The quest for circulating biomarkers in neuroendocrine neoplasms : a clinical perspective

The Quest for Circulating Biomarkers in Neuroendocrine Neoplasms: A Clinical Perspective

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Opinion statement

Given the considerable heterogeneity in neuroendocrine neoplasms (NENs), it appears unlikely that a sole biomarker exists capable of fully capturing all useful clinical aspects of these tumors. This is reflected in the abundant number of biomarkers presently available for the diagnosis, prognosis and monitoring of NEN patients. Although assessment of immunohistochemical and radiological markers remains paramount and often obligatory, there has been a notable surge of interest in circulating biomarkers over the years given the numerous benefits associated with liquid biopsies. Currently, the clinic primarily relies on single-analyte assays such as the chromogranin A assay, but these are far from ideal because of limitations such as compromised sensitivity and specificity as well as a lack of standardization. Consequently, the quest for NEN biomarkers continued with the exploration of multianalyte markers, exemplified by the development of the NETest and ctDNA-based analysis. Here, an extensive panel of markers is simultaneously evaluated to identify distinct signatures that could enhance the accuracy of patient diagnosis, prognosis determination and response to therapy prediction and monitoring. Given the promising results, the development and implementation of these multianalyte markers are expected to usher in a new era of NEN biomarkers in the clinic. In this review, we will outline both clinically implemented and more experimental circulating markers to provide an update on developments in this rapidly evolving field.

Introduction

Neuroendocrine neoplasms (NENs) are a heterogeneous group of tumors that arise from neuroendocrine cells and can occur anywhere in the body, with the lungs and gastrointestinal tract being the most common sites[1, 2]. Besides their variable anatomical location, NENs also display histological, clinical, and molecular heterogeneity[3, 4]. Based on histological differences, NENs can be classified into the often indolent, well-differentiated neuroendocrine tumors (NETs) and the more aggressive, poorly-differentiated neuroendocrine carcinoma (NECs)[5]. Moreover, NENs can also be differentiated based on functionality into functional NENs (F-NENs) characterized by hypersecretion of specific hormones, such as insulin and gastrin, that can give rise to symptoms associated with a clinical syndrome, and non-functional NENs (NF-NENs) that do not exhibit this hypersecretion and are mostly asymptomatic[5]. All this variability results in widely varying clinical presentations, prognoses and responses to therapy[6, 7]. Over the years, a multitude of NEN biomarkers have been investigated including immunohistochemical, radiological and circulating markers, some of which are already implemented in daily clinical practice[7]. Despite the plethora of markers, it remains challenging in clinical practice to (i) accurately and timely diagnose patients, (ii) determine prognosis, and (iii) predict and monitor response to therapy, because of limitations in sensitivity, specificity and accuracy indicating a great need for new markers[8, 9]. Therefore, in recent years, more and more research is conducted into more complex, omics-based biomarkers in liquid biopsies, that allow real-time monitoring of tumor evolution[10-12]. Given the rapid advances in the field of NEN biomarker development, this review aims to provide a comprehensive summary of the currently existing circulating biomarkers and their clinical impact (figure 1, table 1).

Circulating peptide and protein biomarkers

Standard histopathological and immunohistochemical studies require tissue samples obtained through biopsies or resections to diagnose NEN patients. However, these only provide a snapshot of tumor heterogeneity, and their invasive nature makes repeated sampling unfeasible[13-15]. Consequently, there has been increasing interest into non-invasive, highly reproducible markers for diagnosis, prognosis and follow-up[13]. An appealing feature of the neuroendocrine cells is their ability to produce, store and secrete a wide variety of peptides and biogenic amines[16]. These secretory products can serve as biomarkers that can be detected in blood, urine or other body fluids that can be obtained in a minimally- to non-invasive way. General and type-specific biomarkers can be distinguished, with the former theoretically found in all NENs and the latter only detectable in a limited fraction of NENs[17].

