

# Predictive factors of response to mTOR inhibitors in neuroendocrine tumours

Maria Chiara Zatelli<sup>1</sup>, Giuseppe Fanciulli<sup>2</sup>, Pasqualino Malandrino<sup>3</sup>, Valeria Ramundo<sup>4</sup>, Antongiulio Faggiano<sup>5</sup>, and Annamaria Colao<sup>4</sup> on behalf of NIKE Group

<sup>1</sup>Section of Endocrinology and Internal Medicine, Department of Medical Sciences, University of Ferrara, Via Aldo Moro 8, 44124 Cona – Ferrara, Italy

<sup>2</sup>Neuroendocrine Tumours Unit, Department of Clinical and Experimental Medicine, University of Sassari – AOU Sassari, Sassari, Italy

<sup>3</sup>Endocrinology Unit, Garibaldi Nesima Medical Center, Catania, Italy

<sup>4</sup>Department of Clinical Medicine and Surgery, “Federico II” University of Naples, Naples, Italy

<sup>5</sup>Thyroid and Parathyroid Surgery Unit, Istituto Nazionale per lo studio e la cura dei tumori “Fondazione G. Pascale” – IRCCS, Naples, Italy

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Correspondence should be addressed to M C Zatelli  
**Email**  
ztlmch@unife.it

## Abstract

Medical treatment of neuroendocrine tumours (NETs) has drawn a lot of attention due to the recent demonstration of efficacy of several drugs on progression-free survival, including somatostatin analogs, small tyrosine kinase inhibitors and mTOR inhibitors (or rapalogs). The latter are approved as therapeutic agents in advanced pancreatic NETs and have been demonstrated to be effective in different types of NETs, with variable efficacy due to the development of resistance to treatment. Early detection of patients that may benefit from rapalogs treatment is of paramount importance in order to select the better treatment and avoid ineffective and expensive treatments. Predictive markers for therapeutic response are under intensive investigation, aiming at a tailored patient management and more appropriate resource utilization. This review summarizes the available data on the tissue, circulating and imaging markers that are potentially predictive of rapalog efficacy in NETs.

### Key Words

- ▶ mTOR inhibitors
- ▶ neuroendocrine tumours
- ▶ predictors
- ▶ response to treatment

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## Introduction

Neuroendocrine tumours (NETs) consist of a spectrum of malignancies that can arise from neuroendocrine cells throughout the body. Despite NETs having been considered rare for a long time, large epidemiological studies have reported that the observed incidence has increased from 1.09 to 5.25/100,000 from 1973 to 2004 (Yao *et al.* 2008a). Therapeutic tools for NETs include surgery, somatostatin analogs (SSA), peptide receptor radionuclide therapy, interferon alfa, tyrosine kinase inhibitors, chemotherapy, as well as loco-regional

treatments (such as radiofrequency ablation and selected transcatheter arterial chemoembolization) (Modlin *et al.* 2010a, Oberg *et al.* 2010, Frilling *et al.* 2012, Pavel *et al.* 2012). Mammalian target of rapamycin (mTOR) inhibitors are emerging among the new targeted therapies as powerful tools in NET medical therapy.

mTOR is a serine/threonine protein kinase found in two major complexes: mTORC1 and mTORC2. mTORC1 is sensitive to the inhibition by rapamycin and mainly controls the energy status of the cell. This occurs also by

