

ORIGINAL ARTICLE

A multigenomic liquid biopsy biomarker for neuroendocrine tumor disease outperforms CgA and has surgical and clinical utility

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Background: Biomarkers are key tools in cancer management. In neuroendocrine tumors (NETs), Chromogranin A (CgA) was considered acceptable as a biomarker. We compared the clinical efficacy of a multigenomic blood biomarker (NETest) to CgA over a 5-year period.

Patients and methods: An observational, prospective, cross-sectional, multicenter, multinational, comparative cohort assessment. Cohort 1: NETest evaluation in NETs ($n = 1684$) and cancers, benign diseases, controls ($n = 731$). Cohort 2: ($n = 1270$): matched analysis of NETest/CgA in a sub-cohort of NETs ($n = 922$) versus other diseases and controls ($n = 348$). Disease status was assessed by response evaluation criteria in solid tumors (RECIST). NETest measurement: qPCR [upper limit of normal (ULN: 20)], CgA (EuroDiagnostica, ULN: 108 ng/ml). Statistics: Mann-Whitney U-test, AUROC, chi-square and McNemar' test.

Results: Cohort 1: NETest diagnostic accuracy was 91% ($P < 0.0001$) and identified pheochromocytomas (98%), small intestine (94%), pancreas (91%), lung (88%), gastric (80%) and appendix (79%). NETest reflected grading: G1: 40 ± 1 , G2 (50 ± 1) and G3 (52 ± 1). Locoregional disease levels were lower (38 ± 1) than metastatic (52 ± 1 , $P < 0.0001$). NETest accurately stratified RECIST-assessed disease extent: no disease (21 ± 1), stable (43 ± 2), progressive (62 ± 2) ($P < 0.0001$). NETest concordance with imaging (CT/MRI/⁶⁸Ga-SSA-PET) 91%. Presurgery, all NETs ($n = 153$) were positive (100%). After palliative R1/R2 surgery ($n = 51$) all (100%) remained elevated. After curative R0-surgery ($n = 102$), NETest levels were normal in 81 (70%) with no recurrence at 2 years. In the 31 (30%) with elevated levels, 25 (81%) recurred within 2 years. Cohort #2: NETest diagnostic accuracy was 87% and CgA 54% ($P < 0.0001$). NETest was more accurate than CgA for grading (chi-square = 7.7, OR = 18.5) and metastatic identification (chi-square = 180, OR = 8.4). NETest identified progressive disease (95%) versus CgA (57%, $P < 0.0001$). Imaging concordance for NETest was 91% versus CgA (46%) ($P < 0.0001$). Recurrence prediction after surgery was NETest-positive in >94% versus CgA 11%.

Conclusion: NETest accurately diagnoses NETs and is an effective surrogate marker for imaging, grade, metastases and disease status compared to CgA. A multigenomic liquid biopsy is an accurate biomarker of NET disease.

Key words: NETest, biomarker, neuroendocrine tumor, NET, Chromogranin

INTRODUCTION

Effective blood biomarkers can provide an easily available, non-invasive, irradiation-free, real-time appreciation of the disease status of a patient.¹ In the past, monoanalytes that assessed single aspects of a disease (e.g. myeloma protein)

or secretory products (e.g. insulin) provided useful information. Such tools, however, measure a single aspect of tumor biology. The evolution of both molecular biology and scientific tools to identify and quantify multiple regulatory pathways have facilitated development of multianalyte biomarkers.² This has been advanced by mathematical deep-learning strategies. Such strategies in blood have led to the recent consideration of liquid biopsies as real-time, diagnostic and clinical management tools.³

Neuroendocrine tumor (NET) biomarkers have focused on measurements of secretory products, e.g. gastrin, serotonin

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or chromogranin A (CgA).⁴ Guidelines consider such biomarkers as either not useful or as controversial (Level III evidence).⁵ A recent overview by Caplin et al., emphasized the urgent requirement for novel NET biomarkers given that CgA has a 'less-than-ideal diagnostic sensitivity and specificity'.⁶

In NETs, a multigenomic blood assay (NETest) is considered a more accurate biomarker than CgA.⁷⁻¹⁰ The assay has been described in detail and independently validated.^{8,9,11} It comprises a 51-gene expression-based liquid biopsy for NETs that utilizes PCR-technology and multianalyte algorithmic analyses. Output is scored 0-100 with values >20 abnormal. Stable disease is 21-40 and progressive 41-100.¹⁰ A meta-analysis by Oberg et al., concluded the NETest had ~95% diagnostic accuracy and was an effective (>80%) monitor of treatment efficacy.¹⁰ The study noted that a multigenomic biomarker assay could provide a significant fiscal advantage in standard medical practice.

The inability to accurately monitor NETs using an effective pan-NET biomarker is associated with substantial financial costs.¹² Here, we report the results of NETest in 1684 consecutive, prospectively collected NETs assessed over 5 years for diagnostic and surgical utility. Additionally, we examined the NETest directly compared to CgA in a sub-cohort of 922 NETs in whom both biomarker measurements were available.

METHODS

Study design

This is a prospective, multicenter, multinational, cross-sectional study, which comprises 1684 gastroenteropancreatic, lung and other NETs as well as 256 controls and 475 non-NETs. We enrolled and evaluated all patients (irrespective of disease extent or pathological grade) over a 5-year period (1 October 2015-30 September 2020, study design: [Supplementary Table S1](https://doi.org/10.1016/j.annonc.2021.08.1746), available at <https://doi.org/10.1016/j.annonc.2021.08.1746>). No exclusion criteria were used. Samples were collected for NETest and, where requested, for CgA measurements. When multiple samples were collected, only the initial sample was evaluated in this study.

Patient demographics are included in [Table 1](#). Primary outcome was based on a NET diagnosis. Variables included clinical data (diagnosis, grade, staging, imaging, status, surgery effectiveness) and biomarkers (NETest, CgA). Data were collected (Western Institutional Review Board #20150174) and evaluated as per Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.¹³ Evaluations included utility as a diagnostic using area under the receiver curve (AUROC) analyses, correlation with imaging and relationship to tumor organ site, histological grade, clinical status and metastases. In all individuals, a single blood sample per patient was evaluated. For surgical evaluation, two samples were collected, a pre- and post-operative blood sample, and NETest and CgA levels were assessed. This enabled demonstration of the effectiveness of surgical intervention. For matched biomarker assessment, we calculated the

relative risk (RR) using the McNemar's test and odds ratio (OR) or undertook chi-square analyses.

