Role of the tumor microenvironment in digestive neuroendocrine tumors

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Abstract

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) represent a group of heterogeneous tumors whose incidence increased over the past few years. Around half of patients already present with metastatic disease at the initial diagnosis. Despite extensive efforts, cytotoxic and targeted therapies have provided only limited efficacy for patients with metastatic GEP-NETs, mainly due to the development of a certain state of resistance. One factor contributing to both the failure of systemic therapies and the emergence of an aggressive tumor phenotype may be the tumor microenvironment (TME), comprising dynamic and adaptative assortment of extracellular matrix components and non-neoplastic cells, which surround the tumor niche. Accumulating evidence shows that the TME can simultaneously support both tumor growth and metastasis and contribute to a certain state of resistance to treatment. In this review, we summarize the current knowledge of the TME of GEP-NETs and discuss the current therapeutic agents that target GEP-NETs and those that could be of interest in the (near) future.

Introduction

Human gastroenteropancreatic neuroendocrine tumors (GEP-NETs) represent a heterogeneous group of tumors emerging from cells producing glycopeptides and biogenic amines (Klöppel 2011). They account for approximately 0.5% of all human cancers (Modlin et al. 2008, Lawrence et al. 2011, Hallet et al. 2014, Dasari et al. 2017). Pancreatic neuroendocrine tumors (PNETs) represent around 10% of the neuroendocrine tumors (NETs) seen in the clinic, while the distributions of small intestine neuroendocrine tumors (SI-NETs) and rectal NETs differ between region of the world, genders and ethnicities (Fig. 1) (Hauso et al. 2008, Yao et al. 2008, Ito et al. 2010, Landerholm et al. 2010, Taghavi et al. 2013, Hallet et al. 2014).

The current treatment of GEP-NETs consists of a multimodal approach. Surgery remains, if feasible, the first therapeutic option that can result in a complete cure of the disease (Partelli et al. 2017). Besides surgery, locoregional treatment, chemo- and/or radiotherapy, as well as targeted therapies, represent alternative options, which are generally discussed in a case-by-case approach (O’Toole et al. 2016). The medical treatments are primarily represented by long-acting somatostatin analogs (SSAs, octreotide long-acting release (LAR) and lanreotide Autogel), mTOR (mammalian target of rapamycin) inhibitors and sunitinib malate, an inhibitor of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) receptors.
behavior, either via soluble factors released within the TME (Straussman et al. 2012) or due to the physical barrier formed by the tumor stroma, which can hamper drug delivery (Olive et al. 2009, Jacobetz et al. 2013). There are also several lines of evidence that the metastatic process can be the consequence of molecular interactions and dysregulation of mechano-reciprocity between the tumoral cells and their microenvironment (Butcher et al. 2009, Ungefroren et al. 2011).

Until now, the role of the TME in GEP-NETs is underexposed, even though it may constitute both a key driver of tumor growth and a critical modulator of the response to treatment. Identifying new therapeutic targets within the TME or establishing novel therapeutic strategies, such as the so-called co-targeting approach, is a key point to improve the management of such tumors.

**Angiogenesis: a common feature in the microenvironment of neuroendocrine tumors**

**Molecular basis of angiogenesis in GEP-NETs**

NETs are characterized by a high vascular supply owing to the histological organization of endocrine glands with fenestrated epithelium lining the blood vessels to facilitate hormone secretion and dumping into the bloodstream (Turner et al. 2003, Scoazec 2005).

In adult life, angiogenesis occurs through two fundamental mechanisms: the growth of new vessels from capillaries and the enlargement of pre-existing collateral vessels, also known as adaptive arteriogenesis (Risau 1997). In GEP-NETs, a permanent vascular supply is ensured through the formation of new blood vessels from existing ones by a multistep process involving multiple angiogenic factors that are simultaneously counterbalanced by antiangiogenic molecules (Fig. 2). Among proangiogenic factors, VEGF also referred to VEGF-A, is the best known and characterized (Ferrara 2004). Its action is mediated through binding to two highly related tyrosine kinase receptors, VEGFR-1 and VEGFR-2, which are predominantly restricted to endothelial cells where they promote angiogenesis once activated (Kanno et al. 2000), as demonstrated in vitro by a VEGF-induced formation of endothelial fenestrae (Esser et al. 1998). On the other hand, an effective inhibition of the VEGF signaling resulted in a striking decrease of vascular density (Inai et al. 2004), an effect rapidly reversed by discontinuation of VEGF inhibition (Mancuso et al. 2006).

In human GEP-NETs, the VEGF/VEGFR system is, generally speaking, overexpressed compared to the

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**Figure 1**

Schematic distribution of neuroendocrine tumors in the human body. Based on the compilation of data from Hauso et al. (2008), Yao et al. (2008), Ito et al. (2010), Landerholm et al. (2010), Taghavi et al. (2013), Hallet et al. (2014).

In spite of the substantial progress that has been made in the early diagnosis and the therapeutic management of GEP-NETs, about half of the patients already present metastasis, mainly in the liver, at the initial diagnosis or develop metastases during the follow-up (Hallet et al. 2014, Dasari et al. 2017). The limited in vivo efficacy of targeted therapies to inhibit tumor progression or to induce tumor stabilization strikingly contrasts with their efficacy in vitro in experimental models (Kulke 2007). Such a discrepancy may, at least partially, arise from influences exerted by the direct microenvironment surrounding the tumor, referred to as the tumor microenvironment (TME) (Ungefroren et al. 2011).

The TME is a complex nebula comprising multiple cell types, supportive matrix and soluble factors, which promotes the tumor growth and influence tumor behavior (Hanahan & Weinberg 2011). Whereas it appears to be highly specific from one tumor type to another, recent studies underlie the critical role played by the TME in the induction of a tumor ‘resistance to treatment’
normal tissue (Turner et al. 2003). In contrast to the normal pancreas, where expression of VEGF is limited to particular endocrine cells, such as the gastrin-secreting cells or the pancreatic polypeptide-secreting cells in the islets of Langerhans (Kuroda et al. 1995), a marked expression of VEGF has been observed by immunohistochemistry in human GEP-NETs (Itakura et al. 2000, La Rosa et al. 2003). Around 50–80% of PNETs are immunopositive for VEGF with a strong immunoreactivity observed in the tumoral cell compartment (Kuroda et al. 1995, Terris et al. 1998, La Rosa et al. 2003, Angelescu et al. 2013). The corresponding receptors, VEGFR-1 and -2, are detected in both the tumoral compartment and the surrounding endothelial cells, which suggest an autocrine/paracrine action of VEGF such as the one described in human pancreatic adenocarcinomas (von Marschall et al. 2000).

At the biological level, higher plasma concentrations of circulating VEGF have been found in patients with metastatic GEP-NETs compared to their non-metastatic counterparts (Cigrovski Berković et al. 2016); however, this does not mean per se that VEGF is the inescapable factor that permits the tumor cells to metastasize. Animal studies conducted in the PNET-predisposing RipTag2 mouse model, albeit far from human observations, also showed that upregulation of VEGF was a critical step in the development of the tumor (Folkman et al. 1989, Christofori et al. 1995, Bergers et al. 1999, Inoue et al. 2002).

Other factors are indirectly involved in the process of angiogenesis and its maintenance. Among them, angiopoietin-2 (ANG-2) which binds the endothelial-specific receptor tyrosine kinase 2 (TIE2) and acts either as a negative regulator of ANG-1/TIE2 signaling during angiogenesis (Eklund & Saharinen 2013) or, under certain conditions, as promoter of angiogenesis (Felcht et al. 2012). In human PNETs, an eight-fold upregulation

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**Figure 2**

Schematic representation of angiogenesis in GEP-NETs and the main molecular factors that regulate it, as described in this review.
of ANG-2 detected by microarray expression profiling, as compared to the non-tumoral pancreatic tissue, was observed (Durkin et al. 2004). Moreover, a tumor-specific immunopositivity (i.e. present in tumor and absent in surrounding normal pancreas) for ANG-2 is observed in a majority of PNETs (Durkin et al. 2004, Detjen et al. 2010). In contrast, ANG-1 and TIE2 did not show differential expression between tumor and normal pancreas (Durkin et al. 2004). These observations suggest that the ANG-2 system activation may arise during the tumorigenesis process. Experimental data using ANG-2-overexpressing human PNET cell lines BON-1 (by transfection) showed a significant development of neoangiogenesis in orthotopic NET xenografts, compared to WT BON-1 xenografts (Detjen et al. 2010). Although not used routinely as a biomarker of disease activity or aggressivity, plasma concentrations of ANG-2 are significantly higher in patients with GEP-NETS, compared to healthy controls, and even further elevated in patients with metastatic disease, compared to patients without metastasis (Srirajaskanthan et al. 2009, Detjen et al. 2010, Figueroa-Vega et al. 2010).

