Management of Neuroendocrine Tumors of the Pancreas and Digestive Tract

From Surgery to Targeted Therapies: A Multidisciplinary Approach

Eric Raymond · Sandrine Faivre
Philippe Ruszniewski *Editors*

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Editors
Eric Raymond
Sandrine Faivre
Medical Oncology
Bichat-Beaujon University Hospitals
Clichy
France

Philippe Ruszniewski
Gastroenterology and Pancreatology
Bichat-Beaujon University Hospitals
Clichy
France

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Preface

Complexity of Patient Care in Neuroendocrine Tumors of the Digestive Tract

Neuroendocrine tumors (NETs) have emerged as paradigm tumors for which multidisciplinary care is required. NETs are known as rare tumors. However, the increasing incidence of NET renders it likely that physicians caring for cancers may have either already faced or may be certainly exposed during their career to the challenging issues of discussing the case of a patient with NET. During the last 5 years, several novel therapeutic options have emerged for NET, profoundly challenging practices that had been previously set for decades. This moving field has generated some confusion, leading to novel treatment algorithms to guide medical decisions. To either better understand or handle the multidisciplinary approaches that are required for optimizing the care of NET patients, physicians are now looking for references from experts and comprehensive reviews summarizing the current knowledge on treatments of patients with NET.

NETs are fascinating multifaceted diseases that can primarily localize in many organs with various presentations. Few patients may present with symptomatic tumors at diagnosis due to endocrine secretions and/or bulky tumor masses. In some instances, emergency care may even be required to speedup diagnosis and therapy. More frequently, NETs are diagnosed at late stages due to the lack of symptoms and the relative indolence of the disease, even in the presence of multiple metastases. Therefore, the vast majority of patients with NET may present at diagnosis with advanced primary and already developed metastasis, the liver being the primary site of digestive NET dissemination. Although only a small number of patients may undergo surgical resection, surgery remains the only curative approach and shall therefore be discussed along with other options even in the presence of metastases. Since most patients will develop multiple non-operable liver metastases early on during the natural history of their disease, curative surgery is often impossible and instead debulking liver-resection and liver-directed therapy, such as chemoembolization of radiofrequency ablation, may have palliative benefits for patients with liver-dominant metastases. Interestingly, NET cells often express somatostatin receptors that can control hormonal secretions and stimulate tumor proliferation. Somatostatin analogs, inhibiting somatostatin

receptor functions, are often prescribed to relieve symptoms resulting from hormonal hypersecretion in functioning tumors such as diarrheas and flushing episodes. Recently, data also demonstrated that somatostatin analogs could also delay tumor progression in selected patients with carcinoid tumors, although this demonstration has not yet been fully demonstrated for patients with pancreatic NETs (PNETs). Taking advantage of the presence of somatostatin receptors at the surface of cancer cells, somatostatin analogs loaded with radionucleotides have been used to selectively target cancer cells and deliver metabolic radiotherapy to disseminated NET metastases. Based on large retrospective clinical experiences, Peptide Receptor Radionucleotide Therapy (PRRT) is now frequently proposed to patients with advanced NET. Although evidences suggest activity of PRRT in NET, the overall benefit and long-term safety of this therapeutic approach remains to be validated prospectively. For patients with advanced NET, chemotherapy has been an important part in the history of treatment for NET. Chemotherapy was the first treatment option demonstrating significant benefits, delaying tumor progression, controlling symptoms, and in some circumstances improving overall survival. While midgut carcinoid tumors showed poor sensitivity to chemotherapy, PNETs have been acknowledged to be more sensitive to chemotherapy. Chemotherapy, such as streptozocin, either combined with doxorubicin or fluorouracil, has been the only systemic treatment approved for many years in advanced PNETs, though the magnitude of benefit has been often challenged in recent publications. Temozolomide, an oral methylating chemotherapy with mechanisms of action similar to DTIC, has been evaluated in retrospective series. Temozolomide demonstrated evidence of activity, possibly related to the lack of methyl guanine transferase expression, the enzyme that repairs DNA insults caused by temozolomide. More recently, large prospective trials using sunitinib and everolimus demonstrated that progression of PNET could be delayed using small molecules targeting cell signaling. Inhibition of mTOR using everolimus may cause inhibition of cancer cell proliferation and can alter metabolic function of NET cancer cell, delaying tumor progression in advanced well-differentiated tumors. In addition, sunitinib, inhibiting NET angiogenesis at the level of endothelial cells and pericytes was also shown to delay tumor progression in well-differentiated PNET. These two drugs have been recently approved in advanced PNET and now offer more opportunities in the NET armamentarium to delay progression. While treatment options have progressed, imaging techniques and endoscopy have also gained in precision allowing earlier diagnosis, better sensitivity in the detection of metastases, and more efficient criteria for evaluating drug efficacy. Considering the multiple treatment options in PNET, strategies are now required to optimize the sequential use of somatostatin analogs, PRRT, chemotherapy, and targeted therapies in patients with advanced PNETs that are not amenable to curative surgery. Another important issue in the care of patients with NET shall also consider how quality of life could be impacted by treatment decisions.

