Clinical applications of (epi)genetics in gastroenteropancreatic neuroendocrine neoplasms : moving towards liquid biopsies

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ABSTRACT

High-throughput analysis, including next-generation sequencing and microarrays, have strongly improved our understanding of cancer biology. However, genomic data on rare cancer types, such as neuroendocrine neoplasms, has been lagging behind. Neuroendocrine neoplasms (NENs) develop from endocrine cells spread throughout the body and are highly heterogeneous in biological behavior. In this challenging disease, there is an urgent need for new therapies and new diagnostic, prognostic, follow-up and predictive biomarkers to aid patient management. The last decade, molecular data on neuroendocrine neoplasms of the gastrointestinal tract and pancreas, termed gastroenteropancreatic NENs (GEP-NENs), has strongly expanded. The aim of this review is to give an overview of the recent advances on (epi)genetic level and highlight their clinical applications to address the current needs in GEP-NENs. We illustrate how molecular alterations can be and are being used as therapeutic targets, how mutations in DAXX/ATRX and copy number variations could be used as prognostic biomarkers, how far we are in identifying predictive biomarkers and how genetics can contribute to GEP-NEN classification. Finally, we discuss recent studies on liquid biopsies in the field of GEP-NENs and illustrate how liquid biopsies can play a role in patient management. In conclusion, molecular studies have suggested multiple potential biomarkers, but further validation is ongoing.

Keywords

Gastroenteropancreatic Neuroendocrine Neoplasms – Genetics – Epigenetics – Biomarkers – Liquid Biopsies

Abbreviations

AUC	Area Under the Curve
CAPTEM	Capecitabine and temozolomide
cfDNA	Cell-free DNA
CgA	Chromogranin A
CIMP	CpG island methylator phenotype
CNV	Copy number variation
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA
ddPCR	Droplet digital PCR
DFS	Disease-free survival
DSS	Disease-specific survival
EPCAM	Epithelial cell adhesion molecules
FISH	Fluorescence in situ hybridization
G	Grade
GEP-NEN	Gastroenteropancreatic neuroendocrine neoplasm
HIF	Hypoxia inducible factor
IHC	Immunohistochemistry
MEN1/4	Multiple endocrine neoplasia 1/4
miRNA	MicroRNA
MLP	Metastasis-like primary
NEN	Neuroendocrine neoplasm
NF1	Neurofibromatosis Type 1
NGS	Next-generation sequencing
NKA	Neurokinin A
NSE	Neuron-specific Enolase
OS	Overall survival
PD-NEC	Poorly differentiated neuroendocrine carcinoma
PFS	Progression-free survival
PNEC	Pancreatic neuroendocrine carcinoma
PNEN	Pancreatic neuroendocrine neoplasm
PNET	Pancreatic neuroendocrine tumor
PRRT	Peptide receptor radionuclide therapy
PST	Pancreastatin
RFS	Relapse-free survival
siNEN	small intestinal neuroendocrine neoplasm
SEER	Surveillance, Epidemiology, and End Results program
SSA	Somatostatin analog
SSTR	Somatostatin receptor
TCGA	The Cancer Genome Atlas
TS	Tuberous Sclerosis
VHL	von Hippel Lindau
WD-NET	Well-differentiated neuroendocrine tumor
WES	Whole-exome sequencing
WGS	Whole-genome sequencing

