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Blood Molecular Genomic Analysis Predicts the Disease Course of Gastroenteropancreatic Neuroendocrine Tumor Patients: A Validation Study of the Predictive Value of the NETest®

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Keywords
Gastroenteropancreatic neuroendocrine tumors · Biomarkers · Liquid biopsy · Survival · Chromogranin A

Abstract
Reliable prediction of disease status is a major challenge in managing gastroenteropancreatic neuroendocrine tumors (GEP-NETs). The aim of the study was to validate the NETest®, a blood molecular genomic analysis, for predicting the course of disease in individual patients compared to chromogranin A (CgA). NETest® score (normal ≤20%) and CgA level (normal <100 µg/L) were measured in 152 GEP-NETs. The median follow-up was 36 (4–56) months. Progression-free survival was blindly assessed (Response Evaluation Criteria in Solid Tumors [RECIST] version 1.1). Optimal cutoffs (area under the receiver operating characteristic curve [AUC]), odds ratios, as well as negative and positive predictive values (NPVs/PPVs) were calculated for predicting stable disease (SD) and progressive disease (PD). Of the 152 GEP-NETs, 86% were NETest®-positive and 52% CgA-positive. NETest® AUC was 0.78 versus CgA 0.73 (p = ns). The optimal cutoffs for predicting SD/PD were 33% for the NETest® and 140 µg/L for CgA. Multivariate analyses identified NETest® as the strongest predictor for PD (odds ratio: 5.7 [score: 34–79%]; 12.6 [score: ≥80%]) compared to CgA (odds ratio: 3.0), tumor grade (odds ratio: 3.1), or liver metastasis (odds ratio: 7.7). The NETest® NPV for SD was 87% at 12 months. The PPV for PD was 47 and 64% (scores 34–79% and ≥80%, respec-

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tively). NETest® metrics were comparable in the watchful waiting, treatment, and no evidence of disease (NED) subgroups. For CgA (>140 ng/mL), NPV and PPV were 83 and 52%. CgA could not predict PD in the watchful waiting or NED subgroups. The NETest® reliably predicted SD and was the strongest predictor of PD. CgA had lower utility. The NETest® anticipates RECIST-defined disease status up to 1 year before imaging alterations are apparent.

Introduction

Overall survival and progression-free survival (PFS) rates diverge widely between the different subtypes of gastroenteropancreatic neuroendocrine tumors (GEP-NETs), with type of tumor, tumor grade, and stage as independent predictors for tumor progression [1–3]. Despite these parameters, it remains very difficult for clinicians to predict the clinical course in an individual patient [4–6]. Clinical management decisions are often driven by combining the features of the tumor, such as grade and stage, with the course of the disease as assessed by radiological examinations. Therefore, even several years after the initial diagnosis, in many patients clinical decision-making is based on the original pathological examination of a small tissue sample that no longer represents the current biological status of the heterogeneous and polyclonal tumor that has evolved with time and as a consequence of treatment.

In patients with local or locoregional disease, surgery remains the fundamental component of all management strategies. However, even after surgery with curative intent, postoperative surveillance is still necessary for many years to exclude residual or metastatic disease [6], with current techniques confined to imaging and chromogranin A (CgA) measurement. Nevertheless, a significant proportion of GEP-NETs are metastatic at diagnosis [2, 7], and management strategies in these tumors predominantly focus on symptom control and inhibition of tumor growth [8]. In nonfunctional GEP-NETs several guidelines consider a watchful waiting strategy after diagnosis as appropriate to enable estimation of the propensity for growth. In the event of progressive disease (PD), various therapeutic modalities are available to regain tumor growth control and enable maximal PFS [9, 10].

It has thus become self-evident that continual assessment of the disease status remains the fundamental basis of the management of GEP-NET disease [6]. Up to now, a combination of symptomatology, functional and anatomical imaging, and biomarkers is utilized. Despite this multimodal assessment strategy there are well-documented limitations for each parameter [11]. Current biomarkers are considered insufficient for providing accurate reproducible information in respect to the aggressive and proliferative capacity of an individual tumor [12]. Nevertheless, CgA is used both as a prognostic marker at diagnosis and as a marker for disease progression or disease recurrence during surveillance [13]. Although CgA correlates with tumor burden [14], reports on the ability to predict the course of the disease are equivocal [15–19].

Therefore, over recent years, research in GEP-NET disease as in other oncological disciplines has focused on the development of alternative tools that delineate the biological characteristics of this heterogeneous group of tumors [20, 21]. In particular, it is now recognized that multianalyte assessment of tumor biology is more effective than monoanalyte evaluation of membrane antigens (PSA or CEA) or secretory products such as serotonin or CgA [12]. Circulating molecular information from GEP-NETs (circulating tumor DNA or cells and mRNA) can possibly be used as a liquid biopsy to provide information on individual tumor behavior and prediction of the clinical course. With such real-time information the management and treatment of GEP-NETs could directly be adapted to the individual patients' needs.

One of the emerging biomarkers in GEP-NETs is the NETest®. This test is a multianalyte algorithmic analysis intending to provide the biological signature of an individual tumor, quantified by a "NET activity score." This score is based on the gene expression of 51 marker genes and the differential analyses of specific gene clusters (omes) which differentiates stable disease (SD) from PD. Available data on the different applications of the NETest® and its clinical utility have recently been systematically reviewed and analyzed [22]. In this review it was described that the NETest® is diagnostic and appears to have clinical utility in monitoring therapeutic efficacy. Therefore, the authors concluded that the NETest® has a significant advantage over CgA. Currently, only three previous studies illustrated the utility to predict the natural course of disease in GEP-NETs. These studies all had methodological shortcomings. One study with a long-term follow-up assessed utility only in a small group of patients (n = 34) [23]. The other two studies included different types of NETs and had short median follow-up of only 6 and 8 months [24, 25]. Moreover, in one of these studies, clinicians could use the NETest® at their discretion for clinical management [24]. Although encouraging, these results require validation before the clinical utility for predicting the course of disease in individual patients can be judged.

In order to specifically address the clinical utility of the NETest® and compare it to CgA, we investigated the two biomarkers in a well-defined large prospective cohort of patients with well-differentiated GEP-NET with long-term follow-up. We assessed the effectiveness for prediction of PFS, identification of disease recurrence, and all-cause mortality in individual GEP-NET patients.

Methods

Consecutive patients with histologically proven, well-differentiated sporadic GEP-NETs were approached for inclusion between March 2014 and March 2017 at the Netherlands Cancer Institute (Amsterdam, The Netherlands), ENETS Center of Excellence. At inclusion, central standardized pathology review was performed for all patients. All NETs were graded according to the World Health Organization (WHO) 2017 grading system [26].

