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REVIEW ARTICLE

Predictive Markers of Response to Everolimus and Sunitinib in Neuroendocrine Tumors

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Abstract Neuroendocrine tumors (NETs) represent a large and heterogeneous group of malignancies with various biological and clinical characteristics, depending on the site of origin and the grade of tumor proliferation. In NETs, as in other cancer types, molecularly targeted therapies have radically changed the therapeutic landscape. Recently two targeted agents, the mammalian target of rapamycin inhibitor everolimus and the tyrosine kinase inhibitor sunitinib, have both demonstrated significantly prolonged progression free survival in patients with advanced pancreatic NETs. Despite these important therapeutic developments, there are still significant limitations to the use of these agents due to the lack of accurate biomarkers for predicting tumor response and efficacy of therapy. In this review, we provide an overview of the current clinical data for the evaluation of predictive factors of response to/efficacy of everolimus and sunitinib in advanced pancreatic NETs. Surrogate indicators discussed include circulating and tissue markers, as well as non-invasive imaging techniques.

Key Points

Everolimus and sunitinib are widely investigated targeted cancer therapies, and they are both globally approved by regulatory authorities for the treatment of pancreatic NETs.

The establishment of predictive markers of response to everolimus and sunitinib in NETs is of extreme importance for their efficient use.

Most efforts to define predictive biomarkers have failed, with the exception of chromogranin-A and neuron-specific enolase for advanced pancreatic NETs treated with everolimus.

1 Introduction

Neuroendocrine tumors (NETs) comprise a heterogeneous group of malignancies originating from the diffuse endocrine system. Even though NETs are considered a rare malignancy, representing about two new cases per 100.000 persons per year, their incidence and prevalence seem to be rising steadily [1]. Some of the possible reasons for the increasing incidence

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of NETs include improved diagnostic techniques and a deeper understanding of the disease.

Pancreatic neuroendocrine neoplasms are classified as tumors (pNETs) and carcinomas (pNECs) in accordance to the 2010 WHO classification [2]. The former category comprises neoplasms with a low proliferation index of ≤ 20% Ki-67 and the latter with > 20% Ki-67. As for morphology, all NETs with ≤ 20% Ki-67 are classified as well/moderately differentiated (WD/MD), whereas the vast majority of NETs with Ki-67 > 20% are classified as poorly differentiated (PD).

The growing knowledge of NET biology has led to clinical studies exploring several targeted therapies with direct or indirect anti-angiogenic properties, including tyrosine kinase inhibitors (TKIs) and mammalian target of rapamycin (mTOR) inhibitors, and both approaches have recently demonstrated clinical benefits. Everolimus and sunitinib were both investigated in patients with WD or MD NETs, mainly of pancreatic origin. Everolimus (RAD001 [Afinitor®; Novartis]) and sunitinib (sunitinib malate [Sutent®; Pfizer]) both received global approval for the treatment of metastatic or unresectable, progressing, WD/MD pNETs, after showing progression free survival (PFS) benefits in placebo-controlled randomized phase III trials [3, 4]. In addition, everolimus has recently also been approved for non-functioning gastrointestinal (GI) and WD lung NETs on the basis of significantly improved PFS compared with placebo in the RADIANT-4 trial [5].

The approval of everolimus and sunitinib in these settings has established new active treatment options for pNETs patients. However, the preferred treatment sequence is still uncertain, and while some biomarkers have shown correlations with clinical outcomes (Tables 1 and 2), robust and validated predictive markers of clinical benefit are clearly necessary for personalized patient management and more appropriate resource utilization. Despite the massive amount of research that has addressed the discovery and validation of new cancer biomarkers, a very limited number of these has actually been introduced into clinical practice over the last years, probably because most of the newly discovered markers are inferior in terms of sensitivity and specificity to the conventionally used ones.

Recently, a multinational and multidisciplinary Delphi consensus meeting assessing current biomarkers and imaging strategies in NETs concluded that both imaging and current single-analyte biomarkers still have important limitations in predicting response to therapy [24]. Although the combination of imaging and molecular information provided by blood-based multi-analyte transcript analysis was suggested to have the potential to integrate future strategies that predict the response to treatment, prospective studies that may validate this type of methodology are lacking.

In this review, we summarize the recent developments in circulating, tissue, and imaging markers of clinical benefit to targeted therapies in NETs, with emphasis on everolimus and sunitinib, based on a non-systematic review.

2 Methodology

Research data for the present analysis was primarily retrieved by a keyword-based PUBMED and OVID search, using relevant keywords (predictive OR marker OR biomarker) AND (everolimus OR mtor OR m-TOR OR sunitinib OR VEGF OR targeted OR antiangiogenic OR anti-angiogenic) AND (neuroendocrine). In addition, abstracts from major scientific meetings were searched manually. The search was last updated on October 15, 2016, and only human studies that were published in English over the last 10 years were considered. After the exclusion of 86 duplicates, a total of 302 publications were assessed for a full-text evaluation. The papers were filtered according to observational studies, prospective clinical trials (in any phase), systematic reviews, and abstracts of major scientific meetings. Case reports and publications with abstracts not relevant to the current study were removed and at the end of the selection process, 54 publications were included (Fig. 1).

3 Predictive Markers of Response to Everolimus

3.1 mTOR Signaling Pathway and Everolimus Efficacy in NETs

Everolimus is an oral mTOR inhibitor that selectively blocks mTOR complex (mTORC)-1, leading to an increase in mTORC-2 activity. This results in a positive feedback activation of AKT by phosphorylation on Ser473 (Fig. 2), and an inhibition of S6K negative feedback [25].

Everolimus was investigated in pNET patients pre-treated with chemotherapy in the open-label stratified phase II RADIANT-1 trial. One-hundred and sixty patients with advanced, low or intermediate-grade pNETs and progressive disease during or after chemotherapy (according to RECIST criteria) were enrolled. Median PFS was 9.7 months for patients receiving everolimus alone (stratum A) and 16.7 months for those receiving the everolimus + octreotide long acting repeatable (LAR) combination (stratum B) [26]. The multicenter, randomized, placebo-controlled, phase III RADIANT-3 trial confirmed clinical efficacy of everolimus in patients with radiologically progressing WD or MD advanced pNETs, showing a PFS of 11 months with everolimus therapy versus 4.6 months with placebo (hazard ratio [HR] 0.35; $P < 0.001$) [3].

