

Perspectives on the diagnostic, predictive and prognostic markers of neuroendocrine neoplasms (Review)

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Abstract. Neuroendocrine neoplasms (NENs) are a heterogeneous group of rare tumors with different types of physiology and prognosis. Therefore, prognostic information, including morphological differentiation, grade, tumor stage and primary location, are invaluable and contribute to the formulation of treatment decisions. Biomarkers that are currently used, including chromogranin A (CgA), serotonin and neuron-specific enolase, are singular parameters that cannot be used to accurately predict variables associated with tumor growth, including proliferation, metabolic rate and metastatic potential. In addition, site-specific biomarkers, such as insulin and gastrin, cannot be applied to all types of NENs. The clinical application of broad-spectrum markers, as it is the case for CgA, remains controversial despite being widely used. Due to limitations of the currently available mono-analyte biomarkers, recent studies were conducted to explore novel parameters for NEN diagnosis, prognosis, therapy stratification and evaluation of treatment response. Identification of prognostic factors for predicting NEN outcome is a critical requirement for the planning of adequate clinical management. Advances in ‘liquid’ biopsies and genomic analysis techniques, including microRNA, circulating tumor DNA or circulating tumor cells and sophisticated biomathematical analysis techniques, such as NETest or molecular image-based biomarkers, are currently under investigation as potentially novel tools for the management of NENs in the future. Despite these recent findings yielding promising observations, further research is necessary. The present review therefore summarizes the existing knowledge and recent advancements in the exploration of biochemical markers for NENs, with focus on gastroenteropancreatic-neuroendocrine tumors.

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1. Introduction

Neuroendocrine neoplasms (NENs) are a heterogeneous group of rare malignancies that arise from neuroendocrine cells distributed throughout the body and produce peptide hormones and/or biogenic amines (1). NENs can be dichotomised into ‘functional’ (F) and ‘non-functional’ (NF) tumors (2). The F-NENs category represents 33% of all NENs and is mainly characterized by well-defined clinical symptoms that are caused by the over secretion of their circulating products (2). Examples of F tumors include NENs in the midgut (such as the small intestine), which are classically associated with carcinoid syndrome (CS) due to the overproduction of serotonin (3). Symptoms of CS have been previously reported to be associated with the overproduction of serotonin by F NENs. By contrast, NF NENs are more common in terms of incidence and can cause mechanical symptoms, including ischemia or obstruction, the complications of which from local tumor growth can ultimately result in mortality (2).

Due to the substantial heterogeneity in these NENs both in terms of clinical aggressiveness and response to therapy, management of patients with such diseases is a significant challenge (4). In total, 60-80% of NENs have already metastasized on diagnosis (5). Prognostic and predictive markers have been intensively investigated to explore the optimal clinical management strategy for this category of neoplasms (6). Investigation into prognostic markers contribute valuable information in understanding the physiology of NENs and their natural course. They reveal beneficial mechanistic information underlying the aggressive properties of the disease and the risk of recurrence or death. By contrast, predictive markers can be used to estimate the benefit of a certain therapy compared with their corresponding condition at baseline, which can assist in implementing the optimal treatment strategy for the most favorable outcome (7).

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At present, the most important prognostic factor for NENs is morphological differentiation characteristics, which can be divided into the well- or poorly-differentiated categories, corresponding to neuroendocrine tumors (NETs) or neuroendocrine carcinomas (NECs) (8). Furthermore, NENs display a varying degree of proliferation known as grade (G), which is approximated using the Ki-67 proliferation index and mitotic count (9). Specifically, the latest 2019 World Health Organization (WHO) classification of gastro-entero-pancreatic (GEP)-NENs distinguishes three grades of NENs that are classified into the low (G1), intermediate-(G2) and high-grade (G3) categories based on the degree of differentiation, with the mention that poorly differentiated NECs are considered definitively G3 (10,11). Although the WHO classification of tumors is considered to be the gold standard tumor classification, it differs depending on their primary site in the body. For example, the 2015 WHO Classification of Tumors of the Lung, Pleura, Thymus and Heart does not make use of the Ki-67 proliferation index (11). However, the current consensus is to improve the management of NENs by adopting an uniformized nomenclature system towards different organs (12). It is hoped that this new classification will serve as a novel grading tool that can be introduced into common clinical practice to reveal essential information regarding NENs (12).

In addition to adequate classification and grading, tumor staging also carries prognostic significance (13). European Neuroendocrine Tumors Society (ENETS) and tumor, nodes and metastases (TNM) classification of the American Joint Committee on Cancer (AJCC) are currently used staging systems that present the classification criteria but are not identical and are site specific (14,15). A number of studies have reported comparative data between the two systems for the classification of pancreatic neuroendocrine tumors (Pan-NETs), suggesting a similar prognosis being found by the two systems in ENETS and TNM of the AJCC, both regarding progression free-survival (PFS) and overall survival (OS) (16,17). However, further studies are needed, as the ENETS staging system appeared to be superior in stratifying prognosis for each stage of pancreatic NENs according to a previous report (18).

However, in terms of the formulation of concrete guidelines for the clinical management of these tumors, numerous issues persist due to the lack of comprehensive databases and registries (9). In addition, an insufficient number of lesion types have been studied despite wide variations in tumor heterogeneity (9). The 5-year survival rate ranges between 15 and 95%, depending on the location of the tumor primary site, the level of metastatic spread at diagnosis, the available treatment options and the geographical site of care (19-21).

Several methods have been reported to predict OS and PFS, including nomograms taking into account the number of liver metastases, tumor size and the Ki-67 index (22), the blood neutrophil-lymphocyte ratio, Ki-67 index and the lymph node ratio (23) or phosphorylated histone H3 (24). However, further research is required.