Clinical data

Disease status was assessed by clinical review, imaging [computed tomography/magnetic resonance imaging (CT/MRI) or ⁶⁸Gallium labeled somatostatin analogues (⁶⁸Ga-SSA-studies)], grade, and metastases. Status (i.e. stable or progressive disease) was determined using response evaluation criteria in solid tumors (RECIST) 1.0 (CT/MRI) or 1.1 (NETspot).¹⁴ Surgical efficacy was evaluated in a subset ($n = 153$) followed for 24 months. Surgery included R0 ($n = 102$), R1 ($n = 29$) and R2 ($n = 22$) resections ([Table 2](#)).

Blood collection/send-out. Whole blood samples (3 ml) were collected for NETest¹⁵ and plasma for CgA. Samples were shipped to a clinically-certified US laboratory (Wren Laboratories CL-0996, CLIA 07D2081388, NYSDOH PFI: 9138) for measurements. All blood tubes were anonymized, and measurements were blinded.

NETest measurement

Transcripts [messenger RNA (mRNA)] were isolated and real-time PCR carried out.¹⁵⁻¹⁷ Targets were normalized and quantified (population control).¹⁵⁻¹⁷ Final results are expressed as an activity index from 0-100.¹⁵⁻¹⁷ NETest-positive: >20, progressive disease 41-100,¹⁰ abnormally elevated ≥ 80 (predictor of progression).⁸

CgA measurement

CgA measurement was undertaken using the NEOLISA™ kit (EuroDiagnostica), upper limit of normal (ULN):108 µg/l.

Statistical analysis. Analyses were carried out using Prism 9.0 (GraphPad 9.0.0-Windows, La Jolla, CA, www.graphpad.com) and MedCalc (bvba v19.6, Ostend, Belgium; www.medcalc.org; 2013). Descriptive statistics and intergroup analyses included two-tailed non-parametric tests (Mann-Whitney U-test), two-tailed chi-square (Yates correction), McNemar's test (matched NETest and CgA samples) and AUROC-analyses. All data are presented as mean \pm standard error of mean (SEM). $P < 0.05$ was considered significant.

Power analysis (NETs and controls, power = 0.95, $\alpha = 0.05$) adequate to differentiate NETest scores (dichotomous output, assuming 75% incidence in NETs and 25% incidence in controls) was calculated. Requirements were a minimum of 23 per group (total = 46). Overall, 2415 patients (~50 \times) were evaluated including 1684 NETs.

RESULTS

Demographics

NETs. The study included 1684 NETs ([Table 1](#)). All organ sites including neural tumors were evaluated. Median ages ranged from 36 years (pheochromocytomas, paragangliomas [PPGL]) to 62 years (lung, small intestine). Sex

Table 1. NET patient demographics

Site	No.	Age, years Median (range)	Sex (M/F)	Grade					Mets		IPD		Clinical Status				CgA ^a			
				G1	G2	G3	TC	AC	ND	N	%	N	%	NED/CR	SD	PD	ND	N	%	
Appx.	56	42 (18-56)	14 : 42	28	11	4 ^b				13	11	20	23	41	19	31	6	0	26	46
Colon	23	59.5 (38-83)	7 : 16	8	7	1				7	18	78	19	82	2	11	9	1	10	43
CUP	81	62 (25-82)	38 : 43	11	27	9				34	60	74	75	93	4	32	31	14	41	51
Duod.	36	57 (30-87)	20 : 16	17	5	1				13	23	64	30	83	4	21	7	4	25	69
Stomach	76	60 (11-88)	24 : 52	45	13	7				11	16	21	25	33	50	15	10	1	56	74
Lung	301	62 (18-87)	107 : 194				3	142	131	25	129	43	256	85	37	125	123	6	137	44
Pancreas	359	58 (15-87)	179 : 180	105	152	30				72	233	65	309	86	29	133	152	26	253	70
PPGL	121	36 (10-64)	58 : 63	10	2					119	35	29	117	97	4	81	36	0	0	0
Rectum	88	56 (21-82)	47 : 38	52	18	3				15	29	33	39	44	42	19	20	7	65	74
SI	521	62 (22-87)	257 : 264	272	139	7				103	443	85	464	89	36	254	172	11	296	57
Others ^c	22	66.5 (38-77)	7 : 15	2	7	3				9	19	86	21	95	1	9	11	1	13	59

AC, atypical carcinoid; Appx., appendix; CgA, Chromogranin A; CR, complete remission; CUP, carcinoid of unknown primary; Duod., duodenum; IPD, image-positive disease; Mets, metastases; ND, no data; NED, no evidence of disease; NET, neuroendocrine tumor; PD, progressive disease; PPGL, pheochromocytomas and paragangliomas; SD, Stable disease; SI, small intestine; TC, typical carcinoid.

^a These are included in Cohort 2.

^b 3 were goblet cell carcinoids.

^c Including ovarian, pituitary, testicular and thymic NETs.

ranged from almost parity (pancreas 179 : 180) to female-predominant (appendix: 75% women). Histology was evaluable in 75% ($n = 1263$). The incidence of metastases was stomach (21%), lung (43%), pancreas (65%) and small

intestine (85%). Appendiceal, gastric and rectal NETs were typically collected after endoscopy or surgery. Individual demographics (staging, size) for these tumors are included in [Supplementary Table S2](https://doi.org/10.1016/j.annonc.2021.08.1746), available at <https://doi.org/10.1016/j.annonc.2021.08.1746>.

Image-positive disease (IPD) was identified in appendix (41%), rectal (44%), stomach (33%), lung (85%), pancreas (86%), small intestine (89%) and PPGLs (97%). Clinical status was assessable in 91% ($n = 1536$). Matched CgAs were available in 922 NETs (Cohort 2) [controls ($n = 105$), benign disease, CgA ($n = 131$) and non-NET neoplasia ($n = 112$)].