Several observations support the idea that mutations in the mTOR pathway are related to the pathogenesis of pNETs. For instance, loss of function mutations of *TSC1* and *TSC2* (tumor

Table 1 Markers evaluated as predictive factors of response to everolimus in NETs (PFS, progression-free survival; OS, overall survival; RR, response rate; PR, partial response; TTP, time to progression)

Study	Drug	Setting	N° patients	Biomarker	Type of marker	Type of study	Endpoints
Yao JC, 2011 [6]	**Everolimus**	pNETs	114	CgA, NSE	**Circulating**	Prospective	**PFS, OS**
Baudin E, 2011 [7]	**Everolimus**	NETs	429	CgA, 5-HIAA	**Circulating**	Prospective	**PFS**
Yao JC, 2012 [8]	**Everolimus**	pNETs	393	Baseline VEGF-A, PlGF and sVEGFR1/2	**Circulating**	Retrospective	**PFS**
Bellister SA, 2013 [9]	**Everolimus**	pNETs and extra-pancreatic NETs	32	L1016S SNP in PHLPP2	**Circulating**	Prospective	**PFS, OS, RR**
Serra S, 2012 [10]	**Everolimus**	pNETs	17	FGFR4-G388R SNP	**Circulating**	Prospective	**PR, PFS, OS**
Meric-Bernstam F, 2012 [11]	**Everolimus and Rapamycin**	NETs	60	PIK3CA, PTEN mutations; basal pAkt	**Tissue**	Retrospective	**PFS**
Spada F, 2013 [12]	**Everolimus**	GEP NETs	36	PTEN, pAkt, p-mTOR, p70S6K, pS6K, p4-EBP1; PCR-based exons 7 and 9 PI3K mutations	**Tissue**	Retrospective	**PFS**
Gagliano T, 2013 [13]	**Everolimus**	Bronchial NETs	21	Total and p-mTOR; basal and p-Akt; total and p-p70S6K; total and p-ERK1/2	**Tissue**	Retrospective	**Cell-viability**
Casanovas O, 2014 [14]	**Everolimus**	GI NETs	38	IGFR-1, p-mTOR, p-S6	**Tissue**	Retrospective	**PFS**
Cros J, 2015 [15]	**Everolimus**	GEP NETs	41	Gly388ArgFGFR4 Polymorphism	**Tissue**	Retrospective	**PFS, OS, TTP**
Benslama N, 2016 [16]	**Everolimus**	GEP NETs, bronchial, kidney and unknown	53	P-P70S6K; CgA; clinical markers	**Tissue**	Retrospective	**PFS, OS**
Falletta S, 2016 [17]	**Everolimus**	pNETs	16	p-IGF1 R, p-Akt, p-mTOR, and p-4EBP1	**Tissue**	Retrospective	**Cell viability**
van Asselt SJ, 2015 [18]	**Everolimus**	GEP NETs, bronchial, and unknown	14	89Zr-bevacizumab PET scan; VEGF-A (basal and 2 weeks treatment)	**Circulating, tissue, imaging**	Prospective	**PFS**
Yao JC, 2015 [19]	**Everolimus**	GEP NETs, bronchial and unknown	39	Perfusion CT	**Imaging**	Prospective	**BF, MTT, and PS**

GEP, gastroeneteropancreatic; CgA, chromogranin A; NSE, neuron-specific enolase; 5-HIAA, 5-hydroxyindoleacetic acid; VEGF-A, vascular endothelial growth factor A; PlGF, placental growth factor; sVEGFR1/2, soluble VEGF receptor-1 and 2; SNP, single nucleotide polymorphisms; FGFR4, fibroblast growth factor receptor 4; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTEN, phosphatase and tensin homolog; pAkt, phosphorylated-Akt; pmTOR, phosphorilated-mTOR; p70S6K; ribosomal protein S6 kinase beta-1; p4-EBP1, phosphorylated-4EBP1; IGF1 R,insulin-like growth factor-1 receptor; BF, blood flow; MTT, mean transit time; PS; permeability surface

Table 2 Markers evaluated as predictive factors of response to sunitinib in NETs (ORR, objective response rate; SD, stable disease; PFS, progression-free survival; OS, overall survival; PR, partial response)

Study	Drug	Setting	N° patients	Biomarker	Type of marker	Type of study	Primary endpoint
Bello CL, 2006 [20]	**Sunitinib**	pNETs	109	VEGF, sVEGFR-2/3, IL-8	**Circulating**	Prospective	**ORR, SD**
Zurita AJ, 2015 [21]	**Sunitinib**	NETs	105	VEGF-A, sVEGFR-2/3, IL-8, SDF-1, WBC and monocyte subsets, CECs	**Circulating**	Prospective	**PFS, OS**
Dreyer C, 2016 [22]	**Sunitinib**	NETs	31	PDGFR-b, VEGFR-2, Carbonic Anhydrase 9, Ki-67, p-AKT	**Tissue**	Prospective	**Sunitinibs' activity (RECIST 1.1)**
Faivre S, 2012 [23]	**Sunitinib**	pNETs	10	Choi criteria	**Imaging**	Retrospective	**PR**

VEGF, vascular endothelial growth factor; sVEGFR-2/3, soluble VEGF receptor-2 and 3; IL-8, Interleukin-8; VEGF-A, vascular endothelial growth factor A; SDF-1, stromal cell-derived factor 1; WBC, white blood cells; CECs, circulating endothelial cells; PDGFR-b, beta-type platelet-derived growth factor receptor

suppressor genes that inhibit mTOR) were identified in tuberous sclerosis (TS), a hereditary syndrome that is associated with the development of pNETs [27]. Furthermore, phosphatase and tensin homolog (*PTEN*), which regulates the activity of mTOR through the AKT pathway together with *TSC2*, are down-regulated in approximately 75% of pNETs and associated with shorter disease-free survival (DFS) and overall survival (OS) [28].

