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RESEARCH ARTICLE

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Measurement of circulating transcript levels (NETest) to detect disease recurrence and improve follow-up after curative surgical resection of well-differentiated pancreatic neuroendocrine tumors

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Background: Recurrence of pancreatic neuroendocrine tumors (pNET) after surgery is common. Strategies to detect recurrence have limitations. We investigated the role of clinical criteria and the multigene polymerase chain reaction–based NETest during post-operative follow-up of pNET.

Methods: We studied 3 groups of resections: R0 with no recurrence (n = 11), R0 with recurrence (n = 12), and R1 with no recurrence (n = 12). NETest levels (>40%) were compared with chromogranin A (CgA) and clinicopathological criteria (CC; grade, lymph node metastases, size). Nonparametric, receiver operating characteristics, logistic regression, and predictive feature importance analyses were performed.

Results: NETest was higher in R0 with recurrence (56 ± 8%) compared with R1 with no recurrence (39 ± 6%) and R0 with no recurrence (28 ± 6%, $P < .005$). NETest positively correlated with recurrence (area under the curve: 0.82), CgA was not (area under the curve: 0.51 ± 0.09). Multiple regression analysis defined factor impact as highest for NETest ($P < .005$) versus CC ($P < .03$) and CgA ($P = .23$). NETest gave false positive or negative recurrence in 18% using a 40% cutoff. Logistic regression modeling of CC was 83% accurate; it was 91% when the NETest was included. Combining CC and NETest was approximately 2× more effective than individual CC alone (increase in R^2 value from 43% to 80%).

Conclusions: A multigene blood test facilitates effective identification of pNET recurrence, prediction of disease relapse, and outperforms CgA.

KEYWORDS
liquid biopsy, NETest, neuroendocrine tumors, pancreas, recurrence

1 | BACKGROUND

Although the majority of patients with pancreatic neuroendocrine tumors (pNET) are diagnosed at an advanced stage, improvements in imaging modalities, awareness of the disease, and pathological recognition have contributed to the improvement in detection of localized disease.[1-3] For patients with nonfunctioning pNET ≥20 mm in size without distant metastasis, complete surgical resection is

recommended as the primary curative strategy.[4,5] Thereafter, effective follow-up programs are designed to detect recurrence at an early stage, given that treatment of limited disease has the most favorable outcome.[6-8] However, data on post-curative surgical recurrence remains limited, making it challenging to determine the best follow-up strategy. In general, recurrence is thought to occur sporadically, yet some studies report rates up to 48%.[9] Furthermore, recurrence is known to be an independent predictor for a poor 10-year disease-specific survival.[10]

A key unmet need in improving outcome is the early detection of recurrent disease and the timely initiation of treatment after pNET resection. In many cases, early detection of recurrence offers more favorable treatment options, sometimes with curative intent, such as resection of the remnant pancreas or solitary liver metastases.[11,12] Liver-directed, locally ablative procedures are recommended for patients with limited, nonresectable tumor burden.[13-15] When recurrence is discovered with an extensive disseminated disease, systemic treatment is often the only option.[16] Despite the variety of treatment options, there is uncertainty with regard to the optimal treatment regimen. Newly introduced molecular-based markers, along with clinical trials comparing the efficacy of treatment modalities, offer a chance to move the treatment of neuroendocrine tumor disease toward personalized patient care. Given the multiple treatment options available for NET disease, the early detection of recurrence and the judicious introduction of therapy should be considered to optimize pNET outcome.

Current guidelines to evaluate tumor recurrence recommend radiological examinations and biomarker evaluation during follow-up.[4,5] Chromogranin A (CgA) used to be considered as the most useful biomarker for detection of metastases after curative resection of pNET.[17] However, its low sensitivity of 67% and specificity of 68%, as well as controversy regarding technical criteria of the assay, have led to a significant diminution in enthusiasm for its clinical utility.[17,18] Overall, the general accuracy of CgA is moderate, given its poor metrics as a biomarker and the high false positives noted.[19-21] Although some reports describe an increased diagnostic accuracy with a high tumor load, this is of limited value in an early detection and treatment strategy.[17,22] The limited clinical utility of CgA and the overall lack of efficacy of monoanalyte peptide or amine secretory biomarkers has led to considerable interest in developing novel and effective tools for the surveillance of neoplasia. In this respect, considerable attention has focused on the evaluation of the molecular characteristics of cancer and the development of sensitive techniques to define the molecular biology of the tumor as opposed to measuring its secretory products. The term liquid biopsy has been coined to describe the technique of detecting a tumor in blood and has been effectively used in other cancers including breast and colon.[23,24] Recently, a liquid biopsy strategy for neuroendocrine tumors has been described.[25] It comprises a multianalyte polymerase chain reaction–based blood test specific for neuroendocrine tumors (the NETest) with a sensitivity and specificity of >93% for diagnosis.[25] This multianalyte biomarker tool has been successfully used to demonstrate residual disease and the early detection of recurrent disease after surgical resections in small bowel and lung NETs.[26,27]

This prospective surgical cohort study aimed to determine the prognostic accuracy of neuroendocrine transcript expression in blood, compared with CgA and other known clinical criteria associated with pNET recurrence, to determine its usefulness as a biomarker for assessment of surgical efficacy and detection of recurrence after curative resection.

2 | MATERIALS AND METHODS

2.1 | Patient selection

All patients that were operated on for pNET in the Academic Medical Center Amsterdam between 2006 and 2015 were screened for inclusion (Figure 1). The pathology reports of all pancreatic resections in the selected period were reviewed for the diagnosis of pNET. Only patients with histologically confirmed diagnosis of a pNET were eligible for enrollment to the study. Included were 35 patients of 18 years or older and surgically treated for Grade I or II localized pNET (*per* pNET Ki-67 cutoff classification[28]) without distant metastases or hereditary syndromes at initial diagnosis. The study group demographics and clinicopathological characteristics are included in Table 1.