Controls and non-NETs ($n = 831$). This comprised controls, benign disease and non-NET malignancies.

Controls ($n = 256$). Asymptomatic, in good health, no identified malignancy or were being treated for disease. Median age was 37 (range: 20-78) years with a sex distribution of 144 : 111. The ages of controls were significantly ($P < 0.0001$) younger than NETs, individuals with benign diseases and non-NET neoplasia.

Benign diseases ($n = 138$). Including gastro-esophageal reflux disease, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, chronic pancreatitis, benign pancreatic cysts and hypertension. The median age was 65 (22-87) years and predominantly male (92:46).

Non-NET neoplasia ($n = 337$). Including colon, esophageal, gallbladder, lung (adenocarcinoma, squamous cell cancer), pancreatic, rectal, and small intestinal adenocarcinomas. Median age was 67 (20-88) years with a sex distribution of 188 : 121.

NET diagnosis and correlation with imaging

NET diagnosis. In Cohort 1 ($n = 2415$), the NETest diagnosed NETs (accuracy: 91%, area under the curve: 0.97, $P < 0.0001$) compared with controls and non-NET diseases including neoplastic and benign disease. The NETest was

Table 2. Surgical cohort: clinical and biomarker details

Specifics	R0	R1	R2
N	102	29	22
Age, years: median (range)	59 (27-84)	50 (26-85)	60 (19-74)
Sex: M/F	49/53	15/14	12/10
Sites:			
Lung	26	1	0
Pancreas	45	7	5
Small Bowel	29	16	17
Appendix	0	1	1
Stomach	2	1	0
Duodenum	0	2	2
Grade:			
G1	40	19	17
G2	32	10	5
G3	3 ^a	0	0
AC	9	1	0
TC	17	0	0
Staging			
T			
1	38	0	0
2	34	16	5
3	20	13	11
4	10	0	6
N			
0	55	7	0
1	44	22	22
x	3 ^b	0	0
M			
0	98	18	10
1	4	11	12
NETest (DO) median (range) ^a	67 (27-100)	87 (40-100)	87 (33-100)
NETest positive, n (%) ^a	102 (100%)	29 (100%)	22 (100%)
CgA (DO) median (range) ^b	55 (16-1158)	67 (35-156)	75 (40-158)
CgA positive n (%) ^b	18 (22%)	2 (18%)	3 (33%)

AC, atypical carcinoid; CgA, Chromogranin A; DO, pre-operative NETest score; M, metastasis; N, node; T, tumor; TC, typical carcinoid.

^a Measured in Cohort 1.

^b Measured in Cohort 2.

detected in all NET types: 98% PPGL, 96% lung, 94% small intestine, 91% pancreas, 80% gastric, 79% appendiceal and 45% rectal. This ranged from 4% in controls to 22% in benign diseases to 36% in other neoplasia ($P < 0.0001$).

In the different tumor types, levels were: pheochromocytomas (80 ± 4), paragangliomas (65 ± 3), small intestinal (50 ± 1), pancreatic (49 ± 1), bronchopulmonary NETs (45 ± 2), appendiceal (34 ± 3), gastric (30 ± 2) and rectal (30 ± 3) (Figure 1A). These were significantly elevated ($P < 0.001$) compared with controls (8 ± 1), benign disease (21 ± 2) and other neoplasia (20 ± 1).

In a sub-analysis of the significance values of the appendiceal ($P < 0.05$), gastric ($P < 0.001$) and rectal ($P < 0.0001$) NETs, NETest levels were identified to significantly ($r = 0.98$) correlate with tumor stage and size (Supplementary Figure S1 and Supplementary Table S3, available at <https://doi.org/10.1016/j.annonc.2021.08.1746>).

In Cohort 2 ($n = 1260$), the NETest (87%) was a significantly more accurate diagnostic than CgA (54%, $P < 0.0001$, McNemar: 254, OR = 8.6; 95% CI: 5.2-9.3). An evaluation of individual tumor organ sites identified a better RR for diagnosis (compared to non-NETs: $n = 388$) than CgA (Figure 1B). Analysis and comparison of the different organ sites identified that carcinoid of unknown primary (CUPs), small intestinal, pancreatic and lung NETs (>80% NETest-positive) exhibited RR > 3.0. In comparison, CgA was positive in 30%-70% and the RR ranged from 1.5 to 2. Gastric NETs exhibited similar metrics, irrespective of biomarker tested.

Imaging. In Cohort 1 ($n = 1684$ NETs), 1380 (82%) exhibited image-detectable disease using CT/MRI/ ^{68}Ga -SSA-PET. NETest levels in image-detectable disease were 51 ± 1 . In those with no detectable disease, levels were 21 ± 1 ($P < 0.0001$). Imaging concordance was 91% (Figure 2A).

In Cohort 2 ($n = 922$ NETs), image-detectable disease was NETest-positive in 91% compared to 46% CgA-positives (chi-square = 232, OR = 9.1) (Figure 2B). In the surgical subgroup analysis (R1 image-negative cohort, $n = 81$), the NETest was positive in 64/81 (79%) compared to CgA in 25% ($P < 0.0001$).

Correlation with clinical parameters: disease status, histological grade and metastasis

Cohort 1 ($n = 1684$). The NETest stratified image-negative (21 ± 1) from stable (43 ± 1) from progressive disease (62 ± 1) ($P < 0.0001$) (Figure 3A). In the image-negative cohort ($n = 157$), 83 (53%) exhibited microscopic disease. The demographics of this cohort are included in Supplementary Table S4, available at <https://doi.org/10.1016/j.annonc.2021.08.1746>. The NETest was positive in 64 (77%). In the 'true' negatives (image-negative and no evidence of disease per histopathology: $n = 74$), the NETest was positive in 7 (9%, score: 56 ± 8) (Figure 3B). A sub-analysis of the data identified the NETest accurately correlated (83%) with microscopic disease irrespective of tumor

site (Supplementary Table S4, available at <https://doi.org/10.1016/j.annonc.2021.08.1746>).

