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Sensitivity and Specificity of the NETest: A Validation Study

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1 **Title:** Sensitivity and Specificity of the NETest: A Validation Study

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13 **Short title:** NETest: A Validation Study

14 **ClinicalTrials.gov Identifier:** NCT02948946

15 **Keywords:** neuroendocrine tumor, carcinoid tumor, NETest, biomarkers, tumor marker

16 **Word Count:** 2121

17 **Abstract**

18 **Background:** Secretory tumor markers traditionally measured in patients with neuroendocrine
19 tumors (NET) are lacking in sensitivity and specificity, and consequently of limited clinical utility.
20 The NETest, a novel blood multigene RNA transcript assay, has been found to be highly sensitive
21 and specific. We sought to validate the sensitivity of the NETest in a population of metastatic well-
22 differentiated NETs of gastroenteropancreatic and lung origin and evaluate the specificity in a
23 mixed population of metastatic non-NET gastrointestinal (GI) malignancies and healthy
24 individuals.

25 **Design and Methods:** 49 patients with metastatic NETs, 21 patients with other metastatic
26 gastrointestinal cancers, and 26 healthy individuals were enrolled. Samples were sent in a blinded
27 fashion to a central laboratory and a NETest value of 0-13% was considered normal.

28 **Results:** Using the upper limit of normal (ULN) of 13%, the sensitivity of the NETest was 98%
29 (95% CI, 89% - 100%). The overall specificity was 66% (95% CI, 51% - 79%), with 16 false
30 positive results. Specificity was 81% (95% CI, 62% - 92%) among 26 healthy individuals and 48%
31 (95% CI, 26% - 70%) among patients with other GI malignancies. Using an updated normal range
32 of 0-20%, sensitivity was unchanged, but specificity improved to 100% among healthy
33 participants, and 67% among patients with other cancers.

34 **Conclusions:** The sensitivity of the NETest is exceptionally high (>95%) in a population of
35 metastatic, well-differentiated NETs. Specificity within a healthy population of patients is
36 exceptionally high when using a normal range of 0-20% but relatively low when evaluating
37 patients with other GI malignancies.

38 **Introduction**

39 Neuroendocrine tumors (NETs) are a heterogenous group of neoplasms characterized by a
40 relatively indolent rate of growth and propensity to secrete a variety of hormones and vasoactive
41 peptides. Although they arise in a variety of organs, they predominantly originate within the
42 gastroenteropancreatic (GEP) tract and lungs.[1] Recent epidemiological data suggest a rising
43 incidence of NETs and increased survival durations, however the long-term outcome of patients
44 with advanced-stage disease remains poor.[2] A National Cancer Institute (NCI) summit held in
45 2007 focused on key research areas to be prioritized in NETs and noted biomarker limitations to
46 be a crucial unmet need in the management of these tumors. In fact, currently available
47 monoanalyte biomarkers (e.g. chromogranin A, urine 5-hydroxyindoleacetic acid [5-HIAA]) have
48 limited sensitivity, specificity, and predictive ability. Some novel biomarkers are in advanced
49 clinical development for NETs, including miRNAs, circulating tumor cells, and a multianalyte
50 whole blood RNA signature (NETest).[3]

51 The NETest is a novel biomarker encompassing 51 separate gene expressions which define NET
52 biology. It is a PCR-based test, which utilizes a 2-step protocol of RNA isolation and cDNA
53 production. Using a specific algorithm, the NETest provides tumor activity scores ranging from 0-
54 100% in 16 distinct categories (0, 7, 13, 20, 27, 33, 40, 47, 53, 60, 67, 73, 80, 87, 93, 100).
55 Thresholds of 0-13 or 0-20 are generally considered within normal range, >20-40 is considered
56 low range, and high-risk scores have been defined as $\geq 80\%$. [4-6] Elevated NETest scores have
57 been reported to correlate with clinical progression in bronchopulmonary NETs, predict disease
58 relapse after curative surgical resection of well-differentiated pancreatic NETs, and predict disease
59 progression in GEP-NETs. [7, 5, 8, 9].