An important obstacle in clinical practice is the scarcity of a set of sensitive and specific tumor biomarkers (25). The 'perfect' biomarker would ideally be characterized by high sensitivity in diagnosing NENs, good prediction of disease evolution and response to therapy (26). Currently available

NETs biomarkers belong to the mono-analyte class and vary in the rates of sensitivity and specificity for indicating the biological characteristics, such as primary tumor site, or functional or non-functional type secretion. In addition, these mono-analyte measurements are unable to define the state of disease progression or efficacy of therapy (27), which frequently do not correlated well with radiological evaluation data (28). To overcome these shortcomings, interest in the molecular profiling of NENs is increasing. Recent studies have demonstrated the utility of molecular imaging as a viable option for predicting the prognosis of NENs (29,30). Since the density of somatostatin receptors (SSTRs) can also be quantitatively assessed using immunohistochemistry (IHC) in the surgical specimens, this can be used to predict treatment response (31-33). Furthermore complementary imaging modalities or early radiological biomarkers, such as the tumor grade rate (TGR), have been proposed to contribute valuable prognostic information (34,35).

Molecular diagnostics have previously been performed on biopsies of solid tumor tissues (13). As one branch of this technique, 'liquid' biopsy is a non-invasive strategy that can provide the opportunity to investigate the molecular genomic mechanism of tumor cells in circulating blood (the 'tumor circulome') to characterize the circulating molecular profiles, which may be useful for subsequent diagnosis and monitoring (36,37). Emerging biochemical and therapeutic markers have become increasingly popular since 2004 and their relevance are expected to increase exponentially by 2050 (13,38,39). Identifying novel parameters is important for optimally predicting the prognosis and treatment response for each patient. Therefore, the present review summarizes some of the current and future directions in this field of research with an emphasis on GEP-NENs.

2. Aim and search strategy

The aim of the present review is to summarize the available data on the diagnostic, prognostic and predictive biomarkers for NENs. The review is structured into the following two parts: i) Discussion of the markers most commonly used in clinical practice at present; and ii) potential future diagnostic, prognostic and predictive biomarkers.

Search strategy. Published data in the SCIENCE DIRECT (<https://www.sciencedirect.com/>) and PUBMED (<https://pubmed.ncbi.nlm.nih.gov/>) databases were collected and analyzed. Publications from the past 5 years were prioritized and selected using the following relevant key words either alone or in combination: 'Neuroendocrine neoplasms', 'neuroendocrine tumors', 'biomarkers', 'liquid biopsies' and 'molecular imaging'. Additional studies were identified by reviewing the references of all selected articles, whereas publications from major scientific meetings were searched manually. After the exclusion of 52 duplicates, 353 articles were scanned, following which 99 titles were removed because they didn't satisfy the subject of the present review. We included A total of 254 publications were selected for a full-text evaluation. The inclusion criteria were clinical and practical relevance in the management of NENs. Full articles and English language published original and review papers were included that

addressed the diagnosis and especially the prognostic and predictive markers in NENs, with different degrees of statistical power due to the rarity of these neoplasms. Exclusion criteria were case reports and publications with abstracts not relevant for the present review were removed (case studies removed, four; publications removed, 129), so that at the end of the selection process, 121 publications were included (Fig. 1).

3. Currently available biomarkers

A number of biomarkers are currently applied for the diagnosis of NENs. However, they are frequently not correlated with diagnosis or clinical outcomes, mainly due to sensitivity and specificity issues (Table I).

Chromogranin A (CgA). Human CgA is a glycoprotein that belongs to the family of chromogranins and is localized in the secretory granules alongside peptide hormones and catecholamines throughout the neuroendocrine system (40). Recognition of CgA as the default NET biomarker is widely applied over the past decade due to its broad-spectrum nature, with high levels being found in both F and NF NENs (41-43). Yet, CgA detection and reliability are still based on different non-standardized assays (44). However, CgA is highly expressed in NETs tissue, but can also be measured in serum or plasma as a widely used circulating tumor marker (40). High levels of CgA are mainly observed in well-differentiated NETs and are associated with larger tumor burden, especially in gut NETs (5,45). By contrast, CgA suffers from low sensitivity for poorly-differentiated tumors, where its production is less pronounced (40,46).

As a result, enthusiasm for the application of CgA as a marker is waning, which is compounded by the accumulating evidence of its low utility (43,47). The specific cut-off value (identified by the receiver operating characteristic analysis between different assays) (44), primary location of the NEN (48), endocrine-associated syndrome (5), disease spread, liver metastases (49), false-positive elevations in CgA typically caused by proton pump inhibitors, atrophic gastritis and kidney failure (13), can all influence the accuracy of this test.

However, a recent systematic review and meta-analysis evaluated the role of CgA in bronchopulmonary NENs because of the scarcity of evidence in this field (43). This previous study reported the clinical utility of CgA for the diagnosis of lung NENs, especially in small cell lung cancer (SCLC) with a mean diagnostic specificity of 79.5 ± 3.1 and sensitivity of $59.9 \pm 6.8\%$. Still, this finding requires further validation (43).

