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ORIGINAL ARTICLE

Measurement of circulating transcripts and gene cluster analysis predicts and defines therapeutic efficacy of peptide receptor radionuclide therapy (PRRT) in neuroendocrine tumors

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Abstract

Background Peptide receptor radionuclide therapy (PRRT) is an effective method for treating neuroendocrine tumors (NETs). It is limited, however, in the prediction of individual tumor response and the precise and early identification of changes in tumor size. Currently, response prediction is based on somatostatin receptor expression and efficacy by morphological imaging and/or chromogranin A (CgA) measurement. The aim of this study was to assess the accuracy of circulating NET transcripts as a measure of PRRT efficacy, and moreover to identify prognostic gene clusters in pretreatment blood that could be interpolated with relevant clinical features in order to define a biological index for the tumor and a predictive quotient for PRRT efficacy.

Methods NET patients (n=54), M: F 37:17, median age 66, bronchial: n=13, GEP-NET: n=35, CUP: n=6 were treated with ^{177}Lu-based-PRRT (cumulative activity: 6.5-27.8 GBq, median 18.5). At baseline: 47/54 low-grade (G1/G2; bronchial typical/atypical), 31/49 ^{18}FDG positive and 39/54 progressive. Disease status was assessed by RECIST1.1. Transcripts were measured by real-time quantitative reverse transcription PCR (qRT-PCR) and multianalyte algorithmic analysis (NETest); CgA by enzyme-linked immunosorbent assay (ELISA). Gene cluster (GC) derivations: regulatory network, protein:protein interactome analyses. Statistical analyses: chi-square, non-parametric measurements, multiple regression, receiver operating characteristic and Kaplan–Meier survival.

Results The disease control rate was 72 %. Median PFS was not achieved (follow-up: 1–33 months, median: 16). Only grading was associated with response (p<0.01). At baseline, 94 % of patients were NETest-positive, while CgA was elevated in 59 %. NETest accurately (89 %, χ^2=27.4; p=1.2×10^{-7}) correlated with treatment response, while CgA was 24 % accurate. Gene cluster expression (growth-factor signalome and metabolome) had an AUC of 0.74±0.08 (z-statistic=2.92, p<0.004) for predicting response (76 % accuracy). Combination with grading reached an AUC: 0.90±0.07, irrespective of tumor origin. Circulating transcripts correlated accurately (94 %) with PRRT responders (SD+PR+CR; 97 %) vs. non-responders (91 %).

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Conclusions Blood NET transcript levels and the predictive quotient (circulating gene clusters+grading) accurately predicted PRRT efficacy. CgA was non-informative.

Keywords Neuroendocrine tumor · Chromogranin · ^{68}Ga-PET · Gene transcripts · NETest · PRRT

Introduction

Neuroendocrine tumors (NETs) are frequently identified when they are metastatic or locally advanced. Their pathobiology supports multiple treatment modalities, which are individualized through a multidisciplinary approach. In principle, the choice of therapy depends on individual tumor characteristics and ranges from complete surgical eradication to a "watch and wait" approach [1].

Peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-DOTA-Tyr^3-Thr^8-octreotide (^{177}Lu-octreotate) is an established effective therapeutic modality that has been used for 15 years in the treatment of unresectable or metastatic gastroenteropancreatic (GEP) and bronchopulmonary (BP) NETs. Objective response rates of 15–35 % are common, with modest toxicity in the majority of cases if the necessary precautions (e.g., renal protection) are undertaken [2]. Of particular significance is outcome; both progression-free survival (PFS) and overall survival compare favorably with somatostatin analogues (SSA), chemotherapy and new "targeted" therapies [3].

Combinations of anatomical (CT/MRI) and functional imaging (OctreoScan® or ^{68}Ga-SSA-PET/CT–SRI) techniques are used to monitor therapy [4]. However, the low-dimensional alterations typical of these tumors, coupled with the resolution limits of both CT and PET scanners, limit the early and accurate detection of lesion alterations, particularly for large-volume, multicentric or poorly demarcated disease, rendering RECIST criteria largely inadequate for capturing the effects of therapy in a timely and accurate fashion [4].

A variety of blood markers, particularly chromogranin A (CgA), have been proposed to facilitate the determination of therapeutic efficacy and disease status. None, however, has shown adequate sensitivity and specificity or met the metric standards of accuracy [5–7]. Nevertheless, despite reservations regarding its efficacy, CgA remains the default measurement [8]. Given the key unmet need for sensitive and accurate biomarkers to define therapeutic efficacy, innovative solutions including circulating tumor cell (CTC) analysis [9], miRNA measurement [10] and multigene assays with algorithmic analyses (MAAA) have been proposed [11, 12]. The last of these assesses NET biological activity using gene inference technology and cancer hallmark prediction [12]. The set of circulating transcripts that defines this "fingerprint" exhibits high sensitivity (98 %) and specificity (97 %) for detecting NETs, is standardized and reproducible (inter- and intra-assay CV <2 %) and outperforms other GEP-NETs biomarkers, including CgA [11].

Blood gene transcript analysis in patients treated with SSA therapy or surgery has demonstrated a significant advantage in the early detection of residual disease [13] and in the assessment of somatostatin analog response [14]. Furthermore, combinations of circulating NET transcripts in conjunction with ^{68}Ga-SSA-PET/CT have demonstrated that molecular imaging parameters (SUV_{max}) could be integrated into a predictive quotient of tumor status [15]. We recently demonstrated that the segregation of circulating NET transcripts into gene clusters using unbiased protein:protein interactome approaches, in addition to regulatory network and evaluation of published data, was successful in defining the NET cancer "hallmarks" [12, 16]. With this strategy, we identified nine genomic clusters, or "omes," governing the various cellular functions (SSTRome, proliferome, growth factor signalome, metabolome, secretome, epigenome, plurome, apoptome) that captured the biological activity of the tumor. Inclusion of these "omes" provided a predictive activity index for defining tumor behavior and outcome [12]. A recent consensus publication considered gene transcript analysis to be the biomarker strategy most likely to provide clinical utility [17].

The purpose of the present study was to evaluate the efficacy of PRRT with ^{177}Lu-octreotate using circulating NET transcripts and CgA. In addition, we sought to identify whether circulating NET transcripts or gene clusters in the pretreatment blood, which provide biologically relevant information on the individual tumor, could be interpolated with relevant clinical features and used to predict the response to PRRT.

Materials and methods

Patients

Patients with GEP and BP NETs (n=78) that exhibited somatostatin receptors (identified by receptor imaging) and who were candidates for PRRT with ^{177}Lu-octreotate were enrolled from July 2012 to April 2015. The present study is an interim analysis of 54 individuals with complete follow-up (at least 6 months post-therapy) as of April 2015.