The multiple options for treatment of patients with NET require multidisciplinary approaches and discussions from experts from various specialties to select

the best treatment choice for each individual case. Multidisciplinary boards developed in expert centers are aiming to encompass the various needs for care of patients with NET and should be promoted, eventually using networking though teleconferences in centers that cannot develop expertise in all the domains. In this book, we have aimed to keep the spirit of multidisciplinary board meetings, asking experts to deliver chapters where readers may find data to make their own opinions. Authors have been selected from centers of expertise for NET in Europe and in the United States. Authors have been requested to provide updated information about current knowledge for various aspects of treatment of patients with NET. We expect that readers will find inspiring ideas and information that may help them to better understand options and optimize the care of patients with NET.

Eric Raymond
Sandrine Faivre
Philippe Ruszniewski

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Chapter 1
Scintigraphy in Endocrine Tumors of the Gut

Rachida Lebtahi

Abstract This review provides an overview of the currently used nuclear medicine imaging modalities and ongoing developments in the imaging of neuroendocrine tumors (NETs). Most NETs overexpress the somatostatin receptor mainly sst2. Somatostatin receptor scintigraphy with ^{111}In-DTPA-0octreotide has proven its role in the diagnosis and staging of gastroenteropancreatic NETs. The use of ^{68}Ga-labeled analogs of octreotide for PET imaging, with of different radiolabelled somatostatin analogues with higher affinity and different affinity profiles to the somatostatin receptor subtypes such as DOTATOC, DOTANOC, and DOTATATE, are in clinical application in nuclear medicine. The development PET tracers for NET imaging include Fluorodihydroxyphenylalanine (^{18}FDOPA) and fluorodeoxyglucose (^{18}FDG). ^{18}FDOPA-PET appears to be a major tool for the management of carcinoid tumors with excellent diagnostic performances. The role of ^{18}FFDG PET-CT in the prognosis of neuroendocrine tumors should be evaluated.

Keywords Neuroendocrine tumors · Somatostatin receptor scintigraphy · ^{68}Ga-DOTATOC · ^{68}Ga-DOTANOC · ^{68}Ga-DOTATATE · ^{18}FDOPA-PET

Introduction

Nuclear imaging procedures of neuroendocrine tumors (NETs) consist in images performed with a hybrid camera combining single-photon emission computed tomography with computed tomography (SPECT-CT) and/or images with a positron emission tomography camera (PET).

R. Lebtahi (✉)
Department of Medical Oncology (INSERM U728—Paris 7 Diderot University),
Beaujon University Hospital, Assistance Publique—Hôpitaux de Paris,
100 Boulevard du Général Leclerc, 92110 Clichy, France
e-mail: rachida.lebtahi@bjn.aphp.fr

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The first imaging procedure used radiolabeled somatostatin analogs for the detection of NETs [1, 2]. A high density of somatostatin receptors with high affinity for octreotide (somatostatin analog) has been demonstrated in almost all NETs [3]. Five subtypes of somatostatin receptor were identified (from sst1 to sst5 subtypes) [4]. In the same tumor, different subtypes of receptors may be expressed, and most NETs express more than one of five somatostatin receptor subtypes. For the detection of NETs, Krenning et al. [1] and Lamberts et al. [2] reported the first results of somatostatin receptor scintigraphy using radiolabeled somatostatin analogs. The technique most often used today is somatostatin receptor scintigraphy with SPECT-CT using ^{111}In-DTPA-octreotide (Octreoscan®) [5–7]. The uptake of ^{111}In-DTPA-octreotide is based on a specific receptor mechanism. Octreoscan® can therefore visualize tumors which express these receptors, such as NETs. With Octreoscan®, the uptake within the tumor depends on the presence of somatostatin receptors (mainly sst-2), and the intensity of this uptake is related to the density of sst-2 receptors [3, 8, 9]. The localization of the tumor and determination of the extent are essential for the management of patients with NETs [6, 7].

Somatostatin Receptor SPECT-CT

Octreoscan® scintigraphy has been proven useful in functional or nonfunctional neuroendocrine tumors. The sensitivity for the detection reported by the literature is estimated at 70–100 % [6–8]. Scintigraphy permits staging workup and/or the follow-up after treatment [5–7, 10, 11]. The sensitivity of Octreoscan® scintigraphy for detecting neuroendocrine tumors of the gut has been well studied [5–7, 12, 13]. The major diagnostic value of this method is to be complementary to other conventional imaging techniques. Almost all studies demonstrated that scintigraphy has greater sensitivity for detecting both hepatic and extrahepatic metastases. The Octreoscan® scintigraphy confirms known lesions and reveals lesions not visualized by other imaging techniques [11]. It suggests the character of an endocrine tumor already revealed by conventional imaging. The positivity of somatostatin receptor scintigraphy has been reported to be a strong predictive factor of response to treatment with radiolabeled analogs. More recently, it has been used to select patients likely to receive peptide-receptor radionuclide therapy (PRRT) [12]. Its positivity suggests that it is a good prognosis marker of the neuroendocrine nature of a tumor [9, 13]. The recommended protocol is intravenous injection of about 200 MBq of ^{111}In-pentetreotide (with 10 μg of the somatostatin analogs) [5–7]. Images should be performed at 4 and 24 h post injection, using planar images and systematically abdominal SPECT-CT at 24 h post injection. Normal imaging results show a physiologic low-level uptake in the pituitary, thyroid, and breasts. The accumulation is also shown in the liver (with always homogeneous repartition), the kidneys, and the spleen. In addition, the gallbladder is often visualized. The visualization of pituitary, thyroid, and spleen is due to specific receptor binding. There is a predominant kidney clearance, and the