The activity of the mTOR pathway is frequently enhanced in neoplastic cells, and thus mTOR-inhibitors such as everolimus are promising anticancer agents [29, 30]. However, despite the encouraging results on tumor progression, everolimus demonstrates a limited effect on tumor shrinkage, owing to its cytostatic rather than cytotoxic activity [31]. In addition, everolimus efficacy in NETs can also vary according to the patient and in relation to the development of rapalog resistance, this mechanism being unclear for the time being [32]. Therefore, the identification of predictive biomarkers of clinical benefit is crucial for a more efficient use of everolimus in selected patients.

3.2 Circulating Biomarkers

Recent advances suggest that blood-based analysis could replace invasive surgical procedures, representing a major achievement for disease monitoring and allowing the identification of clinically useful biomarkers.

In the setting of NETs, some circulating markers of response have been analyzed. The predictive value of chromogranin A (CgA) and neuron-specific enolase (NSE) were evaluated in a study with 145 pNET patients of the phase II RADIANT-1 trial [6]. The authors showed that elevated baseline CgA levels, defined as CgA > 2× upper limit of normal (ULN) of 36.4 ng/mL, were associated with significantly shorter PFS (8.34 vs. 15.64 months; $P = 0.03$) and OS (16.95 months vs. not reached; $P < 0.001$) in patients with advanced pNETs treated with everolimus 10 mg/d orally. These results were consistent with previous studies in which the baseline CgA levels were considered useful in evaluating the risk of disease progression and survival in patients with advanced pNETs [33]. For NSE, elevated baseline levels, defined as NSE > 2xULN of 8.6 ng/mL, were significantly associated with worse PFS (7.75 months vs. 12.29 months; $P = 0.01$) and OS (13.96 months vs. 24.90 months; $P = 0.005$) [6]. Additionally, Yao et al. observed that patients with early CgA or NSE response (defined as ≥30% decrease from baseline or normalization at the 4th week of everolimus treatment), had longer PFS than those who did not. Therefore, early CgA or NSE responses may also be considered as a potential predictor of response to treatment for patients with advanced pNETs [6]. It is important to underline that these findings are substantially relevant because they were obtained from a prospective analysis of a large cohort of patients with