Study design and procedures

PRRT protocol Patients were treated according to two protocols, depending on previous treatments and risk factors for delayed toxicity [18, 19] (Table 1). The first protocol was developed for PRRT-naïve patients. Two different levels of intended cumulative therapeutic activity, 18.5 (1A) or 27.8 (1B) GBq divided into four cycles (4.6 and 6.5 GBq each,

Table 1 PRRT protocols. Rationale for choosing the protocol was based on PRRT naiveté and the presence of "risk factors" for delayed toxicity. Cycles were administered 2 months apart

Status prior to PRRT	PRRT protocol	Intended cumulative activity (GBq)	Intended activity per cycle (GBq)	Patients treated
PRRT naïve, risk factors	1A	18.5 in 4 cycles	4.6	22
PRRT naïve, no risk factors	1B	27.8 in 4 cycles	6.5	17
PRRT pre-treated	2	14.8 in 4 cycles	3.7	15

respectively), were administered 2 months apart based upon kidney function and bone marrow reserve. The second protocol (14.8 GBq divided into four cycles of 3.7 GBq each, administered 2 months apart) was utilized in individuals pretreated with PRRT (^{177}Lu-octreotate or ^{90}Y-octreotide) [20]. All participants provided informed consent for PRRT and translational analysis, which was authorized by the ethics committees (PRRT: IRST 100.06, EudraCT: 2011-002891-18, 04/08/2011; NETest: IRST B007 [70/12], 10/10//2012).

Assessment of therapeutic response CT (or MRI) was performed at baseline (within 3 months from the start of PRRT) and 3 and 6 months after PRRT to define disease status and assess response according to RECIST 1.1 criteria (Table 2) [21]. Baseline status was defined according to RECIST criteria, based on a comparison with CT/MRI obtained within 1 year from enrolment. ^{68}Ga-SSA-PET or OctreoScan® was performed at baseline and at 6-month follow-up. Subsequent imaging was performed per standard clinical practice.

Transcript analysis Samples of 10 ml of whole blood were collected in 2 × 5 ml tubes and snap-frozen at baseline and after each administration of ^{177}Lu-octreotate, and at 3- and 6-month follow-up after treatment completion (Table 2). Plasma CgA were collected at the same time points. A two-step protocol (RNA isolation, cDNA production and PCR) was used [11, 12]. Analyses were carried out using the MATLAB Statistics and Machine Learning Toolbox (MATLAB R2011a; MathWorks, Natick, MA, USA). The NETest mathematically delineates disease activity risk on a scale of 0 to 100 %, as follows: minimal, <14 %; low, 14–47 %; high, >47 %. The NETest also includes biologically relevant gene expression measurements from the different "omes" (SSTRome, proliferome, metabolome, secretome, epigenome and plurome) that differentiate progressive from stable disease [12].

Table 2 Schedule of blood sampling and diagnostic exams (morphologic and functional)

	Assessment
1st cycle	Blood for transcript analysis and CgA CT/MRI SRI*
2nd cycle	Blood for transcript analysis and CgA
3rd cycle	Blood for transcript analysis and CgA
4th cycle	Blood for transcript analysis and CgA
3-month follow-up	Blood for transcript analysis and CgA, CT/MRI
6-month follow-up	Blood for transcript analysis and CgA, CT/MRI SRI*

*SRI: ^{68} Ga-SSA-PET or OctreoScan

CgA assay CgA was measured using NEOLISA™ Chromogranin A kit (Euro Diagnostica AB, Malmö, Sweden). The upper limit of normal was 108 ng/ml [11].

Toxicity evaluation Hematological, renal, and liver toxicity was evaluated using Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 (NCI, Bethesda, MD, USA).

Statistical analysis Sensitivity comparisons between the NETest and CgA were conducted using chi-square, non-parametric measurements and receiver operator curve (ROC) analysis with determination of the area under the curve (AUC). Prism 6.0 for Windows (GraphPad Software, La Jolla CA USA, www.graphpad.com) and MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) were utilized. Multiple regression analyses were undertaken to identify significant clinical parameters, e.g., ^{18}FDG-positivity. As reported in the literature, pre-therapy CgA> 600 ng/ml was used as one measure to predict response and PFS [3, 22].

An "Ome Index" was derived from a summation of gene cluster expression that included the "growth factor (GF) signalome" and the "metabolome" as measured in the pre-treatment blood. These were chosen based on expression levels and AUC values >0.65. Ome index values were separated into two groups based on their ability to predict response. On the basis of >85 % specificity for predicting disease response (see "Results"), cut-off values of 5.9 separated "Ome (high)" (>5.9) from "Ome (low)" (<5.9). A "Prediction Quotient" comprising the grade (low grade=G1/G2, well-differentiated, bronchial typical or atypical carcinoid"; high grade=G3, "poorly differentiated") and Ome Index (see

"Results") was developed. In order to add further specificity to the Prediction Quotient, we separated the components into two groups—Low Grade/High Ome and High Grade/Low Ome—to assess clinical utility (prediction of disease response and PFS). The mathematical indices evaluated included the NETest (algorithm of 9 "omes" and 51 NET genes), the Ome index (GF signalome+metabolome) and the Prediction Quotient (comprising Ome index+grade). The predictive accuracy of each of the mathematical indices (Fig. 1) was compared using ROC curve analyses, and the sensitivity, specificity, and the AUC were calculated (MedCalc) [23]. AUCs were compared and the Z-statistic derived [24] (MedCalc). Kaplan-Meier survival curves (PFS) were generated and analyzed in Prism. Log-rank (Mantel–Cox) and hazard ratios (Mantel–Haenszel) were calculated. Z-statistic scores >1.96 are significant ($p<0.05$). For other metrics, e.g., accuracy, 80 % is generally accepted as the acceptable cut-off. [6, 7] All data were expressed as mean±standard error of mean.

Results

A. Clinical audit As of April 2015, 78 GEP and BP NET patients were consecutively enrolled, 60 of whom had definitive data. Six were excluded because of withdrawal of consent (three subjects), death during PRRT for causes other than NET (two subjects) or missing data before dropping out due to progression (one subject). The remaining 54 were included in the analysis. Follow-up ranged from 1 to 33 months; the median was 16 months (Table 3).

B. Treatment response and PFS Cumulative administered activity of ^{177}Lu-octreotate was 6.5–27.8 GBq (median 18.5). Fifty-one of the 54 patients (94.4 %) received ≥75 % of the intended cumulative activity. PRRT was well-tolerated, without serious side effects. Severe hematological toxicity was limited to a minority of patients (8 % G3; no G4), while moderate (G2; no G3/4) creatinine toxicity occurred in 5 %. Response to PRRT included complete response (CR) in one subject (1.9 %), partial response (PR) in nine (16.7 %), stable disease (SD) in 29 (53.7 %), and progression (PD) in 15 (27.8 %), with a disease control rate (CR+PR+SD) of 72.2 % (no significant differences between the two PRRT protocols). At the time of the analysis, the median PFS had not been not reached (Fig. 2a). While no significant differences were noted by site ($p=0.11$) (Fig. 2b), the highest objective response rate occurred in pancreatic NETs.