1. INTRODUCTION

Neuroendocrine neoplasms (NENs) are rare malignancies that arise from neuroendocrine cells present in many organs, including lung, pancreas, small intestine and skin. Despite some common features, such as expression of endocrine and neural markers, they are very heterogeneous in biological behavior [1, 2]. Therefore, this review will focus only on NENs originating in the gastrointestinal tract and pancreas, termed gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs). GEP-NENs include esophageal, gastric, small intestinal, pancreatic, colorectal and appendiceal NENs. Their combined annual age-adjusted incidence rate is 3.56 per 100,000 persons, according to the Surveillance, Epidemiology, and End Results (SEER) program, and has increased approximately 4-fold in the last 40 years [1, 3]. NENs can be divided into functional (25%) and non-functional (75%) neoplasms, based on clinical symptoms associated with hormone secretion [4, 5]. Prognosis is highly variable, with 5-year survival rates ranging from 30% to 100%, largely depending on tumor site, grade, stage and functionality, with functional tumors having a better prognosis due to earlier detection [1, 6]. However, prognosis of individual patients remains difficult to predict. GEP-NENs are graded according to the World Health Organization (WHO) classification system, which is based on proliferation markers Ki-67 and mitotic count. Since WHO2017, differentiation grade was added as an additional parameter for pancreatic NEN (PNEN) classification. Well-differentiated PNENs are termed pancreatic neuroendocrine tumors (PNETs) and can be Grade (G) 1, G2 or G3 based on their proliferation rate, while neuroendocrine carcinomas (NECs) are poorly differentiated neoplasms with a high proliferation rate (G3). If other GEP-NENs are highly proliferative, e.g. G3, they are still by definition NECs, while G1 and 2 GEP-NENs are termed NETs [7]. However, the adaptations implemented for PNENs are expected to be extended to the other NENs as well [8]. G3 neoplasms have a bad prognosis [1]. Surgical resection is the primary treatment in locoregional GEP-NENs, and the only curative treatment option. However, more than 50% of cases present with unresectable disease at time of diagnosis [9-11]. For these advanced cases, different therapeutic strategies are available, which are mostly a combination of ablative surgery, peptide receptor radionuclide therapy (PRRT) and medical treatment.

The aim of this review is, to give an overview of the recent advancements in the (epi)genetic characterization of GEP-NENs and highlight their potential clinical applications. As research has primarily focused on small intestinal NENs (siNENs) and PNENs, (epi)genetic knowledge of other GEP-NENs is still scarce and further research is needed. Hence, this review will focus mainly on siNENs and PNENs. We will first discuss mutations and copy number variations (CNVs) in familial and sporadic GEP-NENs and focus on their role as therapeutic targets and

prognostic/predictive biomarkers. Subsequently, we will discuss epigenetic alterations identified in GEP-NENs and their potential clinical applications. We will conclude with a short overview on liquid biopsies for GEP-NEN patients and illustrate how this can be combined with the discussed (epi)genetic alterations.

2. MUTATIONS AND COPY NUMBER VARIATIONS

Most GEP-NENs are sporadic, but they can also develop as part of genetic syndromes. Approximately 10% of the GEP-NENs, mainly PNENs and in lesser extent gastric or siNENs, arise in the context of a genetic syndrome [12].

2.1 Genetic syndromes and familial GEP-NEN cases

Multiple familial syndromes exist in which GEP-NENs can develop. The syndrome with the highest risk for PNENs (60%) is multiple endocrine neoplasia 1 (MEN1), which is caused by inactivating mutations in the *MEN1* gene [13]. *MEN1* encodes for the protein Menin, which is mainly localized in the nucleus, has many interaction partners and plays a role in multiple pathways, including PI3K/Akt/mTOR, chromatine remodeling, DNA repair and cell cycle control [14]. The von Hippel Lindau (VHL) syndrome is caused by germline mutations in the *VHL* gene. VHL functions within a complex that regulates activity of hypoxia inducible factors (HIFs) which can stimulate angiogenesis [15]. Other syndromes include MEN4, Neurofibromatosis Type 1 (NF1) and Tuberous Sclerosis (TS), caused by mutations in *CDKN1B, NF1* and *TSC1* or *TSC2*, respectively [16-18]. Gene products of *NF1*, *TSC1* and *TSC2* all play a role in the PI3K/Akt/mTOR pathway. Remarkably, sequencing of sporadic PNEN cases led to the identification of likely pathogenic germline mutations in cancer susceptibility genes in 10-16% of patients, including *MEN1, VHL, CDKN1B, APC, TSC2, MUTYH, CHEK2* and *BRCA1*, suggesting that a higher than anticipated proportion of patients may have inheritable disease [19, 20].