Urinary 5-hydroxyindoleacetic acid (5-HIAA). 5-HIAA is a metabolite of serotonin, which is excessively produced by serotonin-secreting tumors and is excreted in the urine (13). Prior to interpreting the 5-HIAA test measurement in the urine as a result, pharmacological and dietary artifacts must first be ruled out, typically by avoiding the intake of tryptophan- and serotonin-rich foods (50). Measurement of urinary 5-HIAA excretion is more practical for patients with primary midgut (jejunoileal, appendiceal and ascending colon) NETs, which produces the highest levels of serotonin (51). For carcinoid syndrome (CS), this test has a reported sensitivity of $>90\%$ and a specificity of 90% (52). Additionally, a level of 5-HIAA

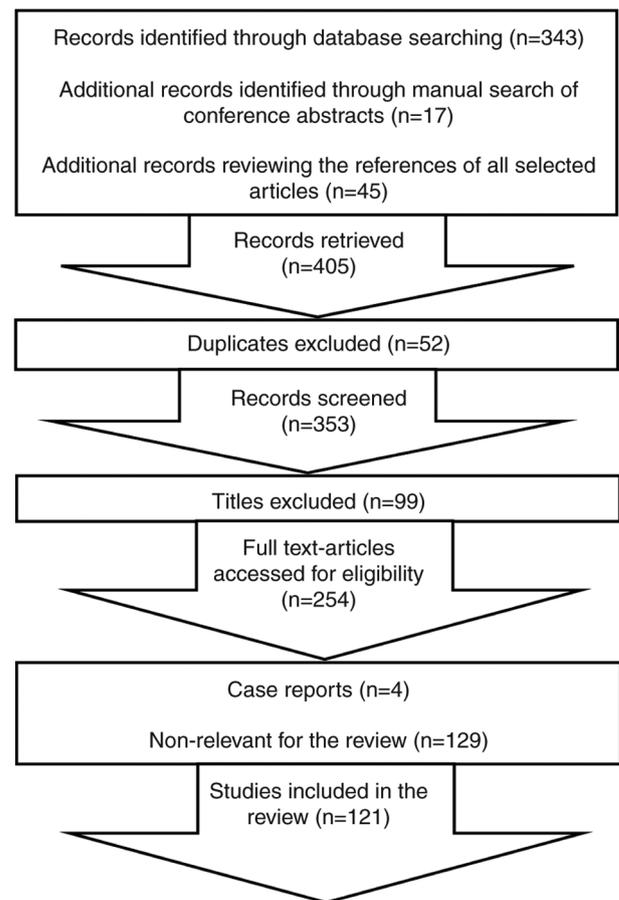


Figure 1. Flow chart for the selection of studies discussed in the present review.

>300 mmol/24 h and three flushing episodes per day can be considered to be a predictive factor of carcinoid heart disease (CHD) (53). There is also evidence that 5-HIAA in combination with N-terminal probrain natriuretic peptide (NT-proBNP) can be accurately used in screening for CHD (54). Nevertheless, its prognostic role remains controversial.

N-terminal Pro-Brain Natriuretic Peptide (NT-proBNP). NT-pro BNP is a peptide that is released by myocardial cells as a result of an increase in heart volume and pressure and is used especially for CHD prediction, which has been previously correlated with patient survival (55,56). A high sensitivity and specificity for CHD, 92 and 91%, respectively, has been reported at the cut-off value of 260 pg/ml (57). In addition, measurement of the level of serum NT-proBNP has been recommended for intestinal NENs and CS for the diagnosis and follow-up of CHD (54).

Neuron specific enolase (NSE). NSE is the main enolase-isoenzyme present in neuronal and neuroendocrine tissues (58). Although mainly expressed poorly-differentiated NET cells, measurement of serum NSE cannot be used to distinguish among different subtypes of NENs (59). Consequently, although elevated levels of NSE seems to have a degree of prognostic value for poor outcome and correlate with tumor burden, NSE is now rarely used in clinical practice since it is inferior to CgA in terms of the information that can be extracted (59).

Table I. Sensitivity and specificity of current biomarkers.

First author/s, year	Tumor marker	Primary tumor location	Sensitivity %	Specificity %	(Refs.)
Oberg <i>et al</i> , 2015	Chromogranin A	Nonspecific	60-90	<50	(2)
Feldman, 1986	Urinary 5-hydroxyindole acetic acid	Midgut	70	90	(51)
Bhattacharyya <i>et al</i> , 2008	N-terminal probrain natriuretic peptide	Midgut: carcinoid heart disease	92	91	(57)
Baudin <i>et al</i> , 1998	Neuron specific enolase	Nonspecific	33	68	(59)
Walter <i>et al</i> , 2012	Pancreatic polypeptide	Pancreas	No data	84	(60)
Laskaratos <i>et al</i> , 2017	Connective tissue growth factor	Midgut: right ventricular dysfunction	88	69	(61)

However, NSE is currently applied as the default tumor marker for SCLC diagnosis, prognosis and follow-up, even though elevated levels of NSE can also be found in non-small cell lung cancer (NSCLC) (58).

Pancreatic polypeptide (PP). PP is serum marker produced by the neuroendocrine cells of the colon and pancreas (60). PP is considered to be a non-specific marker for NENs, where its levels has a modest degree of accuracy as a diagnostic marker (13). However, it appears that an >50% increase in the serum levels of PP in GEP-NETs patients has a direct proportional relationship with the increase in tumor according to RECIST 1.1 criteria on conventional imaging (CT scan ± MRI scan) imaging (60).

Connective tissue growth factor (CTGF). CTGF is a modular secreted protein that serves an important role in complex biological mechanisms, including angiogenesis, tumorigenesis and wound healing (61). In addition, CTGF can regulate various types of fibrosis formation, such as cardiac fibrosis (62). Therefore, CTGF has a reported sensitivity of 80% in detecting heart disease (62). For NETs, previous studies have reported that CTGF expression measured in plasma is more likely to be found in small-bowel ileal rather than in bronchial, pancreatic or rectal NETs (63,64). However, a high level of CTGF was found to be an independent predictive factor for right ventricular dysfunction (61).