transducing PI3K/Akt-dependent growth factor signalling, such as insulin and IGF-1, and thus participating in the regulation of cell growth, survival and proliferation. mTORC2, which is rapamycin-insensitive, mainly influences the actin cytoskeleton, determining cell shape and modulating cell motility (Hay & Sonenberg 2004, Wullschleger *et al.* 2006). Rapamycin and its analogs ('rapalogs') bind the FK506 binding protein (FKBP12) in the cytoplasm, which, in turn, binds and inactivates mTORC1 and related downstream signalling (Meric-Bernstam & Gonzalez-Angulo 2009). Rapalogs have been shown to modulate cell proliferation, metabolism and angiogenesis in several *in vitro* models, including NETs. Zitzmann *et al.* (2007) showed that everolimus, a rapalog currently available for the medical therapy of NET of pancreatic origin (pNET) (Pavel 2013), dose-dependently reduces growth and causes apoptosis as well as arrest in the G0/G1 phase of the cell cycle in a human pNET cell line. This cell line, the BON1 cells, displays constitutive activation of the Akt/mTOR pathway due to an autocrine IGF-I loop (von Wichert *et al.* 2000). Zitzmann also provided evidence that selective mTORC1 inhibition by everolimus induces Akt signalling upregulation, a mechanism possibly responsible for rapalog resistance. Similar findings have also been shown by Grozinsky-Glasberg *et al.* (2008) in a rat insulinoma cell line, where everolimus was capable of inhibiting TSC2, mTOR and p70S6K but not Akt phosphorylation, with no additive effect when used in combination with the SSA octreotide. This drug was unable to enhance the anti-proliferative effects of rapamycin in two NET cell lines: the BON1 cell line and a cell line derived from a human bronchial carcinoid, the NCI-H727 cells. In these settings, rapamycin stimulated Akt phosphorylation, an effect that octreotide failed to overcome, further indicating that the Akt activating loop may cause rapalog resistance (Moreno *et al.* 2008). However, the molecular determinants of rapalog resistance are still unclear, despite the extensive investigation of the mTOR pathway, where several alterations have been described in NETs (Missiaglia *et al.* 2010).

The main rapalogs employed in clinical trials involving NETs are represented by everolimus and temsirolimus (Marotta *et al.* 2013, Chan & Kulke 2014). On the basis of the results of randomized placebo-controlled studies on patients with advanced pNETs, which showed improved progression-free survival (PFS; Yao *et al.* 2008b, 2010), everolimus has been approved for the treatment of patients with advanced pNETs. In addition, the RADIANT-4 trial has recently shown that everolimus reduces the risk of progression and prolongs PFS in

advanced, progressive, non-functioning NETs originating from the gastrointestinal tract and from the lungs (ESMO 2015, Yao *et al.* 2015; ClinicalTrials.gov ID: NCT01524783).

Despite promising results on tumour progression, everolimus shows a limited impact on tumour bulk, in keeping with the cytostatic rather than cytotoxic action of rapalogs. Indeed, everolimus efficacy in NETs may vary depending on the patient and on the development of rapalog resistance. Early detection of responder vs non-responder patients would thus be crucial to avoid ineffective and expensive treatments, shifting to alternative therapies early after treatment initiation.

## Aim

This review summarizes the available data on tissue, circulating and imaging markers of mTOR inhibitor efficacy in NETs that may help to identify patients who may benefit from treatment with mTOR inhibitors.

## Methodology

Four of the authors (M C Z, G F, P M and V R) independently searched MEDLINE (PubMed database) to identify potentially relevant articles on the predictive factors of efficacy of mTOR inhibitors in NET treatment. The search was last updated October 30th, 2015. Only articles published in the English language were considered. The search strategy included the following terms: 'neuroendocrine tumour', 'neuroendocrine carcinoma', 'predictive', 'response', 'everolimus', 'temsirolimus' or 'rapamycin'. Additional studies were identified by reviewing the references of all selected articles. Different article types were considered and only Editorials and Letters were excluded.

Overall, 147 articles were identified and collected in a single file, which was sent to all authors. Potentially relevant factors for predicting the response to mTOR inhibitors were divided into three main topics: tissue markers, circulating markers and imaging. Articles were selected by screening the title and abstract to identify only those that dealt with at least one of these three topics. The selected abstracts were then further assessed for a full-text evaluation.

## Tissue markers

An activated PI3K/AKT/mTOR pathway has been described in different NETs, including pancreatic,

gastrointestinal and lung tumours (Shida *et al.* 2010, Righi *et al.* 2010). Since an evident association between the expression levels of the PI3K/AKT/mTOR pathway and the survival of patients with NETs has been reported, it is reasonable to speculate that the analysis of this pathway may be useful to predict the clinical behaviour of NETs and possibly their response to rapalog treatment.