At inclusion, samples (6 mL of EDTA-collected whole blood) were thoroughly mixed and immediately stored on ice. Samples were stored at –80 °C within 2 h after collection according to standard molecular diagnostics protocols for PCR-based studies [27]. Baseline samples for NETest® assessment were sent in different anonymized batches to Wren Laboratories, Branford, CT, USA from October 2015 to October 2018. Samples were always drawn in combination with CgA and radiological imaging studies. Patients were followed in a standardized manner according to the ENETS guidelines. Study design and analysis plan were defined and agreed upon before the start of the study.

Biomarkers

Details of the PCR methodology, mathematical analysis, and validation have previously been described comprising a two-step protocol (RNA isolation/cDNA production and qPCR) [28–31]. Target transcript levels are subsequently normalized and quantified versus a historical (2014) population control [29]. NETest® outcomes are expressed as an activity index from 0 to 100% [28]. NETest® outcomes are classified in different categories. The upper limit of normal (ULN) has previously been set at 20% [14]; SD is defined as ≤40% and PD as an activity score >40% with 41–79% as intermediate tumor activity and scores ≥80% as high tumor activity [23, 24].

CgA was measured with B·R·A·H·M·S Chromogranin A, an automated immunofluorescent assay for the quantitative determination of CgA in human serum using the KRYPTOR instrument (BRAHMS GmbH, Hennigsdorf, Germany). The ULN was established as 100 μg/L. CgA levels were determined at the Netherlands Cancer Institute (Clinical Laboratory).

All samples were anonymized and coded, and laboratory investigators at both sites were blinded to clinical diagnosis and disease status.

Disease Status

Disease status at entry and follow-up – the primary outcome – was evaluated at consecutive imaging according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [32]. All imaging studies were reassessed in a standardized manner by two independent senior radiologists who were blinded to the biomarker results. Both radiologists were equally expert in the different imaging modalities involved in this study. Patients with a minimum follow-up of 6 months and a minimum of two consecutive imaging modalities appropriate to reliably measure disease status were included. According to protocol, patients underwent anatomical imaging every 3–12 months, alternated with functional imaging once every 1–2 years, depending on their clinical condition and response to treatment. The preferred anatomical imaging for the assessment of the outcome measure was computed tomography (CT) if multiple imaging modalities were available in the same surveillance period. Magnetic resonance imaging (MRI) or ultrasound (US) was used in some individuals due to patient or tumor characteristics. US was only used in some accessible patients (n = 4) who underwent curative surgery as surveillance for recurrence or liver metastasis. US was always alternated with MRI and/or functional imaging. Outcomes of functional imaging (^{68}Ga-DOTATATE PET with low-dose CT [DOTA PET CT]) were used in cases where conventional radiological imaging was not available. Since the sensitivity of DOTA PET CT is superior compared to conventional imaging modalities, new lesions on the first DOTA PET CT after previous conventional imaging were not taken into consideration in the determination of disease status. New lesions identified on conventional imaging had to be confirmed as present on consecutive imaging.

Patients were considered to have measurable disease if a tumor lesion was visualized on consecutive imaging modalities. No evidence of disease (NED) was defined as negative consecutive imaging (minimum two) after surgery with curative intent.

Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 25. Statistical significance was defined at a p value ≤0.05. To describe clinical characteristics, NETest® scores, and CgA levels, the mean ± standard deviation or median with range were calculated in normally and nonnormally distributed data, respectively (Kolmogorov-Smirnov test).

Only blood samples collected at baseline were used in this study. The utility of both biomarkers to predict PFS according to RECIST 1.1 was the primary outcome of this study. PFS was calculated as the time between the baseline measurement and the first date patients were considered to have PD. Baseline imaging was compared to previous imaging procedures (if available), to estimate the disease status (SD or PD) at inclusion to accurately estimate time to progression. Predictive values are described by area under the receiver operating characteristic curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the originally described cutoffs of both tests and the optimal cutoffs for both tests. Optimal cutoffs for both biomarkers were assessed by using the AUC. The McNemar test was used to compare the NETest® with CgA. Kaplan-Meier analysis and log-rank test for PFS were performed to estimate differences in PFS between the cutoff points.

Secondary outcomes were all-cause mortality and recurrence of disease after intended curative surgery. Spearman correlation was used to assess the correlation between biomarkers and outcome measures. Univariate analyses were performed to identify predictors for tumor progression within 12 months of follow-up. Identified covariates for PD in the literature were included [4]. Significant parameters were included stepwise in a multivariate logistic regression analysis.

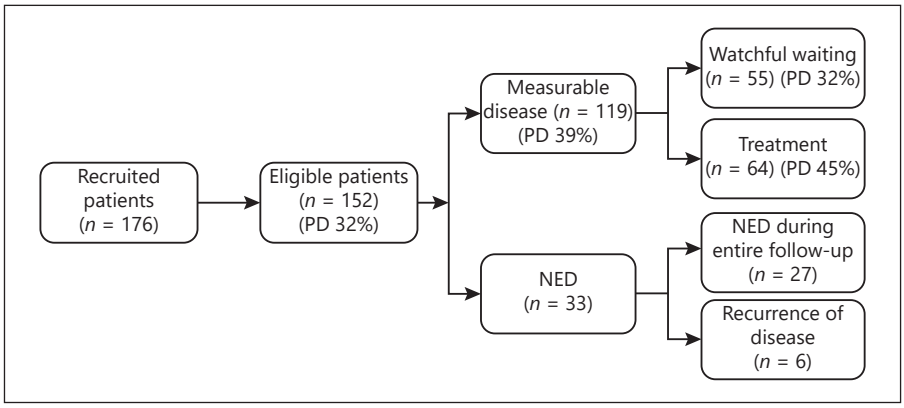

Fig. 1. Overview of the study population and different subgroups. The proportion of patients that showed PD within 12 months is given in parentheses. NED, no evidence of disease; PD, progressive disease.

Results

A total of 152 out of 176 patients were eligible for inclusion in this study. Twelve patients were lost to follow-up or referred back to their referral hospital within 6 months. Seven patients had metastasized disease that could not be used for the primary outcome (e.g., peritoneal metastases). One patient was excluded because of a metastasized second malignancy, and 4 patients were excluded because of curative surgery (3) or peptide receptor radionuclide therapy (1) shortly after baseline and therefore were considered to have an "unnatural alteration" in the course of disease. An overview of the population and different subgroups at baseline is illustrated in Figure 1.

