REVIEW

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.

Key Words

- ▶ neuroendocrine tumors
- ▶ microenvironment
- ▶ angiogenesis
- ▶ growth factors

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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.

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 underline the critical role played by the TME in the induction of a tumor 'resistance to treatment'

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 adaptative 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

Figure 2

Schematic representation of angiogenesis in GEP-NETs and the main molecular factors that regulate it, as described in this review.

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

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 adaptative 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 a 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

TGF α 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 TGF α 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 TGF α 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 TGF α to these primary cultures of SI-NETs resulted in tumor growth stimulation (Nilsson *et al.* 1995), which confers to the TGF α , 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).

TGF β 1 is another cytokine, which binds TGF β R-1 and TGF β R-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 TGF β , whereas TGF β R-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 TGF β 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 TGF β 1 (10 ng/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 TGF β on BON-1 proliferation may involve an upregulation of both the expression of p21^{waf1/cip1} (a cyclin-dependent kinase inhibitor) (Pickup *et al.* 2013) and the secretion of somatostatin by

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.

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).

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 TGF β (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 Nintedanib, 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 (TNF α) (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 TGF β 1 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.

Figure 4

Carcinoid syndrome. (A) Biosynthesis of serotonin and renal excretion of 5HIAA. (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.

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

heparanase 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 β 2-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-1 β (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 (pembrolizumab, nivolumab, atezolizumab) has led to remarkable clinical responses in patients with many

Figure 5

Schematic representation of the PD-1/PD-L1 system in the setting of innate (A) or acquired (C) immune resistance and the effect of the immune checkpoint inhibitor anti-PD-L1 (B). MHC, major histocompatibility complex; TCR, T-cell receptor.

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, 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.

Targeted therapies	Pharmacological target(s)	Study (phase)	Intervention/comparator	Trial identifier	Population	N	Primary endpoint(s)	Status	Result	Ref.
Antiangiogenic agents Axitinib	VEGFR-1	II	Axitinib 5 mg × 2/day	NCT01435122	Unresectable/metastatic extrapancreatic NET (G1/G2)	30	PFS	Completed	mPFS = 26.7 months.	Strosberg <i>et al.</i> (2016)
	VEGFR-2						PFS rate		12-month PFS rate = 74.5%	
	VEGFR-3	II	Axitinib 5 mg × 2/day + OCT LAR 30 mg/28 day vs Placebo + OCT LAR 30 mg/28 day	NCT01744249	Unresectable/metastatic extrapancreatic NET (G1/G2) + PD	80	PFS	Ongoing	NA	NA
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 <i>et al.</i> (2015)
	VEGFR-2									
	VEGFR-3	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 <i>et al.</i> (2015)
	PDGFR-α and -β Stem cell factor receptor (c-Kit)	II	Pazopanib 800 mg/day	NCT01099540	Locally unresectable/ metastatic CT or PNETs (G1/G2/ G3) + progressive disease	37	ORR	Completed	ORR: 18.7% (7/37)	Ahn <i>et al.</i> (2013)
Sorafenib	TKI: VEGFR-2 PDGFR-β c-Raf	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%	Hobday <i>et al.</i> (2007)
		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	Global PFSr: 90.9%	Castellano <i>et al.</i> (2013)
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)	NCT00569127	Metastatic well-differentiated CT	44	18 weeks-PFSr OS	Completed	PFSr: 68% (OCT + IFN) vs 95% (OCT + BVZ) (P = 0.02)	Yao <i>et al.</i> (2008)
			After 18 weeks or PD: OCT + IFNα2b + bevacizumab							
		III	(1): OCT LAR (20 mg/21 day) + BVZ (15 mg/kg/21 day) (2): OCT LAR (20 mg/21 day) + IFNα2b (5 MIU × 3/week)	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 <i>et al.</i> (2015)
		II	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 <i>et al.</i> (2016)

