age (years) (range/SD)	Gender (M:F)	MEN-1 status, n (%)	Metastases, n (%)
Bronchopulmonary carcinoids (n = 118)	58.2 (21–79/12.3)	45:73	2 (2)	50 (42)
Other lung neuroendocrine neoplasia (n = 13)	63 (47–73/9.8)	7:6	0 (0)	ND
Lung cancers (n = 49)	64 (41–74/7.5)	32:17	0 (0)	45 (92)
COPD (n = 18)	61.5 (22–83/13.5)	6:12	ND	
Controls (n = 90)	44 (20–80/17.4)	58:32	ND	

Bronchopulmonary carcinoids: atypical, n = 51; typical, n = 67; disease free, n = 6; stable, n = 74 and progressive, n = 38. Other neuroendocrine neoplasia: LCNEC, n = 9 and SCLC, n = 4. Lung cancers: adenocarcinomas (n = 28) and squamous cell carcinoma (n = 24). COPD: chronic obstructive pulmonary disease; F: female; M: male; MEN-1: multiple endocrine neoplasia Type 1; ND: no data; SD: standard deviation.

stable (n = 74; 63%) at blood draw; 38 (32%) had progressive disease (PD) and 6 (5%) were surgical 'cures' (disease free). The majority (>95%) of AdenoCa and SCC had disseminated disease.

Surgical cohort (n = 28). Blood samples were prospectively collected (March 2016–March 2017) from a single institution (Turin). Samples included carcinoids (n = 16: typical carcinoid 12 and atypical carcinoid 4), LCNEC (n = 3), lung AdenoCa (n = 8) and SCC (n = 1). Inclusion criteria included histological confirmation of disease. Sample size for this ongoing study was based on differences in mean NET multigene blood test (NETest). Target is 25 carcinoids and 15 neoplasia (power = 80%, $\alpha = 5\%$). Patient demographics are listed in Table 2. All patients underwent surgical resection and systematic lymphadenectomy. Eighteen of 19 (95%) neuroendocrine surgeries were R0, and 8 of 9 (89%) surgeries for non-NE lung cancers were R0. Each group had 1 patient who underwent R1 surgery.

Biochemical assays

Normal NETest ($6 \pm 5.6\%$) and CgA (58 ± 20 ng/ml) have previously been published in a healthy control cohort [12].

Neuroendocrine tumour multigene blood test. A 2-step protocol [RNA isolation, complementary DNA and polymerase chain reaction (PCR)] was used [12]. Transcripts (mRNA) were

isolated from ethylenediaminetetraacetic acid-collected whole blood samples (mini blood kit, Qiagen, Valencia, CA, USA), and real-time PCR was performed [12] (Fast Universal PCR Master Mix, Life Technologies). PCR values were normalized to house-keeping genes, and expression was quantified against a population control [12]. Expression levels were converted to an activity score ranging from 0 (low activity) to 100% (high activity) [13]. The upper limit of normal: 14%.

Chromogranin A enzyme-linked immunosorbent assay.

CgA was measured using NEOLISA™ CgA kits (Euro Diagnostics, Malmo, Sweden) [17, 18]. A cut-off of 108 ng/ml defined the upper limit of normal.

Statistical analysis

Intergroup analyses were undertaken using 2-tailed non-parametric tests (Mann-Whitney U-test or Wilcoxon-signed rank test for paired samples). Receiver operating characteristic curve analysis was used to determine the diagnostic accuracy of each biomarker. Area under the receiver operator curve (AUROC) comparison and derivation of the Z-statistic [19] were derived from data in the same patients. Decision curve analysis [20] was used to directly compare clinical benefits of NETest and CgA. Prism 6.0 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and MedCalc

