

# NET Blood Transcript Analysis Defines the Crossing of the Clinical Rubicon: When Stable Disease Becomes Progressive

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## Key Words

Biomarker · Carcinoids · Chromogranin · Multianalyte · Neuroendocrine tumor · NETest · Polymerase chain reaction · Prognostic · Transcript

## Abstract

**Background/Aims:** A key issue in gastroenteropancreatic neuroendocrine tumors (GEP-NETs) is early identification and prediction of disease progression. Clinical evaluation and imaging are limited due to the lack of sensitivity and disease indolence. We assessed the NETest as a predictive and prognostic marker of progression in a long-term follow-up study. **Methods:** GEP-NETs (n = 34) followed for a median 4 years (2.2–5.4) were evaluated. WHO tumor grade/stage grade 1: n = 17, grade 2: n = 14, grade 3: n = 1 (for 2, no grade was available); 31 (91%) were stage IV. Baseline and longitudinal imaging and blood biomarkers were available in all, and progression was defined per standard clinical protocols (RECIST 1.0). The NETest was measured by quantitative PCR of blood and multianalyte algorithmic analysis (disease activity scaled 0–100% with low <40% and high activity risk cutoffs >80%); chromogranin A (CgA) was measured by radioimmunoassay (normal <150 µg/l); progression-free survival (PFS) was analyzed by Cox proportional-hazard regression and Kaplan-Meier analysis. **Results:** At baseline, 100% were NETest positive, and CgA was elevated in 50%. The only

baseline variable (Cox modeling) associated with PFS was NETest (hazard ratio = 1.022, 95% confidence interval = 1.005–1.04; p < 0.012). Using Kaplan-Meier analyses, the baseline NETest (>80%) was significantly associated (p = 0.01) with disease progression (median PFS 0.68 vs. 2.78 years with <40% levels). The NETest was more informative (96%) than CgA changes (≥25%) in consistently predicting disease alterations (40%, p < 2 × 10<sup>-5</sup>,  $\chi^2 = 18$ ). The NETest had an earlier time point change than imaging (1.02 ± 0.15 years). Baseline NETest levels >40% in stable disease were 100% prognostic of disease progression versus CgA ( $\chi^2 = 5$ , p < 0.03). Baseline NETest values <40% accurately (100%) predicted stability over 5 years (p = 0.05,  $\chi^2 = 3.8$  vs. CgA). **Conclusion:** The NETest correlated with a well-differentiated GEP-NET clinical status. The NETest has predictive and prognostic utility for GEP-NETs identifying clinically actionable alterations ~1 year before image-based evidence of progression.

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## Introduction

Over the last decade, sustainable and demonstrably effective clinical advances in the management of gastroenteropancreatic neuroendocrine tumor (GEP-NET) disease have included a well-defined classification system,

introduction of novel therapeutic agents, imaging advances and multidisciplinary strategies to coordinate management [1–4]. Similarly, an appreciation of the limitations of support for neuroendocrine oncology has escalated attention in what was previously a clinically and scientifically underserved disease process [5]. Despite this, there remain two critical areas that limit advances. The first is the relative paucity of knowledge in respect of the molecular and mechanistic basis of the disease [6]. The second is the difficulty in accurately identifying alterations in the disease state from an indolent biology or stability to a progressive or a micrometastatic phenotype.

GEP-NET assessment is currently represented by an amalgam of clinical judgment, imaging and, to a lesser extent, biomarker measurement [7]. Irrespective of clinical astuteness, the often indolent nature of the disease, the limitations of imaging and the absence of credible biomarkers hinders the identification of disease evolution and presents a challenging management obstacle. Disease progression and recurrence or therapeutic responses are usually defined using a combination of anatomical and functional imaging superimposed upon alterations in symptoms or perturbations in biomarkers [7]. Anatomical imaging using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria, however, exhibits well-documented limitations in NETs [8–10]. Functional imaging with somatostatin receptor-based strategies, e.g. <sup>68</sup>Ga-somatostatin analog (SSA) PET/CT, is of value [11] but resolution sensitivity (~5–6 mm) and partial volume effects limit the ability to detect small changes in tumors; the discriminant index of progression is below an effective management threshold [12, 13]. FDG-PET, though providing useful predictive clinical information, is not established as an early harbinger of disease progression [14]. Current imaging strategies in NETs therefore remain suboptimal [15, 16].

Although biomarkers are used in conjunction with imaging as accessories for clinical decision-making, ‘biochemical’ responses are nonconcordant with image-based assessments [1]. This is particularly disappointing since information is readily accessible from blood or urine without either the complexities and limitations of imaging or the risks of invasive and repetitive tissue sampling [17]. To date, identification of an exemplary biomarker(s) in NET oncology has eluded disease stakeholders because of a reliance on monoanalytes, limitations in technology or a lack of understanding of the molecular basis of the disease. Candidates have proven to be insensitive, nonspecific or, if accurate, of perfunctory usage in only one disease (e.g. gastrin, insulin) [18].

Chromogranin A (CgA) represents the best-described biomarker but its limitations in terms of assay reproducibility, sensitivity and specificity have been extensively documented with resultant clinical skepticism as to its utility [19]. Other disciplines of oncology have gravitated to the conclusion that exocytotic or protein products fail to capture the biology of a neoplastic cell and that a dynamic and panoramic view of an evolving neoplasm can best be captured by a multidimensional assessment of the cell’s molecular genomic machinery. This strategy rejects the concept that a monoanalyte measurement shows accurate biomarkers since its unidimensionality cannot mathematically encompass the diversity of the neoplastic environment. Multiple analyte measurements and mathematical algorithmic analyses that accurately capture the magnitude of the biological information [20] provide the basis for acceptable biomarker strategies [21].

In GEP-NET disease, circulating tumor cells, microRNA levels and circulating transcript analysis have been evaluated. The former represents an intriguing nascent technology but current methodological limitations hinder clinical applicability, though considerable potential remains to be assessed pending the development of single cell genomic analysis [22]. MicroRNA measurement is of interest though confounding factors are disease specificity and the immense heterogeneity of the molecules and their proxies [23]. Currently, the most widely investigated biomarker tool is represented by blood-based multianalyte transcript analysis [24, 25]. Blood gene expression closely correlates with tumor tissue expression levels, and inferential gene analysis of relevant clusters captures the biology of neuroendocrine neoplasia facilitating the accurate definition of clinical status [26].

The multianalyte-derived NET gene signature encompasses the expression of 51 genes assessed by 4 different prediction algorithms. This is then scaled to a disease/tumor activity (0–100%) score [26], using expressions that specifically capture the hallmarks of neoplasia [27], and may be used to provide direct information about the tumor, its pathophysiology, and its state of evolution from stability to progression. To assess this, we prospectively collected blood samples from NET disease patients at a single center (ENETS Center of Excellence – Charité, Berlin) over a ~5-year time period and appraised the utility of biomarkers and imaging in defining the bandwidth of the disease spectrum that embraces that most critical clinical decision-making fulcrum: when stability and disease remission become progression.