Tissue specimens allow a reliable assessment of both mutational status and expression levels of the mTOR pathway components through DNA/protein studies and immunohistochemistry (IHC) (Ali *et al.* 2011, Kasajima *et al.* 2011). Moreover, tissue analysis can be useful in the characterization of unresectable NETs undergoing bioptic procedures, which sometimes retrieve only small amounts of tumour tissue. In such cases, the sample may not be sufficient for an appropriate immunohistochemical characterization, but may be suitable for molecular studies, allowing to isolate somatic DNA, RNA or proteins.

DNA studies may then be performed, since they do not need huge amounts of tissue nor a demanding storage procedure. NET mutational profiles have been extensively investigated (Jiao *et al.* 2011, Oberg *et al.* 2013, Francis *et al.* 2013, Fernandez-Cuesta *et al.* 2014, Kidd *et al.* 2015a), leading to the observation that they are extremely variable depending on the site. Somatic mutations have also been intensively investigated to possibly predict sensitivity or resistance to mTOR inhibitors. Previous studies have shown that the presence of oncogenic variants of the phosphoinositide-3-kinase catalytic subunit (*PIK3CA*) and of Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) may influence the response of breast cancer cells to everolimus. Mutations in the PI3K pathway components were found in human cell lines that respond to rapalogs in terms of antiproliferative effects (Di Nicolantonio *et al.* 2010). The *PIK3CA* gene, however, is rarely mutated in pNETs (Jiao *et al.* 2011) and *PI3K-p85a* subunit mutations as well as *PI3K* amplifications have not been reported in NETs, so far (Briest & Grabowski 2014). Data on *KRAS* mutations in pNETs are also controversial: none of the 44 pNETs belonging to a Caucasian cohort was found to harbour *KRAS* somatic mutations (Gilbert *et al.* 2013), which were on the contrary reported in four out of 37 consecutive Chinese pNET patients (Yuan *et al.* 2014). In addition, *KRAS* somatic mutations have not been reported in Goblet cell NETs of the appendix (Dimmler *et al.* 2014), while Sahnane *et al.* (2015) identified *KRAS* mutations in 13 colorectal and two in gastric neuroendocrine carcinomas (NEC) among 53 cases of gastro-entero-pancreatic NEC. *KRAS* somatic oncogenic mutations characterize cancer patients who do not benefit from everolimus

treatment, indicating that *KRAS* and *PIK3CA* mutations may represent useful biomarkers to predict the efficacy of mTOR inhibitors. Indeed, *KRAS* mutations have been reported to confer resistance to everolimus treatment, even in the presence of *PI3K* mutations. Di Nicolantonio *et al.* (2010) observed everolimus resistance in human cancer cells with both *PIK3CA* and *KRAS* mutations; sensitivity to everolimus was restored after genetically deleting *KRAS* mutations. Moreover, they confirmed this finding in clinical settings when evaluating the response to everolimus in metastatic cancer patients: lack of response to everolimus was associated with oncogenic *KRAS* mutations. Clinical studies reporting higher patient numbers are needed to better estimate the prevalence of *KRAS* somatic mutation in NETs and its role in predicting the response to the treatment with rapalogs.

Mutations in Phosphatase and Tensin Homolog (*PTEN*) gene, leading to a reduced protein expression, have also been reported to characterize the NET cell lines that are sensitive to the antiproliferative effects of rapalogs (Meric-Bernstam *et al.* 2012). Indeed, mTOR inhibitors can effectively control *PTEN*-deficient tumour growth in several models (Neshat *et al.* 2001, Shi *et al.* 2002, DeGraffenried *et al.* 2004, Steelman *et al.* 2008), but *PTEN* loss failed to predict sensitivity to everolimus in glioblastoma orthotopic xenografts. *PTEN* mutations, as well as altered *PTEN* expression, have also been reported in pNETs (Missiaglia *et al.* 2010), especially those showing an aggressive clinical behaviour, suggesting that *PTEN* may be useful as a predictive marker in pNET. This is supported by the observation that loss of *PTEN* is associated with a shorter time to progression in patients with NEC treated with temsirolimus (Duran *et al.* 2006).