NETest levels reflected grading: G1 40 ± 1 , G2 50 ± 1 , and G3 52 ± 4 (Kruskal-Wallis statistic 49.4, $P < 0.0001$) (Figure 3C). A separate evaluation of the NETest and Ki67 index identified a significant correlation ($r = 0.16$, $P < 0.0001$, Supplementary Figure S2, available at <https://doi.org/10.1016/j.annonc.2021.08.1746>).

NETest scores were significantly higher in metastatic (52 ± 1 , $P < 0.0001$) than localized disease (38 ± 1). Mean levels in individual tumor types were: gastric (40 ± 7 versus 26 ± 3 , $P = 0.07$), lung (46 ± 2 versus 43 ± 2 , $P = 0.06$), pancreas (53 ± 2 versus 37 ± 3 , $P < 0.0001$), small bowel (52 ± 1 versus 31 ± 4 , $P < 0.0001$) and PPGL (72 ± 4 versus 61 ± 4 , $P = 0.02$) (Figure 3D). A sub-analysis of appendiceal, gastric and rectal NETs, confirmed stage IV disease to exhibit significantly ($P < 0.05$) elevated levels versus stage I disease (Supplementary Figure S1 and Table S3, available at <https://doi.org/10.1016/j.annonc.2021.08.1746>).

Cohort 2 ($n = 922$). The percentage with a positive NETest and progressive disease was 95% compared to CgA (57%, $P < 0.0001$) (Figure 4A). For stable disease, the values were 78% and 35%, respectively ($P < 0.0001$). For grading, the NETest was more accurate than CgA with individual grades exhibiting McNemar's chi-square OR ranging from 5.03 (G1) to 18.5 typical carcinoid (TC) (Figure 4B). For metastatic detection, the NETest was more accurate with an OR = 8.4. Metastatic disease was 87% NETest-positive compared to CgA (48% positive) (Figure 4C).

NETest and surgery

Pre-surgical NETest levels were elevated (68 ± 3) in all 153 (100%). After R2 surgery, there was no significant decrease (60 ± 6 , $P = \text{NS}$) while R1 (45 ± 4 , $P = 0.001$) and R0 (22 ± 2 , $P < 0.0001$) were associated with significant decreases (Figure 5A). All R2 and R1 surgeries were NETest-positive at post-operative day 30 compared to 31 (31%) of the R0 group. In the R0 group ($n = 71$) with a normal NETest none (0%) recurred while 25 (81%) with an elevated level recurred within 24 months (chi-square = 17.1, $P < 0.0001$, Figure 5B).

In the surgical subgroup of Cohort 2 ($n = 122$), all had an elevated NETest preoperatively compared to 28% ($n = 34$) for CgA ($P < 0.0001$). In R0 ($n = 69$), 19 (28%), had elevated post-operative NETest compared to 5 (7%) for CgA. All 19 (100%) with elevated NETests recurred compared to 11% with elevated CgA ($P < 0.0001$, chi-square = 15.5).

DISCUSSION

This study demonstrates that a blood-based, multigenomic signature is an effective diagnostic for NETs and has clinical utility in management since it is concordant with imaging, accurately stratifies disease status (stable versus progressive) and predicts tumor recurrence after surgery. It significantly outperformed CgA in all clinical parameters.

