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Liquid biopsy and preclinical tools for advancing diagnosis and treatment of patients with pancreatic neuroendocrine neoplasms

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ABSTRACT

Pancreatic neuroendocrine neoplasms (pNENs) are rare cancers with broad challenges for their management. The main clinical obstacles are the high rate of patients diagnosed at advanced stages, lack of prognostic markers for early detection of disease recurrence in resected patients, significant limitations in identifying those who will benefit from adjuvant therapy, and timely recognition of treatment response. Therefore, the discovery of new prognostic and predictive markers is necessary for patient stratification and clinical management. Liquid biopsy, which has revolutionized the field of clinical oncology, is extremely under-investigated in pNENs. This review highlights its potential and the recent advances in related technologies, as candidates for the delivery of the new tools that can help to refine pNEN diagnosis and to personalize treatment. In addition, the opportunities and limitations of available preclinical research models with regard to biomarker research are discussed in light of pNEN clinical needs.

1. Introduction

Neuroendocrine neoplasms (NENs) represent a diverse group of tumors as they can form in most epithelial organs, with gastroenteropancreatic neuroendocrine tumors accounting for approximately half of them (Takayanagi et al., 2022). Their incidence has increased over the years, rising to 5.25 cases per 100,000 inhabitants in the last 30 years, likely due to their incidental diagnosis because of improved imaging techniques. Unfortunately, at diagnosis or during follow-up more than a third of the patients present metastatic or locally advanced disease.

Pancreatic neuroendocrine neoplasms (pNENs) are heterogeneous neoplasms that on the basis of histopathologic findings, are separated into poorly differentiated small cell or large cell pancreatic neuroendocrine carcinomas (pNECs), or well-differentiated pancreatic neuroendocrine tumors (pNETs) (Klimstra et al., 2019). pNETs originate from neuroendocrine cells in the pancreatic islets and were first described in 1869 as a subgroup of NENs that have a relatively distinct biological

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behavior and clinical management compared with pancreatic ductal adenocarcinoma (PDAC) (de Herder et al., 2016). They are further divided into three grades based on both mitotic index (number of mitoses per 2 mm^2) and Ki-67 index (Assarzadegan and Montgomery, 2021). Neoplasms showing the coexistence of a neuroendocrine and non-neuroendocrine component are classified as mixed neuroendocrine non-neuroendocrine neoplasms (MiNENs) (La Rosa et al., 2016). The most common neuroendocrine component in pancreatic MiNENs is a pNEC, thus, survival is relatively poor (Nießen et al., 2021).

pNETs can also be classified based on hormone hypersecretion to specific clinical syndromes. Functional pNETs (F-pNETs) include insulinomas, gastrinomas, VIPomas, glucagonomas, and somatostatinomas. In contrast, non-functional pNETs (NF-pNETs) are hormonally silent and more common (60–90%) (Rindi et al., 2018).

Based on epigenetic, transcriptomic, and mutational profiles (Boons et al., 2020; Cejas et al., 2019; Di Domenico et al., 2020; Chan et al., 2018), an emerging pNENs classification with three molecular subtypes has been proposed. The first group, which resembles beta cells, is characterized by the absence of copy number alterations and *MEN1*, *ATRX,* and *DAXX* mutations, with a favorable prognosis. The second group resembles alpha cells and has low copy number alterations and *MEN1* mutations, without *ATRX/DAXX* mutations. The last group is an intermediate group that resembles more alpha than beta cells, and is molecularly characterized by significant copy number alterations, *MEN1* and *ATRX/DAXX* mutations. This intermediate group has a poor prognosis with increased liver metastases compared to the others (Dreijerink et al., 2022; Rindi et al., 2022). In addition, insulinomas, the far most frequent F-pNET, have been further divided into molecular subgroups based on copy number alterations, *YY1,* and *ATRX/DAXX* mutations (Hong et al., 2020; Rindi et al., 2022).

Although pNEN tumors are usually sporadic, approximately 10% are associated with genetic syndromes such as multiple endocrine neoplasia type 1 (MEN1), von Hippel-Lindau syndrome (VHL), tuberous sclerosis (TSC1/TSC2), and neurofibromatosis (NF1) (Basuroy et al., 2016). As their natural history is different, their mortality risk also differs, with some tumors presenting a reasonable survival even in the presence of metastatic disease, while others are very aggressive with a poor prognosis. Thus, survival statistics for pNENs depend on several factors, including histopathological diagnosis, cancer stage, tumor grade, patient age, mode of treatment, and time since diagnosis. Based on an analysis of 5287 cases included in the SEER US National Database from 1973 to 2013, the life expectancy of pNENS patients, although reduced from normal, was 4.1 years, which is considerably longer than the median of 6-month for the more common PDAC (Brooks et al., 2019). However, in a more recent analysis of 8944 patients registered in SEER from 2000 to 2016, the median overall survival for the entire cohort was increased to 5.66 years (Sonbol et al., 2020). Nevertheless, the authors emphasize the importance of early detection, as patients with localized disease amenable to surgery have a near-normal life expectancy. Still, due to the lack of reliable biomarkers and specific symptoms, the majority of patients are diagnosed in an advanced stage when most systemic therapies lack an objective response, and the disease causes severe morbidity. Systemic treatment only stabilizes the disease, most likely because of poor delivery of therapeutic agents to the pancreas or because of inherent or acquired drug resistance. Reliable biomarkers, allowing a decrease of false positive diagnoses, identification of patients benefiting from adjuvant treatment, early detection of recurrence after curative resection, and treatment response or failure are the main clinical needs in pNEN patients. Although our understanding of pNEN biological behavior may have improved substantially in the last decade, many issues regarding etiopathology and the mechanisms involved in their evolution remained undefined. The rarity of pNENs and the consequent lack of suitable preclinical models hamper basic research, biomarkers discovery, and more extensive clinical trials.

In this review, we will discuss the current state of pNEN biomarkers and the potential clinical application of liquid biopsy. In addition, existing pNEN translational research resources will be considered, including tumor models, biobanks, and databanks.

2. Currently available pNEN biomarkers

pNEN diagnosis often begins with the biochemical quantification of circulating biomarkers. However, their specificity is rather low, and reliable assessment of patients with suspected pNEN requires additional diagnostic tests, with the gold standard being their expression evaluation in tissue following an invasive approach. This validation, available upon biopsy or surgery, and the fact that most of the biomarkers were discovered in tissues and blood of advanced-stage patients, prevent their use for early diagnosis. Moreover, limited data are available on treatment monitoring and the selection of patients benefitting from adjuvant therapy. Neuroendocrine markers can be divided into two main groups, specific, found in specific functional syndromes, and non-specific, produced by most pNETs (Table 1).

2.1. Circulating analytes

2.1.1. Specific biomarkers

Gastrin (GAS), insulin (INS), glucagon (GCG), somatostatin (SST), and vasoactive intestinal peptide (VIP) belong to the group of specific biomarkers in F-pNETs. GAS is implicated in chloride acid release from parietal cells in the stomach, gastric motility, and pancreatic secretion, and plasma concentration $>$ 300 pg/mL correlates with gastrinoma. INS is secreted by the beta cells of the pancreatic islets, but as its concentration increases in various conditions, it is not a reliable screening marker for insulinoma. In combination with otherwise unexplained hypoglycemia, elevated insulin levels are diagnostic of insulinoma. On the other hand, GCG is secreted by pancreatic alpha cells in a negative correlation to blood glucose levels, opposing insulin actions by stimulating hepatic glucose synthesis and mobilization. A concentration $>$ 500 pg/mL is indicative, although several other conditions, including cirrhosis, untreated diabetes mellitus, prolonged fasting, sepsis, burns, and acromegaly, could result in increased levels. Even so, its concentration is used in clinics as an indicator of liver metastases (Eldor et al., 2011). SST is a hormone secreted by pancreatic delta cells, APUD (amine precursor uptake and decarboxylation) cells, and gastric antrum D cells, but is characterized by low specificity, and the changes in concentration are not sufficient to diagnose somatostatinoma. VIP is a hormone released by pancreatic and brain cells functioning as a vasodilator and regulator of smooth muscle activity, stimulator of water and electrolyte secretion from the intestinal tract, and inhibitor of gastric acid secretion. Serum VIP level $>$ 200 pg/mL (reference range is $<$ 190 pg/mL) confirms the diagnosis of VIPoma in patients presenting secretory diarrhea (Abdullayeva, 2019).

2.1.2. Non-specific biomarkers

Biomarkers of NF-pNETs include chromogranin-A (CHGA), pancreatic polypeptide (PP), neuron-specific enolase (NSE), human chorionic gonadotropin (HCG), and alpha-fetoprotein (AFP) (Bocchini et al., 2020), whereas NSE and CA19–9 are biomarkers for pNEC.

CHGA is a soluble glycoprotein that belongs to the family of granins, constituting major components of secretory granules of endocrine and neuroendocrine cells. Its sensitivity and specificity vary between 62% and 75% and 60–100%, respectively, since its increase can also be associated with several other cancers and non-pathological conditions or non-neoplastic factors (Modlin et al., 2010). Despite its limitations, CHGA is the only circulating biomarker recommended by the European Neuroendocrine Tumour Society (ENETs) (Modlin et al., 2010; Pavel et al., 2012), and it is routinely used in clinical practice since its decrease is considered a surrogate marker for response to treatment (Nehar et al., 2004; Nikou et al., 2008; O'Toole et al., 2009). Circulating CHGA has been reported to correlate with tumor burden, progression, and presence of metastases (Modlin et al., 2010; Hofland, et al., 2018). All other

Table 1
Overview of currently available circulating biomarkers.