Table 2: Demographics of Set II: prospective, surgical cohort

	Lung neuroendocrine neoplasia (n = 19) ^a	Lung cancers ^b (n = 9)
Age (mean/range)	62 (34–82)	72 (63–82)
Gender (M:F)	8:11	7:2
MEN-1 status, n (%)	2 (11)	0 (0)
pTNM, n (%)	pT1aN0M0: 4 (21) pT1aN2M0: 1 (5) pT1bN0M0: 5 (26) pT1bN1M0: 1 (5) pT1cN0M0: 2 (11) pT1N1M0: 0 (0) pT1cN2M0: 0 (0) pT2aN0M0: 5 (26) pT2aN1M0: 0 (0) pT2aN2M0: 0 (0) pT3N1M0: 0 (0) pT3N2M0: 1 (5) pT4N0M0: 0 (0)	pT1aN0M0: 0 (0) pT1aN2M0: 0 (0) pT1bN0M0: 0 (0) pT1bN1M0: 0 (0) pT1cN0M0: 2 (22) pT1N1M0: 0 (0) pT1cN2M0: 1 (11) pT2aN0M0: 1 (11) pT2aN1M0: 2 (22) pT2aN2M0: 1 (11) pT3N1M0: 1 (11) pT3N2M0: 0 (0) pT4N0M0: 1 (11)
Lymph node involvement, n (%)	3 (16)	5 (56)
Surgery, n (%)		
Right	RP: 2 (11) RUL: 3 (16) RLL: 2 (11)	RP: 0 (0) RUL: 3 (33) RLL: 2 (22)
Left	LP: 2 (11) LUL: 3 (16) LLL: 4 (21)	LP: 1 (11) LUL: 0 (0) LLL: 1 (11)
Other	Middle lobectomy: 1 (5) Inferior bilobectomy: 1 (5) Segmentectomy: 1 (5)	Middle lobectomy: 1 (11) Inferior bilobectomy: 1 (11)

No significant differences were noted between the 2 surgical groups with respect to gender, but the neuroendocrine group was significantly younger ($P < 0.04$, 2-tailed Mann-Whitney test).

^aSixteen carcinoids (12 typical carcinoid and 4 atypical carcinoid) and 3 large-cell neuroendocrine carcinoma (LCNEC).

^bAdenocarcinoma, $n = 8$ and squamous cell carcinoma, $n = 1$.

F: female; M: male; LLL: left lower lobectomy; LP: left pneumonectomy (1 LCNEC, 1 atypical carcinoid and 1 recurrent adenocarcinoma); LUL: left upper lobectomy; MEN-1: multiple endocrine neoplasia Type 1; RLL: right lower lobectomy; RP: right pneumonectomy (in LCNEC); RUL: right upper lobectomy.

Statistical Software version 16.2.1 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2017) were utilized. Data were presented as mean \pm standard deviation [median:(interquartile ranges)].

RESULTS

Diagnostic markers: bronchopulmonary neuroendocrine tumour and controls (Set I)

Neuroendocrine tumour multigene blood test versus chromogranin A as a diagnostic for bronchopulmonary carcinoids. The NETest was positive in all BP carcinoids (100%) and its levels were significantly elevated [48.7 \pm 27.4% (33%:(27–80))] compared with the controls [5.7 \pm 6.1% (6%:(0–7)), $P < 0.001$] (Fig. 2A). The AUROC for differentiating carcinoids from controls

was 0.98 [95% confidence interval (CI) 0.96–1.00] (Fig. 2B). CgA was elevated in 44 of 118 (37%) carcinoids. Levels were 887 \pm 247 ng/ml [85:(49–380)] vs 58 \pm 30 ng/ml [54:(31–87)] in the controls ($P < 0.001$, Fig. 2C). The AUROC was 0.68 (95% CI 0.61–0.76) (Fig. 2D). Comparison of AUROCs identified that the NETest was better than CgA (difference between areas: 0.29 \pm 0.04; Z-statistic: 7.6, $P < 0.001$). Evaluation of diagnostic metrics for each biomarker demonstrated the NETest exhibited >88% for all parameters. CgA had low sensitivity (36%) and negative predictive values (55%).

Clinical value of the neuroendocrine tumour multigene blood test as a diagnostic (Set I).

Decision curve analysis quantified the clinical benefit of the NETest (Fig. 3). The NETest exhibited >80% standardized net benefit up to a risk threshold of 90%. The clinical benefit of CgA was only 20% across comparable risk thresholds.

Diagnostic markers: identifying disease status (Set I).