Familial clustering of siNENs, apart from a hereditary syndrome, has been observed and having a first-degree relative with an siNEN increases the relative risk 3.6 times [21]. However, the causal mutations of these familial forms are only beginning to be uncovered. In 2015, Sei et al. identified mutations in the IMPK gene [22]. Based on a study in 15 families, Dumanski et al. suggested that *MUTYH* and potentially other members of the same DNA excision-repair pathway, such as *OGG1*, could be involved in familial siNENs [23]. One family with gastric NENs has been described, in which a homozygous mutation in the *ATP4A* gene was identified as causal [24].

2.2 Sporadic Grade 1-2 GEP-NENs

Our understanding of the genetic constitution of sporadic, non-familial, GEP-NENs is supported by a growing body of evidence, mainly based upon next-generation sequencing (NGS) data. With the introduction of NGS, it became possible to sequence multiple genes simultaneously and even perform unbiased whole-exome sequencing (WES) and whole-genome sequencing (WGS). Most NGS studies have focused on G1 and G2 GEP-NENs.

2.2.1 Pancreatic NENs

CNV analysis of PNENs, at first via DNA arrays and later via NGS, showed frequent alterations, including whole or partial loss of chromosomes 1, 2, 3, 6, 8, 10, 11, 15,16, 21 and 22, gain of chromosomes 5, 7, 12, 14 and 17 and also loss of heterozygosity [20, 19, 25]. Initial sequencing analysis of sporadic PNEN cases has identified somatic mutations in the *MEN1* gene, previously identified in familial PNENs [26]. Thereafter, two landmark studies have strongly advanced the understanding of genetics of sporadic PNENs, being the WES study of Jiao et al. in 2011 and the WGS study of Scarpa et al. in 2017 [27, 19]. Jiao et al. detected mutations in *MEN1* (44%) and in PI3K/Akt/mTOR pathway genes (15%), in line with previous studies in PNENs, and identified *DAXX* (25%) and *ATRX* (18%) as new frequently mutated genes [27, 26, 28]. Due to the critical role of the PI3K/Akt/mTOR pathway in PNEN oncogenesis, the mTOR-inhibitor everolimus has been tested as a treatment modality for advanced PNENs within the RADIANT trials [28]. Since everolimus extended progression-free survival (PFS), it was approved for the treatment of advanced PNENs [29]. Scarpa et al. performed WGS on 102 sporadic cases, which were mainly early stage. In 2018, Raj et al. performed targeted sequencing of 80 metastatic PNENs and showed that the mutational burden in these metastasized tumors was 2.95 mutations/megabase, which is higher than the 0.82 mutations/megabase identified by Scarpa [20, 19]. Molecular alterations in PNENs were associated with four main pathways, being (1) chromatin remodeling *(*including *MEN1, SETD2*), (2) DNA damage repair (including *MUTYH, CHEK2*), (3) activation of mTOR signaling (including *TSC2, PTEN*) and (4) telomere maintenance (including *DAXX, ATRX*). However, also genes implicated in cell cycle regulation were affected, including *TP53, CDKN2A* and *CDKN1B* [20, 19]. In addition, based on an integrative analysis of 57 PNEN cases using shallow WGS, WES, RNA sequencing and DNA methylation analysis, Lawrence et al. conclude that aneuploidy, i.e. abnormal chromosome numbers, is more important than single mutations in tumor development [30]. Sequencing of insulinomas, a type of functional PNENs, has led to the discovery of a hotspot mutation in transcription factor *YY1* present in 30% of a Chinese, 0% of an Indian and 8%-33% of Western/Caucasian insulinoma populations [31-36]. This suggests that functional and non-functional tumors may also differ genetically. By using a deep targeted resequencing approach on 38 PNENs, Vandamme et al. recently showed that some mutations are only present in a subset of the tumor cells [37]. These mutations strongly suggest the existence of genetic intratumor heterogeneity and could possibly be proliferation-driving and therapy resistance-causing mutations. However, a more extensive study in PNENs, perhaps including multiregion sequencing, could potentially provide additional valuable insights regarding the role of subclonal mutations.