Ki-67 proliferation index /mitotic count. Mitotic count, Ki-67 index and necrosis are typically used for the grading of NENs (9). While mitotic count is recommended to be reported as mitoses per mm² area, in clinical practice this process could be affected by limited areas available for counting. On the other hand, Ki 67 is determined using IHC measured in the most mitotically active areas of the pathological specimen, but due to the intratumoral heterogeneity this process can be affected (9). However, this manner of scoring can be time-consuming, which is compounded by the lack of consensus in the optimal method for determining the proliferative rate (9,65). In clinical practice, when there is a discordance between these two types of measurements in assigning the grade, higher 'grade counts' are normally negatively associated with poorer prognosis (65-67). For example, in ~33% of well-differentiated Pan-NETs, a G1

grade is determined based on the mitotic count, whereas the G2 grade is determined using the Ki-67 proliferation index (66). In addition, the same percentage (33%) of G2 Pan-NETs are found for after mitotic count, but G3 according to the Ki-67 proliferation index (64). Therefore, the use of Ki-67 IHC staining is essential for accurately grading well-differentiated NETs (65,66)

The latest 2019 WHO classification of GEP-NENs (10,67) now recognizes the category of high-grade tumors as those with a Ki-67 index of >20%, of which well-differentiated GEP-NENs represent ≤7% of all NENs (68). In addition, tumor grade based on the Ki-67 index cut-off values has been demonstrated to correlate with patient survival independent of tumor stage in primary and metastatic GEP-NENs (69,70). In particular, the Ki-67 index has also been considered to be a viable prognostic marker for recurrence after the resection of PanNETs (71). In liver metastases, the Ki-67 index has a predisposition to be higher compared with that in primary GEP-NETs, where a proportional relationship between the size of the metastasis and the Ki-67 index was found (72-74)

In clinical practice, detection of multiple primary tumors, multiple lymph node metastases and/or multiple distant metastases are recommended for evaluating tissue blocks with the biggest focus of tumor due to the intratumoral heterogeneity (75). Furthermore, the Ki-67 index may present a variability during the course of the disease or between primary tumor and metastasis (76). In a previous study, the Ki-67 index was evaluated in 103 GEP-NETs, of which 24% presented with higher grades in the metastasis, 10% with higher grades in the primary tumor and 66% with same grade between the metastasis and the primary tumor (77). The PFS and OS were found to be identical for both G1 primaries and G2 metastasis categories, and also for G2 primaries and metastasis; however, these are worse compared with the G1 primary tumors only (G1 stable category). Therefore, any G2 tumors (in primary or in metastases of NETs) influences patient survival.

The Ki-67 index also hold promising potential for clinical application in NECs, since a Ki-67 index with a 55% cut-off for response was found to be associated with poorer prognosis, favorable response to platinum-based chemotherapy (CHT) (78) and adverse reactions to temozolomide CHT (79).

4. Potential novel biomarkers

The current demand in this field is to improve the methodology for the diagnosis, treatment and prognosis of NENs. Therefore, novel markers have been evaluated over the past decade.

Genetic mutations. Recent studies have benefited from advances in sequencing technology for characterizing the complex molecular landscape of NENs (80,81). Regarding PanNETs, the current trend is mainly focused on determining the roles of *multiple endocrine neoplasia 1 (MEN1)*, *death-domain associated protein (DAXX)*, *α -thalassemia* and *mental retardation syndrome X-linked (ATRX)* genes in the alternative lengthening of telomeres (ALT) axis and components in the mTOR and DNA damage pathways (82,83). In particular, molecular alterations have been consistently associated with the following four events: DNA damage repair; cell cycle regulation; PI3K/AKT/mTOR signaling; and telomere maintenance (84).

DAXX and ATRX expression and ALT. Recent studies have revealed increasingly consistent findings despite the lower mutation burden of PanNETs (82) as mutations in the *MEN1*, *ATRX* and *DAXX* genes were frequently observed in this type of tumors (85-87). In total, up to 40% of well-differentiated NENs in the pancreas exhibit somatic mutations in the *DAXX* and *ATRX* genes (82). These two genes encode proteins that interact and have multiple cellular functions, including modulating telomeric chromatin. *ATRX* and *DAXX* interact to deposit histone H3.3-containing nucleosomes in the centromeric and telomeric regions of the genome and may interact to suppress the ALT pathway under normal circumstances (88,89). *ATRX/DAXX* mutations result in loss of nuclear expression of their proteins, as detected by IHC, in tumor tissue, which correlates with the suppression of ALT (90). A negative expression of *ATRX/DAXX* is typically associated with well-differentiated NENs, and correlate with worse survival in Pan-NETs (90,91). ALT is a telomerase-independent telomere maintenance mechanism that has been previously studied using fluorescence *in situ* hybridization (92). Altered telomeres are a key process frequently found in PanNETs (93). As such, positive ALT status in liver metastases of NENs was found to associate with worse survival and increased risk of recurrence (94,95). It has been previously shown that there is 100% concordance among the ALT phenotype, *ATRX/DAXX* mutations and/or protein loss (92), where the presence of ALT-positive and *ATRX/DAXX*-negative (inactivation mutation) in well-differentiated PanNETs is associated with a significantly higher grade, size, grading, vascular/perineural invasion, metastatic disease and with reduced relapse-free and tumor-specific survival (96). Therefore, this profile can be applied as a marker of more aggressive PanNET phenotypes for patient stratification (97,98).

Application of fine-needle aspiration (FNA) makes it possible to detect the loss of *ATRX/DAXX* and the presence of ALT as a non-invasive method to sample tumors. As aforementioned, somatic mutations of *ATRX/DAXX* genes can be detected using IHC to indicate a loss of nuclear expression of their respective proteins (90). In addition, ALT can be assessed using telomere-specific fluorescence *in situ* hybridization (92).