The baseline characteristics of the included patients are described in Table 1. The median follow-up was 36 months (4–56 months). The median NETest® score was 33%. The NETest® was positive (>20%) in 92% of all patients with measurable disease and in 76% with NED. The median NETest® score in patients with NED was 27% (7–100%) and 33% (13–93%) in patients with measurable disease ($p < 0.01$). The median CgA level was 107 μg/L. CgA was positive (>100 μg/L) in 58% of all patients with measurable disease and in 30% with NED. The median CgA in patients with NED was 71 μg/L (19–798 μg/L) compared to 146 μg/L (12–44,150 μg/L) in patients with measurable disease ($p = 0.001$).

Predictive Value for PFS

Disease progression was identified in 17, 32, 38, and 45% of all included patients after 6, 12, 18, and 24 months of follow-up, respectively.

Figure 2 shows the distribution of NETest® and CgA in those with or without progression within the first year after baseline. The highest accuracy for the NETest® to

Table 1. Baseline characteristics of the included patients

Number of patients	152
Age, years	163 (25–81)
Sex	
Male	82
Female	70
Primary tumor	
Small intestine	104
Pancreas	25
Gastric/duodenal	5
Appendiceal	5
Colon/rectum	5
Unknown	8
Grade	
Grade 1	105
Grade 2	44
Grade 3	2
Missing	1
Disease stage	
No evidence of disease	33
Locoregional	1
Distant metastases	118
Current treatment	
None	88
SSA	60
Everolimus	3
CAPTEM	1
NETest® score	33 (7–100)
Negative	12 (8%)
Low scores	93 (61%)
Intermediate scores	26 (17%)
High scores	33 (22%)
CgA level	107 (12–44,150)
Normal	72 (47%)
Elevated	79 (52%)
Missing	1 (1%)

Values are presented as *n*, *n* (%), or median (range). CgA, chromogranin A.

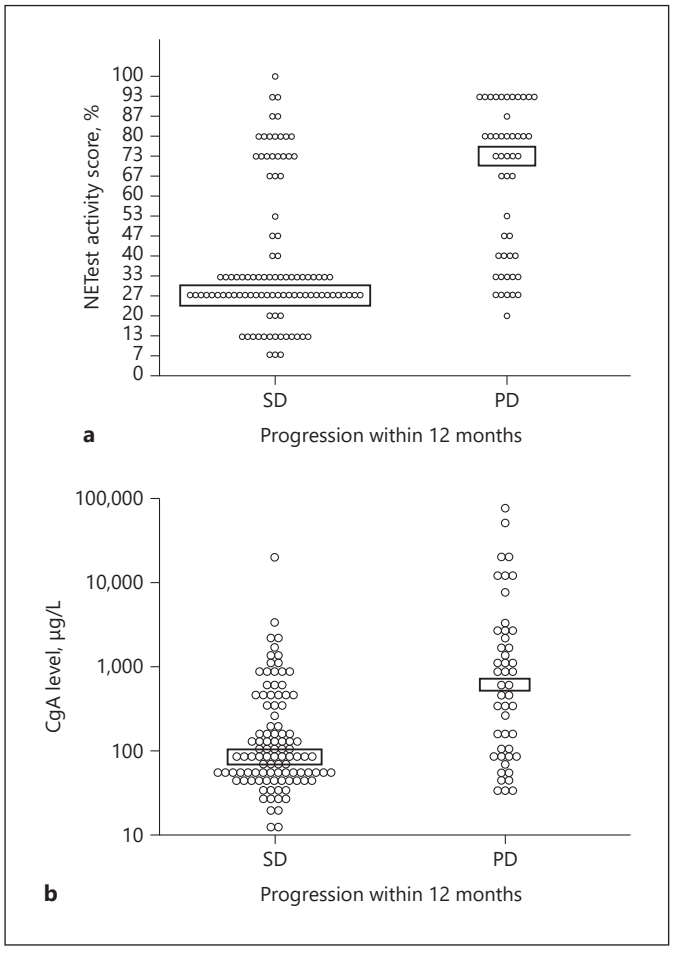

Fig. 2. Distribution of individual NETest® scores (**a**) and CgA levels (**b**) between patients with and without progression during the first 12 months of follow-up. The median NETest® score in the SD group was 27 versus 73% in the PD group (horizontal bar). The median CgA level was 78 versus 483 µg/L (horizontal bar). **b** The *y* axis is logarithmic. CgA, chromogranin A; PD, progressive disease; SD, stable disease.

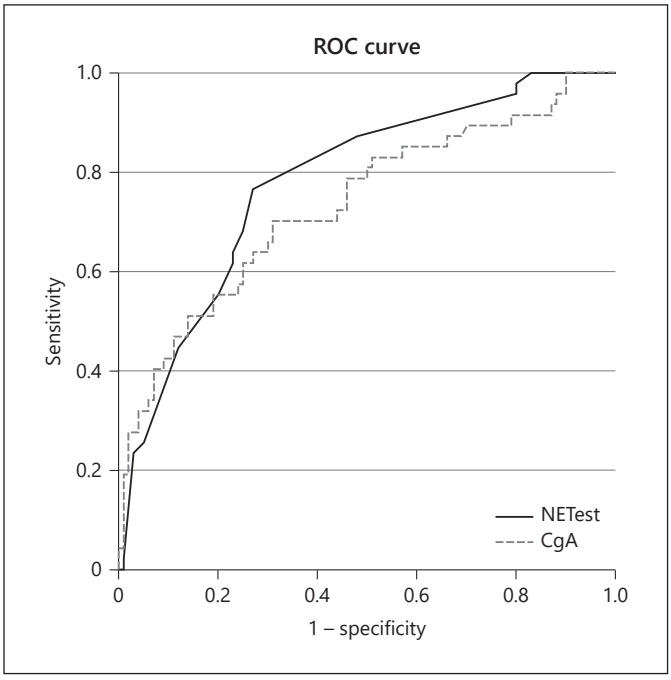

Fig. 3. AUC for both the NETest® and CgA. The NETest® accuracy to predict disease status at 12 months was 0.78 (95% CI 0.70–0.86) compared to 0.73 (95% CI 0.64–0.83) for CgA (p = ns). AUC, area under the receiver operating characteristic curve; CgA, chromogranin A; ROC, receiver operating characteristic.

predict disease status was demonstrated at 12 months of follow-up. The AUC for predicting disease status (SD vs. PD) up to 12 months from baseline was 0.78 for the NETest® (95% CI 0.70–0.86) and 0.73 for CgA (95% CI 0.64–0.83; p = ns) (Fig. 3). Of the 101 patients who were considered to have SD at this time interval, 74% had a NETest® score ≤40% compared to 57% for CgA (ULN: 100 µg/L, $p < 0.01$). Of the patients with PD, 68% had an elevated NETest® score and 70% had an elevated CgA outcome (p = ns). The median NETest® score in the SD group was 27 versus 73% in the PD group ($p < 0.001$). The median CgA level was 78 versus 483 µg/L ($p < 0.001$). The PFS for the previously established NETest® categories (≤40% [low tumor activity], 41–79% [intermediate tumor activity], and ≥80% [high tumor activity]) and CgA (ULN: 100 µg/L) is illustrated in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000509091). A significant difference was observed between the low- and high-activity NETest® categories ($p < 0.001$). The PPV for the intermediate and high tumor activity categories was 44 and 64%, respectively; the NPV was 83%. The PFS of patients was also significantly different between normal and elevated CgA levels ($p = 0.04$), with a PPV and NPV of 43 and 80%, respectively (Table 2). No difference in AUC was observed in the subgroups of patients with pancreatic NETs and small intestine NETs.