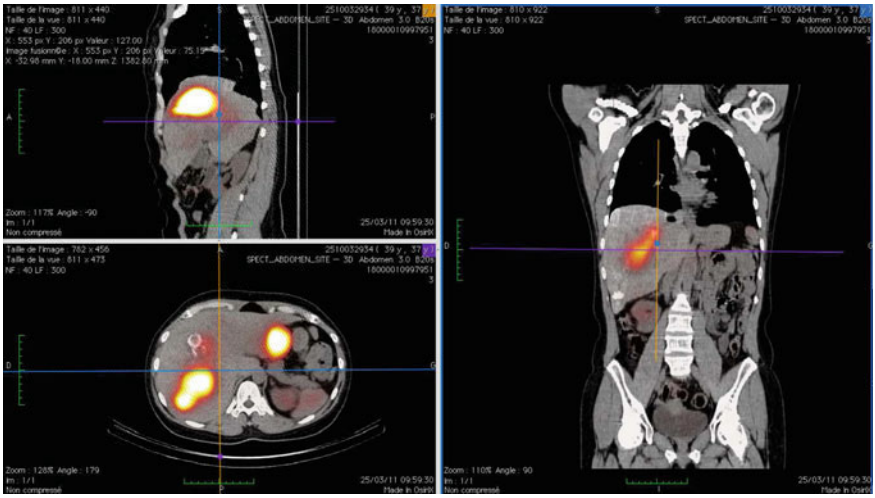

Fig. 1.1 Patient with well-differentiated neuroendocrine tumors: Octreoscan® SPECT-CT showed liver metastases

renal uptake is related to reabsorption of the radiolabeled peptide in the renal tubular cells. Hepatobiliary clearance into the bowel also occurs, leading to the acquisition of delayed abdominal images or the use of laxatives in order to differentiate tumoral from physiologic uptake (Fig. 1.1).

Despite greater sensitivity, limitations of Octreoscan® scintigraphy should be noted. The methodology clearly influenced the sensitivity of the examination. Routine use of planar images and SPECT-CT images of the abdomen (24 h after injection) rather than whole body images are recommended. Octreoscan®x cannot provide information on the size of the tumor. The density and type of the somatostatin receptors vary with the histologic type of the tumors: Insulinomas have a low affinity for octreotide, related to a low expression of sst subtype-2 [5]. Garin et al. [13] reported that negative Octreoscan® scintigraphy in well-differentiated endocrine tumors is negative prognostic factor.

Specificity of Octreoscan® should be noted. Some other tumoral and nontumoral diseases can show positivity of Octreoscan® [7].

Somatostatin Receptor PET-CT

Positron emission tomography (PET) scan is becoming more widely used and may be a useful localizing modality for neuroendocrine tumors as different radiolabeled substances can be used as metabolic substrate. After the development of a PET tracer for somatostatin analogs, ^{68}Ga-DOTA-NOC (tetra-azacyclododecane

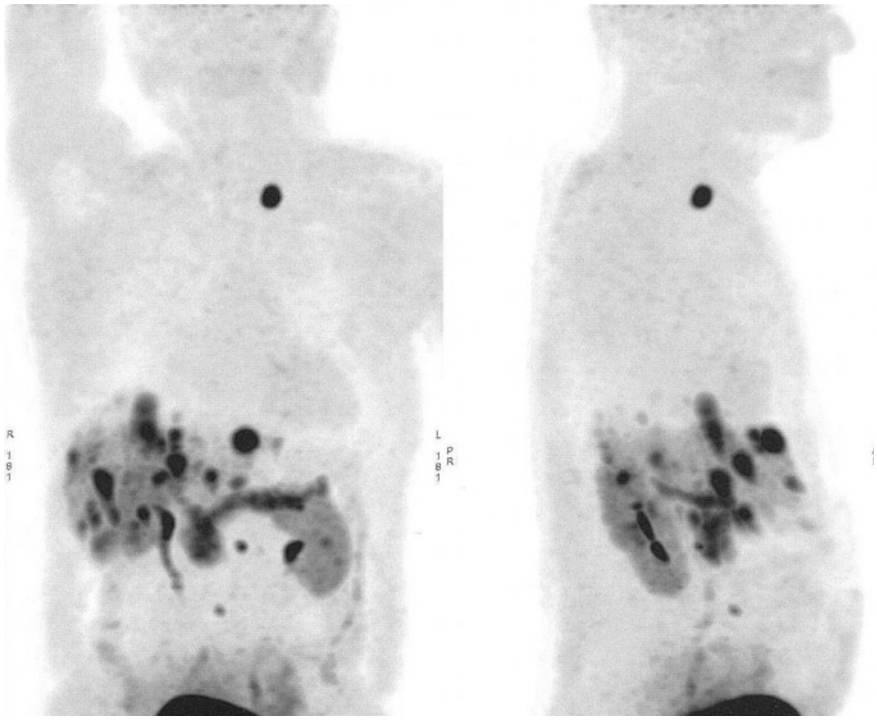

Fig. 1.2 Patient with well-differentiated neuroendocrine tumors: ^{18}FDOPA PET-CT showed multiple liver metastases and sus-clavicular left lymph node

tetra-acetic acid-[1-Nal3]-octreotide) has been introduced. This compound for PET imaging has a high affinity for sst2 and sst5 and has been used for the detection of NETs in preliminary studies. The uptake of ^{68}Ga-DOTA-NOC is based on a receptor mechanism and although this has not yet been adequately assessed, it seems to have higher sensitivity for NETs than Octreoscan®, thereby increasing diagnostic accuracy. Additionally, it has several advantages over Octreoscan®: increased spatial resolution and the possibility of images with a short uptake time (60 min), and relatively easy synthesis [14].