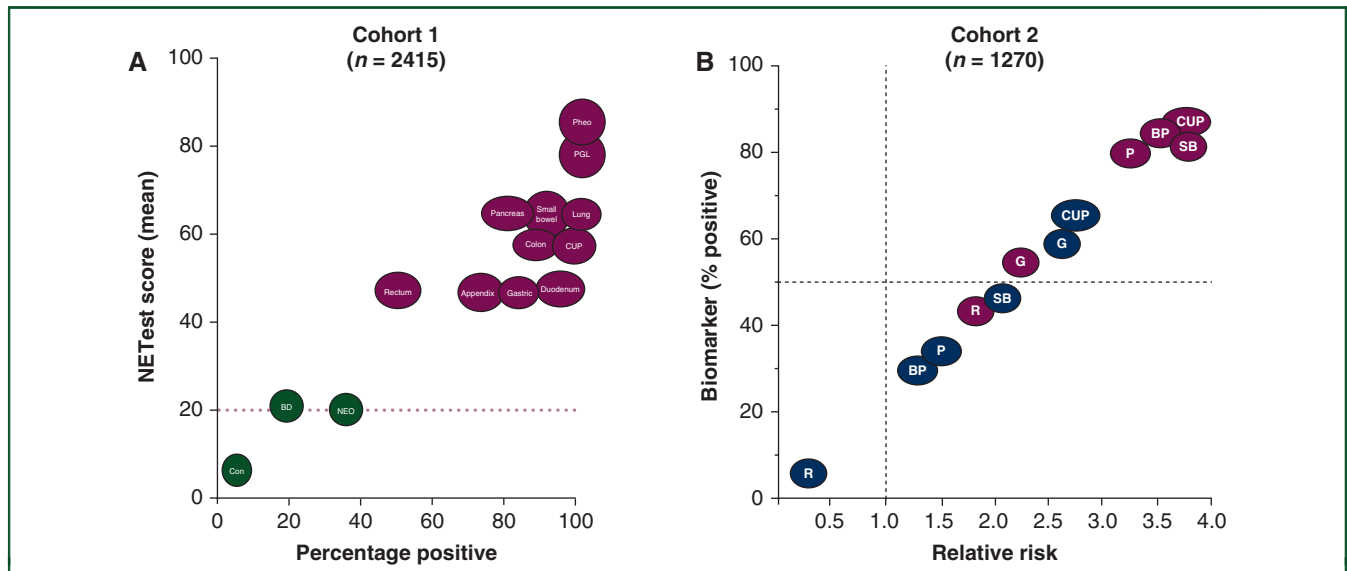


Figure 1. Biomarker diagnostic utility. (A) Cohort 1 (n = 2415): NETest scores (y-axis) and the percentage positive cases (x-axis) irrespective of disease status (includes CR/NED) for the principal organ sites. Upper limit of normal = 20 is expressed by the dotted line. The majority of NETs (>80%) exhibit an elevated NETest with mean scores >20. (B) Cohort 2 (n = 1270): Relationship between the percentage positive cases and the calculated relative risk (versus the controls, benign and malignant diseases: n = 348) for the principal sites. NETest (burgundy circles) and CgA (dark blue circles). Pancreatic (P), small bowel (SB), CUP and lung (BP—bronchopulmonary) exhibited relative risks of >3 for the NETest. CgA exhibited lower performance metrics especially for rectal (R), lung (BP) and pancreatic (P) NETs (all with <40% positive samples, RR < 1.5). Dotted lines reflect a 50% cut-off for test positive (horizontal line) and a RR of 1.0 (vertical line). BD, Benign disease; CON, control; CR, complete remission; CUP, carcinoid of unknown primary; G, gastric; NED, no evidence of disease; NEO, other neoplasia; NETs, neuroendocrine tumors; PGL, paraganglioma; Pheo, pheochromocytoma; R, rectal.

Study strengths include a real-time, multicenter, multinational experience of the NETest in clinical day-to-day usage. The centers involved were ENETs Centers of Excellence and assessments of disease consistent with the published standards of the group. All samples were deidentified and analyzed blind by a central USA CLIA-approved laboratory. This study is highly-powered (50× higher enrollment than required) to reject the null hypothesis and demonstrate that NETest is a more effective biomarker than CgA.

Study limitations include heterogeneity of imaging studies, no centralized pathological review (in ~25%,

histopathology was not available) and dependence on individual centers to provide clinical information. It should be noted that an additional study design limitation was that we did not evaluate the NETest as either a continuous monitor or as a prognostic for overall survival. Separate studies addressing these questions are ongoing. We anticipate substantial follow-up will be required to adequately evaluate survival and assess the impact of monitoring and prognostic analysis.

Circulating biomarkers are important in oncology management. In NETs, monoanalytes e.g. secretory amines and

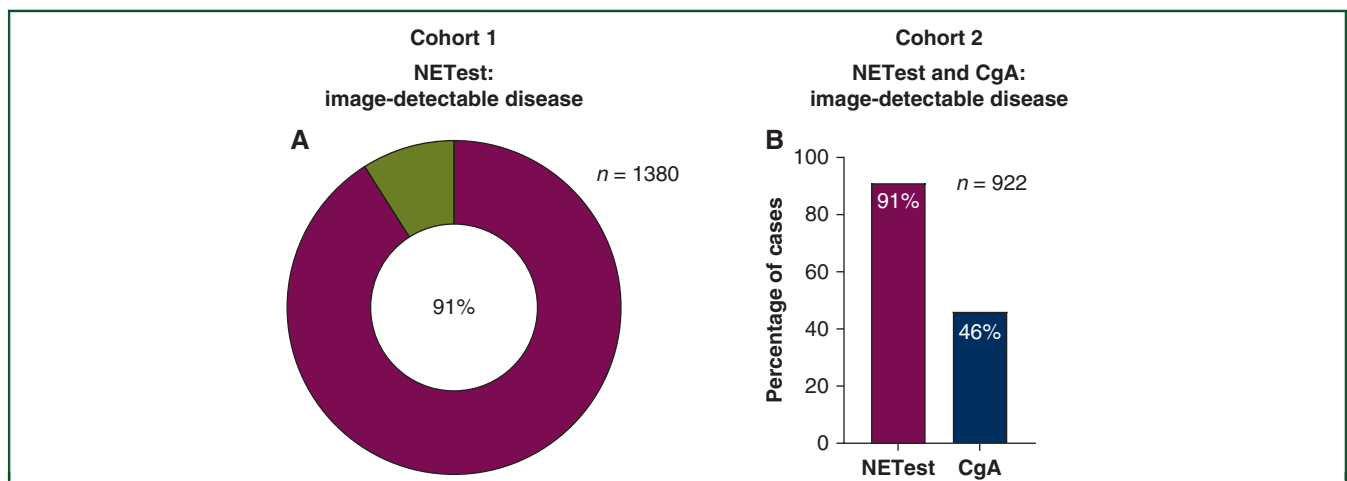


Figure 2. NETest versus CgA: imaging utility. (A) Cohort 1 (n = 1684). NETest was elevated in image-positive disease (n = 1380) in 91%. (B) Cohort 2 (n = 922). NETest-positive 91% positive compared to CgA elevated in 46%. NETest is significantly more accurate than CgA (P < 0.0001). CgA, chromogranin A.