Other genetic mutations have been evaluated as putative biomarkers to predict rapalog sensitivity. A single-nucleotide polymorphism (SNP) in the fibroblast growth factor receptor isoform 4 gene (*FGFR4*), causing a conversion of guanine to adenine at position 1.217 in exon 9 (*FGFR4-G388R*), has been reported to associate with a worse prognosis in several human cancers (Morimoto *et al.* 2003, Wang *et al.* 2004, Thussbas *et al.* 2006, da Costa Andrade *et al.* 2007, Sasaki *et al.* 2008, Falvella *et al.* 2009). Serra *et al.* (2012) showed that, in pNET patients, the *FGFR4-G388R* allele (assessed at germline level) associates with a tumour diameter > 2 cm, local invasiveness, lymphovascular invasion, lymph node and liver metastases. In addition, BON1 xenografts (Evers *et al.* 1991), over-expressing *FGFR4-R388*, display a very aggressive behaviour in the animal model and a reduced responsiveness to everolimus. Similarly, pNET patients

displaying at least one *FGFR4-G388R* allele show a statistically significant reduction in response to everolimus *in vivo*. On the other hand, a retrospective study recently reported that the presence of an *FGFR4-G388R* allele does not influence PFS, overall survival (OS) and mTOR pathway components expression in patients with NET of the small bowel or of the pancreas (Cros *et al.* 2015). The Authors concluded that *FGFR4-G388R* allele does not predict everolimus sensitivity in pNETs. Therefore, the role of the *FGFR4-G388R* allele as a predictive marker of pNET rapalog sensitivity is still very controversial. In conclusion, since NETs apparently display few relevant mutations, DNA profiling may not help in predicting therapeutic responsiveness. On the other hand, epigenetic approaches may be more relevant since most of the mutated genes are involved in chromatin remodelling (Pipinikas *et al.* 2015).

Protein studies may be more difficult due to the scant amount of tissue which is often available. Nevertheless, biopsies in patients with NETs have shown that baseline AKT activation not only characterizes an aggressive clinical course, but also associates with an increased PFS under treatment with everolimus and octreotide (open-label phase II trial NCT00113360) (Ghayouri *et al.* 2010, Meric-Bernstam *et al.* 2012, Zitzmann *et al.* 2012). Similarly, it has been previously demonstrated that phosphorylated mTOR protein levels differentiate human bronchial carcinoids that are sensitive from those that are resistant to everolimus treatment *in vitro*. In addition, AKT/mTOR pathway signalling molecules in their active form (i.e. phosphorylated), such as basal mTOR, p70S6K, AKT and ERK1/2, are expressed at higher levels in human bronchial carcinoids responding to everolimus treatment *in vitro*, as compared to those resistant (Gagliano *et al.* 2013). Therefore, these markers may be useful to identify human NETs that may benefit from medical therapy with mTOR inhibitors. However, the potential predictive value of such markers has not been tested yet in other NETs, indicating the need for further validation studies to test the predictive value towards rapalog sensitivity of phosphorylated AKT, 4EBP1, S6K1 and S6 as well as of the presence of *PTEN* or *PIK3CA* mutations in NETs (Wander *et al.* 2011).