Endostatin is an endogenous peptide that inhibits the migration and proliferation of vascular endothelial cells, and recombinant human endostatin showed a significant therapeutic impact in metastatic melanomas at the clinical level (Cui et al. 2013). However, no significant tumor regression was observed following treatment with recombinant human endostatin in patients with advanced GEP-NETS (Kulke et al. 2006).

In summary, angiogenesis appears as an essential mechanism for the NET growth and maintenance, especially in well-differentiated and low-grade tumors where markers of angiogenesis are overwhelmingly found as compared to high-grade and/or undifferentiated forms of GEP-NETS, a condition known to define the ‘neuroendocrine paradox’ (in contrast with other models of epithelial tumors in which the highest degree of vascularization generally reflects a more aggressive tumor) (Poncet et al. 2009, Scoazec 2013, Yazdani et al. 2014). In well-differentiated NETs, it is still unknown whether marked angiogenesis increases the risk of distant metastasis. Another source of uncertainty related to angiogenesis concerns the adaptive mechanisms that occur during the antiangiogenic therapy regimen of certain patients with GEP-NETS, responsible for an escape and, ultimately, a resistance to this treatment. In the following section, we will discuss the concept of resistance to antiangiogenic therapy with regard to tumor microenvironment.

### Tumor microenvironment and resistance to antiangiogenic therapies in GEP-NETS

In the setting of GEP-NETS, VEGF pathway inhibitors have shown only temporary beneficial effects at best, generally followed by a restoration of tumor growth and progression (Shojaei & Ferrara 2008) whose mechanisms have been described on the basis of preclinical studies although not validated clinically (Bergers & Hanahan 2008):

- A pre-existing state of resistance, also called intrinsic resistance, observed in a minority of patients with PNETs and treated with the VEGFR inhibitor, sunitinib. These patients failed to show any transient clinical benefit from the treatment, namely no tumor shrinkage, no cessation of tumor growth (stasis) and not even retardation in the growth rate (Raymond et al. 2011). In human GEP-NETS, the involvement of the TME in the intrinsic resistance to treatment possibly occurs by the local production of other-than-VEGF alternative proangiogenic signals overcoming the VEGF pathway inhibition and maintaining the angiogenic process in the tumor niche (Bergers & Hanahan 2008) (see below).

- The second described mode of resistance to antiangiogenic therapies referred to as acquired resistance, which means that the tumor progressively became resistant to the treatment after an initial phase of sensitivity. Acquired resistance in GEP-NETS results from the activation of alternative ways that sustain tumor growth whereas the specific therapeutic target of the antiangiogenic drug remains inhibited (Bergers & Hanahan 2008, Tijeras-Raballand et al. 2012). In GEP-NETS, one well-described mechanism of acquired resistance is the hypoxia state the tumor undertakes, directly generated by the effect of antiangiogenic therapy (Allen et al. 2011, Yao & Phan 2011). By entering within a hypoxic state, the tumor cells released high amounts of hypoxia-inducible factor 1-alpha (HIF-1A), the master transcriptional regulator of cellular response to hypoxia (Semenza 1998). In turn, HIF-1A induces or upregulates alternate proangiogenic growth factors (Tijeras-Raballand et al. 2012). Apart from the synthesis of hypoxia-induced proangiogenic factors, other mechanisms contribute to the acquired resistance to antiangiogenic therapies like the increase in pericyte coverage; however, their role in the field of GEP-NETS remains to be clarified (see review: Bergers & Hanahan 2008).
Interactions between tumor and stroma in GEP-NETs

Secretion of growth factors and cytokines within the TME: putative role and function

Tumor cells and cells that are part of the TME, including vascular cells, stromal fibroblasts and inflammatory cells, are constantly exposed to various growth factors and cytokines that modulate tumor growth, invasiveness and metastasis (Hanahan & Weinberg 2011). These growth factors and cytokines mostly act in a paracrine fashion on tumor cells to stimulate their proliferation, sensitivity to treatment, chemoresistance, motility, invasion, etc. Given the numerous factors that have been reported so far in the TME of GEP-NETs, we focused on those that may have a significant impact, and for which several studies have been conducted with relevant conclusions (Fig. 3).

Platelet-derived growth factor: a pivotal factor in the TME of GEP-NETs

PDGF is an ubiquitous growth factor released by many cell types (including platelets) whose expression has been observed in about 70% of human GEP-NETs (Chaudhry et al. 1992, 1993). It binds two types of receptor tyrosine kinases (RTK), PDGFR-α and PDGFR-β (Fredriksson et al. 2004), the latter being expressed by a majority of SI-NETs and PNETs and even further in case of liver or lymph node metastasis as compared to corresponding primary tumor sites (Funa et al. 1990, Fjällskog et al. 2007). Within the tumoral niche, this expression can be either exclusively limited to stromal cells (especially endothelial cells and pericytes) or sometimes observed in both tumoral and stromal cells (Funa et al. 1990, Welin et al. 2006, Fjällskog et al. 2007). Moreover, a decreasing level of PDGFR-β expression is noted from the tumor to its periphery such that the closer the stromal cells are from the tumor, the higher PDGFR-β expression they display (Funa et al. 1990). Interestingly, this expression was closely correlated with the density of capillary blood vessels, in line with the proangiogenic activities of PDGF isoforms as previously described (Risau et al. 1992, Cao et al. 2002, Cao 2013).

The second type of PDGF receptor, PDGFR-α, is also expressed in clusters of tumor cells and occasionally on adjacent stroma of human GEP-NETs (Chaudhry et al. 1993).

In summary, the PDGF/PDGFR system is widely expressed in GEP-NETs, with a higher expression level close to the tumor epicenter. These data suggest that PDGF likely acts locally in a paracrine and/or autocrine manner, an assumption which is supported by its rapid clearance from the plasma with an half-life of less than 2 min (Fredriksson et al. 2004). Whether PDGF expression enters and participates to the tumorigenesis process remains elusive since no correlation has been properly established yet between the tumoral status and its degree of differentiation on the one hand, and the local expression of PDGF/PDGFR on the other hand (Fjällskog et al. 2003).

Transforming growth factors alpha, beta and epidermal growth factor

TGFα is a growth factor characterized by its close analogy to EGF and therefore its capability to bind the EGF receptor (EGFR). A majority of NETs co-express TGFα and EGFR (Nilsson et al. 1995, Krishnamurthy & Dayal 1997, Srivastava et al. 2001), a condition which is believed to confer growth advantage to tumor cells (Wong et al. 1989). In vitro, a significant amount of TGFα was detected in the medium of primary cultures of SI-NETs and its secretion dramatically decreased by the addition of octreotide to the cells (Nilsson et al. 1995). Noteworthy, the addition of TGFα to these primary cultures of SI-NETs resulted in tumor growth stimulation (Nilsson et al. 1995), which confers to the TGFα, proliferative properties in human SI-NETs cells that can be counteracted via somatostatin receptors activation. At the receptor level, EGFR mRNA was almost exclusively detected in cases of gastrinomas and sparsely in other types of GEP-NETs (Wulbrand et al. 1998).

TGFβ1 is another cytokine, which binds TGFβR-1 and TGFβR-2, and paradoxically serves as a growth inhibitor at the beginning of tumor development but later becomes a growth accelerator for transformed tumors (Blobe et al. 2000, Roberts & Wakefield 2003). In human GEP-NETs, both the tumor and the surrounding mesenchyme display a strong expression of TGFβ, whereas TGFβR-1 and -2 expression was exclusively observed on the tumoral cells (Chaudhry et al. 1994, Wulbrand et al. 1998, Wimmel et al. 2003). This peculiar pattern of expression suggests that a local TGFβ loop of activation initiates from the tumor and the stroma to serve the tumor compartment. Thus, in BON-1 and QGP-1 human PNET cell lines, the addition of TGFβ1 (10ng/mL) resulted in a significant and time-dependent inhibition of BON-1 proliferation while no effect was observed in QGP-1 cells (Wimmel et al. 2003, Leu et al. 2008). The mechanism underlying the negative effect of TGFβ on BON-1 proliferation may involve an upregulation of both the expression of p21WAF1/CIP1 (a cyclin-dependent kinase inhibitor) (Pickup et al. 2013) and the secretion of somatostatin by
BON-1 cells, a negative regulator of proliferation (Leu et al. 2008). On the other hand, TGFβ1 demonstrated proliferative action and an enhancement of the migration capability in the KRJ-I cells, a human-derived SI-NET cell line, due to loss of E-cadherin expression (Kidd et al. 2007a).