Marker	Sensitivity	Specificity	Pros	Cons	References
Specific					
GAS	94%	100%	Associated with age and pNENs functional status	↑ in various conditions	(Qiu et al., 2016; Sansone et al., 2019; Shibata et al., 2013)
INS	52 – 94%	92 – 100%	Associated with ↓survival (in patients with MHS)	Other conditions lead to ↑ serum insulin	(de Mestier et al., 2015; Granberg, 2015; Oberg et al., 2017)
GCG	N/A	N/A	↑ concentrations are diagnostic for glucagonomas (distinguish from other pNENs)	Several conditions ↑ glucagon levels (e.g. cirrhosis, diabetes mellitus, fasting, sepsis)	(Appetecchia et al., 2018; Batcher et al., 2011; Sansone et al., 2019)
SST	N/A	N/A	Potential use of SST analogs as adjuvant therapy	Not sufficient to diagnose somatostatinoma	(Sansone et al., 2019; Tran et al., 2021; Wang et al., 2021)
VIP	N/A	N/A	Confirms the diagnosis of VIPoma in patients with secretory diarrhea	VIPoma occurs in several tissues	(Bocchini et al., 2020; Smith et al., 1998)
Non-specific					
CHGA*	62–75%	60–100%	Surrogate marker for response to treatment	↑ associated with several other cancers and non-pathological diseases	(Hofland, et al., 2018; Mandair, et al., 2021; Modlin et al., 2010; Pavel et al., 2012)
PP	31–63%	67%	Sensitivity ↑ up to 80% in metastatic disease	↑ by many non-NEN factors	(Herrera-Martínez et al., 2019; Kanakis and Kaltsas, 2012; Oberg, 2011)
NSE	33%	73%	CHGA/NSE provide prognostic information on PFS and OS	↑ in several non-NENs	(Herrera-Martínez et al., 2019; Nobels et al., 1997; O'Toole et al., 2009)
HCG	N/A	N/A	Could improve the efficacy of the measurement	Limited clinical application (CHGA is more sensitive and specific)	(Grimaldi et al., 2014; Heitz et al., 1987; Shah et al., 2008)
AFP	N/A	N/A	↓ is a sign of adequate treatment	Validity in the context of pNENs is not definite	(Shah et al., 2008)
NETest	80%	94%	Validated for early detection of residual disease or recurrence after surgery	Do not necessarily correlate with tumor size	(Modlin et al., 2013; Partelli et al., 2020)

Abbreviations: GAS – gastrin; INS –insulin; GCG – glucagon; SST – somatostatin; VIP – vasoactive intestinal peptide; CHGA – chromogranin-A; PP – pancreatic polypeptide; NSE – neuron-specific enolase; HCG – human chorionic gonadotropin; AFP – alpha-fetoprotein; NETest – neuroendocrine gene transcripts; PFS - progression-free survival; OS - overall survival; MHS - metachronous hormonal syndromes; N/A - data not available, * also analyzed in tissues

non-specific biomarkers have low sensitivity or specificity and therefore limited clinical application. An in-depth description and categorization of all circulating pNEN biomarkers can be found in Bocchini et al. (2020).

Experts from different disciplines further underline in a consensus statement that circulating biomarkers have substantial limitations in NENs, highlighting the potential of circulating multianalyte measurement strategies (Oberg et al., 2015). In this regard, a more reliable indicator for evaluating the diagnosis and prognosis of pNENs has been recently introduced, a blood test panel of 51 NET marker genes derived from the transcript profile of NET cells (NETest). This molecular signature has been validated by a number of research groups, showing superior diagnostic utility for NENs, as it does not depend on a patient's characteristics, such as age, ethnicity, or treatment. Increased NETest levels at baseline (at least >33–40%) correlate with disease progression, a decrease or stability after treatment predicts response to treatment, while an increase indicates the emergence of minimal residual disease after surgery (Puliani et al., 2022). NETest score represents a multivariate biomarker of circulating mRNA, with a diagnostic sensitivity of 80–100% combined with a specificity of 94% in pNENs (Modlin et al., 2013; Partelli et al., 2020). Its introduction into clinical practice can help to identify patients at high risk of recurrence after radical surgery (Puliani et al., 2022).

2.2. Tissue biomarkers

Grading and correct histological diagnosis are important regarding pNEN treatment strategy. Grading based on the Ki-67 index has been proven to be the most reliable factor in the prognostic evaluation of pNENs (Klöppel and La Rosa, 2018). Histopathological analysis of tumor tissues resected during surgical or endoscopic biopsies can confirm the diagnosis of pNENs based on morphological evaluation and the demonstration of neuroendocrine differentiation markers by immunohistochemistry, even without the presence of high levels of a specific biomarker in serum (Table 2) (Oberg et al., 2017). CHGA and synaptophysin (SYP) are the most commonly used markers (Annaratone et al., 2014; Sainio et al., 2018). SYP, a transmembrane glycoprotein involved in synaptic transmission (Erickson and Lloyd, 2004), is highly sensitive but not extremely specific, as it is expressed in some non-neuroendocrine carcinomas. In contrast, CHGA, a precursor of several functional peptide hormones such as pancreastatin and vasostatin, is highly specific but less sensitive than SYP. The combination of CHGA with SYP represents the first multistep approach to confirm the tumor's neuroendocrine nature. Recently, insulinoma-associated 1 (INSM1), a zinc finger transcription factor involved in the terminal steps of neuroepithelial development, has emerged as a new neuroendocrine marker with increased sensitivity and specificity (Rosenbaum et al., 2015; Zhang et al., 2021). Other second-generation neuroendocrine markers have been studied in pNEN, such as insulin gene enhancer protein-1 (ISL-1), neuroendocrine secretory protein 55 (NESP 55), secretagogin, and syntaxin. Although, data regarding their diagnostic utility and prognostic value are still limited (Bocchini et al., 2020; Juhlin et al., 2020; Kővári et al., 2020).

Transcriptional factors are a fruitful source of tissue biomarkers in cancer. Pancreatic and duodenal homeobox 1 (PDX1) is a transcriptional factor implicated in pancreas development and insulin-producing beta cell differentiation. It is repressed in glucagon-producing alpha cells, in contrast to aristaless-related homeobox (ARX), required for terminal alpha cell differentiation. The combination of PDX1 and ARX1 expression assessed by immunohistochemistry have been proposed as surrogate markers to replicate the molecular classification. The first published reports described that PDX1 expression was associated with better relapse-free survival (Cejas et al., 2019; Di Domenico et al., 2020). However, a more recent analysis comprising 561 NF-pNETs found that tumor classification based on PDX1 and ARX expression was not independently correlated with relapse-free survival (Hackeng et al., 2022).

Table 2
Overview of the most relevant tissue pNETs biomarkers available.

Marker	Positivity	Pros	Cons	References
Neuroendocrine differentiation biomarkers				
SYP	≈ 95–100%	Universal clinical availability	Less specific than CHGA	(Juhlin et al., 2020; Rindi et al., 2022)
CHGA	≈ 90–95%	Universal clinical availability	Less sensitive than SYP	(Juhlin et al., 2020; Rindi et al., 2022)
INSM1	99%	↑Sensitivity and specificity	Limited clinical availability	(Juhlin et al., 2020; Zhang et al., 2021)
ISL-1	82–93%		Less sensitive than SYP and CHGA	(Koo et al., 2012; Juhlin et al., 2020)
Secretagogin	80–100%		Less sensitive than SYP and CHGA	(Juhlin et al., 2020; Lai et al., 2006)
Syntaxin	80%		Less sensitive than SYP and CHGA	(Kővári et al., 2020)
NESP 55	41–56%		Less sensitive than SYP and CHGA	(Jakobsen et al., 2003; Srivastava and Hornick, 2009)
Pancreatic NET origin biomarkers				
PAX6	65–74%	Could indicate pancreatic origin of metastatic NETs	Expressed in duodenal and rectal NETs	(Falconi et al., 2016; Lai et al., 2015; Sangoi et al., 2011)
PAX8	50–88%	Could indicate pancreatic origin of metastatic NETs	Expressed in duodenal, rectal, thymic, parathyroid, and skin rectal NETs. Wide variability between monoclonal and polyclonal antibodies	(Koo et al., 2012; Lai et al., 2015; Liau et al., 2016)
PDX1	≈ 30%	Could indicate pancreatic origin of metastatic NETs	Expressed in gastroduodenal, rectal and appendiceal NETs. Not expressed in alpha cell related pNETs.	(Srivastava and Hornick, 2009; Di Domenico et al., 2020)
Prognostic biomarkers				
SSTR2a	≈ 75%	SSTR2a can be considered for both diagnostic and therapeutic purposes	Higher sensitivity only in multianalyte combination	(Modlin et al., 2015; Zimmermann et al., 2016; Cavalcanti et al., 2017; Liverani et al., 2018)
PD-L1	≈ 20%	Represent a predictive biomarker for NEN patients who may benefit from immunotherapy	Low positivity in higher grade GI-NENs (only 10%)	(Cavalcanti et al., 2017; Kim et al., 2016; Bösch et al., 2019; Ali et al., 2020)
PR	40–75%	Suitable predictive factor and inversely correlated with tumor progression	Low positivity in high-grade and metastatic pNENs	(Doglioni et al., 1990; Yazdani et al., 2015; Chai et al., 2018)

Abbreviations: SYP – synaptophysin; CHGA – chromogranin-A; INSM1 - insulinoma-associated 1; ISL-1 – insulin gene enhancer protein-1; NESP 55 – neuroendocrine secretory protein 55; PAX6 – paired box 6; PAX8 – paired box 8; PDX-1 –pancreatic and duodenal homeobox 1; SSTR2a –somatostatin receptor 2a; PD-L1 – programmed cell death ligand; PR – progesterone receptor; GI-NENs - gastrointestinal neuroendocrine neoplasms

Other biomarkers with clinical value are somatostatin receptors (SSTRs). This is a family of specific membrane-bound receptors with five subtypes (SSTR1-SSTR5), the SSTR2 subtype has two splice variants (SSTR2A and SSTR2B). Most pNETs express more than one SSTR, SSTR2 and SSTR5 being the most frequently expressed (Gomes-Porras et al., 2020). Moreover, patients with pNETs expressing SSTR2 or SSTR5 by immunohistochemistry showed better disease-specific survival than those without SSTR2 or STTR5 (Song et al., 2016). SSTR overexpression has been used clinically in diagnosis and treatment. pNET primary location and metastases can be detected by using imaging techniques such as SSTR scintigraphy and SSTR emission tomography/computed tomography (SSTR PET/CT), in which a synthetic somatostatin analog is labeled with a radionuclide (Clift et al., 2020). Concerning treatment, SSTR2 is the main receptor for somatostatin analogs such as octreotide and lanreotide, the first-line treatment in pNETs (Gomes-Porras et al., 2020). Moreover, peptide receptor radiotherapy using somatostatin analogs labeled with β-emitting radionuclides has been demonstrated to improve progression-free survival (PFS) and overall survival (OS) (Hicks et al., 2017). Initially, SSTR assessment was proposed to have a possible predictive value before treatment initiation since immunohistochemistry SSTR2A expression correlated with SSTR radionuclide uptake (Brunner et al., 2017; Kaemmerer et al., 2015). However, further studies demonstrated that SSTR2A immunoexpression had no additional value compared with SSTR2-radionuclide imaging techniques in predicting prognosis and tumor response after peptide receptor radiotherapy using ^{17}Lu-octreotate (Brunner et al., 2017; van Adrichem et al., 2016).

The immune landscape has been scarcely analyzed in pNENs mainly because they are considered cold tumors with very few infiltrating lymphocytes. Available evidence shows that higher expression of immune checkpoint markers such as PD-1, PD-L1, and tumor-infiltrating lymphocytes (TILs) are associated with higher grade (Bösch et al., 2019; Cavalcanti et al., 2017). Moreover, high TILs and PD-1 expression are significantly associated with shorter patient survival (Bösch et al., 2019). Thus, grade 3 pNETs and pNECs may benefit from immune checkpoint treatments, as some clinical trials are currently assessing (Mehnert et al., 2020). Moreover, many other biomarkers are currently under evaluation. For example, metachronous hepatic metastasis and poor survival were related to low PAX6 expression, as documented by Kudo et al. (2020). Although such evidence makes these biomarkers potential candidates for new therapeutic strategies, they have not yet been routinely validated in pNENs management. They represent only a small fraction of available studies and are provided here as an example of recently reported investigations (Table 2).