The NETest was significantly elevated [73 \pm 22% (80%:(58–89))] in PD compared with stable disease [SD, 36 \pm 19%, (33%:(27–33)); $P < 0.001$]. Surgically 'cured', disease-free patients [10 \pm 5% (13%:(5–13))] were the same as controls [$P < 0.001$] (Fig. 4A). The AUROC for differentiating BP pathology (benign or neoplastic) from 'cured' (disease free) was 0.99 (95% CI 0.97–1.00) ($P < 0.001$) and for PD from SD was 0.91 (95% CI 0.87–0.96) ($P < 0.001$; Fig. 4B). CgA levels were higher in SD [966 \pm 350 ng/ml (82:(43–379))] than PD [548 \pm 171 ng/ml (88:(55–369))] but was not statistically different from disease-free patients [55 \pm 24 ng/ml (48:(41–72)), Fig. 4C]. The AUROC for differentiating BP neuroendocrine disease from 'cured' was 0.71 (95% CI 0.58–0.85) ($P = 0.08$) and for PD from SD was 0.52 (95% CI 0.40–0.64) ($P = 0.75$; Fig. 4D). The NETest was significantly more effective at distinguishing 'cures' (difference between areas: 0.27 \pm 0.07; Z-statistic: 3.8, $P < 0.001$) and defining PD (difference between areas: 0.39 \pm 0.07; Z-statistic: 5.4, $P < 0.001$) than CgA.

Evaluation of the neuroendocrine tumour multigene blood test and chromogranin A in other lung pathology (Set I).

We next evaluated COPD, AdenoCa, SCC and SCLCs and LCNEC neoplasia. The NETest was significantly elevated [58.5 \pm 10% (80%:(17–84))] in neuroendocrine neoplasia (LCNEC/SCLC) compared with COPD [23 \pm 4% (23:(23–26)); $P < 0.05$], AdenoCa [19 \pm 3%, (18%:(0–33)); $P < 0.001$] and SCC [18 \pm 4%, (16%:(0–33)); $P < 0.01$] (Fig. 5A). CgA levels in neuroendocrine neoplasia [867 \pm 683 ng/ml (76:(45–383))] were not significantly different from COPD [150 \pm 158 (89:(52–261))], AdenoCa [68 \pm 38 (51:(43–83))] and SCC [63 \pm 40 (47:(38–78)), Fig. 5B].

Utility of biomarkers in surgical resection of bronchopulmonary neuroendocrine tumours (Set II).

The NETest and CgA were prospectively evaluated in patients with lung neuroendocrine neoplasia ($n = 19$) and lung cancers ($n = 9$) who underwent surgery. NETest levels were elevated in this cohort of lung neuroendocrine neoplasia (69 \pm 28%) compared with Set I (50 \pm 28%, $P < 0.01$). This reflects the increased number of PD patients (100%) compared with Set I (39%). No differences were noted for lung cancers or CgA in the 2 sets. In lung neuroendocrine neoplasia, the NETest was significantly reduced from 69 \pm 28% [70%:(30–98)] to 29 \pm 9% [27%:(27–33)] ($P < 0.001$, Fig. 6A) on POD30. This represents an average decrease of 59%. Surgery had no effect on NETest scores in lung cancers (Fig. 6B). CgA was not significantly affected by surgery [174 \pm 63 ng/ml