Summarizing, a limited number of studies has focused on the role of the TGFβ system in human GEP-NETs and the majority of these studies were observational, depicting whether this cytokine is expressed or not in the tumoral as well as in peritumoral tissues. The interest which is currently brought to the development of TGFβ inhibitors is based on the pleiotropic effect of this cytokine known to regulate, beyond its direct effect on tumor proliferation, immune cell function in normal and tumor-associated lymphocytes and tumor-associated fibrosis. As such, TGFβ inhibitors could represent an ‘ideal’ drug for treating simultaneously tumor and TME in GEP-NETs (Herbertz et al. 2015, de Gramont et al. 2017).

Figure 3
Schematic overview of the tumor microenvironment in digestive neuroendocrine tumors. Putative role and functions of the different components of the tumoral microenvironment, as described in this review.
**Placenta growth factor and the neuropilin system**

Placenta growth factor (PIGF) is a VEGF-homolog angiogenic growth factor, which supports pathological angiogenesis by binding to VEGFR-1 (also known as Flt1), neuropilin-1 (NRP-1) and -2 (NRP-2) (Parr et al. 2005, Wei et al. 2005, Fischer et al. 2008). Circulating plasma levels of PIGF are higher in patients with either PNETs or SI-NETs compared to controls (Hilfenhaus et al. 2013). In vitro, PIGF demonstrated capability to stimulate both proliferation and migration of BON-1, QGP-1 and KRJ-I. In human PNET samples, PIGF protein is almost exclusively detected within the TME (i.e. stromal cells such as endothelial and inflammatory cells) and occasionally in the tumoral compartment. In contrast, no staining is observed in both the normal endocrine and exocrine pancreatic tissues, suggesting that PIGF is de novo expressed during the process of tumorigenesis (Hilfenhaus et al. 2013).

Neuropilins, which bind PIGF, also act as co-receptors for the semaphorins, a class of proteins involved in the process of axon guidance during the nervous system maturation and in regulation of cell migration (Prud’homme & Glinka 2012). More specifically, NRP-1 and NRP-2 are co-receptors for class-3 semaphorins (SEMA3), a potent angiogenesis inhibitor (Neufeld et al. 2005). A recent study showed that expression of semaphorin 3F (SEMA3F) is almost undetectable in a series of human SI-NETs (primitive site and metastasis), while conserved in the normal neuroendocrine tissue (Bollard et al. 2015). The authors further showed that upregulation of SEMA3F in the enteroendocrine cell lines STC-1 and GluTag led to a reduced ability of the cell line to form tumors and liver metastasis in a xenograft model of SI-NET liver metastasis (Bollard et al. 2015).

**Connective tissue growth factor**

Connective tissue growth factor (CTGF/CCN2) is a 349 amino acid, which promotes fibroblast proliferation, migration, adhesion and extracellular matrix (ECM) formation (Moussad & Brigstock 2000). The latter is illustrated by the development of fibrosis when CTGF is overproduced under the regulation of TGFβ (Grotendorst 1997). For instance, highest plasma concentrations and tumor expression of the full-length CTGF protein was observed in patients with NETs and clinically documentable fibrosis (Kidd et al. 2007b). Similarly, Bergestuen et al. reported an important elevation of plasma CTGF levels in patients with NETs and right unresectable dysfunction by valvular regurgitation, one known complication of serotonin-induced fibrosis (Bergestuen et al. 2010). At the tumoral level, CTGF is highly overexpressed in human SI-NETs compared to other types of NETs, with an especially pronounced immunoreactivity in the tumor cells adjacent to the surrounding fibrovascular stroma (Cunningham et al. 2010, Kaltsas et al. 2011). In vitro, CTGF demonstrated proliferative properties in KRJ-I, but had no effect on BON-1 (Siddique et al. 2009).

**Basic fibroblast growth factor**

Basic fibroblast growth factor (bFGF) belongs to a 22-member family of factors that bind to the fibroblast growth factor receptor (FGFR) and trigger carcinogenesis signaling pathways (Touat et al. 2015).

In human GEP-NETs, a strong immunostaining for bFGF was observed in the stromal component of tumor samples, especially in the macrophage subpopulation of cells (Chaudhry et al. 1993). The FGFR expression was also prominent in the stroma part of the tumor and almost undetectable in the tumoral cell compartment (Wulbrand et al. 1998). This particular pattern of expression could suppose that the FGF/FGFR system may be primarily active in an autocrine loop within the stroma to itself, rather than between the tumor and its stroma. Whether FGF acts as a promoter of fibrosis in the TME of GEP-NETs is unclarified yet. Comparable plasma levels of FGF2 expression were found in patients with SI-NETs as compared to their matched controls and, in patients with SI-NETs, whatever the existence of an extensive mesenteric sclerosis (Zhang et al. 2004, Pavel et al. 2005). To date, there are no phase III clinical trials with selective targeted agents against FGFR, which have been conducted and/or approved. A phase II clinical trial using Nindetanib, a small-molecule tyrosine kinase inhibitor targeting vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR), is currently ongoing in patients with well- or moderately differentiated, unresectable, and/or metastatic, extrapancreatic NETs (ClinicalTrials.gov Identifier: NCT02399215).

**Insulin-like growth factors**

Several lines of evidence have been accumulated that both insulin-like growth factors, IGF-1 and IGF-2, and their receptor, IGF1R, are involved in the development and progression of cancer, including GEP-NETs in which a constitutive expression of IGFs and IGF receptors has been shown (Wulbrand et al. 2000, Kaltsas et al. 2011, van Adrichem et al. 2013). In gastrinomas, an increased expression of both IGF-1/IGF1R by the tumor was...
associated with a worse outcome in terms of curability and metastatic presentation (Furukawa et al. 2005).

In BON-1, experimental data showed that IGF-1 acts as an autocrine regulator of chromogranin A secretion and cellular growth (von Wichert et al. 2000, 2005, Mergler et al. 2005) and its effect could be reversed by the use of the IGF1R inhibitor, NVP-AEW541 (Höpfner et al. 2006). Our group recently showed that dopamine receptor subtype 2 agonists and the multiple somatostatin receptor analog, pasireotide, blocked the release of IGF-2 by BON-1 which suggests, in this model, an interaction between the IGF/IGF1R system and signaling pathways downstream dopamine and somatostatin receptors (van Adrichem et al. 2016). Monoclonal antibodies that target the IGF1/IGF1R system recently entered clinical trials in patients with GEP-NETs (see below).

### Chromogranin A: equivocal function in the TME of GEP-NETs

Chromogranin A (CGA) and its fragments vasostatin-1 (VS-1) and -2 (VS-2) are specific markers of neuroendocrine cells that have previously demonstrated proliferative effects in NET cells (Giovinazzo et al. 2013). They could further influence the synthesis of TME components, as well as the vascularization dynamic phenomenon (Corti 2010). For instance, VS-1 exerts antiangiogenic (anti-VEGF) effects in human umbilical vein endothelial cells and in mice models while CGA acts as a preventive factor of vascular leakage induced by tumor necrosis factor α (TNFα) (Ferrero et al. 2004). In addition, CGA seems to endorse inhibitory functions over fibroblast adhesion in the TME, as suggested in non-invasive breast carcinomas where the number of CGA-immunoreactive neuroendocrine cells was shown to be higher in comparison to invasive breast carcinomas (Kimura et al. 2002).

In experimental models of GEP-NETs, Giovinazzo et al. showed that (i) CGA mRNA and protein levels were both increased in SI-NETs compared to normal enterochromaffin cells, (ii) silencing of CGA, as well as prohormone convertase (which cleaves CGA to VS-1), decreased the proliferation of SI-NET cell lines (KRJ-I and STS cell lines) and eventually, (iii) SI-NET cell line (H-STS) proliferation was stimulated by VS-1 (Giovinazzo et al. 2013).