2.3. Molecular biomarkers

Published data demonstrate that pNETs and pNECs differ significantly in genetic aberrations. pNETs frequently display mutations in chromatin remodeling genes such as *DAXX/ATRXX* and *MEN1*; together with mutations in genes from the mTOR pathway such as *TSC1/2* and *PIK3CA* in one-sixth of tumors (Chan et al., 2018). Still, as mentioned previously, approximately 10% of all cases that manifest as a cancer-prone syndrome are caused by germline mutations in *MEN*, *VHL, NF1*, or *TSC* genes (Rindi et al., 2007). Whole-genome/exome studies of pNENs identified *PTEN* and *TSC1/2* as potential driver mutations in their development and in samples processed *via* somatic whole-genome sequencing and telomere length, a high rate of gene rearrangements and novel fusion genes were identified, including the Ewing sarcoma 1 (*EWSR1*) gene (Jiao et al., 2011; Scarpa et al., 2017). Combined alterations in *TP53, MYC,* and *RB1* have been confirmed as driver mutations for pNECs, whereas *KRAS* and *SMAD4* mutations are less common. These molecular alterations are particularly important for distinguishing pNECs from G3 pNETs when immunostaining is insufficient (Crippa et al., 2016). Noteworthy, Rb loss and p53 overexpression are more frequent in small cell NEC than in large cell NEC (Couvelard and Cros, 2022).

Concerning pNETs, the presence of *ATRX* and/or *DAXX* mutations can identify a subgroup of patients with worse prognosis and frequent liver metastases (Hackeng et al., 2022; Singhi et al., 2017). Furthermore, these mutations can be clinically screened by ATRX/DAXX

immunoexpression (Hackeng et al., 2022; Hechtman et al., 2019). *ATRX/DAXX* mutations correlate with alternative lengthening of telomeres (ALT), a telomerase-independent telomere maintenance mechanism, which can be assayed by a specific FISH (Heaphy et al., 2011). Both the study of ATRX/DAXX loss by immunohistochemistry and/or the presence of ALT are recommended in pNET, as they have demonstrated to be independent prognostic factors of reduced relapse-free survival in a large series of 561 NF-pNETs (Hackeng et al., 2022).

Copy number (CN) variants have also been detected in NF-pNETs, including losses of 11q (together with *MEN1* gene at 11q13) and gains in chromosomes 1p, and 1q, 3p, 6q, 7p and 7q, 9p, and 9q, 10q, 11p, 17q, 20q (Capurso et al., 2012). CN losses of chromosomes 3, 6q, and 1 and a CN gain of chromosome 17q have been associated with an increased risk of liver metastases (Ebrahimi et al., 1999; Speel et al., 2001). Among insulinomas, the most frequent F-pNETs, mutations in ATRX/DAXX defined a subgroup with worse prognosis as occurs in NF-pNETs. In contrast, mutations in *YY1* are found in a favorable subgroup characterized by neutral CN variations (Hong et al., 2020). This supports ATRX/DAXX immunohistochemistry assessment in insulinomas, the analysis of these markers in the context of liquid biopsy is discussed in subsequent sections. In contrast, gastrinomas present low rates of CN variants, whereas in 44% of sporadic cases somatic mutations are reported, especially in *MEN1*, which is known to affect DNA methylation (Tirosh and Kebebew, 2020).

In addition to mutation events, epigenetic alterations have also been reported to contribute to neuroendocrine transformation, underlining the role of DNA methylation in driving tumorigenesis and stratifying prognosis in pNENs. Three pNEN subgroups were identified with distinct DNA methylation patterns (Lakis et al., 2021), particularly between NF-pNET subgroups (Tirosh et al., 2019). Aberrations in DNA methylation have been found in *RASSF1A, GSTP1, CASP8, HIC1, TIMP3, VHL, MGMT,* and other genes, including *LINE-1* repetitive sequences (Stefanoli et al., 2014).

3. Liquid biopsy concept

Liquid biopsy represents a promising alternative to the tissue biopsy, which, as mentioned previously, is currently the gold standard for pNEN diagnosis. However, tissue biopsy has certain limitations due to its invasive nature, preventing patients from being tested on an ongoing or repetitive basis. Although in the beginning, the liquid biopsy was only used to detect CTCs in peripheral blood, at present, it is extensively used to obtain additional tumor-derived biomarkers, including ctDNA, circulating microRNAs (miRNAs), long non-coding RNAs (lncRNAs), extracellular vesicles (EVs) and tumor proteins (De Rubis et al., 2019), revolutionizing the initial concept. Liquid biopsy represents a minimally invasive approach, well tolerated by most patients, allowing to obtain and analyze tumor-derived material present in peripheral blood or other body fluids in a timely fashion. It offers flexibility, which is especially important when managing the emergence of tumor heterogeneity, metastasis, and resistance to therapy, issues that are widely recognized to be among the most important in cancer management. However, it has been rarely applied in pNEN, except for NETest, and its clinical potential for this disease remains practically unknown.

3.1. Circulating tumor cells (CTC) and disseminated tumor cells

The multistep metastasis process consists of tumor cell local infiltration into the adjacent tissue, intravasation, dissemination into the distant sites, extravasation, and proliferation in competent organs. Individual tumor cells shed passively or actively from the solid tumors into the vasculature are known as CTC or, when found in clusters, as circulating tumor microemboli (CTM). This population of tumor cells can gain a more aggressive phenotype *via* epithelial-to-mesenchymal transition (EMT). Due to this process, heterogeneous CTC populations evolve from originally epithelial tumors such as CTC EMT, hybrid (epithelial/EMT+), irreversible EMT+ tumor cells, and circulating tumor stem cells, presenting different phenotypes, biological values, and prognostic potential (Grover et al., 2014). CTM also exhibit distinct phenotypic and molecular characteristics with enhanced stemness and higher metastatic potential, and they are often associated with higher metastatic risk. They can be both homotypic and heterotypic as they often form clusters with immune cells and cancer-associated fibroblasts (Huh et al., 2010; Saini et al., 2019).

Even though CTC are often considered surrogate biomarkers in the case of solid cancers and many studies have anticipated their great potential in clinical applications, their inclusion in the present clinical guidelines is limited, with the exception of CTC being part of the TNM breast cancer staging, where cMo(i +) represents the presence of cancer cells in blood, bone marrow, or lymph nodes as detected by laboratory tests, although there is no sign of cancer on physical examination, scans or x-rays. According to several studies, there is evidence of a correlation between CTC number and tumor stage. However, the clinical value of CTC for early diagnosis is still controversial and remains a significant technological challenge and hot topic for translational research. The formation of resistant tumor subclones, as well as tumor evolution, are driven by genomic instability that can be studied by serial blood sampling accessible by liquid biopsy (Lin et al., 2021). Nevertheless, small numbers and heterogeneous profiles make CTC identification challenging. CTC are usually disguised within a high background of white and red blood cells (approx. 1 CTC per 10^5-10^7 leukocytes) (Allan and Keeney, 2010). Although it is currently accepted that CTC express epithelial cell adhesion molecule (EpCAM) and cytokeratin but not CD45, CTC detection using antibodies against EpCAM is considered limited and insufficient to detect just a small epithelial CTC subpopulation. Thus, combining the advantages of different technologies and tumor-specific markers has received more attention from the research community (Lin et al., 2021).

CTC have been detected in patients with pNENs, but their level of accuracy, including their optimum threshold, is still under investigation (Mandair, et al., 2021), as available data are limited, and they are mainly derived from studies in NEN tumor cohorts (Table 3). CTC in pNENs have been shown to express EpCAM. The first study addressing this issue was by Khan et al. (2011) in NENs of the pancreas, midgut, and bronchopulmonary origin. In this study, the reported load for CTC derived from midgut tumors by the CellSearch® System (Janssen Diagnostics) was greater than five CTC/7.5 mL of blood in 47% of cases, but only 21% of the 19 pNET patients had at least one (ranging between 0 and 11) EpCAM-positive CTC. A high correlation between CTC number and liver metastases burden was found, whereas no or low correlation was identified between CTC number and Ki-67 and serum CHGA levels, respectively. In a second study published by the same group in 2013, the use of the EpCAM-based CellSearch® platform in a cohort of 42 pNEN patients using 7.5 mL of blood enabled the identification of > 50 CTC in 12 of these cases, whereas more than one CTC (ranging from 0 to 430) at baseline was found in 36 patients (Khan et al., 2013).

Several studies have shown CTC positivity associated with treatment response and survival in pNEN patients. Post-treatment CTC count as a predictive biomarker for OS and PFS was investigated in a study by Khan et al. (2016). Blood was collected at baseline, three to five weeks, and 10–15 weeks after starting therapy from 31 pNEN patients in addition to 81 midgut and 12 bronchopulmonary NENs (Khan et al., 2016). The development of progressive disease was associated with CTC positivity at baseline and at the first post-treatment time point in metastatic NENs. In patients with no CTC at both time points, metastasis occurred in 8% in contrast to 60% in those with less than a 50% CTC reduction post-treatment. Poor outcome groups were defined by the presence of more than eight CTC at 3–6 weeks after therapy or by less than a 50% fall or rise in CTC number. In the multivariate analysis, changes in CTC number had the strongest association with OS ($HR = 4.13$, $p = 0.0002$). Although data for individual NEN subgroups were missing, results achieved for NENs suggest that CTC may be a useful surrogate marker of

Table 3
Circulating tumor cells as pNEN biomarkers.