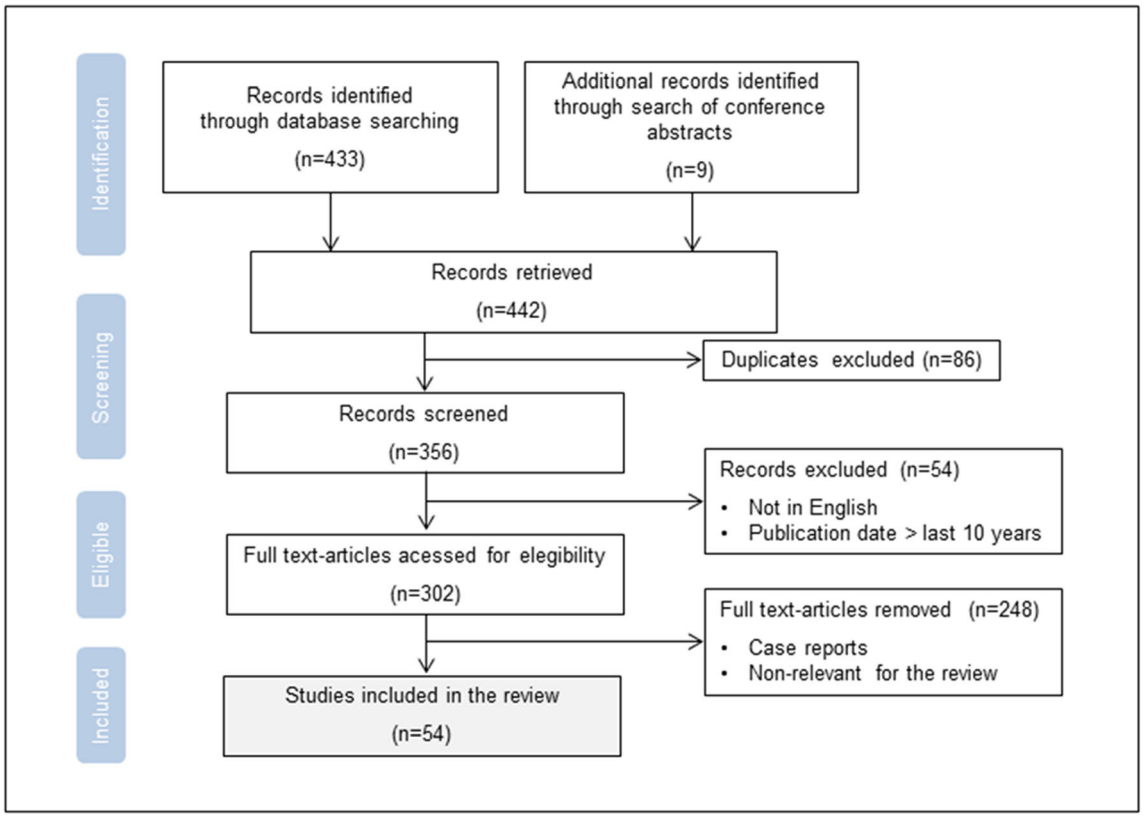

Fig. 1 Flow chart for the selection of studies discussed in this review

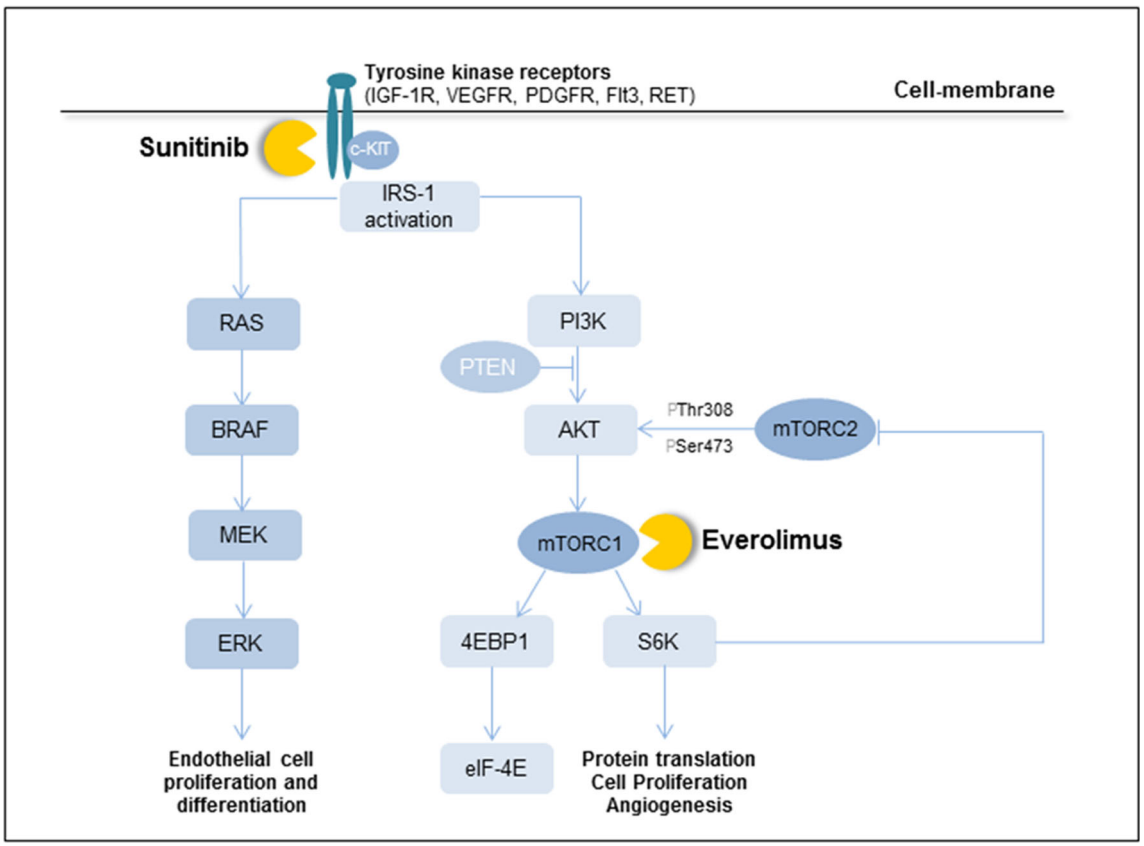

Fig. 2 Mechanism of action of sunitinib and everolimus and overview of the tyrosine kinase receptors and PI3K/AKT/mTOR pathways

advanced pNETs. However, they must be interpreted with caution, since some of the included patients achieved treatment benefit without early CgA or NSE responses.

Baudin et al. evaluated the predictive value of CgA and 5-hydroxyindoleacetic acid (5-HIAA) levels in 416 patients with advanced pNETs from the phase III RADIANT-2 study [7]. The numbers of patients with early CgA and 5-HIAA responses (≥ 50% reduction at the 4th week of treatment) were superior in the everolimus *plus* octreotide group to the placebo *plus* octreotide group (24.5% and 24.0% vs. 16.5% and 17.5% for CgA and 5-HIAA, respectively). Moreover, patients with an early 5-HIAA response had longer median PFS than those without, but without statistical significance (18.3 vs. 13.6 months; HR 0.71; $P = 0.139$). As in the RADIANT-1 trial [25], the analysis of early CgA reduction in the study of Baudin et al. was associated with significantly improved PFS, suggesting that CgA may be considered as a potential marker for a longer PFS in patients receiving everolimus [7].

Yao et al. presented their results of baseline plasma levels of several VEGF pathway biomarkers in the multicenter, double-blind RADIANT-3 trial, which included 410 patients with advanced pNETs, at the 37th ESMO Congress [8]. The optimal cut-off values defined for these markers were 246 pg/mL (VEGF-A), 32.06 pg/mL (PlGF), 226.2 pg/mL (sVEGFR-1), and 24,503.1 pg/mL (sVEGFR-2). While PFS was significantly improved to a similar extent in patients receiving everolimus compared with patients who received placebo, regardless of baseline levels of markers, a significant improvement of PFS in patients with lower baseline VEGF-A, PlGF, and sVEGFR1 levels was observed, suggesting that these markers have a prognostic value in pNETs [8].

The predictive effect of single nucleotide polymorphisms (SNPs) has also been investigated. Bellister et al. analyzed the predictive value of an L1016S SNP inactivation in PHLPP2 (a phosphatase that inhibits phosphatidyl inositol 3 kinase (PI3K)-Akt-mTOR signaling), as a predictive factor of response in a phase II single-arm trial of 32 patients with pancreatic and extra-pancreatic NETs treated with everolimus [9]. The PHLPP2 SNP was not predictive for a significant difference in PFS (median PFS in wild type patients of 16.8 vs. 11.3 months in patients positive for the SNP; $P = 0.154$). However, a subset analysis showed that wild type PHLPP2 patients with extra-pancreatic NETs treated with everolimus had a significantly prolonged PFS compared to patients with the SNP (median PFS of 16.8 vs. 7.7 months; $P = 0.002$). Finally, the SNP did not influence OS in this trial [9].

Serra et al. genotyped 17 patients with G1 and G2 pNETs included in the RADIANT-1 trial, isolating DNA from normal somatic tissue or blood. They verified that 11 (65%) patients were homozygous for *FGFR4-G388* and 6 (35%) carried only one *FGFR4-R388* allele [10]. The assessment of best-percentage change in RECIST tumor measurements demonstrated greater reductions among patients homozygous for *FGFR4-G388* (mean percentage change of 25% vs. 9%; $P = 0.049$). It was also observed that patients homozygous for *FGFR4-G388* had a median PFS of 16.6 months, compared with those harboring one *FGFR4-R388* allele, who presented a PFS of 4.8 months ($P = 0.40$). Moreover, patients homozygous for *FGFR4-G388* presented a higher OS of 40 months, in comparison to those harboring only one *FGFR4-R388* allele, of 9.3 months ($P = 0.54$). Therefore, no statistically significant reductions in PFS and OS in patients with at least one *R388* allele were observed [10].