60 The NETest is associated with very encouraging sensitivity and specificity (>90%) in patients with
61 grade 1 and 2 GEP-NETs.[10] Based on this information, a validation study was designed to
62 determine the performance metrics (sensitivity and specificity) of NETest in a real-world,
63 heterogenous cohort of NET patients compared to a cohort of controls consisting of healthy
64 subjects (without known diagnosis of cancer) and patients with other gastrointestinal (GI)
65 malignancies.

66 **Patients and Methods**

67 *Patient Selection*

68 This study was a prospective, blood collection study comprised of two cohorts: NET and non-NET
69 patients. The study protocol (NCT02948946) was approved by the Advarra Institutional Review
70 Board and conducted in accordance with Good Clinical Practice principles. Written informed
71 consent was obtained from all study participants.

72 The study was designed to enroll 100 NET and 100 non-NET subjects in two stages. In the first
73 stage, 50 NET and 50 non-NET subjects would be enrolled, and if the false positive or false
74 negative rate was <25%, the study would continue to the second stage of recruitment. A NETest
75 score of ≤ 13 was initially prospectively defined as normal. We also examined a higher cut-off of
76 20 since recent publications have reported this to be a better discriminant. [11, 9, 12]

77 Patients were eligible for the NET cohort if they had histologically confirmed NET of GEP or lung
78 origin (only stage IV, well-differentiated tumors in first stage of the study), had disease
79 documented on a diagnostic scan, were off cytotoxic chemotherapy or PRRT for at least 4 weeks
80 prior to date of blood collection, and had no other active malignancy within 3 years of enrollment,
81 with the exception of adequately treated basal cell or squamous cell skin cancer, in situ cervical
82 cancer, or any treated stage I or II cancer from which patient was in complete remission.

83 Non-NET patients were eligible if they were either healthy subjects or patients with any
84 histologically or cytologically proven diagnosis of other active GI malignancies. Patients with GI
85 malignancies with histological evidence of neuroendocrine differentiation were excluded. The plan
86 called for equal enrollment of healthy subjects, and patients with other GI malignancies.

87 *Blood Collection*

88 After written informed consent was obtained and eligibility of subjects confirmed, a single blood
89 sample was collected per patient. Blood samples were collected in 9mg K₂EDTA tubes (BD
90 Vacutainer Venous Blood Collection Tubes, BD Diagnostics, Franklin NJ). Aliquots of whole
91 blood were stored at -80°C within 2 hours of collection, per standard molecular diagnostic
92 protocols for PCR-based studies. Specimens were stored with individual information, however
93 coded and deidentified prior to shipment for analysis. Samples were sent in batches and analyzed
94 by Wren Laboratories. NETest results were sent to the principal investigator only and no
95 information was communicated to study participants.

96 Data was collected on Chromogranin A (CgA) for patients who had tumor markers drawn at or
97 around the time of NETest blood sample collection. Samples collected prior to 9/2017 were
98 analysed via the Enzyme Linked Immunosorbent Assay (ELISA) by QuestDiagnostics™. Samples
99 collected from 9/2017 onward were analyzed by ARUP labs using the Cisbio CGA-ELISA-US
100 kit.

101 *Statistical Considerations and Sample Size Calculation*

102 The primary objective of this protocol was to determine the performance metrics (sensitivity and
103 specificity) of the NETest in a real-world, heterogenous cohort of NET patients. Descriptive
104 statistics were used for patient demographics. Receiver operating characteristics (ROC) curve
105 analysis was used to assess the performance metrics of the NETest. Exact 95% confidence intervals

106 (CIs) were calculated for each proportion of interest. All tests were one-sided, and statistical
107 significance was declared at a p value of .025 or less. Statistical analysis was conducted using
108 IBM® SPSS version 25.