Patients were divided into 3 groups based on the pathological assessment of the pancreatic resection margins and the clinical disease status at the time of the blood draw. Resection margins were classified according to the Royal College of Pathologists. Completely excised tumors were classified as R0, whereas tumors with microscopic margin involvement <1 mm were classified as R1. Assessment of completeness of surgical resection and disease staging at the time of initial diagnosis or during follow-up was based

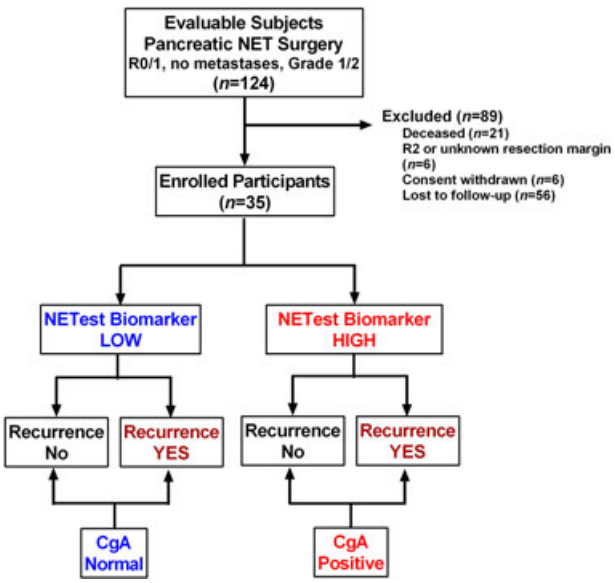

FIGURE 1 STARD diagram outlining the study. NETest Low (scores <40%) and NETest High (scores >40%). CgA normal: values <108 ng/mL; CgA positive: values >108 ng/mL. CgA, chromogranin A; STARD: Standards for Reporting Diagnostic Accuracy [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Patient and tumor characteristics

	R0 with no recurrence (R0NR) (n = 11)	R0 with recurrence (R0R) (n = 12)	R1 resection with no recurrence (R1NR) (n = 12)
Male:Female	4:7	6:6	6:6
Age, median (IQR)	63 y (59-65)	62,5 y (61-64)	57 y (50-59.5)
F:NF	0:11	1:11	5:7
Tumor location			
Head	6	4	7
Body	0	3	4
Tail	5	5	1
Surgical resection (n)			
Pancreaticoduodenectomy	6	3	1
Central pancreatectomy	0	2	3
Left pancreatectomy	5	6	2
Total pancreatectomy	0	0	0
Enucleation	0	1	6
Grade I	73% (8/11)	42% (5/12)	100% (12/12)
Grade II	27% (3/11)	58% (7/12)	0% (0/12)
Tumor size, median (IQR)	20 mm (17-53)	45 mm (27-63.8)	15 mm (7.8-27.5)
Lymph node metastases	27% (3/11)	75% (9/12)	8% (1/12)
Perineural invasion	50% (3/6)	67% (4/6)	0% (0/3)
Vascular invasion	50% (4/8)	91% (10/11)	0% (0/5)
Follow-up, median (IQR)	31 months (24-47)	105 mo (54.8-125.3)	92.5 mo (61.8-115.8)
NETest score (%), median (SD)	27 (6.4)	50 (26)	27 (22)
CgA level (ng/mL), median (SD)	67 (439)	62 (268)	81 (315)
Time from surgery to blood collection, median (IQR)	18 mo (1-33)	104 mo (44.3-125.3)	91.5 mo (60-105.8)
Recurrence	-		-
Local		25% (3/12)	
Regional		17%% (2/12)	
Distant		58% (7/12)	
Time to recurrence, median (IQR)	-	37.5 mo (26-58.3)	-
Follow-up after recurrence, median (IQR)	-	50 mo (20.8-94.8)	-
Time from recurrence to blood collection, median (IQR)	-	49.5 mo (4.5-93.8)	-
Time from blood collection to the last follow-up, median (IQR)	18 mo (6-22)	5.5 mo (0-15)	2.5 mo (0.3-9)

CgA, chromogranin A; IQR, interquartile range; SD, standard deviation; mo, months.

upon anatomical imaging (computed tomography [CT]/magnetic resonance imaging [MRI]). Tumor recurrence was defined as local recurrence in the remnant pancreas, new localization in lymph nodes (LNs), or the development of distant metastases after initially being rendered free of disease, and was diagnosed in accordance with RECIST 1.0 criteria.

The 3 groups comprised of: (1) R0 resection and no signs of recurrence during follow-up (R0NR); (2) R0 resection and evidence of recurrent disease on imaging during follow-up (R0R); (3) R1 resection and therefore residual tumor in situ, without evidence of recurrence on imaging (R1NR).

The medical records, pathology reports, radiological imaging reports, and operation reports were reviewed for the demographics and clinicopathological data, including patient's age at the time of surgery, sex, tumor functionality, tumor location within the pancreas, type of surgery, tumor size (based on post-operative pathology), grade, LN involvement, and perineural and vascular invasion. Radiological imaging consisted of abdominal CT or MRI scans and in some patients,

endoscopic ultrasonography of the pancreas and/or functional imaging (Octreoscan or ^{68}Ga-DOTATATE PET/CT) were performed. Use of proton pump inhibitors (PPIs) was determined. A control group of healthy volunteers was included. The local Medical Ethics Committee approved the study (protocol number: NL50925.018.15).

2.2 | Sample collection

Two blood samples per patient were collected according to the local protocol of the laboratory in the outpatient clinic of the Academic Medical Center Amsterdam. Following informed consent, each patient donated two 5 mL whole blood samples collected into ethylenediaminetetraacetic acid tubes. The blood draw was combined with other regular blood tests performed during the follow-up. After sampling, 1 blood specimen was immediately stored at −20°C, whereas the second ethylenediaminetetraacetic acid tube was spun at 800 rpm (10 minutes) to separate plasma dedicated for CgA measurement by enzyme-linked immunosorbent assay, as previously described.[29,30] Thereafter, both samples were stored at −80°C within 2 hours from blood collection.

2.3 | NETest blood measurement

Details of PCR methodology, mathematical analysis, and validation have been previously published in detail.[31-34] In brief, this comprises a 2-step protocol (RNA isolation, complementary DNA production, and PCR)[29,31,32] from ethylenediaminetetraacetic acid–collected whole blood.[29,31,32] Target transcript levels are normalized and quantified versus a population control.[31] Thereafter, multianalyte algorithm analyses are undertaken. Final gene expression results are expressed as an activity index score from 0% to 100%,[33] based on the integration of the majority vote and summated expression of 5 gene clusters that include the proliferome, epigenome, growth factor signalome, and genes involved in pluripotency.[33]

2.4 | CgA enzyme-linked immunosorbent assay

CgA was measured using the NEOLISA™ CgA kit (EuroDiagnostica, Malmo, Sweden).[35] CgA enzyme-linked immunosorbent assay normal values were ≤108 ng/mL.[34]

2.5 | Data analysis

The primary outcome was obtaining the NETest score of the patients in the 3 various pNET groups as specified above and the control group. A NETest score between 0% and 100% was obtained and a value of >20% was considered as a positive test.[25] Scores ranging between 0% and 20% were considered as negative. Previous studies have identified that a cutoff of 40% differentiates low activity (stable) disease from active (progressive) disease.[34,36] Therefore, scores ranging between 41% and 100% were evaluated as predictive of disease recurrence. Scores for each patient and each group were collated and assessed. In addition, CgA levels were compared with the NETest score. Data are presented as mean ± standard error of mean (median:[interquartile ranges]).