## Materials and Methods

### Patients

Thirty-four GEP-NETs (collected between May and October 2009) were histopathologically confirmed as well differentiated (n = 31; grade 1 or 2), 1 was grade 3, and 2 were not available when studied. Patient demographics are summarized in table 1. Thirty-one had radiologically demonstrable disease (stage IV), ascertained within 6 months prior to study initiation; 3 exhibited no evidence of disease. All patients provided informed consent for the blood translational analysis authorized by the local ethics committee (authorization EA2\_064\_09). Patients were followed up for a median of 4 years (2.2–5.4 years), and imaging was undertaken as dictated by their clinical condition and response to therapy.

### Blood Sampling Schedule

Whole blood (10 ml) for transcript analysis was collected at baseline and thereafter at clinically defined points during the follow-up. Plasma for CgA analysis was obtained at exactly the same time points. Overall, blood samples were collected a median 2.5 times per patient (range: 2–5).

### Image Analysis

Anatomical imaging (CT/MRI) was used to evaluate patients at study entry and at appropriate time intervals until progression occurred. RECIST 1.0 criteria were used to assess therapy response. The consensus for the therapeutic response as stable disease (including partial response or complete remission, CR) or disease progression during follow-up was confirmed by a NET Tumor Board Group (M.P., H.J., V.P.). Staging was undertaken a median 7 times per patient (range: 3–15).

### PCR-Based Transcript Analysis NETest

The NETest assesses biological activity using gene inference technology and cancer hallmark prediction [26]. Details of the PCR methodology, mathematical analysis and validation have been published in detail [24, 26, 28, 29]. The procedure utilizes a 2-step protocol (RNA isolation, cDNA production and PCR) [24, 28] from EDTA-collected whole blood [24, 28]. The expression of 51 NET marker genes includes analysis of clusters of biologically relevant genes that constitute the different ‘omes’ (SSTRome, proliferome, metabolome, secretome, epigenome and pluromes) [26] which define the NET ‘fingerprint’. Expression was normalized to housekeepers and quantified versus a population control [24]. Multianalyte algorithm analysis was undertaken (support vector machine, linear discriminant analysis, K-nearest neighbor, and Bayes classifier) for categorization into different groups using the ‘majority vote’ [24]. This results in a 0–8 score [24, 28] which is converted to an activity ranging from 0 (low activity) to 100% (high activity) based on the expression of ‘omic’ genes [26]. Elevated expression of these genes is used to weight the score such that a high score, e.g. ‘8’, when combined with elevated ‘omes’ (identified to differentiate progressive disease, PD, from stable disease [26]) is scaled to 100% (high activity). A score of ‘8’ with a low ‘ome’ is weighted to 53%. We determined the ranges that conform to clinical disease assessment: minimal activity = <0–14%, low activity = 14–40%, and intermediate-high activity = >40–100% [26]. In this study, we used ≥80% as a cutoff for progressive disease, i.e. a NETest score of 80–100% as indicative of highly active disease. This has previously been used as a cutoff to predict disease progres-

**Table 1.** Patient demographics

| Characteristic            | Entire cohort (n = 34) |
|---------------------------|------------------------|
| Mean age, years           | 60.2 (43–83)           |
| Gender (M:F)              | 17:17                  |
| Grade                     |                        |
| 1                         | 17                     |
| 2                         | 14                     |
| 3                         | 1                      |
| Unknown                   | 2                      |
| Site                      |                        |
| Gut                       | 25                     |
| Pancreas                  | 9                      |
| Stage                     |                        |
| CR <sup>1</sup>           | 3                      |
| Stage IV                  | 31                     |
| Status                    |                        |
| Stable                    | 18                     |
| Progressive               | 9                      |
| Disease free              | 3                      |
| Partial remission         | 1                      |
| Disease status not known  | 3                      |
| Treatment at T0           |                        |
| None                      | 14                     |
| SSA                       | 16                     |
| SSA + everolimus          | 1                      |
| STZ/5-FU                  | 3                      |
| Treatment during study    |                        |
| Everolimus                | 7                      |
| PRRT                      | 5                      |
| TEMCAP                    | 4                      |
| STZ/5-FU                  | 2                      |
| Surgery                   | 2                      |
| Sunitinib                 | 1                      |
| Telotristat etiprate      | 1                      |
| TAE                       | 1                      |
| Median baseline CgA, µg/l | 157 (67–58,600)        |
| Median baseline NETest, % | 40 (6.7–93.4)          |

Figures in parentheses indicate ranges. CR = Complete remission; T0 = time of first blood sample; STZ = streptozotocin; 5-FU = 5-fluorouracil; PRRT = peptide receptor radionuclide therapy; TEMCAP = temozolomide + capecitabine; TAE = transarterial embolization.

<sup>1</sup> Three patients were not included in the group analysis: 45 years old, F, grade 1, duodenum, surgery (disease-free for entirety of study); 69 years old, M, grade 1, duodenum, surgery (disease-free for entirety of study); 63 years old, M, grade 3, pancreas, surgery (disease-free at start but recurrence at 2.2 years).

sion in SSA-treated patients [29]. We also evaluated a lower value (≥70%) to assess whether this would function as a more effective/sensitive predictor of disease progression. This is based on a preliminary affinity propagation algorithm analysis [30] undertaken on the sample set that identified a value ≥70% to be informative for predicting disease progression.

### CgA Assay

Serum CgA was measured using a competitive radioimmunoassay (CGA-RIACT, Cisbio Bioassays) [31]. This is a solid-phase 2-site immunoradiometric assay; two monoclonal antibodies are used against sterically remote sites on CgA. The assay can detect whole and fragmentary CgA species and is a standard assay at the Charité. The reference range was 19–150 µg/l; 150 µg/l was used to define the upper limit of normal (ULN). Values >150 µg/l signified an elevated CgA, while values  $\geq 300$  µg/l ( $2 \times$  ULN) were used to signify abnormally elevated CgA [32]. In subjects with elevated CgA, an increase  $\geq 25\%$  between any two time points was used as a measure to predict disease progression. This is based on a previous retrospective study which found that this value exhibited a sensitivity and specificity of >85% for predicting disease progression during patient monitoring [33].

### Grading

Tumors were graded (1, 2, or 3) according to the WHO classification, utilizing the Ki-67 values obtained from the original histopathological reports [34].