The two other compounds most often used in functional imaging with PET are ^{68}Ga-DOTATOC and ^{68}Ga-DOTATATE. ^{68}Ga-DOTATOC and ^{68}Ga-DOTA-TATE possess similar diagnostic accuracy for detection of NET lesions. The increasing availability of ^{68}Ga somatostatin analogs PET-CT now offers superior accuracy for localization and functional characterization of NETs. However, studies are needed to enable imaging of NET with optimal targeting of tumor receptors.

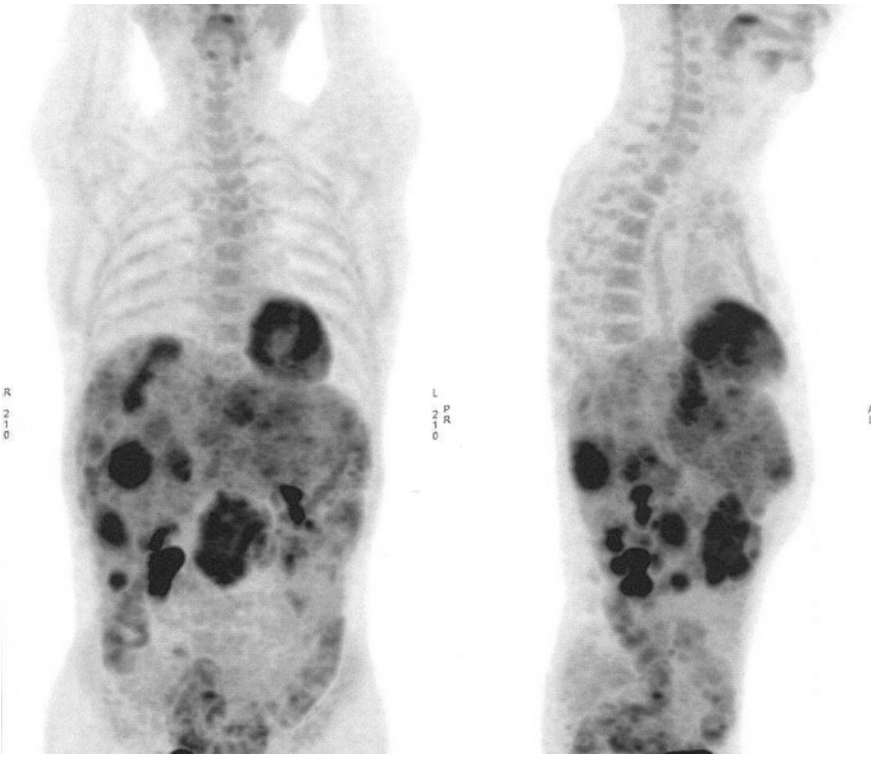

Fig. 1.3 Patient with well-differentiated neuroendocrine tumors grade 2 (KI 67: 15 %).^{18}FDG-PET-CT showed liver metastases and mesenteric lymph node

^{18}F-DOPA PET-CT

Fluorodihydroxyphenylalanine-(^{18}F-FDOPA) PET is a recent imaging modality used to localize neuroendocrine tumors [15]. These tumors have the ability to produce biogenic amines and polypeptide hormones, and they take up and decarboxylate their amine precursors, L-dihydroxyphenylalanine. ^{18}FDOPA-PET appears to be a major tool for the management of carcinoid tumors with excellent diagnostic performances (65–96 %) related to these capacities to concentrate amino acids inside the vesicules of cytoplasmatic space through metabolic mechanism. ^{18}FDOPA-PET is less sensitive and less useful for the management of noncarcinoid tumors (Fig. 1.2).

^{18}FDG PET-CT

Although ^{18}F-2-Deoxy-D-glucose (^{18}F-FDG) PET is the most widely used and accepted type of PET in clinical oncology, it has limited use in well-differentiated tumors such as NETs due to their low expression of glucose transporters and low proliferative activity. However, several studies have evaluated ^{18}F-FDG PET-CT in well-differentiated NET [13, 16, 17]. Garin et al. reported that ^{18}FDG uptake is a poor prognostic factor in NETS, in relation to tumor aggressiveness and is related to a lower overall survival (Fig. 1.3).

Conclusions

All of these performances highlight the significant contribution of the scintigraphic procedures from a diagnostic point of view and the management of therapy of patients with NETs. PET imaging could be of major interest for the diagnosis, evaluation of progression and treatment response in NETs. ^{18}FDG-PET even though still not validated, carries major prognostic information and may influence determination of the optimal therapeutic strategy. The role of ^{18}F-DOPA is clearly recommended before surgery for the detection of carcinoid tumors. The different new somatostatin analogs with ^{68}Ga radiolabeling must be evaluated.