109 The study was designed to test the null hypotheses that the NETest has a sensitivity and specificity
110 of 70% or less in NET patients. The sample size calculation was based on the assumption that a
111 sensitivity and specificity of greater than 90% would generate further interest in the test for
112 unselected NET patients. Power and type 1 error were 99% and 5%, respectively. Under this
113 model, 80 or more positive tests in a cohort of 100 NET patients would lead to the rejection of the
114 null hypothesis, suggesting that the NETest is sensitive. Likewise, 80 or more negative tests in 100
115 non-NET patients would suggest that the NETest is specific. An interim analysis was set to be
116 conducted after enrollment of 100 patients, with plans to discontinue enrollment if a false positive
117 or false negative >25% was observed. In other terms, if more than 12 false positives or negatives
118 were observed among the first 100 subjects (50 with NET and 50 without), the study would be
119 suspended.

120 **Results**

121 49 patients with metastatic (stage IV), well-differentiated NETs of GEP or lung origin, 21 patients
122 with other active metastatic gastrointestinal cancers, and 26 healthy individuals were included in
123 this analysis. Table 1 represents the NET patient demographics. A NETest value of 0-13% was
124 prospectively considered within normal range, but an alternate NETest value of 0-20% was also
125 evaluated, given several recent reports using this cutoff.[11, 9, 12] By ROC curve analysis (Figure
126 1), sensitivity was 98% (95% CI, 89-100%) for both 13% and 20% cutoff ranges, corresponding
127 to a single false-negative result in a patient with widely metastatic, somatostatin receptor positive

128 rectal NET, and confirming that the NETest was a sensitive assay. NETest scores for all
129 participants are depicted in Figure 2.

130 Using the 0-13% cutoff, specificity was 66% (95% CI, 51% – 79%) for all non-NET participants,
131 corresponding to 16 false positive tests among 47 patients with either other GI cancers or no cancer
132 (Table 2). Among healthy participants, the specificity was 81% (95% CI, 62% - 92%),
133 corresponding to 5 mildly elevated NETest results out of 26 patients. Among 21 patients with other
134 GI cancers, the specificity was 48% (95% CI, 26% – 70%), corresponding to 11 false positive tests
135 which included metastatic adenocarcinomas of the colon (n=6), pancreas (n=2), stomach (n=1),
136 esophagus (n=1) and appendix (n=1). Using the cut-off of 13, we could not reject the null
137 hypothesis. The assay specificity therefore was too low using this score and confirms the reports
138 supporting the use of a higher (20) cutoff level.

139 Using the 20% cutoff (Table 3), however, the specificity was 85% (95% CI, 72% - 94%). It was
140 100% (95% CI, 87% – 100%) among healthy participants and 67% (95% CI 43% - 85%) among
141 patients with other cancers. In total, 7 patients, all with other GI cancers (including colon [n=5],
142 pancreas [n=1] and esophagus [n=1]), had false positive results, whereas no healthy subjects had
143 false-positive results.

144 Of the 48 NET patients who had true positive NETest results, there was a dichotomous distribution
145 of results with 15 patients having a score of 27% and 14 having a score of 93% (Figure 2). Tumor
146 burden and disease status of the TP NETest patients are represented in Table 4. Tumor burden was
147 assessed by investigator. High tumor burden corresponded to >20% liver involvement and/or
148 bulky extrahepatic disease, low tumor burden corresponded to <10% liver involvement and
149 minimal extrahepatic disease, and patients who fell into neither category were defined as having
150 moderate tumor burden.

151 Among patients with low-risk scores of 27% and 33% (n=17), 11 had stable disease and 6 had
152 progressive disease at time of blood collection. Among patients with high risk scores of 87%, 93%
153 and 100% (n=19), 13 had stable disease and 6 had progressive disease at time of NETest blood
154 collection.

155 29 of the NET patients (small bowel [n=22], pancreatic [n=5], unknown primary [n=1]) had serum
156 Chromogranin A (CgA) drawn at or around the same time of NETest blood collection. 16 patients
157 had elevated CgA and 13 had normal (false negative) CgA, resulting in a sensitivity of only 55%.
158 There was no statistically significant correlation between NETest score (high vs. low) and CgA
159 results (elevated vs. normal) (p=0.832).