### Statistical Analyses

Analyses included  $\chi^2$  (Fisher's, 2-tailed), nonparametric (Mann-Whitney, 2-tailed) measurements, Kaplan-Meier survival curves (progression-free survival, PFS), event curve analysis (based on Kaplan-Meier curves), and multivariate analysis (Cox proportional-hazard regression). Prism 6.0 for Windows (GraphPad Software, La Jolla Calif., USA; www.graphpad.com) and MedCalc Statistical Software version 16.2.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) were utilized. PFS of the cohort over the extent of the study (i.e. time from baseline to image-verified disease progression) was assessed to provide a descriptive overview of the cohort. The utility of baseline biomarkers for predicting PFS based on imaging (RECIST) was examined at two different time points (baseline = time 0 and Time 1 = a subsequent follow-up time when the majority of the patients, i.e.  $n = 29$ , had blood sample collections). For NETest predictive accuracy assessment, cutoffs of 70 and 80% were evaluated. CgA levels >150 and  $\geq 300$  µg/l ( $2 \times$  ULN) were evaluated. For the event curve analyses, the time difference between a blood sample and image-based evidence for disease progression was identified. For individual biomarkers, the data are presented as means  $\pm$  SEM.

## Results

### Patient Demographics

Thirty-four patients (stable  $n = 18$ , 53%; PD  $n = 9$ , 26%; disease free on CT/MRI, i.e. CR,  $n = 3$ , 9%; partial remission  $n = 1$ , 3%; disease status not known  $n = 3$ , 9%) were included (table 1). Disease types included small intestine ( $n = 24$ ), pancreas ( $n = 7$ ), 2 multiple endocrine neoplasia/Zollinger-Ellison syndrome and 1 cancer of unknown primary. Most cases were TNM stage IV (31/34), and 3 were CR (no evidence of disease on CT/MRI) following (curative) surgery. Subjects were a median 57 years old at the time of enrollment (range 43–83 years) with a similar gen-

der ratio of M:F 17:17. The WHO grading distribution was grade 1 = 17, grade 2 = 14, grade 3 = 1, while 2 had no grade available. Previous therapy included surgery ( $n = 23$ ), SSAs ( $n = 9$ ) and streptozotocin/5-fluorouracil ( $n = 2$ ). At enrollment, 16 were receiving SSAs, 1 a combination of SSA and everolimus, 3 streptozotocin/5-fluorouracil and 14 were not being treated. Treatment modalities thereafter involved use of everolimus ( $n = 7$ ), peptide receptor radionuclide therapy ( $n = 5$ ), temozolomide + capecitabine ( $n = 4$ ), streptozotocin/5-fluorouracil ( $n = 2$ ), sunitinib ( $n = 1$ ) or telotristat etiprate ( $n = 1$ ). Two patients received surgery; 1 received transarterial embolization. These were all undertaken after disease progression had been identified (typically 2–3 years into the study). These patients were not excluded from the group analysis because blood samples had already been collected prior to this therapy. After a median 4.4-year (range 2.2–5.4) observation period, 27 were alive, 5 were lost to follow-up and 2 had died.

Two were excluded from group analysis because they had CR and remained thus for the duration of the study (patient 15, with Zollinger-Ellison syndrome/duodenum, <1%, surgery: 4.8 years; patient 21, with duodenum, <1%, surgery: 3.2 years). A third patient was also excluded due to CR at the start of the study (patient 26, pancreas, 30%, surgery: 2.2 years before disease recurrence).

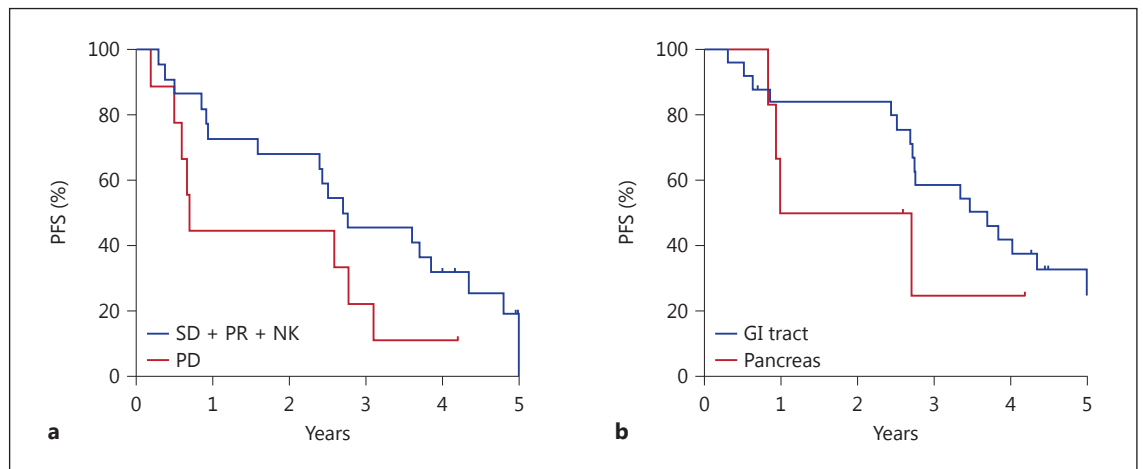
The median PFS for the patient cohort ( $n = 31$ ) was 2.59 years. Patients with PD ( $n = 7$ , PD and mixed progression:  $n = 2$ ) had a lower median survival, 0.7 years, than those with stable disease (i.e. stable disease, partial response and not known; 2.7 years,  $p = 0.11$ ; fig. 1a). Grade was not related to PFS. Disease site influenced PFS (fig. 1b), with pancreatic tumors exhibiting a PFS of 1.84 years compared to 3.67 years for gastrointestinal NETs ( $p = 0.27$ ).

### Baseline Biomarker Parameters

#### CR Patients ( $n = 3$ )

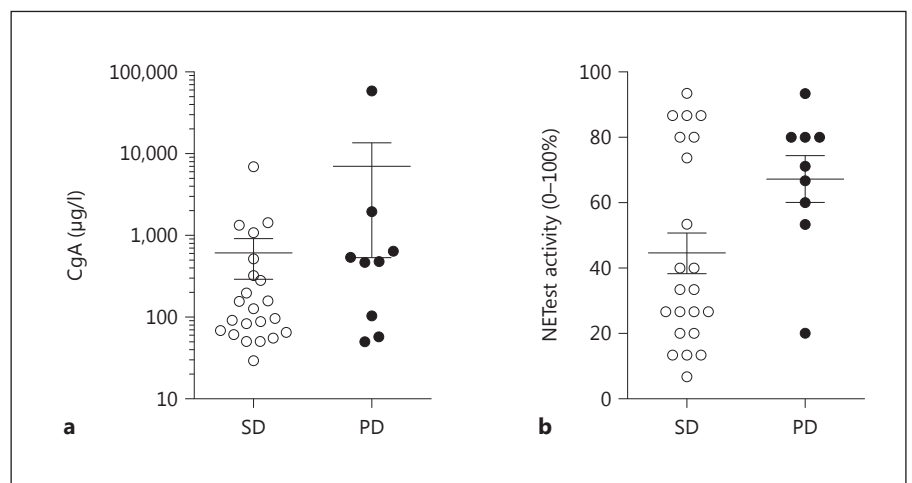
The baseline NETest for the 2 patients who were surgically 'cured' was 27 and 14%, while CgA was 343 and 381 µg/l, respectively. Two sequential measurements at 2.6 and 3.9 years (in patient 15) were 33 and 33%; CgA was 95 µg/l. For patient 21, the NETest at 3.2 years was 20%, CgA was 248 µg/l. The third patient developed recurrence after 2.2 years. This was a grade 3 (Ki-67 = 30%) pancreatic NET. The NETest was 14% at baseline, and the CgA level was 83.1 µg/l. The subsequent NETest level was 66.7% when disease recurrence was noted. CgA was normal (95 µg/l). An additional measurement was lower (NETest 40%; CgA 124 µg/l) when the disease was adequately treated with temozolomide + capecitabine (RECIST – stable).