General peptide and protein biomarkers

Chromogranin A

The most widely used general circulating biomarker is chromogranin A (CgA), an acidic glycoprotein that is highly expressed in neuroendocrine tissue and co-secreted with peptide hormones in the systemic circulation upon stimulation[18, 19]. Both European Neuroendocrine Tumor Society (ENETS) and North American Neuroendocrine Tumor Society (NANETS) guidelines recommend CgA measurements for the diagnosis and follow-up of NENs[20, 21]. Despite these recommendations, there is a lack of a recognized, standardized CgA assay which makes it extremely challenging to compare measurements from different studies as there are currently many different commercially available kits[22, 23]. Moreover, the clinical utility of CgA determination for the diagnosis, prognosis and follow-up of NEN patients remains a matter of debate[19]. The application of CgA measurements as a diagnostic marker is hampered by the widely varying sensitivity (43-100%) and specificity (10-96%) reported in literature[19, 24, 25]. This can be explained by the fact that CgA can be elevated in patients who receive antisecretory drugs such as proton pump inhibitors, or suffer from other types of cancer or even non-cancer related conditions (e.g. renal failure, pregnancy, untreated hypertension, certain medication etc.)[17, 18], which limits specificity. Sensitivity is limited because normal levels of CgA are observed in 30-50% of patients[26].

The use of CgA levels for prognostic purposes remains controversial as well. Although there is a clear relationship between CgA levels and tumor burden (i.e. the main predictor of clinical outcome), this does not always appear to be clinically relevant[19, 27]. Clinicopathological features such as functional status can influence CgA secretion, which explains, for example, why equally high CgA levels can be detected in patients with non-metastatic gastrinomas, a gastrin-producing tumor, as in patients with

metastatic non-functional NETs originating from the pancreas[19]. Studies that examined the direct effect of CgA levels on survival, were mainly retrospective, focused on patients with advanced disease[19, 28] and reported a shorter overall survival (OS) in patients with high CgA levels[29, 30]. Recently, one study also demonstrated that in a cohort of grade 1 and 2 gastroenteropancreatic NETs (GEP-NETs), high CgA baseline levels (i.e., prior to treatment) could serve as an independent factor to predict worse OS[30]. Besides the numerous retrospective studies, the prognostic value of CgA was also prospectively studied during the RADIANT-1[31], RADIANT-2[32] and RADIANT-3[33] trials on everolimus treatment in NEN, each demonstrating that low levels (<2*36.4ng/mL) were a favorable prognostic factor for OS, independent of treatment[31-33]. However, in these trials low baseline CgA levels were not found to be predictive of an effect of everolimus on OS[32, 33]. Nevertheless, in the RADIANT-1, it could be shown that patients with early CgA response (i.e. normalization or reduction of ≥30% at week 4) had longer progression-free survival (PFS) and OS, suggesting that CgA levels could potentially be useful for prognostication of patients under everolimus treatment[34]. In addition, several studies[30, 35, 36] also demonstrated that changes in CgA concentration during follow-up reflected a therapeutic response or probability of tumor progression. However, these studies applied different cutoff values to indicate significant changes[30, 35, 36]. Finally, some studies also reported that changes in CgA levels could not be used to predict response to PRRT[37, 38]. All of these findings question the wide application of this marker in clinical practice and whether the current guidelines on the clinical implementation of this biomarker should be revised.

Chromogranin B and pancreastatin

The diagnostic ability of another granin namely chromogranin B (CgB) was investigated as well because of its structural similarities to CgA[39, 40]. However, Monaghan et al. showed that elevated CgB levels could only be detected in 16.6% of NET patients and that determination of CgB levels in addition to standard CgA measurements did not improve diagnostic accuracy, limiting its clinical utility[41]. Contrarily, pancreastatin, an enzymatic cleavage product of CgA, appeared to be a more promising marker[24, 40, 42, 43]. Several studies reported that pancreastatin has a higher sensitivity and specificity than CgA and is also better at detecting progression[43-45]. In addition, it correlates with survival as it was demonstrated that patients with levels above the reference range (80 pg/mL) had worse PFS and OS[42-44, 46]. Pancreastatin also holds promise as a predictive and follow-up marker, with a reduction in pancreastatin levels after transarterial chemoembolization and surgery correlating with a lower risk for disease progression[42].

Neuron-specific enolase

Another general NEN marker is the neuron-specific enolase (NSE), a glycolytic enzyme produced in neurons and neuroendocrine cells of the central and peripheral nervous system[40]. Elevated NSE levels are most often found in poorly differentiated NECs and have a negative prognostic value[8, 24, 47]. The diagnostic potential of this marker is rather low since NENs and non-NENs can only be distinguished with a sensitivity of 39-43% and specificity of 65-73%[48-50]. Consequently, its role in clinical practice is rather limited and is usually combined with CgA to increase the reliability of the potential NEN diagnosis[51, 52].