2.6 | Statistical analysis

Statistical analysis was performed using SPSS Statistics for Windows version 23.0 (IBM Corp., Armink, NY), Prism 6.0 for Windows (GraphPad Software, La Jolla, CA; www.graphpad.com), and MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; www.medcalc.org; 2013). Sensitivity comparisons using Fisher's exact test, nonparametric tests, and receiver operating characteristics analysis were made between the NETest and CgA and/or other selected tumor clinicopathological characteristics known as predictive for recurrence. The accuracy of each of the variables separately was compared with the NETest, as well as in various combinations, using receiver operating characteristics curve analyses and the sensitivity, specificity and area under the curve (AUC) were calculated. Area under the curves were compared and the *Z*-statistic (values >1.96 are significant) derived and the Youden J index (performance of a diagnostic) was calculated. Multiple regression and logistic regression analyses were undertaken to identify which parameters were associated with recurrence. The odds ratio (OR), $\chi 2$ value, and Nagelkerke R^2 coefficient (coefficient of determination) were derived to assess the strength of the association or "relatedness" of each factor or combination of factors to recurrence.[37] Predictive feature importance analysis (FIA) was undertaken to define the "importance value" for each factor (biomarker or clinical criterion) alone or in combination. Importance values were derived using a random forest approach that evaluates the output from decision tree algorithms used to define the relationship of a variable for example, a biomarker, to an output for example, recurrence. A random forest model is generated with 10-fold cross-validation and examined to determine mean decreases in the Gini coefficient.[38] The Gini coefficient provides a measure of how each variable contributes to the structure of a random forest plot. Variables that result in nodes with higher purity (ie, more accurately model disease recurrence) have a higher decrease in Gini coefficient.[39] As such, the greater the decrease in the Gini coefficient, the greater the "Importance" value and better the relation to predicting recurrence. Biomarkers (CgA, NETest) and 3 clinical variables criteria (tumor grade, size, and LN involvement) were each evaluated to determine which factor (or combination of factors) had the highest "Importance" value score.

3 | RESULTS

3.1 | Demographics and follow-up

Patients and tumor characteristics are presented in Table 1. In total, 35 patients were included: 11 patients with R0NR, 12 patients with R0R, and 12 patients with R1NR. Duration of follow-up from surgery to the last hospital visit was significantly shorter for patients in the R0NR group compared with R0R and R1NR ($P < .002$; Figure 2). Three patients died during follow-up, of which 1 death was pNET related. The healthy control group consisted of 6 male and 5 female volunteers, with a median age of 42 years (interquartile range, 32.5-53.5).

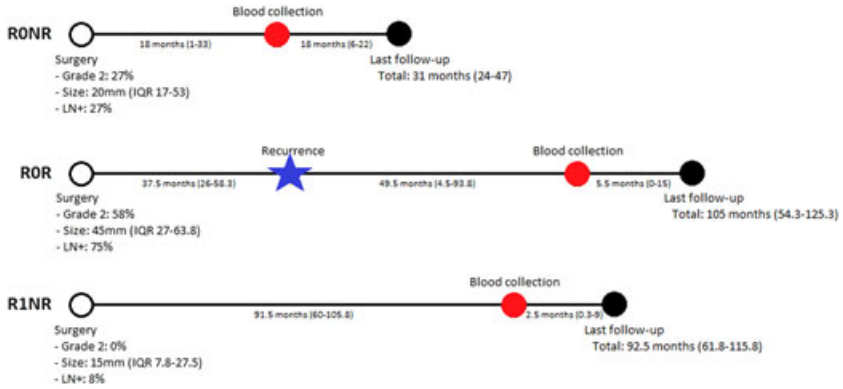

FIGURE 2 Diagram defining the median times of blood collection, last follow-up, and recurrence. LN, lymph node; R0NR, R0 with no recurrence; R0R, R0 with recurrence; R1NR, R1 resection with no recurrence [Color figure can be viewed at wileyonlinelibrary.com]

Nineteen (54%) of the 35 patients were using PPIs at the time of blood collection; 7/11 (64%) R0NR, 7/12 (58%) R0R, and 5/12 (42%) R1NR. Ten of the 12 (83%) with recurrence received therapy to treat the disease relapse. This included somatostatin analogs (n = 5), chemotherapy (n = 2), embolization (n = 1), metastasectomy (n = 1) and peptide receptor radionuclide therapy (PRRT) (n = 1). Collection of blood samples was either after or during these treatments. Two patients did not receive therapy for their recurrence. All patients without recurrence did not receive any systemic therapy between resection and collection of the blood samples.

3.2 | Biomarker evaluation in controls and pNET cohorts

The NETest scores were significantly elevated (56 ± 8%: [50%:28.8-85.25]) in the R0R cohort compared with R0NR (28 ± 2%: [27%:20-40], $P = .004$) (Figure 3A). Levels were not significantly different in R0R compared with R1NR (39 ± 6%: [27%:27-40], $P = .08$). All pNET cohorts, irrespective of recurrence, had higher levels ($P < .05$) than controls (19 ± 5%: [20%:13-20]). CgA levels were not significantly different ($P = .62$-$.94$) between any of the 3 pNET cohorts (mean: 205-244 ng/mL; median 62-18 ng/mL; Figure 3B). Levels were higher in the nonrecurrence (NR) cohorts ($P < .05$) than in controls (44 ± 5 [40:32-56]); however, CgA was not significantly different ($P = .14$) between R0R and controls.

3.3 | Correlation between biomarkers and recurrence

The NETest cutoff of 40%[36] determined that the area under receiver operating characteristic curve (AUROC) for differentiating recurrence from NR was 0.82 ± 0.08, Z-statistic: 4.19; $P < .0001$ (Figure 4). The Youden index (J) was 0.64. Using the upper limit of normal (108 ng/mL) as the cutoff for CgA, the AUROC was 0.51 ± 0.09. The Z-statistic was 0.14 and the Youden index (J) of 0.02 are not significant ($P = .88$). A comparison of the NETest and CgA identified the AUC was significantly better for the former. The difference between areas was 0.31 ± 0.14; Z-statistic: 2.01; $P = .044$.