### The ECM of GEP-NETs: the structural support of the TME

**Serotonin and fibrosis in the ECM of SI-NETs**

Fibrosis that occurs either local to or distant from the unresectable tumor is one of the hallmarks of SI-NETs (Druce et al. 2009). Fibrosis is likely to occur in case of serotonin oversecretion (also known as 5 hydroxytryptophan (5-HT)), a biogenic amine whose synthesis depends on the tryptophan hydroxylase enzyme. When secreted in excess, patients can present with a plethora of vasoactive symptoms (e.g. diarrhea, flushing), together known under the name of carcinoid syndrome (Fig. 4). In vitro, serotonin demonstrated mitogenic properties in numerous cell types (Nemecek et al. 1986, Seuwen et al. 1988), including KRJ-I, in which it also triggers, by binding the 5-HT$_{2B}$ serotonin receptor, the release of serotonin by the cells, in an autocrine manner. (Drozdov et al. 2009). In parallel, serotonin induced both proliferation and synthesis of profibrotic factors, namely CTGF, FGF2 and TGFβ1 in the HEK293 cell line, a cell line assumed by the authors of this study to be a fibroblast-like cell (Svejda et al. 2010). Unlike KRJ-I, in this cell type, the effect of serotonin was mediated by the 5-HT$_{2A/C}$ serotonin receptor. Subsequently, the authors demonstrated that, using a specific inhibitor of 5-HT$_{2B}$ in a coculture experiment of KRJ-I and HEK-293 (where exchanges between the two cell types were permitted only through a semi-permeable membrane) an inhibition of both proliferation of HEK293 cells and synthesis of profibrotic factors by the cells occurred, underlying a molecular crosstalk between the two different cell types (Svejda et al. 2010). Such an experiment mimicks what is likely to happen in situ between the NET cells and the fibroblast component of the TME; however, to date, to the best of our knowledge, there are no studies on the functional role of serotonin in the TME of human GEP-NETs.

In summary, the understanding of the 5-HT/5-HT receptor system in the field of SI-NETs is still based on results obtained in cell lines which, as previously mentioned, have limitations, and care should be taken to extrapolate these data to human NETs. Currently, research concerning serotonin in the setting of human GEP-NET mainly focuses on the control of high serotonin plasma levels rather than to elucidate its specific action within the TME. In this context, the new tryptophan hydroxylase inhibitor, telotristat ethyl, showed promising results in terms of control of carcinoid symptoms and reduction of serotonin metabolite, namely urinary 5-hydroxyindole acetic acid (u5-HIAA) particularly in patients with carcinoid syndrome refractory to usual somatostatin analogs (Kulke et al. 2014, 2017). The impact of this new agent on the fibrotic matrix surrounding the tumor remains unknown.
The remodeling of ECM in GEP-NETs: heparanases and matrix metalloproteinase enzymes represent potential therapeutic targets

ECM remodeling is a critical process during multiple stages of tumor development and facilitates cancer cell proliferation, angiogenesis, invasion and ultimately metastasis. This process involves a plethora of factors among which the heparan sulfate proteoglycans (HSPGs) that consist of a protein core with covalently attached heparan sulfate (HS) side chains. In addition to provide structural integrity, HSPGs act as a storage depot for a variety of proteins that bind HS, including growth factors, angiogenic proteins and chemokines (Vlodavsky et al. 2012). In human GEP-NETs, a differential regulation of a subgroup of HSPGs (i.e. syndecan 2, glypican 1 and 5), which depends on the tumor differentiation and its grading was shown (García-Suárez et al. 2014). For instance, syndecan 2 acts as a cell surface receptor (or ‘docking’ receptor), able to interact with many extracellular molecules within the TME. Its downregulation in high-grade compared to low-grade NETs may be one of the reasons that explain a high capability of tumor spread and metastasis in high-grade cases (García-Suárez et al. 2014). Likewise, the chondroitin sulfate proteoglycans (CSPGs), which are also able to interact with molecular and protein partners within the TME, are highly expressed in the tumor stroma compared to the normal stroma, which suggests (i) a certain degree of induction during the tumorigenesis and (ii) that they might serve in this situation as docking proteins to promote local growth of the tumor by the release of various growth factors and/or cytokines (García-Suárez et al. 2014). The release of growth factor by the processing of HSPGs is performed exclusively by enzymes called heparanase whose activity can therefore lead to both physical remodeling of the ECM and the conversion of tethered growth factors to soluble bioactive mediators once released (Vlodavsky et al. 2007). Heparanase is upregulated during multistage tumorigenesis in the RipTag2 mice (Joyce et al. 2005) and more recently, a significant correlation between high

Figure 4
Carcinoid syndrome. (A) Biosynthesis of serotonin and renal excretion of SHIAA. (B) Abdominal CT scan showing an ileal neuroendocrine tumor revealed by a fibrotic retractile mesenteritis (arrow). (C) Flushing occurring in a patient with carcinoid syndrome. *Vesicular monoamine transporter. Telotristat etiprate is a new tryptophan hydroxylase inhibitor. Written consent to publish was obtained from the patient.
heparyanase expression/staining and a more advanced tumor stage, a higher tumor grade and the presence of distant metastasis in patients with PNETs was reported (Hunter et al. 2014). In RipTag2 mice, the overexpression of heparanase by tumor-associated macrophages (TAMs) is associated with a higher incidence of invasive tumors (micro- and macro-invasive), compared to either the WT mice or the heparanase knock-out RipTag2 mice (Hunter et al. 2014). Interestingly, the latter model was further marked by an increase in angiogenesis, pericyte coverage and a wider vascular network, which is more generally associated with low-grade tumors in human GEP-NETs as discussed earlier.

Matrix metalloproteinases (MMPs) are proteinases that also contribute both to the degradation, as well as the modeling of the ECM (Kessenbrock et al. 2010). Additionally, in the RipTag2 mice, MMPs, especially MMP9, seem to be a critical factor that promotes the angiogenic switch of the PNET carcinogenesis (Shchors et al. 2013). When absent or deleted, MMP9-deficient mice exhibited more aggressive/invasive PNETs, suggesting, once again, that angiogenesis is a feature of ‘low-grade’ NET and blocking angiogenesis may promote in the end the emergence of more invasive tumors, presumably by reactivating other signaling pathways such as the ones involved in hypoxia metabolism.

The cellular actors of the TME: immune cells and fibroblasts

**Immune cells** The immune response to tumors has been extensively described and analyzed for many years and forms the basis for the development of immunotherapy (Blattman & Greenberg 2004).

In human GEP-NETs, several tumor-associated antigens (TAA), which are cancer-specific surface molecules generally overexpressed and recognized by the immune system, have been identified, like for instance chromogranin A- or VMAT1 (vesicular monoamine transporter 1)-derived TAA (Wuttke et al. 2009). In addition, NETs were shown to have an infiltration of lymphocytes CD3+/CD4+, CD3+/CD8+ - immunopositive cells (Vikman et al. 2009, Katz et al. 2010). In SI-NETs, this infiltrate was accompanied by an important infiltration of T regulator lymphocytes (Tregs) that fight against recruitment of anti-tumor effector T cells within the tumor niche with a more important infiltrate observed in metastasis as compared to the primitive tumor site (Katz et al. 2010). Whether this T cell infiltrate is ‘immunologically’ efficient remains difficult to establish. In addition to an infiltration of Treg lymphocytes, a loss of expression of HLA class I components (e.g. the β2-microglobuline), necessary for epitope presentation to CTLs, was identified in 10/11 PNET samples, suggesting that despite the presence of both TAA and CTLs, the tumor-specific immune response can be hampered through a different mechanism (Ryschich et al. 2003).

Besides a direct modulation of the cytotoxic response, others aspects potentially contributing to a non-proper immune response within the tumor niche have been described such as a reduction of systemic Th-1 promoting cytokines, like IL-1β (Vikman et al. 2009) or the local release of soluble factors by the tumor cells that can completely block the maturation and function of dendritic cells (Katsenelson et al. 2001).