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Chapter 2
Profiling mTOR Pathway in Neuroendocrine Tumors

S. Cingarlini, M. Bonomi, C. Trentin, V. Corbo, A. Scarpa and G. Tortora

Abstract The serine/threonine kinase mammalian target of rapamycin (mTOR) plays a central role in regulating critical cellular processes such as growth, proliferation, and protein synthesis. The study of cancer predisposing syndromes within which neuroendocrine tumors (NETs) may arise has furnished clues on the involvement of mTOR pathway in sporadic diseases so far. Recent comprehensive analyses have definitely shown activation of mTOR pathway in both experimental and human sporadic NETs. Upstream regulators of mTOR (PTEN and TSC2) have been found mutated in sporadic PNETs. Activation of mTOR pathways in NETs is already demonstrated by expression profiles analysis that revealed downregulation of TSC2 gene and alterations of TSC2 and PTEN protein expression in the vast majority of tumors well-differentiated tumors. Moreover, a global microRNA expression analysis revealed the overexpression, in highly aggressive tumors, of a microRNA (miR-21) that targets PTEN reducing its expression and therefore leading to mTOR activation as well. Overall, these clues have furnished the rationale for the use of mTOR inhibitors the treatment for PNETs. With the recent approval of everolimus (mTOR-targeted drug) for the treatment of advanced PNETs, this paradigm has been effectively translated into the clinical setting. In this review, we discuss mTOR pathway involvement in NETs, the clinical evidence supporting the use of mTOR inhibitors in cancer treatment, and the current clinical issues that remain to be elucidated to improve patients' management.

The pathway of the mammalian target of rapamycin (mTOR) plays a central role both in cell proliferation and in the survival rate. Physiologically, it finely tunes anabolic and catabolic processes according to the available energy sources to

S. Cingarlini · M. Bonomi · C. Trentin · G. Tortora (✉)
Medical Oncology, Department of Medicine, University of Verona and Azienda Ospedaliera Universitaria Integrata, P.le Scuro 10, 37134 Verona, Italy
e-mail: giampaolo.tortora@univr.it

V. Corbo · A. Scarpa
ARC-NET Research Centre and Department of Pathology and Diagnostics, University of Verona and Azienda Ospedaliera Universitaria Integrata, Verona, Italy

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warrant cell proliferation and homeostasis [1]. mTOR is also involved in many pathological conditions other than cancer such as diabetes, neurodegeneration, and obesity. Aberrant signaling caused by molecular alterations within the cascade may contribute to cancer development and progression [2–4].

The great amount of extracellular and intracellular inputs converging on it (or on its singular components) makes mTOR a crucial crossroad whose outputs influence essential cellular functions (such as protein/lipid synthesis, autophagy, or cytoskeletal organization). Growth factors stimuli (acting on mTORC1 and triggering the downstream anabolic signaling), energy depletion and low oxygen levels (activating mainly AMPK and thereby inhibiting mTOR complex either directly or through TSC2), DNA damage (which leads to a PTEN- and TSC2-mediated inactivation of mTOR), and amino acids levels (whose presence is essential for mTOR signaling but whose exact mechanism of action is still unraveled) are some of the most significant examples of the plethora of inputs and outputs coming to and from mTOR [1].

In neuroendocrine tumors (NETs), nearly all the members of PI3K/Akt/mTOR pathway, from the upstream RTK inducers to its final effectors, can be molecularly altered and one or more than one of the above-mentioned alterations can be detected in the same cancer cell. The involvement of mTOR pathway in neuroendocrine tumorigenesis is suggested by a series of evidences:

- Familial syndromes such as multiple endocrine neoplasia type 1 (MEN1), von Hippel–Lindau (VHL) syndrome, type 1 neurofibromatosis (NF1), and tuberous sclerosis complex (TSC). Single pathogenic molecular alterations may trigger the development of NETs with a higher incidence if compared to the generic population. Inactivation of VHL is associated with an increased steady-state level of HIF-1, whose expression is dependent on mTOR-mediated translational regulation [5, 6]. Loss of NF1 is associated with constitutive mTOR activation (depending upon Ras and PI3K) [7]. Loss of function mutations of either TSC1 or TSC2, whose encoded proteins form the TSC complex, can negatively regulate mTOR.
- Sporadic disease: The majority of primary pancreatic neuroendocrine tumors (PNETs) show reduced protein levels of either one or both of the two main inhibitors of the mTOR pathway, TSC2 and PTEN [2]. Allelic loss of PTEN at the level of the chromosome arm 10q is frequent, and somatic inactivating mutations affecting PTEN and TSC2 genes have been reported in nearly 10 % of PNETS [8–10]. Reduced PTEN expression may also be ascribed to the miR-21 overexpression, a noncoding microRNA regulating protein expression on a post-transcriptional level [11]. Oncogene mutations affecting mTOR pathway are rarely, if ever, observed [12, 13].
- A phase III clinical trial showing that the mTOR inhibitor everolimus gave a clinically meaningful benefit in treated patients.

Alterations of mTOR Pathway and Therapeutic Opportunities

The engagement of upstream RTKs by growth factors switches on PI3K signaling axis. **PI3K** is then recruited to plasma membrane-anchored receptors and activated; its activation status leads to phosphorylation of PIP2 to PIP3. **Akt**, through its pleckstrin homology (PH) domain, binds PIP3 activating **mTOR**, as part of the mTORC1 complex, by suppressing the suppressor **TSC 1/2 complex**. The two best-established substrates of mTORC1, S6K1 and 4EBP1, control various aspects of translation. **p-S6K1** leads to activation of eIF3 translation complex; substrates of p-S6K1 includes other translation-related proteins such as S6, eIFB4, eEF2K, PDCD4, CBP80, and SKAR. By contrast, phosphorylation of **4EBP1** by activated mTORC1 leads to a "loss of function" of its translation repressor physiological activity; 4EBP1 phosphorylation-mediated dissociation from eIF4E allows eIF4G and eIF4A to assemble with eIF4E, a complex known as eIF4F, and to initiate translation. PI3K/Akt/mTOR pathway is regulated by main proteins. PTEN seems to be one of the main negative regulators of this pathway with its phosphatase activity on both protein and lipid substrates. In particular, it antagonizes PI3K, taking a phosphate away from PIP3, thereby partially switching-off Akt activity [1].