Pancreatic polypeptide

Pancreatic polypeptide (PP), predominantly produced in the pancreas, is also considered a general NEN marker as it is also released by NENs of various origins[40, 53]. However, PP levels can be falsely elevated due to physical exertion, hypoglycemia and non-NEN related conditions (i.e. inflammatory processes, chronic kidney disease and diarrhea)[51, 54] affecting its specificity. The sensitivity is rather low, namely 41-63% in pancreatic NETs (PNETs) and 18-53% in gastrointestinal NENs causing the marker to be rarely used in daily clinical practice[53, 55].

Human circulating progastrin

Lastly, in a recent study[56], Chauhan et al. proposed human circulating progastrin (hPG80) as a new promising general NEN marker. Plasma hPG80 values were found to be significantly higher in the NEN cohort (n=95) compared to controls (n=389). Diagnostic sensitivity ranged from 47.37% (NENs vs. 50-80 year old control group) to 62.11% (NENs vs. 18-25 year old control group), both at 90% specificity. Sensitivity was highest for the small cell lung cancer (SCLC) subgroup vs. 18-25 year-olds (69.23%) and lowest for the NEC subgroup vs. 50-80 year-olds (37.50%). It is important to note that the majority of the NET patients (84%) had advanced disease (stage IV), so conclusions on the performance of the marker at early stages cannot be made. Currently, the role of this marker for disease monitoring is being evaluated in a clinical trial (ETCTN10450)[56].

Type-specific circulating peptide markers

Patients presenting with a clinical syndrome associated with NEN related hormone secretion, so-called functioning NENs (F-NENs) could be diagnosed and monitored by certain type-specific peptide biomarkers next to general NEN biomarkers[16].

Serotonin and 5-HIAA

Serotonin (5-hydroxytryptamine) is a biogenic peptide released by various types of cells including the enterochromaffin cells in the gastrointestinal tract to regulate motility. It is excessively produced in a fraction (15-20%) of GEP-NENs which usually clinically present with carcinoid syndrome characterized

by diarrhea, abdominal cramps, flushing, fatigue and asthma-like shortness of breath[40, 52, 57, 58]. If carcinoid syndrome is clinically suspected, biochemical confirmation is required[52], but the ENETS guidelines recommend assessing this marker in all patients with advanced intestinal NET, lung/ovary NET of any stage and in NETs of unknown origin[59]. However, determining serotonin levels is difficult because of circadian rhythm related and interindividual fluctuations[40, 52]. Consequently, it is recommended to instead determine the concentration of 5-hydroxyindole acetic acid (5-HIAA), the main metabolite of serotonin, in 24-hour urinary samples[16, 52]. In the presence of carcinoid syndrome, high sensitivity (70%) and specificity (90%) are achieved for diagnosis[60], but sensitivity decreases sharply (38-73%) when 5-HIAA is used as a general NEN marker (including for NF-NENs)[16, 57]. Moreover, false positives can be observed due to malabsorption, celiac disease and after ingestion of tryptophan-rich foods, while renal insufficiency can in turn lead to false negatives[13, 52, 61]. Another disadvantage of urinary 5-HIAA analysis in daily clinical practice is that it is very time-consuming and patient unfriendly[61]. Therefore, several studies examined whether plasma and serum 5-HIAA measurements could serve as valid alternatives and reported that results obtained through plasma and serum correlated very strongly with those obtained from urine samples[61-63]. In addition, the potential prognostic value of 5-HIAA was also investigated. For example, Zandee et al. demonstrated that 5-HIAA overexcretion (>10 times upper limit of normal (ULN); 46.8µmol/24h) was a negative predictor for OS in univariate analysis, but in multivariate analysis this effect disappeared[64]. Moreover, Bhattacharyya et al. reported that 5-HIAA levels >300 µmol/24h and >3 episodes of flushing/day are independent predictors of the development of carcinoid heart disease (CHD) where mainly right sided valvular dysfunction is observed caused by fibroblast deposits on the tricuspid and pulmonary valve[65]. Consequently, the clinical utility of 5-HIAA is currently largely limited to diagnosing serotonin-producing NENs and predicting CHD.