Besides the lymphocyte population, the role of TAMs in tumor initiation and progression is complex, with variable pro- and anti-tumor-promoting effects in different types of cancers (Lewis & Pollard 2006). In Rip-Tag2 mice, a decrease in TAMs infiltrates is associated with less PNETs foci (Pyonteck et al. 2012). In 27 human PNETs tissue sample, a higher infiltration of TAM, assessed by CD68 immunostaining, correlated with a higher tumor grade and stage and more frequent liver metastases (Pyonteck et al. 2012). Mast cells also raised interest in the field of tumor-associated immune cells. Their importance in the tumor expansion of islet cell tumors is underlined by Soucek et al. who showed that the recruitment of mast cells was required for tumor expansion and, reciprocally, that inhibition of mast cell function led to hypoxia and both tumor and endothelial cell death (Soucek et al. 2007, Theoharides 2008). Of interest, the same group showed that treatment of insulinoma-bearing mice with a novel inhibitor of Bruton tyrosine kinase (Btk) that blocks mast cell degranulation (PCI-32765), resulted in tumor vasculature collapse and tumor regression (Soucek et al. 2011).

Over the past few years, the program death 1 (PD-1) and its ligands PD-L1/PD-L2 pathway progressively became one of the most interesting immune check points for the development of immune therapies in solid tumors, including GEP-NETs (Chen & Han 2015). The PD-1 pathway is a negative immune checkpoint that anergizes the Th1 cytotoxic immune response and, therefore, counteracts the cytotoxic action of T lymphocytes against tumoral cells (Fig. 5). Its upregulation is one key immunosuppressive mechanism by which cancer avoids its eradication by the immune system. Blockade of this pathway with antibodies to PD-1 or its ligands (pembrozilumab, nivolumab, atezolizumab) has led to remarkable clinical responses in patients with many
different types of cancer (Ferris et al. 2016, Reck et al. 2016, Bellmunt et al. 2017). In human GEP-NETs, the expression of PD-L1 was observed only in a subset of patients (22%), irrespective of the primary tumor site (Kim et al. 2016). However, its expression was significantly higher in grade 3 tumors according to the 2010 WHO classification. Interestingly, a significant expression of PD-L2 (the second ligand for PD-1) was observed in both PNET and SI-NET (da Silva et al. Poster B3, NANETS 2016). These encouraging data could pave the way for the use of current immune checkpoint inhibitors in GEP-NETs (Ghiotto et al. 2010). There are currently several clinical trials testing the PD-1 antibody pembrolizumab (ClinicalTrials.gov Identifier: NCT02939651, NCT03290079, NCT03012620, NCT03043664) and nivolumab (ClinicalTrials.gov Identifier: NCT03420521) in different stages of GEP-NETs (mostly high-grade tumors).

**Fibroblasts** Fibroblasts are an integrated component of the TME and emerge progressively as a cornerstone actor of the stroma by ensuring the production of growth factors, chemokines, ECM and by facilitating the angiogenic recruitment of endothelial cells and pericytes. Via a modification of their phenotype, the ‘activated’ fibroblasts, referred to as cancer-associated fibroblasts (CAFs), play an important role in the malignant progression of the cancer and represent an important target for cancer therapies (see review: Kalluri & Zeisberg 2006). While the implication of CAF in the TME of ductal pancreatic cancer has been extensively studied and described (Feig et al. 2012), much less is known about their role in the field of GEP-NETs.

In the early 90s, Beauchamp et al. observed that conditioned medium of the BON-1 cell line stimulates DNA synthesis and the fibroblast colony growth (Beauchamp et al. 1991). In this study, the BON-induced proliferation of fibroblasts was related to the release of TGFβ, a cytokine known to considerably influence the functionality of the fibroblast within the TME in a multimodal way (Pickup et al. 2013). We similarly identified through conditioned medium experiments that both BON-1 and QGP-1 were able to stimulate the proliferation of human fibroblast cell lines and vice versa (personal unpublished data). Through a proteomic approach, Bowden et al. screened in vitro a panel of 55 proteins with known carcinogenic associations and potentially secreted by CAFs in NETs. Five out of 55 proteins were identified as being significantly up or downregulated, compared to the supernatant of cultured normal human fibroblasts, including an elevated secretion of IL-6, MCP-1 and VEGF and suppressed levels of IL-8 and bFGF (Bowden et al. 2014). Of note, both fibroblast-mediated secretion of IL-6 and VEGF were reported to be notably increased under TGFβ actions in the neuroendocrine TME (Pickup et al. 2013).

In summary, several lines of evidence strongly support the existence of interactions within the TME of GEP-NETs. In the past years, studies have focused in particular on the study of growth factors and their respective receptors, because they were known to be involved in the pathogenesis of other tumor models. These multiple and diverse targets within the TME currently constitute the rationale for the use of pharmacological targeted therapies and the rationale for the development of future pharmacological agents.
Table 1 Summary of past and current clinical trials targeting components of the TME in GEP-NETs.

<table>
<thead>
<tr>
<th>Targeted therapies</th>
<th>Pharmacological target(s)</th>
<th>Study (phase)</th>
<th>Intervention/comparator</th>
<th>Trial identifier</th>
<th>Population</th>
<th>N</th>
<th>Primary endpoint(s)</th>
<th>Status</th>
<th>Result</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Angiogenic agents</td>
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<tr>
<td>Axitinib</td>
<td>VEGFR-1</td>
<td>II</td>
<td>Axitinib 5 mg × 2/day</td>
<td>NCT01435122</td>
<td>Unresectable/metastatic extrapancreatic NET (G1/G2)</td>
<td>30</td>
<td>PFS</td>
<td>Completed</td>
<td>mPFS = 26.7 months. 12-month PFS rate = 74.5%</td>
<td>Strosberg et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>VEGFR-2</td>
<td>II</td>
<td>Axitinib 5 mg × 2/day + OCT LAR 30 mg/28 day vs Placebo + OCT LAR 30 mg/28 day</td>
<td>NCT01744249</td>
<td>Unresectable/metastatic extrapancreatic NET (G1/G2) + PD</td>
<td>80</td>
<td>PFS</td>
<td>Ongoing</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

| Pazopanib         | VEGFR-1                   | II            | Pazopanib 800 mg/day   | NCT00454363      | Locally unresectable or metastatic CT or PNETs (G1/G2) | 52 | ORR | Completed | ORR: 21.7% (PNETs) No response in CT | Phan et al. (2015) |
|                    | VEGFR-2                   | II            | Pazopanib 800 mg/day   | NCT01280201      | Locally unresectable/ metastatic CT or PNETs (G1/G2) + PD | 44 | Clinical benefit rate (CBR) (CR + PR + SD) | Completed | CBR: 59.5% (4 PR, 21 SD) | Grande et al. (2015) |
|                    | VEGFR-3                   | II            | Pazopanib 800 mg/day   | NCT01099540      | Locally unresectable/ metastatic CT or PNETs (G1/G2) + PD | 37 | ORR | Completed | ORR: 18.7% (7/37) | Ahn et al. (2013) |

| Sorafenib         | TKI: VEGFR-2              | II            | Sorafenib 400 mg × 2/day | NCT00131911      | CT (n = 50)/PNETs (n = 43) | 93 | ORR | Completed | PR: 10% CT PR: 14% PNETs Grade 3–4 toxicity: 43% Global PFSR: 90.9% | Hobday et al. (2007) |
|                    | PDGF-β c-Raf              |               |                         |                  |                         |     |       |       |        |        |      |
|                    |                           | II            | Sorafenib 200 mg bid on days 1–5 + bevacizumab 5 mg/kg once/2 weeks | NA | Locally unresectable/ metastatic CT or PNETs (G1/G2) | 44 | 6 months PFSr | Completed |                | Castellano et al. (2013) |