CTC positivity	CTC counts	Sample size	Prognostic role	Correlation with other markers	Method	References
21%	0–11	19	Correlation with liver metastases burden	Low correlation with serum CHGA	CellSearch®	(Khan et al., 2011)
85.7% (33/42)	0–430	42	Correlation with metastases, nonsignificant association with OS	Association with tumor grade	CellSearch®	(Khan et al., 2013)
N/A	N/A	31	N/A	N/A	CellSearch®	(Khan et al., 2016)
36%* (43/119)	0–430	119	Association with tumor grade and bone metastases	Previous treatments did not affect CTC count	CellSearch®	(Rizzo et al., 2019)
N/A	N/A	11	N/A	N/A	ODEP-based microfluidic system (Chiu et al., 2016)	(Hsieh et al., 2019)

Abbreviations: CTC - Circulating tumor cells; CHGA - chromogranin A; OS - overall survival; N/A – not available; ODEP - optically induced dielectrophoretic; * metastatic patients

response to treatment in this tumor type. A significant difference in OS between patients with and without CTC at baseline (hazard ratio, HR 3.88; 95% CI, 2.15 – 7.00; $p < 0.001$) has been demonstrated (Khan et al., 2016). Although the prognostic value of CTC has also been investigated in an Asian NEN population, analysis stratified for individual cancer types was not possible, as only 11 pNET patients were enrolled (Hsieh et al., 2019). More recently, CTC positivity (36%) was significantly correlated with the presence of bone metastases ($p < 0.0001$) in a group of 119 pNEN patients (Rizzo et al., 2019). Association of CTC positivity with poor prognosis was also reported in a cohort of 90 pNEN patients, while the absence of CTC at baseline was strongly related to stable disease (Mandair, et al., 2021). More than one CTC per 7.5 mL of blood was predictive of 12-month progression (odds ratio, OR = 6.69, $p < 0.01$) and death at 36 months (OR = 2.87, $p < 0.03$). Patients with no detectable CTC had a significantly longer OS (HR = 3.16, $p < 0.01$) and PFS (OR = 2.6, $p < 0.01$) compared to CTC-positive cases, similar to the predictive value of histological grade. The number of CTC detected in this study was low, with 30/90 (33%) of patients with one CTC or more, while 60/90 (66%) cases were negative.

3.1.1. *CTC detection and characterization*

Efficient CTC isolation represents a significant technical challenge since CTC in circulation are extremely rare. Sequential procedures consisting of cell enrichment and cell characterization for detecting CTC have been employed to overcome the problem of cell rarity. Size, density, electric charge, or the biological profile represent factors upon which enrichment techniques have been developed (Tables 4, 5). Therefore, there is a wide range of approaches to choose from when performing CTC isolation. Enrichment methodologies include density-based methods (*e.g.* density gradient centrifugation) and size-based methods, immunocapture methods (based on epithelial cell surface marker expression) for positive or negative selection (*e.g.* immunodensity cell separation), whereas cell characterization can be achieved by flow cytometry, immunofluorescence, quantitative polymerase chain reaction (qPCR), microarrays, or sequencing (Alix-Panabières and Pantel, 2013; Neves et al., 2021). However, the CellSearch® System remains the only CTC platform approved by the Food and Drug Administration (FDA) for CTC enumeration. More details concerning the available approaches to separate CTC from whole blood can be found in the review article of Bankó et al. (2019).

Nanomaterial-based technologies for CTC isolation and detection

Table 4
Different approaches except for microfluidics for CTC enrichment and isolation.

Enrichment method	Company	Technology	Single-cell technology	PC preclinical studies* *	PC clinical studies* **
Size	Rarecells	Iset® Technology	NO	N/A	N/A
	Creatv MicroTech	CellSieve™	NO	N/A	N/A
Piezoelectricity	ScreenCell	ScreenCell®	NO	(Kulemann et al., 2015)	N/A
Density gradient	Greiner Bio-One	OncoQuick®	NO	N/A	N/A
Density gradient with immunofluorescence and mechanical process	RareCyte	AccuCyte® CyteFinder® with CytePicker®	YES	N/A	N/A
	Qiagen	AdnaTest	NO	N/A	N/A
	Meranini Silicon Biosystems	*CellSearch®	NO	(Dotan et al., 2016)	N/A
Immunomagnetic	Miltenyi Biotech	MACSQuant® Analyzer, MACS Cell Separation technology	NO	N/A	N/A
	Stemcell Technologies	EasySep™	NO	N/A	N/A
Immunomagnetic and size	SurExam Biotech	CanPatrol™	NO	(Zhao et al., 2019)	N/A
	Stemcell Technologies	RosetteSep™	NO	(Buscail et al., 2019)	NCT02349867
Immunocapture	Hangzhou Watson Biotech	Cytosorter®	NO	N/A	N/A
	Gilupi	CellCollector® (*in vivo*)	NO	N/A	N/A
Immunofluorescence	Epic Sciences	Epic CTC Assays	NO	N/A	N/A
Immunofluorescence and manual spiking	CytoTrack	CytoTrack™ and CytoPicker™	YES	N/A	N/A
Functional (adhesion)	Vitatex	Vita-Assay™	NO	(Tulley et al., 2016)	N/A

Abbreviations: PC – Pancreatic cancer, N/A - data not available
*FDA approved; * * Pubmed.ncbi.nlm.nih.gov (filtered by Technique and Company); * **Clinicalgov.com (filtered by Technique and Company)

Table 5
Microfluidic-based methodologies for CTC enrichment.

Enrichment method	Company	Technology	Single-cell technology	PC preclinical studies* *	PC clinical studies* **
	Angle Plc	Parsortix®	NO	N/A	N/A
	Clearbridge BioMedics	ClearCell® FX1 System with CTChip® FR	YES	N/A	N/A
	VyCAP	Puncher Technology	YES	N/A	N/A
	Celsee Diagnostics	Celsee™ PREP Single Cell	YES	N/A	N/A
Size	Cytena	Single-Cell Printer™	YES	N/A	N/A
	Cytogen	SmartBiopsy™ (HDM Chip)	NO	N/A	N/A
	Vortex Biosciences	Vortex HT Chip	NO	N/A	N/A
	Clinomics	CD-Prime™	NO	(Park et al., 2021)	N/A
	Siemens Healthineers	Automated Siemens Healthineers filtration device	NO	(Brychta et al., 2017)	N/A
Piezoelectricity	NanoCellect Biomedical	WOLF® Cell Sorter and N1 Single-cell disperser	YES	N/A	N/A
Dielectrophoresis (size)	Meranini Silicon Biosystems	DEParray™	YES	N/A	N/A
	Precision For Medicine (Apocell)	ApoStream™	NO	N/A	NCT02349867
Immunomagnetic	Fluxion Biosciences	IsoFlux ™CTC system	NO	(Brychta et al., 2017; Wu et al., 2020)	N/A
	Cynvenio Biosystems	LiquidBiopsy®	NO	N/A	N/A
	Biocept	OncoCEE®	NO	N/A	N/A
Immunocapture	CytoLumina Technologies Corp.	Nanovelcro chip (CytoTrapNano™)	NO	(Ankeny et al., 2016; Court et al., 2018)	N/A
	Biofluidica	BioFluidica CTC Detection System	NO	N/A	N/A
	CellMax Life	CellMax (CMx®)	NO	N/A	N/A
Immunofluorescence	Fluidigm Corporation	C1™ - IFC	YES	(Xin et al., 2016)	N/A

Abbreviations: PC – Pancreatic cancer, N/A - data not available
* * Pubmed.ncbi.nlm.nih.gov (filtered by Technique and Company); * **Clinicalgov.com (filtered by Technique and Company)

have also been developed and successfully applied over the past years. Besides others (Bai et al., 2014; Gribko et al., 2019; Cheng et al., 2020a; Ming et al., 2017; Wang et al., 2019; Yoon et al., 2014), the review articles of Cheng et al. (Cheng et al., 2020b) and Farahinia et al. (2021) present a very clear overview of the novel microfluidic approaches to CTC separation and sorting, which led to the recent development of microfluidic-based technologies. Microfluidics represents a promising approach allowing to isolate CTC with simplicity, including lower cost and increased speed, high capture efficiency, and cell purity, overcoming the common problems of clogging and adhesion often present in mechanical apparatus and allowing their use in everyday clinical practice. It permits single CTC isolation by trapping them depending on different physical and biological properties: size, density, electricity, immunoaffinity, or adhesion (Table 5). A technique using magnetic particles, which are bound to specific cell surface proteins on the target cell, allows specific cell isolation of CTC from heterogeneous mixtures (Wu et al., 2020). Several operations can be done simultaneously by analyzing single CTC with microfluidic devices: pretreatment, enrichment, detection, and diagnosis, thus increasing the efficiency of CTC technology (Coumans et al., 2013; Hvichia et al., 2016; Cho et al., 2018; Magbanua et al., 2017). Moreover, CTC can remain viable after capture and can be used for further analysis. The development of microfluidic devices has revolutionized the field of CTC enrichment, especially with the incorporation of capture agents, including antibodies and nucleic acid aptamers (Bankó et al., 2019). Portability, cost-effectiveness, low volume of reagents and samples required, speed, high recovery rate, purity, and precision are the main advantages of these new technologies (Gwak et al., 2018; Chen et al., 2014).

Microfluidic devices are easy to design, fabricate and modify and have been widely recognized as a powerful tool that will play an essential role in future medical applications to meet large-scale and high-throughput requirements. Most microfluidics applications are intended to diagnose prostate, breast, and colorectal cancers by capturing, isolating, and enumerating CTC with the use of ferrofluid nanoparticles labeled with antibodies targeting EpCAM. As CTC are highly heterogeneous, CTC analysis using microfluidics at the single-cell level represents the most up-to-date technological approach. The pros and cons of microfluidic enrichment methods are summarized in Fig. 1. As mentioned above, studies on pNEN CTC are limited, and so is the bibliography on the use of commercialized devices to detect CTC or follow pNEN patients based on microfluidics. Therefore, this is a field that needs to be further explored.

3.1.2. *Single-cell omics*

With the emergence of single-cell technologies, it has been possible to identify molecular aberrations on a single-cell level (Bond et al., 2020). These high-resolution approaches allow systematic profiling of the CTC genome, transcriptome, proteome, or methylome (Gkountela et al., 2019; Krebs et al., 2014). Although immunohistochemistry and CTC enumeration remain the main tools for predicting cancer progression and therapeutic response, this technological revolution began to unravel unprecedented opportunities in our understanding of the metastatic process. Omics studies have already revealed considerable heterogeneity among individual CTC subpopulations, reflecting multi-clonal origins of the tumors (Hwang et al., 2018; Lim et al., 2019; Riebensahm et al., 2019). Moreover, emerging research demonstrates the existence of different CTC subpopulations with diverse phenotypes and metastatic potential in individual patients. Although it currently remains challenging to achieve high genome coverage, low allele dropout, and low amplification errors, single-CTC genomics has the potential to become a powerful diagnostic tool.

Besides mutations and chromosomal rearrangements, the cancer cell epigenome harbors abnormalities that could serve as potential biomarkers or be targeted by novel therapeutic strategies. Altered DNA methylation signatures found in stem cell genes and increased pluripotency network expression were associated with disseminated cells' metastatic potential (Gkountela et al., 2019). Analysis of CTC methylation status can provide information about hybrid EMT states related to the more aggressive phenotypes.