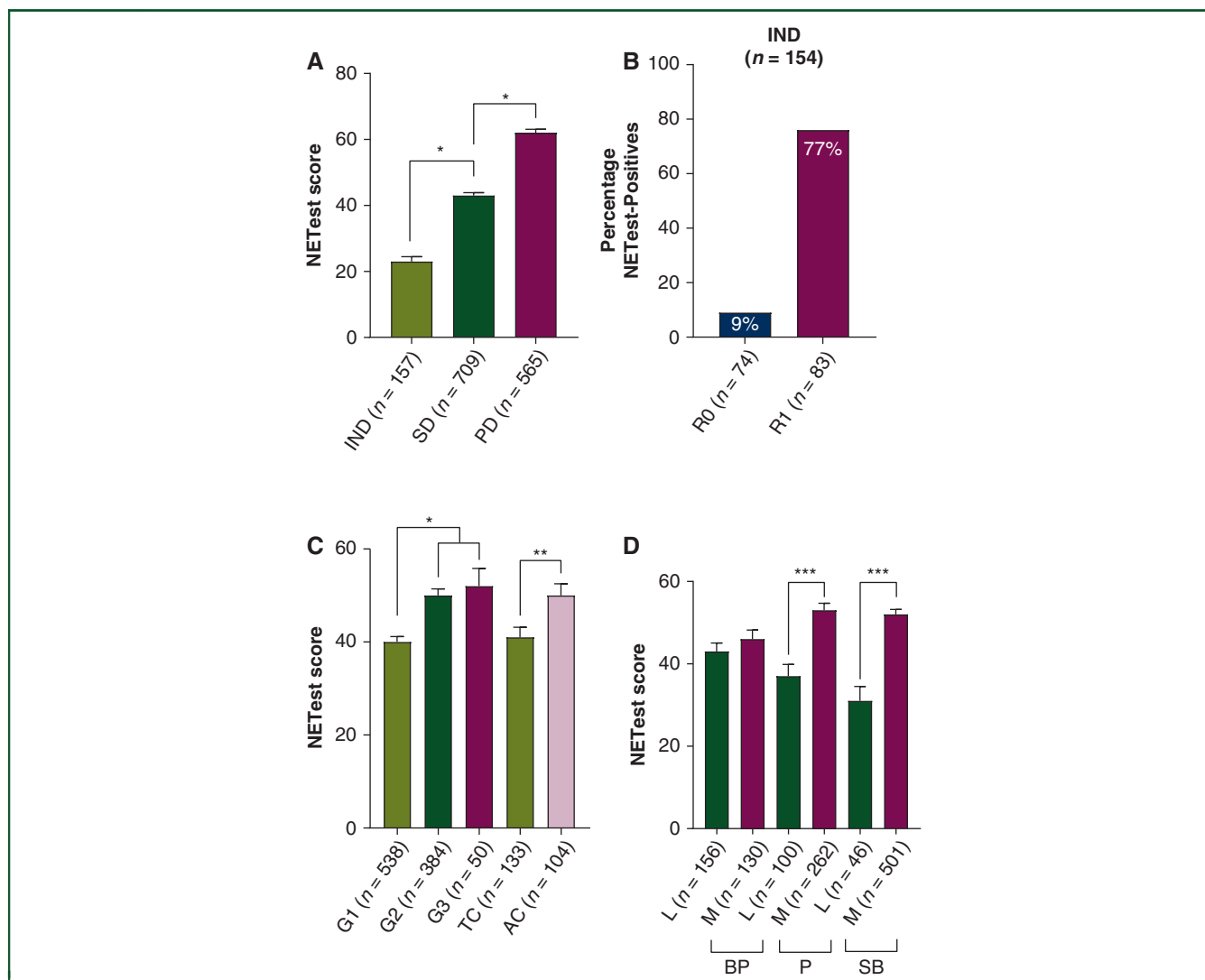


Figure 3. Correlations with clinical parameters.

(A) Clinical Disease status. NETest scores were significantly elevated in those with SD versus IND and in PD versus SD. (1-way ANOVA: $F = 191.3$, $P < 0.01$). (B) Image Negative Disease. In the IND cohort with microscopic disease (R1) the percentage positive NETest was 77% versus 9% in those with R0 resection (chi-square = 64, $P < 0.0001$). (C) Grading. NETest scores were significantly elevated in G2 and G3 tumors compared to G1. In BPNETs the AC group NETest levels were similar to G2 and G3 and were elevated compared to TC (not different from G1). (D) Disease Extent. NETest scores were significantly elevated in metastatic versus localized disease. Mean \pm standard error of mean.

AC, atypical carcinoid; BP, bronchopulmonary; IND, image-negative disease; L, localized; M, metastatic; NETs, neuroendocrine tumors; P, Pancreas; PD, progressive disease; SB, small bowel; SD, stable disease; TC, typical carcinoid.

* $P < 0.01$, ** $P < 0.05$, * $P < 0.0001$.

peptides like CgA were of value in the past. Measurement, however, reflected functionality and $\sim 50\%$ of tumors are 'non-functional' without evaluable markers.⁴ A further limitation is that little information about biological or clinical behavior is provided.² Recent European and USA consensus statements conclude that CgA is controversial and has little benefit.^{5,18}

The NETest is a next-generation, NET-multigenomic blood biomarker developed by transcriptomic analysis using deep learning strategies to specifically identify tumors with a neuroendocrine genotype.⁷ This was developed to capture the NET molecular signature² and provide real-time information regarding the biological status of a tumor. The amalgam of 51 NETest genes have been confirmed as bona fide neuroendocrine markers in a large The Cancer Genome Atlas (TCGA) dataset of $>10\,000$ samples.¹¹

In this study, the NETest diagnosed NET disease with an accuracy $>91\%$ compared to CgA ($<50\%$). NETest blood levels correlated significantly with other clinical parameters including imaging, tumor grade, the Ki-67 index, tumor size, American Joint Committee on Cancer staging, the detection of metastatic disease, and disease status. The area where NETest and CgA were comparable was gastric NETs (RR for NETest 2.0 versus 2.5 for CgA). We interpret this to reflect that gastric NETs are a pan-mucosal disease and occur against a background of antral G cell hyperplasia. Elevated CgA levels have two sources in gastric NETs: diffuse mucosal enterochromaffin-like (ECL) cell hyperplasia based on gastrin-drive and antral G-cell proliferation and secretion. Of note, stage I tumors (T1N0M0) were NETest positive in 26/46 (57%) of cases. We consider this consistent with detection by the NETest of mucosal ECL cell hyperplasia.