<p>| Bevacizumab (BVZ) | Anti-VEGF monoclonal antibody | II           | First 18 weeks: OCT + IFNα2b (0.5 µg/kg/week) or OCT + BVZ (15 mg/kg/21 day) After 18 weeks or PD: OCT + IFNα2b + bevacizumab | NCT00569127 | Metastatic well-differentiated CT | 44 | 18 weeks-PFSr | Completed | PFS: 68% (OCT + IFN) vs 95% (OCT + BEV) (P = 0.02) | Yao et al. (2008) |
|                    |                           | II           | OCT LAR (20 mg/21 day) + BVZ (15 mg/kg/21 day) | NCT00569127 | Locally unresectable/ metastatic CT or PNETs (G1/G2) + PD | 402 | PFS | Completed | mPFS: 16.6 months (1) vs 15.5 months (2) (NS) | Yao et al. (2015) |
|                    |                           | III          | OCT LAR (30 mg/28 day) + BVZ 15 mg/kg/21 day + pertuzumab 420 mg/21 day | NCT01121939 | Locally unresectable/ metastatic CT or PNETs (G1/G2) + PD | 43 | ORR | Completed | ORR: 16% | Bendell et al. (2016) |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunitinib</td>
<td>III</td>
<td>Lan 120 mg/28 day + oral sunitinib 37.5 mg/day</td>
<td>Placebo + oral sunitinib 37.5 mg/day</td>
<td>mPFS (months): 11.4 vs 5.5; HR 0.42 (0.26–0.66); P &lt; 0.001</td>
</tr>
<tr>
<td>Sulfatinib</td>
<td>III</td>
<td>Sulfatinib 300 mg/day (28-day cycle)</td>
<td>Placebo 300 mg/day</td>
<td>mPFS (months): 16.82 vs 16.52; HR 0.991 (0.636–1.543); P = 0.488</td>
</tr>
<tr>
<td>Everolimus + mTOR inhibitors</td>
<td>III</td>
<td>Everolimus 10 mg/day + OCT LAR 60 mg/28 day + pasireotide LAR 60 mg/28 day</td>
<td>Placebo + OCT LAR 60 mg/28 day</td>
<td>mPFS (months): 16.82 vs 16.52; HR 0.991 (0.636–1.543); P = 0.488</td>
</tr>
<tr>
<td>Everolimus</td>
<td>III</td>
<td>Everolimus 10 mg/day + OCT LAR 60 mg/28 day + metformin</td>
<td>Placebo + OCT LAR 60 mg/28 day</td>
<td>mPFS (months): 16.82 vs 16.52; HR 0.991 (0.636–1.543); P = 0.488</td>
</tr>
<tr>
<td>Everolimus + OCT LAR</td>
<td>II</td>
<td>Everolimus 10 mg/day + OCT LAR 60 mg/28 day</td>
<td>Placebo + OCT LAR 60 mg/28 day</td>
<td>NA</td>
</tr>
<tr>
<td>Everolimus + OCT LAR</td>
<td>II</td>
<td>Everolimus 10 mg/day + OCT LAR 60 mg/28 day</td>
<td>Placebo + OCT LAR 60 mg/28 day</td>
<td>NA</td>
</tr>
<tr>
<td>Everolimus + OCT LAR + metformin</td>
<td>II</td>
<td>Everolimus 10 mg/day + OCT LAR 60 mg/28 day + metformin</td>
<td>Placebo + OCT LAR 60 mg/28 day</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: PFS = Progression-free survival; ORR = Overall response rate; mPFS = Median progression-free survival; HR = Hazard ratio; CI = Confidence interval; DQ = Disease control rate; OS = Overall survival; DFS = Disease-free survival; mORR = Median overall response rate; mOS = Median overall survival; P = 0.003; NA = Not applicable; OCT = Octreotide; LAR = Long-acting release; mTOR = Mammalian target of rapamycin; OCT = Octreotide; LAR = Long-acting release; NA = Not applicable; OCT = Octreotide; LAR = Long-acting release; NA = Not applicable; OCT = Octreotide; LAR = Long-acting release.
<table>
<thead>
<tr>
<th>Targeted therapies</th>
<th>Pharmacological target(s)</th>
<th>Study (phase)</th>
<th>Intervention/comparator</th>
<th>Trial identifier</th>
<th>Population</th>
<th>N</th>
<th>Primary endpoint(s)</th>
<th>Status</th>
<th>Result</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temsirolimus</td>
<td>mTOR complex 1</td>
<td>Phase II</td>
<td>Temsirolimus 25mg intravenously (IV) once/week + BVZ 10 mg/kg IV once/2 weeks</td>
<td>NCT01010126</td>
<td>Unresectable and/or metastatic CT and PNETs (G1/G2)+PD</td>
<td>58</td>
<td>6-months PFS ORR</td>
<td>Completed</td>
<td>ORR: 41% 6-months PFS: 79%</td>
<td>Hobday et al. (2015)</td>
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<tr>
<td>Somatostatin analogs</td>
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<tr>
<td>Octreotide (OCT)</td>
<td>Somatostatin receptor subtype 2 (SSTR2)</td>
<td>III</td>
<td>(1): OCT LAR (30mg/28 day) vs (2): Placebo PROMID trial</td>
<td>NCT00171873</td>
<td>Unresectable and/or metastatic midgut NETs (G1/G2)</td>
<td>85</td>
<td>PFS</td>
<td>Completed</td>
<td>mPFS (months) = 14.3 (1) vs 6 (2) (HR 0.34, 95% CI 0.2–0.59; P=0.000072)</td>
<td>Rinke et al. (2019)</td>
</tr>
<tr>
<td>Lanreotide (LAN)</td>
<td>SSTR2</td>
<td>III</td>
<td>(1) LAN ATG (120mg/28 day) vs (2): Placebo (CLARINET trial)</td>
<td>NCT00333496</td>
<td>Unresectable and/or metastatic well-differentiated NETs from pancreas, midgut or hindgut (G1/G2) (Ki67 &lt;10%)</td>
<td>204</td>
<td>PFS</td>
<td>Completed</td>
<td>mPFS (months): (1) not reached vs (2) 18.0 months (HR 0.47, 95% CI 0.3–0.73; P&lt;0.001)</td>
<td>Caplin et al. (2014)</td>
</tr>
<tr>
<td></td>
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<td>(1): LAN ATG (120mg/28 day) as maintenance therapy vs (2): Placebo (REMINET trial)</td>
<td>NCT02288377</td>
<td>Unresectable and or metastatic well-differentiated duodeno-pancreatic NETs (G1/G2)+PD</td>
<td>222</td>
<td>6-months PFS rate</td>
<td>Recruiting</td>
<td>NA</td>
<td>NA</td>
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<td></td>
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<td>II</td>
<td>LAN ATG (120mg/28 day) + temozolomide (SONNET trial)</td>
<td>NCT02231762</td>
<td>Unresectable and/or metastatic CT and PNETs (G1/G2)+PD</td>
<td>57</td>
<td>6-months DCR</td>
<td>Completed</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>Pasireotide LAR 60 mg/28 day vs (2): OCT LAR 30 mg/28 day</td>
<td>NCT00690430</td>
<td>Patients with carcinoid tumors and symptoms (diarrhea and flushing) that are not adequately controlled by somatostatin analogs</td>
<td>186</td>
<td>Patients (%) who achieved clinical symptom improvement</td>
<td>Completed</td>
<td>(1): 20.9% (2): 26.7% (14.6–41.9)</td>
<td>NA</td>
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<tr>
<td></td>
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<td></td>
<td>Pasireotide LAR IM 60 mg/28 day</td>
<td>NCT01235161</td>
<td>Unresectable and/or metastatic CT and PNETs (G1/G2)+PD</td>
<td>29</td>
<td>PFS</td>
<td>Completed</td>
<td>Overall mPFS = 11 months</td>
<td>Cives et al. (2015)</td>
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<td></td>
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<td></td>
<td>Peptide receptor radionuclide therapy (PRRT) 177Lu-DOTA0-Tyr3-Octreotate</td>
<td>SSTR2</td>
<td>Unresectable and/or metastatic and NETs (G1/G2)+PD</td>
<td>229</td>
<td>PFS</td>
<td>Completed</td>
<td>mPFS (months): not reached (1) vs 8.4 (2) (HR 0.29, 95% CI 0.129–0.388; P&lt;0.0001)</td>
<td>Strosberg et al. (2017)</td>
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Table 1 Continued.
### Immunotherapy – interferon