Dysregulation of miRNA expression at a single CTC level is evolving and has the potential to identify miRNAs that are essential for cancer evolution, paving the way for personalized therapies. Single-cell

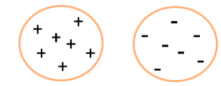
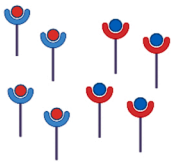
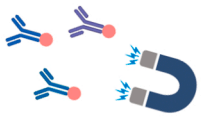
		PROS	CONS
 **PHYSICAL properties-based enrichment** 	**Size** (based on cell size)	➢ Cells not labeled and unmodified ➢ Simple ➢ Low cost	➢ Loss of smaller CTC ➢ Interference with leukocytes
	Dielectrophoresis (based on electrical properties of CTC)	➢ Quick and simple ➢ Cells not labeled	➢ Loss of smaller CTC ➢ Cells can have complicated dielectric properties ➢ Buffers are required
	Density gradient (separation depending on differential density)	➢ Cells not labeled ➢ Fast and cheap ➢ High efficiency	➢ Loss of smaller CTC ➢ Interference with leukocytes
 **BIOLOGICAL properties-based enrichment** 	**Immunocapture** (specific antigens that are only expressed on the surface of CTC)	➢ High specificity and sensitivity ➢ Possibility to use several antibodies to capture more subpopulations	➢ Difficulty in retrieving cells after enrichment ➢ Loss of CTC subpopulations due to the heterogeneity of CTC
	Immunomagnetic (magnetic beads with antibodies)	➢ High specificity and sensitivity ➢ Possibility to use several antibodies to capture more subpopulations ➢ Solution for the difficulties in recovering CTC after enrichment using immunocapture	➢ Loss of CTC subpopulations due to the heterogeneity of CTC
	Cellular adhesion (based on cellular adhesion with ligands or matrix)	➢ Cells not labeled	➢ Inability to use whole blood because of blood complexity

Fig. 1. Pros and cons of the main CTC microfluidic-based enrichment methods.

transcriptomics provides high-resolution information on cellular differences that cannot be revealed using bulk RNA sequencing. Although various approaches have been implemented for whole-transcriptome analysis, certain biases still exist. Among the main limitations is the inability to achieve a full-length sequence from single-cell and capture low-abundance transcripts.

Furthermore, single-cell proteomics techniques are indispensable for studying cellular mechanisms and processes such as signal transduction and regulation of transcription, cytokine and chemokine secretion, and cell migration and invasion. However, proteomic studies are more challenging than nucleic acid analyses due to the complexity of the proteome. The study of CTC by this approach can help clarify metastatic progression processes and discover novel markers and mechanisms behind CTC immune escape (Mohme et al., 2017). Therefore, even though CTC detection and analysis remain a challenge due to their low abundance, current technological advances imply the potential of CTC phenotyping in everyday clinical practice, allowing more personalized management of cancer patients. However, the application of single-cell omics in NENs is limited, and no data have been published on pNENs.

3.2. Circulating cell-free DNA

Cell-free DNA (cfDNA) was first reported in 1977 (Leon et al., 1977). Taking advantage of the currently available technologies, studies focusing on the analysis of cfDNA to estimate tumor burden in patients have attracted considerable attention. Plasma, among other body fluids, is a good resource for cfDNA, released in healthy individuals mainly because of apoptosis, active cellular release, autophagy, and necrosis. The majority of cfDNA in plasma is derived from hematopoietic cells. cfDNA is double-stranded, characterized by a rapid turnover, and highly fragmented, with most molecules being approximately 150 bp long, which matches the length of DNA occupied by a nucleosome. Larger fragments start from 320 bp, and DNA wrapped around two nucleosomes with a length of up to 1000 bp can also be found (Thierry et al., 2010; Volik et al., 2016).

In the presence of malignancy, affected tissues release their DNA into peripheral blood, referred to as circulating tumor DNA (ctDNA). While the trend is that cfDNA levels in blood serum are higher in cancer patients compared with healthy individuals, cfDNA concentration varies considerably (Diehl et al., 2008; Figg and Reid, 2013;; Fleischhacker and Schmidt, 2007; Perkins et al., 2012). It has to be noted that ctDNA accounts for only 0.1–10% of the total cfDNA, and the levels might depend on tumor load, stage, and response to treatment. Still, an increase of cfDNA is not always a sign of malignancy and can be detected in healthy individuals, especially after intense exercise or in the presence of inflammation (Wan et al., 2017). High rates of necrosis with phagocytosis of necrotic neoplastic cells in advanced tumors are considered to contribute to an increased release of uneven, longer DNA fragments into the circulation of cancer patients. Elevated amounts of shorter mitochondrial DNA molecules have also been observed (Jiang et al., 2015; Underhill et al., 2016). Consequently, it is not only the levels of nucleotide fragments with a DNA double-helical structure that are altered in the presence of malignancy but also their integrity. The reported difference in the fragmentation profile of ctDNA compared to cfDNA from healthy cells led to the use of repetitive DNA elements such as Alu or LINE-1 amplicons for the analysis of cfDNA integrity (Fawzy et al.,

2016). cfDNA derived from neoplastic tissues is considered to exhibit a higher degree of fragmentation, and some studies have reported ctDNA fractions to be 20–50 base pairs shorter than cfDNA sequences (Mouliere and Rosenfeld, 2015).

In addition to fragmentation profile, methylation pattern seems to be different in ctDNA compared to cfDNA. It is well established that in human genome, DNA methylation occurs mainly on CpG dinucleotides located in repetitive sequences of non-coding DNA, whereas in a wide range of pathologies, including cancer, a global DNA hypomethylation occurs (Ivanov et al., 2015). On the other hand, hypermethylation of gene promoters enriched with CpGs (CpG islands) inducing tumor suppressor silencing is a common hallmark of human cancer (Esteller, 2007).

The analysis of ctDNA samples from plasma of cancer patients reported mutations in oncogenes such as *KRAS*, *EGFR* (Lone et al., 2022), and *TP53* (Lam et al., 2021). Studies have revealed that ctDNA provides a much more holistic view of tumor characteristics and progression emanating from primary and metastasized tumor foci (Murtaza et al., 2015). However, high degradation, the low quantity, and high mixture of cfDNA in ctDNA pose a major challenge for the development of sensitive and robust detection workflows. The issue is complicated by the fact that tumors are characterized by multiple subclonal populations, but only a subset of somatic mutations shared among all cells (Andor et al., 2016). A priori knowledge of specific DNA aberrations (short insertions/deletions and mainly mutations) and recurrent "hotspot" mutations allows the implementation of sophisticated PCR-based methods for their detection in plasma or serum from cancer patients. Historically, these approaches had low DNA requirements and low noise levels and were shown to be quite efficient in cancers where few genomic alterations are sufficient for patient stratification. One of the first applications for PCR-based analysis of ctDNA was the detection of high-level amplification of oncogenes. Targeted approaches include PCR-based methods such as digital droplet PCR and BEAMing that present remarkable sensitivity of 1–0.001% in detecting somatic point mutations, personalized analysis of rearranged ends (PARE), tagged-amplicon or CAPP-Seq deep sequencing (TAm-Seq or CAPP-Seq). In addition, untargeted approaches offering a broader analysis and monitoring of the tumor genome, independent of any prior data, have been applied, such as massively parallel sequencing of ctDNA.

Currently, ctDNA analysis represents a cost-effective tool characterized by a high sensitivity, which is used to analyze predefined genomic mutation hotspots associated with defined drug-related patterns in malignant neoplasms. Nevertheless, it should be taken into consideration that it can derive from dead cells that may or may not affect disease progression. Still, it is necessary to evaluate its sensitivity, specificity, and limitations in large cohorts of patients, including patients with benign tumors, before it can be fully incorporated into clinical practice.

Furthermore, a consensus on the best practices for sample retrieval (plasma *vs.* serum) and handling (preanalytical factors like clotting, freeze-thawing, DNase activity of blood, and temperature in ctDNA analysis) to assure cfDNA quality is needed (El Messaoudi et al., 2013, Barra et al., 2015; Chan et al., 2005; Parpart-Li et al., 2017).

The application of cfDNA in pNEN patients is under consideration since they present a relative heterogeneity in recurrent mutations in comparison with other tumors (Table 6), as already mentioned in Section 2.3. Next-generation sequencing (NGS) of ctDNA from liquid biopsies identified genomic alterations in *TP53, KRAS, EGFR, PIK3CA, BRAF, MYC,* and *CCNE1* genes in 280 (87.5%) of 320 NEN samples (Zakka et al., 2020). Even though analysis of circulating cfDNA reflects the presence of tumor-specific genetic alterations that could have a prognostic role, an official consensus has not yet been met (Mafficini and Scarpa, 2018; Rizzo and Meyer, 2018). However, recently, NGS analysis of cfDNA isolated from a 50-year-old female with a metastatic pNEC at diagnosis was used to determine the most appropriate available therapeutic strategy, resulting in an improvement of the patient's general condition. This case report of advanced pNEC demonstrates the utility of NGS analysis of cfDNA for personalized treatment (Wang et al., 2017).

3.3. Circulating non-coding RNAs

Non-coding RNAs represent another widely studied class of liquid biopsy biomarkers (Anfossi et al., 2018). They are primarily classified into two main groups - small non-coding RNAs (sncRNAs) with a length between 18 – and 200 nt and long non-coding RNAs (lncRNAs) with a length over 200 nt (Watson et al., 2019). sncRNAs can be further divided into five well-characterized subgroups: miRNAs, small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), piwi-interacting RNAs (piRNAs), and tRNA-derived small RNAs (tsRNAs) (Watson et al., 2019). It was perceived for some time that genome regions containing non-coding RNAs were "junk" DNA. However, it has been proven that both sncRNAs and lncRNAs perform a vast array of regulatory functions within cells,

Table 6
Genetic alterations potentially suitable as ctDNA biomarkers.

Gene	Mutation	Positivity	Potential role	Notes	References
DAXX/ ATRXX	Loss of protein expression	43%	Associated both with better and poor prognosis	Potential role in driving metastatic disease; pNET tumor suppressor genes	(Jiao et al., 2011; Marinoni et al., 2014; Singhi et al., 2017)
MEN-1	Inactivating mutations	44.1%	Better prognosis	Causes MEN1 syndrome; role in chromosome maintenance	(Jiao et al., 2011; Scarpa et al., 2017; Sonoda et al., 2020)
PTEN	Inactivating mutation	7.3%	Clinical application (selection of patients for mTOR inhibitors therapy)	Negative regulators of mTOR signalling	(Jiao et al., 2011; Scarpa et al., 2017)
TSC1/2	Inactivating mutation	8.8% 6.2%			(Jiao et al., 2011; Scarpa et al., 2017)
VHL	Germline mutation with age-dependent penetrance	5–17% of pNENs developed in VHL patients	Associated with increased pNET risk	Causes hereditary VHL syndrome	(Anlauf et al., 2007; Binkovitz et al., 1990; Cheng et al., 1997; Libutti et al., 1998; Scarpa et al., 2017)
TP53	Inactivating mutations	3% WD 57% PD	Characteristic for PD-pNEN (potential diagnostic benefit)	Higher frequency in HG neuroendocrine tumors (but not in LG)	(Nagtegaal et al., 2020; Puccini et al., 2020; Yachida et al., 2012)
RB1	Inactivating mutation	0% WD 71.4% PD			(Nagtegaal et al., 2020; Puccini et al., 2020; Yachida et al., 2012)
KRAS	Activating mutations	0% WD 28% PD	Typical marker of PDAC (unusual for pNENs)		(Puccini et al., 2020)
SMAD4	Inactivating mutation	0% WD 19.5% PD	Distinguishing PD-NENs from G3 WD-pNENs	Present in PD-pNEC (not identified in WD-pNETs)	(Tang et al., 2016; Yachida et al., 2012)
BCL-2	Overexpression	18.2% WD 73.7% PD	Correlates with higher mitotic rate and Ki-67 index	Increases resistance to chemotherapy	(Fisher et al., 1993; Yachida et al., 2012)

Abbreviations: MEN1 - Multiple endocrine neoplasia link type 1; mTOR - The mammalian target of rapamycin; VHL - von Hippel-Lindau syndrome; WD - well-differentiated pNENs; PD - poorly differentiated pNENs; HG - high grade; LG - low grade; PDAC - pancreatic ductal adenocarcinoma

including gene expression regulation (Ling et al., 2015). For this reason, non-coding RNAs have gained an interest in the field of cancer research as the altered levels of their expressions can correlate with tumor characteristics (Yan and Bu, 2021).