160 We discontinued the study after the planned interim analysis due to the false positive rate of >25%
161 using the 13% cutoff per original protocol. Using the updated cut-off of 20, the false positive rate
162 (18%) would have allowed us to continue the study.

163 **Discussion**

164 NETs are a heterogeneous group of tumors predominantly originating from the GEP tract and
165 lungs, with a high propensity to secrete various hormones and vasoactive peptides, and recently
166 increasing in incidence and survival durations. Approximately 40% of patients with NETs are
167 diagnosed with stage IV disease. Given the heterogeneity of this disease, the available biomarkers
168 have poor sensitivity, specificity and predictive ability. The NETest has been associated with high
169 levels of sensitivity and specificity, and our study was designed to determine the performance
170 metrics in a real-world, heterogeneous cohort of NET patients compared to non-NET patients
171 comprised of both healthy participants and those with other GI malignancies.

172 We found that the NETest was highly sensitive (98%) for patients with metastatic NETs of varying
173 sites of origin, with no difference using either cutoff range ($\leq 13\%$ or $\leq 20\%$). The sensitivity of

174 corresponding CgA levels was 55%. Using the 13% cutoff, we found that the NETest was
175 moderately specific, with a specificity of 63% among non-NET patients as a whole, and more
176 specific among healthy participants with a specificity of 81% as opposed to 44% among patients
177 with GI cancers. We found that using the $\leq 20\%$ cutoff, which has been reported in several recent
178 manuscripts, resulted in a higher specificity among all patients (82%): 100% specificity among
179 healthy participants, and 64% specificity among patients with other GI cancers. The large majority
180 of false-positive results in this study comprised of patients with other GI cancers. This is not
181 surprising given the fact that several of the NETest parameters, such as proliferation and
182 metabolism transcripts, are not unique to NETs. Similar observations regarding elevated NETest
183 scores in non-neuroendocrine tumors of the lung have been noted. [5, 9] However, the relatively
184 low specificity observed in our study with respect to other GI cancers stands in contrast to a prior
185 evaluation of the NETest in patients with carcinomas of the GI tract and pancreas in which only
186 3/54 patients had positive NETest results (Specificity 94%). [4]

187 The limitations of this study are its relatively small sample size and single blood sample collection.
188 Measurement of serial blood samples at various timepoints during a patient's treatment (pre- and
189 post-progression) may give a better indication as to whether or not NETest score was indicative of
190 progressive trends in tumor burden as has been demonstrated in other studies.[13, 7] Evaluating
191 the NETest score in patients with non-NET malignancies of various stages and disease status may
192 also lead to a more definitive understanding of the specificity of the test and its clinical utility in
193 these tumors. Finally, CgA assays vary in sensitivity and while use of a higher sensitivity assay
194 may have improved the accuracy of the test, our findings reflect a real-world evaluation of CgA
195 measurements.

196 **Conclusion**

197 The sensitivity of the NETest is exceptionally high in a population of metastatic well-differentiated
198 NETs. Specificity within a healthy population of participants is exceptionally high when using a
199 normal range of 0-20% and moderately high when using a normal range of 0-13%. Specificity is
200 relatively low when evaluating patients with other GI malignancies.

201

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203 contributions to study design, analysis of the blood specimens, and interpretation of the data.

204 **Statement of Ethics:**

205 The protocol was approved by the institutional review board and the study was conducted in
206 accordance with Good Clinical Practice principles. Written informed consent was obtained from
207 all participants.

208 **Disclosure Statement:**

209 Dr. Strosberg has consulted for Novartis and has received honoraria from Ipsen and Lexicon.

210 Tiffany Valone has received honoraria from Abbvie, Genentech, Ipsen, Lexicon and Novartis.

211 None of the other authors declares a personal or financial conflict of interest which could affect
212 the outcome of this study.

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214 & Research Institute

215 **Author contributions:** J.S. and M.C. contributed to the conception and design of the protocol.

216 T.V. and K.B. contributed to the acquisition of the data/enrollment of patients. J.S., M.C., and

217 T.A., contributed to data acquisition, analysis, interpretation of data, drafting and revising the

218 manuscript. All authors reviewed and approved the final version of this manuscript, and agree to

219 be accountable for all aspects of the work and its accuracy.