Several studies investigated the relationship between clinical outcome and IHC scores for mTOR signalling pathway proteins, but findings are discordant and only few studies analysed the efficacy of IHC in predicting the response to mTOR inhibitors in patients with NETs. In addition, the capability of these markers to predict survival is still very controversial (Zhou *et al.* 2011,

Qian *et al.* 2013, Ruza *et al.* 2014). The expression of the mTOR pathway components may be highly heterogeneous among different types of NETs, depending on both the primary site and the grading. Higher mTOR expression and activity have been found in foregut than in midgut NETs, also depending on the presence of metastases (Kasajima *et al.* 2011). The expression of *phospho-mTOR* and its downstream targets has been reported to be significantly different between low-to-intermediate grade tumours (i.e. typical and atypical carcinoids) and high grade tumours (i.e. large cell neuroendocrine carcinomas and small cell lung cancers) (Righi *et al.* 2010). Moreover, a strong expression of *phospho-mTOR* was observed more frequently in poorly differentiated as compared to well differentiated gastroenteropancreatic NETs (Shida *et al.* 2010, Catena *et al.* 2011). Bollard *et al.* (2013) observed a strong expression of the two major mTOR effectors (*phospho-p70S6K* and *phospho-4EBP1*) in six human tissue samples of NECs. They also investigated the effect of everolimus in a xenograft model of two NET cell lines (STC-1 and GluTag cells) in nude mice and found that the tumours derived from these cell lines mimicked NEC behaviour *in vivo*. In addition, treatment of xenografted mice with everolimus caused a significant reduction in tumour volume, which correlated with mTOR signalling inhibition. Duran *et al.* (2006) evaluated mTOR pathway components by IHC in 13 paired biopsies (obtained before and after 2 weeks of temsirolimus therapy) in patients with advanced NEC. Higher baseline expression of *phospho-mTOR* was predictive of tumour response. In addition, they found that after 2 weeks of treatment with temsirolimus an increased time to progression was associated with increased *phospho-AKT* and decreased *phospho-mTOR* expression. Spada *et al.* (2014) observed that among 36 patients with metastatic gastro-enteropancreatic NETs treated with everolimus 10 mg once daily, patients with Ki-67  $\leq 20\%$  (30/36) displayed a longer PFS when *phospho-mTOR* IHC score was positive as compared to those with negative *phospho-mTOR* IHC. These data suggest that mTOR pathway components IHC score in combination with Ki-67 labelling index may predict the response to the treatment with mTOR inhibitors. On other clinical grounds, two trials reported similar benefit from everolimus treatment in both well and moderately differentiated NETs, regardless of the neuroendocrine differentiation grade (Pavel *et al.* 2011, Yao *et al.* 2011a).

Sensitivity to mTOR inhibitors is not always directly related to PI3K/AKT/mTOR signalling. Because of compensatory feedback loops and cross talk between the PI3K/AKT/mTOR cascade and other pathways

(Markman *et al.* 2010, Burris 2013), resistance to mTOR inhibitor drugs is not a rare event. This is confirmed by observations both in trials and in real-world clinical settings. For instance, the PI3K/AKT/mTOR pathway may be activated upstream by a mutated and constitutively activated RAS/MAPK pathway. Indeed, adaptive resistance to everolimus monotherapy has been observed in a genetically engineered mouse model of pNET, where a significant regression in tumour burden was documented by combining everolimus with erlotinib, which acts through the inhibition of the epithelial growth factor receptor. (Chiu *et al.* 2010). Further studies are required to confirm these findings. In addition, controversies may come from well-known limitations of IHC, such as the possibility of different methods of staining evaluation and threshold values for distinguishing between negative and positive samples. IHC analysis is often performed on tissues obtained at diagnosis; subsequently, patients are treated with one or more types of antineoplastic therapies and tumour biology may change, no longer correlating with the initial mTOR pathway status.

In summary, IHC analysis of NETs allows the identification of patients with hyperactivated PI3K/AKT/mTOR pathway components, thus hypothetically leading to select which patient may benefit from treatment with mTOR inhibitors. However, the available evidence to support the utility of this evaluation for predicting the response to mTOR inhibitors is weak. Further studies with a better selection of patient cohorts (to reduce selection and measurement bias) and combined therapies targeting different signalling pathways (to overcome drug resistance) are warranted. At present, evaluating PI3K/AKT/mTOR pathway components by IHC is unlikely to achieve a satisfying and reliable predictive value (Delbaldo *et al.* 2011).