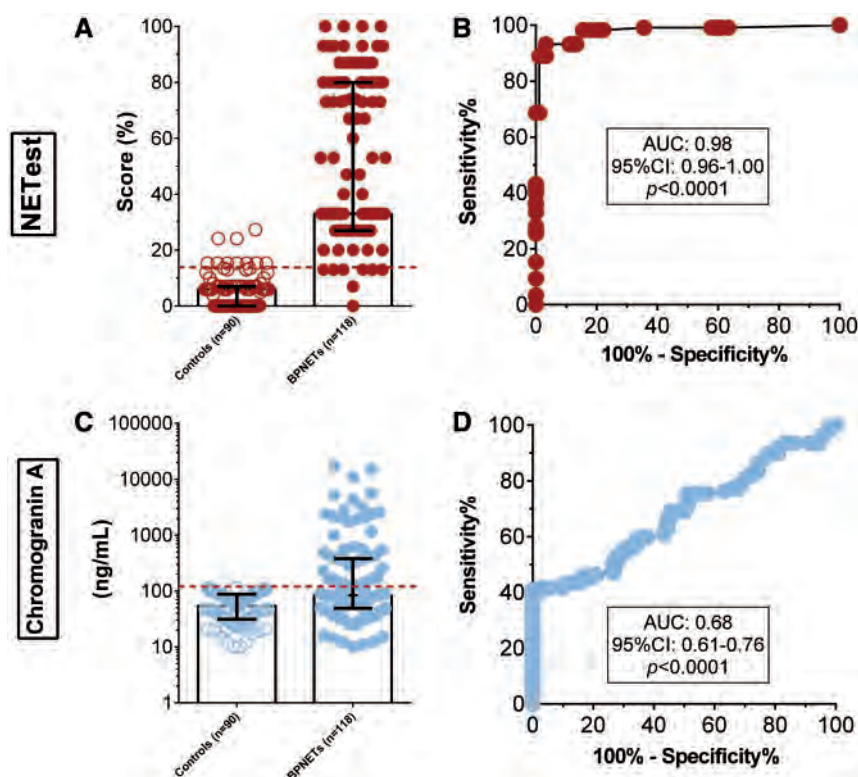


Figure 2: Biomarker values in carcinoids (BPNETs) and controls (Set I). NETest: (A) NETest scores were significantly elevated ($P < 0.001$) in BPNETs ($n = 118$) compared with the controls ($n = 90$). (B) The AUROC for differentiating controls from BPNETs was 0.98 (0.96–1.00), $P < 0.001$. Chromogranin A (CgA): (C) CgA levels were only elevated in 37% of carcinoids. In this group, they were statistically higher ($P < 0.001$) than controls. (D) The AUROC for CgA to differentiate controls from BPNETs was 0.68 (0.61–0.76), $P < 0.001$. Median and interquartile ranges are indicated in black. AUC: area under the curve; AUROC: area under the receiver operator curve; BPNETs: bronchopulmonary neuroendocrine tumours; CI: confidence interval; NETest: neuroendocrine tumour multigene blood test.

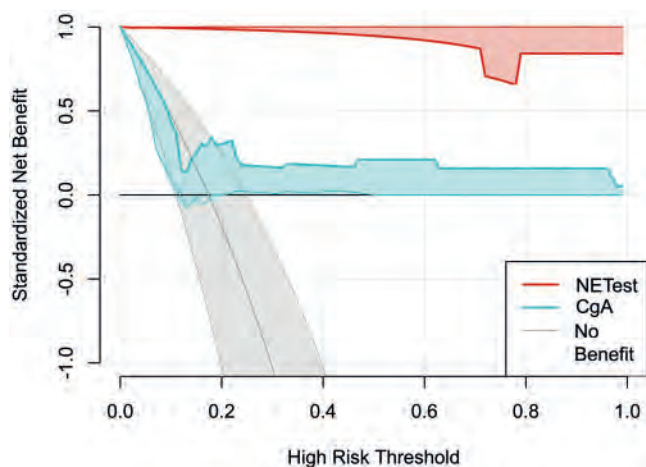


Figure 3: Decision curve analysis comparing the efficacy of NETest and CgA as diagnostic markers of bronchopulmonary neuroendocrine tumours (Set I). The standardized NET benefit line (y axis) reflects the clinical usefulness of a biomarker, 1.0 reflects 100% useful, 0.5 is 50% useful and negative values reflect 'harm' (negative benefit) related to an intervention based on a biomarker. The x axis demonstrates the probability (risk) of disease. When a biomarker is not used for intervention, the standardized NET benefit = 1.0 and the risk threshold is 0 (none). The grey line (labelled as No Benefit) reflects the overall benefit of introducing an intervention to all individuals irrespective of biomarker value. In the diagnostic cohort of 90 controls and 118 carcinoids, the clinical benefit for the NETest (red line) is $> 80\%$ up to a disease risk threshold of 0.99. This indicates that the NETest has significant diagnostic benefit in bronchopulmonary carcinoids. In contrast, CgA expression levels (blue line) do not display any clinical benefit. Quantitatively, $< 20\%$ of CgA defined patients benefit at a risk threshold < 0.95 . CgA: chromogranin A; NET: neuroendocrine tumour; NETest: neuroendocrine tumour multigene blood test.

(58:(47–108)) vs 140 ± 52 ng/ml (46:(38–63)), Fig. 6C]. In lung cancers, CgA, however, increased from 82 ± 36 ng/ml [79:(53–112)] preoperatively to 176 ± 142 ng/ml [110:(70–344)] (Fig. 6D).

DISCUSSION

The management of BP carcinoids can be problematic since histological differentiation between typical and atypical BPNETs can be difficult [7], especially in small biopsies or cytology [2]. Tumours evolve with time, develop clonal heterogeneity [21], locally recur and metastasize; hence, measuring the proliferative index (Ki67) is not as effective as in GEPNETs [2]. Furthermore, anatomical/structural imaging cannot prognosticate tumour behaviour, and molecular imaging is not always available. The latter strategies (18F-fluorodeoxyglucose-PET and ^{68}Ga -SSA PET/CT) have limitations in the individual prediction and prognostication of tumour behaviour. Blood biomarkers such as CgA and pancreastatin have been considered as ineffective clinical tools [22]. Furthermore, repeated biopsy is problematic for accurate assessment and has risks [6]. The need for a tool to identify and define BPNET disease and monitor its clinical course is an important unmet clinical need. Early identification of recurrent or residual disease after surgical resection is key to timely institution of therapeutic strategy.