Optimal Cutoff

The highest accuracy for the NETest® to predict PD was demonstrated at 12 months of follow-up with an activity score >33% as optimal cutoff (combining the optimal sensitivity and specificity). Using a low activity category of 0–33%, an intermediate activity category of 34–79%, and a high-risk category of ≥80%, PD was observed in 13, 47, and 64% after 12 months of follow-up, respec-

Table 2. Overview of metrics

Test (cutoff)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
NETest® (33%)	77 (62–88)	72 (62–81)	56 (47–65)	87 (80–92)
NETest® (40%)	68 (53–81)	74 (65–82)	55 (46–64)	83 (76–89)
NETest® (80%)	45 (30–60)	86 (80–94)	64 (49–76)	77 (72–82)
CgA (140 μg/L)	70 (55–83)	69 (59–78)	52 (43–60)	83 (76–89)
CgA (100 μg/L)	70 (55–83)	57 (47–67)	43 (36–51)	80 (72–87)

Metrics for the predictive ability for SD (NPV) and PD (PPV). The NETest® has three categories and therefore the upper limit for low tumor activity and the lower limit for high tumor activity are presented. For both biomarkers, the original and the optimal cutoff are demonstrated. CgA, chromogranin A; NPV, negative predictive value; PD, progressive disease; PPV, positive predictive value; SD, stable disease.

tively. For 24 months of follow-up, this was 24, 54, and 79%, respectively. Figure 4a demonstrates the course of disease in relation to these NETest® categories. The NETest® categories significantly differed in median PFS: 55 months compared to 18 and 11 months, respectively ($p < 0.001$; intermediate-high: $p = 0.08$).

The recalculated optimal cutoff for CgA to predict progression at >12 months of follow-up was 140 μg/L. Figure 4b illustrates the median PFS for this CgA cutoff. An elevated CgA (ULN 140 μg/L) predicted PD in 52% of patients at >12 months of follow-up compared to 17% of patients with a CgA outcome ≤140 μg/L. For 24 months of follow-up, this was 59 and 31%, respectively. The median PFS was 55 versus 12 months, respectively ($p < 0.001$).

The metrics (original and optimal cutoffs) for NETest® and CgA to predict SD and PD at 12 months are shown in Table 2. The NETest® had overall better metrics compared to CgA. Patients with a NETest® outcome >33% had an almost 9 times higher chance of PD compared to those with an outcome ≤33% (odds ratio: 8.6). Patients with an optimized CgA outcome >140 μg/L had a 5.2 times higher chance of PD compared to those with a lower outcome.

Predictors for Disease Progression

In multivariate analysis, NETest®, CgA, tumor grade, and presence of liver metastases were independent predictors for PD. The model explained 58% (Nagelkerke R^2) of the variance in disease progression and correctly classified 82% of the cases. The NETest® was the strongest predictor. Intermediate scores (34–79%) were associated with a 5.7 (95% CI 1.7–18.5) times increased likelihood for patients to develop tumor progression. High scores (≥80%) increased the risk of tumor progression by 12.6-fold (95% CI 3.7–43.1). Tumor progression was 3.0 (95% CI 1.3–6.9) times more likely for every 10-fold elevation of CgA. Patients with grade 2 tumors were 3.1 (95% CI 1.0–9.5) times more likely to progress within 1 year compared to patients with grade 1. Patients with liver metastases were 7.7 (95% CI 1.6–37.4) times more likely to progress compared to patients with nonliver metastases. There was no predictive association with age or sex.

Combination of CgA and NETest®

Figure 5 demonstrates the cumulative PFS when the outcomes of NETest® and CgA were combined. When both tests were below the optimal cutoff level (NETest®: ≤33%; CgA: ≤140 μg/L), a large proportion of patients remained stable over a long period of time (log-rank test $p = 0.02$). The NPV was 96% (95% CI 87–99). The PPV for PD was 69% (95% CI 56–79).

Watchful Waiting Strategy versus Treatment in Patients with Measurable Disease

Fifty-five patients with measurable disease had no treatment at inclusion – the watchful waiting group. Fifty of those patients (91%) had a positive NETest® (>20%) and 33 patients (61%) had a positive CgA level (>100 μg/L; $p = 0.001$). Thirty-two percent of patients in the watchful waiting cohort developed PD within 1 year after inclusion. Of the patients with low NETest® scores (≤33%), only 16% had PD in the first 12 months of follow-up, compared to 50 and 54% in the intermediate (34–79%) and high (≥80%) activity categories, respectively ($p = 0.02$; intermediate-high: ns). This significant difference between survival curves was sustained during the entire follow-up period (Fig. 6). The AUC of the NETest® in this subgroup was 0.70 (0.55–0.85) and the cutoff combining the optimal sensitivity and specificity was 33%.