This procedure is becoming increasingly important in clinical practice, through which ALT or the loss of *ATRX* or *DAXX* expression can be verified with higher degrees of confidence during the prognostic process (92).

mTOR signaling pathway. Somatic gene alterations are currently studied in PanNETs (98,99), of which two processes have been frequently observed to be affected: chromatin remodeling and activation of PI3K/Akt/mTOR signaling (100). The mTOR pathway regulates cell proliferation, cell cycle and apoptosis; however, in tumor cells, an abnormal activation of mTOR pathway influences the tumor to grow and metastasize (101). Since 14.7% of PanNET cases exhibit mutations in the PI3K/Akt/mTOR pathway, this finding that can be exploited to select patients for treatment with mTOR inhibitors (100,102).

Mutations in the tuberous sclerosis complex 2 (*TSC2*) and *PTEN* genes are suppressors of the Akt/mTOR pathway, which are present in up to 11% of sporadic PanNETs (100). Reduced expression of both *PTEN* and *TSC2* was found to be associated with more aggressive phenotypes, presence of liver metastases and reduced disease-free survival and OS in a cohort of 72 primary PanNETs according to microarray analysis (99). However, further studies are warranted before mutations of components in the PI3K pathway can be considered as a biomarker of response.

Retinoblastoma protein 1 (RB1) and p53 in NECs but not in NETs. The most recent 2019 WHO classification of GEP-NENs made a clear distinction between NET G3 and NEC (10). NET G3 lacks consensus-based recommendations and is associated with longer OS (98). In certain cases, histology is not sufficient for differentiating between highly proliferative NETs and NECs (103), where it is hoped that molecular profiling can amend this deficiency. Abnormal immunolabeling of *p53* and *RB1* pathways are currently being proposed for the distinction of well-differentiated NETs, especially NETs G3 from poorly differentiated NECs during diagnosis (103). Although *p53* and *RB1* are considered to be two key drivers of NECs, but in NETs these mutations are rarely observed (104-107). Double inactivation of the *TP53* and *RB1* genes is one of the genetic signatures of SCLC (108). The presence of these inactivated mutations is used as a predictive marker of the response to platinum-based CHT in lung and GEP-NECs (104,105,108). In addition, the loss of *RB1* is associated with superior responses to platinum salts in both lung and pancreas NECs (104,109), whilst the presence of *p53* staining in colorectal NECs is associated with weaker responses to platinum-based CHT and worse prognosis (105). In GEP-NECs, *p53* IHC is currently under examination as a possible diagnostic, prognostic and predictive method for GEP-NECs, which was reported in several studies with frequencies of *p53* immunoreactive cells ranging from 20-100% (103), where mutations in *TP53* associates poor survival, moreover *TP53* being the most prevalent mutation in NECs (105,110).

Expression markers

Insulinoma-associated protein 1 (INSM1). *INSM1* is a zinc-finger family of transcription factors that serves a role in neurogenesis and neuroendocrine cell differentiation (111).

INSM1 confers certain advantages in NECs as it more sensitive compared with traditional general neuroendocrine markers, such as CgA or synaptophysin, since the clear nuclear expression pattern of INSM1 facilitates its accurate interpretation and quantification whilst reducing the incidence of staining artifacts (65).

The Notch/hairy and enhancer of split-1 (Hes-1) signaling pathway serves a key role in tumor growth and development, which has also been reported to inhibit INSM1 (112). INSM1 has recently been reported to be an important biomarker in the diagnosis of SCLC as it serves as an important factor in ASCL1-driven pathways. ASCL1 is needed for the protein expression of NE molecules and in the development of lung NE cells (113,114). Furthermore, functional interactions between ASCL1 and the Notch1-Hes1 pathway have been reported (113). As aforementioned, the Notch1-Hes1 pathway is involved in the suppression of INSM1 expression (112). Moreover, the implication of INSM1 suppression is being studied as a modulator factor in PanNETs, which could be connected to the Notch1-Hes1 signaling pathway (112,115).

Tanigawa *et al* (112) revealed that INSM1 expression was positive in all cases of PanNETs but negative in cases of pancreatic ductal adenocarcinoma (PDAC), rendering it a potential marker for distinguishing between PanNETs and PDAC. In addition, INSM1 has also been found to be a potential marker for SCLC, since a number of studies reported that INSM1 expression was present in 97-100% SCLC cell lines, which appeared to be more sensitive and specific compared with CgA and L-3,4-dihydroxyphenylalanine decarboxylase (111,113). Furthermore, recent studies reported the superior sensitivity of INSM1 for the diagnosis of NENs regarding their neuroendocrine origin (65,116). Bellizzi *et al* (65) studied a cohort of 93 NECs, where 95% sensitivity was found for INSM1 compared with 83 and 82% for CgA and synaptophysin, respectively.

NETest—a transcriptomic signature of NETs. The NETest uses multianalyte assays with algorithmic analyses (MAAAs), which is a novel method including procedures that incorporate results derived from multiple assays and can increase both the sensitivity and specificity (117). This assay involves mRNA isolation, cDNA synthesis followed by the subsequent quantitative-PCR measurement of 51 circulating NET marker genes (117). These genes were chosen based on the analyzed microarray datasets containing the cellular profiles of fresh frozen tumor samples and whole blood samples from patients diagnosed with NET to characterize the expression patterns (117). Finally, a multi-analyte liquid biopsy representative for NETs was used to determine the biological activity of the tumor and therefore clinical status of the patient (118). Results are presented as the numeric score (NET score) that include the following three categories: i) Low, ≤ 21 -40%; ii) intermediate, 41-79%; and iii) high (biologically aggressive), ≥ 80 %. The PCR test is standardized and reproducible; moreover, diet, proton pump inhibitors medication, age, sex and ethnicity do not interfere with the accuracy of the test (119,120). In addition, MAAA is reproducible at multiple time points, which provides real-time NET scores (118,120).