<table>
<thead>
<tr>
<th>Study</th>
<th>Designation</th>
<th>Groups</th>
<th>Drugs/Concomitant Treatments</th>
<th>Comparator</th>
<th>Unresectable/metastatic</th>
<th>Time to Treatment Failure</th>
<th>Time to Treatment Failure and Long-term Survival</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arnold et al.</td>
<td>II OCT 200 µg × 3/day vs OCT</td>
<td>NR</td>
<td>Treatments to PD</td>
<td>OCT 200 µg × 3/day + IFNα 4.5 × 10^6 MU × 3/week</td>
<td>109 midgut or duodenopancreatic NETs (G1/G2) + PD</td>
<td>completed</td>
<td>did not differ significantly between the 2 groups</td>
<td></td>
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</table>
| Kölby et al.   | NA (1) OCT 100 µg × 2/day–200 µg × 3/day vs (2) OCT 100 µg × 2/day–200 µg × 3/day + IFNα 3 × 10^6 MIU × 3/week
(3) OCT 100 µg × 2/day–200 µg × 3/day + IFNα 3 × 10^6 MIU × 3/week | NR     | Treatments to PD            | OCT 100 µg × 2/day–200 µg × 3/day + IFNα 3 × 10^6 MIU × 3/week | NR midgut NETs + PD | 68 5-year OS/time to tumor progression | completed               | no significant difference in survival |
| NA             | (1): LAN SC 1 mg × 3/day (2): IFNα 5 × 10^6 × 3/week (3): LAN SC 1 mg × 3/day + IFNα 5 × 10^6 × 3/week | NR     | Treatments to PD            | IFNα 5 × 10^6 × 3/week | 80 1-year tumor progression rate | completed               | No significant difference in rates of partial remission, stable disease or tumor progression among treatment groups |
| Dahan et al.   | III Arm 1: 5FU-STZ/6 week Arm 2: IFNα 3 × 10^6 × 3/week | NR     | Treatments to PD            | IFNα 3 × 10^6 × 3/week | 64 PFS well-differentiated digestive NETs + PD | completed               | p = 0.008 |
| Kulke et al.   | III During 12 weeks: Arm 1: Telotristat etiprate 250 mg × 3/J Arm 2: Telotristat etiprate 500 mg × 3/J Arm 3: Placebo From 12 to 36 weeks (extension phase): All groups: Telotristat etiprate 500 mg × 3/J (TELESTAR trial) | NR     | Treatments to PD            | Placebo | NCT01677910 well-differentiated metastatic NETs + carcinoid syndrome with ≥4 bowel movements per day (BMD) during the run-in period + receiving stable-dose somatostatin analog (SSA) therapy | 135 Change from baseline in number of BMD (12 weeks) with rate of patients with durable response (≥30% reduction in BMD over at least half days of study period) | completed               | Durable response: Arm 1: 44% patients Arm 2: 42% patients Arm 3: 20% patients, P < 0.040 vs Arm 1 and 2 During extension phase: All patients from the placebo arm experienced a significant decrease of BMD |
| Kulke et al.   | (Continued)                  |        |                             |            |                        |                          |                                               |       |

**Serotonin inhibitor**

<table>
<thead>
<tr>
<th>Study</th>
<th>Designation</th>
<th>Groups</th>
<th>Drugs/Concomitant Treatments</th>
<th>Comparator</th>
<th>Unresectable/metastatic</th>
<th>Time to Treatment Failure</th>
<th>Time to Treatment Failure and Long-term Survival</th>
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<tr>
<td>Kulke et al.</td>
<td>III during 12 weeks Arm 1: Telotristat etiprate 250 mg × 3/J Arm 2: Telotristat etiprate 500 mg × 3/J Arm 3: Placebo From 12 to 36 weeks (extension phase): All groups: Telotristat etiprate 500 mg × 3/J (TELESTAR trial)</td>
<td>NR</td>
<td>Treatments to PD</td>
<td>Placebo</td>
<td>NCT01677910 well-differentiated metastatic NETs + carcinoid syndrome with ≥4 bowel movements per day (BMD) during the run-in period + receiving stable-dose somatostatin analog (SSA) therapy</td>
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<td>Durable response: Arm 1: 44% patients Arm 2: 42% patients Arm 3: 20% patients, P &lt; 0.040 vs Arm 1 and 2 During extension phase: All patients from the placebo arm experienced a significant decrease of BMD</td>
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<tr>
<td>Kulke et al.</td>
<td>(Continued)</td>
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Therapeutic targets in the TME of GEP-NETs: clinical trials and future perspectives

Angiogenesis and mTOR pathway inhibitors

In clinical trials, small molecules have been developed to target VEGF signaling, such as tyrosine kinase inhibitors (TKIs) directed toward many receptors, including VEGF-Rs, humanized anti-VEGF antibody (bevacizumab), aflibercept) and inhibitors of the mTOR signaling pathway (mTORi) (Cao 2014) (Table 1). Sunitinib malate, a TKI directed against PDGF-R (α and β), VEGF-R (2 and 3) and c-kit (stem cell factor receptor), showed both antitumor and angiogenic effects in RipTag2 mice (Pietras & Hanahan 2005). Clinically, the drug significantly improved progression-free survival (PFS) and the objective response rate as compared with placebo among patients with advanced PNETs (Raymond et al. 2011). As discussed earlier, blocking the VEGF-R axis in preclinical models of GEP-NETs gives rise to hypoxia, which is one of the mechanisms of acquired resistance to angiogenic therapies. Based on results from animal models, another approach could be to simultaneously block VEGF and other factors released in the TME of GEP-NETs such that it has been shown for FGF blockade (Casanovas et al. 2005, Allen et al. 2011) or the cMET receptor (Sennino et al. 2012, 2013). The hepatocyte growth factor (HGF), the ligand of cMET, has been recently identified as a critical promoter of a resistance to treatment state toward melanoma human cell lines treated with BRAF inhibitors (Straussman et al. 2012). Although the cMET receptor is expressed in human samples of PNETs (Fig. 6), its functional role in the TME of GEP-NETs requires further investigations.

Besides specific antiangiogenic drugs, SSAs previously demonstrated antiangiogenic properties as well (Albini et al. 1999). Inhibition of angiogenesis through repression of endothelial cells proliferation is mediated either via the somatostatin receptor subtype 1 (SSTR1) (Bocci et al. 2003, Walter et al. 2011) or SSTR3 (Florio et al. 2003, Adams et al. 2004). On the other hand, in an in vivo experimental animal model of intrahepatic dissemination of the somatostatin-producing endocrine cell line STC-1, no effect of octreotide on tumor growth and intratumoral microvascular density was observed, suggesting a potential adaptive mechanism by the microenvironment (Walter et al. 2011). In human GEP-NETs, there are no studies that focused on a measurable antiangiogenic impact of SSAs, independent from a direct anti-tumor effect.

The PI3K/AKT/mTOR has also been a target of interest in the treatment of GEP-NETs, especially since the mTOR
pathway was shown to be dysregulated in a subset of PNET (Jiao et al. 2011) and because the PI3K/AKT/mTOR pathway is connected to angiogenesis through HIF (hypoxia inducible factor)-dependent and -independent mechanisms (Karar & Maity 2011). Accordingly, in animal models of either gastric or colorectal cancer, the mTOR inhibitor everolimus significantly decreased microvessel density, suggestive of a direct effect on the TME (Onoyama et al. 2013, Yuge et al. 2015). Major clinical trials have been published over the past few years with the use of mTOR inhibitor everolimus in the field of NETs with significant impact over the PFS of treated patients vs the placebo group (Yao et al. 2011, 2016, Pusceddu et al. 2016).

**IGF-1/IGF-1R blockade**

In the field of GEP-NETs, a pilot study investigated the effect of NVP-AEW541, an IGF1R tyrosine kinase inhibitor able to block the activation of both PI3K/Akt/ mTOR and RAF/MEK/ERK pathways, in BON-1 and in an insulinoma cell line. NVP-AEW541 dose-dependently inhibited the proliferation of NET cells by inducing both apoptosis (assessed by the caspase 3 activity) and cell cycle arrest. Moreover, its anti-neoplastic effect was also detected in primary cultures of human neuroendocrine gastrointestinal tumors (Höpfner et al. 2006).

Subsequently, phase-I/II studies have been conducted with monoclonal antibodies directed against the IGF-1R in patients with well-differentiated GEP-NETs, however, without significant results in term of objective response rate (Table 1). The resistance to IGF-1R inhibitor in vivo, despite convincing results observed in vitro, may be, at least partially, explained, once again by an adaptive mechanism elicited by the TME, as suggested by Lee et al. where they showed in a murine model of orthotopic breast tumor that treatment with IGF-1R antibody paradoxically accelerates tumor infiltration of stromal cells and metastatic tumor growth through a STAT3-dependent transcriptional upregulation of IGF-2 by the cancer cells (Lee et al. 2015).