In this study, the clinical utility of a 51-gene circulating mRNA marker panel as a biomarker in neuroendocrine lung diseases was evaluated. Blood levels of the multianalyte marker were significantly elevated in lung neuroendocrine neoplasia and could differentiate pulmonary carcinoids from controls with an area under the curve (AUC) > 0.98 . Levels were also significantly

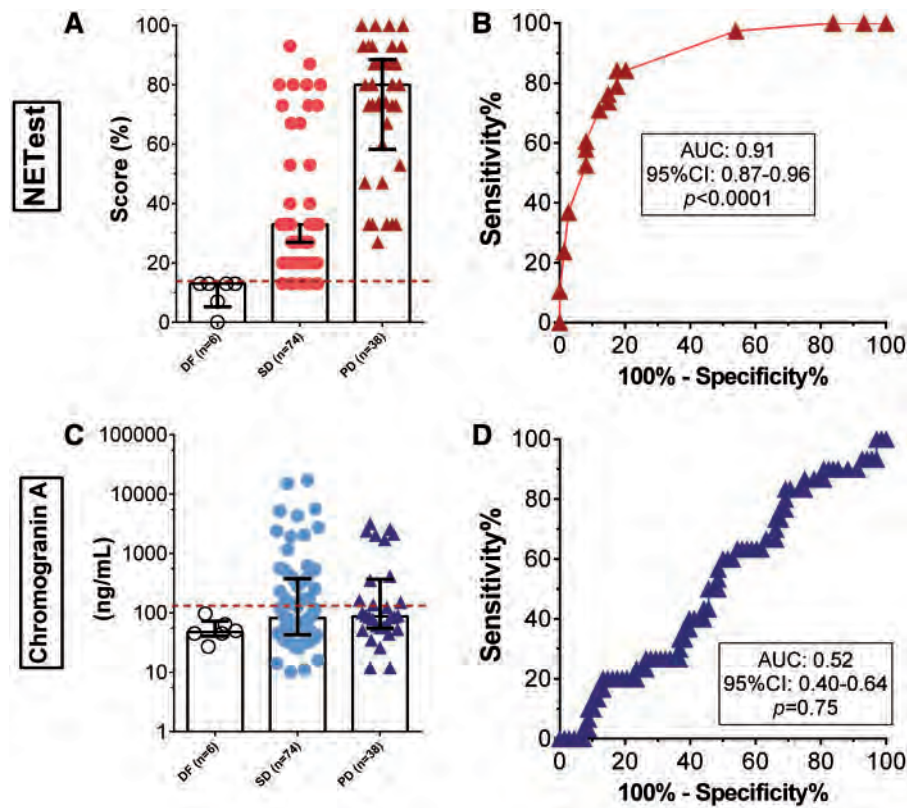


Figure 4: Relationship between biomarker levels and disease status in carcinoids (bronchopulmonary neuroendocrine tumours) (Set I). NETest: (A) NETest scores were significantly elevated ($P < 0.001$) in PD ($n = 38$) and SD ($n = 74$) compared with DF subjects ($n = 6$). Levels were also significantly elevated in PD versus SD ($P < 0.001$). (B) The AUROC for differentiating PD from SD was 0.91(0.87–0.96), $P < 0.001$. Chromogranin A: (C) Chromogranin A levels were not significantly elevated in any of the 3 clinical groups, DF, SD and PD. (D) The AUROC for differentiating PD from SD was 0.52(0.40–0.64), $P = 0.75$. Median and interquartile ranges are indicated in black. AUC: area under the curve; AUROC: area under the receiver operator curve; CI: confidence interval; DF: disease free; NETest: neuroendocrine tumour multigene blood test; PD: progressive disease; SD: stable disease.

elevated in poorly differentiated neuroendocrine carcinomas, hence the biomarker may have some utility as a diagnostic tool for identifying clinically aggressive disease. This is of clinical relevance since the majority of lung NETs are poorly differentiated tumours [23, 24]. In contrast, CgA exhibits low levels in poorly differentiated neuroendocrine carcinomas compared with well-differentiated tumours [25]. Surgical resection significantly reduced the circulating signature indicating that neuroendocrine neoplasia was the source of the blood signal. In contrast, CgA levels did not decrease suggesting that it is a poor biomarker for BPNET disease. CgA levels also exhibited no relationship with disease status or progression.