3.3 Tumor Tissue Markers

Tissue analysis of NETs has suggested that PI3K-AKT-mTOR signaling constitutes a promising target for therapy. In a phase II trial of everolimus and octreotide LAR in 60 patients with advanced low and intermediate grade NETs [11], all tumors expressed phosphorylated mTOR and almost all expressed PTEN. There were not significant differences in PFS based on the expression of other evaluated markers including pAKT S473, p4E-BP1 T37/46 or pS6 S235/236 on archival samples. The authors found that high pAKT T308 levels in baseline, as well as in on-treatment fine needle-biopsies, correlated with PFS (baseline $R = 0.4762$, $P = 0.0533$; on-treatment $R = 0.6041$, $P = 0.0102$). In addition, patients who had a partial response (PR) to everolimus treatment had significantly higher probability to have an increase in pAKT T308 than patients who had stable disease (SD) or progressive disease (PD) ($P = 0.0146$), suggesting that pAKT increases more in responders, compared to non-responders [11].

Few SNPs have been evaluated as putative tissue markers to predict everolimus sensitivity. Cros et al. retrospectively assessed the predictive role of the *FGFR4-388G/R* polymorphism, its impact on the activation of mTOR and on the expression of several mTOR pathway molecules (PTEN, pPTEN, pAKT, pmTOR, pS6, p4EBP1) in a group of 41 patients with pancreatic and small bowel NETs treated with everolimus [15]. The authors confirmed that the presence of at least one *388R* allele did not affect median time to progression (TTP) in the whole cohort and in the pNETs subgroup, likewise it did not influence PFS, OS, or the expression of mTOR pathway components. In light of the results from the study of Serra et al. that showed (statistically non-significant) differences in PFS and OS in patients with at least one *388R* allele [15], the impact of the *FGFR4-388G/R* polymorphism as a predictive indicator of everolimus sensitivity in pNETs is still questionable.

Spada et al. observed a longer PFS in patients who showed a positive pmTOR immunochemistry (IHC) score, compared to those showing a negative pmTOR score, in a cohort of 36 patients with metastatic gastro-enteropancreatic (GEP) NETs treated with everolimus [12]. The results suggested that pmTOR, in combination with the Ki-67 labeling index may

be predictive for response in patients treated with mTOR inhibitors. In the same study, the IHC score was additionally used to evaluate PTEN, pAKT, and p4E-BP1, however, no significant correlation was identified between positive and negative staining [12].

Including a small number of samples derived from 21 patients, the results obtained in the study of Gagliano et al. were consistent with previous analyses, demonstrating that pmTOR protein levels distinguish human bronchial carcinoids that are sensitive to everolimus treatment in vitro from those that are resistant [13]. Additionally, AKT/mTOR pathway signaling molecules in their phosphorylated form, for example basal mTOR, p70S6K, AKT, and ERK1/2, were expressed to higher levels in human bronchial carcinoids that responded to everolimus treatment in vitro, in contrast to those that were resistant [13].

Contrary to previous studies, Benslama et al. reported conflicting data showing that in a cohort of 53 patients treated with everolimus for metastatic (pancreatic and non-pancreatic) NETs, 68% within a clinical trial, high tumoral expression of pp70S6 K was associated with shorter PFS under everolimus (HR = 2.51; P = 0.013) [16]. The results were comparable with other studies suggesting that a higher expression of mTOR pathway components is associated with poorer OS in NETs [34, 35]. Casanovas et al. also reported conflicting data about the implications of mTOR activation, having demonstrated that pmTOR IHC positivity was associated with a worse PFS in a small group of 38 patients with GI-NETs on everolimus plus somatostatin analogs (SSA) [14].

A cell viability study reported by Falletta et al. demonstrated that everolimus induced apoptosis in primary cultures derived from tissue samples of 16 patients with pNETs [17]. The authors observed that the proliferative and anti-apoptotic effects of IGF1 were both blocked by everolimus, suggesting that the IGF1 pathway could take a relevant part in the development of everolimus resistance. These results are of the utmost importance, as it was previously stated that owing to the development of primary or secondary acquired resistance, some patients do not demonstrate advantage from everolimus therapy [32]. Falletta et al. also suggested that the IHC characterization of pAKT can be helpful in identifying patients with pNETs who can benefit from everolimus therapy, as the response to the drug in vitro was associated with an active AKT/mTOR pathway and appeared to be related to superior clinical aggressiveness, demonstrating that primary pNET cultures can be considered as a suitable model for testing medical treatment [17].

Although these results were clearly different from the ones obtained by Benslama et al., it must be taken into account that the latter study included a very heterogeneous group of NETs of different origins, so the results might not adequately reflect pNETs behavior [16].

3.4 Imaging Markers

VEGF is one of the main factors responsible for the angiogenesis process, thus being considered as an attractive candidate for predicting response to anti-angiogenic therapies. In fact, rapalogs have been shown to inhibit AKT signaling in endothelial cells, which not only results in the inhibition of VEGF-driven endothelial cell proliferation but also in the suppression of VEGF production. Moreover, it was already verified that mTOR inhibition reduced VEGF-A excretion in NET cell lines [36].

The assessment of angiogenesis in NETs has led to the development of encouraging strategies using radiolabeled antibodies such as 89Zr-labeled bevacizumab. Van Asselt et al. developed both single-photon emission computed tomography (SPECT) and positron emission tomography (PET) radiopharmaceuticals to noninvasively visualize VEGF-A [18]. They used 89Zr-bevacizumab PET in a group of 14 patients with advanced progressive well-differentiated pNETs on everolimus therapy to examine whether neoplastic lesions could be visualized, and also investigated the effect of everolimus on the tumor uptake of the radioactive-labeled VEGF-A antibody bevacizumab. It was observed that everolimus reduced 89Zr-bevacizumab tumor accumulation by 7% at the 2nd week (P = 0.09) and by 35% at the 12th week of treatment (P < 0.001). It was also observed that the maximum standardized uptake value (SUVmax) at the 2nd and 12th weeks significantly correlated with the percentage change in the sum of the target lesion diameters (according to RECIST 1.1), assessed by computed tomography (CT) after 6 months (P < 0.05 and P < 0.01, respectively) [18]. The multiple anti-tumor effects of everolimus, including the reduction of tumor VEGF-A production were also noticed in this study. Although no correlation between the percentage change in serum levels of VEGF-A and the reduction of tumor uptake (ΔSUVmax) after the 12th week of treatment was observed, the authors verified that VEGF-A levels were 25% inferior at the 12th week than at baseline (P < 0.05). In respect to CgA, they observed that in patients with high baseline levels (over 100 μg/L), the percentage change of this biomarker after 3, 6, 9, and 12 months did not correlate with ΔSUVmax after 12 weeks of treatment with everolimus [18].