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261

262 **Figure and Table Legends**

263 **Table 1.** NET Patient Demographics

264 **Table 2.** Specificity using 13% cut-off

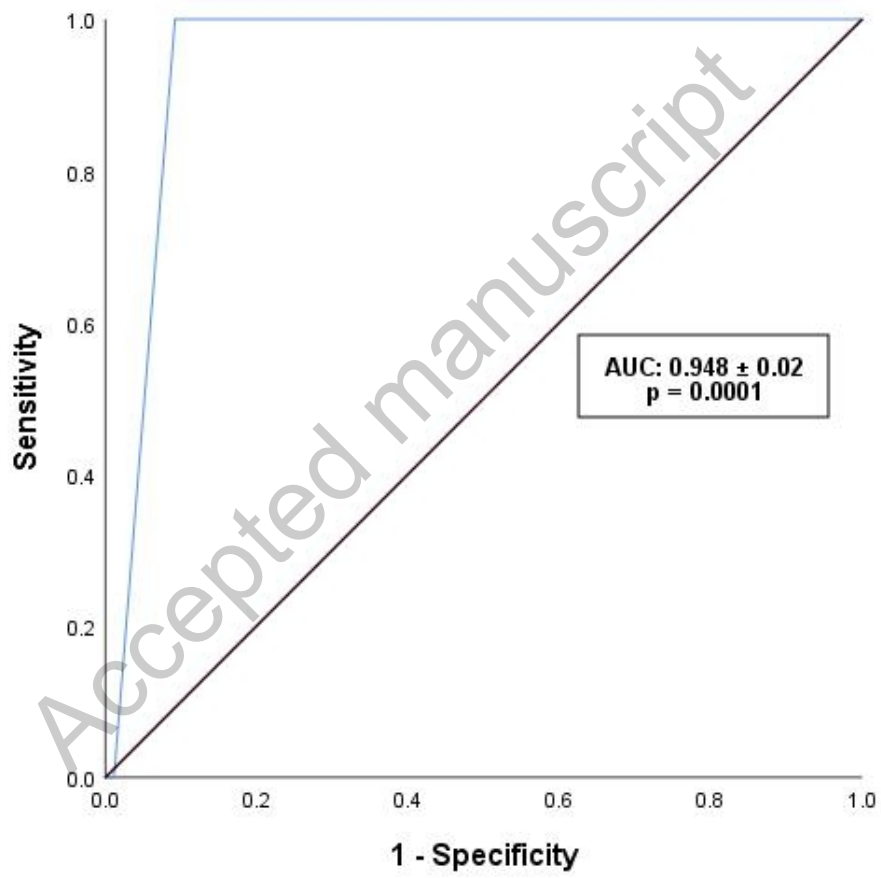
265 **Table 3.** Specificity using 20% cut-off

266 **Table 4.** Disease status and tumor burden of true-positive NET patients.