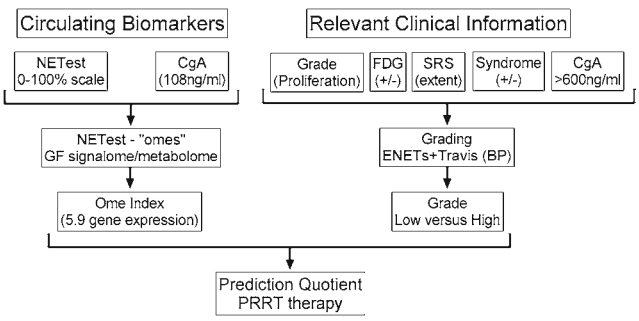

Fig. 1 Development of the Predictive Quotient for assessing PRRT therapy. Graphic demonstrating the development of the Prediction Quotient from an assessment of circulating biomarkers as well as clinically relevant information

Regression analysis of clinical parameters at baseline (demographics, SSA use, primary location, metabolic status at ^{18}FDG PET/CT, symptoms, time since diagnosis, baseline ECOG status) identified that no clinical parameters were associated with treatment response (odds ratios: 0.5 [SSA, $p=0.35$)] – 2.0 [ECOG status, $p=0.35$]) except for grading (OR: 8.75, $p=0.004$) (Table 4). Further analysis confirmed that grading was associated with outcome ($R^2=0.19$, F-ratio: 11.7, $p=0.0012$).

C. Biomarker assessment NETest and CgA were examined as a function of cycle and follow-up time in order to evaluate the relationship to treatment response (Table 2). Outcome was defined by RECIST 1.1 criteria as responders (SD+PR+CR; $n=39$) or non-responders (PD; $n=15$).

C1. NETest The NETest (mean pre-therapy time 0, prior to Cycle I) was 42.9±4.6 %. This was elevated (>14 %) in 94 % (Fig. 3a). At 3-month follow-up, this was reduced (33.2±5.6 %, $p=0.08$), and at the 6-month follow-up, the reduction was significant (25±3.7 %, $p=0.012$) in responders. At 3 and 6 months, levels were increased in non-responders (3 months: 45.3±8 %, $p=0.32$ vs. pre-therapy, $p=0.11$ vs. responders and 6 months: 58.2±6.2 %, $p=0.04$ vs. pre-therapy, $p<0.0001$ vs. responders). The NETest decreased in 88 % of responders (no change in 12 %) and was increased in 90 % (no change in 10 %) of non-responders at 6 months.

C2. CgA Levels prior to therapy were 2426±611 ng/ml (Fig. 3b). Elevated (>108 ng/ml) CgA, however, was only noted in 57 %. The decrease (to 689±215 ng/ml) in responders (at 6 months) was not significant. CgA was unchanged in 47 %, decreased in 21 % and elevated in 32 % of responders at 6 months. Levels were significantly decreased in non-responders (202±117 ng/ml) ($p=0.045$). CgA was unchanged in 33 %, decreased in 40 % and was elevated in 27 % of the non-responders. No significant differences were noted at the 3-month or 6-month follow-up time points ($p>0.3$).

C3. NETest vs CgA The NETest correlated significantly with pre-therapy status (94 % vs. 57 %, $p=0.0004$, chi-square) (Fig. 3c) in comparison to CgA, while changes in levels more consistently correlated with treatment response than in CgA (responders: $p=0.0002$, non-responders: $p=0.0068$)

Table 3 Patient Demographics and Disease Characteristics

Patients	(*n*=54)
Age, median (range) in years	66 (43–83)
Gender, *n*	37 M, 17 F
Time since diagnosis, in months (range)	3–265
Mean (SD)	58 (57.5)
Median	37
Length of follow up, median (range), in months	16 (1–33)
NET origin, *n* (%)	
Broncho-pulmonary [26]	**13 (24.1 %)**
Typical carcinoids	*1 (1.9 %)*
Atypical carcinoids	*7 (13 %)*
NOS carcinoids	*1 (1.9 %)*
High-grade	*4 (7.4 %)*
GEP	**35 (64.8 %)**
Stomach	*1 (1.9 %)*
Pancreas	*14 (25.9 %)*
Small intestine	*17 (31.5 %)*
Colon	*1 (1.9 %)*
Rectum	*2 (3.7 %)*
Unknown	***6 (11.1 %)***
GEP NETs, Tumor grade (WHO 2010 [25]), *n* (%)	
G1 (Ki-67 0–2 %)	6 (17.1 %)
G2 (Ki-67 3–20 %)	20 (57.1 %)
G3 (Ki-67>20 %)	3 (8.6 %)
Non-specified (well-differentiated)	6 (17.1 %)
Initial clinical stage, *n* (%)	
Stage IV	54 (100 %)
Liver	*45*
Lymph nodes	*31*
Bone	*18*
Peritoneum	*9*
Lung	*4*
Other sites (e.g. adrenal, pleura, pericardium)	*9*
Baseline tumor status	
Progressive disease	39 (72.2 %)
Stable disease	13 (24.1 %)
Response to previous chemotherapy	2 (3.7 %)
Extent of disease§, *n* (%)	
Limited	7 (13 %)
Moderate	26 (48.1 %)
Extensive	21 (38.9 %)
Intensity of uptake*, *n* (%)	
Grade 1	1 (1.9 %)
Grade 2	6 (11.1 %)
Grade 3	16 (29.6 %)
Grade 4	31 (57.4 %)
^{18}FDG PET/CT	
Negative	18 (33.3 %)
Positive	31 (57.4 %)
Not tested	5 (9.3 %)
CgA	
Normal	23 (42.6 %)
Elevated	31 (57.4 %)
Previous therapy, *n* (%)	
Surgery	**32**
Primary tumor surgery	*30*
Liver surgery	*7*
Non-resective surgery	*1*
Somatostatin Analogs	**44**
Pharmacotherapy	
Chemotherapy	*21*
Everolimus	*5*
Sunitinib	*1*
Interferon alpha	*1*
Other	
PRRT	*16*
Radiotherapy	*6*
TACE	*4*

§Extension of uptake according to Krenning scale [32]

*Intensity of uptake according to Krenning scale either on OctreoScan [32] or modified criteria applied to ^{68}Ga-PET

(Fig. 3d–e). Neither baseline (pre-treatment levels) of the NETest nor of CgA were predictive of outcome (NETest: OR=0.98, p=0.3; CgA: OR=1.0, p=0.18).

C4 Metrics The metrics for biomarkers and outcome identified that the NETest had 89 % accuracy, 75 % sensitivity, 100 % specificity, 100 % PPV and 83 % NPV (Fig. 4a). CgA, in contrast, had 24 % accuracy, 17 % sensitivity, 40 % specificity, 40 % PPV and 17 % NPV (Fig. 4b). The NETest significantly outperformed CgA (chi-square=27.4; $p=1.2\times10^{-7}$).

D. Derivation of outcome predictors We next examined each of the biomarkers or constituent factors, e.g., gene clusters or "omes" and clinical parameters or combinations thereof, to predict response. We specifically focused on pre-therapy blood.