2.2.1 Small intestinal NENs

In 2001, separate groups have shown that loss of chromosome 18 is a frequent event in siNENs (67-80% of cases) [38-41]. A WES study on 48 siNENs provided the first insights into single gene mutations and has identified protein-altering variations in several genes, including *MEN1*, *VHL* and *SMAD1*, despite the very low average mutation rate in siNENs [42]. Recurrent loss of chromosomes 11 and 18, and gains of chromosomes 4, 5, 19 and 20 were identified, confirming previous findings using DNA arrays [38-41, 43, 44]. Integrative analysis of mutations and CNVs identified the following pathways as recurrently altered: chromatin remodeling, DNA damage, apoptosis, RAS signaling, and axon guidance. In addition, amplification of *AKT1* or *AKT2* in several patients implicates the involvement of the PI3K/Akt/mTOR pathway in siNENs [42]. A WES and WGS study has identified both recurrent mutations and deletions in *CDKN1B*, implicating a role for this tumor suppressor gene in siNEN tumorigenesis [45]. Multiregion sequencing of primary tumor and synchronous liver metastases has recently been performed for 5 siNEN patients, which indicated strongly varying degrees of intratumor heterogeneity in different patients. However, the same degree of intratumoral heterogeneity was found for CNVs and single nucleotide variations within every patient [46].

2.3 Sporadic Grade 3 GEP-NENs

Classifying a neoplasm as NET or NEC is often not straightforward because of limited tissue material, poor microscopic slide quality, tumor heterogeneity, difficulties with assessing differentiation grade of PNENs or due to overlap in morphological features [47]. In addition, there are also interobserver inaccuracies, when performing Ki-67% or mitotic index estimations. Due to the big differences in tumor behavior, prognosis and patient management between different classes, it is very important that a correct diagnosis is made. Molecular analysis of NETs and NECs has indicated differences on the genetic level between the two entities and these differences might provide an interesting additional tool to distinguish and classify them.

For esophageal and appendiceal NENs, genetic data are lacking. Although data are available on G1 and G2 siNEN, data on G3 NECs of the small intestine are lacking, as these neoplasms are uncommon. For gastric NENs the available genetic data are limited, but multiple studies have highlighted mutations in or loss of TP53 in NECs, while it is unaffected in NETs. In addition, mutations were found in *KRAS*, *RB1*, *SMAD4* and *BRAF* in gastric NECs, but only in a limited number of cases, so validation of these mutations is still required [48-52]. The largest part of the colorectal NENs are NECs, and colorectal NECs were found to be mutated in *APC*, *KRAS*, *BRAF* and

TP53 and often have microsatellite instability [49, 52]. Mutations in these genes were not observed in colorectal NETs [53].