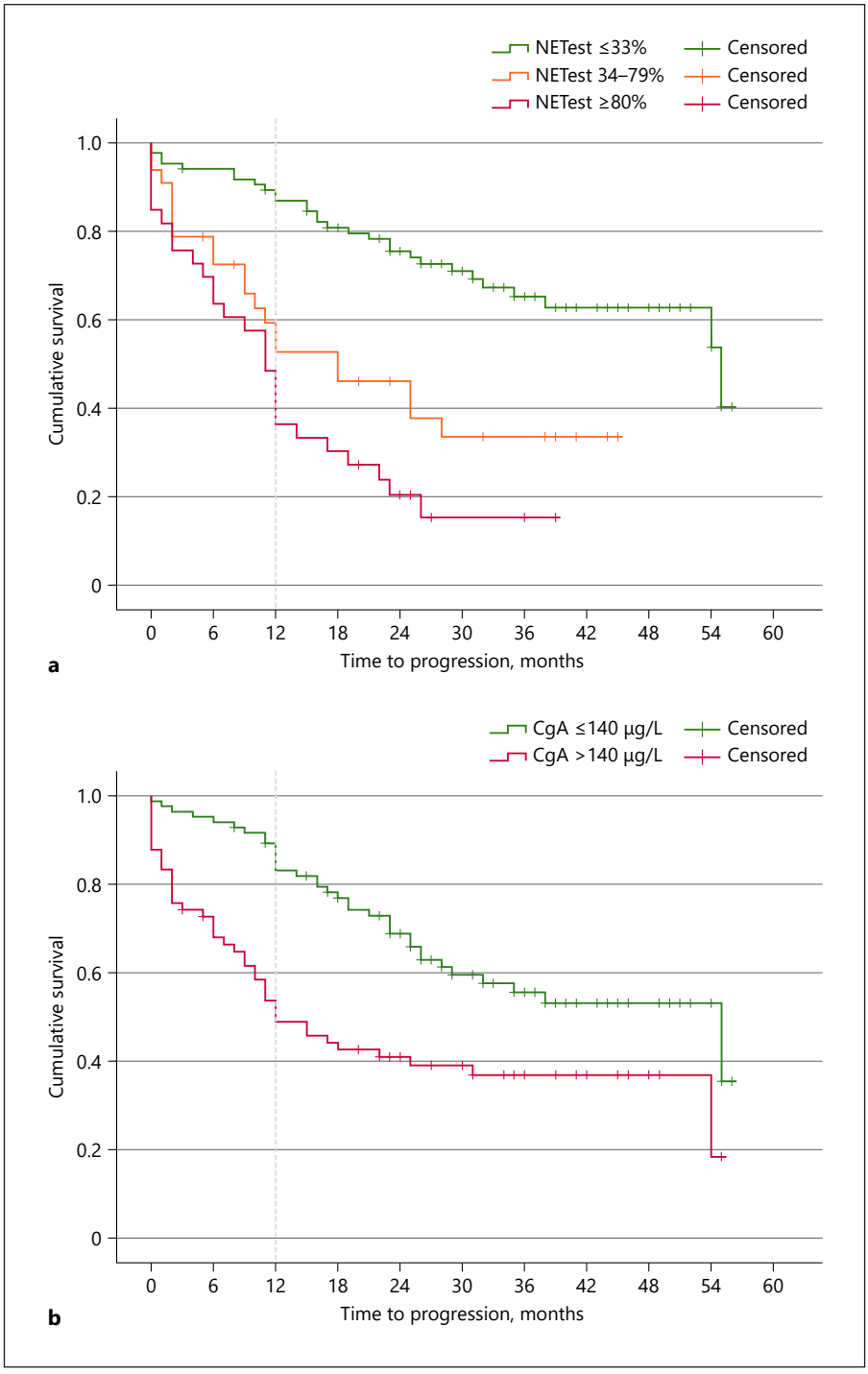

Fig. 4. a Categories in which the threshold for low tumor activity decreased to 33%. A significant difference in median PFS between the low- and higher-activity categories was observed: 55 months compared to 18 and 11 months, respectively ($p < 0.001$; intermediate-high: $p = 0.08$). **b** Kaplan-Meier curve for CgA (ULN 140 µg/L) with significant difference in median PFS between the two curves: 55 versus 12 months ($p < 0.001$). CgA, chromogranin A; PFS, progression-free survival; ULN, upper limit of normal.

The NPV and PPV were also calculated for 24 months: 70% still had SD in the NETest® low-activity category at 24 months. Patients with intermediate and high activity scores showed disease progression in 50 and 74%, respectively, at 2 years of follow-up.

CgA failed to predict the course of disease in the watchful waiting subgroup. The AUC for CgA was 0.64 (0.47–0.82) and the optimal cutoff was 140 µg/L. PD was observed in 21% with low CgA outcomes (≤140 µg/L), compared to 41% with elevated CgA levels (p = ns).

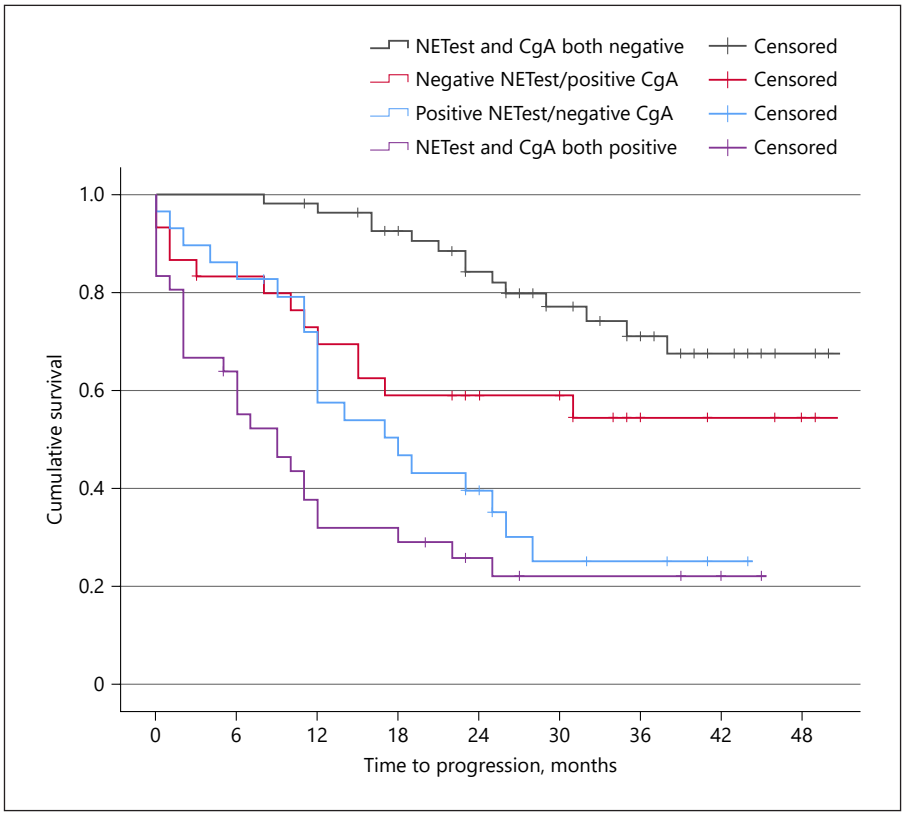

Fig. 5. PFS for the combined outcome of CgA and NETest®. When both tests were negative (–), a large proportion of patients remained stable over a long period of time (log-rank test $p = 0.02$). Patients with positive results (+) in both tests had a significantly lower PFS compared to patients with only a positive CgA level (CgA+/NETest®–: red line; $p = 0.04$), but not compared to NETest®+/CgA– (blue line; ns). The median PFS was 55 months (both tests negative), 54 months (NETest®–/CgA+), 18 months (NETest®+/CgA–), and 9 months (both tests positive). CgA, chromogranin A; PFS, progression-free survival.