In a cohort of 100 patients with three different types of tumors (GEP, lung bronchopulmonary NETs and unknown origin tumors), the NETest test had a reported diagnostic

sensitivity of 96%, which was >90 % effective in combination with diagnostic imaging for guiding treatment decisions (121). This previous study also demonstrated that a low NET score (≤ 40 %) was associated with superior outcomes, where the PFS was not reached, whilst intermediate to high NETest scores (41-100%) were associated with significantly shorter PFS and treatment failures (121). In addition, the NETest has been documented to be a useful tool for the detection of lung, thymic, pancreatic and gastrointestinal tract NETs, as well as paragangliomas and pheochromocytomas with ≥ 90 % accuracy (39). Moreover, the NETest can predict aggressive tumor behavior in other NENs (122,123), or the outcome following tumor resection and efficacy of medical treatments, such as somatostatin analogues (SSAs) or peptide receptor radionuclide therapy (PRRT) (39).

A recent meta-analysis was conducted to assess the eligibility of NETest as a biomarker tool in the field of oncology (124). The results supported the utility of this test as a diagnostic tool for GEP and bronchopulmonary NETs, which reported an accuracy of 85% in differentiating between stable and progressive disease whereas a specificity of ~ 90 % was reported (124).

Aristaless related homeobox (ARX)/pancreatic and duodenal homeobox 1 (PDX1) expression. ARX and PDX1 are regulatory proteins involved as epigenetic modifiers in pancreatic development (86). The assessment of ARX/PDX1 expression has been previously studied as a potential pre-operative risk stratification marker for PanNETs (125). Since >50 % PanNETs already have liver metastases at first presentation, novel strategies are emerging for the other 50% with aims to reduce the risk of metastasis (126). Although the choice of surgery for the primary tumor can reduce the risk of metastasis, it is also associated with increased risk of post-surgery morbidity and mortality. Therefore, apart from tumor size, transcription factors ARX and PDX1 alongside ATRX/DAXX mutations and the status of ALT were studied as prognostic markers in resected NF PanNETs (96). Cytological specimens of PanNETs obtained by endoscopic FNA was previously studied using the IHC staining of ARX, PDX1 and telomere-specific fluorescence *in situ* hybridization to detect ALT (125). Positive ALT activity and ARX expression in the tumor coupled with negative PDX1 staining was documented to predict metastatic phenotype for the stratification of patients into the low- or high-risk groups preoperatively (125).

MicroRNA (miRNA or miR) markers. miRNAs are a class of RNAs that do not encode protein and are ~ 22 nucleotides in length (127). They typically regulate post-transcriptional gene expression by targeting mRNA molecules (127). Circulating miRNAs can be used as minimally invasive biomarkers of ovarian, cervical or breast cancer as they are readily detectable in a wide variety of biofluids, including plasma, serum and saliva (128).

The global microRNA expression patterns were studied in normal pancreas, PanNETs and acinar carcinomas in order to assess the role of microRNAs in malignant transformation and progression (129). miR-103 and miR-107 expression levels were found to be higher whereas the level miR-155 expression was lower in PanNETs compared with the normal

pancreas (129). Furthermore, miR-21 expression level appears to be higher in the plasma of patients with PanNETs compared with that in patients with chronic pancreatitis (129). Therefore, it can be applied both as a diagnostic and a prognostic tool for PanNETs, since higher levels of miR-21 expression and high Ki-67 proliferation index were associated with the presence of metastases (129,130).

It was recently reported that in tumors without a known primary site, miRNA expression can be used to facilitate diagnosis (131). miRNA expression profiles were previously analyzed in four pathological types of GEP-NETs, including samples from pancreatic, ileal, appendiceal and rectal NETs (131). The results were promising, as the midgut NETs (ileum and appendix) could be discriminated from non-midgut NETs (rectum, pancreas) according to miR-615 and miR-92b expression (131). In addition, ileal NETs could be discriminated from appendiceal NETs according to miR-125b, miR-192 and miR-149 expression, whilst rectal NETs could be distinguished from pancreatic NETs based on miR-429 and miR-487b expression (131).

Due to their stability in the circulation and abundance, cell-type and disease stage specificity and their reported roles in a number of biological processes, miRNAs have been investigated in various studies. However, due to the lack of consistency in the reported signatures (between results that used tissue and those that used circulating blood) for the same disease and the lack of standardization methods with accurate techniques, further research is required (132,133).

Methylation markers. Epigenetic events occur depending on the subtype of NETs (134). DNA hypermethylation is an early event that frequently occurs during cancer initiation and can dictate the rate of disease development and progression (135). The 'Hypermethylation phenotype' was associated with poorer OS and with more progressive disease in PanNETs (135).

O-6-methylguanine-DNA methyltransferase (MGMT) for NETs but not for NECs. The role of the MGMT enzyme is to repair DNA lesions as a result of alkylating agents usually used in NENs. Loss of MGMT function occurs as a result of epigenetic events, such as hypermethylation, of the MGMT gene promoter (136). This in turn leads to the loss of MGMT protein expression, which can be detected using IHC or detected on the gene level by methylation analysis (for example using methyl-specific PCR or pyrosequencing). Analysis of the MGMT status can be used to predict the prognosis and response to alkylating agents that induce DNA damage in well-differentiated NENs (107). Previous studies showed that reduced MGMT expression is associated with increased rates of treatment response to temozolomide, dacarbazine and streptozotocin CHT in digestive and lung NENs (136,137).