### Circulating markers

In addition to tumour samples, putative markers to predict sensitivity to rapalogs could be evaluated in peripheral blood, ensuring that these agents are delivered to those patients that are most likely to respond. The role of blood-based biomarkers has been recently addressed in a consensus review, which underlined the important limitations of monoanalyte biomarkers and the potential importance of circulating multianalyte biomarkers in predicting treatment efficacy (Oberg *et al.* 2015).

Chromogranin A (CgA), a circulating peptide, is usually considered the most helpful marker in patients with NETs. Elevated CgA levels are known to associate

with poor PFS and OS in NET patients (Modlin *et al.* 2010b, Lawrence *et al.* 2011). Neuron-specific enolase (NSE), on the contrary, has a scant clinical applicability, since it demonstrated low sensitivity and specificity as a NET biomarker (Baudin *et al.* 1998, Vinik *et al.* 2009). Yao *et al.* (2011b) provided information regarding the prognostic role of CgA and NSE in patients with advanced pNETs under treatment with everolimus in the RADIANT-1 (enrolling patients with low to intermediate grade advanced pNET) (Yao *et al.* 2010) and in the MDACC US-52 study at The University of Texas MD Anderson Cancer Center (enrolling patients with low to intermediate grade advanced carcinoid tumours and pNETs). The analysis of the biomarker pattern in patients under treatment with everolimus suggests that an early reduction in CgA or NSE level may predict a longer PFS in patients with pNET. Early modifications in CgA and NSE levels are therefore potentially important markers of response in pNET patients treated with everolimus, an issue that needs to be confirmed in prospective, randomized studies (Yao *et al.* 2011b).

Circulating tumours cells are detectable in patients with midgut NETs and with pNETs (Khan *et al.* 2011), where they could be considered a prognostic marker (Khan *et al.* 2013). Indeed, a recent study provides information as concerns the role of CTC count as predictive of response to treatment with several different approaches (including SSA, chemotherapy, peptide receptor radionuclide therapy, transarterial embolization, radiofrequency ablation, sunitinib, interferon alpha and surgery) that did not include rapalog treatment (Khan *et al.* 2015). Further research is needed to fully exploit the potential predictive role of CTCs in the context of NET medical therapy by means of rapalogs.

The expression profile of microRNAs (miRNAs), small noncoding RNAs involved in gene expression regulation, has been reported to be quite specific in pNETs (Roldo *et al.* 2006), but data on serum miRNAs as potential biomarkers of clinical behaviour as well as of response to medical treatment are not available, to date (Modlin *et al.* 2014a).

It has been recently suggested that Multianalyte Algorithmic tests (MAAAs) may perform much better as compared to monoanalyte markers, taking advantage of the simultaneous evaluation of several markers. To test this hypothesis, Modlin *et al.* (2013) developed a gene biomarker assay that includes several genes selected on the basis of the results of microarray data and by means of a computational strategy. This test has been applied to identify candidate marker genes in peripheral circulation

of patients with NETs with high performance (Modlin *et al.* 2014b) independent of age, gender, ethnicity, fasting or proton pump inhibitor treatment and identified affected patients with high sensitivity and specificity (Modlin *et al.* 2014c). Along the same line, Kidd *et al.* (2015b), matched the results of the evaluation of the 51 circulating transcripts identified by MAAA with the analysis of tumour tissue transcripts, divided into different gene clusters, and applied an MAAA/cluster integrated algorithm. The latter was capable of accurately separate NETs with progressive disease from those displaying stable disease after different lines of therapy, predicting the disease status by means of a disease activity NET score, the NETest. The NETest appears to be a very promising tool not only for the diagnosis but also for the follow-up of NET patients, although to date no information is available concerning a predictive role for the NETest as concerns rapalog sensitivity.