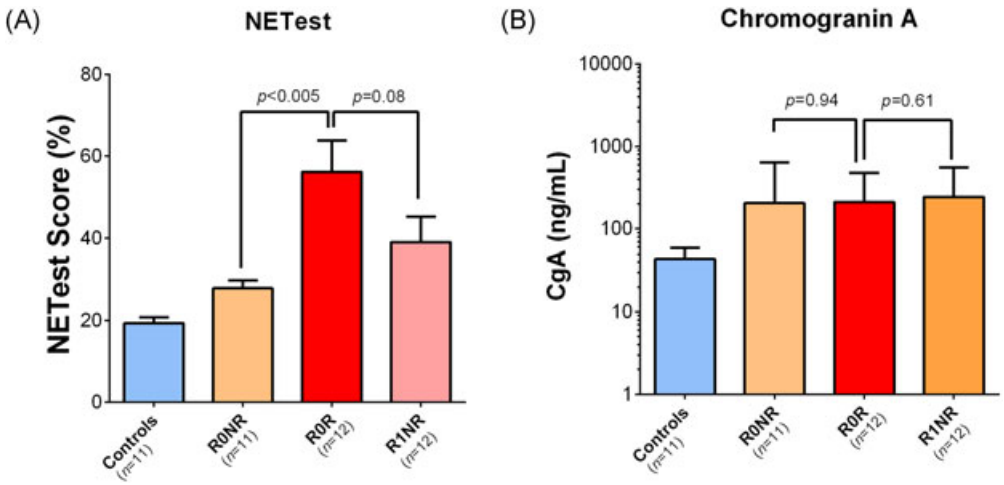

FIGURE 3 NETest expression and CgA levels in controls and cohorts. (A) NETest scores were significantly elevated in the R0 resection cohort with recurrence (R0R) compared with the resection cohort that did not recur (R0NR). Levels were similar between R0NR and R1 resection with no recurrence (R1NR: $P = .08$). (B) CgA levels were not significantly different between any of the pNET cohorts irrespective of the presence of recurrence or no recurrence. Mean and standard error of mean are indicated. CgA, chromogranin A; pNET, pancreatic neuroendocrine tumors [Color figure can be viewed at wileyonlinelibrary.com]

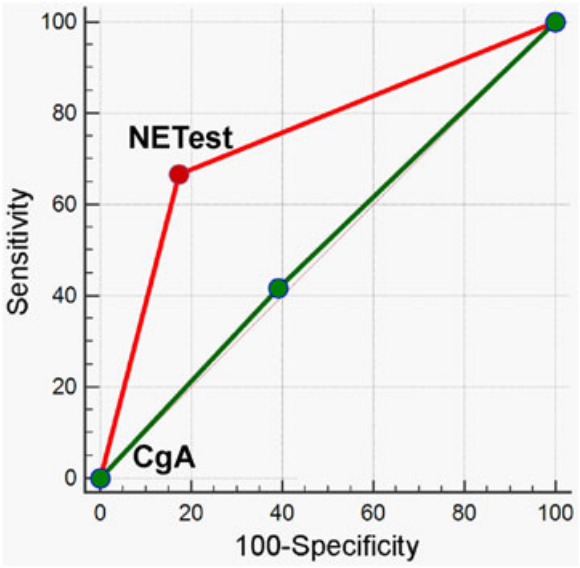

FIGURE 4 Receiver operator curve analysis for the identification of recurrence. NETest: The AUROC for differentiating recurrence from no recurrence using NETest was 0.82 ± 0.08; 95%CI, 0.65-0.93; $P < .0001$. CgA: The AUROC for differentiating recurrence using CgA (normal versus elevated) was 0.51 ± 0.09; 95%CI, 0.34-0.69; $P = .88$. NETest (red line); chromogranin A (CgA; green line). An AUC of 0.5 is indicated by the thin diagonal line behind the CgA AUC line (green). AUC, area under the curve; AUROC, area under the receiver operator characteristic [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Evaluation of PPIs, tumor burden at recurrence, and biomarkers

NETest levels were not different in those using PPIs (44 ± 6%: [33%:27-73]) compared with nonusers (38 ± 6%: [27%:27-41]). CgA levels were higher in those using PPIs (278 ± 86 ng/mL: [142:55-451]) compared with nonusers (153 ± 69 ng/mL: [55:37-93]). This did not reach statistical significance ($P = .059$).

In the R0NR group, CgA was elevated in 3 of 7 (43%) on PPI and in 1 of 4 (25%) not using a PPI. CgA was therefore elevated by PPI in 43% of surgically "cured" patients when it was used.

In the R1NR group, CgA was elevated in 3 of 5 (60%) on PPI and in 2 of 7 (29%, no PPI). This indicates that approximately 17% of all R1NR have a "true" elevated CgA level, that is, CgA related to neuroendocrine tumor disease. All patients with residual disease had single LN-positive disease. This indicates that an elevated CgA is associated with <20% of lymph node–positive disease. The NETest was low (but positive >20%) in all R1NR patients consistent with accurate disease detection.

In the ROR group, CgA was elevated in 5 of the 7 (71%) on PPI. All who were not on PPI (5/5) had normal CgA levels. An evaluation of tumor burden identified that 2 patients exhibited a single LN recurrence. One had normal CgA, and the other had elevated CgA—both were using PPIs. One had an elevated NETest and the other had a low NETest. In 1 patient in which a local recurrence was identified, CgA was normal (no PPI); the NETest was elevated. In 9 patients who developed a distant metastatic disease, 4 had elevated CgA levels. All 4 were on PPIs. The NETest was elevated in 6 of the 9. Low NETest levels were ascribed to effective SSA use (n = 2) and streptozotocin/5-fluorouracil (FU) treatments (n = 1) at the time of the blood draw. CgA elevation in ROR was therefore related to PPI use.

3.5 | Evaluation of biomarkers and clinical factors as predictors of recurrence

Multiple regression analysis identified that the following biomarkers and the tumor clinicopathological characteristics were associated with recurrence: NETest score >40% ($P < .001$), tumor grade ($P < .03$), positive LNs ($P < .03$), and tumor size >20 mm ($P < .02$; Table 2). The R^2 coefficient was 0.65, the F-ratio was 13.9, and $P < .0001$. CgA levels alone had no association with recurrence ($P = .23$).

Examination of the individual factors identified the NETest was overall 83% accurate for disease status (Figure 5A). For clinical criteria, this ranged between 69% and 80%. Most factors were strongly associated with no recurrence (83%-91%) except for the tumor size, which was poorly associated (57%; χ^2: $P < .05$ vs. LN positivity and NETest). Normal CgA levels were identified in 61% of patients with no recurrence and in 59% of those with recurrence ($P = 1.0$). CgA levels were therefore unhelpful in the detection of recurrence ($P < .005$ vs. all other factors: Fisher's exact test; 2-tailed).