In tissue analysis of pNENs, most studies have focused on miRNAs since other ncRNAs such lncRNAs, piRNAs, tsRNAs, snoRNAs, and snRNAs have been generally understudied in cancer research. For example, an increased level of miRNA miR-196a in tumors indicates a poor prognosis in pNENs as it is associated with more aggressive tumors and relapse (Lee et al., 2015). miR-27b is yet another miRNA that is reported to be associated with pNEN reoccurrence (Kang et al., 2014).

Currently, there is evidence for the presence of all aforementioned non-coding RNA types in bodily fluids (Pös et al., 2018; Savelyeva et al., 2017; Wang et al., 2022), and their levels may correlate with the state of the disease, especially in tumors, as they can be secreted by the tumor itself (Wang and Chen, 2014). They are relatively stable in bodily fluids, either bound by proteins (argonautes, lipoproteins) or carried by EVs that protect them from degradation by RNases (Pös et al., 2018; Cui et al., 2019). So far, studies on circulating non-coding RNAs in pNENs are scarce, with few published articles focusing on miRNAs (Table 7) (Kövesdi et al., 2020; Xu et al., 2016).

miRNAs are the most studied of the circulating non-coding RNAs. They are vital components of gene expression regulation within the cells, involved in post-transcriptional gene expression regulation by targeting the 3' untranslated region of the mRNAs and inducing their degradation *via* the argonaute proteins (O'Brien et al., 2018). In cancers, miRNAs can also be secreted outside the cell, affecting the gene expression profile of nearby cells within the tumor microenvironment and further stimulating tumor development (Kai et al., 2018). One of the first studies that evaluated circulating miRNAs in pNENs was conducted by Xu et al. (2016). While the study mainly focused on the more common form of pancreatic cancer, PDAC, the authors discovered four miRNAs (miR-126–3p, miR-26b-3p, miR-938, and miR-19b-3p) that were upregulated in the plasma of pancreatic cancer patients compared to pNENs (Xu et al., 2016). In a more recent study by Kövesdi et al., circulating miRNAs were evaluated using the NGS approach with the aim to identify miRNAs that could improve the diagnostic accuracy of the CHGA test (Kövesdi et al., 2020), which is greatly influenced by the use of both short- and long-term applications of proton pump inhibitors (PPIs) and other acid-suppressive medications, thus decreasing its clinical utility in the management of NETs (Nehra et al., 2018; Raines et al., 2012). The study discovered 33 circulating miRNAs in serum that could distinguish pNEN from controls. For validation by qPCR, the authors selected six underexpressed miRNAs (let-7b-5p, let-7i-5p, miR-143–3p, and miR-30d-5p, miR-451a, miR-486–5p) of which all six were downregulated in pNENs compared to healthy controls. Furthermore, in receiver operating characteristic analysis, it was found that a combination of CHGA and let-7b-5p, let-7i-5p, miR-143–3p, miR-30d-5p had a higher diagnostic accuracy than CHGA alone in scenario where pNEN patients were compared to healthy controls under PPI treatment. This study provided one of the first clues for the development circulating miRNA-based panel to improve the accuracy of CHGA test to diagnose pNENs (Kövesdi et al., 2020).

Table 7
Long-noncoding RNA in pNET preclinical research.

Non-coding RNA	Study group	Biofluid	Expression	Control group	Reference
miR-126b-3p			↑		(Xu et al., 2016)
miR-26b-3p	PC (n = 156)	Plasma	↑	pNEN	
miR-938			↑	(n = 27)	
miR-19b-3p			↑		
let-7b-5p			↓		(Kövesdi et al., 2020)
let-7i-5p	pNEN (n = 25)	Serum	↓	Healthy controls w/ PPI	
miR-143–3p			↓	(n = 29)	
miR-30d-5p			↓		

Abbreviations: PC – pancreatic cancer; pNEN – pancreatic neuroendocrine neoplasms; PPI – proton pump inhibitor treatment

Unlike miRNAs, lncRNAs have a much broader spectrum of mechanisms to regulate gene expression. They are involved in histone modifications, chromatin structure modifications, and DNA methylation regulation. They can even act as "sponges" for miRNAs by containing multiple miRNA binding sites, ultimately limiting their interactions with target mRNAs (Dahariya et al., 2019). Similar to miRNAs, circulating lncRNAs are also either encapsulated by EVs or bound to protein complexes (Moonmuang et al., 2021). Currently, lncRNA involvement in pNENs has only been evaluated in tissue studies, and two well-characterized lncRNA candidates have been reported (Ji et al., 2019; Modali et al., 2015). Meg3 is a lncRNA that activates *MEN1* product menin and suppresses the expression of protooncogene *HGFR* (Modali et al., 2015). It has been shown that the loss of Meg3 in pNENs facilitates tumorigenesis, indicating that the Meg3 is a tumor-suppressive lncRNA in pNENs (Modali et al., 2015). On the other hand, lncRNA H19 has a tumor-promoting effect in pNENs as the overexpression of H19 is associated with tumor growth and metastasis by interacting with neuroendocrine-specific protein VGF (Ji et al., 2019).

3.4. Extracellular vesicles

EVs are important mediators of cell-to-cell communication due to their cargo consisting of various molecules, including DNA, mRNA, miRNA, siRNA, and proteins. Exosomes are a subset of EVs, the smallest in size with a diameter of 40–150 nm, discovered and named in 1987 (Johnstone et al., 1987). They originate from endosomes through a dynamic process, maturing into multivesicular bodies and fusing with the plasma membrane. They are released as vesicles having a double-layered lipid membrane with the same orientation as the originating cell's plasma membrane. Orientation and structure are crucial to exosomes for their ability to efficiently mediate cell-cell interactions. Following their release, they can remain near their cell of origin or travel through body fluids from which they can be isolated.

Their separation from other small EVs is considered a challenging procedure. Classically, exosomes are separated by high-speed differential ultracentrifugation, where steps to clear cells, cell debris, and larger microvesicles are incorporated (Théry et al., 2006). Currently, various commercial kits for exosome isolation are available and widely used. In addition, a methodology that combines acoustic waves and fluidic approaches has also been developed (Wu et al., 2017). Following isolation, exosomes should be quantified and assessed for purity. Although rough quantification is possible by protein quantification, direct quantification is proposed to be performed using either nanoparticle tracking analysis, such as NanoSight, or flow cytometry after binding exosomes to larger beads (Dragovic et al., 2015). Electron microscopy is the first choice regarding purity and quality of exosomes but probing for the presence of exosome structural molecules, tetraspanins TSG101, CD81, andCD9, and the absence of histone proteins should also be used to confirm no cellular contamination (Théry et al., 2006). Exosome cargo can consist of cell-type-specific exosomal proteins primarily of cytoplasmic origin, lipids, and nucleic acids, including genomic and mitochondrial DNA and non-coding RNAs. Initially, the protein content of various origin exosomes was characterized by low-content approaches such as western blotting and flow cytometry, whereas nowadays, mass spectrometry-based analyses have been incorporated in exosome research. The NGS-based analysis also provided numerous data.

Currently, several databases, such as Vesiclepedia, exRNA, and Exo-Carta, have been created to assist the more comprehensive understanding of exosome biology.

EVs are important mediators of cell-to-cell communication due to their cargo, consisting of various molecules, including DNA, miRNA, siRNA, and proteins. Numerous studies have underlined their essential role in transferring signals relating to cell growth, transformation, and survival between cancer cells. Specifically, the pancreatic cell line's exosomes were shown to promote cancer survival by modifying apoptosis *via* the Notch-1 signaling pathway (Ristorcelli et al., 2009). Recently their role as a component of tumor microenvironment signaling has also received considerable attention (Li and Nabet, 2019). Furthermore, they seem to have a prominent role in the metastatic process.

As our understanding of exosome biology is growing, EVs are increasingly considered promising, sensitive, and specific biomarkers that can provide holistic information about the tumor. Total circulating exosome burden has been shown to have prognostic relevance, with an increase shown to be associated with unfavorable outcomes (Dai et al., 2020). It has been suggested that exosomal content can be an early marker for disease diagnosis, as indicated for glypican-1 and ALIX proteins in studies in pancreatic cancer (Melo et al., 2015). In the latter study, 73 proteins were found to be differentially expressed in exosomes from pNEN patients compared to healthy control subjects, and 33 were different in comparison with pancreatic cancer patients (Yang et al., 2021) which is encouraging for further investigations. In the NANETS