As a diagnostic test, the NETest was only significantly elevated in carcinoids and lung neuroendocrine neoplasia (LCNEC and SCLC). The AUC for differentiating carcinoids from controls was 0.98. This metric was significantly elevated compared with CgA (Z-statistic 7.5, $P < 0.001$). Moreover, significantly more carcinoid samples were positive (NETest >14%) using the NETest (110 of 118; 93%). CgA was only elevated in 45 (38%) of the carcinoid cohort. Thus, CgA was normal in approximately 60% of carcinoids. This is consistent with other studies that reported low-CgA detection efficacy in pulmonary carcinoids [26, 27]. Although SCLC may present with elevated CgA, levels are typically much lower than those measured in well-differentiated tumours, e.g. midgut carcinoids, supporting the notion that CgA may be less useful in undifferentiated neuroendocrine neoplasms [25]. In the latter, neuron-specific enolase has been proposed to support the diagnosis in blood [25]. In general, however, the metrics of neuron-specific enolase fail to meet accepted criteria as an effective biomarker.

The NETest metrics for detecting carcinoids were 93% sensitivity and 88% specificity. These values are comparable to diagnostic studies in GEPNETs, where the NETest exhibits 89 and 98% sensitivity and specificity, respectively [12]. Biomarker metrics are considered acceptable if the measurement is specifically associated with a particular tumour type as well as differentiating normal from the disease, while performance metrics should be $\geq 80\%$ [28]. The data collected in this study reflect numerous observations in the literature that CgA is an unreliable biomarker, not only in GEPNET disease but also in BPNETs [26, 27]. Overall, the CgA metrics ranged from 36% to 95%. Enthusiasm over the use of CgA as a biomarker has been diminished both by the sensitivity (<40%) of the assay for detecting pulmonary carcinoids and by the low proportion (37%) of BPNETs that have detectable levels. This is consistent with other studies demonstrating low CgA accuracy in detecting lung neuroendocrine neoplasia [22].

Apart from the diagnostic issue, we separately assessed the clinical utility of the NETest to evaluate the clinical status of lung carcinoid disease. Levels were significantly elevated in PD compared with SD, and the AUC for differentiating these 2 conditions was 0.91. It is noteworthy that all image-negative subjects had normal transcript levels and could be differentiated from diseased subjects (AUC = 0.99) 5 years after complete resections. In contrast, CgA had no clinical utility as a diagnostic parameter for malignant disease. CgA was neither differentially elevated in PD/SD versus controls, nor were the levels significantly different from surgically cured (disease-free) patients. The NETest was significantly more effective at distinguishing 'disease-free' status and defining PD than CgA.

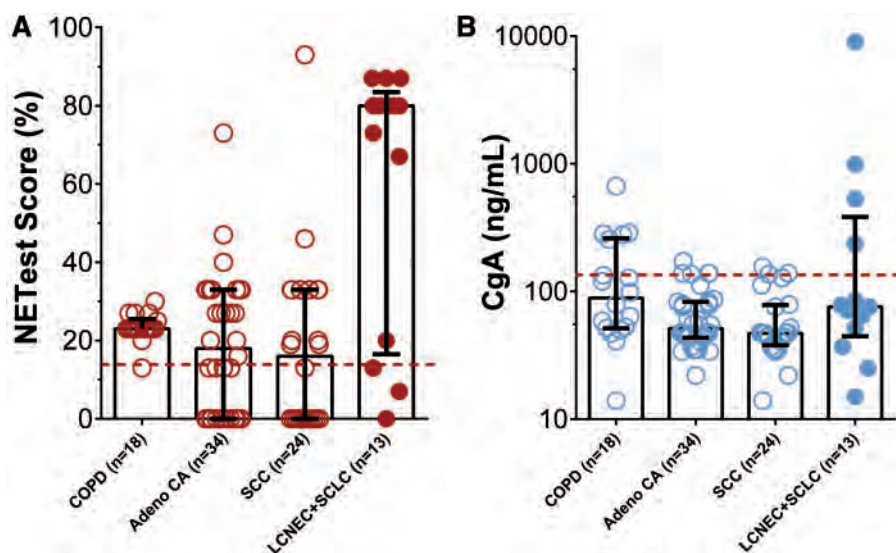


Figure 5: Biomarker values in other lung pathology (Set I). **(A)** NETest scores were significantly elevated ($P < 0.05$) in LCNEC ($n = 9$) and SCLC ($n = 4$) compared with COPD ($n = 18$), adenocarcinomas ($n = 34$) and SCCs ($n = 24$). **(B)** CgA levels were not significantly elevated in any of the groups. Median and interquartile ranges are indicated in black. AdenoCa: adenocarcinoma; CgA: chromogranin A; COPD: chronic obstructive pulmonary disease; LCNEC: large-cell neuroendocrine carcinoma; NETest: neuroendocrine tumour multigene blood test; SCC: squamous cell carcinoma; SCLC: small-cell lung carcinoma.