**Fig. 1.** PFS in the cohort (n = 31). **a** Groups defined by imaging had PD at baseline (n = 9) compared to those with stable disease (SD) or identified as partial response (PR) to therapy. The 3 patients who were undefined (not known: NK) were included in the stable

disease group. The median PFS was 0.7 years in the PD group versus 2.71 years (p = 0.11). **b** PFS assessed by tumor site. Pancreatic NETs had a PFS of 1.84 years compared to gastrointestinal (GI) tract tumors with 3.70 years (p = 0.27).



**Fig. 2.** Baseline blood-based biomarker expression (n = 31). **a** Groups had PD at baseline (n = 9) or stable disease (SD; n = 22). CgA levels tended (p = 0.12) to be higher in the PD group at baseline. Means ± SEM. **b** NETest levels were significantly elevated in the PD group at baseline (p < 0.05). Means ± SEM.

#### Total CgA Levels (n = 34)

At baseline, CgA was  $2,261 \pm 1,720$  µg/l. Levels were higher in pancreatic NETs ( $9,935 \pm 8,160$  µg/l) than gut NETs ( $313 \pm 77$  µg/l, p < 0.006), were increased in grade 2 versus grade 1 ( $4,767 \pm 4,144$  vs.  $591 \pm 378$  µg/l, p < 0.05) but were not different by stage (stage IV vs. CR:  $2,617 \pm 2,014$  vs.  $495 \pm 184$  µg/l, p = n.s.).

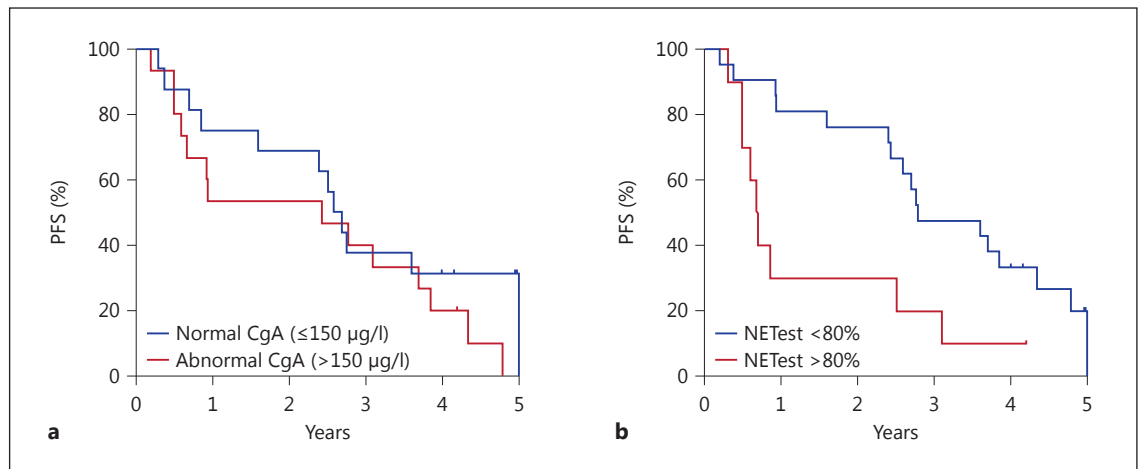
#### CgA Levels in the Patient Cohort (n = 31)

Levels tended to be higher in PD ( $6,989 \pm 6,454$  vs.  $603 \pm 315$  µg/l, p = 0.12, fig. 2a). Sixteen (50%) exhibited elevated (above the ULN = 150 µg/l) CgA levels. Twelve of the 16 (75%) had a baseline CgA  $\geq 300$  µg/l. Elevated CgA

was not associated (p = 0.26) with the eventual development of progressive disease compared to normal levels (fig. 3a). Abnormally high CgA ( $\geq 300$  µg/l) was not associated with outcome either, although a shorter median survival was noted (1.24 vs. 2.76 years).

#### Total NETest (n = 34)

At baseline, the NETest was  $48.4 \pm 5\%$ . Levels were not different between sites (pancreatic NETs:  $57.8 \pm 7.3\%$  vs. gut NETs:  $42.7 \pm 5.9\%$ , p = 0.28) or grade (grade 1:  $45.3 \pm 7\%$  vs. grade 2:  $47 \pm 7\%$ , p = n.s.) but were different by stage (stage IV vs. CR:  $48.7 \pm 5$  vs.  $16 \pm 6\%$ , p = 0.05).



**Fig. 3.** Relationship between survival and baseline biomarker levels. **a** PFS was not associated with baseline CgA in this cohort; normal CgA levels ( $\leq 150 \mu\text{g/l}$ ) were associated with a median survival of 2.65 years compared to 2.43 years in those with elevated ( $>150$

$\mu\text{g/l}$ ) levels ( $p = 0.26$ ). **b** NETest activity at baseline ( $<80\%$ ) was associated with PFS of 2.78 years versus 0.68 years ( $p < 0.02$ ) in those with  $>80\%$ .

#### NETest in the Patient Cohort ( $n = 31$ )

At baseline, the NETest was  $51 \pm 5\%$ . Levels were significantly higher in the PD group ( $67.2 \pm 7.1$  vs.  $41.6 \pm 5.8\%$ ,  $p < 0.05$ ; fig. 2b). An elevated NETest (defined as  $\geq 80\%$ ) was significantly associated ( $p = 0.014$ ) with the development of PD compared to lower NETest values (fig. 3b). The median survival was 0.68 years (NETest  $\geq 80\%$ ) compared to 2.78 years.

Analysis of the 7 patients (clinically stable at baseline) who had an intermediate or high NETest ( $>40\%$ ) identified that all 7 (100%) developed PD in a mean of 24 months (range: 3.6–57.5). Conversely, the 7 with stable disease, who never developed disease progression during the duration of the follow-up, all exhibited a low-activity ( $\leq 40\%$ ) baseline NETest (mean follow-up: 52 months, range: 32–60.5 months).