Insulin

Insulinomas are NETs arising from the β-cells of the pancreas that secrete the peptide hormone insulin. They are classically characterized by the so-called "Whipple's triad", a specific diagnostic hallmark consisting of 3 main features that indicate the likely presence of an insulinoma[66]. These are: (i) symptoms of hypoglycemia (e.g. hunger, dizziness, tremor, etc.), (ii) episodes of low blood sugar (≤45mg/dL), and (iii) normalization of glucose levels and relief of symptoms after glucose administration[18, 54, 66, 67]. Any clinical suspicion of an insulinoma should be confirmed using the 72-hour fasting test which is the gold standard for diagnosing insulinoma given its sensitivity of 100%. During this test, a blood sample is drawn every 6 hours for glucose and insulin level determination until blood glucose levels drop to ≤45mg/dL and insulin levels simultaneously reach ≥36pmol/L[18, 52, 66]. In 80% of the patients, these levels are reached within 24 hours[18]. When the outcome is not

conclusive, it is recommended to perform a glucagon stimulation test immediately thereafter since an increase in glucose after administration of glucagon (1mg) indicates sufficient stores of glycogen and consequently confirms the presence of an insulinoma[18, 52].

Glucagon

Glucagonomas are NETs arising from the α-cells of the pancreas that secrete glucagon which exerts the opposite function of insulin in the glycometabolism[51, 68]. This tumor is consequently often characterized by symptoms such as weight loss, diabetes mellitus and necrolytic migratory erythema[51, 68]. An increase in concentration (≥10-20 times ULN; 50-100pg/mL) in the circulation may indicate the presence of a glucagonoma[16, 68]. Upon clinical suspicion based on history and clinical examination, the diagnosis is ultimately confirmed based on measurement of glucagon levels in fasting state (usually ≥500pg/mL)[16, 52, 68].

Gastrin

Overproduction of gastrin, owing to the presence of a gastrinoma in the pyloric antrum, duodenum or pancreas, can induce the Zollinger-Ellison syndrome (ZES)[16, 51, 58]. ZES is characterized by the development of severe diarrhea, gastric ulcers and gastroesophageal reflux disease caused by excessive secretion of gastric acid, triggered by elevated gastrin levels[58]. Upon clinical suspicion, patients are diagnosed by assessing fasting gastrin levels, but elevations can be observed in other situations as well[52, 58]. Most commonly, this is due to physiological hypergastrinemia caused by the ingestion of antacids to treat chronic atrophic gastritis, for example, which ultimately results in an increased gastrin release. In addition, the use of proton pump inhibitors (PPIs) such as omeprazole, that function as strong antacids, causes increased levels of gastrin[58]. Consequently, it is important to determine fasting levels of gastrin in a gastric acid hypersecretion state, so it is recommended that treatments with PPIs are paused 10-14 days before measurements in patients with an acceptable risk of gastric ulcers upon cessation of PPI [16, 52, 58]. A ZES diagnosis is confirmed by gastrin levels >1000pg/mL, while intermediate levels (100-1000pg/mL) require an additional secretin test for confirmation[16, 51]. If, after secretin administration, gastrin baseline levels rise to ≥120pg/mL, the test is positive and a ZES diagnosis is still confirmed[16]. If this test is negative, a calcium or glucagon stimulation test is recommended[52].

Somatostatin

Somatostatinomas are a rare type of NETs arising from δ- and D-cells, located in the pancreatic islets and stomach respectively, that secrete the cyclic peptide hormone somatostatin[69]. In case of a functional somatostatinoma, clinical presentation varies widely due to the widespread effect of

somatostatin throughout the body, but often patients present with cholelithiasis and diabetes mellitus. Diagnosis can be confirmed by a fasting serum somatostatin concentration >14mmol/L. Importantly, levels can also be falsely elevated in medullary thyroid cancer, lung cancer, pheochromocytoma and paraganglioma[70].

Vasoactive intestinal peptide

Lastly, hypersecretion of vasoactive intestinal peptide (VIP) is characteristic of VIPomas, a rare type of F-PNETs that present with very specific clinical symptoms such as watery diarrhea, hypokalemia and achlorhydria also known as the Verner-Morrison syndrome[16, 51, 52]. Assessment of VIP levels is often sufficient to diagnose a patient with VIPoma since elevated VIP levels (> 60 pmol/L) achieve a specificity of 100%[71].