D1. Biomarkers Pre-therapy (T0 cycle I) NETest or CgA levels alone were not significantly predictive of outcome (see Section C3). ROC analysis identified AUCs of 0.65± 0.09 (p=0.09) and 0.58±0.08 (p=0.32), respectively. CgA levels (>600 ng/ml) were also not associated with outcome or PFS (<600 ng/ml: 16.9±1.2 months PFS vs. 13.8 ±.4 months, p=0.19). There was no significant difference in NETest (p=0.26-0.71) or CgA (p=0.06-0.6) levels between tumor sites, e.g., bronchopulmonary versus GEP-NET.

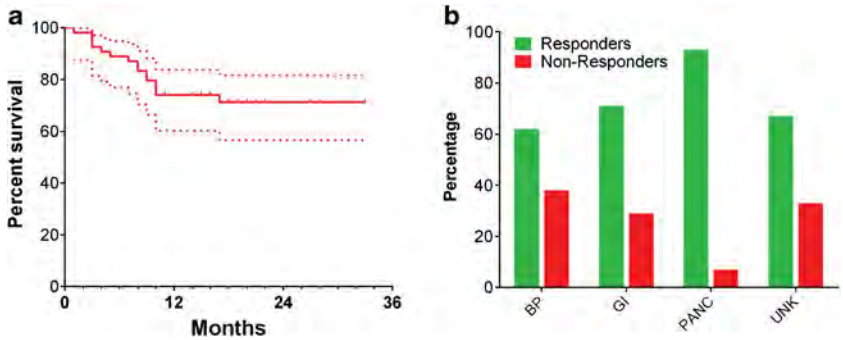

Fig. 2 PRRT outcome by site assessed by overall RECIST objective response. **a**) Progression-free survival curve (*solid red line*) with 95 % confidence interval (*dotted red lines*) indicates that a median value was not reached. **b**). Responders (SD+PR+CR) were 62 % bronchopulmonary, 71 % GI, 93 % pancreas and 67 % unknown. Chi-square analysis identified no significant differences (p=0.11) in outcome based on site. BP, bronchopulmonary (n=13); GI, gastrointestinal (n=20); PANC, pancreas (n=15), UNK=unknown site (n=6)

D2: "**Omes**" An assessment of gene expression in the nine different "omes" captured by the NETest identified no significant difference between primary sites (p=0.10–0.98). Pre-treatment blood levels of growth factor signaling (GF signalome) and metabolism, however, were different between responders and non-responders. Specifically, subsequent PRRT responders exhibited significantly elevated growth factor signaling (9.4±1.3 vs. 5.3±0.7, p=0.05) and metabolomic gene expression (4.37 vs. 2.3±0.6, p=0.03) at T0 (Cycle I, i.e., prior to therapy) compared to non-responders (Fig. 5a). Gene expression of the other "omes", e.g., somatostatin receptor-ome "SSTRome", were not different (49.5±11.6 vs. 29.7±8.5, p=0.15). ROC analysis (Fig. 5b) identified AUCs of 0.67 and 0.7 and z-statistics of 2.44 and 2.26 for the GF signalome and metabolome, respectively. An integration of the two "clusters" (GF signalome+metabolome) in an "Ome Index" resulted in an AUC of 0.74±0.08 (z-statistic=2.915, p=0.0036). A cut-off of 5.9 (normalized gene expression) exhibited >85 % specificity for predicting response (>5.9 predicted subsequent PRRT responders).

Table 4 Clinical Variables and Response*

Variable	Logistic Regression Analysis		Multiple Regression Analysis
	OR (95 % CI)	p Value	
Age	1.01 (0.94–1.078)	0.85	
Gender	0.89 (0.25–3.17)	0.86	
Site			
Bronchopulmonary	0.73 (0.17–3.1)	0.67	
Pancreas	0.64 (0.15–2.7)	0.54	
Gastrointestinal tract	2.29 (0.68–7.69)	0.18	
Unknown	0.49 (0.05–4.54)	0.53	
Time since diagnosis	1.01 (0.997–1.02)	0.17	
Grading	8.75 (2.01–38.14)	**0.004**	R^2=0.19, F-ratio 11.7, p=0.0012
Baseline status	1.78 (0.423–7.48)	0.43	
ECOG	2.00 (0.475–8.42)	0.35	
Syndrome	1.56 (0.47–5.22)	0.47	
Extent of disease	1.57 (0.62–3.96)	0.34	
Intensity of uptake	0.81 (0.38–1.73)	0.58	
FDG	1.24 (0.35–4.44)	0.74	
SSA use	0.5 (0.12–2.11)	0.35	

*RECIST 1.1 Criteria

D3. Clinical parameters Multiple regression analysis of clinical parameters at baseline identified that only grading [low grade: G1/G2 (WHO 2010 [25]), bronchial typical or atypical carcinoids [26]]; high grade: G3, poorly differentiated) was associated with outcome (coefficient: 0.598±0.19, p=0.0035). Individually, the coefficient for G1 was −0.71 (p=0.004) and for G 2 was −0.66 (p=0.0043). Variables including syndrome, SRI parameters (extension of disease, intensity of uptake [Rotterdam Scale proportions] pre-PRRT), concurrent SSA use, baseline status or syndrome were not significantly associated with PRRT response in this cohort (Tables 4 and 5).[18]FDG positivity, although not associated with outcome (OR: 1.24, p=0.74), was associated with PFS (chi-square=15.9, p<0.0001, log rank: 5.1). Low-grade tumors typically responded to therapy (77 %), while 50 % of high-grade lesions exhibited a response (Fig. 5c). While grade per se, was not significantly different (p=0.12), grading did exhibit an AUC of 0.66±0.06 (p=0.08) for predicting response (Fig. 5d).

E. Derivation of a clinically useful "Prediction Quotient" The combination of the Ome Index ("GF signalome"+"metabolome", Fig. 5b) with the grade had a significantly