#### Predictive Utility of Biomarkers ( $n = 31$ )

The study design allowed us to examine the predictive utility of the NETest compared to CgA at defined, clinically chosen, image-based follow-up times using multivariate and Kaplan-Meier analyses.

#### Multivariate Analysis

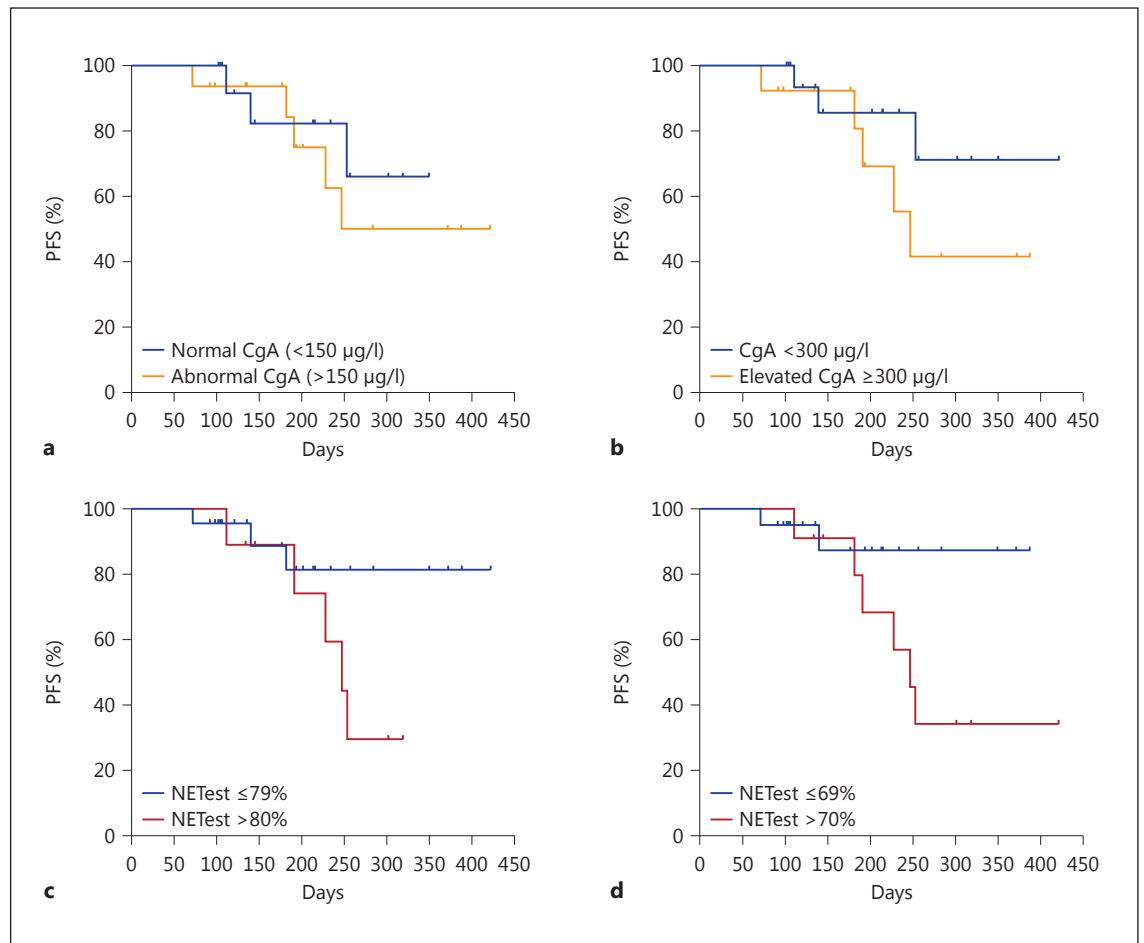
We initially investigated which baseline variables were associated with PFS. Cox proportional-hazard regression was fitted to the baseline data. Baseline CgA ( $\mu\text{g/l}$ ) and NETest (%) levels as well as histological grade (grade 1 vs. 2) and site (gut vs. pancreas) were evaluated. The only significant covariate in the model was the NETest (hazard

ratio, HR = 1.022, 95% confidence interval, CI = 1.005–1.04,  $p < 0.012$ ). Neither grade (grade 1 vs. 2; HR = 1.095, 95% CI = 0.473–2.54,  $p = 0.83$ ) nor site (HR = 0.72, 95% CI = 0.239–2.17,  $p = 0.56$ ) or CgA (HR = 1.00, 95% CI = 1.00–1.00,  $p = 0.89$ ) were predictive of PFS in this cohort.

#### Kaplan-Meier Analyses (T0 to First Restaging)

The mean follow-up time from baseline to the first restaging scan was 194 days (range: 72–422). Assessments included: histological grade, NETest ( $\geq 80\%$ ), normal versus elevated ( $\geq 150 \mu\text{g/l}$ ,  $n = 16$ ) and abnormally elevated CgA ( $\geq 300 \mu\text{g/l}$ ,  $n = 12$ ). Values were assessed to predict disease progression at the initial restaging. CgA was not informative. The median survival with CgA  $\geq 150 \mu\text{g/l}$  was 335 days (vs. undefined for normal levels; fig. 4a), while it was 247 days in the 12 with CgA  $\geq 300 \mu\text{g/l}$  (fig. 4b). The only predictive biomarker was the NETest which exhibited a trend toward significance ( $p = 0.07$ ) with outcome at this time point (fig. 4c, d). The HR for an elevated NETest was 3.3 (95% CI = 0.78–14.3). The median survival was 247 days (not reached for NETest  $\leq 79\%$ ) after 422 days (fig. 4c). Lowering the NETest cut-off to 70% improved the HR to 3.5 (95% CI = 0.87–14.0). The median survival remained 247 days (fig. 4d).

During the follow-up, 29 patients had a second blood sample (both NETest and CgA) and thereafter image-based restaging. The mean time between blood measurement (T1) and restaging was 183 days (range: 26–814). CgA was not informative when either assessed in the entire cohort (normal levels vs. elevated levels, fig. 5a, me-