Sunitinib	VEGFR-1 VEGFR-2 VEGFR-3 PDGFR- α PDGFR- β , c-Kit, FLT-3 CSF1R	III	(1): oral sunitinib 37.5 mg/day vs (2): Placebo	NCT00428597	Locally unresectable/ metastatic well- differentiated PNETs + PD	171	PFS	Completed	mPFS (months): 11.4 (1) vs 5.5 (2) HR 0.42 for progression or death; 95% CI 0.26–0.66; $P < 0.001$ NA	Raymond <i>et al.</i> (2011)
		II	(1): LAN 120 mg/28 day + oral sunitinib 37.5 mg/day (2): Placebo + oral sunitinib 37.5 mg/day (SUNLAND trial)	NCT01731925	Locally unresectable/ metastatic CT (G1/ G2) + PD + functional	104	PFS	Active, not recruiting	NA	NA
Sulfatinib	VEGFR-1 VEGFR-2 VEGFR-3 FGFR	Phase III SANET-p	(1): Sulfatinib 300 mg/day (28-day cycle) (2): Placebo 300 mg/day (28-day cycle) (SANET-p trial)	NCT02589821	Locally unresectable and or metastatic well-differentiated PNETs (G1/G2) + PD	195	7-months PFS	Recruiting	NA	NA
		II	(1): Everolimus 10 mg/day (2): Everolimus 10 mg/day + OCT LAR ≤ 30 mg/28 day (RADIAN-1 trial)	NCT00363051	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	160	ORR	Completed	ORR = 9.6% (1); 67.8% SD ORR = 4.4% (2); 80% SD	Yao <i>et al.</i> (2010)
mTOR inhibitors Everolimus		III	(1): Everolimus 10 mg/day + OCT LAR 30 mg/28 day vs (2): Placebo + OCT LAR 30 mg/28 day (RADIAN-2 trial)	NCT00412061	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	429	PFS	Completed	mPFS (months) = 16.4 (1) vs 11.3 (2) (HR 0.77, 95% CI 0.59–1.00; $P = 0.026$)	Pavel <i>et al.</i> (2011)
		III	(1): Everolimus 10 mg/day + best supportive care vs (2): placebo + best supportive care (RADIAN-3 trial)	NCT00510068	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	410	PFS	Completed	mPFS (months) = 11.0 (1) vs 4.6 (2) (HR 0.35, 95% CI 0.27–0.45; $P < 0.001$)	Yao <i>et al.</i> (2011)
		III	(1): Everolimus 10 mg/day + best supportive care vs (2): placebo + best supportive care (RADIAN-4 trial)	NCT01524783	Unresectable and/or metastatic non- functional digestive or lung NETs (G1/G2) + PD	302	PFS	Completed	mPFS (months) = 11.0 (1) vs 3.9 (2) (HR 0.48, 95% CI 0.35–0.67; $P < 0.0001$)	Yao <i>et al.</i> (2016)
		II	Everolimus + OCT LAR + metformin (Met-NET1 trial)	NCT02294006	Well-differentiated PNETs (G1/G2)	43	PFS	Recruiting	NA	Pusceddu <i>et al.</i> (2016)
		II	(1): Everolimus 10 mg/ day + pasireotide LAR 60 mg/28 day vs (2): Everolimus 10 mg/day (COOPERATE-2 trial)	NCT01374451	Well-differentiated PNETs (G1/G2)	160	PFS	Completed	mPFS (months) = 16.82 (1) vs 16.52 (2) HR 0.991, 95% CI 0.636–1.543; $P = 0.488$	Kulke <i>et al.</i> (2017)
		II	Everolimus in patients with PNET metastatic to the liver previously treated with surgery	NCT02031536	Metastatic PNETs to the liver Surgery of liver metastasis (G1/G2) + PD	150	DFS	Recruiting	NA	NA
		III	Efficacy and safety of everolimus and (STZ-5FU) given one upfront the other upon progression in advanced pNET (SEQTOR trial)	NCT02246127	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	180	Second PFS	Recruiting	NA	NA

(Continued)

Table 1 Continued.