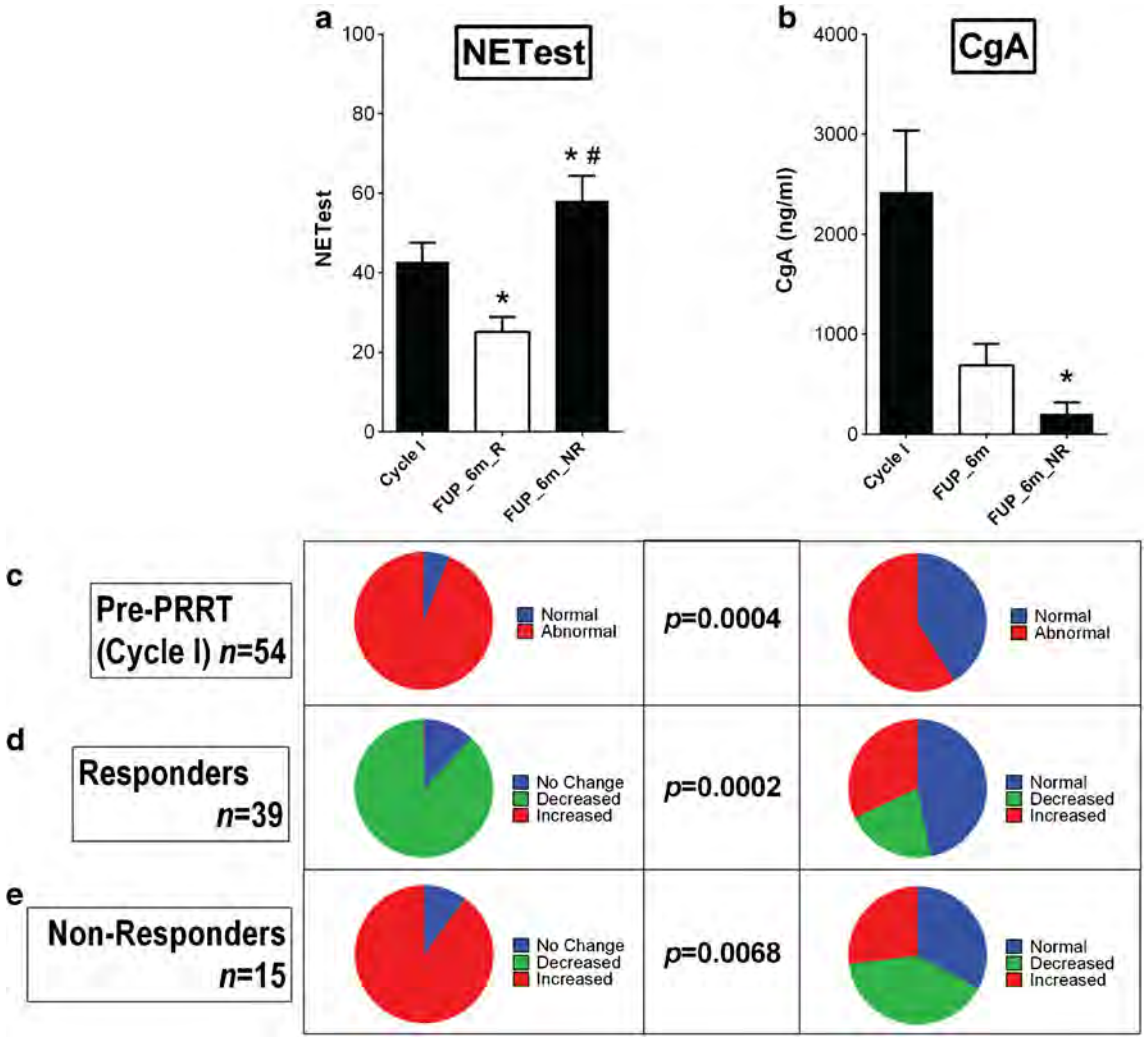

Fig. 3 NETest and CgA levels in responders and non-responders. **a**) NETest was significantly reduced at 6-month follow-up compared to pre-treatment values. Non-responders (NR) exhibited significantly elevated levels at 6 months (FUP_6m). This was also significantly higher than in responders (R). **b**) A significant alteration was noted only for CgA in non-responders (NR) at 6 months. **c**) *Pre-PRRT*: NETest scores were elevated in 94 % and CgA in 57 % (p=0.0004). **d**) *Responders:* NETest decreased in 88 % of responders (no change in 12 %). CgA was unchanged in 47 %, decreased in 21 % and was elevated in 32 % (p=0.0002 vs. NETest). **e**) *Non-Responders:* NETest increased in 90 % (no change in 10 %). CgA was unchanged in 33 %, decreased in 40 % and was elevated in 27 % (p=0.0068 vs. NETest). For the NETest, no falsely decreased or increased values occurred in responders or non-responders, respectively. Mean±SEM. *p<0.05 vs. Cycle I (T0); #p<0.05 vs. responders (6-month follow-up)

better AUC (0.90±0.06) than the grade alone (AUC=0.66, difference between areas 0.23, z-statistic 2.25, p=0.024) (Fig. 6a) for predicting response. This Prediction Quotient was also clinically useful. The high-grade/low-Ome tumor group had significantly lower PFS (17 months) than the low-grade/high-Ome group (undetermined, log-rank: 26.8; p<0.0001: Fig. 6b). The hazard ratio was 53.26. A separate analysis of both BP-NETs and GEP-NETs showed that the Prediction Quotient exhibited an AUC=1 (vs. 0.94 for the Ome Index and Grade: 0.75) for the former while this was 0.88 (vs. 0.85 for the Ome Index and Grade: 0.63) for GEP-NETs. The overall metrics for the Prediction Quotient were: accuracy of 94 %, sensitivity 79 %, specificity 100 %, PPV 100 % and NPV 93 % (Fig. 6c).

Discussion

Our study was designed to assess whether a circulating multianalyte 51-gene NET signature could be used as a surrogate measure of clinical responses to PRRT when assessed at 3- and 6-month follow-up. We also evaluated whether integration of the NET gene signature (gene cluster analysis) measured in pre-treatment blood and clinical factors (e.g., tumor grading, pre-PRRT FDG positivity or pre-PRRT SRI uptake) could be used to predict outcome.

Overall, PRRT was effective (72 % disease control rate) and well-tolerated in advanced metastatic NETs, even when risk factors for delayed renal or hematological toxicity were present, including individuals previously treated with PRRT

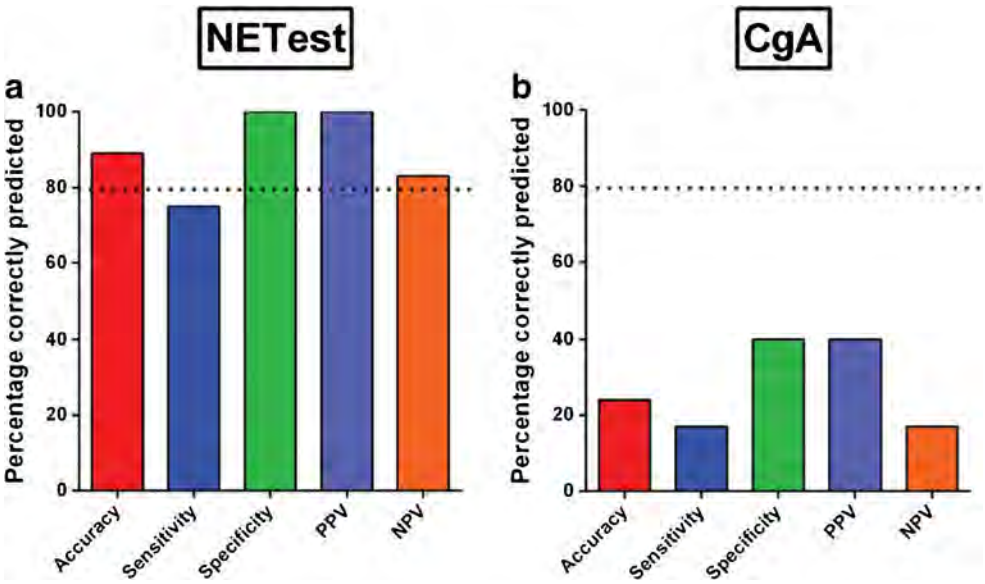

Fig. 4 Metrics for the NETest and CgA in responders and non-responders. **a**) The NETest: accuracy 89 %, sensitivity 75 %, specificity 100 %, PPV 100 % and NPV 83 %. **b**) CgA: accuracy 24 %, sensitivity 17 %, specificity 40 %, PPV 40 % and NPV 17 %. The *dotted line* (**3A-B**) represents 80 % (standard cut-off level for biomarkers) [6, 7]

[20, 27]. Stability was included in the positive outcome, given the intrinsic malignant nature of these tumors. Responses occurred within 2–8 months (median 3), consistent with previous observations [28, 29]. Median PFS was not reached, and PFS was significantly longer in low-grade (undefined vs. 10 months in high-grade lesions) and in ^{18}FDG-negative (undefined vs. 15 months in FDG-positive) tumors.