PI3K

Jiao et al. [12] by sequencing the exome of nearly 18,000 protein-coding genes in a set of ten PNETs and with the validation in 58 additional ones found mutations along mTOR pathway in nearly 15 % of the tumors. **Mutations** in PI3KCA (p110α) was identified in 1.4 % of PNETs (1/68). This percentage faces with higher ones described in other histotypes (breast 27 %, endometrial 24 %, colon 15 %, etc.) [14, 15]. No p85α mutations are to date described in NETs contrary to other histotypes (8 % glioblastoma, 8 % colon cancer, 17 % pancreatic cancer, 2 % breast cancer). **PI3K amplification** was detected in 53 % of lung squamous cell carcinomas, 69 % of cervical tumors, and 32 % of head and neck squamous cell carcinomas. To date, no data relative to PI3K amplification are available in NETs.

Preclinical studies in NETs with first-generation **PI3K inhibitors** outlined the evidence that PI3K signaling plays a role in in vitro neuroendocrine cell growth. *LY294002* alone, a morpholine derivative of quercetin and a potent PI3K inhibitor, reduced tumor cell proliferation both in lung (NCI-H727) and in GI (BON) neuroendocrine tumor cell lines, together with a consensual decrease in pAkt levels [16]. *LY294002* treatment of murine endocrine cell lines synergize with rapamycin in inhibiting cell growth [17]. In other neuroendocrine tumor cell lines (BON, GOT-1, and NCI-H727), *BEZ235*, a dual PI3K and mTOR inhibitor, is similarly able to limit the triggering of MAPK cascade [18]. These data are in agreement

with the evidence that MAPK pathway activation occurs during mTOR inhibition through a PI3K-mediated feedback loop [19].

Neither clinical experience has so far been reported with pure **PI3K** inhibitors nor with dual PI3K/mTOR inhibitors in NETs.

Akt

Analysis of gene copy number shows the relation between **amplification** of Akt family members and cancer. Akt2 amplifications in particular were reported in 14, 20, and 30 % of ovarian, pancreas, and head–neck cancers, respectively [20, 21]. Akt1 gene amplification was detected in a single gastric carcinoma out of a series of more than 200 human malignancies [22]. No literature data are to date available concerning Akt amplification in NETs. A comprehensive screening of human malignancies for genetic **mutations** in the catalytic domain of nearly 240 Ser/Thr kinases did not reveal any mutations in Akt1, Akt2, and Akt3 exome sequences. A further analysis, instead, showed a unique mutation in the PHD of Akt1 (E17K) in 8, 6, and 2 % of breast, colorectal, and ovarian cancers, respectively [23, 24]. Genome-wide analysis of a set of ten PNETs did not reveal alteration in Akt-coding genes.

Activation of Akt is described in many human tumors; the phosphorylation rate of Akt ranges from 61–76 % in two different series including GEP-NETs [25]. Activated status was not in relation to grading, dimension, or stage of the disease.

Different kinds of **Akt inhibitors** have been described, and an increasing number of new molecules are under way. Among them: (a) *Phosphoinositides analogues* able to replace PIP3 at the Akt PH site, thereby preventing plasma membrane localization and phosphorylation of Akt; the perifosine belongs to this class of inhibitors, for which encouraging phase II data have been obtained in renal cell carcinoma, colorectal cancer, and multiple myeloma. Recently, the pan-Akt inhibitor, perifosine, shows very effective inhibitory activity on Akt phosphorylation and on NET tumor cells viability [26]. (b) *Substrate analogues* work as Akt inhibitors, but no clinical data are to date available with such inhibitors. (c) *ATP-competitive ligands* represent another class of new molecules. GDC-0068 is an highly selective pan-Akt inhibitor that paradoxically increases phosphorylation of Akt in cells while locking it in a nonfunctional state [27]. The preferential targeting of activated ATP-bound Akt by such an inhibitor can lead to an increase in the therapeutic index (i.e., drug more active against tumor cells with highly activated Akt rather than normal cells showing low Akt activity). An open-label phase Ib, dose-escalation study assessing safety, tolerability, and pharmacokinetics of GDC-0068 in combination with docetaxel or fluoropyrimidines in patients with advanced solid tumors is ongoing. (d) A small *pan-Akt inhibitor*, named triciribine, is able to inhibit the cell growth and increase apoptosis in human cancer cells that harbor constitutive activation of Akt due to overexpression of Akt or other genetic aberrations such as PTEN inactivation. In vitro experiences with triciribine on