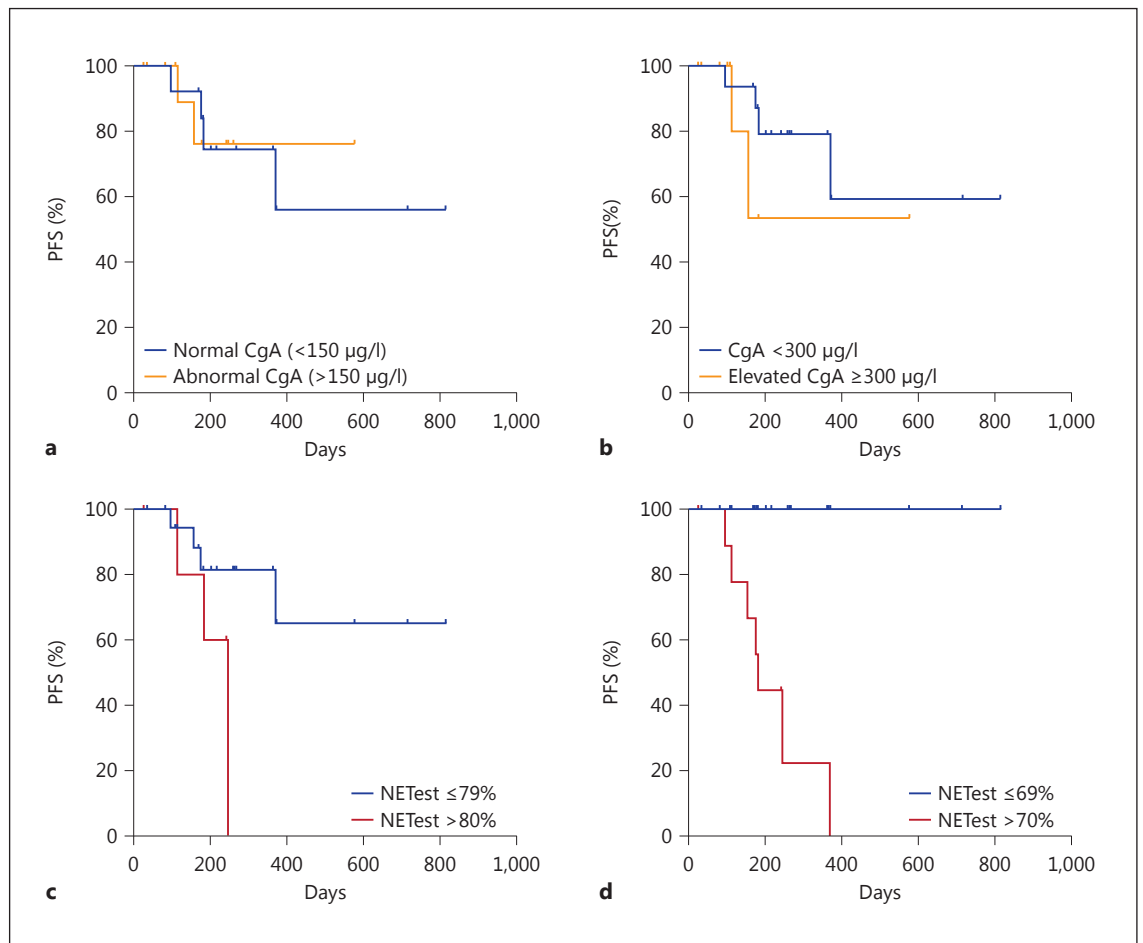
**Fig. 4.** Blood-based biomarkers and relationship with survival (baseline time point: T0, n = 31) assessed using Kaplan-Meier analysis. **a** PFS was not associated with elevated (>150 µg/l) CgA levels. **b** PFS was not linked to abnormally elevated CgA (≥300 µg/l) at baseline in the 12 patients with elevated CgA. **c** PFS was associated

with baseline NETest activity in this cohort; the median survival was 247 days in those with NETest >80% (p = 0.07). **d** PFS was associated with baseline NETest activity in this cohort; the median survival was 247 days in those with NETest >70% (p = 0.06).

dian survival not reached in either cohort) or using 300 µg/l (fig. 5b). In the latter, the median survival with an elevated CgA was undefined. Assessment of the NETest (using cutoffs of ≥70 or ≥80%) found that this was predictive (fig. 5c, d). The HR for the NETest (≥80%) was 5.5 (95% CI = 0.77–38.9) with a median survival of 246 days (vs. not reached, p = 0.07; fig. 5c). Using a cutoff of 70%, the HR was 23.5 (95% CI = 4.7–116.5), and the median survival was 183 days (not reached for NETest ≤69%, p = 0.0009; fig. 5d).

The NETest was more informative than elevations in CgA. NETest alterations (a rise to ≥80% in progressive disease or remaining low (≤40%) in stable disease) occurred more consistently (24/25, i.e. 96%) than alterations in CgA (e.g. elevation ≥25% or no change in SD:

10/25, i.e. 40%,  $p < 2 \times 10^{-5}$ ,  $\chi^2 = 18.1$ ). The time point at which the NETest (≥80%) was measured prior to image evidence of PD was significantly earlier ( $1.02 \pm 0.15$  years) than for alterations in CgA ( $\Delta 25\%$ :  $0.51 \pm 0.11$  years, p = 0.034). Elevated CgA (≥300 µg/l) occurred at  $0.52 \pm 0.13$  years before indication of disease (p = n.s. vs. CgA  $\Delta 25\%$ ). If a NETest ≥70% was used, the time point was even earlier. Progression was predicted  $1.85 \pm 0.27$  years prior to image confirmation. This was significantly earlier than for either NETest (≥80%, p < 0.02) or for CgA ( $\Delta 25\%$ , p = 0.003). Event curves for each of the biomarkers (fig. 6a, b) show that the median times prior to image-confirmed disease progression for CgA were 0.51 years ( $\Delta 25\%$ ) and 0.60 years (≥300 µg/l), and for the NETest they were 0.76 years (≥80%) and 1.62 years (≥70%).



**Fig. 5.** Blood-based biomarkers and relationship with survival (second blood time point: T1, n = 28) assessed using Kaplan-Meier analysis. **a** PFS was not associated with elevated (>150 µg/l) CgA levels. **b** PFS was not linked to abnormally elevated CgA (≥300

µg/l) at the second blood time point (T1). **c, d** PFS was associated with baseline NETest activity in this cohort; the median survival was 246 days (cutoff >80%) or 183 days (cutoff >70%).

## Discussion

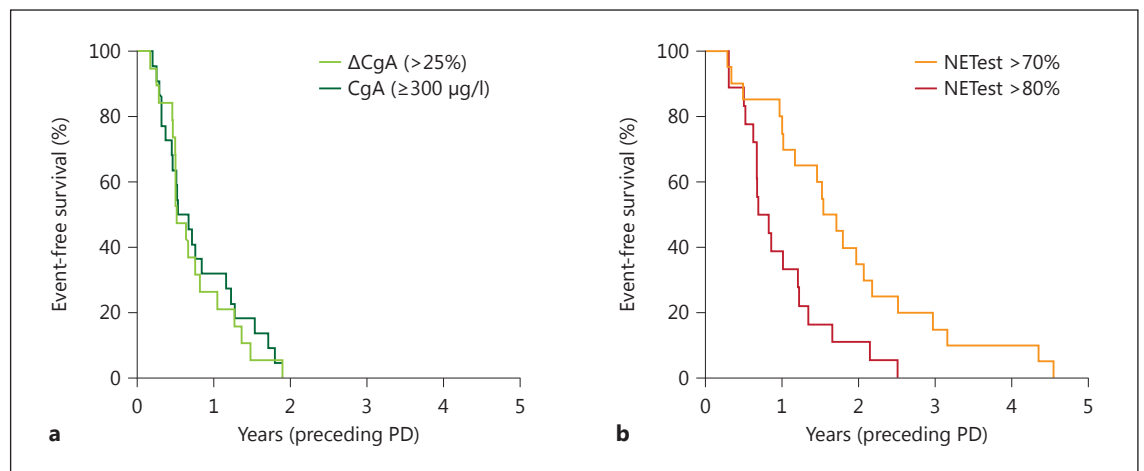
A critical unresolved issue in GEP-NET management is the early identification of disease progression [30]. Effective prognostic biomarkers are not robust, and imaging is relatively insensitive [10, 35]. The current consensus is that an accurate circulating biomarker that captures the biological activity of a NET and predicts its clinical behavior would provide an optimal method for the early detection of disease progression [1]. We evaluated the role of a blood-based multigene transcript analysis as such a predictive and prognostic marker and assessed whether the performance was more effective than standard biomarkers providing added clinical utility.