Circulating transcripts

Multiple studies already demonstrated that the transcriptome in NENs is altered[72, 73]. Since tumor-derived transcripts can be detected not only in tissue but also in the systemic circulation, they represent an interesting source of non-invasive biomarkers[16].

Circulating microRNAs

Interesting candidate biomarkers are the microRNAs (miRNAs) which are a group of short, non-coding RNA transcripts involved in gene regulation at the post-translational level and whose regulation is highly dysregulated in neoplasms[74, 75]. Up until now, over 100 miRNAs have already been identified that exhibit differential expression in NENs of which the majority (~90%) are specific to the tissue of origin[74]. Bowden et al. identified 31 miRNAs that were similarly expressed in tissue and plasma of SINET patients and evaluated their expression in an independent cohort after which they were left with 4 miRNAs that were subsequently validated in a large panel of 120 cases and 120 controls. Ultimately, they found an association between the presence of metastatic SINETs and the levels of miR-22-3p, miR-21-5p and miR-150-5p[76]. More recently, Malczewska et al. proposed four other miRNAs (miR-125-5p, miR-362-5p, miR-425-5p and miR-500a-5p) that could differentiate serum samples of SINET patients from those of healthy controls with an area under the curve (AUC) of 0.951[77]. A study conducted by Kövesdi et al. revealed that in PNET patients with normal CgA levels, a set of miRNAs could improve the diagnostic accuracy (AUC: 0.904)[78]. Moreover, a significant correlation between CgA and the relative miR-29b levels, a miRNA that has been identified in several other cancer types as well, was observed[79]. Only limited data is available about the prognostic value of circulating miRNAs. Expression levels of miR-21-5p, miR-22-3p and miR-150-5p have been reported to be associated with OS[76], while another study found downregulation of miR-375 to be a strong

predictor for shorter OS[80]. Recently, Bocchini et al. demonstrated the prognostic power of hsa-miR-5096 as an accurate, independent predictor of PFS in PNETs where levels above 70 can stratify ^{18}F-FDG-PET/CT positive patients with often metabolic aggressive PNETs. Their findings suggest that in PNET cells with high hsa-miR-5096 levels, SSTR2 expression is down-modulated, leading to reduced response to SSTR-targeted therapies such as PRRT. Consequently, they hypothesized that a treatment aimed at inactivation of hsa-miR-5096 could potentially increase SSTR expression thereby sensitizing PNET cells to PRRT or other SSTR-targeted therapies[81]. Nevertheless, it remains difficult to estimate the true value of miRNA as a potential circulating biomarker. At the moment, numerous methodologies are being used for isolation and detection of miRNA and sample sizes are rather low in most studies. This makes it hard to compare between studies and to define consistent panels that are suited for diagnostic or prognostic purposes in clinic[75].

NETest

Analysis of tumoral circulating transcripts appears more promising and forms the basis of an innovative assay called NETest. This multigenomic blood-based assay measures the gene expression of 51 NET-related genes by q-PCR and uses the obtained expression profiles as input for machine-learning algorithms[10, 82]. Ultimately, a NETest or tumor activity score is calculated ranging from 0 to 100%, with a score of >20% indicating the presence of a NET[10]. A recent comprehensive meta-analysis showed that the NETest is an effective diagnostic tool with a sensitivity, specificity and accuracy of 93.2%, 98.4% and 95.6%, respectively[83]. Through direct comparison, both Modlin et al. and Malczewska et al. were able to show that the NETest has a higher accuracy (84%-96%) than the current standard marker, CgA (58%-67%)[84, 85]. However, in individuals with image negative disease, accuracy was lower in NETest (67%) compared with CgA (78%)[85], whereas previous studies reported strong concordance with both anatomic (92%), functional (94%) and combined (96%) imaging[86]. This may be explained by the presence of microscopic disease in a proportion of image negative patients that is only picked up by NETest and consequently considered to be false positive[85, 87].

Numerous studies were able to demonstrate the prognostic function of the baseline NETest score as it allows to distinguish stable and progressive disease[88]. Different cutoffs were applied, but it is generally accepted that a NETest score of >40% is indicative of progressive disease[83, 88]. However, van Treijen et al. recently reported that the prognostic accuracy of the NETest score decreases over time. In a cohort of 132 GEP-NET patients, they observed a decrease in AUC from 0.74 (baseline) to 0.55 and 0.45 for the first and second follow-up sample, respectively. They showed that scores fluctuated significantly in patients with RECIST confirmed stable disease (SD) or no evidence of disease, questioning the use of NETest as a follow-up tool to reliably predict disease progression[89].