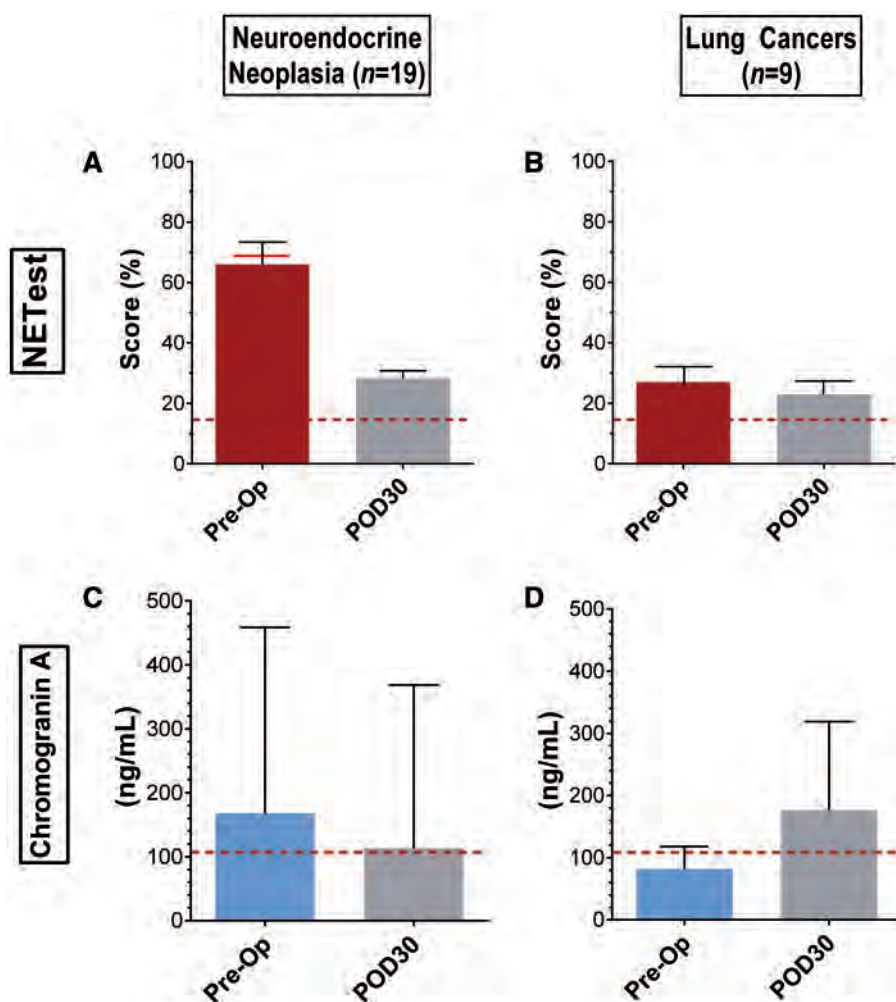


Figure 6: Effect of surgery on NETest and CgA levels in bronchopulmonary neuroendocrine tumours and lung cancers (Set II). **(A)** In neuroendocrine neoplasia, the NETest scores were significantly decreased ($P < 0.001$) ($n = 19$) on POD30 compared with presurgery. **(B)** In lung cancers ($n = 9$), the NETest was not affected by surgery. **(C)** CgA levels were not significantly decreased by surgery in neuroendocrine neoplasia. **(D)** CgA levels were not significantly altered by surgery in lung cancers. NETest: neuroendocrine tumour multigene blood test; POD30: postoperative Day 30; pre-op: preoperative.