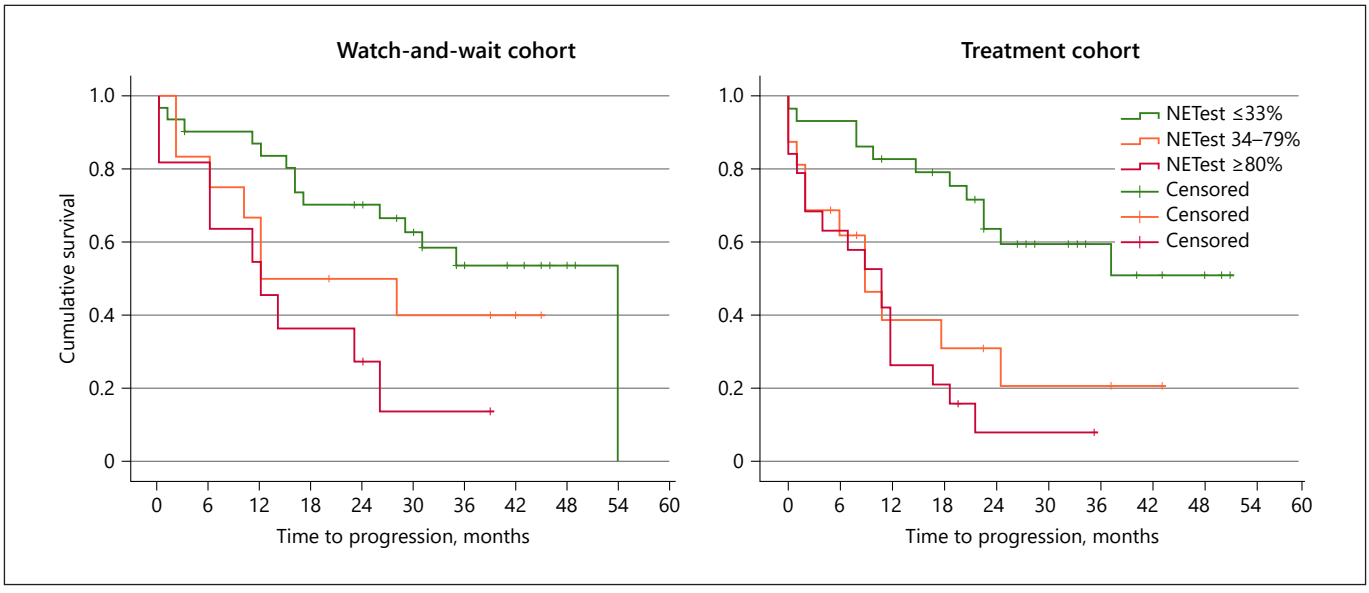

Fig. 6. Cumulative PFS in each of the three NETest® categories in the watch-and-wait cohort (left) and in patients on treatment at baseline (right). In the watch-and-wait cohort the median PFS for the low-activity (NETest® ≤33%) group was 54 months compared to 12 months in intermediate-activity group (34–79%; $p = 0.015$) and 12 months in the high-activity group (≥80%; intermediate-high: ns). In the treatment group, median PFS was not reached for the low-activity group, compared to 9 and 11 months for the intermediate- and high-activity categories ($p < 0.001$; intermediate-high: ns). PFS, progression-free survival.

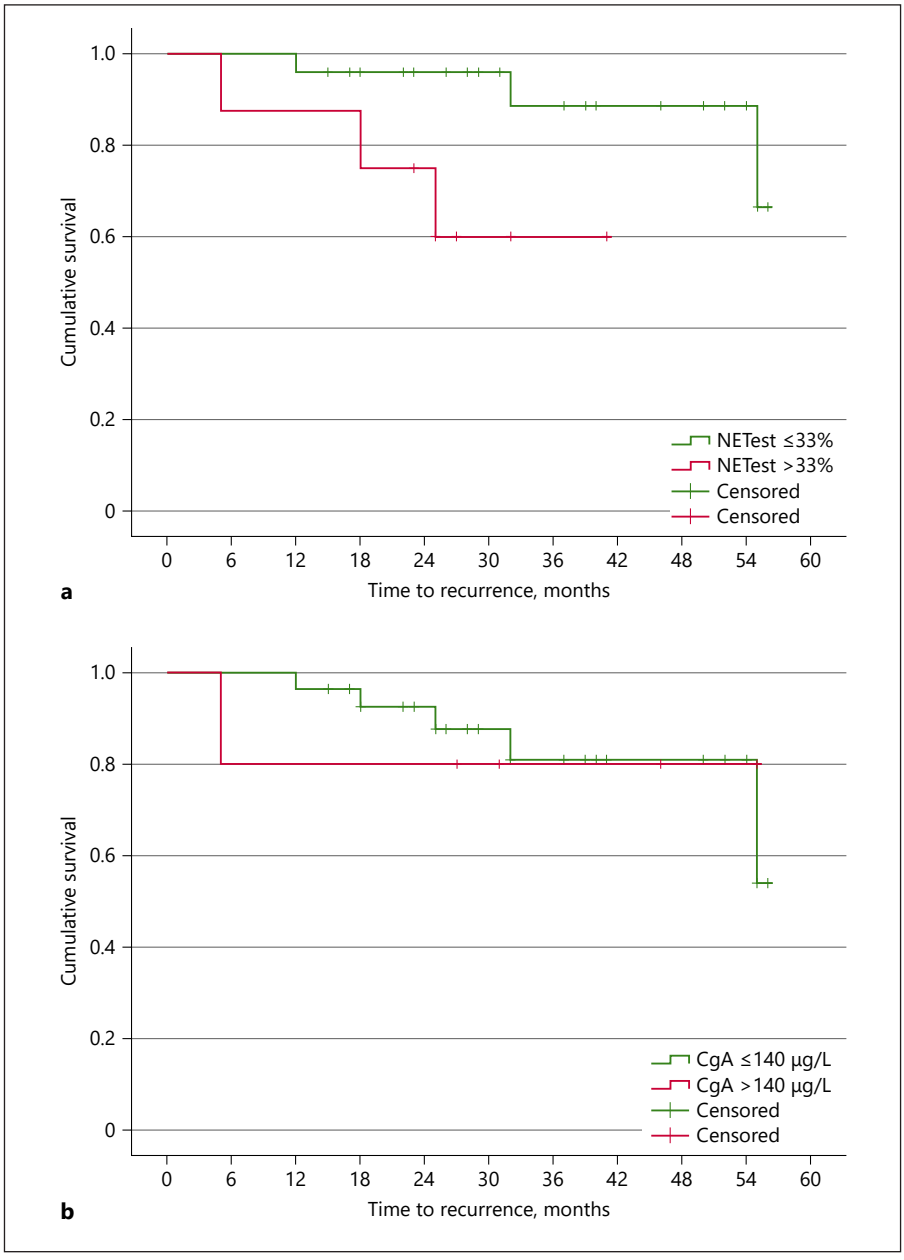

Fig. 7. a Proportion of recurrent disease between patients with low NETest® activity scores and those with high scores. All patients (n = 33) had NED at baseline. **b** Proportion of patients with recurrent disease for normal and elevated CgA outcomes. There was no significant difference between the groups. CgA, chromogranin A; NED, no evidence of disease.