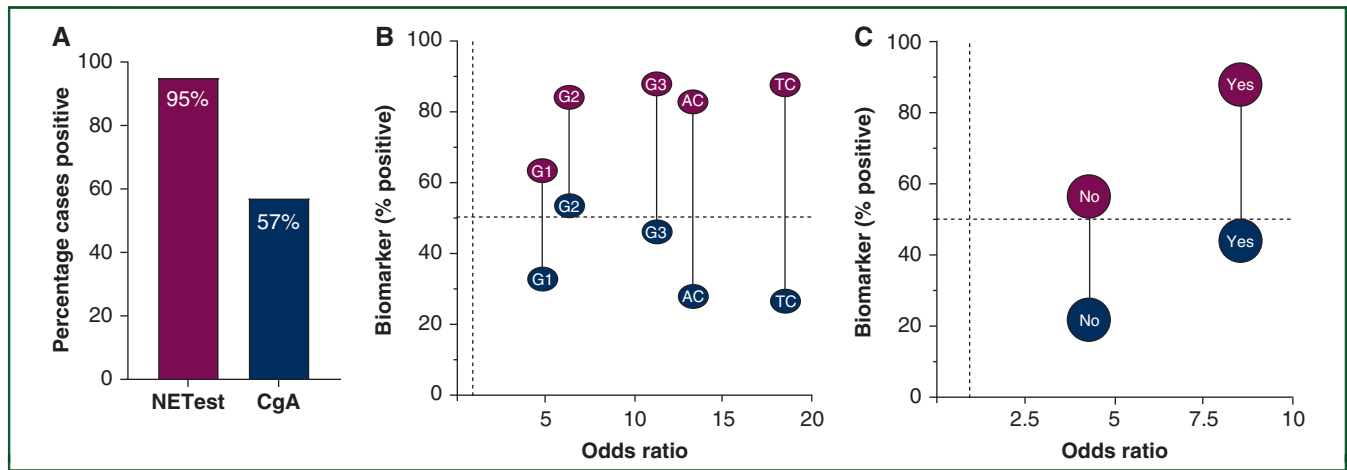


Figure 4. Cohort 2 NETest/CgA correlation with clinical parameters (n = 922).
 (A) Progressive disease. NETest 95% positive was compared to CgA 57% ($P < 0.0001$). (B) Grade analysis by McNemar test of the relationship between the percentage positivity and Odds Ratio (OR) for grades and NETest/CgA. The OR ranged from 5.03 for G1 tumors (NETest positive: 63% versus CgA-positive 33%) to 18.5 (TC: NETest positive 87% versus 27% for CgA). Overall, the NETest (burgundy circles) is 5-20x more accurate than CgA (dark blue circles) for grade. (C) Metastasis assessment using the McNemar’s test. For no metastases, the OR was 3.9. NETest was elevated in 60% versus 20% for CgA. For metastases, the OR was 8.4 (87% elevated NETest, 47% elevated CgA). Overall, the NETest (burgundy circles) is 8x more accurate than CgA (dark blue circles) for identifying metastasis. Dotted lines reflect 50% who are biomarker positive (horizontal line, 4B-C) and an OR of 1.0 (vertical line: 4B-C). AC, atypical carcinoid; CgA, Chromogranin A; TC, typical carcinoid.

Further evidence of NETest utility was provided by surgical data. This cohort provides decisive surgical and pathological evidence of tumor presence or removal and definitively identifies whether a blood biomarker represents tumor presence or absence. In preoperative assessments, the NETest was 100% elevated compared to CgA (28%). Tumor removal as judged by pathological criteria (R0/R1/R2) resulted in normalization of NETest levels in the R0 group as opposed to continued elevated levels after R1/R2 surgery (known residual disease). In 30% of the R0 group,

where the NETest failed to normalize, 74% had post-operative radiological-recurrence within 12 months and this was 84% by 24 months. At 24 months, 0% of those with normal NET levels had recurred. Post-operatively, CgA had no relationship to tumor resection or recurrence. Recent European Society for Medical Oncology guidelines (2020) noted the NETest might have clinical utility as a marker defining complete surgical removal.¹⁸ Our current study confirms this and demonstrates that a post-operative NETest can be used to accurately (94%) predict disease

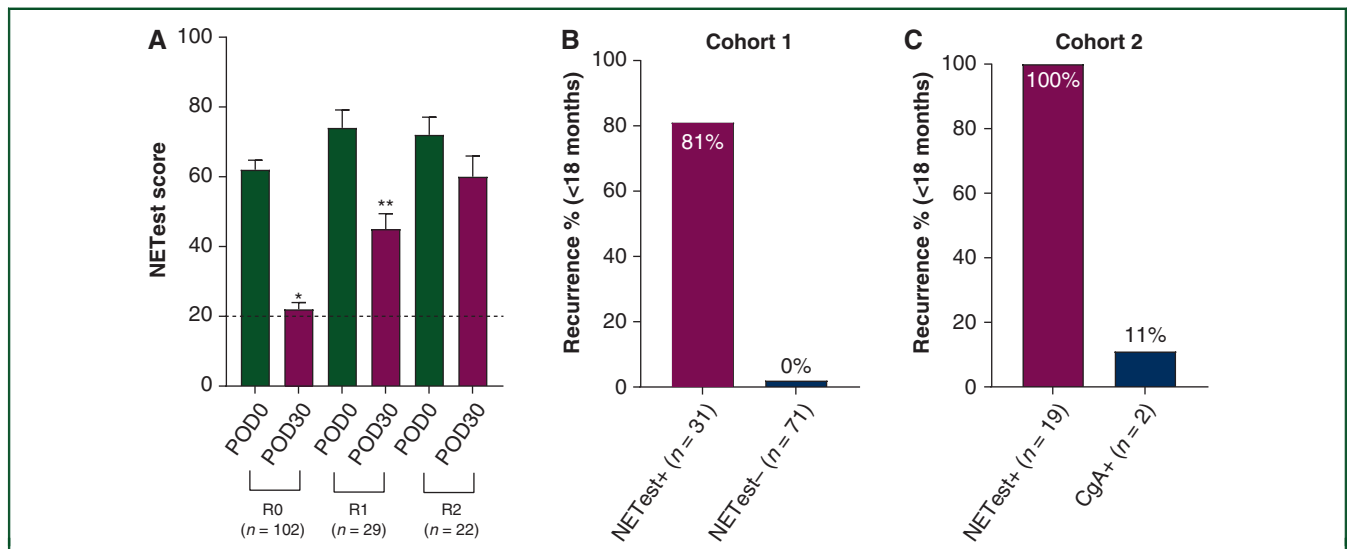


Figure 5. NETest and prediction of surgical recurrence.
 (A) Surgical strategies. R0 and R1 surgery significantly decreased NETest levels (respectively $P < 0.0001$, $P < 0.002$). R0 NETest levels were significantly decreased ($P < 0.0001$) compared to R1. R2 resection failed to decrease NETest levels. (B) Cohort 1, R0 percentage recurrence rate within 24 months. Of the 71 with a normal NETest, recurrence was 0% (0/71). Of the 31 with elevated NETest levels 25 (81%) recurred. (C) Cohort 2, R0 percentage recurrence rate within 24 months. All nineteen (100%) with an elevated NETest recurred compared to 11% (2/19) with an elevated CgA. Mean \pm SEM. Black dotted line, upper limit of normal. CgA, chromogranin A; POD0, pre-operative NETest score; POD30, post-operative day 30 NETest score. * $P < 0.0001$, ** $P < 0.002$.

recurrence. Although guidelines query CgA usage, it is reimbursed and is currently used on an *ad hoc* basis for surgery follow-up.¹⁹

Small, microscopic or minimal residual disease (MRD, <2-3 mm) cannot be detected by current imaging modalities, including ⁶⁸Ga-PET-SSA.²⁰ Previous reports have demonstrated that the NETest can identify image-negative disease.²¹ We note that ~80% of image-negative subjects with R1 disease have a positive NETest score. Given advances in detection technology, it seems likely that 'molecular' evidence of non-imageable disease is a NET reality that will need further consideration as therapeutic paradigms evolve.