The results on baseline 89Zr-bevacizumab PET might be related to the fact that only a subset of patients with NETs benefit from anti-angiogenic therapies [37–39]. Accordingly, 89Zr-bevacizumab PET could probably be useful as an early predictive biomarker of anti–VEGF treatment in patients with NETs, however, more prospective studies are needed to validate this.

Yao et al. evaluated some parameters of perfusion CT as potential biomarkers of anti-tumor activity of everolimus and bevacizumab, among patients with advanced NETs [19]. The study included 39 patients who received a 3-week cycle of

15 mg/kg bevacizumab on day 1, or 10 mg of everolimus daily, and following cycles comprised the combination of both drugs. It was observed that in the group of patients receiving everolimus first, single agent everolimus was associated with a 12% decrease in tumor blood flow ($P = 0.16$). Furthermore, the combination of everolimus and bevacizumab was associated with an additional 32% reduction at week 9 compared with everolimus alone ($P = 0.004$). The mean transit time was also significantly increased in everolimus treated patients, however, no considerable modifications in blood volume ($P = 0.87$) or in permeability surface ($P = 0.43$) were observed. Although single-agent everolimus therapy was not associated with a significant change in tumor blood flow, nine weeks after adding everolimus to patients already receiving bevacizumab, a 29% decrease in this parameter was noted [19]. As previous studies did not show a further decrease on tumor blood flow after longer exposure to bevacizumab [37, 40], the results obtained indicate that the observed decrease was associated with the addition of everolimus [19].

Also in the study of Yao et al. no significant correlation between perfusion CT parameters and PFS was observed. However, pre-treatment permeability surface, percentage of reduction in blood flow, blood volume and post-treatment mean transit time correlated with RECIST-based tumor response, which can suggest that the anti-tumor activity is associated to the effects on vasculature [19]. According to the obtained data, perfusion CT may also be considered as a possible resource to help in selecting patients that are expected to benefit from everolimus.

4 Predictive Markers of Response to Sunitinib

4.1 Activity of Sunitinib and the Role of VEGF Signaling in NETs

Sunitinib is an oral multi-targeted inhibitor of a range of receptor tyrosine kinases (RTKs) (Fig. 2) that are associated with growth, proliferation, and metastatic spread, including KIT, PDGFR-α and -β, and VEGFR-1, −2 and −3, Fms-like tyrosine kinase-3 receptor, and the receptor encoded by the ret proto-oncogene (RET) [41, 42].

Sunitinib was evaluated in a randomized, double-blinded, placebo-controlled phase III study of 171 patients with well-differentiated metastatic or unresectable pNETs [4]. A significant benefit in PFS was found for sunitinib-treated patients (11.4 months vs. 5.5 months; HR 0.42; $P < 0.001$). Due to the higher rates of serious adverse events (SAEs) and death in the placebo group, as well as greater PFS in the sunitinib group, the study was discontinued early. The objective response rate (ORR) for sunitinib was 9.3% vs. 0% for placebo ($P = 0.007$) however, due to the early interruption of the study and to the inadequacy of RECIST to precisely evaluate the anti-tumor effects of sunitinib, the obtained ORR did not include some patients that reached unconfirmed PRs [4]. More recently, Raymond et al. presented the preliminary results from an open-label phase IV clinical trial (NCT01525550) in patients with progressive, well-differentiated, unresectable advanced or metastatic pNETs that received sunitinib 37.5 mg once daily. Investigator–assessed median PFS (mPFS) was 13.2 months and ORR was 24.5% [43]. These results support the outcomes of the pivotal phase III study of sunitinib in pNETs and confirm its activity in this setting.

Although angiogenic growth factors such as VEGF-A, PDGF, basic fibroblast growth factor (bFGF), PIGF, hepatocyte growth factor (HGF), and interleukin 8 (IL-8) are examples of possible biomarkers that have been already proposed [44], they are not definitely established to evaluate the efficacy of sunitinib. Such biomarkers are needed in order to identify responsive and non-responsive patients, define optimal doses, and predict outcomes from the actual validated regimen.

4.2 Circulating Markers

Previous studies suggested that the activation of VEGF and its receptors could induce neoplastic progression. Although some of these biomarkers have been identified in other types of tumors as molecular predictors of response to antiangiogenic therapies [45], none has been conclusively validated as a predictor of response to sunitinib in patients with NETs.

Bello et al. evaluated the plasmatic levels of VEGF, sVEGFR-2, sVEGFR-3, and IL-8 in 109 patients with metastatic pNETs enrolled in a multicenter phase II trial, who received sunitinib in 6-week cycles, with a 50 mg/day regimen for 4 weeks, followed by 2 weeks without treatment [20]. The results suggested that these circulating proteins may be useful as pharmacodynamic biomarkers of sunitinib activity in patients with advanced NETs. It was observed that at the end of the first cycle, VEGF levels increased more than 3-fold over baseline in approximately 50% of the patients. In respect to sVEGFR-2 and sVEGFR-3 levels, these were significantly decreased by ≥30% in 60% and 70% of all patients, respectively ($P < 0.0001$). The reported reduction in sVEGFR-3 levels in the first cycle was superior in the group of patients with PR compared to the others (45% vs. 38%). However, it was observed that VEGF, sVEGFR-2, and sVEGFR-3 levels had a trend to return to near-baseline after 2 weeks without treatment. Moreover, there was a 2.2-fold average increase in IL-8 levels by the end of cycle 1, and a larger proportional increase in IL-8 levels in patients exhibiting decreases in tumor size, who were also likely to have inferior baseline IL-8 levels [20]. Thus, the results reported by Bello et al. provided a new perspective regarding the properties of sunitinib, in order to induce changes in circulating plasma proteins, suggesting that such modifications may be exploited as pharmacodynamic activity markers.