NETest has also been evaluated several times as a tool to predict response to therapy. For example, Liu et al. observed in a watch-and-wait cohort (n=45) that 93% of low-score (<80%) patients remained stable and consequently continued without treatment, while in 71% of high-score individuals the therapeutic strategy was modified[90]. Similarly, in the treated group (84% with somatostatin analogs (SSA)), all patients with a low score continued their therapy after a 6-12 month follow-up, while interventions were undertaken in 86% of individuals with a high score[90]. This was consistent with previous findings, already showing that NETest scores ≥80% are indicative of SSA non-responders[91]. Similarly, van Treijen et al. recently confirmed that NETest could predict response to systemic therapy (i.e. PFS ≥12 months) with an accuracy of 73%. However, they observed that patients who exhibited a low NETest score (<40%) before the start of systemic treatment had a significantly shorter PFS compared to those who displayed increased scores (10 vs. 31 months). They hypothesized that tumors with an increased expression of genes involved in several neoplastic processes, and hence an elevated NETest score pre-treatment, are more sensitive to systemic treatment resulting in a significantly longer PFS[89]. Multiple studies also investigated the ability of the NETest to assess efficacy of PRRT treatment. Bodei et al. found that by applying a cutoff score of 40%, PRRT response could be correctly predicted in 93% of cases[38]. Furthermore, a specific predictive biomarker was developed, the so-called Positive Predictive Quotient (PPQ), which demonstrated an accuracy of 95-97%[38, 92]. In 92% of patients who were positive for this biomarker a stable disease score was observed during follow-up, while 75% of PPQ-negative patients showed an increase in NETest score of which 80% even had a progressive disease score[38]. Similarly, after resection, patients with a significant decrease remained disease-free and those without significant changes presented with recurrent disease 6 months after surgery[93, 94]. These results were not obtained after R2 surgery in which macroscopic residual tumor is known to remain in the patient's body[95].

Based on the findings described above, it can be concluded that the NETest may be used primarily for diagnostic purposes and to predict response to PRRT treatment using the PPQ. However, prior to clinical implementation, more clarity should be provided on the availability and the costs associated with the test[40].

Circulating tumor cells

Circulating tumor cells (CTCs) have been extensively studied as a potential biomarker in a wide range of tumors as they can provide important diagnostic and prognostic information[16, 96]. In 2011, Khan et al. were the first to demonstrate the presence of CTCs in patients with midgut (43%), pancreatic (21%) and bronchopulmonary (31%) NET via the CellSearch® platform[97]. This platform uses ferromagnetic beads coated with epithelial cell adhesion molecules (EpCAM) that allow to distinguish

CTCs from white blood cells[96]. In a follow-up study, CTCs were recovered in 49% of NET patients[98], and a similar percentage of CTC positive NET patients was reported by Ehlers et al.[99]. Presence of CTCs is suggested to have prognostic relevance as well, as it was found to be associated with progressive disease[97, 98]. One study by Rizzo et al, for example, found that the presence of CTCs was associated with bone metastases in NET patients[100]. Furthermore, a significant association could be observed between altered CTC counts and response to therapy or OS, indicating a possible role as a follow-up biomarker[101]. During the phase II PAZONET study, patients with low baseline CTC levels showed improved response and longer median PFS albeit not significant[102]. However, within the CALM-NET phase IV study, no statistical difference in response rate was reached between patients with and without baseline CTCs, and there was also no notable effect on the PFS in patients receiving lanreotide treatment[103]. Recently, increased attention is being paid to the properties of the CTCs rather than merely looking at the presence and quantity of cells. For example, Childs et al. succeeded in detecting SSTR-2 and -5, two therapeutic targets for SSAs and PRRT, on CTCs, thereby illustrating the predictive value of CTCs[104]. Moreover, whole genome sequencing and subsequent copy number analysis proved that the copy number alterations (CNAs) detected in CTCs mirrored those in tissue and could potentially serve as surrogates for tissue biopsies[105].