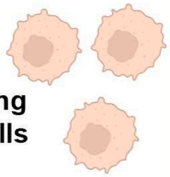
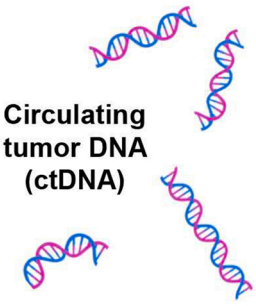
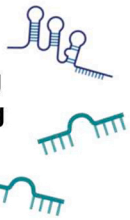
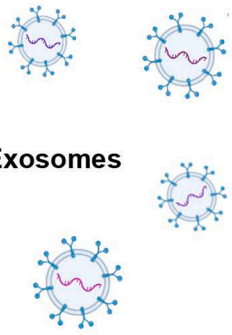
	PROS	CONS
 **Circulating tumor cells (CTC)**	➢ Prognostic in PNENs ➢ Intact cells could be resistant clones thus providing valuable information for therapeutic decisions ➢ Can be used for functional assays (DNA, RNA, proteins) ➢ Can be cultured in vitro or in vivo ➢ Can be classified by intrinsic subtypes ➢ Can be used for response to treatment prediction	➢ Technically challenging ➢ Costly ➢ Sampling bias (affinity-based, size-based selection) ➢ Single-cell sequencing technically challenging (heterogeneity observed could be biological or technical bias)
 **Circulating tumor DNA (ctDNA)**	➢ Technically easier to isolate than CTC ➢ DNA more stable than RNA ➢ Proportion of ctDNA related to tumor burden and overall survival ➢ High sensitivity and dynamic range ➢ More sensitive than CellSearch® CTC platform	➢ Can only be dissected at the genomic level ➢ Limitations of available material ➢ Blood cell death could spike cfDNA fraction ➢ Source not clear - lysis, apoptosis, CTC-derived ➢ Large background of normal cfDNA ➢ Unclear if ctDNA is released from cancer cells dying from therapy or resistant cells
 **Circulating non-coding RNAs**	➢ High abundance and stability in body fluids ➢ Multiplexed ➢ Quantitative	➢ Wide variability of results and lack of reproducibility ➢ Influence of other factors ➢ Complexity in the comprehensive analysis ➢ Their source in the circulation is unclear ➢ Occurrence of similar non-coding RNAs in various diseases ➢ Lack of standardization
 **Exosomes**	➢ Rich source of biomarkers ➢ Ability to cross the blood-brain barrier ➢ Higher stability, greater resistance to degradation ➢ Longer half-life ➢ Increased capacity to travel long distances in comparison with free proteins, lipids, and nucleic acids in the cytoplasm ➢ Permanence in the blood ➢ Low toxicity ➢ Non-immunogenic ➢ Therapeutic potential	➢ Few unique cell-specific proteins ➢ Challenging to determine their tissue of origin ➢ No state-of-the-art technology to isolate extracellular vesicles ➢ Lack of reproducibility

Fig. 2. Pros and cons of various liquid biopsy biomarkers.

2020 symposium, exosomal Survivin expression was found to be promising as a response biomarker in GEP-NETs, although the sample size was limited (Gupta et al., 2021). Recently, exosomal caveolin-1 expression was correlated to OS and PFS in metastatic pancreatic cancer patients (Bittoni et al., 2020). In addition, a growing body of research arises from the role of exosomes as biologically active carriers leading to exciting clinical applications and therapies despite the hidden challenges (Kamerkar et al., 2017).

Exosomes have a great potential to deliver drugs for cancer treatment and monitor cancer progression. Recently a study was implemented to develop EVs tagged both anti-SSTR2 and anti-C-X-C motif chemokine receptor 4 (CXCR4) monoclonal antibodies. They were found by both live-cell confocal microscopy and *in vivo* imaging to target specifically and accumulate in NEN cells and xenografts. These EVs were then loaded with verrucarin, a natural cytotoxic compound, and the microtubule polymerization inhibitor mertansine. The growth inhibition effect of these dual-targeted EVs-delivered combined therapies was promising and broadened the applications of EVs for the targeted delivery of chemotherapeutic compounds (Si et al., 2020) (Fig. 2).

4. Implementation of the liquid biopsy into the clinic

There are many challenges related with the implementation of the liquid biopsy into the clinic, including ethical issues, standardized protocols and correct interpretation of the findings. The shift of current oncology from traditional biopsies to liquid biopsy despite applied technological issues gives rise to ethical challenges in the clinical scenario, which involve many aspects, including informed consent, communication, and patient-physician encountering. As the use of liquid biopsy becomes more widespread, protocols are being reviewed for ethical standing, especially in the case where they are performed within clinical trials, research projects or for biobanking purposes. In every case, the minimum amount of material required to perform planned analyses should be collected using the safest procedure possible. Technical training and skills of all personnel involved should be ensured in order to minimize procedure time, participant risk, and sampling error. This is especially important when endoscopic ultrasound is applied to access the portal circulation, a novel well-promising technique, since portal circulation content directly reflects the output from the spleen, pancreas, and gastrointestinal system, including the microbiome (Ryou and Stylopoulos, 2020). This approach has already been used for the detection of CTC in pancreatic cancer patients (Catenacci et al., 2015). Biomedical research and its clinical applications raise substantial issues towards the improvement of the therapeutic strategies adopted nowadays, still the moral obligation of treating human beings with respect for their individuality and safeguarding the fundamental rights of the individual patient according to the international legal conventions is primary. In the case of biobanking, comprehensive documentation such as informed consent forms, material transfer agreements and other procurement records, equipment maintenance records, and notes on sample handling and storage conditions are required to ensure biospecimen quality. In the informed consent process, it should be clearly stated that the collected material will be kept for biobanking and future unspecified research, with no clinical benefit to participants from the procedure, but with the potential to generate useful information and produce a downstream benefit. All data storage, accessibility, and exchange should strictly follow FAIR (Findable, Accessible, Interoperable, and Reusable) data principles.

In order for biomarkers to be introduced into the clinical practice, the biomarker must achieve several requirements, which have been recently reviewed (Ignatiadis et al., 2021). First, a clinical and analytical validation, ensuring the clinical benefit of applying the marker in clinical practice. Second, obtain approval for its application in the clinic and incorporate the use of this marker into current guidelines. Finally, implementation of the workflow into the clinical framework, with specific infrastructure and trained personnel to manage sample collection analysis according to clearly defined standard operating procedures. The prospective-specimen collection, retrospective-blinded-evaluation (PRoBE) design is currently the best model for biomarker validation, with a blinded case-control approach (Pepe et al., 2008). There are several preanalytical issues associated with the liquid biopsy, such as sample type, sample collection tube, and processing time. The Cancer Institute (NCI) Biorepositories and Biospecimen Research Branch Biospecimen Evidence-Based Practices (BEBP) have recently published guidelines related to the use of cfDNA in the clinic, which is currently the most used new age liquid biopsy marker applied in the clinic (Greytak et al., 2020).

5. Preclinical tools for the discovery of new therapeutic approaches for pNENs

Cancer biomarkers obtained from blood or other body fluids have a significant translational impact. However, genetic complexity and environmental variability can influence the serum proteome and sometimes hinder blood-based biomarker identification. *In vitro* and *in vivo* experimental systems provide the advantage of controlled experimental conditions, thus reducing variability critical for biomarker discovery. Moreover, low specificity of potential biomarkers, which turn out to be nonspecific markers of angiogenesis, inflammation, fibrosis or other pathologies irrelevant to cancer, could be, in some cases, attributed to the lack of high-quality matched cases and controls. In this regard, it is critical that mouse models can recapitulate the neoplastic evolution of human cancer, which allows early detection research to be performed, which is challenging under clinical conditions.

Although transgenic and knockout techniques revolutionized the development of animal models for translational cancer research, they are still far from being ideal. The challenges are associated with the selection of the most fitting model, reflecting the given tumor system.

The research identifying tumor markers, revealing development mechanisms, and potential treatment strategies requires biological samples of the respective tumor patients that could be used for in-depth studies and validation of the obtained results (Matzke and Watson, 2020; Patil et al., 2018). Specifically, in the case of pNEN, where any biological sample is of high value due to the rarity of the disease, the most sustainable way of performing research is to establish interlaboratory collaboration and integrate biological tissues or blood samples into a common biobank. As biobanks usually obtain broad informed consent from the study participant, the stored biological samples could be used for various research purposes in many studies and generate added value to the pNEN research field.

It is well established in most pathology departments to keep tumor tissue in the form of formalin-fixed paraffin-embedded blocks or as fresh frozen material. The stored material enables studies of tumor genetics or assessment of tissue immunohistochemical properties (Coppola et al., 2019), but these approaches have many crucial limitations since they contain non-viable cells. The main drawbacks include the inability to modulate the *in vivo* environment for interaction studies, search for molecular targets, or perform drug screens.

5.1. *In vitro pNEN models*

In cancer research, failure to translate promising drug candidates from the bench to the bedside can often be attributed to inadequate model systems for human cancer research, such as two-dimensional cancer immortalized cell lines, which have been used in basic cancer research for decades. This indicates that these models lose their ability to recapitulate the *in-vivo* properties of the tumor. Although they were a valuable tool in preclinical *in vitro* research, cell lines failed to reflect several important features of human tumors, such as subclonal architecture, intratumoral heterogeneity, or phenotypic complexity. However, only a few pNEN *in vitro* and *in vivo* models exist for translational research in contrast to other cancer types (Table 8). The QGP-1 cell line

Table 8
Overview of preclinical models available in pNEN research.

Type	Success rate	Origin	Characterization	References
Cell lines				
QGP-1	N/A	Pancreatic islet cell carcinoma	RNA, protein and miRNA expression, WES	(Boora et al., 2015;Kaku et al., 1980;Luley et al., 2020;Vandamme et al., 2015
BON-1	N/A	Metastatic pNEC	RNA, protein and miRNA expression, WES	(Boora et al., 2015; Luley et al., 2020; Townsend et al., 1993; Vandamme et al., 2015)
NT-3	N/A	Resected lymph node from a patient with WD-pNET	RNA, protein and microRNA expression, panel sequencing	(Benten et al., 2018; Luley et al., 2020; Viol et al., 2022)
CM	N/A	Ascitic fluid taken from a patient with a pNET	N/A	(Gueli, 1987)
Organoids	37.5% 3 of 8 pNEN samples	pNENs (2 pNEC and 1 pNET)	Multi-omics analyses (WGS, RNA-seq, MMA), functional characterization	(Kawasaki et al., 2020)
PDXs	80% (with stimulation of MET signaling)	pNENs patient's lymph node metastasis	Subcutaneously transplanted tumor fragments to NSG mice	(Krampitz et al., 2016)
	N/A	Liver metastasis from advanced WD-pNET producing insulin	Subcutaneous implantation of tumor tissue into female athymic nude mice	(Chamberlain et al., 2018)
	20% (1/5)	Primary pNEN	Subcutaneously implanted in the subcutaneous pocket of NOD SCID mice	(Pham et al., 2021)
GEMMs				
Hz *MEN1* inactivation	N/A	N/A	Range of endocrine tumors, including pNET	(Bertolino et al., 2003)
Ho *MEN1* inactivation	N/A	N/A	pNET development	(Shen et al., 2009)
MEN1/PTEN loss	N/A	N/A	WD G1/G2 pNETs	(Wong et al., 2020)

Abbreviations: GEMMs – genetically engineered mouse models; Hz – heterozygous; Ho – homozygous; MMA – methylation microarray analysis; NOD SCID – non-obese diabetic severe combined immune-deficient mice; NSG – NOD scid gamma mice; PDXs – patient-derived xenografts; PE – pancreatic endocrine; pNEC – pancreatic neuroendocrine carcinoma; pNEN – pancreatic neuroendocrine neoplasm; pNET – pancreatic neuroendocrine tumor; RNA-seq – RNA sequencing; WD – well-differentiated; WES – whole-exome sequencing; WGS – whole-genome sequencing

isolated in the 1980 s (Kaku et al., 1980) was established from a human pancreatic islet cell carcinoma. The BON-1 cell line was isolated from a peripancreatic lymph node in 1986 from a patient with a metastatic pNEN (Townsend et al., 1993). Both cell lines carry atypical pNET mutations (eg. *RAS*, *TP53*) and show a very high proliferation rate, thus making it difficult to classify them as pNET or pNEC, although as BON-1 cells carry *MEN1* mutation, this makes them more a pNET cell line, whereas QGP-1 is sometimes regarded as pNEC. The NT-3 cell line, established from a resected lymph node, was derived from a patient with well-differentiated pNET (Benten et al., 2018). CM cell line, isolated in 1986 from ascitic fluid, was taken from a patient with a pancreatic mass identified as a neuroendocrine tumor in a biopsy sample (Gueli, 1987). BON-1, QGP-1, and NT-3 have been extensively characterized at the molecular level, including neuroendocrine, epithelial, developmental endocrine-related gene expression at the RNA and protein level, and NGS analysis of miRNA (Luley et al., 2020), with whole-exome sequencing data for BON-1 and QGP-1 (Vandamme et al., 2015). Although cell lines provide models that have features common with original tissue, their inability to mimic the heterogeneity of human cancer cells, the cancer microenvironment, and the stromal compartment hinders the understanding of tumor biology.