TABLE 2 Multiple regression analysis of NETest, CgA, and clinicopathological parameters associated with recurrence

Independent factors	Coefficient	Standard error	$r_{partial}$	t	P	VIF
(Constant)	−0.1639					
NETest score	0.4550	0.1208	0.5940	3.765	**.0009**	1.168
Tumor size >20 mm	0.3903	0.1459	0.4647	2.676	**.0127**	2.001
Positive lymph nodes	0.3106	0.1292	0.4263	2.403	**.0237**	1.528
Grade (GI vs. GII)	0.3119	0.1329	0.4181	2.347	**.0268**	1.413
Site of tumor (head vs. corpus/tail)	−0.1392	0.1099	−0.2410	−1.266	.2166	1.182
CgA	0.1318	0.1064	0.2361	1.239	.2264	1.064
Tumor size >40 mm	−0.1934	0.1536	−0.2397	−1.259	.2192	2.219
Nonfunctional status	0.1769	0.1537	0.2203	1.151	.2601	1.314
LNR	−0.7323	0.4464	−0.3795	−1.641	.1204	2.569

LNR, lymph node ratio (=number of positive lymph nodes/all dissected lymph nodes).

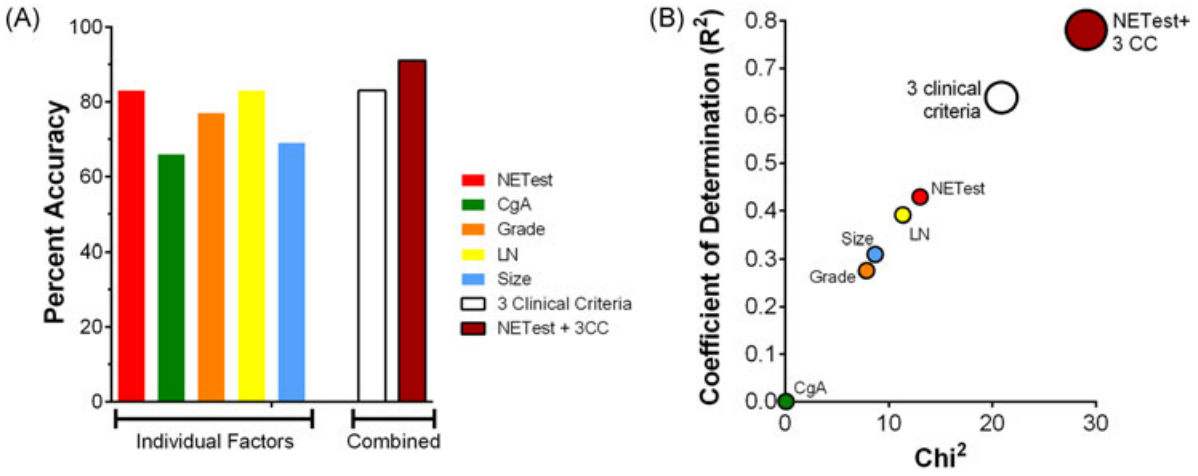

FIGURE 5 Association between biomarkers, clinical criteria, and disease recurrence. (A) Accuracy of individual factors for the assessment of disease recurrence. CgA accuracy was 66%, tumor size >20 mm: 69%, grading: 77%, lymph node (LN) involvement 80%, and NETest 83%. NETest and LN positivity were significantly more accurate than both size and CgA ($P < .05$). Combining the 3 clinicopathological characteristics had the same accuracy as the NETest alone (83%). The inclusion of the NETest to the 3 clinical criteria increased the accuracy to 91%. (B) Logistic modeling for strength of association (χ^2) versus the coefficient of determination (Nagelkerke R^2) to disease recurrence. CgA levels exhibited almost no relationship ($\chi^2 = 0.02$, $R^2 = 0.0008$) to recurrence compared with other clinical criteria. The individual clinical criteria exhibited χ^2 and R^2 values of: grading 7.8/0.28, size 8.7/0.31, and lymph nodes 11.5/0.39. The NETest χ^2 value was 13 and the R^2 0.43 (43%). The combination of all 3 clinical criteria increased the χ^2 from an average of 9.3 to 21.7 and R^2-value from an average of 33% to 64%. The addition of the NET blood transcript information to the 3 clinical criteria increased the χ^2 value from 13 to 30 and the R^2 value from 43% to 80%. The combination of 3 clinical criteria and the NETest exhibited the greatest association with disease recurrence. CC, clinicopathological characteristics; CgA, chromogranin A; LN, lymph node involvement; NET, neuroendocrine tumors. The coefficient of determination is a measure of "relatedness" (Nagelkerke R^2)

The OR for each of the individual factors and recurrence in the regression models was CgA: 1.11 (P = NS), grade: 9.3 ($P = .005$), LN: 14.3 ($P = .002$), and size: 14.3 ($P = .003$). The OR for the NETest was 21 ($P < .0001$).

Individual $\chi 2$ values were CgA: 0.02, grade: 7.8, LN: 11.4, and size: 8.7. The χ^2 value for NETest was 13.

Nagelkerke R^2 (relatedness—coefficient of determination) were CgA: 0.0008, grade: 0.27, size: 0.31, and LN: 0.39 (Figure 5B). For the NETest, R^2 was 0.43.

Combining all 3 clinical criteria resulted in a $\chi 2$ value of 21.7 with a relatedness value of 0.64. Different combinations of the NETest and individual clinical criteria exhibited $\chi 2$: 15.5-24.2 and R^2: 0.49-0.69. The combination of the 3 clinical criteria and the NETest in a logistic regression model provided the best fit with a $\chi 2$ of 30.31 ($P < .0001$), a relatedness value of 0.80, and an AUC of 0.96 ± 0.04. The model accuracy was 91.4%. Twenty-two of 23 (96%) NR patients were correctly classified and 10 of 12 (83%) recurrences were correctly identified.

FIA (see Section 2) was then undertaken to further examine the importance of individual biomarkers and clinical criteria in respect of disease recurrence. The NETest (1.8) was 4.5× more important than CgA (0.4) and identified as the predominant variable related to recurrence (Figure 6). A combination of all 3 clinicopathological characteristics yielded an importance value of 2.3. FIA assessment of the value of inclusion of the NETest to the clinicopathological characteristics increased the importance numerator to 4.3. The measurement of tumor transcript levels in blood (NETest)