Circulating biomarkers

Cell-free DNA (cfDNA). The importance of tumor-specific alterations in cell-free DNA (cfDNA) in liquid biopsies is becoming increasingly recognized, which can either complement or replace tissue biopsies for several types of cancer, including NSCLC with mutations in the *EGFR* gene (138,139). cfDNA consists of a proportion of circulating tumor DNA (ctDNA) in the blood plasma, which originates from the tumor following

apoptosis, necrosis and active secretion (140,141). cfDNA can be used as a biomarker for cancer, as patients with patients are reported to have greater levels of plasma cfDNA compared with tumor-free controls (142); in addition, high levels of cfDNA are also described in other diseases, such as autoimmune disorders (143). However, further studies in NENs are required to verify the utility of cfDNA as a profiling tool. Boons *et al* (141) first reported that the presence of ctDNA through the identification of copy number variations and tumor specific point mutations using shallow whole genome sequencing and droplet digital PCR, respectively can be used to differentiate between metastatic and localized PanNET. The results of this study demonstrated that ctDNA is found in the plasma samples of patients with metastatic disease. This was shown by tumor-specific variants that were obtained through whole exome sequencing (WES) analysis of primary tumor tissue and germline DNA, in comparison with localized PanNETs where when genotyping variants in cfDNA, the variants could not be detected. Therefore, cfDNA is a candidate as an alternative biomarker to tissue biopsies for molecular profiling.

Circulating tumor cells (CTCs). CTCs are typically released into the blood of patients who have undergone epithelial and mesenchymal transition, which cause metastatic disease (13,144). The presence of CTCs at the moment of recruitment/initial evaluation in the blood samples of patients with midgut, pancreas, bronchopulmonary and of unknown primary metastatic NENs was previously associated with worse PFS and OS (145). In addition, CTCs are associated with higher tumor grades and burden, high levels of CgA and higher Ki-67 indices in G1 and G2 midgut and pancreatic NETs (146). Their potential predictive role was previously studied in a cohort of 138 patients with metastatic NENs, where 41 (29.7%) received long-term SSAs. Changes in repeat CTC count at 3-5 weeks after initiation of therapy were associated with both progressive disease and OS (147). Improved survival was recorded in patients who did not have CTCs at baseline and after therapy in addition to those who presented with >50% CTC reduction after treatment (147). However, CTC lacks sensitivity and specificity as a diagnostic tool for different types of NENs (13). This limitation is currently under investigation in a multicenter, exploratory CalmNET phase IV study (ClinicalTrials.gov Identifier, NCT02075606), which is monitoring a relatively homogeneous group of patients with F G1-G2 midgut NETs treated with lanreotide autogel. This study is attempting to evaluate the predictive power of CTC count on clinical outcome, PFS and quality of life (148).

Molecular imaging as biomarkers

Somatostatin receptors agonists. Expression of SSTR in the majority of NENs, particularly subtype 2, can be imaged by labelling SSAs with a radionuclide, which is typically ⁶⁸Ga, using a chelate, such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (149,150).

At present, two types of molecular imaging that can be used to target SSTR in clinical practice: ¹¹¹In-pentetreotide (OctreoScan) and ⁶⁸Ga-DOTA-Phe¹-Tyr³-Octreotide (TOC), ⁶⁸Ga-DOTA-NaI³-Octreotide (NOC) or ⁶⁸Ga-DOTA-Tyr³-Octreotate (TATE). ⁶⁸Ga-DOTA-TATE/TOC/NOC-PET/CT confers higher scanner

sensitivity, superior spatial resolution and require lower radiation doses (151). Although all three radiotracers (DOTA-TOC, DOTA-NOC and DOTA-TATE) have good affinity for SSTR types 2 and 5, ^{68}Ga -DOTA-NOC exhibits higher affinity for SSTR type 3 (152). However, ^{68}Ga -DOTA-TATE confers superior diagnostic precision for the nuclear imaging of NENs and is currently used in USA, whilst ^{68}Ga -DOTA-TOC-PET is mainly used clinically in the European Union (153,154).

^{68}Ga -DOTA-TATE-PET has previously demonstrated its superiority over Octreoscan for the location of primary GEP-NETs, with a 95.1% detection rate compared with 30.9 and 45.5% from other conventional imaging modalities, respectively (155). This outcome changed the medical recommendation in 32.8% of the patients (155). Results from ^{68}Ga -DOTA-TOC-PET/CT imaging also demonstrated a high affinity to SSTR2 expression derived from IHC and can serve as a predictive marker for patient response to treatment with PRRT (156,157). In addition, ^{68}Ga -DOTA-TOC-PET/CT can be used to select patients who may potentially benefit from SSAs and PRRT, as a high tumor uptake of ^{68}Ga -DOTA-TOC-PET/CT can be useful in the treatment selection of the patients (158).

PRRT is a key second-line treatment option for G1 or G2 midgut NETs with disease progression on SSA treatment (91). PRRT can be used to identify radiation delivered by radionuclides, such as lutetium-177 (^{177}Lu) or yttrium-90 (^{90}Y), to NET cells following internalization after binding to SSTR (151). However, the expression of SSTRs is currently being studied as a predictive marker for treatment response (13). In the NETTER-1 prospective randomized phase 3 clinical trial (ClinicalTrials.gov Identifier, NCT01578239), ^{177}Lu -DOTA-TATE demonstrated its superiority compared with high-dose octreotide in prolonging the PFS in patients with midgut NETs (PFS at month 20, 65.3% for ^{177}Lu -DOTATATE; 10.8% for high-dose octreotide group) (159).

Somatostatin receptors antagonists. Higher tumor uptake of radiolabeled somatostatin receptor antagonists their potential role has been studied for diagnostic and therapeutic approach in NETs (160). Previous studies reported higher sensitivity and diagnostic accuracy with increased image contrast for ^{68}Ga -NODAGA-JR11 (^{68}Ga -OPS202), a SSTR-2 antagonist, compared with agonists ^{68}Ga -DOTA-TATE and ^{68}Ga -DOTA-TOC, for staging G1 and G2 GEP-NETs (ClinicalTrials.gov identifier, NCT02162446) (160,161). A ‘theragnostic pair’- $^{68}\text{Ga}/^{177}\text{Lu}$ -DOTA-JR11 combination was also investigated in a single-center study (ClinicalTrials.gov identifier, NCT02609737). Although there are indications that it binds to more cell types compared with DOTA-TATE or DOTA-TOC in low-grade NETs, this investigation remain in progress at present. Similarly, a peptide ligand, ^{68}Ga -DOTA-bombesin, which can bind to the gastrin realizing peptide receptor in prostate cancer cells (162), is another example of this receptor system that is under evaluation for NENs. Its first application for PET imaging in humans for prostate and breast cancer has been previously reported (162).