## Imaging

Tumour measurement by computed tomography (CT) using the Response Evaluation Criteria in Solid Tumours (RECIST) (Therasse *et al.* 2000, Therasse *et al.* 2006) has been considered for a long time the gold standard to evaluate the effect of antineoplastic drugs. However, the effect of cytostatic drugs, whose antineoplastic action may not be immediately followed by a reduction in tumour size, can represent a limit in the use of RECIST criteria (Benjamin *et al.* 2007). Positron Emission Tomography (PET) is emerging as a powerful tool, capable of overtaking CT measurement limits, offering additional information useful for treatment monitoring (Eisenhauer *et al.* 2009) and predicting the response to chemotherapy at an earlier stage (Jensen *et al.* 2010).

## Studies in animal models

18F-fluorodeoxyglucose (FDG) PET is the most widely employed PET in oncology. FDG uptake mirrors cell glycolytic activity and may correlate, in some tumours, with early treatment responses (Weber & Wieder 2006).

18F-fluorothymidine (FLT) PET, on the other hand, reflects proliferation (Shields *et al.* 1998). FLT is a thymidine analog, similar to the nucleotide usually incorporated during DNA synthesis (Kong *et al.* 1992). In humans, 18-FLT PET has recently shown its usefulness for predicting the response to carbon ion radiotherapy in subjects with melanoma (Inubushi *et al.* 2013). Johnbeck *et al.* (2014) investigated in animal models (mice) the

ability of 18F-FDG PET and 18F-FLT PET to predict tumour response to everolimus. Mice were inoculated with a human NET cell line (lung carcinoid) and tumours were allowed to grow for 2 weeks. Then, the experimental animals underwent CT for tumour size measurements, and scanning with 18F-FDG and 18F-FLT PET (baseline). Mice were then treated with either everolimus (5 mg/kg daily, subcutaneously) or with placebo for 10 days. CT as well as 18-FDG and 18-FLT PET for measurements of tumour size were then performed. The study showed that early 18F-FDG uptake (day 3) significantly correlated with tumour diameter at a later time point (day 10). Similarly, early 18F-FLT uptake (day 1) correlated with tumour growth at day 7, and 18F-FLT uptake at day 3 correlated with tumour growth at later time points (day 7 and 10). The authors conclude that early 18F-FLT uptake may predict later tumour growth and propose that 18F-FLT PET uptake could potentially be employed as an imaging biomarker for tailoring NET therapy. Moreover, 18F-FDG may represent a possible alternative in subjects with FLT-negative NETs. However, as outlined by the authors, the study was performed by using a single lung NET cell line and cannot demonstrate that early prediction of everolimus effect on tumour growth by PET imaging can hold for all NETs.

## Studies in humans

Angiogenesis is a well-known hallmark of tumour growth (Hanahan & Weinberg 2011). Vascular endothelial growth factor A (VEGF-A), in particular, plays a pivotal role in angiogenesis. The effect of everolimus on the reduction of VEGF-A production by tumour cells (Huynh *et al.* 2009) could potentially offer an early detection of responders vs non-responders. A PET method obtained by coupling the anti VEGF-A antibody bevacizumab to a radionuclide (89Zr-bevacizumab) has been shown to detect human neoplastic cells (Nagengast *et al.* 2007). van Asselt *et al.* (2014) investigated the performance of 89Zr-bevacizumab PET to predict tumour response to everolimus in patients showing progression of well-differentiated NETs. Fourteen patients underwent 89Zr-bevacizumab PET scanning before everolimus treatment, and then 2 and 12 weeks after treatment initiation. Ten out of 14 patients showed positive 89Zr-bevacizumab PET scan findings. In this subset of patients, the Authors observed that the tumour Maximum Standardized Uptake Value (SUVmax) decreased in seven patients while it increased in three. Interestingly, after 6 months of therapy, the sum of target lesion diameters (measured by CT) showed a correlation

with SUV max at 2 and 12 weeks (compared to SUV max at baseline). The authors concluded that sequential <sup>89</sup>Zr-bevacizumab PET scan might be employed to predict early the effects of everolimus. However, none of the 14 patients (including four <sup>89</sup>Zr-bevacizumab PET negative scan patients) experienced progressive disease after 6 months of everolimus treatment. Thus, patients cannot be excluded from everolimus treatment on the basis of a negative <sup>89</sup>Zr-bevacizumab PET scan