RECIST criteria are the current default for defining therapeutic responses although their limitations are well

documented [8–10]. Local confounders such as necrosis, hemorrhaging or fibrosis complicate assessment [36] while the spatial resolution of CT/MRI (~2 mm) approaches the limits of tumor measurement, particularly for recurrent or micrometastatic disease. This issue together with observer-dependent accuracy (low kappa) further confounds accuracy. Such difficulties are amplified by the often indolent growth rates of well- and moderately differentiated NETs.

Biomarkers such as CgA define tumor secretion and do not reflect biological activities including cell proliferation, growth factor signaling or any of the ‘hallmarks of cancer’ [37]. A variety of metrics have been assessed to evaluate the use of these markers. For example, a 30% decrease in CgA (from pretreatment levels) is considered predictive of SSA efficacy [38], while an increase in three





**Fig. 6.** Time before blood-based biomarker elevations preceded image-based evidence for PD. **a** Changes in CgA ( $\Delta$ CgA, >25%) occurred at a time point (0.51 years) similar to an elevated CgA ( $\geq 300 \mu\text{g/l}$ : 0.60 years) prior to image-detected disease progression. Only 50% of patients (16 of 32) exhibited elevated CgA at

baseline with a subset of 10 exhibiting abnormally elevated ( $\geq 300 \mu\text{g/l}$ ) CgA. **b** The median times prior to image-confirmed disease progression for the NETest were 0.76 years (>80%) and 1.62 years (>70%). All patients were NETest positive (>14%).

consecutive measurements is considered to anticipate relapse after midgut surgery [39]. Some authors proposed that alterations of  $\geq 25\%$  in CgA may have good sensitivities (>75%) and specificities (>85%) for predicting disease events [33] while others suggested that levels twice the ULN ( $\sim 300 \mu\text{g/l}$  or  $300 \text{ ng/ml}$ ) [32] or higher ( $\geq 600$ ) [40] are effective predictors of disease progression. Irrespective of the cutoff levels proposed, numerous technical issues, e.g. low reproducibility and disease confounders (drugs, diseases), limit its utility. CgA and other single (mono)analyte biomarkers have not met the expectations of the clinical community [19, 41, 42].

Investigation has focused on the development of multianalyte assays that can identify multiple key elements of neoplastic cell function and be interfaced with sophisticated mathematical models (multianalyte algorithm analysis). This approach has been acknowledged as necessary to define and predict the exquisite complexity of the neoplastic conundrum [43]. Defining the bandwidth of the genomic regulation of neoplasia requires the simultaneous assessment of numerous parameters that circumscribe diverse aspects of tumor biology and facilitate the ‘framing’ of clinical behavior [44–46]. Numerous successful applications of this principle have advanced the disease management of neoplasia in other sites including breast, liver, colon, prostate and lung cancer [44, 47–50].

Multianalyte algorithm analysis strategies based upon a 51-gene signature (NETest) have been reported in NETs [24, 26, 28, 29, 51–53]. This defines the circulating NET

‘fingerprint’ [54] and exhibits a higher sensitivity and specificity (98 and 97%, respectively) than secretory markers for identifying neoplasia [25, 51]. The assay is standardized and highly reproducible (inter- and intra-assay coefficient of variation <2%), and is independent of tumor heterogeneity [28]. Gene expression is captured in a 0–8 score derived from 4 different prediction algorithms that is mathematically scaled to disease activity (0–100%) by interpolating the expression of ‘omic’ transcripts that define specific biological components (hallmarks) of neoplasia [27]. HR-derived analyses enable the derivation of scores that define disease activity (0–14%, minimal activity; 14–40%, low activity; >40%, intermediate and high activity) [26]. Individual activity levels correlate accurately with clinically stable disease or PD and effectively facilitate the determination of treatment efficacy [26].

In the current study, all patients were NETest positive, e.g.  $\geq 14\%$  at baseline. NETest levels were significantly ( $p < 0.05$ ) elevated in 8 (90%) of the 9 patients identified with PD. One patient (terminal ileum, grade 1) had a score of 20%, and was termed as ‘mixed’ progression, suggesting the low score reflected a response to therapy (SSA). An analysis of the 7 (defined as clinically stable at baseline) who did not have a low tumor activity NETest (i.e. scores >40%) showed that all developed PD with a mean of 24 months (range: 3.6–57.5 months). Conversely, 7 stable (at baseline) patients who never developed disease progression exhibited a low-activity (<40%) NETest

(mean follow-up: 52 months, range: 32–60.5 months). While this shows some discordance between the NETest and imaging at a particular point in time (50% of patients had low activity, 50% high activity despite all 14 exhibiting disease ‘stability’), the NETest accurately (100%) predicted outcome. This is predictable given the limitations of imaging in terms of sensitivity and defining biological activity. An image cannot *intrinsically* provide dynamic information about tumor activity whereas a circulating molecular fingerprint that defines neoplastic biology is specifically designed to identify and quantify such information. These data suggest that the use of blood-based tumor-derived molecular information in conjunction with standard imaging may provide added clinical value in defining disease status and guiding therapy.