The study of Zurita et al. explored circulating cytokines and monocyte subpopulations as biomarkers associated with sunitinib activity in 105 patients with pNETs and carcinoid tumors, using specimens from a phase II study [21]. The analysis included VEGF, sVEGFR-2, sVEGFR-3, IL-8, stromal cell-derived factor (SDF)-1, and circulating endothelial cells (CECs), owing to preceding studies that suggested the prognostic value of the majority of these biomarkers in patients on sunitinib therapy [46, 47]. The results obtained at baseline did not demonstrate differences in sVEGFR-3 and IL-8 levels between tumors, however there were significantly higher SDF-1α and sVEGFR-2 concentrations in patients with pNETs compared with patients with carcinoid tumors [21]. Interestingly, it was reported for the first time that sVEGFR-2 concentration was more elevated in patients with pNETs with longer OS, which corroborate the data obtained by Grande et al. in 44 patients with NETs, treated with a different TKI (pazopanib) [48].

Zurita et al. also verified that in patients with carcinoid tumors, low pre-treatment IL-8 levels predicted longer PFS and longer OS, suggesting that IL-8 might be considered as a possible predictive marker of response. In addition, both low baseline concentrations of sVEGFR-3 and SDF-1α were associated with longer PFS and OS in a subgroup of patients with pNETs and carcinoid tumors [21]. The last marker (SDF-1α) was previously associated to pNETs with high rates of tumor growth and metastasis [49]. At the end of the first cycle with sunitinib, Zurita et al. reported a significant increase from baseline in 3 of the evaluated biomarkers, VEGF, IL-8, and SDF-1α, and a decrease in sVEGFR-2 and sVEGFR-3, without differences between tumor types [21], consistent with previous studies in mRCC [47]. Also at the end of the first cycle, Zurita et al. observed a significant correlation between plasmatic sunitinib levels and changes in VEGF ($P < 0.001$), sVEGFR-3 ($P < 0.001$), and IL-8 ($P = 0.014$) in pNETs patients. However, in patients with carcinoids, only a correlation between sVEGFR-3 and the drug level was observed. White blood cells and monocyte subsets were also analyzed in this study and, with the exception of lymphocytes and basophils, significant changes during the first 3 cycles of sunitinib were identified. The more remarkably decrease was observed on monocytes, which was negatively correlated with the plasmatic concentration of sunitinib. Concerning the evaluation on peripheral blood mononuclear cell specimens, it was observed during therapy that particularly CD14+ monocyte subpopulations co-expressing VEGFR-1 or CXCR4 decreased, suggesting that they may also be suitable indicators of sunitinib response and biological activity [21].

4.3 Tumor Tissue Markers

Several circulating proteins have been proposed as potential biomarkers of sunitinib response in patients with pNETs. However, only one study has reported tissue-based molecular biomarkers that can predict response to this targeted agent [22].

In patients with mRCC undergoing sunitinib therapy, previous studies have demonstrated that strong VEGFR-2 expression was significantly associated with tumor response. This pattern may reflect the fact that although sunitinib has inhibitory effects on multiple tyrosine kinases, its ability to inactivate VEGFR-2 is particularly relevant [50].

Recently, Dreyer et al. presented the results of a prospective phase II trial that evaluated potential biomarkers correlating with sunitinib activity in 31 patients with advanced WD G3 NET or PD NECs [22]. The authors evaluated a panel of biomarkers (PDGFR-b, VEGFR2, carbonic anhydrase 9, Ki67, and pAKT) in tumor tissues by IHC and correlated them with response by RECIST. It was observed that only Ki67 correlated with sunitinib activity, with a median Ki67 of 20% in patients with clinical benefit versus 77.5% in non-responders ($P = 0.002$). Sunitinib also demonstrated more pronounced activity in patients with Ki67 < 47% [22]. Thus, in accordance with the obtained results, Ki67 may be considered as a possible marker of response to sunitinib in NETs, but more prospective studies are needed for validation.

4.4 Imaging Biomarkers

When exposed to sunitinib, target lesions in NETs reveal limited variations in tumor size, but reasonably detectable alterations in tumor density [47].

Faivre et al. evaluated 10 patients previously included in the sunitinib phase III trial and observed that after 4 weeks of treatment, two of the patients were considered as responders according to RECIST, while six patients had a PR according to Choi criteria and presented an improved TTP compared with non-responders [23]. Despite the small sample size of the study, the obtained results suggest that tumor density on CT scan should be considered as a surrogate marker of response and as an alternative method to RECIST, particularly in patients treated with targeted therapies such as sunitinib for advanced NETs. Indeed, Choi criteria - which include both tumor size and tumor density - have previously more effectively identified GIST patients with clinical benefits from imatinib than RECIST [51].

The difficulties of assessing early response to sunitinib with conventional CT were also reported by Vercellino et al. [52]. The authors assessed the accuracy of 18-fluorodeoxyglucose–positron emission tomography/computed tomography (FDG-PET/CT) in evaluating early response to sunitinib in a group 12 of patients with mRCC who completed at least two cycles of treatment. After the first cycle of sunitinib, FDG-PET/CT findings were consistent with CT results obtained at the end of the second cycle in 9 of the patients [52]. Accordingly, the data suggest that FDG-PET/

CT may be considered as a convenient tool for the early evaluation of response to sunitinib. However, prospective and larger studies are required to endorse these preliminary results and to establish a predictive value for PET/CT in patients with advanced NETs treated with sunitinib.

5 Concluding Remarks

Nowadays, cancer treatment is more and more turning into a personalized approach centered on the importance of the molecular characterization of the tumor. However, the field of cancer biomarkers seems to be relatively stationary and most of the recently discovered biomarkers have not been clinically endorsed, as they did not reach the criteria required for application in the clinical setting.

The reasons for this failure have been extensively debated and classified into different sorts [53]. According to Dimandis et al., tumor biomarker failures are common and can be classified into three different categories: fraudulent reports, discovery of biomarkers with weak clinical performance, and false discovery or artefactual biomarkers, which include markers that, despite having a favorable original performance, present several weaknesses during the validation phase [54]. This supports the fact that the development of effective biomarkers needs coordinated attempts and that the required phases in the translation procedure have to be respected.