PNECs and PNETs can also be distinguished based on their genetic profiles. Even G3 PNETs, which show overlap with PNECs regarding Ki-67 values, show a distinct genetic profile. The most frequently affected genes in PNECs are *TP53, KRAS, RB1* and *CDKN2A/p16* while in PNETs, *MEN1, DAXX, ATRX* and mTOR pathway genes are most frequently affected [54, 27, 19]. Mutations in these genes also resulted in an altered protein expression detectable via immunohistochemistry (IHC) [55]. Some genes, however, have been found to be mutated in both, including *LRP1B, ARID1A, CDKN2A, APC* and *TP53* [54]. In 2016, Tang et al. studied 33 G3 NENs and described that in 66% of the cases pathologists didn't reach consensus on the differentiation state of the NENs based on morphologic analysis alone [47]. The use of a Ki-67 cut-off of 55%, where lower levels would indicate a well-differentiated NET (WD-NET) and higher levels a poorly differentiated NEC (PD-NEC), did not enable discrimination of WD-NETs and PD-NECs, leading to a misclassification of approximately 30% of WD-NETs and 30% of PD-NECs [56, 47]. Therefore, they have studied the additional value of performing IHC staining for p53, SMAD4, Rb, ATRX and DAXX expression, next to extended pathological review. DAXX or ATRX loss allowed correct classification in 50% of the morphologically ambiguous WD-NET cases, while abnormal expression of p53 or Rb allowed correct classification of 90% of the morphologically ambiguous PD-NEC cases. SMAD4 evaluation didn't provide additional value when p53 and Rb were evaluated. Tang et al. demonstrated that, next to additional clinical information and presence of G1/2 regions which points towards a G3 WD-NET, also IHC analysis can aid in making the correct diagnosis. In more than 60% of the cases IHC analysis could differentiate between a WD-NET and a PD-NEC [47].

As discussed, genetic differences have been found between NETs and NECs, which could be interesting as markers in cases were histology is inconclusive. However, additional studies, with larger sample sizes, will be required to further assess the potential of molecular analysis to differentiate between NETs and NECs and thereby guide patient management. In addition, overlap between genetic profiles of exocrine tumors and NECs has been described, especially in pancreas and colon, suggesting that NECs might have an exocrine genetic signature, which might be relevant for treatment and should be explored further [53, 54].

2.4 Copy number variations and mutations as biomarkers

2.4.1 Copy number variations as prognostic biomarker

CNVs are frequently observed in GEP-NENs and some alterations have been associated with prognosis. Genome-wide loss of heterozygosity was found to be associated with inferior survival in PNENs [20]. CNV profiles also allow subgrouping of PNENs in groups characterized by different mutational profiles and clinical features, including different metastatic potential [19, 57]. PNEN patients with a higher level of chromosomal instability show a trend towards longer survival [58]. Loss of only chromosome 18 has been found in siNEN patients with a better survival, while gain of chromosome 14 was predictive of poor survival in siNENs [58-60]. In contrary to PNENs, a higher level of chromosomal instability was associated with worse survival in gastrointestinal NENs [58].

2.4.2 Mutations in DAXX/ATRX as prognostic biomarker

A large fraction of PNENs has mutations in *DAXX* and *ATRX* which correlates with loss of DAXX and ATRX protein expression [27, 61, 62]. PNENs usually have mutations in either *DAXX* or *ATRX*, which can be readily understood as their encoded proteins function in the same pathway, where they form a complex. The DAXX/ATRX complex has an important role in maintaining telomeric chromatin by deposition of the Histone H3.3 variant at the telomeres [63]. Loss of DAXX/ATRX has been reported in 20%-79% of PNEN cases [62, 64-68]. These diverse prevalences could be explained by a different ethnic background of the patients or by differences in the composition of the study population regarding, for example, tumor stage. DAXX/ATRX loss associated with higher grade, higher Ki-67 index, larger size and tumor stage [65, 69]. Furthermore, it has been shown that loss of DAXX/ATRX was a late event in MEN1-associated PNEN development as 47 microadenomas (<0.5cm) showed no ATRX/DAXX loss [70]. Singhi et al. showed that DAXX/ATRX status is concordant between primary and metastatic tissue of 52 sporadic PNEN cases [69]. This suggests that, although loss of DAXX/ATRX expression is a late event, it still occurs prior to development of metastatic disease and it might therefore also play a role in driving tumor metastasis.