^{18}F -fluoro-deoxyglucose (FDG)-PET/CT (^{18}F -FDG-PET/CT). ^{18}F -FDG-PET/CT avidity, especially for detecting G3 NET, was reported to be an indirect marker of proliferative activity in the

tumor, showing a higher sensitivity (87.5%) than somatostatin receptor scintigraphy (87.6%) for detecting rapidly progressive disease (163). In a retrospective study, ^{18}F -FDG avidity was measured quantitatively as a potential prognostic marker in a cohort of 89 patients with metastatic GEP-NETs (164). These patients were divided into three groups based on the ratio of standardized uptake value (SUV) max of the lesion that had the highest FDG uptake as compared to normal liver uptake of FDG (tumor-to-liver T/L SUV ratio) (ratio ≤ 1 , 1-2.3 and >2.3). These three categories associated positively with OS (median OS not reached after 114 months for patients with T/L SUV ratio ≤ 1 vs. 55 months for patients with T/L SUV ratio of 1-2.3 vs. 13 months for patients with T/L SUV ratio >2.3) (164).

NET-PET score. Somatostatin receptor imaging (SRI) is currently considered the gold standard for detecting well-differentiated NETs. However, it has a number of limitations for detecting high grade NETs due to the possibility of false-negatives, since these types of tumors do not express SSTRs (156). Although ^{18}F -FDG-PET can be used for staging G3 NECs, it is more suited for predicting the prognosis of well-differentiated NETs, where higher levels of uptake were associated with an increased risk of early progression (163). By contrast, lower levels of uptake is associated with a less aggressive phenotype of the tumor (163). Chan *et al* (165) therefore proposed a grading system combining these two nuclear imaging techniques (SRI and ^{18}F -FDG-PET) as a single parameter, named ‘NET-PET’, which was found to associate with OS. This scoring system is designated into five risk category groups: i) Grade P0, negative uptake for both scans; ii) grade P1, purely SSTR-positive lesions without FDG uptake above background; iii) grades P2-P4, intermediate categories; and iv) grade P5, presence of significant FDG-positive/SSTR-negative disease (166). The NET-PET score may influence the initial management method of patients with well-differentiated metastatic midgut NET, since the ENETS guideline (167) recommends CHT or SSA as the first-choice treatment option. During the initial phases of SSA therapy, there is no consensus on the upper cut-off value of the Ki-67 proliferation index (165). In this case, the NET-PET score would favor SSA treatment if there is high SRI uptake and low FDG uptake, whilst the contrary would favor CHT (168). The same line of reasoning can be made during patient selection for PRRT, where high SRI uptake but low FDG uptake would suggest PRRT as a viable treatment option, whilst higher FDG uptake and low SRI uptake would indicate likelihood of PRRT resistance (169). Since this was a retrospective analysis, it remains to be elucidated how the individual intermediate classifications can influence the prognosis and treatment decision in a prospective study.

Tumour growth rate as an early biological marker. Accumulating data suggest that the response evaluation criteria in solid tumors (RECIST 1.1) has several limitations in predicting the response to different types of systemic treatments (ST) (34,170). According to RECIST 1.1, the majority of patients with G1-2 GEP-NETs would be classified as having ‘stable disease’ (170). However, even non-responders can survive for a long period without disease progression, since it is known that well-differentiated GEP-NETs have a relatively

slow growth rate (34). Therefore, is important to discover a tumor marker that can identify patients who are at high risk of disease progression at the early stage (171). Previous studies reported TGR to be a dynamic marker, which analyzes images from two examinations and the time between the examinations and can reveal beneficial quantitative information regarding the percentage of change in the tumor volume each month (171,172). The GREPONET I study confirmed that the TGR measured at 3 months (TGR_{3m}) after starting ST or watch and wait (WW) treatment with a cut-off of 0.8%/m (m, percentage of the change of the tumor size in one month), could be used in clinical practice to monitor the treatment response in NETs for the early prediction of PFS (34). In addition, an increased TGR_{3m} ($\geq 0.8\%/m$) was found to be associated with shorter PFS, whilst a decreased TGR_{3m} ($< 0.8\%/m$) was found to be associated with longer PFS (34). This finding suggests that patients with high TGR_{3m} should be followed up more regularly, whilst those with lower TGR_{3m} can receive imaging less regularly to avoid unnecessary radiation (34). Subsequently, the GREPONET II study explored whether beginning any ST including WW can induce any changes in TGR, which was defined as $TGR_{3m} - TGR_0$ (TGR_0 was calculated by comparing the baseline and imaging examination performed within 1 year before the baseline scan) (35). Since it was expected that TGR can be used for monitoring treatment change with no impact on PFS, further study is required.

5. Conclusions

Selection of the optimal treatment option for patients with NEN is difficult due to the heterogeneity in the tumor physiology and varying degrees of aggressiveness. There is a demand for multidisciplinary tumor management guidelines driven by data derived from modern radiology and molecular profiling techniques, to inform the optimal medical decision. Overall, further studies integrating a combination of markers based on tumor genomics and a large spectrum of radiological techniques, such as molecular imaging, would be better placed for shaping the future of clinical NEN research. The present review highlights the importance of a second opinion for improving the method of prognostic stratification and choice of personalized treatment strategies.

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Competing interests

The authors declare that they have no competing interests.

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