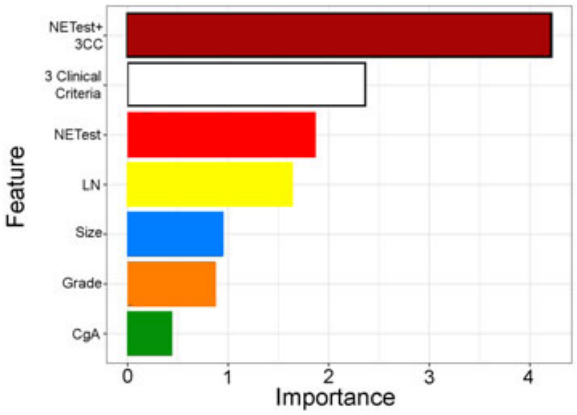

FIGURE 6 Feature performance analysis defining the relative importance of biomarkers and clinicopathological characteristics for disease recurrence. CgA was the least important feature (0.4) for identifying disease recurrence. Individual clinical criteria grade (0.8), tumor size (0.92), and LN involvement (1.6) were 2-4× more important than CgA. The NETest (Importance: 1.8) was the single most important individual feature (4.5× > CgA). The combination of the 3 clinicopathological characteristics had an importance value of 2.3. The inclusion of the pNET blood transcriptome increased the importance to 4.3. A combination of 3 clinicopathological characteristics and the NETest exhibited the greatest importance value. CC, clinicopathological characteristics; CgA, chromogranin A. LN, lymph node; pNET, pancreatic neuroendocrine tumors [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Clinical characteristics of misclassified patients (n = 6)

No.	Cohort	Sex	Age	F/NF	Surgery	Resection margin	Grade	LNR	Size, mm	Post-surgery treatment	NETest score (%)	CgA, ng/mL	Total FU, y	TTR	TTRB
1	NR	F	66	NF	Enucleation	R1	I	0/0	6	Nil	93	497	6.3	0	0
2	NR	F	67	NF	Central pancreatectomy	R1	I	0/0	10	Nil	73	55	9.5	0	0
3	R	M	47	NF	Left pancreatectomy	R0	II	6/1	115	RFA/embolization, NVP-BEZ235, S/5-FU	27	31	8.2	1.9	5.1
4	R	M	74	NF	Corpus resection	R0	II	3/1	17	Prostate cancer (chemotherapy)	33	210	10.7	2.8	7.9
5	R	F	64	NF	Pancreaticoduodenectomy	R0	II	5/2	55	SSA, S/5-FU	27	451	2.6	0.3	1.1
6	R	M	71	NF	Left pancreatectomy	R0	II	6/0	25	SSA	27	188	9.9	8.5	0.2

F/NF, functional or nonfunctional; FUP, follow-up (years); LNR, lymph node ratio (=number of positive lymph nodes/all dissected lymph nodes); NR, nonrecurrence; R, recurrence; RFA, radiofrequency ablation S/5-FU, streptozotocin/5-fluorouracil; SSA, somatostatin analog; TTR, time to recurrence (years) TTRB, time from recurrence to blood draw (biomarker evaluation; years).

significantly improved the utility of grade, tumor size, and LN metastases in the accurate prediction of recurrence by almost 2-fold.

3.6 | NETest and recurrence/NR

Six patients (17%) could overall be considered incorrectly classified by the NETest (Table 3 and Figure 7). In the NR group (n = 23), the NETest incorrectly identified 2 patients. Patient 1, 66-year-old female, nonfunctional, 6 mm G1 tumor, underwent enucleation. Six years after surgery had an increased NETest and CgA—93% and 497 ng/mL—respectively. An MRI 18 weeks thereafter identified no recurrent disease. Patient 2, a 67-year-old female, with a nonfunctional 10 mm G1 tumor underwent a central pancreatectomy. Biomarkers were measured 8.9 years after surgery and the NETest was elevated—73%. CgA was normal. A ^{68}Ga-DOTATATE-PET-CT was undertaken 31 weeks later, and no tumor was detected. Both patients were designated R1 resections and neither had post-operative therapy.

In the recurrence group (n = 12), 4 individuals were incorrectly identified by the NETest. All 4 patients had nonfunctional tumors and all underwent R0 resections. Patient 3 was a 47-year-old male with a 115 mm G2 pNET and positive LN (1/6), who underwent left pancreatectomy. He had completed a phosphoinositide 3-kinase inhibitor clinical study (NPV-BEZ235) and also undergone radiofrequency ablation/embolization for liver metastases. NETest and CgA were measured 5.1 years after surgery before initiation of a third line therapy, streptozotocin/5-FU treatment. The NETest was 27% (positive) and CgA was 31 ng/mL (normal). Abdominal MRI, 68 weeks after biomarker measurement, identified persistent recurrent disease. Patient 4, a 74-year-old male with a 17 mm G2 tumor, positive LN (1/3) and perineural invasion, underwent a pancreatic corpus resection. Biomarkers were measured 8 years after surgery. The NETest was 33% (elevated) and CgA was 210 ng/mL (elevated). CT and an Octreoscan, 1 week after biomarker evaluation, identified a small LN deposit, which was confirmed at excisional biopsy. Patient 5, a 64-year-old female, with a 55 mm G2, 2/5 LN pNET underwent a pancreaticoduodenectomy. Biomarkers were evaluated 1.4 years after surgery during somatostatin analog therapy. The NETest was 27% (elevated) and CgA was 451 ng/mL (elevated). CT identified liver disease, which was then treated by streptozotocin/5-FU. Follow-up imaging 64 weeks later (MRI) was interpreted as stable disease. Patient 6, 71-year-old male, underwent a left pancreatectomy for a 25 mm G1 pNET with perineural invasion and LN (0/6). Biomarker evaluation 9.8 years after surgery was NETest: 27% (elevated) and CgA: 188 ng/mL (elevated). CT identified local recurrence, and he was treated with somatostatin analogs. Follow-up MRI 68 weeks later identified stable disease.

4 | DISCUSSION

To improve the outcomes of patients who have been surgically treated for a localized G1/G2 pNET, post-operative management needs to be optimized to detect recurrences at an early stage. Such an identification or effective prediction of disease recurrence would