## Conclusions

Multiple putative predictors of response to mTOR inhibitors have been proposed (Table 1), but the attempts to standardize these biomarkers have been mostly unsuccessful, possibly because PI3K/mTOR pathway complexity includes several feedback loops that may result in unpredictable effects. In addition, standardization of IHC and molecular techniques is very challenging, and genomic as well as epigenetic methods are promising but need accurate validation for clinical applications. Imaging, such as <sup>18</sup>F-FLT or <sup>18</sup>F-FDG PET, may represent

a more attractive and clinically useful means to predict therapeutic responses to rapalogs, but validation studies on greater patient numbers are needed. Circulating markers are easier to assess, but reproducibility has not been strongly documented. Nevertheless, CgA and NSE levels may be assessed in NET patients undergoing rapalog therapy, until more powerful indicators will be identified. Indeed, single biomarkers are unlikely to achieve the goal to correctly predict NET responsiveness to rapalogs. On the other hand, new multimodal approaches, such as MAAA, may be more successful, also because they are based on easily accessible patient material, i.e. blood (associated or not to the corresponding tumour sample). The evaluation of the disease activity NET score at baseline may help in patient follow-up, and future studies will test the potential of the NETest to predict NET response to rapalogs.

In conclusion, the field of research for reliable predictive markers of response to rapalogs in NETs remains wide open, with the future perspective to identify basic and clinical predictors possibly useful to prospectively select patients who may benefit from rapalog treatment.

**Table 1** Markers evaluated as predictive of response to mTOR inhibitors in NETs.

Marker/method	Experimental group	Drug (dose)	NET subtype	Reference
<b>Tissue markers</b>				
KRAS	Human cancer cell lines	Everolimus	–	Di Nicolantonio et al. (2010)
PTEN	Human cancer cell lines; n=43	Rapamycin (100 nM)	–	Meric-Bernstam et al. (2012)
pAKT	Human; n=60	Octreotide (30 mg/28 days) + Everolimus (5–10 mg/day)	Carcinoid and islet cell NETs	Meric-Bernstam et al. (2012)
PTEN	Human; n=36	Temsirolimus (25 mg i.v./week)	Advanced progressive NEC	Duran et al. (2006)
FGFR4-G388R	Human; n=17	Octreotide (30 mg/28 days) + Everolimus (10 mg/day)	G1-G2 pNET	Serra et al. (2012)
FGFR4-G388R	Human; n=41	Everolimus (10 mg/day)	G1-G3 pNET, small bowel NET	Cros et al. (2015)
mTOR, p70S6K, AKT, and ERK1/2	Human primary cultures; n=17	Everolimus (100 nM)	Well differentiated lung NETs	Gagliano et al. (2013)
phospho-mTOR, phospho-AKT	Human; n=13	Temsirolimus (25 mg i.v./week)	G3 NECs	Duran et al. (2006)
phospho-P70s6k, phospho-4EBP1	Animal (mice); n=34	Everolimus (1.5 mg/kg per day)	Intestin cell line (STC-1 and GluTag cell line)	Bollard et al. (2013)
Ki-67, Phospho-mTOR	Human; n=36	Everolimus (10 mg/day)	G1-G3 pNET, ileal NET, other	Spada et al. (2014)
<b>Circulating markers</b>				
CgA, NSE	Human; n=115	Everolimus (10 mg/day)	G1-G2 pNET	Yao et al. (2010)
<b>Imaging</b>				
<sup>18</sup> F-fluorodeoxyglucose PET and <sup>18</sup> F-fluorothymidine PET	Animal (mice); n=20	Everolimus (5 mg/kg per day, subcutaneously)	Well differentiated lung NET (human cell lines)	Johnbeck et al. (2014)
<sup>89</sup> Zr-bevacizumab PET	Human; n=14	Everolimus (10 mg/day)	Advanced progressive G1-G2 NETs	van Asselt et al. (2014)

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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