Circulating tumor DNA

Another interesting minimally invasive biomarker is circulating tumor DNA (ctDNA), the fraction of cell-free DNA (cfDNA) that is released into the circulation by tumor cells via apoptosis, necrosis and active secretion[15]. Although ctDNA cannot be selectively isolated, its detection is possible due to the presence of tumor-specific molecular alterations[106]. Consequently, it represents an interesting and extensively researched alternative to the highly invasive tissue biopsies with great potential for various clinical applications[40]. In 2018, Boons et al. were the first to detect ctDNA in the plasma of metastatic PNET patients via customized digital droplet PCR (ddPCR), but could not recover tumor-specific single nucleotide variants (SNVs) in patients with localized disease[106]. Zakka et al.[107] evidenced that targeted ctDNA next generation sequencing (NGS) testing in NEN patients was possible and reported mutational changes in 280 of 338 samples analyzed. More than half (52%) of the mutations were located in *TP53*[107]. Moreover, Knappskog et al. recently showed that liquid biopsies in GEP-NEC patients constitute a good alternative approach to characterize tumor mutation status in patients in whom tissue biopsies cannot be acquired[108]. Despite these findings, it is rather unlikely that SNV or mutation analysis in ctDNA can serve as a general diagnostic biomarker in NENs since tumor mutational burden is rather low in NENs and a general signature, as used in NETest, is lacking.

Boons et al. [12] investigated genome wide CNV profiles in cfDNA and found these to be similar between different PNET patients and significantly correlated to those identified in tumor tissue of corresponding patients[12]. Based on these findings, the biomarker potential of CNV cfDNA analysis was further investigated in a cohort of 43 NET patients. Presence of ctDNA in plasma was found to be significantly associated with higher WHO grade, higher levels of CgA and worse OS was observed in ctDNA-positive patients[12]. In addition to the prognostic potency, it was also demonstrated that CNV profiles in ctDNA could be employed to differentiate PNETs from the more frequent pancreatic adenocarcinoma with a sensitivity, specificity and AUC of 62%, 86% and 79%, respectively. Moreover, PFS was associated with changes in tumor fractions (i.e., amount of ctDNA relative to total amount of cfDNA) during longitudinal measurements[12]. This study was the first to demonstrate the diagnostic, prognostic and follow-up potential of ctDNA analysis without a need for prior knowledge of tumor tissue.

Besides genetic alterations, several studies in tissue also reported the importance of epigenetic changes in NENs[109, 110]. Alterations in DNA methylation occur early in carcinogenesis and hence represent an interesting biomarker for early diagnosis. In a recent publication, Mettler et al. evaluated the integrity and methylation status of cfDNA in 63 patients with an advanced metastatic NEN. They reported that the combination of higher cfDNA concentration, decrease in DNA integrity and global hypomethylation was strongly associated with disease burden and worse prognosis. Based on these cfDNA characteristics, metastatic NEN patients could be distinguished from cured NEN patients and healthy individuals with an AUC of 91% and 69%, respectively[111]. These results provide the first important indication of the power of the methylome as a circulating biomarker in NENs which will logically prompt more extensive studies.

Figure 1. Overview of the circulating NEN markers. The most well-known and used markers are peptides and proteins secreted by the neuroendocrine cells, from which NENs originate. A distinction is made between the general markers that are theoretically produced by all types of neuroendocrine cells and the type-specific markers which are only released by a certain subpopulation of neuroendocrine cells. Besides these peptides and proteins, NENs, like other tumor types, also excrete cell-free nucleic acids that exhibit molecular changes specific to the tumor. Moreover, whole tumor cells may also be released into the circulation which have a diagnostic and prognostic value on their own, but in addition may release other markers (peptides, proteins and nucleic acids) after cellular degradation. This figure was created with BioRender.com.

Table 1. Summary of the diagnostic sensitivity and specificity of the most widely used and promising NEN markers for which these parameters are known.