NET cell lines (BON, CM, STC-1) showed that inhibition of Akt conferred a growth inhibitory effect together with a consensual reduction of pAkt levels in sensitive cell lines (STC-1 and CM). BON cells are resistant to *in vivo* effective doses of drug; lower basal level of pAkt and higher level of PTEN compared to sensitive cells are probably related with insensitivity to Akt inhibition [28]. (e) *Allosteric inhibitors* represent the last generation, isoenzyme-specific Akt inhibitors; the inhibitory properties result from a change in the shape of Akt active site after their binding to an allosteric Akt site. In NET cell lines, knockdown models blocking Akt isoforms 1 and 3 seemed to have the highest efficacy in lowering Akt phosphorylation and inhibiting cell tumor growth. According to these preclinical data, selective targeting of Akt-1 and/or Akt-3 in NETs seems to be a promising approach. In two carcinoid cell lines (i.e., pancreatic carcinoid BON and bronchopulmonary H727), the treatment with MK-2206, an allosteric inhibitor of Akt, was able to suppress AKT phosphorylation and significantly reduced cell proliferation in a dose-dependent manner. MK-2206 leads to an increase in the levels of cleaved PARP and cleaved caspase-3, with a concomitant reduction in the levels of Mcl-1 and XIAP, indicating that its antiproliferative effect probably occurs through the induction of apoptosis [29].

A first in human **clinical trial** with an allosteric Akt inhibitor (MK-2206), including, among other histotypes, three NETs, has been recently published. Two of these NETs bearing patients achieved tumor shrinkage of −13 and −17 % and both remained on trial for 32 weeks. Ras mutations and PTEN loss were described among partial responding patients with other histotypes. Recently, a new trial has just started with MK-2206 in PNET [30].

mTOR

In NETs, there is evidence that **mutations** and other genetic alterations can affect PI3K/Akt/mTOR pathway (i.e., PTEN and TSC2 loss/mutations, PI3KCA mutations) [12, 31].

Despite the importance of mTOR activation in human cancer, activating **mutations** in its coding gene were only recently reported. By mining cancer genome database, Sato et al. [32] identified ten mutations in the mTOR gene from 750 cancer samples. Among them, two different mutations (S2215Y and R2505P in colon and kidney cancers, respectively) are able to confer growth factors-independent mTORC1 activation. These mutations have not yet been reported to have a transforming activity, besides the "promoting" one, remains unclear [31, 32]. No data are now available in NETs with regard to mTOR genetic defects.

Phosphorylation status of "nodal" proteins, having many putative specific phosphorylation sites, cannot be investigated with an antibody specific to only one of them. mTOR in particular possesses four known phosphorylation sites (i.e., Ser^{2448}, Ser^{2481}, Thr^{2446}, and Ser^{1261}), each one having a cognate "phosphorylator" and a different biological significance. Phospho-mTOR (pmTOR) for example was

analyzed by Righi et al. [33] in a series of 218 surgically resected lung NETs using an antibody specific for Ser^{2448}, originally believed to be an "Akt-restricted" phosphorylation site but recently identified as "S6K1-cognate" one. In this series, mTOR **activation** was significantly higher in low-to-intermediate grade tumors as compared to high-grade ones, although no correlation with survival was showed. mTOR and pmTOR expressions were also detected, respectively, in 70 and 61 % of PNETs in a series of 34 patients described by Zhou et al. [34]. In a series reported by Kasajima et al. [35], mTOR positivity was also detected in 67 % of gastric and pancreatic NETs compared to 16 % of duodenal NETs.

In a preclinical setting, the reduction in tumor cell viability after the treatment with **mTOR inhibitors** supports the hypothesis of an important biological role for mTOR in tumor cell biology. There are to date two different classes of mTOR inhibitors:

(a) *Rapamycin analogues*, allosteric inhibitors of mTORC1 which, by forming a complex with the intracellular receptor FKBP12, bind to mTOR and inhibit mTORC1 downstream signaling. They are partial mTORC1 inhibitors and cell-type-specific mTORC2 inhibitors. Sirolimus, temsirolimus, everolimus, and deforolimus are members of this family. Everolimus treatment leads to NET **cell growth inhibition** in different experimental settings; RAD001 inhibited BON (a human PNET cell line) and INS1 (a rat insulinoma cell line) proliferation in nanomolar ranges [36, 37]. In 24 primary cultures from bronchial carcinoids, a different sensitivity to RAD001 treatment was observed; more aggressive histopathological features (i.e., higher proliferation index and nodal metastatic status) and higher expression of the molecular targets (i.e., mTOR-specific mRNA amount and basal phosphorylated and total mTOR levels) predict response to mTOR inhibition. In another study, PI3KCA and/or PTEN genetic defects, higher basal pAkt, greater inhibition of pS6K, and greater increase in pAkt during the treatment were hallmarks of mTOR inhibition [38].

(b) *Small molecules* mTOR kinase inhibitors. They can act only on mTOR, since they are ATP-competitive inhibitors (i.e., AZD8055 and WYE-354) or mTOR kinase inhibitors (i.e., PP30, PP242, and torin1), or they can be dual PI3K and mTOR inhibitors (i.e., primarily BEZ235 and XL765). As described below and in contrast to FHIT- or VHL-deficient kidney cancers or PTEN-deficient glioblastomas, everolimus has to date a limited clinical activity once tested in clinical trials in the absence of molecular and genetic stratification. This could be related to the inability to prevent mTORC2-mediated activation of Akt. The dual mTORC1/mTORC2 inhibitor CC-223 has recently showed ability to address mTORC2-mediated escape mechanisms; a phase I evaluation in advanced solid and hematologic cancers is ongoing. Also, the dual mTOR/PI3K inhibitor NVP-BEZ235 has proved to be more effective than single inhibitors in limiting NET cell lines growth [39].