Three patients (No. 15, 21, and 26) are worthy of consideration. All were surgically cured (‘complete resection’) at operation. They comprised 2 low-grade (Ki-67 <1%) duodenal NETs (one, patient 15, was a gastrinoma) and a pancreatic tumor. Two of the 3 ‘surgically cured’ individuals (No. 15 and 21) did not exhibit ‘clinical’ evidence of disease (by CT/MRI) for the duration of the study. Their NETest scores at baseline (after surgery) were 27 and 20%, respectively. Two sequential measurements at 2.6 and 3.9 years in patient 15 were 33 and 33%, respectively; for patient 21, the NETest at 3.2 years was 20%. These fall into the ‘low-activity’ range. In a separate 5-year follow-up study of a surgical cohort (n = 12; all with NET grade 1, none with gastrinoma), complete resection was associated with NETest levels <14% [52]. The elevated postsurgical level in the duodenal gastrinoma patient (33%) in the current study is of concern and may reflect microscopic disease not identifiable at imaging. Low-activity, image-negative disease is suggested by the follow-up circulating values (33%). One patient, No. 26, with no disease at baseline, developed recurrence after 2.2 years. This was a grade 3 (Ki-67 = 30%) pancreatic NET. The NETest was 14% at baseline, and CgA levels were normal. Thereafter, NETest levels increased (score >65%) while CgA remained normal. The borderline score (14%) after surgery suggests that the completeness of surgical excision might require re-evaluation. The subsequent rise demonstrates that the tumor recurred. The elevation in the NETest was concordant with disease recurrence and importantly occurred ~6 months before any measureable elevation in CgA.

Cox proportional-hazard modeling identified that of all variables assessed, only the NETest was predictive of PFS. The HR was 1.022 demonstrating that for each percentage point change (increase) in the NETest, the risk of disease progression was 2.2%. NETest values for >70 or

>80% would therefore have HRs of ~2.5 and ~2.8, respectively. In this model, baseline CgA (HR = 1) and site (HR = 0.72) were not associated with PFS. In addition, histological grade was not a significant variable either (HR = 1.095). The low numbers (n = 31) likely contribute to the absence of statistical significance; however, despite this, the NETest was mathematically identified as being significant even in this cohort.

We also performed Kaplan-Meier PFS analysis which identified that the NETest ( $\geq 80$  or  $\geq 70\%$ ) at baseline (T0) exhibited a trend toward significance (p = 0.06) with predicting outcome (RECIST-based assessment after a mean of ~6 months). Proof of principle for the NETest as a prognostic was confirmed by clinical assessment after a second blood sample. The mean time to the second image assessment was also 6 months. In this cohort (n = 29), the NETest ( $\geq 80\%$ ) was associated with a median survival of 246 days (vs. not reached for NETest  $\leq 79\%$ ). Decreasing the cutoff to 70% resulted in an HR of 25.7 (95% CI = 5.2–129). The median survival was 183 days (not reached for NETest  $\leq 69\%$ , p < 0.00001). These analyses (Cox/Kaplan-Meier) show that NETest levels are a significant prognostic variable in GEP-NETs (independent of grade and site) and can accurately predict clinical outcome as well as response to therapy.

CgA, in contrast to the NETest and consistent with its function as a unidimensional measure of secretion, was not an effective biomarker. Firstly, it was not identified in the Cox proportional-hazard model as an informative variable. Secondly, elevated levels, i.e.  $>1 \times$  ULN, were only noted in 50% of patients at baseline, thus half the patients could *ab initio* derive no value from its assessment. This is confirmed by multivariable analysis. Moreover, the 2 ‘surgically cured’ patients (no evidence of disease recurrence) had elevated baseline levels, 343  $\mu\text{g/l}$  (patient 15) and 381  $\mu\text{g/l}$  (patient 21), respectively. Subsequent measurements showed that CgA was only normalized in patient 21. Patient 15, who had a gastrinoma, had elevated levels consistent with the secretory behavior captured by this biomarker. Baseline CgA was not statistically significantly elevated in the PD cohort. A subanalysis of the 16 with elevated baseline found that abnormally high ( $2 \times$  ULN) levels ( $\geq 300 \mu\text{g/l}$ ) were not associated with outcome. Kaplan-Meier analysis did not identify any significant role for CgA as a prognostic marker in this cohort either. Use of higher cutoffs, e.g.  $\geq 600 \mu\text{g/l}$ , was also noninformative.

In head-to-head comparisons, the NETest was significantly more informative than alterations in CgA. Assessment of NETest alterations (elevation to levels  $\geq 80\%$  in PD or remaining low, i.e. <47%, in stable disease) oc-

curred more consistently (96%) than alterations in CgA (e.g. elevation  $\geq 25\%$ ,  $p < 2 \times 10^{-5}$ ). This should be considered in the context that in 50% of patients, CgA was never elevated despite a histological NET diagnosis. Of considerable relevance was the observation that elevations in NETest ( $\geq 80\%$ ) occurred at a significantly earlier time point than image evidence of PD. Such changes occurred  $\sim 1 \pm 0.15$  years before image confirmation of disease, time points similar to that identified for circulating DNA in colorectal cancer (mean = 10 months) [55]. More germane to the early identification of disease progress was the observation that if the cutoff was adjusted downward to  $\geq 70\%$ , the time point of identification of disease progression was significantly earlier. A NET level of  $\geq 70\%$  enabled identification of disease progression  $\sim 2$  years prior to evidence of image-identifiable disease progression. This was significantly earlier than for a  $\geq 80\%$  cutoff ( $p < 0.02$ ). In contrast, alterations in CgA were less informative. A change of 25% or any elevation  $\geq 300 \mu\text{g/l}$  occurred only  $\sim 0.5$  years prior to image-based confirmation and was unidentifiable in 50%.

These data are consistent with the proposal that the NETest had a prognostic role in this cohort and confirms utility in clinical management as has been previously noted [29]. High levels are associated with disease progression while low levels reflect and predict disease stability. Limitations of the study include the relatively small numbers (34 patients, the final analyzed cohort included 31 patients), that this was not a formal prospective study in a homogeneously treated cohort (blood sampling was undertaken on average 2.5/patient and restaging on average  $\sim 7$  occasions/patient) and that some of the data were extracted and confirmed retrospectively (e.g. imaging was re-evaluated according to RECIST, some CgA samples were measured retrospectively). The study strengths in-

clude that it was undertaken at an ENETS Center of Excellence (Charité, Berlin) in a 'real-world' setting with state-of-the-art imaging (single center), follow-up and patient care using standardized biomarker assessments. The concordance in the results between two different statistical approaches, Cox proportional-hazard regression and Kaplan-Meier assessment, substantiate the utility of the NETest as a biomarker. The data further define the increasing awareness of the limitations of CgA measurements.

In conclusion, the NETest is an independent variable predictive of clinical disease status. The measurement of this circulating transcript signature correlates with clinical disease status, and levels ( $\geq 70\%$ ) are prognostic for well-differentiated GEP-NET progression (per RECIST). The NETest  $< 40\%$  correlated with disease stability over  $\sim 5$  years, identifying this molecular signature also as predictive. Patients clinically categorized as stable with high NETest levels ( $\geq 70\%$ ) develop disease progression in 100% of cases within 2 years. The overall utility is emphasized by observations that clinically actionable alterations occurred  $\sim 1$  year before image-based evidence of disease progression. Unlike single analyte secretory measurements, the NETest accurately defines the spectrum of well-differentiated GEP-NET disease and, more critically, can predict when disease stability evolves into progression.

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### Disclosure Statement

There is no conflict of interest.

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