Targeted therapies	Pharmacological target(s)	Study (phase)	Intervention/comparator	Trial identifier	Population	N	Primary endpoint(s)	Status	Result	Ref.
Temsirolimus	mTOR complex 1	Phase II	Temsirolimus 25 mg intravenously (IV) once/week + BVZ 10 mg/kg IV once/2 weeks	NCT01010126	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	58	6-months PFS ORR	Completed	ORR: 41% 6-months PFS: 79%	Hobday <i>et al.</i> (2015)
Somatostatin analogs Octreotide (OCT)	Somatostatin receptor subtype 2 (SSTR2)	III	(1): OCT LAR (30 mg/28 day) vs (2): Placebo <i>PROMID</i> trial	NCT00171873	Unresectable and/or metastatic midgut NETs (G1/G2)	85	PFS	Completed	mPFS (months) = 14.3 (1) vs 6 (2) (HR 0.34, 95% CI 0.2–0.59; $P=0.000072$)	Rinke <i>et al.</i> (2009)
Lanreotide (LAN)	SSTR2	III	(1) LAN ATG (120 mg/28 day) vs (2): Placebo (<i>CLARINET</i> trial)	NCT00353496	Unresectable and/or metastatic well-differentiated NETs from pancreas, midgut or hindgut (G1/G2) (Ki67 <10%)	204	PFS	Completed	mPFS (months): (1) not reached vs (2) 18.0 months (HR 0.47, 95% CI 0.3–0.73; $P<0.001$)	Caplin <i>et al.</i> (2014)
		II/III	(1): LAN ATG (120 mg/28 day) as maintenance therapy vs (2): Placebo (<i>REMINET</i> trial)	NCT02288377	Unresectable and/or metastatic well-differentiated duodeno-pancreatic NETs (G1/G2) + PD	222	6-months PFS rate	Recruiting	NA	NA
		II	LAN ATG (120 mg/28 day) + temozolomide (<i>SONNET</i> trial)	NCT02231762	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	57	6-months DCR	Completed	NA	NA
Pasireotide	SSTR1, 2, 3 and 5	III	(1): Pasireotide LAR 60 mg/28 day vs (2): OCT LAR 30 mg/28 day	NCT00690430	Patients with carcinoid tumors and symptoms (diarrhea and flushing) that are not adequately controlled by somatostatin analogs	186	Patients (%) who achieved clinical symptom improvement	Completed	(1): 20.9% (2): 26.7% (14.6–41.9)	NA
		II	Pasireotide LAR IM 60 mg/28 day	NCT01253161	Unresectable and/or metastatic CT and PNETs (G1/G2) + PD	29	PFS	Completed	Overall mPFS = 11 months	Cives <i>et al.</i> (2015)
Peptide receptor radionuclide therapy (PRRT) 177Lu-DOTA0-Tyr3-Octreotate	SSTR2	III	(1): 177Lu-DOTA0-Tyr3-Octreotate + OCT 30 mg LAR/28 day vs (2): OCT 60 mg LAR/28 day (<i>NETTER</i> trial)	NCT01578239	Unresectable and/or metastatic well-differentiated midgut NETs (G1/G2) + PD	229	PFS	Completed	mPFS (months): not reached (1) vs 8.4 (2) (HR 0.209, 95% CI 0.129–0.338; $P<0.0001$)	Strosberg <i>et al.</i> (2017)
		II	(1): 177Lu-DOTA0-Tyr3-Octreotate vs (2): Sunitinib 37.5 mg/day (<i>OCCLURANDOM</i> trial)	NCT02230176	Unresectable/metastatic PNETs (G1/G2) + PD	80*	12 months PFS	Recruiting	NA	NA

Immunotherapy – interferon Interferon $\alpha 2b$ (IFN α)	Immunomodulation, antiproliferative agent	II	NR	OCT 200 $\mu\text{g} \times 3/\text{day}$ vs OCT 200 $\mu\text{g} \times 3/\text{day} + \text{IFN}\alpha$ 4.5 $\times 10^6$ MIU $\times 3/\text{week}$	Unresectable/metastatic midgut or duodeno- pancreatic NETs (G1/ G2) + PD	109	Time to treatment failure (progression, death, stop of study)	Completed	Time to treatment failure and long-term survival did not differ significantly between the 2 groups	Arnold <i>et al.</i> (2005)
Serotonin inhibitor Telotristat etiprate	Tryptophan hydroxylase inhibitor	III	NR	During 12 weeks: Arm 1: Telotristat etiprate 250 mg $\times 3/\text{J}$ Arm 2: Telotristat etiprate 500 mg $\times 3/\text{J}$ Arm 3: Placebo From 12 to 36 weeks (extension phase): All groups: Telotristat etiprate 500 mg $\times 3/\text{J}$ (TELESTAR trial)	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 <i>et al.</i> (2014) Kulke <i>et al.</i> (2017)
Immunomodulation, antiproliferative agent	Immunomodulation, antiproliferative agent	II	NR	OCT 200 $\mu\text{g} \times 3/\text{day}$ vs OCT 200 $\mu\text{g} \times 3/\text{day} + \text{IFN}\alpha$ 4.5 $\times 10^6$ MIU $\times 3/\text{week}$	Unresectable/metastatic midgut or duodeno- pancreatic NETs (G1/ G2) + PD	109	Time to treatment failure (progression, death, stop of study)	Completed	Time to treatment failure and long-term survival did not differ significantly between the 2 groups	Arnold <i>et al.</i> (2005)
Serotonin inhibitor Telotristat etiprate	Tryptophan hydroxylase inhibitor	III	NR	During 12 weeks: Arm 1: Telotristat etiprate 250 mg $\times 3/\text{J}$ Arm 2: Telotristat etiprate 500 mg $\times 3/\text{J}$ Arm 3: Placebo From 12 to 36 weeks (extension phase): All groups: Telotristat etiprate 500 mg $\times 3/\text{J}$ (TELESTAR trial)	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 <i>et al.</i> (2014) Kulke <i>et al.</i> (2017)

(Continued)

Table 1 Summary of past and current clinical trials targeting components of the TME in GEP-NETs.

Targeted therapies	Pharmacological target(s)	Study (phase)	Intervention/comparator	Trial identifier	Population	N	Primary endpoint(s)	Status	Result	Ref.
IGF-1 receptor inhibitor										
Cixutumumab	IGF-1 receptor inhibitor	I	Single-institution, single-arm, inter-patient, dose-escalation study: everolimus 10mg/day+OCT LAR 20mg/21 day+ Cixutumumab 10 and 15mg/kg every 21 days	NA	Unresectable/metastatic well-differentiated CT and PNETs (G1/G2) + PD	19	Safety and recommended phase II dose	Completed	Median PFS: 43.6 weeks Median OS: 25.5 months	Dasari <i>et al.</i> (2015)
		II	Cixutumumab 10mg/kg every 2 weeks I.V.+OCT LAR	NCT00781911	Unresectable/metastatic well-differentiated CT and PNETs (G1/G2) + PD	43	6-months PFS	Completed	NA	NA
MK-0646	IGF-1 receptor inhibitor	II	MK-0646 10mg/kg once weekly over 1 h	NCT00610129	Metastatic well-differentiated NETs (hindgut, midgut, foregut)	25	ORR	Completed	No PR or CR were observed	Reidy-Lagunes <i>et al.</i> (2012)
Ganitumab (AMG-479)	IGF-1 receptor inhibitor	II	Ganitumab 18 mg/kg every 3 weeks	NCT01024387	Unresectable/metastatic well-differentiated CT and PNETs (G1/G2) + PD	60	ORR	Completed	No partial or complete responses were observed	Strosberg <i>et al.</i> (2013)

CR, complete response; CT, carcinoid tumor; G1/G2, grade 1 and grade 2 (according to the 2010 WHO classification); OS, overall survival; mPFS, median progression-free survival; NA, not available; PD, progressive disease; PFS, progression-free survival; PNET, pancreatic neuroendocrine tumor; PR, partial response; SD, stable disease.

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 anti-tumor and antiangiogenic 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 antiangiogenic 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 showed 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.* 2007, 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 adaptative 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 IGF-BPs. 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.

Figure 6

cMET expression by IHC in a well-differentiated grade 2 human pancreatic neuroendocrine tumor (original magnification $\times 100$). The section was stained with antibody against human cMET (8F11, Leica Biosystems, Newcastle, UK, at 1:60 dilution). Unpublished personal data.

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 500mg three times a day (Kulke *et al.* 2014). Among evaluable telotristat etiprate-treated patients, 5/18 (28%) experienced a $\geq 30\%$ reduction in BM frequency for ≥ 2 weeks, 9/16 (56%) experienced biochemical response ($\geq 50\%$ 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 (IFN α) has also been proposed in the management of NET. However, IFN α 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 IFN α (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 IFN α 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 (β 2-microglobuline) on tumor cells deficient in such expression, as previously shown in SI-NETs (Funa *et al.* 1986). A similar effect of IFN α 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 IFN α 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 takes into consideration the tumor and its microenvironment.

For the elaboration of innovative therapeutical 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 of 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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All authors significantly contributed to the content of this review.

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