Biomarker	Sensitivity	Specificity	AUC	Reference(s)
CgA	43-100%	10-96%	NA	[19, 25]
NSE	39-43%	65-73%	NA	[48-50]
PP	41-63% in PNET 18-53% in GI-NET	NA	NA	[53, 55]
hPG80	47,37-62,11%	90%	NA	[56]
NETest	93,2%	98,4%	95,6%	[83]
ctDNA	62%	86%	79%	[12]

Conclusions

The shift from tissue to liquid biopsy-based biomarkers in the NEN field was prompted by their ability to offer noninvasive biomarker detection and enable repeated sampling, facilitating efficient follow-up. These biopsies tend to be highly informative in NEN as they contain a multitude of general and type-specific peptides and proteins secreted by the tumor into circulation. As a result, single-analyte biomarkers such as CgA and NSE currently form the foundation for circulating markers in NENs and have been employed alongside immunohistochemical and radiological markers in clinical practice for several years. Nevertheless, the available general markers suffer from limited sensitivity and specificity, and a lack of standardized assays, while type-specific markers that exhibit better accuracy are only applicable to a small subset of NENs, significantly curtailing their utility. Consequently, the quest for NEN biomarkers experienced a shift from single to multianalyte markers capable of comprehensively analyzing a molecular panel in a single assay. This has prompted the development of the NETest and important advances in ctDNA research. Clinical application of these multianalyte markers is currently lagging due to a lack of prospective studies validating their reliability in clinical decision making and limitations (in the availability) of specific detection techniques. However, given the numerous promising results, it is expected that in the coming years the remaining, yet crucial obstacles will be overcome and that also newer research fields including methylation in cfDNA, will be further explored. As such, it is expected that in the foreseeable future a new generation of NEN biomarkers will enter daily clinical practice, thereby providing significant improvements for NEN patients.

Compliance with Ethics Guidelines

Conflict of Interest

No potential conflicts of interest relevant to this article were reported.

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References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

• Korotaeva A, Mansorunov D, Apanovich N, Kuzevanova A, Karpukhin A. MiRNA Expression in Neuroendocrine Neoplasms of Frequent Localizations. Non-coding RNA. 2021;7(3). doi: 10.3390/ncrna7030038.

This review gives an extensive overview of the hitherto known miRNAs and their functional characteristics in different types of NENs.

• Modlin IM, Kidd M, Falconi M, Filosso PL, Frilling A, Malczewska A, et al. A multigenomic liquid biopsy biomarker for neuroendocrine tumor disease outperforms CgA and has surgical and clinical utility. Annals of oncology : official journal of the European Society for Medical Oncology. 2021;32(11):1425-33. doi: 10.1016/j.annonc.2021.08.1746.

This article shows in an extensive, international cohort that the NETest is more accurate to diagnose, grade, identify metastases and predict recurrence as compared to CgA.

• Boons G, Vandamme T, Mariën L, Lybaert W, Roeyen G, Rondou T, et al. Longitudinal Copy-Number Alteration Analysis in Plasma Cell-Free DNA of Neuroendocrine Neoplasms is a Novel Specific Biomarker for Diagnosis, Prognosis, and Follow-up. Clinical cancer research : an official journal of the American Association for Cancer Research. 2022;28(2):338-49. doi: 10.1158/1078-0432.ccr-21-2291.

This paper reveals that the CNA patterns in the cfDNA of NEN patients could serve as a novel, highly specific biomarker.

• Mettler E, Fottner C, Bakhshandeh N, Trenkler A, Kuchen R, Weber MM. Quantitative Analysis of Plasma Cell-Free DNA and Its DNA Integrity and Hypomethylation Status as Biomarkers for Tumor Burden and Disease Progression in Patients with Metastatic Neuroendocrine Neoplasias. Cancers. 2022;14(4). doi: 10.3390/cancers14041025.

Research article that demonstrates how the combination of high cfDNA concentrations, decreases in DNA integrity and global hypomethylation can be used as a marker for disease burden and worse prognosis in metastatic NEN patients.

•• Öberg K. Molecular Genomic Blood Biomarkers for Neuroendocrine Tumors: The Long and Winding Road from Berzelius and Bence Jones to a Neuroendocrine Destination. Neuroendocrinology. 2021;111(4):297-303. doi: 10.1159/000508488.

This review summarizes the evolution and progress in the development of biomarkers in NENs.

•• Hofland J, Falconi M, Christ E, Castaño JP, Faggiano A, Lamarca A, et al. European Neuroendocrine Tumor Society (ENETS) 2023 Guidance Paper for Functioning Pancreatic Neuroendocrine Tumour Syndromes. Journal of neuroendocrinology. 2023;n/a(n/a):e13318. doi: https://doi.org/10.1111/jne.13318.

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