Differences decreased after 2 years. The proportions of patients with PD were 37 and 51% for low and high CgA outcomes, respectively, after 2 years of follow-up, and proportions of cumulative PFS converged after 28 months (online suppl. Fig. 2a).

Sixty-four patients were on treatment at baseline (Table 1). In this group, the NETest® was positive (>20%) in 56 patients (88%) compared to 36 patients (56%) with positive CgA (>100 µg/L; $p < 0.001$). PD was observed in 45% within 12 months of follow-up. PD at 12 months of follow-up was observed in 17% of patients with NETest® low activity scores (≤33%). This was a significantly lower proportion compared to the intermediate category (PD: 61%; $p < 0.001$) and high tumor activity (PD: 74%; intermediate-high: ns) (Fig. 6). The AUC for the NETest® in the treatment group was 0.83 (0.73–0.93). At 24 months, 64% of patients with low activity scores exhibited SD. The PPV for the intermediate and high tumor activity categories was 69 and 92%, respectively, at this time interval.

A significant difference was also observed between normal CgA levels (26% PD, ULN 140 µg/L) and elevated

CgA levels (64% PD; $p = 0.03$) (online suppl. Fig. 2b). The AUC for CgA in this subgroup was 0.76 (0.64–0.88). PPV and NPV for each subgroup are illustrated in Table 3.

Patients with NED

Thirty-three patients were considered to have NED at baseline. In 88% of those patients this was based on a combination of anatomical and functional imaging, and in the remaining 12% on a combination of different types of anatomical imaging. The median follow-up in this subgroup of patients was 38 months (12–56 months). Six patients (18%) developed metastases or recurrence of disease. The median NETest® score in patients who still exhibited NED at follow-up was 27% compared to 53% in patients with recurrence ($p = 0.07$). In this patient group, a low NETest® activity score (≤33%) had a high NPV (88%; 95% CI 76–94%). A high disease activity score (>33%; $n = 8$) had a PPV of 38% (95% CI 16–65%) for disease recurrence. Figure 7a illustrates recurrence over time in the 33 patients with NED at baseline as assessed by NETest® (cutoff 33%; $p = 0.032$). No patient with a negative NETest® score (≤20%; $n = 8$) had disease recurrence during follow-up.

CgA could not differentiate between recurrence or continued NED. The median CgA outcome was 60 versus 75 μg/L ($p = 0.46$), respectively. Equivalent proportions were 18% (≤140 μg/L) and 20% (>140 μg/L) exhibiting disease recurrence ($p = 0.97$) (Fig. 7b). An overview of test performances in each subgroup is presented in Table 3.

Mortality

Thirty-one patients died during follow-up. Fifteen patients died within 2 years. Patients with elevated NETest® scores (>33%) had a minor but significantly lower cumulative survival ($p = 0.02$). Differences in all-cause mortality remained significant between the groups when only the first 2 years were analyzed, with 6% (5 of 86) (NETest® score ≤33%) versus 15% (10 of 66) (NETest® score >33%) being deceased ($p = 0.05$). CgA proved to be a stronger predictor. Only 2.4% (2 of 84) of all patients with negative CgA died within 2 years, compared to 19% (13 of 67) of patients with elevated CgA (>140 μg/L; $p = 0.01$).

Table 3. Predictive value in the various subgroups

Population	Disease status at 12 months		Disease status at 24 months	
	NPV	PPV	NPV	PPV
Total population ($n = 152$)				
NETest®	87	47/64	76	54/79
CgA	83	52	69	59
Watch and wait ($n = 55$)				
NETest®	84	50/54	70	50/74
CgA	79	41	63	49
Treatment ($n = 64$)				
NETest®	83	61/74	64	69/92
CgA	74	64	53	74
NED ($n = 33$)				
NETest®	96	13	96	25
CgA	96	20	93	20

Illustrates the PPV (for the NETest®, intermediate-/high-activity category are given) and NPV for the NETest® (ULN 33%) and CgA (ULN 140 μg/L) in our total population and various subgroups. CgA, chromogranin A; NED, no evidence of disease; NPV, negative predictive value; PPV, positive predictive value; ULN, upper limit of normal.

Discussion

In this independent and largest prospective cohort study to date, a low score on the NETest® – a multigene-based blood test measuring circulating transcripts – proved to reliably predict long-term SD in GEP-NET patients. The NETest® predicts RECIST-defined disease status up to 1 year before this is apparent on imaging with a predictive accuracy of 78%. Patients with a low NETest® score (≤33%) had an 87 and 75% chance of SD at 12 months and even 24 months of follow-up, respectively. In addition, there was a clear difference in the course of disease between patients with low and higher scores even for >2 years after baseline. Comparable results were evident in subgroups of patients who were following a watchful waiting strategy (NPV 84%) or were on treatment (NPV 83%). In line with earlier reports, in patients who underwent surgery with curative intent ($n = 33$), very low NETest® outcomes (≤20%) reliably predicted no recurrence of disease in years of follow-up [33, 34]. We also noted that low-activity NETest® scores (≤33%) were associated with a significantly longer time to recurrence compared to NETest® scores >33%. These results illustrate that the NETest® can be used as a "rule-out" biomarker to provide assessment of surgical efficacy. Very low NETest® outcomes (≤20%) could even replace other currently used measures of disease status like CgA and possibly even imaging. However, the subgroup of patients with very low NETest® outcomes was too small ($n = 8$) for drawing firm conclusions.

Furthermore, in multivariate analysis the NETest®

with an almost 6 and 13 times higher chance of disease progression in patients with an intermediate (34–79%) or high (≥80%) NETest® outcome, respectively. Although the AUC of CgA (0.73) was comparable to that of the NETest® (0.78), CgA was unable to predict the course of disease in the watchful waiting subgroup and could not predict recurrence in the NED subgroup.