Of the potential clinical parameters, tumor grading alone had some value in predicting GEP- and BP-NETs most likely to respond to PRRT. [30, 31] The metabolic status (^{18}FDG-PET) predicted PFS but did not reach statistical significance at multivariate analysis in our cohort. This may be due to the low number of patients with negative ^{18}FDG-PET at baseline (18 vs. 31). Neither clinical syndrome nor pre-PRRT SRI uptake was associated with outcome. The latter is in contrast to a previous report [32]. The non-homogeneous patient population, different PRRT dosages and receptor techniques used (scintigraphy, PET) are possible confounders.

With regard to circulating biomarkers and treatment response, changes in NETest accurately (89 %) correlated with treatment response, while CgA was only 24 % accurate. In two previous studies, pharmacological treatment with SSAs or surgery [11, 13], we demonstrated a high correlation between the NETest and tumor response, indicating that this circulating fingerprint effectively captures the tumor response to intervention. To provide further insight into the prediction of treatment efficacy and assessment of response, we developed a gene inference methodology to identify specific "omics" (growth factor signalome and tumor metabolome) relevant to tumor biology (behavior). We integrated these into a predictive quotient. We focused on the GF signalome and metabolome, as they both provide biologically relevant information about the tumor [12]. The former (including *ARAF1, BRAF, KRAS* and *RAF1*) captures information specific to growth factor-mediated signaling. Growth factor expression and signaling is a well-known component of GEP-NET proliferation, and signaling pathways (e.g., the RAS/RAF/MAPK signaling pathway) are typically activated [33, 34]. Moreover, B-Raf expression can be detected by immunohistochemistry (in 75 % of tumors) [35], while RAF1 is directly linked to proliferation [36]. Genes in the metabolome (*ATP6V1H, OAZ2, PANK2, PLD3*) are not as well-characterized in GEP-NETs, but their biological roles are well-described. *ATP6VIH* is involved in regulating neuroendocrine oxidative phosphorylation (pancreatic islets) [37], *OAZ2* is involved in polyamine biosynthesis [38], *PANK2* in metabolism and oxidation [39], and *PLD3* in lipid metabolism and hypoxic signaling [40].

Expression of signaling and metabolic genes in blood prior to initiation of PRRT showed an AUC of 0.74±0.08 (z-statistic=2.92, $p<0.004$) for predicting subsequent treatment response (76 % accuracy). When combined with grading, the accuracy increased significantly (76 to 94 %) and was highly effective as a prognostic marker (AUC: 0.90±0.07), irrespective of tumor origin. Tumors that exhibited significant signalome/metabolome gene elevatins at baseline were responsive (97 %) to PRRT as compared to those who failed therapy. Combining these two clusters in an "Ome Index" and then adding these into a Predictive Quotient with grading provided significantly greater predictive accuracy than that provided by the original parameters. In comparison, CgA (a monoanalyte constitutive secretory protein) was non-informative, and the variations after the completion of PRRT were non-significant and failed to correlate with objective response. Of interest, however, was that CgA was lowest

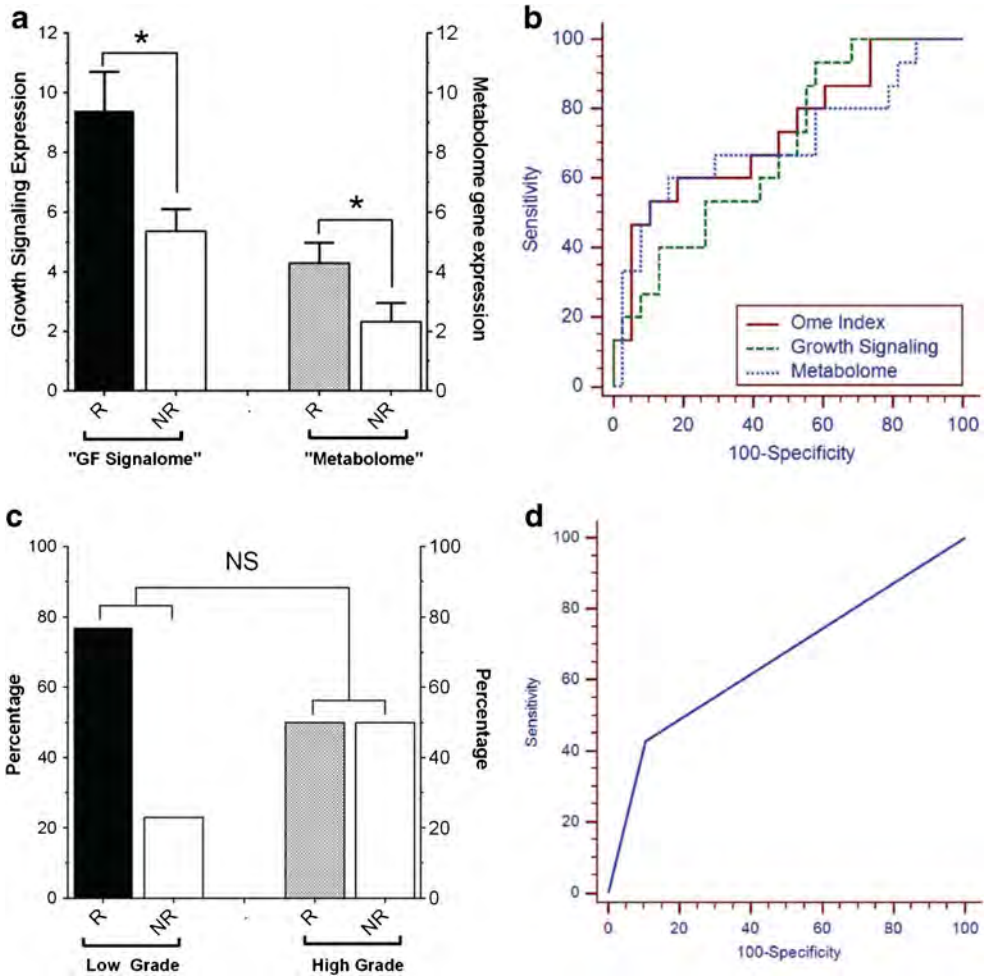

Fig. 5 GF signalome, metabolome and grading in outcome prediction. **a)** Growth factor signaling gene expression ("GF signalome") and genes involved in regulating cell metabolism ("metabolome") were significantly higher in responders than non-responders at T=0 of Cycle I. **b)** ROC analysis identified that each of these "omes" individually could differentiate responders from non-responders. Metrics included growth signaling (AUC=0.67±0.08, z-statistic=2.439, p=0.0147) and metabolome (AUC=0.7±0.09, z-statistic=2.262, p=0.0237). The "Ome Index" (a combination of *GF signalome and metabolome)* exhibited an AUC of 0.74±0.08 (z-statistic=2.915, p=0.0036). This was more significant than each alone. **c)** Low-grade tumors (G1/G2; typical/atypical BP carcinoids) responded to therapy (77 %), while high-grade lesions (G3; BP undifferentiated) were associated with response (50 %). Grade *alone*, however, was not significant (p=0.12), **d)** ROC analysis of grade identified an AUC of 0.66 for treatment response prediction. NR=non-responder, R=responder

in non-responders who also exhibited the greatest decrease. The relevance of this observation is unknown but highlights the limitation of using a marker of secretion as an indicator of tumor progression.