267 **Figure 1.** Performance metrics of the NETest by ROC curve analysis

268 **Figure 2.** NETest Scores

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NETest Scores

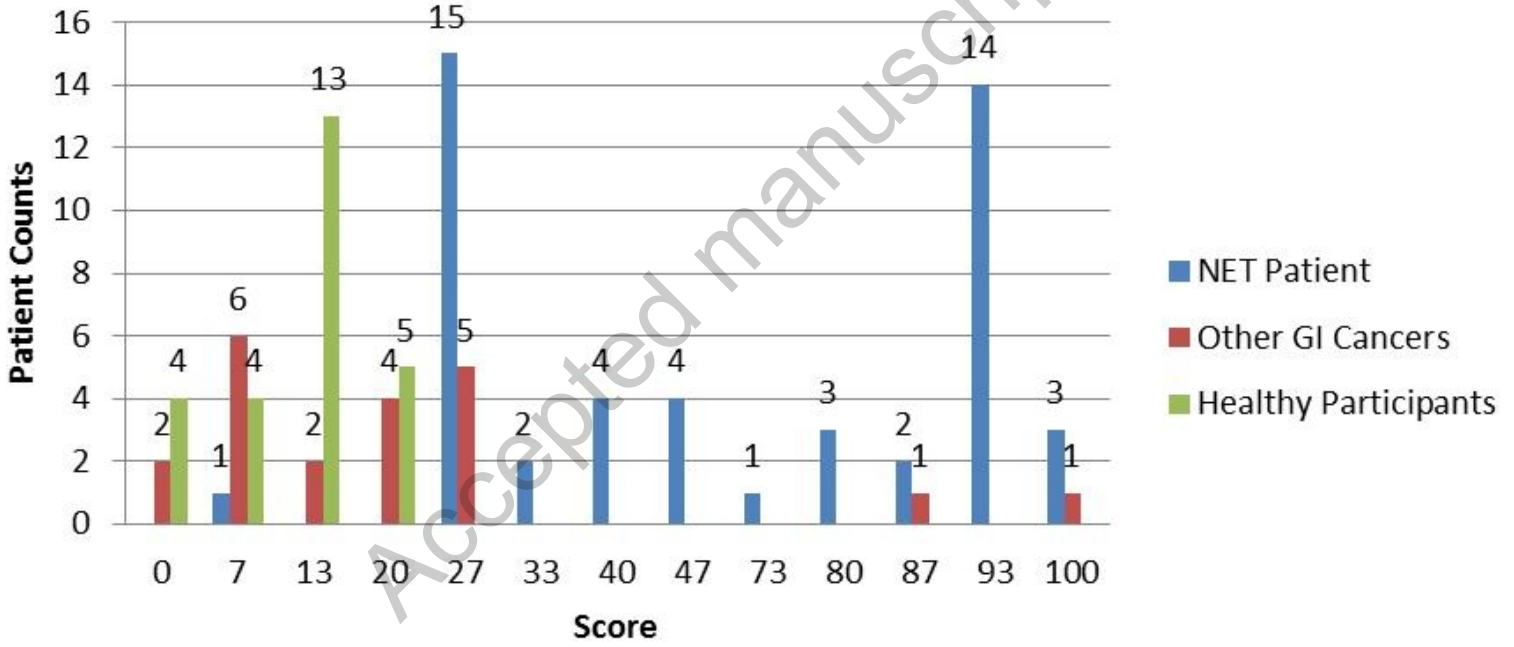


Table 1. NET patient demographics.

	N (%)
Primary tumor	
Small bowel	24(49%)
Pancreatic	18 (37%)
Rectal	3 (6%)
Gastric	2 (4%)
Lung	1 (2%)
Unknown primary	1 (2%)
Tumor grade	
Grade 1 (Ki-67% <3)	20 (41%)
Grade 2 (Ki-67% 3-20)	23 (47%)
Grade 3 (Ki-67% >20)	1 (2%)
Well-differentiated, unspecified	5 (10%)
Disease Status	
Progression	15 (31%)
Stable disease	34 (69%)
Treatment status	
On active treatment	40 (82%)
Observation only	9 (18%)

Table 2. Specificity using 13% cut-off.

<i>*Using 13% cut-off</i>	Specificity	True Negative	False Positive
All Non-NET participants	66% (95% CI 51% - 79%)	31	16
Healthy Participants	81% (95% CI 62% - 92%)	21	5
Other GI Cancers	48% (95% CI 26% - 70%)	10	11

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Table 3. Specificity using 20% cut-off.

<i>*Using 20% cut-off</i>	Specificity	True Negative	False Positive
All Non-NET participants	85% (95% CI 72% - 94%)	42	9
Healthy Participants	100% (95% CI 87% - 100%)	26	0
Other GI Cancers	67% (95% CI 43% - 85%)	14	7

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Table 4. Disease status and tumor burden of true-positive NET patients.

		Disease Status	
		Stable (n, %)	Progressive (n, %)
Disease Burden	Low	19 (40%)	1 (2%)
	Moderate	10 (20%)	6 (12%)
	High	4 (8%)	8 (17%)

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