Interestingly, Jiao et al. described a significant association between *DAXX/ATRX* mutations and an improved survival in metastatic cases [27]. Following these observations, many additional studies have investigated DAXX/ATRX mutation and expression status in relation to prognosis, as summarized in **table 1**. However, controversy is still present. In several populations including between 16 and 347 PNEN patients, DAXX/ATRX

mutations and/or loss were significantly associated with worse survival, including disease-free (DFS), relapse-free (RFS), disease-specific (DSS) and overall (OS) survival [62, 65-69, 71-73]. The worse prognosis in ATRX/DAXX-negative patients might seem contradictory to the findings of Jiao et al. [27]. However, all these studies mainly included early stage patients. In a subset analysis on metastatic patients or in studies including only metastatic patients, DAXX/ATRX-negative tumors showed a trend towards or association with longer survival [62, 74, 65, 68]. Furthermore, *DAXX* mutations have been found to be a risk factor for development of liver metastases in small PNENs [57]. In some studies, DAXX/ATRX status is significantly associated with DFS, but not with OS or DSS in multivariate analysis. This is possibly due to a strong correlation with other prognostic factors, a too short follow-up time or the presence of confounding factors [62, 65, 69]. In a large PNEN cohort (N=269), loss of ATRX/DAXX was an independent prognostic factor for OS when the cohort was limited to patients with synchronous or metachronous metastatic PNENs, with loss of expression being associated with a better OS [65]. In a study on 105 unselected PNENs, loss of ATRX or DAXX was associated with poor OS in univariate analysis, but not in multivariate analysis. However, in a separate analysis of DAXX and ATRX, only ATRX loss was significantly associated with a worse OS in both univariate and multivariate analysis [68]. Targeted sequencing of 80 metastatic PNEN patients showed an improved survival in *DAXX/ATRX*-mutated tumors compared to wild-type tumors, further adding to the evidence that loss associates with better prognosis in metastasic patients [20].

IHC studies for ATRX and DAXX in other GEP-NENs, showed that loss of ATRX/DAXX could also be detected in rectal NEN (80%), gastric NEN (60%) and duodenal NEN (13-27%), although no ATRX/DAXX mutations have been described in these tumor types so far [75, 76]. An analysis of 327, mainly early stage GEP-NENs, including stomach, duodenum, pancreas, liver, appendix and colon NENs, showed that the OS was lower in patients who had lost DAXX/ATRX expression [77].

As an overall conclusion, we can state that the prognostic value of DAXX/ATRX loss depends on disease status, with loss in non-metastatic patients associated with worse survival and loss in metastatic patients associated with better survival. However, a large meta-analysis that includes all cases and makes relevant stratifications, e.g. metastatic versus non-metastatic, might still lead to better insights. A possible explanation for this phenomenon could be that DAXX/ATRX-deficient tumors more easily progress and metastasize, but that because of the intact telomeres, they have less chromosomal instability and might therefore have more difficulties adapting to the new

microenvironment, resulting in a slower growth [65, 78]. In addition, DAXX/ATRX loss might also indicate that micrometastasis has already occurred in some cases without clinical apparent metastasis. In metastatic cases, loss of DAXX/ATRX might indicate a subtype of NENs that is associated with a better prognosis.

2.4.3 Mutations and copy number variations as predictive biomarkers

Everolimus and sunitinib, a VEGF pathway inhibitor, are frequently used targeted therapies for GEP-NENs, but their effects are limited by both primary and acquired resistance and predictive biomarkers are needed. Multiple (pre)clinical studies have therefore focused on the identification of resistance mechanisms, ways to overcome resistance and biomarkers related to these treatments, including in genetically characterized PNEN cell lines [79-82]. A study on 17 PNEN patients by Serra et al. suggested predictive value for everolimus efficacy for the Gly388Arg *FGFR4* polymorphism, with a worse response in *FGFR4-R388* patients, in concordance with preclinical models [83]. However, this polymorphism didn't seem to have prognostic or predictive value for everolimus response in a retrospective study on 35 GEP-NENs [84]. Cell lines with mutations in the PI3K/Akt/mTOR pathway genes *PIK3CA* and/or *PTEN*, were more likely to be sensitive for rapamycin treatment. As everolimus is a rapamycin analog this warranted further research [85]. Genetic analysis of 191 GEP-NEN patients of the phase III RADIANT trials, studying everolimus in GEP-NENs, couldn't evaluate the predictive value of PI3K/Akt/mTOR pathway mutations due to a low number of mutations and inequal distribution over treatment and placebo cohorts [86, 58]. In this analysis, no other mutations or CNVs could be identified to be predictive for therapy response. In conclusion, there are still no prognostic or predictive molecular markers available for everolimus or sunitinib [87, 79].