For further extension of preclinical pNEN research, it is crucial to preserve the tumor microenvironment, hormonal response, inner signaling, tissue integrity, and architecture, which is feasible only by the maintenance of living tissue. Therefore, the establishment of *in vitro* and *in vivo* experimental models from living cell repositories is an essential tool (Luo et al., 2014; Matzke and Watson, 2020; Zohouri and Ghaderi, 2020). Biobanking of viable specimens could enable functional assays that aid in the discovery and validation of therapeutic targets, assessment of drug responses, or resistance to treatment. Living cell repositories include the generation of patient-derived *in vitro* cell models, which retain important features of the original human tumors. The process of creating a living cell repository is complex and involves derivation, propagation, annotation, and characterization (Palechor--Ceron et al., 2019; Sachs et al., 2018; van de Wetering et al., 2015). The establishment of living biobanks for the most common cancer types such as breast, colon, and gastrointestinal cancers (Li et al., 2020; Luley et al., 2020; Sachs et al., 2018; van de Wetering et al., 2015; Vlachogiannis et al., 2018; Zhou et al., 2021) used for next-generation tumor biology and treatment studies was accompanied by the above-mentioned hardships and challenges. There is only one report so far on the establishment of a living biobank containing a pNEN sample collection (Kawasaki et al., 2020). The authors have developed total of 25 organoid lines from various NENs, three of which were pNENs. Due to the rarity of pNENs, the further establishment of living biobanks could greatly benefit the research. Furthermore, patient-derived cell repositories can include a multitude of preclinical model types, such as three-dimensional organoids, conditionally reprogrammed cell lines, cancer-associated fibroblasts, and ideally contain matched models from primary and metastatic tissues for research use that ranges from biomarker discovery to patient-tailored therapy applications (Palechor-Ceron et al., 2019).

Organoids are ex-vivo three-dimensional cultures that can be expanded *in vitro* to provide cancer models that recapitulate the characteristics of the primary tumor. They represent an important advance in cancer cell biology systems that can be used to study several aspects of tumor biology, including treatment resistance and susceptibility. These cultures may be expanded *in vitro* for up to several months and can be passaged and stored in liquid nitrogen, as with other immortalized cell lines, providing a "biobank" of primary tumor organoid models. Organoids overcome the limitation of the availability of primary tumor tissue and provide an alternative to mouse models. However, like any model, they have several disadvantages. Although they are widely used, they are also underinvestigated and poorly validated. The effect of basement membrane gel on cell behavior is still poorly understood, and the validity of their use is influenced by the morphology of the studied tissue. They are heterogeneous cultures, which increases the probability that some clones will dominate. Therefore, it is crucial to understand genotype and phenotype of the culture, including the proportion of individual cells to ensure that the model is fit for application in precision medicine.

A protocol for the establishment of 3D models from pNEN tumors for drug screening in a cost-effective and scalable manner was published recently (April-Monn et al., 2021). Unfortunately, no G1 or G2 organoid models have been reported so far. Three-dimensional *in vitro* models

allow the co-culture of tumor cells and other relevant cells within the primary tumor, being more representative of the *in vivo* situation. Overall, this study has also carried out WGS and transcriptome sequencing of the samples to analyze the entire landscape of molecular data (Kawasaki et al., 2020). The generation of omics accompanied information on living biobank samples is an absolute "must-have" in today's tumor field (Luo et al., 2014) to dissect several levels of tumor heterogeneity and find those personalized patterns to be used for next-generation treatment development. The next step from living biobanks to actual use for preclinical models are intravital biobanks, where tumor tissue from living biobanks is maintained as xenotransplant in immunodeficient mice for different studies (Luo et al., 2014).

5.2. In vivo models

Besides patient-derived organoids, patient-derived xenograft (PDX) models also have the ability to recapitulate the genotypic and phenotypic nature of the tumor and advance translational research (Hou et al., 2022). Although they allow to mimic heterogeneity of tumors and relatively rapid prediction of response to treatment, they provide a less realistic representation of tumor microenvironment. Moreover, their disadvantages include unsatisfactory success rate, limited throughput screening efficiency, they are expensive and technically challenging. Very few PDX models of pNENs are currently available, with none reported for G1 pNET so far. This is not due to the lack of trying but because they are extremely difficult to propagate. To date, only three groups have reported the successful establishment of PDX models derived from pNENs. The first pNEN PDX model was developed in 2016 by Krampitz et al. They subcutaneously transplanted primary tumor fragments obtained from patient's lymph node metastasis into extremely immunodeficient NOD scid gamma (NSG) mice and noticed that without activation of the MET receptor, the xenografts failed to grow. However, upon stimulation of MET signaling *via* HGF analog to activate the HGF/MET signaling axis, palpable tumors developed with an average of 80% efficiency. Their observation that MET activation is critical for tumor growth was confirmed by the study, in which only one tumor developed into xenografts without the aid of MET agonists out of 39 WHO grade 1 and 2 primary pNENs that they transplanted into immunodeficient mice (Krampitz et al., 2016). However, as reviewed by Maharjan et al., the genetic resemblance of the PDXs with the primary tumor tissue nor any follow-up studies with the above-mentioned established PDXs were reported (Maharjan et al., 2021).

Another record of a successful pNEN PDX model was described by Chamberlain et al. (2018). The xenograft was established by subcutaneous implantation of liver metastasis tissue from a patient with an advanced, well-differentiated pNET producing insulin, into female athymic nude mice. The successfully transplanted tumor retained the characteristics of the patient tumor tissue through several rounds of serial transplantation in mice (Chamberlain et al., 2018). Recently, Pham et al. (2021) attempted to create PDXs established from five resected pNENs and succeeded with one that was able to propagate and create stable PDX.

The lamentably low number of successful attempts illustrates how challenging and complicated the task is. Besides PDX models, several genetically engineered mouse models (GEMMs) have been developed for pNEN research. Most of them are associated with loss of *MEN1*, as it has been observed that MEN1 syndrome is related to the development of pNETs. Bertolino et al. (2003) established a conventional MEN1 loss mouse model in which heterozygous mice for *Men1* developed a range of endocrine tumors, including pancreatic tumors. Later, Shen et al. (2009) showed that the Cre-loxP system can be used to generate transgenic mice with homozygous inactivation of the Men1 in mature pancreatic endocrine cells leading to pNET development. Recently, two well-differentiated genetically engineered pNET mouse models were also developed using the Cre-LoxP system (Wong et al., 2020). In these models, the authors showed that Men1 loss combined with Pten loss accelerated tumorigenesis. Both mouse models ($Men1^{flox/flox}$ $Pten^{flox/flox}$ RIP-Cre and $Men1^{flox/flox}$ $Pten^{flox/flox}$ MIP-Cre) developed well-differentiated G1/G2 pNETs at a much shorter latency than Men1 or Pten single deletion alone. Interestingly, the deletion of *DAXX* (a mutation frequently found in pNET) does not lead to pNET development (Sun et al., 2022). Although GEMMs have many advantages, such as enabling the evaluation of the role of tumor microenvironment, immune system or tumor vasculature, they do not reflect the complex heterogeneity of human tumor cells, and tumor development in mice might be slow and variable.

Nevertheless, despite these obstacles associated with *in vivo* models of pNETs, patient-derived cell models generated from surgical biopsy specimens have the potential to functionally complement molecular and pathological tumor analysis and hence are considered desired preclinical tools (Bolck et al., 2019). Furthermore, more detailed annotation of the obtained material, including comprehensive clinical data, omics information (genome, transcriptome, metabolome, proteome), and also the use of the newest cutting-edge technologies like single-cell sequencing and spatial transcriptomics helps not only to prove the representability of the obtained preclinical models but also gain valuable insight on tumor biology itself (Bolck et al., 2019; Luo et al., 2014).

6. Conclusions

Metastasis, locoregional and distant recurrence represent issues affecting long-term survival in many types of cancer, including pNEN, and many studies have been dedicated to unraveling the mechanisms behind it. Recurrence has been observed not only shortly after the treatment but also after many years or even decades after the initial diagnosis and treatment, implying that the cells responsible for recurrence must have the ability to live long and possess all the characteristics required to initiate and form new tumor lesions after a long period of dormancy (Gao et al., 2017). Identifying these cells in pNEN patients is extremely important as in 50–80% of them, metastases occur months or years after the primary tumor is diagnosed and has been successfully treated (Foulfoin et al., 2017). Understanding the mechanisms under which dormant cells switch on, causing late relapse, will open new scientific discovery horizons and expand current clinical control opportunities.

Liquid biopsy could not only facilitate the early detection of changes and monitor disease evolution but allow the identification of patients with a need for frequent follow-up due to the high risk of relapse. The technique is associated with both genomic and proteomic assessment of a wide range of tumor-derived elements such as CTCs, ctDNA, cfRNA, small RNAs/miRNAs, lncRNA, tumor-derived EVs and other markers. Combined with single-cell omics technologies and the establishment of *in vitro* and *in vivo* experimental systems, including ex-vivo cultured CTC lines and CTC-derived xenograft models, these approaches can accelerate research on pNEN biology and speed up the clinical implementation of liquid biopsy biomarkers. Undoubtedly though, liquid biopsy presents a great potential to generate promising data, to better understand the mechanisms behind the development and evolution of pNEN, and to develop an algorithm that, by combining multiple records, will serve in a much more convenient and non-invasive way, to improve the management of pNEN patients at different clinical levels.

CRediT authorship contribution statement

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Conflict of interest statement

All authors declare no conflict of interests.

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