Specifically in the field of NETs, the efforts to generalize the use of some of the proposed biomarkers (Fig. 3) have been substantially ineffective, mostly due to the heterogeneity of NETs, the small number of patients, and also owing to the difficulties in systematizing blood-based research studies, IHC procedures, and molecular analysis approaches, which should be less discrepant between centers. In addition, differences in obtaining blood and tissue samples from patients included in the various studies discussed contribute to the differences in the obtained results and to the growing necessity to standardize methods of harvesting surgical specimens, performing biopsies, and collecting other types of samples.

In respect to circulating biomarkers, they are considered as an accessible and reliable method to evaluate response to therapy. Several studies, including the RADIANT-1 trial, stated that baseline and early response levels of CgA and NSE can predict outcomes in patients with advanced pNETs treated with everolimus, these results being extremely relevant as they were obtained from a prospective trial which included a large cohort of patients. Therefore, we admit that these two markers might be considered in the future for the evaluation of patients with advanced pNETs treated with everolimus.

The recent assessments of circulating biomarkers in patients with advanced pNETs treated with sunitinib demonstrated specific changes on plasmatic levels of VEGF, sVEGFR-2, sVEGFR-3, IL-8, and SDF-1α during therapy. These modifications revealed an important association with the obtained outcomes, suggesting that they may also be considered as appropriate indicators of response to this agent. However, we believe that more prospective studies, including larger cohorts of patients, are needed to validate these markers.

In respect to the available studies on tissue and imaging markers of response, they generally included a smaller number of patients who were retrospectively evaluated, which clearly renders them insufficient to predict the response to therapy in patients with pNETs on everolimus or sunitinib treatment.

On the other hand, current studies using genomic technologies, such as next generation sequencing (NGS)

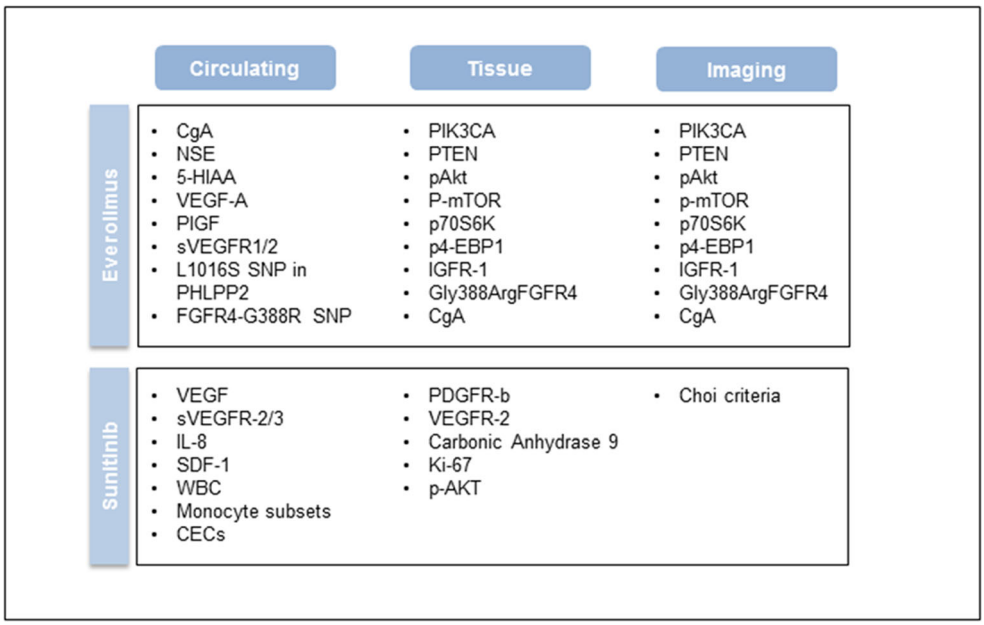

Fig. 3 Biomarkers of response to everolimus and sunitinib in pNETs discussed in this review

methods as well as the detection of circulating tumor cells (CTCs), allow the identification and characterization of new circulating cancer biomarkers. The recent advances in tissue microarrays and NGS methods demonstrated an increasing number of mutations in the mTOR pathway and also in chromatin modeling genes as *MEN1*, *DAXX*, or *ATRX*. However, although NGS is considered as an emerging method to evaluate the mutational landscape of NETs, its importance in clinical practice is still questionable and the experimental attempts to identify factors that are associated with the response to therapy are still ongoing. In addition, the characterization of CTCs as well as the identification of circulating cell-free tumor DNA containing mutations may contribute to significant changes in the care of these patients in the future, providing a complement to histological analysis, and allowing serial evaluations of predictive and prognostic markers through the different stages of disease progression. An alternative cancer biomarker with increasing acceptance and that might also be used as a "liquid biopsy" are microRNAs (miRNA), which constitute fragments of single-stranded non-coding RNAs that regulate a variety of genes by targeting mRNA transcripts and regulating various cell functions. However, prospective trials are also required to clarify and confirm the clinical utility of these techniques.

In conclusion, at the moment we have two molecular targeted agents available in clinical practice for the treatment of patients with advanced NETs, everolimus and sunitinib. Unfortunately, there are no ideal validated markers for these agents predicting tumor response and clinical benefit that could help us in the process of selecting patients. Therefore, there is an urgent medical need to validate biomarkers to spare toxicity in non-responsive patients, to reduce cost, and to avoid unuseful therapies in some patients. One of the reasons for not having validated predictive markers can be an inadequate design of the trials. In addition, retrospective analyses may be affected by a potential unreliability of the biological material. These should be considered as only hypothesis-generating studies and represent the basis of how specific prospective trials should be designed, possibly with homogeneous tumor populations, larger cohorts of patients, centralized pathology and molecular investigations, which may require the coordination and collaboration of an international research agenda.

Compliance with Ethical Standards

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Conflicts of Interest Nicola Fazio has received consulting fees or honorarium for oral presentations from Novartis, Ipsen and Pfizer. All other authors declare no conflict of interest.

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