The strategy of integrating data from different sources such as imaging, clinical and biological parameters into indices or nomograms has previously been used to provide a descriptive predictive tool for NETs. For example, a nomogram composed of 15 variables (e.g., age, symptoms, 5-HIAA, CgA, tumor size, invasion, metastasis, histology, Ki67 index and adopted therapy) has been useful in determining prognosis in small intestine NETs [41]. Subsequently, a tumor size >4 cm combined with grading has been proposed to predict preoperative risk of lymph node metastases in non-functioning pancreatic NETs [42]. More recently, an elevated quotient of gene expression (*MORF4L2*) and SUV_{max} has been reported as predictive of disease status in NET patients undergoing ^{68}Ga-SSA-PET [15]. In the current study, incorporating growth factor signaling genes and those linked to metabolism activity provided added dimensionality to the predictive capacity of the multianalyte algorithm for PRRT. It is essential, however, to continue the search for additional markers, likely genetic in origin, in order to build more accurate models and better characterize individuals with GEP-NETs.

Although PRRT is an effective therapy, the development of tools to facilitate timely and accurate identification of therapeutic responses is needed. This is of particular relevance in the management of NETs, and is crucial

Table 5 Treatment Details

Patient no.	Sex	PRRT protocol	Primary	Carcinoid syndrome	Time since diagnosis (months)	FDG	Baseline status	Cum. activity (GBq)	RECIST response	Protocol termination for PD	Length of Fup (months)
11	M	1A	Bronchial	0	10	Pos	PD	20.4	SD	No	22
16	M	1A	Bronchial	+	113	Pos	PD	18.5	PD	No	10
24	M	1A	Bronchial	0	9	Pos	PD	9.3	PD	Yes	3
31	M	1A	Bronchial	0	128	Pos	PD	18.5	PD	No	18
45	M	1A	Bronchial	+	5	Pos	PD	18.9	SD	No	16
49	M	1A	Bronchial	0	81	NA	PD	18.5	SD	No	13
50	F	1A	Bronchial	0	34	Pos	SD	18.5	SD	No	11
6	M	1A	Pancreas	+	37	NA	PD	18.5	SD	No	18
10	M	1A	Pancreas	0	12	Pos	SD	21.1	SD	No	19
29	M	1A	Pancreas	0	8	Pos	SD	18.5	SD	No	16
44	F	1A	Pancreas	0	66	NA	PD	17.9	SD	No	13
52	M	1A	Pancreas	0	25	Neg	§	18.5	PR	No	15
8	M	1A	Jejunum	0	22	Pos	SD	17.6	SD	No	22
7	M	1A	Ileum	+	18	Pos	PD	18.5	PD	Yes	10
15	M	1A	Ileum	0	49	Neg	PD	18.5	SD	No	18
20	F	1A	Ileum	+	110	Neg	SD	18.5	PR	No	16
21	F	1A	Ileum	+	176	Neg	PD	18.5	SD	No	19
35	M	1A	Ileum	+	14	Pos	PD	18.5	SD	No	18
42	M	1A	Ileum	+	75	Neg	PD	8.3	PD	Yes	3
51	M	1A	Ileum	0	8	Pos	PD	18.5	SD	No	15
5	F	1A	Unknown	0	22	Neg	SD	18.5	SD	No	17
13	F	1A	Unknown	+	9	NA	SD	18.5	SD	No	20
53	M	1B	Bronchial	0	37	Pos	PD	21.3	SD	No	22
3	F	1B	Bronchial	0	14	Pos	SD	27.8	PD	Yes	6
12	M	1B	Bronchial	0	16	Pos	PD	24.1	SD	No	20
32	M	1B	Bronchial	0	21	Pos	PD	25.9	PR	No	16
38	F	1B	Bronchial	0	44	Neg	SD	25	SD	No	16
22	F	1B	Pancreas	0	142	Neg	PD	23.1	SD	No	8
33	M	1B	Pancreas	0	21	Pos	PD	25.9	SD	No	15
34	F	1B	Pancreas	0	9	Pos	§	23.1	PD	No	17
40	M	1B	Pancreas	ZES	30	Neg	PD	25.2	SD	No	17
14	M	1B	Ileum	+	14	Pos	PD	25.9	PD	Yes	9
28	M	1B	Ileum	+	6	Pos	SD	25.9	PR	No	17
36	M	1B	Ileum	+	9	Pos	PD	25.9	SD	No	14
46	M	1B	Ileum	+	37	Pos	SD	25.2	SD	No	13
47	M	1B	Ileum	0	3	Pos	PD	25.9	SD	No	14
1	M	1B	Rectum	0	26	Neg	PD	27.8	CR	No	33
2	F	1B	Unknown	+	19	Neg	PD	25.9	PR	No	28
43	M	1B	Unknown	+	117	Pos	PD	6.5	PD	Yes	1
26	M	2	Bronchial	0	58	Neg	PD	11.1	PD	Yes	5
23	F	2	Gastric	0	55	Pos	PD	14.8	PR	No	10
9	M	2	Pancreas	0	51	Pos	PD	15.7	SD	No	17
25	M	2	Pancreas	0	52	Pos	PD	14.8	SD	No	16
27	M	2	Pancreas	0	59	Neg	PD	14.8	SD	No	19
37	M	2	Pancreas	0	213	Pos	PD	14.8	PR	No	17
48	F	2	Pancreas	0	135	Pos	PD	14.8	SD	No	15
4	F	2	Ileum	+	111	Neg	PD	14.8	SD	No	25

Table 5 (continued)

Patient no.	Sex	PRRT protocol	Primary	Carcinoid syndrome	Time since diagnosis (months)	FDG	Baseline status	Cum. activity (GBq)	RECIST response	Protocol termination for PD	Length of Fup (months)
19	M	2	Ileum	+	65	Neg	PD	14.8	PR	No	17
30	F	2	Ileum	+	128	Pos	SD	14.8	PD	No	13
41	M	2	Ileum	+	154	NA	SD	17	PR	No	17
18	F	2	Colonic	0	49	Pos	PD	14.8	PD	No	19
17	F	2	Rectum	+	56	Neg	PD	14.8	PD	Yes	7
39	M	2	Unknown	0	83	Neg	PD	14.8	PD	No	17
54	M	2	Unknown	0	265	Neg	PD	14.8	PD	No	13