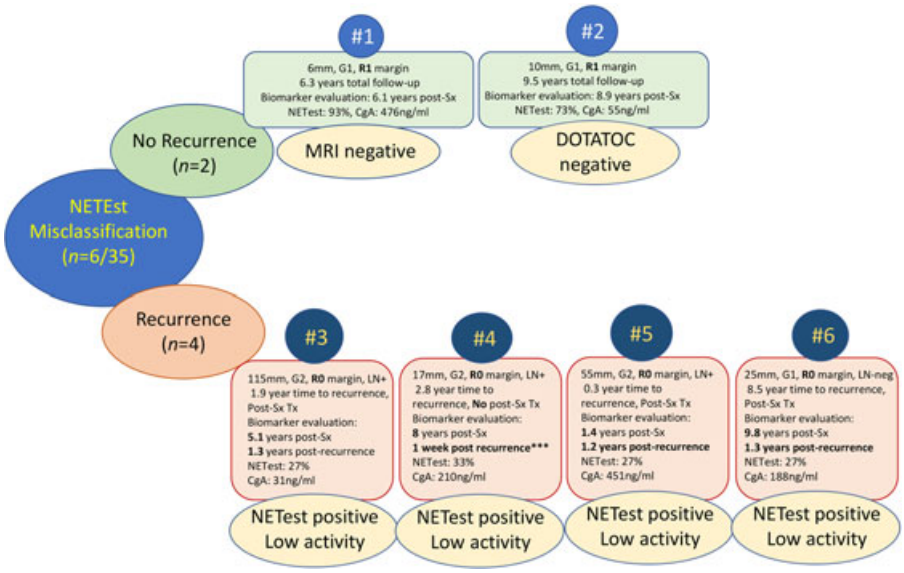

FIGURE 7 Relationship between NETest and false results for recurrence. *Recurrence false positive*: Two patients (1 and 2) were identified with elevated NETest (scores: 93% and 73%) in the absence of image detectable disease. Both had R1 margins and had been followed for 6.3 and 8.9 years, respectively. In case 1, NETest (93%) and CgA (476 ng/mL) were elevated. The time from biomarker measurement to imaging (MRI only) was 18 weeks. In case 2 NETest was 73% and CgA normal. A ^{68}Ga-DOTATATE PET/CT was undertaken 31 weeks after biomarker measurement was negative. The inability to detect disease despite molecular evidence of a NET may reflect the limitations of image sensitivity. *Recurrence false negative*: Four patients (3-6) were identified with positive NETest scores (>20%) but values that are categorized as low activity (ie, <40%) range. All had R0 margins and tumors had recurred 0.3-2.8 years after surgery (in the 3 G2 cases) and within 8.5 years in the G1 case. Three of the 4 (3, 5, and 6) had in the interim undergone a variety of therapies to treat recurrence. Biomarker evaluation was undertaken 1.2-1.3 years after recurrence in these 3 cases. Tumors were all stable at imaging, which is consistent with the low NETest activity. The NETest was measured in case (4), 1 week after surgery (lymph node excision) for recurrent disease. Presumably, the low value in this instance reflects the residual circulating transcript levels from residual low burden disease. Sx, surgery (pancreatic resection); Tx, treatment. chromogranin A; MRI, magnetic resonance imaging; NET, neuroendocrine tumors [Color figure can be viewed at wileyonlinelibrary.com]

facilitate stratification of the patients into those at higher risk, who would benefit from adjuvant treatments. Current clinical biomarkers in use are limited. In the study, we have demonstrated that the NETest effectively detects disease recurrence after curative surgery of pNET, and hence can be used to improve quality of the post-operative follow-up. The NETest has proven to be robust in the discrimination of pNET from healthy controls, therefore confirming its utility as a diagnostic for pancreatic NET disease.[25] As it has been already noted in other surgical series of the lung and GI tract NETs,[26,27] higher NETest scores were evident in patients with recurrence compared to those without. Furthermore, NETest scores >40% were significantly correlated with recurrence, while CgA was not. In fact, the utility of CgA was significantly limited by a number of factors. First, an elevated CgA was only identified in 10 of 24 (42%) patients with residual or recurrent disease. Of these 10, 8 were taking PPIs. Therefore, a total of 2 cases (out of 24 patients) or 8% of individuals with elevated CgA levels could be unequivocally ascribed to pNET disease. Second, PPIs were used in 19 patients (54%). This use elevated CgA in 11 (58%) of them. Third, CgA was not related to disease burden in the recurrent group. Indeed, it was elevated in only 4 of 9 with distant metastases, and all 4 were receiving PPIs. The inconsistent elevation in CgA (presumably due to intermittent use) coupled to the high number of patients in whom it is prescribed, as well as its unreliable relationship to disease burden results in it being of poor clinical value.

Personalization of post-operative care for pNET is under debate by NET experts, but this has not yet led to consensus recommendations on follow-up strategies. One explanation is the limited number of monitoring strategies available to accurately detect recurrence. Imaging, either anatomical or functional, is currently the gold standard for recurrence detection. The blood-based tests currently advised by various guidelines (ie CgA) do not demonstrate reliable accuracy (sensitivity/specificity metrics) to give support to or provide an alternative to imaging. Indeed, the level of evidence for the use of CgA is classified by the National Comprehensive Cancer Network as Type 3 *"Based upon any level of evidence, there is major NCCN disagreement that the intervention is appropriate."*[40]

In agreement with previously published studies, including one from Genc et al,[10] predictors for recurrence have been identified and include tumor size, grade, and LN metastases. In addition, we identified the NETest, but not CgA, as the biomarker that could accurately identify recurrence. Receiver operator curve analysis, logistic regression modeling, and predictive FIA demonstrated that CgA had no value for detecting or predicting recurrence. Indeed, the OR for CgA was 1.11 (1.0 is no association). Similarly, the $\chi 2$ and the Nagelkerke R^2 were both lower than 0.1, indicating no relationship to disease status (ie, pNET disease recurrence) after surgery.

As a component of the clinical criteria for predicting recurrence CgA was identified to have the least importance. Indeed, normal CgA levels were identified in the same proportion of patients in each cohort (59% and 61%, respectively). Furthermore, while some studies describe a correlation between CgA levels and tumor load,[41-43] our results do not support this hypothesis. In contrast, patients with evidence of recurrence on radiological imaging, and therefore tumor tissue in situ (R0R group), showed comparable CgA levels to patients without evidence of tumor tissue on radiological imaging. Indeed, only 4 (of 9) with distant disease exhibited elevated CgA; all 4 were being treated with PPIs. Elevated CgAs were noted in 5 of 12 with known residual (LN positive) disease (R1NR group). Three were taking PPIs. Thus, an elevated CgA may be of relevance in <20% of cases. CgA clearly is not a useful marker for either pNET disease or for recurrence. In contrast, elevated NETest levels were strongly associated with the development of a pNET recurrence. The OR and $\chi 2$ were 21 and 13, respectively, with a relatedness value of 0.43 (43%). Levels were unaffected by PPI use and were significantly elevated irrespective of disease burden. The NETest AUC for recurrence was significantly better (0.82 vs. 0.51, $P < .05$) than CgA. Similarly, predictive FIA demonstrated that the molecular biomarker was almost 5-fold more important than CgA (FIA).

We specifically evaluated a cutoff of 40% for the NETest in this surgical series. This level has been previously demonstrated to differentiate those with "low" risk of disease from those with a moderate or high risk of disease activity.[34,36] NETest scores >40% have been identified to be prognostic (in 100% of cases) for disease progression.[36] Conversely, scores <40% in those with stable disease were 100% consistent with image-confirmation of disease stability.[36] In the current surgical study, the NETest was 83% accurate for pNET recurrence.