As a diagnostic, the NETest was >91% accurate for detection of NETs in comparison with healthy controls and other benign and malignant diseases. This is consistent with reports by Oberg et al.¹⁰ and Malczewska and colleagues²² who assessed NETest diagnostic accuracy in large cohorts and reported values of 94%-95%.

The consideration of the NETest in other disease states including different cancers is relevant since neuroendocrine-like differentiation (NELD) has been reported in diverse neoplasia. These include breast, esophagus, colon and prostate cancers with incidences ranging from 5% (breast) to 51% (colorectal adenocarcinomas). An examination of a 10 224 TCGA tumor database by Chen et al., demonstrated that NETest gene expression and NELD at a tissue level was evident in ~4% of all tumors.¹¹ They noted that up to 32% of colon and lung cancers exhibited the NETest signature.¹¹ In our current study, ~35% of the neoplastic group had NETest scores >20. Individual tumor types ranged from 32% lung (mean NETest = 18) to 47% in pancreas (mean NETest = 23). This is consistent with blood-based detection of NELD-associated tumors expected in the non-NET neoplasia cohort. The biological and therapeutic implications of evidence of neuroendocrine elements in diverse neoplasia requires further consideration. In prostate cancer it is already considered a critical component in determining therapy and prognosis.²³

Given the selection of genes specific to neural and endocrine tumor tissue, the NETest assay detected all NETs irrespective of organ of origin. Nervous system tumors that exhibit neuroendocrine features, e.g. PPGL, expressed high levels of NETest genes and were ~100% detectable. This is consistent with their 'neuroendocrine' genotype. This is supported by the TCGA study that confirmed numerous ($n = 21$) NETest genes in nervous system-derived tumors including meningiomas and glioblastomas.¹¹

In separate reports, van Treijen et al.,⁸ and Liu et al.,⁹ described the accuracy of the NETest to differentiate stable from progressive disease as between 84% and 96%. Our study confirmed the NETest as an accurate disease status biomarker and identified that progressive disease exhibited significantly elevated scores. In all NET types examined, metastatic disease was associated with elevated scores (compared to localized disease). This is consistent with increased 'omic-cluster expression' in metastatic disease and its association with a worse prognosis.²⁴ This

relationship was also reflected in histopathological grading where an increasing grade correlated with elevated NETest blood levels. Grading, as determined by Ki67 (a mono-analyte with proliferative biological associations), has proven useful in stratifying lesions, particularly at the low and high ends of the neoplastic spectrum. The relationship to a multigenomic PCR might therefore be predicted. Our assessment of 877 gastroenteropancreatic-NETs confirmed a weak ($r = 0.09-0.22$) but statistically significant relationship. The clinical associations of the NETest support that tumor biology changes are reflected in a blood measurement and the assay captures alterations in tumor activity (including proliferation) during the evolution of NET disease.

Whether the NETest could be a surrogate for imaging is of interest. The correlation between NETest and image-positive disease (CT/MRI and ⁶⁸Ga-PET-CT) was 91%. This is similar to other studies that identified associations of 86%-95%.^{8,9,22} The NETest was also positive in ~80% of image-negative but microscopic disease-positive disease. The NETest was positive in 71 of 154 image-negative disease subjects. Sixty-four (90%) of these also exhibited MRD.

⁶⁸Ga-PET-CT is highly sensitive since it identifies somatostatin receptors on tumors. This also has theranostic implications and is used for peptide receptor radionuclide therapy (PRRT).³ Identification of receptors does not predict treatment responsiveness since presence of a target does not correspond to radio-sensitivity.³ Indeed, ~30% of PRRT-treated patients are non-responders. PRRT is costly and has well-described renal, hematological and bone marrow toxicity.³ Specific genes identified in the NETest predict PRRT efficacy with ~95% accuracy.²⁵ This provides a basis to identify individuals in whom PRRT treatment will likely be successful. We anticipate that a similar approach can be undertaken with the NETest to develop predictors for other NET treatments such as SSAs, everolimus and sunitinib.

Since there is high concordance with imaging, using the NETest to reduce diagnostic radiation exposure has been proposed.⁹ One study reported a decrease in CT usage in ~40%.⁹ While this study was not designed to assess health economic issues, there appears to be fiscal advantages to using an accurate biomarker. A blood test which costs ~\$500, provides an objective evaluation and decreases imaging modalities associated with radiation exposure (cost \$2000-\$7000) seems an attractive consideration for management. This is especially relevant in NET disease that often has a long, indolent course.

While evaluation of a novel strategy has scientific interest, an assessment of its clinical relevance by comparison to a commonly used clinical strategy (CgA) has practical relevance. Small studies have previously proposed the NETest to be more effective than blood CgA measurement.^{6-9,26} The largest NET/control group included 253 subjects with matched NETest/CgA samples.²⁶ Our study ($n = 1270$) includes other neoplasia and benign diseases and provides a significantly more substantial and diverse clinical evaluation. The large sample size negates any issues related to Type II errors.

This study, in a substantially powered and diverse NET cohort, enrolled over a 5-year time period, demonstrated the NETest provides an accurate, non-invasive strategy for real-time assessment of disease status and surgical treatment efficacy. The consideration of an accurate, non-invasive biomarker to identify disease that can be used as a surrogate, or provide synergistic information with imaging, has attractive clinical and health economic possibilities.

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