In summary, the IGF/IGF-1R system is highly complex and includes, besides the classical IGF-1/IGF-1R couple, the insulin receptors that can also be activated by IGF-1 and many binding proteins called the IGFBPs. As a consequence, it seems unlikely that monotherapies using antibodies specifically directed toward the IGF-1R will be successful, but IGF-1/IGF-1R targeting may still be of interest for a co-targeting therapeutic strategy.

**Inhibition of the serotonin pathway**

Since 2007, telotristat etiprate has emerged as a new therapeutic option for patients with GEP-NETs and refractory carcinoid syndrome (Kulke et al. 2014). Telotristat etiprate is an oral, systemically available, small-molecule inhibitor of peripheral serotonin synthesis by inhibition of the tryptophan hydroxylase, the rate-limiting enzyme in the conversion of tryptophan to serotonin. In a prospective randomized study, patients with evidence of carcinoid tumor and ≥4 bowel movements (BMs)/day, despite stable-dose octreotide LAR depot therapy, were enrolled in sequential escalating cohort and assigned to either placebo or telotristat etiprate at 150, 250, 350 or 500 mg three times a day (Kulke et al. 2014). Among evaluable telotristat etiprate-treated patients, 5/18 (28%) experienced a ≥30% reduction in BM frequency for ≥2 weeks, 9/16 (56%) experienced biochemical response (≥50% reduction or normalization in u5-HIAA) at week 2 or 4 and 10/18 (56%) reported adequate relief during at least 1 of the first 4 weeks of treatment. Similar activity was not observed in placebo-treated patients. In a multicenter trial in 14 patients with metastatic well-differentiated neuroendocrine tumors (66% from the midgut) responsible for a carcinoid syndrome, telotristat etiprate resulted in both a reduction of BM/day in every patient (with a mean decrease of 43.5%) and a 73% decrease of the mean u5-HIAA concentration after 12 weeks of treatment (Pavel et al. 2015). Finally, the clinical utility of telotristat for reduction of BM frequency and u5-HIAA in patients with carcinoid syndrome not adequately controlled by SSAs.
was recently reported in a phase III clinical study (Kulke et al. 2017) (Table 1). The potential impact of this drug over the TME of GEP-NETs remains currently unknown.

Interferon therapy

Because of its antiangiogenic, as well as its immunomodulatory properties (Tompkins 1999, von Marschall et al. 2003), interferon alpha (IFNα) has also been proposed in the management of NET. However, IFNα use has been limited due to toxicity and perceived limited efficacy (Mirvis et al. 2014). In vitro studies conducted in BON-1 provided several lines of evidence that IFN-β was an even more potent inhibitor of proliferation compared to IFNα (Vitale et al. 2006). IFNs exert their effect via the type I IFN receptor (IFNAR-1, IFNAR-2c), but the level of IFNAR expression in GEP-NETs is currently unknown. Several clinical trials using IFNα as monotherapy or in combination have been published in the field of GEP-NETs (see review: Alonso-Gordoa et al. 2014 for details) and its efficacy, even partial, suggests intricate mechanisms that remain poorly understood to date. One of the effects could be the induction of class I antigens (β2-microglobuline) on tumor cells deficient in such expression, as previously shown in SI-NETs (Funa et al. 1986). A similar effect of IFNα on the TME of GEP-NETs was suggested by observation of an antineovascular activity in BON-1 xenografted mice model (Liu et al. 2006). Finally, a direct effect of IFNα on the tumoral cell itself by induction of apoptosis and modulation of cell cycle proteins were reported in several experiments (Detjen et al. 2000, 2002, Zhou et al. 2002).

In summary, the development of the current targeted therapies now available in GEP-NETs marked a little revolution as they were able, by definition, to specifically block a molecular target known to regulate the tumor development and behavior. As such, they were supposed to preserve the patients from the adverse effects usually encountered with intravenous systemic chemotherapies. Based on the clinical experience, it henceforth appears that the influence of the TME over the tumor growth and its capability to escape from the treatment has to be increasingly taken into consideration and two intended goals are currently assumed in that sense: first, to control (and ideally reduce) the primitive tumor growth, and second, to decrease the capability the tumor cells have to metastasize. Sunitinib malate, mTORi and SSA LAR have achieved clinical impact in phase III clinical trials. However, their efficiency is now challenged by the occurrence of resistance to treatment state the tumor can adopt. There could be a rationale to target, alternatively to VEGF, multiple growth factors or their receptors known to promote carcinogenesis such as PDGF, IGF or PIGF, but experimental data focusing on those factors are mostly observational and/or conducted in animal models. Furthermore, one could assume that whatever the number of growth factor pathways blocked, the tumor cells will indefinitely reactivate alternative signaling pathways (including under the influence of their TME) and eventually overcome the treatment efficacy at one point. An innovative approach could be to simultaneously target the tumoral cells and pivotal components of the TME, for example, the combination of antiangiogenic drugs with the cytotoxic action of immune checkpoint inhibitors, which have shown promising results in other models of tumors.

Less is known about the impact of the current treatment over the capability the tumor cells have to metastasize, and a number of innovative targets deserve attention. As discussed earlier, heparanase and/or matrix metalloproteinases within the ECM may constitute innovative targets in the future as it has already been done in an Ewing sarcoma model (Cassinelli et al. 2013). A recent original study conducted by Li and Hanahan showed that glutamate and its receptor N-methyl-D-aspartate receptor (NMDAR) was upregulated at the periphery of PNETs in RipTag2 mice, particularly invasive fronts, and that this invasivity was inhibited by the use of NMDAR inhibitor (Li & Hanahan 2013).

An emerging concept in the future could be the combination of treatment targeting the tumor cell on the one hand, with those directed toward components of the ECM on the other hand. Moreover, by modulating the ECM, an improvement of the drug deliverance to the tumor niche is likely to occur as this was previously demonstrated (Olive et al. 2009).

Summarizing conclusions

The tumor and its microenvironment appear as two inseparable entities, which opens a new concept of pathophysiology and, therefore, require novel treatment strategies. Although significant progress have been achieved in the past years for treating GEP-NETs, a subset of patients will still experience treatment resistance or an escape after an initial period of remission. This issue is really challenging because it suggests the tumoral cell progressively adapts to or bypasses the therapeutic effect of a drug by activating alternative signaling pathways. The latter obviously occurs within the tumoral cell, but also results from the action of numerous ligands released
within and by the components of the TME, a process called acquired resistance. Moreover, growing evidence from preclinical models indicates that when a tumor escapes from the treatment, a substantial modification of its behavior occurs, resulting in increased aggressiveness and a higher propensity to metastasize. Thus, understanding the impact of the TME in the tumor niche may lead not only to approaches to prevent treatment resistance, but also to prevent the emergence of an undifferentiated phenotype of the tumor.

It should be emphasized that many of the results of the studies reported in this review refer to animal models, which may be difficult to extrapolate to human GEP-NETs (Grozinsky-Glasberg et al. 2012). Moreover, relevant differences between GEP-NET cell lines have been recently emphasized by whole-exome sequencing and constitute another source of uncertainty when extrapolating results to the clinical setting (Vandamme et al. 2015). An alternative way could be studies on primary cultures of human GEP-NETs tumor, representing an affordable and individualized approach of the tumor biology and response to treatment from bench to bedside (Mohamed et al. 2017). Alternative options to optimally reproduce the in vivo landscape of the TME could be the use of microencapsulated SI-NET cells (Rokstad et al. 2012), as well as the development of animal models of spontaneous PNETs could be a solution (Yu 2016). In addition, 3D spheroid culturing of NET cells (Wong et al. 2012) or pheochromocytoma cells (D’Antongiovanni et al. 2017), allowing cell–cell interactions, have been successfully performed and give the possibility to perform histological, immunohistochemical and functional analysis and take into consideration the tumor and its microenvironment.

For the elaboration of innovative therapeutic regimens (like the co-targeting strategy) and/or identification of new potential targets of GEP-NETs, it may be difficult to take into consideration the TME. Moreover, the TME of GEP-NETs is likely to change over the natural history of the tumor, under influence from the treatment, and from one tumor site to another. However, the development of whole proteomic as well as genomic approaches in the near future will undoubtedly help to delineate the TME of one given GEP-NET and possibly lead to combination treatments and sequential therapeutic schemes in order to overcome emerging resistance. Recent promising results on the genomics of PNETs (Scarpa et al. 2017) marked a step forward that will open perspectives in the understanding of the TME of such tumors.

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