Two individuals (9%) were incorrectly classified in the NR group (n = 23). Both had undergone R1 resections for small (<20 mm) Grade I tumors. Neither exhibited LN metastases nor were being treated. The NETest score for patient 1 was 93% with an elevated CgA level (497 ng/mL). Patient 2 had a NETest level of 73% and CgA of 55 ng/mL. In patient 1 the time between biomarker measurement and imaging was 18 weeks. In patient 2 the time was 31 weeks. It has previously been noted that NETest scores >70% are associated with a median progression-free survival (PFS) of 0.7 years and that up to 25% may not have image demonstrable disease for up to 3 years.[36] Based on previous experience with NETest sensitivity compared with imaging, we suspect that both patients will exhibit image detectable disease in the future. The image positivity criteria disparity probably reflects the difference in sensitivity of detection between imaging tools (CT and MRI) compared with transcriptomic analysis. It has been noted that functional imaging detects 10% to 30% lesions not identified on an anatomic imaging.[44,45] It is possible that the use of functional (^{68}Ga-PET-CT) would have detected recurrent pNET disease in these patients.[44]

In a group of patients (n = 4) who recurred, the NETest was positive by definition (ie, >20%) but was less than the 40% that had been preselected as the cutoff point to predict disease recurrence. Blood samples were collected 1, 64, 68, and 68 weeks after detection of recurrence. Three of these patients were being treated with somatostatin analog and streptozotocin/5-FU therapy and were identified as exhibiting stable disease. Thus, the low but positive NETest levels likely reflect low activity disease presence (stable). In patient 6, the blood sample was collected 1 week after resection of the LN with microscopic disease; thus, it was predictable that the NETest score would be low (27%). The period of time between biomarker evaluation, the effective post-surgical treatments are consistent with a NETest scores (20%-40%) that fall into the low disease activity range. In essence, they confirm the presence of disease that is stable as might be predicted after effective therapy.

The individual clinical criteria identified by regression analysis exhibited 69% to 80% accuracy for predicting recurrence. While a tumor size >20 mm was strongly associated with recurrence (92% of all recurrences were associated with large tumors), only 52% of tumors >20 mm recurred. Grading itself was not always indicative of recurrence. While 70% of Grade II tumors did recur, only 58% of all recurrences were Grade II tumors. While LN status was associated with recurrence, the LN ratio[28] did not appear useful as an effective marker. Only 6 of 12 (50%) R0 that recurred exhibited LNR >0.2. It can thus be considered that single clinical criteria alone cannot effectively predict recurrence in pNETs. This supports the increasing enthusiasm for generating multiplex scoring systems or nomograms to predict disease recurrence.[10]

Combining the 3 clinical criteria (size, grade, and LN metastases) resulted in a model with an overall accuracy of 83%. Recurrence was predicted in 75% of cases (9 of 12). The inclusion of the NETest further increased the accuracy to 91%. Ninety-six percent of those who did not recur were accurately predicted, whereas 10 of 12 recurrences were identified. This model had the highest coefficient of variation (0.8) identifying that it most accurately captured information related to disease recurrence. Furthermore, evaluation of this model using predictive feature analysis identified it to be >1.8 times more important than individual clinical criteria alone for determining recurrence. This observation would suggest a role for the measurement of blood molecular biomarker in pNET recurrence prediction modeling. More accurate stratification of pNET disease using multiple criteria would also provide a better basis for defining different clinical treatment groups in the evaluation of treatment efficacy.

The NETest+3 clinical criteria model allowed for consideration of a post-operative stratification into 3 risk categories to guide follow-up. Tumors with no unfavorable characteristics (eg, <20 mm, low grade, and no LN metastasis), with a low NETest score (≤40%), could be considered less likely to recur, and hence less intensive resource-dependent post-operative monitoring might be possible. In the presence of a single unfavorable clinical criterion, or a NETest score >40%, more aggressive follow-up protocol with yearly consultations and radiological imaging could be advised. Those with 2 or more unfavorable clinical criteria, and a NETest score >40%, could be considered as high risk and the follow-up frequency intensified. This might involve more frequent imaging particularly utilizing more sensitive nuclear medicine strategies[44] to ascertain disease not identifiable by anatomic imaging.

Currently, patients with small pNETs also undergo intensive follow-up because surgical resection is no longer directly indicated according to current European Neuroendocrine Tumor Society (ENETS) guidelines.[4] During the follow-up of these patients, there is no other possibility to monitor tumor progression besides imaging. Under these circumstances, the NETest might provide an opportunity to better assess the future malignant potential of the disease, particularly as its metrics are significantly better than any other biomarker, including pancreatic polypeptide and pancreastatin.[38] In this respect, an elevated NETest (≥80%) is strongly associated with disease progression[34] and "omic analysis" of transcript measurement in the blood has been reported to be of value in increasing the accuracy of the prediction of tumor progression.[26,33] Thus, the gene cluster (omic analysis) of NET transcript expression in individuals with small pNETs (<20 mm) might be useful to identify those with a high risk of disease progression. Such patients might benefit from pre-emptive surgical resection despite small tumor size. Moreover, elevated levels after surgery are effective prognostic markers and can be used to identify those who would benefit from early intervention because of risk of recurrence.[26,27] Ultimately, we envisage that the NETest could be evaluated at least annually after surgery and included in the algorithm to provide an updatable real-time patient risk status. The frequency of testing would depend on the risk of progression. Cost-effectiveness would be determined through changes (decreases) in imaging as has been recently noted.[46]

The current study confirms that CgA has no role in predicting pNET disease recurrence. It has little accuracy, was the poorest feature for prediction and the OR (1.1) was no different to using no biomarker at all. If the patient is receiving PPIs, the value of CgA is even further obfuscated. It seems likely that use of a molecular blood test as an accurate marker of disease recurrence or progression might be of clinical utility.

The study has some limitations. As with many investigations in the NET field sample sizes for each group are small and patients identified retrospectively. Patients were older than controls but this has not been identified to be relevant to the NETest; no correlation has been noted between age and transcript levels.[29] The follow-up duration of patients in the R0NR group is shorter compared to the other patient groups (31 months vs. 105 and 92.5 months, respectively). As recurrence is typically seen within the first 5 years after surgery, a follow up of 31 months may not be adequate to identify all of those who will recur.

5 | CONCLUSIONS

Identification of, or prediction of pNET recurrence at an early time point is a critical medical necessity to facilitate further treatment and improve survival. Current clinically used criteria are effective, but inclusion of a blood biomarker that will improve accuracy would be of added value. In this respect the NETest has performance metrics that conform to NIH standards and detects and predicts recurrence after curative resection of G1/G2 pNET. Our study indicates that blood transcript analysis of pNETs added biomolecular value to support the current clinical and radiological parameters used in post-operative follow-up. Larger prospective studies are warranted to more fully explore the utility of the NETest in the identification of post-operative residual pancreatic NET disease or recurrence and to help better stratify patients for post-surgical treatment.

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