In the clinical setting, **mTOR** inhibition led to encouraging results in an otherwise daunting scenario. In the first study of the "RADIANT saga" (RADIANT-1), everolimus was given alone or in combination with octreotide LAR if such a treatment was ongoing at baseline. Primary endpoint was response rate in the largest

stratum of everolimus monotherapy ($n = 115$ patients). A RR of 9.6 % was observed in the everolimus "stratum" as against 4.4 % in the everolimus + octreotide one. PFS in the stratum of SSA and everolimus is longer than the one of everolimus alone (PFS 16.7 vs. 9.7 months) [40]. In RADIANT-2 phase III study, the role of everolimus in association with octreotide LAR in patients with low-to-intermediate grade NETs was explored versus placebo. Median progression-free survival by central review was 16.4 months in the everolimus plus octreotide LAR group and 11.3 months in the placebo group [41]. RADIANT-3 study further explored the role of everolimus in the management of advanced PNETs randomizing patients versus placebo; pretreatment with chemotherapy was a stratification criteria and SSA treatment was allowed. The trial design allowed also the crossover at PD. A total of 5 % of patients had PR according to RECIST criteria in the everolimus arm, but a total of 64 % of patients receiving the drug experienced some degree of tumor shrinkage as compared to 21 % in the placebo arm. In addition to this, everolimus reduced tumor proliferation as shown by lowered Ki67 values on paired re-biopsies. But the most striking benefit following the treatment with everolimus is the lengthening of time to disease progression; central review PFS was 11.4 and 5.4 months for the everolimus and placebo arm, respectively, resulting in a reduction of the risk of progression for the experimental arm of nearly 65 %. No subgroup was disadvantaged; neither chemo-pretreated patients nor tumors with a moderately grade of differentiation [42].

TSC2 and PTEN

PTEN and the TSC complex are the major upstream-negative regulators of PI3K-dependent mTORC1 activation. A recent expression profiling of PNETs leads to evidences for a frequent activation of mTOR pathway in primitive disease and the alteration of TSC2 and PTEN protein expression in the vast majority of cases [2]. These observations were confirmed by the finding of mutations in TSC2 or PTEN in about 16 % of cases [12]. Interestingly, altered expression of either TSC2 or PTEN was found in tumors showing an aggressive clinical behavior. The authors commented that the deficiency of one of those genes could help in overcoming the impairment of mTOR activity due to the hypoxic condition in which these aggressive tumors growth. The presence of multiple alterations along the pathway may help to bypass this negative feedback, as suggested by the fact that tumors bearing reduced expressions of both PTEN and TSC2 are those that developed metastases and showed progression of disease. Furthermore, the results of a global microRNA expression analysis revealed overexpression of miR-21, which has PTEN among its targets, in NETs showing the highest proliferation indexes [11, 43].

The development of a molecularly target agent should be sustained by the identification of biomarkers predictive of efficacy to adequately select those patients more likely to benefit from the treatment and thereby optimizing the therapeutic index.

In this setting, the activation status and the molecular alterations of PI3K members (as well as those of downstream effectors or of molecules belonging to parallel and interacting pathways) have been evaluated both on cell lines and *in vivo* with sometimes discrepant results.

- **pAKT** predicts sensitivity to molecular inhibitors both in JFCR39 (a panel of 39 well-characterized cell lines) analyzed in silico and in other in vitro and *in vivo* models [44]. Moreover, pAKT levels positively correlated with sensitivity to everolimus in treated patients, both baseline and during drug administration. In the latter case, there was an evidence of compensatory activation of Akt as a consequence of mTOR inhibition [38].
- Predictive role of **PI3KCA** mutation and **PTEN** loss on breast [45] and neuroendocrine cell lines [38] was not confirmed in other settings [44].
- **KRAS** and **BRAF** mutations showed a negative predictive role for PI3K pathway inhibitors [44]. A single nucleotide polymorphism on the **FGFR** was found to have a negative prognostic and predictive role both in PNETs in preclinical models and patients [46].
- **c-MYC** and **4EIF** amplification were detected in human cells becoming resistant to BEZ235, a dual PI3KCA and mTOR inhibitor [47]. The role of c-MYC (and NOTCH) in PI3K inhibitors resistance was also confirmed in an analysis of breast cancer cell lines [48].

These fragmented evidences, derived from heterogenous preclinical models, are still too immature and limited to draw significant conclusions and to provide for a rationale to design clinical trials on molecularly selected patients.

mTOR-Interacting Pathways and Therapeutic Opportunities

mTOR pathway is part of a complex network. Thousands of molecular interplays occur: synergistic, additive, or (partially) redundant effects of the above-mentioned alterations, associated with positive or negative feedback loops, outline cancer real landscape. Nevertheless, most studies have focused on singular PI3K members and analyzed this signaling pathway as a vertical, one way, straightforward axis. NETs do not represent an exception. This approach does not mirror cancer cell biology and may have been responsible of the so far limited (and sometimes discouraging) results of target therapies in "PI3K-addicted" tumors, either in preclinical or, unavoidably, in clinical setting. In fact, each molecule and each pathway (PI3K included) are part of the complex and dynamic cancer signaling network. The understanding of the interactions between the different signaling intracellular processes is crucial to develop more effective therapeutic strategies.

Examples of such complex interactions in NETs are the following: