REVIEW

Neuroendocrine neoplasms: current and potential diagnostic, predictive and prognostic markers

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Abstract

Some biomarkers for functioning and non-functioning neuroendocrine neoplasms (NENs) are currently available. Despite their application in clinical practice, results should be interpreted cautiously. Considering the variable sensitivity and specificity of these parameters, there is an unmet need for novel biomarkers to improve diagnosis and predict patient outcome. Nowadays, several new biomarkers are being evaluated and may become future tools for the management of NENs. These biomarkers include (1) peptides and growth factors; (2) DNA and RNA markers based on genomics analysis, for example, the so-called NET test, which has been developed for analyzing gene transcripts in circulating blood; (3) circulating tumor/endothelial/progenitor cells or cell-free tumor DNA, which represent minimally invasive methods that would provide additional information for monitoring treatment response and (4) improved imaging techniques with novel radiolabeled somatostatin analogs or peptides. Below we summarize some future directions in the development of novel diagnostic and predictive/prognostic biomarkers in NENs. This review is focused on circulating and selected tissue markers.

Key Words

- neuroendocrine neoplasms
- novel
- markers
- diagnosis
- prognosis

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Introduction

Neuroendocrine neoplasms (NENs) represent a heterogeneous group of rare neoplasms, which originate from enterochromaffin cells that are located throughout the whole body. NENs located in the gastrointestinal tract and pancreas are also referred to as gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) (Modlin *et al.* 2008, Xavier *et al.* 2016). The annual NENs incidence increases over time, although it is not known whether this is a true increase in NEN incidence, the result of increased use of (improved) diagnostic procedures or a combination of both (Xavier *et al.* 2016, Dasari *et al.* 2017).

NENs can be divided into 'functional' and 'nonfunctional' tumors. Functional NENs are able to produce, store and secrete bioactive peptides and can present with specific clinical syndromes related to the biological effects of these substances. Nonfunctional NENs can present with mechanical effects, that is, bowel obstruction or ischemia, but are also frequently discovered by the incidence during diagnostic procedures (Hofland *et al.* 2018). The clinical course of NENs can be highly variable and includes a spectrum ranging from well-differentiated, indolent growing tumors to

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aggressive, highly proliferative tumors. Around 60–80% of NENS are metastasized when diagnosed (Modlin *et al.* 2010*a*). The overall 5-year survival rate of patients with NENs ranges between 35 and 82% in well- to moderately differentiated NENs and between 4 and 38% in poorly differentiated NENs (Pape *et al.* 2004, Yao *et al.* 2008). Survival is specifically determined by several parameters including the localization of the primary tumor (overall 5-year survival: 75.0% for jejunoileal, 42.9% for pancreatic NENs), tumor size, presence of vascular invasion, necrosis, surgical resection margin, metastasis, grade and stage of disease (particularly in G1/G2 NENs with localized, regional and distant disease survival rates of 223, 111 and 33 months have been reported respectively; (Pape *et al.* 2004, Veenendaal *et al.* 2006, Gao *et al.* 2018)). Since several factors are involved in NENs patients' survival, the use of nomograms that combine clinical, biochemical, histological and therapeutic characteristics has been proposed (Modlin *et al.* 2010*b*, Clift *et al.* 2017). These nomograms are mostly not validated yet and currently not used in clinical practice. Appropriate standardized diagnostic procedures are required to assure early diagnosis, monitor disease progression and guide an optimal treatment (Oberg *et al.* 2015). An ideal biomarker should have a high sensitivity for the diagnosis of NENs, to predict tumor clinical behavior and for the response to treatment (Turner *et al.* 2006). To date, only few diagnostic and therapeutic (reflecting treatment response) markers are available with limited performance, but new biomarkers are in development, including peptides/growth factors, DNA/RNA markers and circulating tumor cells. In this review, we describe the currently available and potential future diagnostic, prognostic and therapeutic biomarkers in NEN, with a focus on circulating factors.

Search strategy

We searched the Cochrane Library, MEDLINE and EMBASE up to March 2017. Publications from the past 5 years were predominantly selected. The reference lists of articles identified by this search strategy were screened for relevant publications. Commonly cited and important older publications were also included. Some review articles, but especially original articles, were included.

The following search terms, alone or in combination, were used: 'biomarkers', 'novel markers', 'neuroendocrine tumors', 'NETs', 'neuroendocrine neoplasms', 'NENs', 'circulating markers', 'tissue markers', 'therapeutic markers', 'diagnosis', 'chromogranin A', 'neuron-specific enolase', 'N-terminal pro-brain natriuretic peptide', '5-hydroxyindoleacetic acid', 'pancreatic peptide', 'peptides and growth factors', 'DNA markers', 'epigenetic', 'genomic', 'mutations', 'germline', 'somatic', 'alternative lengthening of telomeres', 'whole-genome sequencing', 'chromatin remodeling', 'DNA repair', 'cell-free DNA', 'RNA markers', 'microRNAs', 'somatostatin receptor expression', 'O-6-methylguanine-DNA methyltransferase', 'mTOR pathway inhibitors', 'everolimus', 'temozolamide', 'alkylant-based chemotherapy', 'somatostatin analogs', 'sunitinib', 'immunotherapy', 'molecular biomarkers', 'epidermal growth factor receptor', 'vascular endothelial growth factor', 'Interleukin-8', 'stromal cell-derived factor-1α', 'circulating tumor cells', 'endothelial cells', 'white cells', 'placental growth factor', 'tuberous sclerosis complex', 'programmed death-1', 'programmed death-1 ligand'.

Currently available biomarkers for NENs

Currently used biochemical markers in NENs are usually hormones or amines secreted by NEN cells, which can be influenced by several factors including co-existent disease(s) and drugs, as shown in Table 1. These biomarkers add to diagnosis, but are insufficient to accurately diagnose NENs, to identify the primary tumor site or to differentiate tumor grading, especially due to sensitivity and specificity issues (Oberg *et al.* 2015). Despite this, some of them are considered for the diagnosis and follow-up of NENs according to several clinical guidelines as shown in Table 2. In Table 3, a summary of the sensitivity and specificity of currently used biomarkers in NENs is depicted.

Chromogranin A

Chromogranin A (CgA) is a protein expressed in the secretory granules of normal and neoplastic neuroendocrine cell types. It is released with peptide hormones and biogenic amines and is also the precursor for functional neuroendocrine peptides (Eskeland *et al.* 1996, D'Amico *et al.* 2014). Several guidelines recommend plasma CgA measurement during diagnosis, treatment and follow-up in GEP-NENs (Table 2). Baseline and serial CgA may predict clinical outcome, prognosis and tumor response (Arnold *et al.* 2008) and may be indicative for local progression in patients with liver involvement (Bajetta *et al.* 1999). Additionally, progressive decrease in CgA levels may be observed in patients with extensive metastatic spread and loss of neuroendocrine differentiation (Zatelli *et al.* 2007).

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Table 1 Foods, drugs and other conditions which interfere with the results of current NENs biomarkers (Schwartz 1983, Batterham *et al*. 2003, Jin *et al*. 2015, Gut *et al*. 2016).

Current marker	False-positive results	False-negative results
5-HIAA	*Foods and drinks:* Fruits (banana, kiwis, avocado, pineapple, plums, tomato, aubergine, figs, grapefruit, melon), red wine, coffee, tea, chocolate, cheese, vegetables (black olives, spinach, broccoli, cauliflower) *Drugs:* Somatostatin analogs, levodopa, methyldopa, heparin, isoniazid, monoamine oxidase inhibitors, methenamine, tricyclic antidepressants, phenothiazines, acetylsalicylic acid	*Drugs:* Paracetamol, naproxen, phenacetin, fluorouracil, testosterone, methysergide, acetanilide, reserpine, atenolol, pindolol, oxprenolol, ephedrine, diazepam, methocarbamol
Cg-A	*Foods and drinks:* 30–90 min after a meal *Drugs:* Proton pump inhibitors, histamine type-2 receptor antagonists *Diseases:* Atrophic gastritis, pancreatitis, chronic hepatitis, liver cirrhosis, impaired kidney function, chronic heart failure, acute coronary syndrome, untreated hypertension, rheumatoid arthritis, irritable bowel syndrome and inflammatory bowel disease *Others:* Strenuous exercise before the test	*Diseases:* Low proliferative, rapidly Proliferating and poorly differentiated NENs
Pancreatic polypeptide	*Foods and drinks:* 30–90 min after a meal *Diseases:* Uncontrolled diabetes mellitus *Others:* Increased age, exercise	*Drugs*: Atropine *Diseases:* Chronic pancreatitis, pancreatic resection

However, CgA is elevated in only 60–80% of patients with NENs and has a limited sensitivity of 60–83% and a relatively low specificity, that is, 72–85% (Table 3; Schurmann *et al*. 1992, Bajetta *et al*. 1999, Seregni *et al*. 2001, Stivanello *et al*. 2001, Nehar *et al*. 2004, Walter *et al*. 2012, Duque *et al*. 2013, Wang *et al*. 2014, Oberg *et al*. 2017). Moreover, proton pump inhibitors, atrophic gastritis and impaired kidney function can induce a rise in CgA levels (Ardill & O'Dorisio 2010, Oberg *et al*. 2017). The combination of CgA with other diagnostic methods, for example, somatostatin receptor scintigraphy, may increase its sensitivity (93%) and specificity (81%) (Kalkner *et al*. 1995, Cimitan *et al*. 2003, Namwongprom *et al*. 2008). Importantly, the sensitivity of CgA depends further on the threshold cut-off (Zatelli *et al*. 2007, Nolting *et al*. 2012, Oberg *et al*. 2017), NEN primary location (Baudin *et al*. 2001, Tomassetti *et al*. 2001, Nolting *et al*. 2012), endocrine-associated syndrome (Modlin *et al*. 2010*a*), disease spread, liver metastases (Zatelli *et al*. 2007, Nikou *et al*. 2008, Nolting *et al*. 2012, Walter *et al*. 2012) and the used assay (Ferrari *et al*. 2004). Despite its use being described in some clinical guidelines, some recent publications suggest a limited applicability as follow-up marker (Marotta *et al*. 2018). Importantly, different analytical properties of the CgA kits give different performances, a fact that must be taken into consideration when comparing results from different clinical studies.

Neuron-specific enolase

Neuron-specific enolase (NSE) is a soluble cerebral protein, which provides information on neural, neuroendocrine and paraneuronal cells (Jorgensen *et al*. 1996). An increase in NSE levels is thought to be related to a high death rate of cells with neuroendocrine differentiation (Bajetta *et al*. 1999). NSE is probably the most reliable tumor marker in diagnosis, prognosis and follow-up of small-cell lung cancer (SCLC) (Isgro *et al*. 2015). This marker may be elevated in 38–40% of GEP-NENs patients, in particular, in those with high-grade tumors (Baudin *et al*. 1998, van Adrichem *et al*. 2016*b*). The specificity of NSE is similar to CgA but with lower sensitivity (Table 3; Grouzmann *et al*. 1990, Nobels *et al*. 1997, Baudin *et al*. 1998). NSE levels have been directly associated with tumor differentiation, aggressiveness and size (Baudin *et al*. 1998, van Adrichem *et al*. 2016*b*). Despite its limited sensitivity, NSE is inversely correlated to overall survival (OS) in ENETS TNM stage IV (van Adrichem *et al*. 2016*b*) and with shorter progression-free survival (PFS), even if CgA levels are normal (Yao *et al*. 2011).

N-terminal pro-brain natriuretic peptide

N-terminal pro-brain natriuretic peptide (NT-proBNP) is a peptide produced by myocardial cells in response to electrolyte and fluid balance. Despite it not being a specific NEN marker, its serum concentration is usually elevated in midgut-NENs with a sensitivity of 87% and a specificity of 80% (Oberg *et al*. 2015, Modlin *et al*. 2016). NT-proBNP is in particular used for evaluating carcinoid heart disease (CHD), and it has been reported that a cut-off value of 260 pg/mL has a sensitivity of 92% and specificity of 91% (Bhattacharyya *et al*. 2008). Interestingly, it has been

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Table 2 Indications of current biomarkers in GEP-NENs according NCCN 2.2015, NANETS 2010, ESMO 2012, ENETS 2009–2016- and UKINETS 2012 guidelines.

Guideline	CgA	NSE	u-5HIAA	Others
NCCN (Kulke *et al.* 2015)	In immunohistochemistry to establish neuroendocrine differentiation For diagnosis (GEP-NENs) For follow-up (GEP-NENs)		For diagnosis (GEP-NENs) For follow-up (GEP-NENs)	*PP:* For PNEN diagnosis
NANETS 2010 (Boudreaux *et al.* 2010, Kulke *et al.* 2010)	For diagnosis (GEP-NENs, bronchial NENs**) For follow-up (GEP-NENs)	Diagnosis bronchial NENs Useful for follow-up (gut-NENs)++	For diagnosis (GEP-NENs) For follow-up (GEP-NENs)	
ESMO 2012 (Oberg *et al.* 2012)	For diagnosis (GEP- bronchial -NENs) For follow-up(GEP-bronchial-NENs)	For bronchial NENs Value as general marker	For midgut, bronchial NENs	*PP:* For non-functioning PNEN diagnosis
ENETS 2009–2016 (O'Toole *et al.* 2009, Niederle *et al.* 2016)	For diagnosis (GEP- bronchial -NENs) For follow-up(GEP-bronchial NENs) Useful in NEC	NEC diagnosis and follow-up	For midgut, bronchial NENs	
UKI NETS 2012 (Ramage *et al.* 2012)	For diagnosis For follow-up		In bronchopulmonary and gut-NENs For follow-up carcinoid syndrome	*NT-proBNP:* To rule out CHD morbidity, midgut NENs *PP:* alternative when CgA is within the reference range NKA: in gut-NENs

**Suggested. ++Limited use.
CHD, carcinoid heart disease; ENETS, European Neuroendocrine Tumor Society; ESMO, European Society of Medical Oncology; Gut-NENs include tumors in the jejunum, ileum, appendix, and cecum; NANETS, North American Neuroendocrine Tumor; NCCN, National Comprehensive Cancer Network; NEC, neuroendocrine carcinoma; NKA, neurokinin A; NSE, plasmatic neuron-specific enolase; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; PNEN, pancreatic neuroendocrine neoplasm; PP, pancreatic polypeptide; u-5HIAA, urinary 5-Hydroxy-indolacetic acid; UKI NETS, UK and Ireland Neuroendocrine Tumour Society.

suggested that patients with elevated NT-proBNP levels combined with increased CgA levels have a worse OS when compared to CgA alone (Korse *et al.* 2009*b*, Oberg *et al.* 2015). Importantly, NT-proBNP is not disease specific; thus, further studies for evaluating its applicability in the progression of CHD are still required (Bhattacharyya *et al.* 2008).

5-hydroxyindoleacetic acid

Serotonin, produced by (midgut) NENs, is the most prominent hormone associated with diarrhea and flushes in carcinoid syndrome. Its metabolite, 5-hydroxyindoleacetic acid (5HIAA), measured in 24-h urine is used as a diagnostic and follow-up marker (Korse *et al.* 2009*a*). Urinary (u) 5HIAA levels are not directly related to the severity of symptoms and large fluctuations within an individual have been described (Zuetenhorst & Taal 2005). The specificity of 5HIAA is around 90%, but the reported sensitivity is 35–68% in patients with NENs (Bajetta *et al.* 1999, Zandee *et al.* 2016, Oberg *et al.* 2017). 5HIAA is mainly used as an indicator of hypersecretory activity in patients with NENs, especially in midgut NENs (Bajetta *et al.* 1999). Its prognostic value, however, is limited. Some studies have related higher urinary 5HIAA levels with mortality (Janson *et al.* 1997), but these results were not reproduced by other studies (Korse *et al.* 2009*a*, Zandee *et al.* 2016). Its combination with other markers also failed to predict OS and for this reason 5HIAA determination is only recommended to assess carcinoid syndrome (Bhattacharyya *et al.* 2008).

Pancreatic peptide

Pancreatic peptide (PP) is a non-specific marker in NENs (Landry *et al.* 2014). Around 63% of pancreas NENs (PNENs) and 18–53% of primary gastrointestinal NENs show increased PP levels (Panzuto *et al.* 2004). Its determination does not seem to increase the diagnostic performance of other markers like CgA, but changes above 50% in PP serum levels seem to correlate with tumor increase on imaging (Walter *et al.* 2012).

Application of currently available biomarkers

Despite the above-mentioned limitations, current biomarkers are regularly used in clinical practice and their accuracy may increase when combined. Current evidence

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Table 3 Sensitivity and specificity of current and novel neuroendocrine biomarkers.

Tumor marker	Primary tumor location	Sensitivity (%)	Specificity (%)
Chromogranin A (Schurmann *et al*. 1992, Bajetta *et al*. 1999, Seregni *et al*. 2001, Nolting *et al*. 2012, Duque *et al*. 2013, Wang *et al*. 2014, Oberg *et al*. 2017)	Non-specific	60–83	72–85
Urinary5-HIAA (Bajetta *et al*. 1999, Zandee *et al*. 2016, Oberg *et al*. 2017)	Midgut	35–68	90–100
Pancreatic polypeptide (Panzuto *et al*. 2004, Metz & Jensen 2008, Oberg *et al*. 2015)	Pancreas, midgut	31–63	~67
Neuron-specific enolase (Baudin *et al*. 1998, Bajetta *et al*. 1999, Oberg *et al*. 2015)	Non-specific	33	73
NT-proBNP (Oberg *et al*. 2015, Modlin *et al*. 2016)	Midgut (non-specific for CHD)	87	80
Pro-GRP (Korse *et al*. 2011)	Lung	43	99
PNMA2 (Cui *et al*. 2010)	SB-NENs	46–50	NDA
DCR (Edfeldt *et al*. 2017)	SB-NENs	AUC: 0.74	
TFF3 (Edfeldt *et al*. 2017)	SB-NENs	AUC: 0.72	
Midkine (Edfeldt *et al*. 2017)	SB-NENs	AUC: 0.71	
Multritranscript genes (Modlin *et al*. 2013, Kidd *et al*. 2015, Bodei *et al*. 2016)	GEP-NENs	75–98	

AUC, area under the curve; NDA, no data available.

suggests that circulating CgA levels should be measured at the diagnosis and during follow-up for evaluating disease course and for evaluating treatment response. NSE may be determined for the diagnosis and follow-up of neuroendocrine carcinomas and to predict outcome in NENs. u-5HIAA measurement is valuable for diagnosis, especially in midgut NENs, and when elevated, it should be determined during follow-up in which it might be used in combination with NT-proBNP (O'Toole *et al*. 2009, Niederle *et al*. 2016).

Importantly, specific comparisons between markers are difficult since several publications are based on heterogeneous cohorts and retrospective analysis. Additionally, the differences between the used assays limit comparisons and solid conclusions. Notwithstanding, several guidelines recommend these biomarkers for the diagnosis and follow-up in NENs (Table 2).

Potential novel diagnostic biomarkers

To improve early diagnosis and follow-up of NENs, several new prognostic and treatment-related biomarkers have been developed in recent years (Fig. 1). Most of them are still under study and not yet available for use in clinical practice. It is aimed to develop high-specific and sensitive circulating biomarkers using DNA, RNA and metabolomic approaches. Combination markers and multianalyte analysis may be more effective than the current use of monoanalytes because of a higher sensitivity (Modlin *et al*. 2013, 2015, Oberg *et al*. 2015), although validation is still needed. A summary of potential novel circulating and tissue biomarkers for diagnosis, prognosis and therapy response prediction, as well as their relation with tumor localization, is shown in Fig. 2. Herein we describe the potential applicability of novel peptides/growth factors, DNA, RNA and therapeutic markers for NENs, in particular, circulating biomarkers.

Peptides and growth factors

Several peptides and growth factors (Table 4) have been studied for a (potential) role as biomarker in NENs and may (1) help to localize primary tumors (e.g. progastrin-releasing peptide in lung NENs, connective tissue growth factor (CCN2), paraneoplastic Ma antigen 2, DcR3, TFF3 and midkine in small intestine NENs (Bergestuen *et al*. 2010, Korse *et al*. 2011, Oberg *et al*. 2015, Edfeldt *et al*. 2017)); (2) predict the outcome in functioning NENs (e.g. α-Internexin in insulinomas (Schimmack *et al*. 2012, Liu *et al*. 2014)) or predict early complications in patients with CHD (CCN2 (Bergestuen *et al*. 2010)) and (3) add information to that provided by other circulating/tissue markers for treatment response evaluation and outcome prediction (e.g. pro-GRP and CgA for predicting outcome/therapeutic response in lung carcinoids; α-Internexin in combination with Ki67 for aggressiveness prediction in insulinomas (Grabowski *et al*. 2005, Korse *et al*. 2011, Fotouhi *et al*. 2016, Fujino *et al*. 2016) or as part of multianalytes tests (Edfeldt *et al*. 2017)).

Although imaging markers are not described in this review, it is important to mention that some peptides may be useful to correlate with imaging techniques. For instance, glucose transporter 1 (GLUT1) expression in NENs is associated with the Ki67 index and

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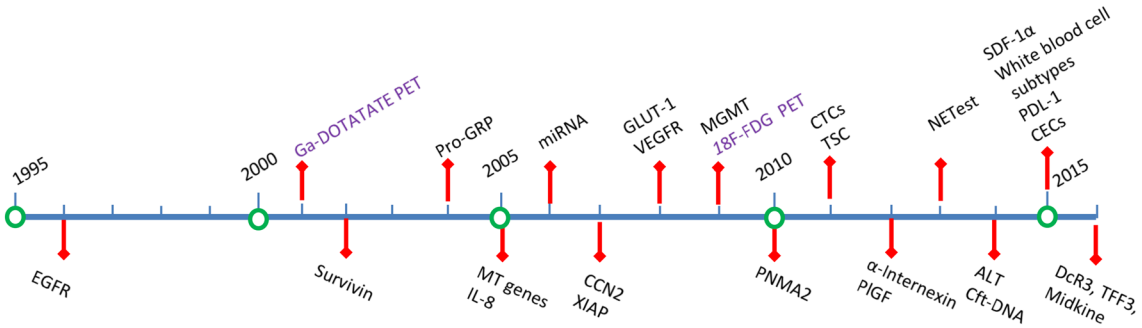

Figure 1
Timeline of the publication of potential novel biochemical and therapeutic markers in neuroendocrine neoplasms during the last two decades. Monoanalytes, transcripts, DNA-, RNA- immune- markers are shown, mostly are still under study and not available for use in clinical practice. Image-based modalities are represented in purple. 18F-FDG PET, 18fludeoxyglucose positron emission tomography; ALT, alternative lengthening of telomeres; CCN2, connective tissue growth factor; CECs, circulating endothelial cells cftDNA, circulating cell-free tumor DNA; CTCs, circulating tumor cells; EGFR, epidermal growth factor receptor; GLUT-1, glucose transporters type 1; IL-8, interleukin 8; MGMT, O-6-methylguanine-DNA methyltransferase; miRNA, microRNA; MT, multitranscript; PD-L, programmed death ligand-1; PlGF, Placental growth factor; PNMA2, paraneoplastic Ma antigen 2; proGRP, progastrin-releasing peptide; SDF-1α, stromal cell-derived factor 1α; TSC, Tuberous sclerosis complex; VEGFR, vascular endothelial growth factor receptor; XIAP, X-linked inhibitor of apoptosis. Imaging techniques, as reference, are presented in purple.

18-fluorodeoxyglucose (FDG) uptake at FDG-positron emission tomography (PET) scans (Binderup *et al.* 2013). GLUT-1 expression may serve as an additional marker for aggressiveness of NENs and may add to a more accurate grading (Binderup *et al.* 2013).

Although some of these peptides have been suggested as promising biomarkers, most of them are non-specific. In addition, their applicability is limited, due to their sensitivity and specificity (Table 3) and the absence of appropriate cut-off levels. In addition, some of them have been described only in single retrospective studies; thus, further validation in larger and longitudinal cohorts is still required. A summary of potential peptide/growth factors markers for NENs is described in Table 4.

Genetic and epigenetic markers

Generally, tissue and circulating tumor DNA markers may provide information on the genetic characteristics of the tumor which may result in better prediction of clinical

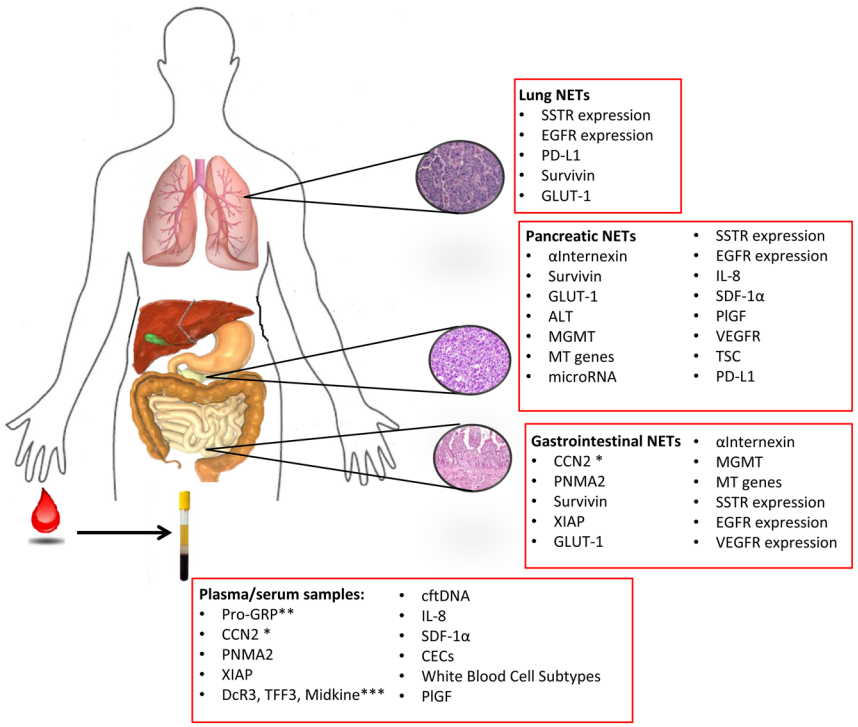

Figure 2
Summary of potential novel diagnostic and therapeutic markers in neuroendocrine neoplasms. Several tumor or plasma/serum biomarkers seem to play a role in the diagnosis or follow-up in lung and GEP-NENs. Its presence may be determined in serum or tissue samples. Please refer to the text for explanation of each tumor marker. *specific for carcinoid heart disease; **only for lung NENs; ***only for small intestine NENs; ALT, alternative lengthening of telomeres; CCN2, connective tissue growth factor for carcinoid heart disease; CECs, circulating endothelial cells; cftDNA, circulating cell-free tumor DNA; EGFR, epidermal growth factor receptor; GLUT-1, glucose transporters type 1; MGMT, O-6-methylguanine-DNA methyltransferase; MT, multitranscript; PD-L, programmed death ligand-1; PlGF, placental growth factor; PNMA2, paraneoplastic Ma antigen 2; proGRP, progastrin-releasing peptide; SDF-1α, stromal cell-derived factor 1α; SST, somatostatin; TSC, tuberous sclerosis complex; VEGFR, vascular endothelial growth factor receptor; XIAP, X-linked inhibitor of apoptosis.

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Table 4 Peptides and growth factors as novel markers in NENs.

Peptide/growth factor	Function	Potential role as marker in NENs
Progastrin-releasing peptide (proGRP)	Precursor of gastrin-releasing peptide, a neuropeptide hormone widely distributed throughout the gastrointestinal and pulmonary tract (McDonald *et al*. 1979)	Primary tumor localization in patients with a metastatic NEN of unknown origin. Complementary marker to CgA in lung NEN for treatment response evaluation and survival (Korse *et al*. 2011, 2015)
Connective tissue growth factor for carcinoid heart disease (CCN2)	CCN2 is an early gene product of the CCN family of matricellular proteins, which are involved in cell proliferation, angiogenesis, tumorigenesis and wound healing. It may be involved in the pathogenesis of carcinoid heart disease (Holbourn *et al*. 2008, Bergestuen *et al*. 2010)	Independent predictor of both reduced right ventricular function and right-sided valve regurgitation (its plasma levels are inversely related to right ventricular function levels) Early predictor of cardiac fibrosis (Bergestuen *et al*. 2010)
Paraneoplastic Ma antigen 2 (PNMA2)	Antineuronal antibodies identified as markers of neurological paraneoplastic syndromes (Schuller *et al*. 2005)	Allows the identification of almost 50% of SB-NENs at the primary stage of the disease Correlation with disease progression and recurrence free survival (Cui *et al*. 2010)
α-Internexin	Cytoskeleton protein involved in tumorigenesis and disease progression (Nathke 2006)	Association with proliferation, ki67 index and malignancy (Schimmack *et al*. 2012)
X-linked inhibitor of apoptosis (XIAP)	Inhibitor of apoptotic cell death in cancer cells (Mihaly *et al*. 2014, Obexer & Ausserlechner 2014)	Potential target therapies (Yap *et al*. 2011, Cingarlini *et al*. 2012, Augeri *et al*. 2016)
Glucose transporters type 1 (GLUT-1)	Mediates the transport of glucose across the cellular membrane and are commonly overexpressed in tumors, probably related with higher metabolism and cell growth (Clavo *et al*. 1995)	Predictor of risk of death in neuroendocrine lung carcinomas and lung carcinoids (Ozbudak *et al*. 2009) Relation with Ki67 index in GEP- and lung NENs (Binderup *et al*. 2013, Benzerdjeb *et al*. 2017) Correlation with the uptake in 18-FDG-PET (Binderup *et al*. 2013)
DcR3	Regulates cytokines which influence tumor growth and reduce apoptotic stimuli (Lin & Hsieh 2011)	DcR3 correlates to liver metastasis and worse survival Predictor of treatment resistant tumors (Edfeldt *et al*. 2017)
TFF3	Protects and repairs epithelial surfaces Enhances migration, angiogenesis, and inhibits apoptosis (Vestergaard *et al*. 2010, Casado *et al*. 2012, Huang *et al*. 2013)	Higher concentrations have been correlated to reduced survival (Edfeldt *et al*. 2017)
Midkine	Promotes tumor cells migration, angiogenesis and reduces apoptosis (Jones 2014)	Predictive marker to chemotherapy response(Hu *et al*. 2010, Wu *et al*. 2015)

outcome and could aid in clinical decision making (Modlin *et al*. 2014*b*). The possibility of performing liquid biopsies is expected to anticipate malignancy of solid lesions in a non-invasive way, but it is still necessary to optimize the detection technique, analysis and interpretation.

An example of a tissue DNA prognostic marker for NENs involves alternative lengthening of telomeres (ALT). A telomerase-independent mechanism to avoid the chromosome end replication was suggested in tumor cells, and in this context, ALT was reported in liver metastasis of NENs (Dogeas *et al*. 2014). In PNENs, whole-exome sequencing studies have demonstrated that inactivating mutually exclusive mutations in X-linked transcriptional regulator (*ATRX*) and death domain-associated protein 6 (*DAXX*) genes (Jiao *et al*. 2011) are associated with the ALT (Heaphy *et al*. 2011, Schwartzentruber *et al*. 2012). However, results on its relationship with clinical features are inconclusive and in some cases contradictory. In this sense, ALT-positive PNENs have been associated with larger tumor size, grading, vascular/perineural invasion and metastasis (Marinoni *et al*. 2014, Kim *et al*. 2017). In addition, an increased risk of recurrence and decreased OS has been reported in ALT-positive NEN metastasis (Marinoni *et al*. 2014, Kim *et al*. 2017). In contrast, other authors found an association with better clinical outcomes (Jiao *et al*. 2011, Dogeas *et al*. 2014) including metastatic disease (Kim *et al*. 2017). Since it is possible to determine ALT, ATRX and DAXX using fine-needle

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aspiration (FNA), they may be used as minimally invasive prognostic markers in NENs (VandenBussche *et al.* 2017), but their clinical significance should still be evaluated in detail.

Additionally, whole-genome sequencing in NENs allow the identification of genomic events related with tumor pathogenesis. In this sense, germline deleterious mutations affecting DNA damage repair in PNENs have been described (in the base-excision-repair *MUTYH* gene or the homologous recombination gene *BRCA2)* (Scarpa *et al.* 2017). Furthermore, mutations in genes involved in chromatin remodeling, DNA repair and mTOR signaling may also play a role in PNEN pathogenesis (Scarpa *et al.* 2017). Several publications have also shown chromosome losses affecting genes related to DNA repair or damage checkpoints (*VHL*, *MEN1*, *ATM*, *PTEN*) in PNENs (Capurso *et al.* 2012, Scarpa *et al.* 2017). In this sense, most recent reviews describe genetic alterations that are consistently related with the loss of *MEN1* function, the activation of the PI3K/mTOR pathway, changes in chromatin remodeling and telomeres alteration (Mafficini & Scarpa 2018).

Importantly, somatic mutations and deletions have also been described in midgut NENs, specifically in the cyclin-dependent kinase inhibitor gene *CDKN1B*. This finding suggests that the p21/p27/p57 family, which is involved in the cell cycle, may also be involved in the pathogenesis of small-bowel NENs (Francis *et al.* 2013). Genetic alterations in PNENs are extensively reviewed in a recent publication by Stevenson and colleagues (Stevenson *et al.* 2018).

Cell-free DNA (cftDNA) from liquid biopsies may become a biomarker in NENs, although to date, no studies have been published yet on the detection of cftDNA in NENs. cftDNA generally contains identical genetic defects as the primary tumor (Diaz & Bardelli 2014), is released after apoptosis (Francis & Stein 2015) and can be detected in serum (Marzese *et al.* 2013, Rothe *et al.* 2014). The analysis of cftDNA may be useful for early detection of (residual or recurrent) disease, to monitor tumor burden, to assess molecular heterogeneity and to predict PFS (Francis & Stein 2015), especially in adenocarcinomas (Francis & Stein 2015). Measurement of cftDNA might be a promising tool in NENs, but needs further investigation (Rizzo & Meyer 2018).The ultimate aim of cftDNA assessment would be to add to or replace tissue biopsies (Khan *et al.* 2013, Sikora *et al.* 2015). Prerequisites for cftDNA analysis are sufficient tumoral DNA release and the presence of tumor-related mutations to identify tumor DNA. These conditions may only be present in a subset of NEN patients, thereby limiting the applicability of cftDNA measurement. Further study is needed to determine the potential role of cftDNA analysis in NEN.

A summary of genetic markers in NENs is shown in Fig. 3.

RNA markers

RNA markers are novel and potentially promising minimally invasive markers used for diagnostic purpose and/or to identify the therapeutic targets of NENs. Specifically, the identification of circulating target gene mRNAs using PCR amplification has been used for determining stage, prognosis, recurrence or new metastasis in several cancers (van't Veer *et al.* 2002, Frederiksen *et al.* 2003, Hess *et al.* 2006). Blood circulating- and tumor tissue-transcripts from GEP- and bronchopulmonary NENs are highly correlated (Cwikla *et al.* 2015, Kidd *et al.* 2015). Modlin *et al.* have developed a PCR-based molecular test using 51 genes for identifying GEP-NENs (Modlin *et al.* 2013). For this so-called NETest, a score, based on tissue and peripheral blood transcriptomes, was developed (Modlin *et al.* 2013) as a prognostic and follow-up tool for NENs (Modlin *et al.* 2013, 2014*a*, 2015, Kidd *et al.* 2015). NETest results were shown to differentiate progressive disease (Kidd *et al.* 2015) and predict tumor response to somatostatin analogs (SSAs) (Cwikla *et al.* 2015). Further prospective validation of this test is awaited.

Additionally, dysregulated miRNAs have been correlated with diagnosis, staging, progression, prognosis and therapeutic response in several tumors, including NENs (Di Leva & Croce 2013). miRNAs are endogenous, small (19–25 nucleotides), non-coding RNAs that regulate post-transcriptional gene expression by binding to mRNA molecules, and they probably modulate the expression of at least one-third of protein-coding genes (Demes *et al.* 2016). miRNAs have the capacity to target different genes implicated in the same pathway and/or in interacting pathways, allowing for the possibility of developing directed therapies that could silence several tumor pathways (Reddy 2015).

Specific patterns of miRNA expression may distinguish tumor tissue from normal tissue and acinar tumors from PNENs (Roldo *et al.* 2006, Vicentini *et al.* 2014). Some miRNAs are upregulated in PNENs (miR-140, miR-210), small-bowel (SB-)NENs (miR-96, miR-182, miR-183, miR-196a, miR-200a) and lung carcinoids (mMR-34a) (Li *et al.* 2013, Demes *et al.* 2016). Also, the downregulation of miRNA-133a, miRNA-1 and miRNA-143-3p has been demonstrated in metastasis when compared to the

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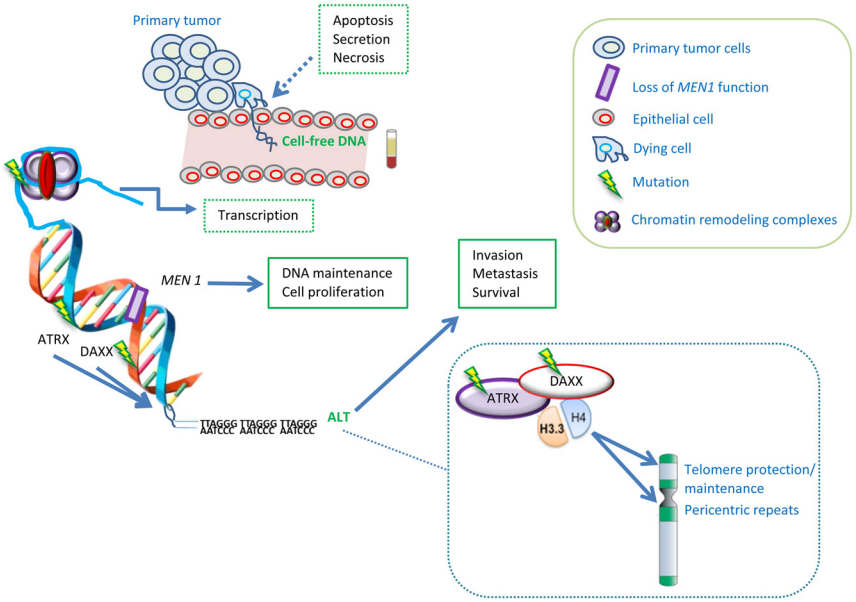

Figure 3
Summary of genetic markers in pancreatic neuroendocrine neoplasms. Genetic markers are presented in green. Tumor cells release small fragments of cftDNA into circulation by multiple mechanisms, cftDNA contains identical genetic defects compared to the primary tumor. Mutations affecting chromatin remodeling and MEN1 function may be related to tumor proliferation. Inactivating mutations in ATRX and DAXX genes are associated with ALT. ALT, alternative lengthening of telomeres; ATRX, X-linked transcriptional regulator; cftDNA, cell-free tumor DNA; DAXX, death domain-associated protein 6.

primary NEN tumor (Ruebel *et al.* 2010, Miller *et al.* 2016). As prognostic marker, miRNA levels have been correlated with Ki-67 (Thorns *et al.* 2014, Arvidsson *et al.* 2018), degree of malignancy (e.g. miR-21 in PNENs; miR-13/miR-204-5p in SB-NENs) (Demes *et al.* 2016, Arvidsson *et al.* 2018) and OS (e.g. the downregulation of miR-375 in SB-NENs (Arvidsson *et al.* 2018)).

miRNAs may also be used for therapeutic goals, for example, inhibition of oncogenic miRNA expression or the introduction of a tumor suppressor miRNA (Vicentini *et al.* 2014). However, the currently available technology is not robust enough to support diagnostic or therapeutic use of miRNAs yet (Oberg *et al.* 2016). Furthermore, dysregulation of miRNAs is not tumor specific and the absence of cut-off levels for differentiating tissue and tumor subtypes, the lack of reproducibility in other NEN cohorts and the difficulties in their interpretation, currently limit their clinical application. Further studies are required to evaluate the application of miRNAs as clinical and therapeutic markers in NENs. This issue is extensively reviewed in recent publications (Zatelli *et al.* 2017, Zimmermann *et al.* 2018, Rizzo & Meyer 2018, Panarelli *et al.* 2019).

Potential novel diagnostic-therapeutic biomarkers

Some (potential) circulating and tissue therapeutic markers are available in NENs. To a certain extent, the currently available markers CgA and NSE can be used for treatment monitoring. Variations in serum CgA levels after treatment with SSAs and PRRT have been reported. Specifically, decreased CgA in stable/responsive tumors has been observed (Caplin *et al.* 2014, van der Zwan *et al.* 2015), but serum CgA may also increase (>20%) due to radiation-induced cell damage or lysis after the first cycle of PRRT. In the latter case, this increase was followed by declined levels usually after 12 weeks (Brabander *et al.* 2017). Furthermore, tumor shrinkage has been associated with CgA or NSE response after treating patients with everolimus (increased PFS in patients with early CgA/NSE response) (Li *et al.* 2011). However, specific cut-off values to define response to different treatment modalities have not been determined yet and novel/specific therapeutic biomarkers are still required. A summary of clinical applicability of novel biomarkers in NENs is presented in Table 5.

Somatostatin receptor expression

Somatostatin receptor (SST) expression by NENs, in particular subtype 2, is used for imaging to diagnose and stage NENs (Wong *et al.* 2012) and is considered to have therapeutic implications for treatment with SSAs and PRRT with radiolabeled somatostatin analogs as Lutetium-177- or Yttrium-90-coupled analogs (Kwekkeboom *et al.* 2010, Strosberg *et al.* 2017). SST expression as imaging marker is not described in this review, but it has been comprehensively evaluated in several publications (de Herder *et al.* 2006, Kwekkeboom *et al.* 2010, Kunikowska *et al.* 2017, Bodei & Weber 2018, Hope *et al.* 2018).

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SST can also be evaluated in NEN tissue samples using immunohistochemistry and qPCR (Fig. 4; Righi *et al*. 2010, Mizutani *et al*. 2012, Lambertini *et al*. 2013, Kanakis *et al*. 2015, Herrera-Martinez *et al*. 2017, 2018). SST expression may help to differentiate normal and tumor tissue in lung carcinoids and GEP-NENs and has been related to vascular/nerve invasion and metastasis (Herrera-Martinez *et al*. 2017, 2018). The tumor SST expression profile may also be helpful for predicting treatment response (Reubi *et al*. 2010, Righi *et al*. 2010), especially in aggressive cases (Righi *et al*. 2010). On the other hand, the immunohistochemical assessment of NEN tissue expression of SST subtype 2 had no additional value compared to the uptake on the OctreoScan in predicting tumor response after PRRT (Korner *et al*. 2012, Bison *et al*. 2014, van Adrichem *et al*. 2016*a*). The tumoral expression of SST3 and SST5 may also be a relevant marker supporting the potential benefit of novel SSAs. Truncated isoforms of SST can also be expressed by NENs, and their presence is associated with aggressive features (Sampedro-Nunez *et al*. 2016). Unfortunately, results are based on retrospective heterogeneous cohorts, which may limit the reproducibility. Additionally, in some of the above indicated studies, SST expression by qPCR was determined in formalin-fixed paraffin embedded tumors, which may affect the expression profile in some samples (Sampedro-Nunez *et al*. 2016, Herrera-Martinez *et al*. 2017, 2018).

Table 5 Clinical applicability of potential new biomarkers in NENs.

	Function	
Clinical diagnosis	Therapeutic role	Prognostic role
ALT	ALT(?)	ALT*
cftDNA	SST expression	Somatic/germline mutations*
microRNAs	MGMT	cftDNA*
SST expression	VEGF	microRNAs***
PlGF (?)	IL-8 (?)	SST expression***
proGRP	PD-1/PD-L1	MGMT**
CCN2	XIAP	EGFR**
PNMA2		VEGF*
DcR3		IL-8***
		(SDF)-1á ***
		PlGF***
		TSC**
		PD-1/PD-L1***
		proGRP with CgA*
		CCN2*
		PNMA2*
		á-Internexin*
		GLUT-1*
		DcR3, TFF3, midkine***

*Disease course predictor; **Intervention outcome predictor; ***Disease and treatment predictor.
ALT, alternative lengthening of telomeres; CCN2, connective tissue growth factor for carcinoid heart disease; cftDNA, cell-free DNA; CTCs, circulating tumor cells; EGFR, epidermal growth factor receptor; GLUT-1, glucose transporters type 1; IL-8, interleukin-8; MGMT, O-6-methylguanine-DNA methyltransferase; PD-1, programmed death-1; PD-L1, programmed death ligand 1; PlGF, placental growth factor; PNMA2, paraneoplastic Ma antigen 2; proGRP, progastrin-releasing peptide; SDF-1α, stromal cell-derived factor-1α; SST, somatostatin receptor; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis.

O-6-methylguanine-DNA methyltransferase (MGMT)

An example of a tissue DNA therapeutic marker for NENs is the methylation pattern of the DNA repair enzyme O-6-methylguanine-DNA methyltransferase (MGMT). Several agents have antitumor effects by inducing DNA methylation at the O6 position of guanine, resulting in apoptosis and tumor cell death (Liu & Gerson 2006). Those lesions can be restored by MGMT, reduction of which may increase the sensitivity of tumor cells to alkylation-induced DNA damage (Christmann *et al*. 2011). The methylation of MGMT promoter and loss of MGMT protein expression have been reported in GEP-NENs (Walter *et al*. 2015). Decreased MGMT expression has been associated to tumor sensitivity to alkylant-based chemotherapy agents, for example, temozolomide, alone or in combination therapy (Gerson 2002). In PNENs, absence of MGMT is more common compared to intestinal or lung carcinoids, which explains the better treatment response in PNENs to temozolomide (Kulke *et al*. 2009, Schmitt *et al*. 2014).

Further, a 'hypermethylator' phenotype, which is characterized by the presence of a high number of methylated genes, has been associated with more progressive disease and shorter survival (Walter *et al*. 2015), whereas this association was not found in well-differentiated PNENs (Raj *et al*. 2017). The predictive value of MGMT status for treatment response in NENs will be evaluated in clinical trials with alkylating agents (NCT03217097).

Molecular biomarkers for treatment with tyrosine kinase inhibitors (TKIs)

In patients with advanced, well-differentiated, progressive PNENs, sunitinib can induce tumor stabilization and improve PFS. Currently, sunitinib is used for progressive disease, but its combination with SSAs, chemotherapeutic agents or neo-adjuvant or adjuvant therapy in earlier stages of resectable PNENs has also been proposed (Delbaldo *et al*. 2012, Mateo *et al*. 2012). The possibility to peripherally measure monoanalytes directly related to

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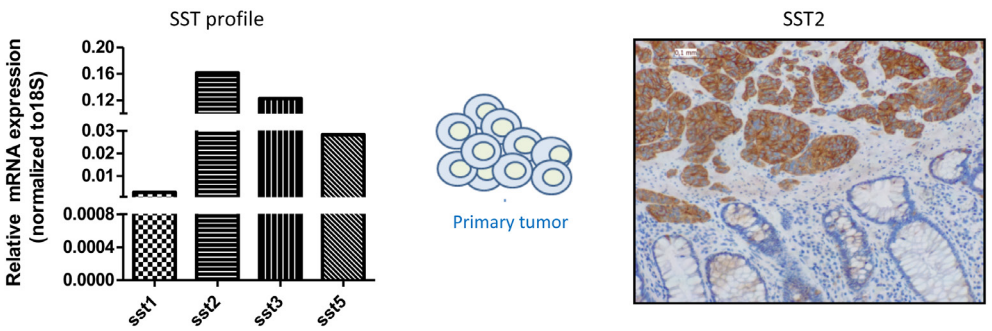

Figure 4
Somatostatin receptors expression for diagnosis. SST expression may be determined in tumor using immunochemistry (sst2 immunostaining in a GEP-NEN; right panel) and/or RT-qPCR (SST subtype mRNA profile in an exemplary GEP-NEN; left panel). Both results may be used for treatment decisions but also to predict outcome. SST, somatostatin.

the drug mechanism of action represents an important approach to predict treatment response; in this sense, some molecular biomarkers have been examined in the last years.

Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is overexpressed in non–small-cell lung cancer and an increased sensitivity to the TKIs erlotinib and gefitinib has been reported when the tyrosine kinase domain of the EGFR has somatic mutations (Paez *et al.* 2004). EGFR expression by immunohistochemistry was demonstrated in 21–28% of typical carcinoids, 29–57% of atypical lung carcinoids (Rusch *et al.* 1996, Rickman *et al.* 2009) and in 40–96% of NENs, while its gene amplification by fluorescent *in situ* hybridization was observed in 55% of cases (Srivastava *et al.* 2001, Shah *et al.* 2006, Rickman *et al.* 2009, Gilbert *et al.* 2010). In addition, activating EGFR mutations have been described in NENs and may be associated with improved treatment response (Costanzo *et al.* 2013, Aroldi *et al.* 2014). Importantly, EGFR mutations are rare in GEP-NENs, and their clinical relevance may be limited as compared with other tumors including lung adenocarcinoma (Park *et al.* 2016). The specific clinical significance of the expression of EGFR, however, is still to be determined.

Vascular endothelial growth factor receptor

Vascular endothelial growth factor (VEGF) is the most important regulatory factor of tumor angiogenesis and has been related to cell survival, growth and metastasis (Niu & Chen 2010). VEGF may be determined in tumor and in FNA samples (Angelescu *et al.* 2013). In general, well-differentiated GEP-NENs express high levels of VEGF and its transmembrane receptors ((VEFGR-1, VEFGR-2, VEFGR-3) (Pavel *et al.* 2005)), which can be detected in peripheral blood (Mateo *et al.* 2012). In this context, a worse clinical course has been reported in NENs with increased VEGF expression (Terris *et al.* 1998, Pavel *et al.* 2005, Zhang *et al.* 2007, Yao *et al.* 2011). Immunohistochemical and molecular expression of VEGFR-1/-2 has been reported in over 50% of GEP-NENs (La Rosa *et al.* 2003, Angelescu *et al.* 2013) without being directly related to tumor malignancy (La Rosa *et al.* 2003).

Some relations between VEGFR and patient outcome have been described; specifically, low VEGFR-1 levels have been related to longer PFS (Yao *et al.* 2012), high baseline VEGFR-2 levels to decreased OS in PNENs (Zurita *et al.* 2015) and low VEGFR-3 levels to longer PFS/OS in carcinoid patients (Zurita *et al.* 2015). Increased VEGF concentrations accompanied by decreased VEGFR-2 and VEGFR-3 levels have been reported after treatment with sunitinib (Deprimo *et al.* 2007), but their levels returned to baseline during the off-treatment period (Zurita *et al.* 2015). Unfortunately, VEGFRs are not tumor specific; additionally, cut-off levels are also necessary to predict outcome. The applicability of VEGFR-1 as a therapeutic predictive marker is currently considered in clinical trials (pazopanib in advanced NENs, NCT01280201), but further studies are still required.

Interleukin-8

Interleukin-8 (IL-8) has proangiogenic, mitogenic and motogenic effects through the activation of the receptors CXCR1 and CXCR2 (Mateo *et al.* 2012). PNENs overexpress not only IL-8 but also its receptor CXCR2 (Tecimer *et al.* 2000, Hussain *et al.* 2010). IL-8 seems to be increased in progressive NENs, while lower IL-8 baseline levels have been associated with longer survival (Pavel *et al.* 2005). Patients with SB-NENs and lower IL-8 levels were shown to

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have disease stabilization or clinical response to sunitinib (Bello *et al.* 2006). It has also been hypothesized that monitoring IL-8 in plasma during sunitinib treatment could be useful to predict drug resistance (Huang *et al.* 2010), but this needs further investigation.

Stromal cell-derived factor (SDF)-1α

SDF-1α plays a role in cell migration, proliferation, survival and angiogenesis (Mateo *et al.* 2012) and is the natural ligand of CXCR4. CXCR4 has been reported in several cancer types (Balkwill 2004), seems to be involved in tumor progression, metastasis, hypoxia adaptation and stem cell survival (Kaemmerer *et al.* 2015) and may serve as a marker of tumor activity and progression (Zurita *et al.* 2015). SDF-1α expression seems to be increased in PNENs compared to other NENs and is inversely correlated with time to disease progression (Zurita *et al.* 2015). In addition, it has been described as a circulating biomarker associated with tumor response to sunitinib (Antonuzzo *et al.* 2013). Arvidsson and collaborators described downregulation of SDF-1α and upregulation of CXCR4 in hypoxic carcinoid cells with consequently higher cell migration, probably due to the activation of the mitogen-activated protein kinase pathway. Based on this, a putative role in antiangiogenic drugs resistance was also suggested, and SDF-1α may serve as a therapeutic target (Arvidsson *et al.* 2010).

Circulating tumor, endothelial and white cells

Circulating tumor cells (CTCs) have been widely used in several tumors as peripheral blood tumor markers (Cristofanilli *et al.* 2005, Cohen *et al.* 2008, Resel Folkersma *et al.* 2012). The identification of cellular expression of the epithelial cell adhesion molecule (EpCAM) allowed the determination of CTCs in NENs (Modlin *et al.* 2016), in which a threshold of 1 CTC (similarly to breast cancer) was demonstrated (Khan *et al.* 2011). CTCs have been associated with higher tumor grade and burden, increased circulating CgA, Ki67 index and worse PFS and OS in grade 1–2 NENs (Khan *et al.* 2011, 2013). Measurement and molecular characterization of CTCs may be helpful to stratify patients for specific therapies in the future (Khan *et al* 2011, Zatelli *et al.* 2017). However, the sensitivity of CTCs varies according to the NEN type, and CTCs are not specific for any subgroup of tumors (Oberg *et al.* 2015).

Additionally, circulating endothelial cells (CECs) were also described in NENs, specifically two different subpopulations: endothelial precursors derived from the bone marrow (CEPs) and mature CECs (Nolan *et al.* 2007). Increased circulating CECs have been related to vessel damage during antiangiogenic treatment and consequently with a longer PFS (Beaudry *et al.* 2005). Theoretically, CEPs should decrease after anti-VEGF therapy (Kalka *et al.* 2000); but in contrast, decreased CECs and stable CEPs were observed in NENs after the first cycle of treatment with sunitinib (Zurita *et al.* 2015).

Moreover, myeloid cells have been related to angiogenesis, disease progression, metastasis and the expression of some receptors related with the VEGF and SDF-1α pathways in other tumors (Fernandez Pujol *et al.* 2000, Condeelis & Pollard 2006). Zurita *et al.* described decreased CD14+ monocytes expressing VEGFR-1 and CXCR4 in NENs treated with sunitinib. It has been postulated that possible relations exist between specific monocyte subpopulations and drug pharmacodynamics, which would be useful as a treatment response predictor (Zurita *et al.* 2015). Because of the heterogeneity of the included patient samples (grading, previous treatments and origin of primary tumor), results remain controversial (Antonuzzo *et al.* 2013). Currently, there is no consensus for supporting the use of CTCs or CECs as an indicator of tumor burden or parameter of treatment response in NENs (Oberg *et al.* 2016). Further prospective, longitudinal studies in this field are still required. A summary of current tumor biomarkers for TKIs is shown in Fig. 5.

Molecular biomarkers for mTOR pathway inhibitors

Increased PFS has been described in patients with advanced metastatic PNENs treated with the mTOR pathway inhibitor everolimus (Yao *et al.* 2010, Pavel *et al.* 2011). Although the combination of everolimus and SSAs does not seem to increase OS, the heterogeneity of the included patients makes these results inconclusive (Pavel *et al.* 2017). Based on this, it would be valuable to develop markers that could early identify those patients who may benefit from everolimus alone or in combination with SSAs.

In this sense, placental growth factor (PlGF) has been related to angiogenesis, tumor burden, presence of metastases and survival (Carmeliet *et al.* 2001, Fischer *et al.* 2008) and is thought to reflect the activation of AKT and ERK pathways (Parr *et al.* 2005, Fischer *et al.* 2008, Wei *et al.* 2009). Elevated levels of circulating PlGF have been demonstrated in PNENs, with increasing levels from grade 1 to grade 3 tumors (Hilfenhaus *et al.* 2013). Decreased circulating PlGF was observed after treatment with everolimus in the RADIANT-III study (Hilfenhaus *et al.* 2013). PlGF levels reflect tumor aggressiveness

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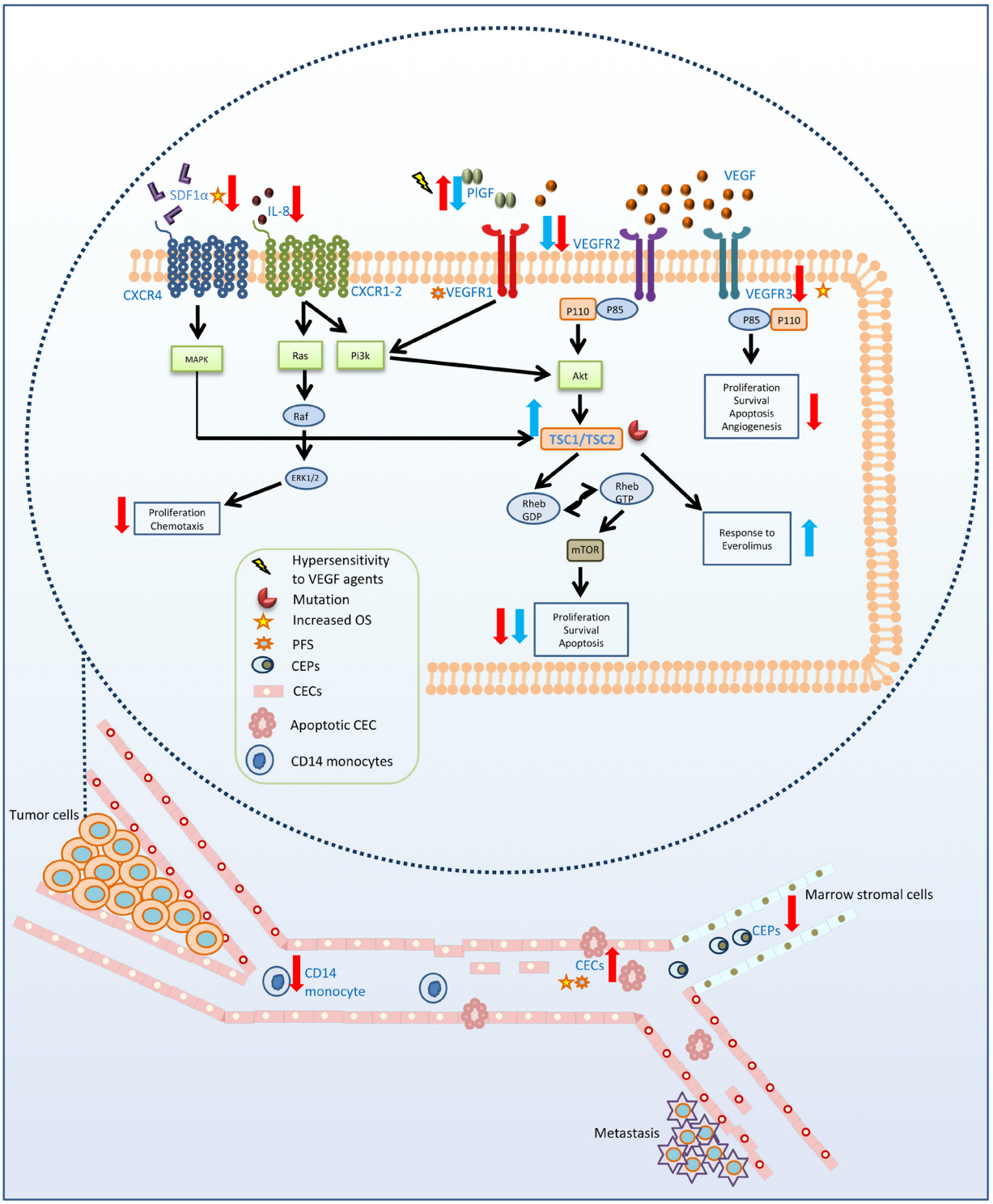

Figure 5

Therapeutic markers: molecular biomarkers for tyrosine kinase and mTOR inhibitors. Blue arrows represent the effect of the mTOR inhibitor everolimus and red arrows the effect of the tyrosine kinase inhibitor sunitinib. Molecular markers are presented in blue. Response to sunitinib has been related to decreased SDF1, IL-8, VEGFR 2-3, CD14 monocytes expressing VEGFR, decreased CEPs, increased CECs and probably decreased PlGF. Response to everolimus has been related to decreased PlGF and VEGFR2. Factors related to progression-free survival (PFS) and OS are also shown. CECs, circulating endothelial cells; CPECs, circulating endothelial precursors derived from the bone marrow; CXCR 1,2,4, chemokine family receptor 1,2,4; IL-8, interleukin-8; PlGF, placental growth factor; SDF-1α, stromal cell-derived factor 1α; TSC 1-2, tuberous sclerosis complex 1-2; VEGF, vascular endothelial growth factor; VEGFR 1–3, vascular endothelial growth factor receptor 1–3.

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(Hilfenhaus *et al.* 2013), whereas others studies suggest that PlGF may also induce the formation of vascular networks that are hypersensitive to anti-VEGF therapy (Hedlund *et al.* 2013). Tumor-derived PlGF was postulated to become a potential predictive marker of anti-VEGF therapy (Hedlund *et al.* 2013).

As previously described for TKIs markers, midgut NENs and PNENs overexpress VEGF and its receptors (Christofori *et al.* 1995, Terris *et al.* 1998, La Rosa *et al.* 2003). In the RADIANT-III trial, a significantly progressive reduction in VEGFR-2 was observed after treatment with everolimus (Yao *et al.* 2012), but the specific clinical relevance and applicability in NENs must still be determined.

In addition, it has been suggested that mTOR-directed therapies may be more effective in tumors with tuberous sclerosis complex 1 (TSC1) somatic mutation, which acts as a regulator of mTOR pathway activation (Iyer *et al.* 2012). TSC is an autosomal dominant multisystem disorder caused by mutations of two tumor suppressor genes, TSC1 and TSC2, which encode for hamartin and tuberin, respectively. The interaction between these proteins is critical for cell growth and proliferation (Dworakowska & Grossman 2009). Additionally, mutations in TSC2 have been described in PNENs with more aggressive features and progressive disease (Bombardieri *et al.* 2013). A schematic overview of current tumor biomarkers for mTOR inhibitors and TKIs is shown in Fig. 5.

Biomarkers for immune therapy

Programmed death-1 (PD-1) is expressed on T-, B- and myeloid cells and downregulates the activation of T-cells in tumors. The programmed death ligand 1 (PD-L1) and PD-1 are overexpressed in tumor-infiltrating lymphocytes in several types of cancer (Dong *et al.* 1999, Freeman *et al.* 2000, Lyford-Pike *et al.* 2013, He *et al.* 2015). PD-1 and PD-L1 induce lymphocyte apoptosis and cytokine secretion, which is crucial in tumor immunosuppression (Fan *et al.* 2016).

Initially, PD-L1 was described in SCLC (Schultheis *et al.* 2015), in lung typical-atypical carcinoids and in large cell neuroendocrine carcinoma (Tsuruoka *et al.* 2017). Subsequently, its presence was described and related with clinical features in other NENs; specifically, PDL-1 has been correlated with tumor stage and histological type in PNENs, as well as with worse survival (Fan *et al.* 2016). In GEP-NENs, PD-L1 has been related with more proliferative and aggressive tumors (Ghebeh *et al.* 2006, 2007, Sabatier *et al.* 2015, Kim *et al.* 2016), probably related to a higher immunogenicity in proliferative tumor cells (Kim *et al.* 2016). Despite PDL-1 and tumor grading being associated in GEP-NENs, no specific relation with the site of origin has been established (Kim *et al.* 2016). Some studies have reported an antitumor effect by drugs targeting PD-1 (nivolumab and pembrolizumab) and PD-L1 (MPDL3280A and BMS- 936559) (Ohaegbulam *et al.* 2015), especially in non-SCLC, which seems to be correlated to PD-L1 overexpression in the tumor (Powles *et al.* 2014). Unfortunately, the applicability in well-differentiated NENs seems to be limited, however. Further studies are required, especially focusing in the relation of PD-L1 expression and response rate in poorly differentiated NENs and carcinomas (Roberts *et al.* 2017).

Conclusions and future directions

The currently available biomarkers for NENs have important limitations, and there is an unmet need for accurate biomarkers that can be used for NEN diagnosis, prognosis and follow-up, therapy stratification and evaluation of treatment response. Currently, several prospective trials are evaluating the effect of novel therapeutic strategies in NENs (^{90}Yt-labeled microspheres, lenvatinib, palbociclib, tremelimumab, bevacizumab, temozolomide, pasireotide, PDR001) and most trials include the evaluation of treatment-related follow-up markers. To date, these new biomarkers include peptides/growth factors, DNA/RNA markers and CTCs.

Circulating markers, as well as, low-invasive techniques for early diagnosis (of disease and related complications) would be valuable to identify a personalized therapeutic sequence and follow-up. Circulating/tissue markers that could predict treatment response could assist to improve treatment-induced PFS. Finally, the combination of markers that enable to better predict the course of the disease, would also allow for better decision making with regard to the therapeutic strategy with the available different treatment options. In this respect, multianalyte measurement based on tumor genomics seems a promising tool for early outcome stratification and decision making. The development of blood-based analysis and liquid biopsy would represent non-invasive methods for diagnosis and prognosis as well. Additional validation studies will establish the definite role for this test. Other new parameters like cftDNA, miRNAs and CTCs and markers that predict response to TKIs and mTOR inhibitors need further investigation before clinical application is possible. Development and validation of novel biomarkers that improve diagnosis, assessment of tumor load, prediction of disease course,

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prognosis and outcome of intervention is still a challenge. In this context, genomics may represent the basis for developing multitranscript biomarkers, additionally, the combination of several markers (that provide multifaceted information), may offer better medical management and use of resources in order to improve diagnosis, treatment, quality of life and survival in NEN patients.

Declaration of interest

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

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