ZES, Zollinger-Ellison syndrome; Neg, negative; Pos, positive; NA, not assessable; ECOG, Eastern Cooperative Oncology Group performance status, PD, progressive disease; CR, complete response; PR, partial response; SD, stable disease; §, patient in response to chemotherapy; PFS, progression-free survival; Fup, follow up

when performing time/labor-intensive/expensive therapies (typically 6–12 months with the intended ^{177}Lu-octreotate cumulative dosage) [28]. While imaging and imaging-based assessment of response (e.g., with RECIST criteria), in conjunction with biomarkers, is currently used to assess treatment response, limitations include the difficulty in capturing volumetric modifications in slow-growing tumors, particularly for large-volume disease, or in defining tumor alterations using CT or MRI when structural changes, such as necrosis, hemorrhage or fibrosis, occur as a result of treatment [43]. Various imaging protocols have been proposed to measure response, including the quantification of SSR density in vivo and assessment of molecular tumor volume before and after PRRT [44]. Quantification criteria (SUV_{max}), however, are not yet prospectively validated [43]. Although the disease course and effectiveness of PRRT can be followed by ^{177}Lu-scans, early predictors of response to PRRT are rare [45, 46]. The only significant factor is SSR density at baseline (OctreoScan; ^{68}Ga-SSA-PET) [47]. An uptake at OctreoScan of grade 4 (higher than that of kidneys and/or spleen) is associated with an objective response in 60 % of cases [32]. Intrinsic tumor and patient characteristics are also involved in response, as demonstrated by other predictors, including performance status, extent of liver involvement and extra-hepatic metastases [3, 28]. As a consequence, a personalized predictive assessment represents a major unmet need.

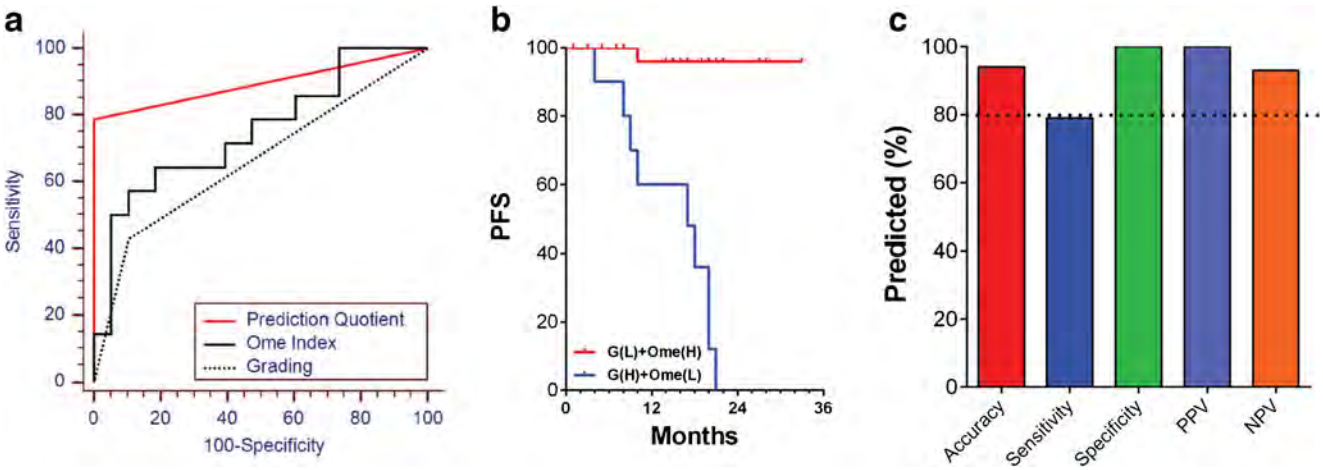

Fig. 6 Derivation of a prediction quotient and clinical utility for predicting responses. **a**) The combination of the "Ome Index" and tumor grading (WHO 2010 classification for GEP NETs; Travis for BP NETs) – the "Prediction Quotient" had metrics of AUC: 0.90±0.06, z-statistic=6.39, $p<0.0001$, for predicting response in all tumors ($n=53$). This was significantly different from grading alone (difference between areas 0.23, z-statistic 2.25, $p=0.024$). **b**) Kaplan–Meier analysis of progression-free survival showed that the Response Quotient identified that tumors that were "low-grade/high-Ome index" had an undefined median survival. The "high-grade/low-Ome index" group had a median progression-free survival of 17 months. This was significant (log rank: 26.8, $p<0.0001$). The hazard ratio was 53.3. **c**) The metrics for the Prediction Quotient were: accuracy: 94 %, sensitivity 79 %, specificity 100 %, PPV 100 % and NPV 93 %. G(L)=low grade, G(H)=high grade, Ome(L)=low ome index, Ome(H)=high Ome index. The *dotted line* represents 80 % (accepted cut-off level for biomarkers) [6, 7]

Currently accepted biomarkers include CgA, despite reservations regarding its performance metrics and confounding features such as renal insufficiency and proton pump inhibitor usage [48]. Moreover, many NET patients (~30-50 %) do not have elevated CgA [49]. In our series, 23 (43 %) of 54 patients had normal CgA levels. Biochemical responses, however, are frequently observed after PRRT [32], and a recent retrospective analysis showed that a baseline CgA>600 ng/ml was predictive of response [50]. This threshold was not predictive in our prospective series. Tumor grading is also a well-recognized prognostic factor, although it is the expression of a higher risk of tumor progression in the history of the patient rather than a predictor of the outcome of a specific treatment [30].

A limitation of this study is the inclusion of a cohort of advanced patients who, in some cases, had to discontinue treatment. These subjects, however, represent the "real-world" situation of advanced NET referral and treatment. In this respect, a qPCR-based blood biomarker test that defines the circulating "fingerprint" of a NET provides a novel strategy for assessing NET disease. A circulating tumor MAAA can be easily acquired (simple blood draw) at multiple time points during periods between sequential imaging assessment. Such information can be combined with imaging results to provide additional evidence regarding tumor behavior as well as therapy response.

In conclusion, this study demonstrated that a circulating NET gene transcript signature accurately correlated with treatment response and that a combination of gene cluster analysis and grading—the predictive quotient—accurately predicted PRRT efficacy. Our study highlights two aspects of the assessment of PRRT efficacy with NET transcript analysis. One is the observation of the phenomena, namely, how the specific NET transcript signature behaves during and after PRRT, and how it correlates with standard morphologic and functional imaging, and hence with the treatment response or outcome to therapy. This information is of clinical utility in more precisely defining tumor growth status, thus confirming the efficacy of therapy. The second aspect is the identification of specific gene clusters in pretreatment blood that are able to predict the subsequent response to PRRT. Confirmation of these observations in larger series will allow identification of likely nonresponders and will better define at a molecular level the natural history of individual neuroendocrine tumors.

Compliance with ethical standards

Disclosure of potential conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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