The present study was set up in a manner to limit potential bias. All eligible consecutive patients with GEP-NETs were recruited for inclusion and therefore represent the population of interest. Furthermore, all patients were followed according to protocol. The disease status of the patients – primary outcome – was reassessed (blinded/anonymized fashion) for this study by independent radiologists using a predefined protocol. The NETest® was performed in the laboratory without any knowledge of the patients' disease status, and clinicians and radiologists were unaware of the NETest® results. As a result of this study setup, we created a robust and independent evidence base for the predictive ability of the NETest® for individual patients with GEP-NETs encountered in daily clinical practice. The unique prospective long-term follow-up leads to new insights into the predictive value even after 24 months.

A recent meta-analysis by Öberg et al. [22] reported a median accuracy of the NETest® to reflect disease status to be 85%. However, this review focused on actual disease status at the time of blood draw and not on predicting the course of disease over time, which was our goal. Therefore, the outcomes are not comparable to the results of the current study.

We are aware of only three studies that assessed the utility of the NETest® to predict the course of the disease in GEP-NET patients [23–25]. The PPV in the high (≥80%) NETest® activity outcome group varies between studies. In our study, 64% of all patients with high tumor activity scores were progressive within 1 year and even 79% at 24 months. Two of the three earlier studies reported comparable PPVs. Pavel et al. [23] reported a PPV of approximately 70% 1 year from baseline in 31 patients. Malczewska et al. [25] calculated a PPV of 70% at a shorter median follow-up of 8 months. In contrast, in a previous US registry-based study, also with a shorter period of follow-up (median 6 months), PPV was 81% [24]. In this particular study, since it was a real-life format, NETest® outcomes could be used at the discretion of clinicians and symptomatology of patients were part of the primary outcome. The variations in outcomes of the individual studies may reflect the different approaches.

The high NPV in our study (87%) is consistent with the calculated values in previous studies [23–25]. A biomarker with a high NPV can be used to alter management strategies, such as imaging frequency or initiation of therapy. However, the predictive value should be well above 90% to ensure that only a very minimal proportion of patients are misclassified. Despite the high NPV found in this study, an individual patient with a low NETest® outcome still had a 13% chance of PD at 12 months of follow-up in our population. It is debatable whether this is acceptable when changes in management, for example a reduction in imaging frequency, are considered based on low NETest® scores, but it certainly remains an attractive possibility.

In our study, the combination of a low NETest® outcome and a negative CgA level had an excellent NPV of 96%. Lowering surveillance frequency and refraining from expensive treatment options such as somatostatin analogs, peptide receptor radionuclide therapy, or everolimus in this patient group would readily be envisaged to result in lower healthcare costs. However, since this was a post hoc analysis, this could be a chance finding. Additionally, the combination of both biomarkers is only useful when both biomarkers are positive or negative. With different analytical performances of CgA assays, these results are difficult to validate and therefore probably have limited clinical application.

In our study CgA performed better when compared to other studies evaluating both biomarkers [23, 24]. This might be explained by the standardized workup and processing of the samples. Since the accuracy of CgA is highly dependent on the used assay, our results on the accuracy of CgA cannot be extrapolated to the general population [35, 36]. Furthermore, to evaluate both biomarkers identically, we also calculated the optimal cutoff for CgA. This resulted in an overestimation of the predictive value compared to the original cutoff, and results are therefore not transferable to other CgA assays and institutions. Despite using the optimal cutoff, CgA results were still contradictory. CgA was positive in only 52% of patients using the standard cutoff of 100 μg/L. Increasing this to 140 μg/L was associated with an even lower positive rate of 44%. False-negative outcomes therefore remain a critical limitation since CgA could not be used in these patients (with measurable disease) as a biomarker that would provide relevant clinical information. Additionally, CgA could not predict recurrence of disease or disease status in the watchful waiting subgroup. Contrarily, CgA was a stronger predictor for mortality. This ambiguity can probably be explained by the previously supposed correlation between CgA and tumor load [14, 37]. CgA is a secretory protein and therefore volumetric marker of disease and is mostly negative in those with microscopic disease or pa-

tients with low tumor burden, while its correlation with hepatic tumor load probably makes it predictive for shorter survival [38]. However, as a result of the limitations, the independent contribution of CgA in daily practice is limited, especially with the ongoing advances in other diagnostics such as imaging that now use multidimensional mathematically calculated tumor volume as outcome measurement. We previously demonstrated that the NETest® does not have a correlation with tumor load [14], which is consistent with observations that it provides a measurement of tumor "activity." A predictive biomarker that reflects biological disease activity as opposed to tumor load creates a new method to delineate disease status and has therefore significant clinical utility.

NETs – like all malignancies – represent dynamic entities with evolution over time. Consequently, RNA levels and gene expression alter based upon tumor evolution and influencing factors like treatment. Determining the molecular alterations of tumors over time is a fundamental requisite of the NETest®. The reliability and reproducibility of serial NETest® measurement over a long period of follow-up is therefore of utmost importance and must be validated. Serial liquid biopsies over years in patients with SD on imaging will give more insight in the dynamic behavior of GEP-NETs. Since NETest® gene expression measurement is based on the quantity of circulating transcripts, factors affecting the quantity of these transcripts in NET patients undergoing treatment (tumor degradation, ischemia) or suffering from comorbidities (other malignancies, benign diseases) need to be assessed. To our knowledge, independent validation of the reliability of serial NETest® and the reflection of the disease status over time is currently limited to only a subgroup of patients in one study [24]. Blind validation of multiple NETest® samples in a prospective study with sufficient sample size and intercurrent interventions (e.g., treatment initiation, [radio]embolization) over a long period of follow-up is therefore needed.

In conclusion, this study shows that the NETest® is currently the strongest predictor for disease progression and predicts RECIST-defined disease status up to 1 year before this is apparent on imaging. The high NPV can support a watch-and-wait management in patients with well-differentiated GEP-NETs. In head to head comparison, novel genomic analysis proved to provide more value than the monoanalyte marker CgA. It is apparent that with the NETest®, personalized medicine in the management of GEP-NETs is one step closer.

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Statement of Ethics

This study was carried out in accordance with the recommendations of the Netherlands Cancer Institute local ethics committee with written informed consent from all subjects in accordance with the Declaration of Helsinki. The protocol was approved by the Netherlands Cancer Institute local ethics committee.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

M.J.C. van Treijen performed the analysis and wrote the manuscript with input from all authors. M.E.T. Tesselaar, C.M. Korse, L.J. Saveur, and W.H.M. Verbeek collected the samples. D. van der Zee and B.C. Heeres reassessed all imaging studies. G.D. Valk and M.J.C. van Treijen supervised the project. All authors discussed the results and contributed to the final manuscript.

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