Finally, the surgical utility of the NETest was evaluated in a prospective setting. NETest and CgA were measured in 28 resections (19 BPNETs and 9 lung cancers) presurgery and on POD30. In neuroendocrine lung disease, the NETest was significantly reduced following surgery. No significant alteration in values was noted in the lung cancer cohort. This is consistent with previous studies that established the tumour transcriptome analysis of the BPNETs to be fully identified by the 51-gene signature measured in blood [16]; resection would be predicted to diminish transcript blood levels. In contrast, CgA was not significantly reduced by surgery. Indeed, CgA normalized in only 1 patient. A further confounding variable is that cardiac and sympathetic stress-related events (e.g. surgery) are known to elevate CgA [29].

In NET disease management, the precise assessment of the disease status and response to therapy has been hampered by the poor performance of contemporary biomarkers and by the limited resolution capacity of imaging. A recent study demonstrated that uptake at ^{68}Ga -SSA PET (standardized uptake value_{max}) correlated with the NET transcripts in blood and that the transcript signature could provide an accurate appraisal of the disease status at the time of the scan [30]. This observation provides the basis for further exploration of strategies to investigate the interface of circulating tumour molecular indices and functional imaging. Correlation of the spatial components of a tumour and circulating biological tumour-derived transcripts are likely to enhance the real-time accuracy of monitoring tumour status.

CONCLUSION

In summary, measurement of neuroendocrine-specific circulating mRNA levels in blood accurately identifies BP neuroendocrine neoplasia. This signature specifically distinguishes carcinoids from controls and other lung diseases and is decreased following surgery. Furthermore, levels can identify individuals with PD and confirm complete surgical resection 'cure'. In contrast, CgA is non-informative as a biomarker for diagnosing or monitoring BPNETs. We propose that blood measurements of neuroendocrine-specific transcripts using a blood-based multianalyte algorithmic analysis could supplement the clinical armamentarium (imaging/biopsy) by providing real-time information to define surgical responses and identify disease recurrence or progression.

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APPENDIX. CONFERENCE DISCUSSION



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Dr. E. Vallieres (Seattle, WA, USA): Pier Luigi, do you have any experience with this test, can it help you separate typical versus atypical carcinoids, any experience with large-cell neuroendocrine tumours and small-cell lung cancers?

Dr. P.L. Filosso (Castellamonte Torino, Italy): We have some experience. As I mentioned before, we operated in this period 2 cases of large-cell neuroendocrine carcinomas (LCNCs). In those cases, the NETest increased in the postoperative course when the patients experienced a recurrent disease. In terms of 1 patient, in fact, he unfortunately died of brain metastasis and the other one had the local tumour recurrence. Both of them were N1T2 T2N1 LCNCs, and that test was strongly able to predict the outcome and the recurrence and the development of recurrences was very, very early. This is the key point of this test. It has been described by the authors Modlin and Coll in the gastroenteropancreatic neuroendocrine tumours that NETest was very, very effective in predicting recurrences prior to surgery. The case I showed to you before it is the only case we have in this population; also in this case, the NETest was very efficient.

Dr. B. Sepesi (Houston, TX, USA): How many of those patients had a DIPNECH syndrome, because the 1 patient that you showed us had carcinoid on

1 side and carcinoid tumour on the other side. Could this be 2 separate carcinoids or some of the patients may actually have had DIPNECH syndrome, which would explain why they had some prolonged biomarker in their blood.

Dr. Filosso: The patient I showed you had in effect dyspnoea, as other 2 patients. I well remember a patient who had T3N2 typical carcinoid with the several tumourlets and dyspnoea. In that case, the last I mentioned, NETest has decreased after intervention, therefore I am not sure that the presence of the tumourlets or dyspnoeal syndrome may influence the result of the NETest itself.

Dr. Sepesi: I guess it would be also important to correlate perhaps tumour volume with this biomarker and another issue with carcinoid tumour is that, let's say you have an elevation biomarker a year later, you only were checking these biomarkers up to 10 days postoperatively. I am not sure really what the half-life is, but I would say that maybe they would have been cleared. But let's say you have an elevation of this biomarker a year later after you resected a carcinoid, but no imaging to support any sort of a therapy and we know that for a typical carcinoid chemo, radiation, are really not as good as surgery, so what is the long-term applicability of this versus follow-up imaging?

Dr. P.L. Filosso: I believe that there should be integration between common follow-up for those patients and biological follow-up. This is what we try to do in the future in close co-operation with the American colleagues from Yale. This is the reason why we would like to improve our series of operated NET patients and to be more effective in the follow-up. To look whether the NET test is able to predict tumour recurrence or tumour metastasis prior to common imaging as it did in the gastroenteropancreatic neuroendocrine tumour.