Response to platinum-based chemotherapy was evaluated in a population of 70 G3 PNEN patients, for which Rb expression and *KRAS* mutation status were also assessed. G3 PNET patients had a low response-rate for platinum-based chemotherapy, while PNEC patients showed a good response. 55% of the PNEC patients had lost Rb expression and 49% had mutations in *KRAS*, while no abnormal Rb expression or *KRAS* mutations were observed in G3 PNETs. Rb expression and *KRAS* mutations were both predictive for reponse to platinum-based chemotherapy in G3 PNENs, while Rb expression was even predictive for response within the PNEC population [88].

3. EPIGENETIC ALTERATIONS

Epigenetic modifications affect gene expression without changing the DNA sequence. DNA methylation is the most studied epigenetic mechanism and it entails the addition of a methyl group to a cytosine in a CpG context. Hypermethylation of a promotor has been shown to inhibit gene expression, while hypomethylation in gene bodies or intergenic regions can lead to chromosomal instability or altered gene expression [89]. Another epigenetic mechanism is the modification of histones, which involves the addition of methyl, acetyl or other groups to histone proteins. However, there are limited data on histone modifications in NENs. In addition, microRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression post-transcriptionally. miRNA expression is therefore often also considered to be an epigenetic mechanism. Cancer cells usually have altered epigenomes. As GEP-NENs have in general a low mutation burden, especially siNENs, other mechanisms driving development and progression might be expected, such as epigenetic changes. Commonly mutated genes in PNENs, e.g. *DAXX*, *ATRX* and *MEN1*, play a role in epigenetic regulation, suggesting its importance as well. In addition, overexpression of DNA methyltransferases DNMT1, -3A and -3B is a common feature of GEP-NEN [90].

3.1 DNA Methylation

The first studies regarding DNA methylation changes in GEP-NENs have focused on one or a few genes. Several tumor-suppressor genes were reported to be hypermethylated in PNENs, including *RASSF1A*, *HIC-1*, *MLH1*, *CDKN2A* and *MGMT* [91, 92]. Furthermore, global hypomethylation has been found in GEP-NENs using the methylation status of LINE-1 and Alu repeats. In general, global hypomethylation is seen more frequently in GEP-NEN compared to normal samples, but the frequency of global hypomethylation differs between types of GEP-NENs [93-95].

With the introduction of DNA methylation arrays, methylation status of many CpGs could be assessed at the same time, providing a more genome-wide DNA methylation profile. A study by How-Kit et al. on 62 GEP-NEN patients showed that DNA profiles differed between siNENs and PNENs, but also between insulinomas, gastrinomas and non-functional tumors, meaning that also on the epigenetic level, heterogeneity within the GEP-NENs is present [96].

3.1.1 PNENs

Genome-wide DNA methylation profiles of 53 and 64 sporadic PNENs were shown to be different depending on the mutation status (e.g. *DAXX*, *ATRX* or *MEN1* mutated) [97, 72]. Promotor hypermethylation of tumor suppressor

genes is a frequent event in both MEN1-related and sporadic PNENs [98]. In a recent study, the genome-wide DNA methylation profile of 9 sporadic, 10 VHL-related and 10 MEN1-related PNENs and 4 pancreatic islets was assessed using the most recent DNA methylation array of Illumina (850k array). Unsupervised clustering resulted in a cluster of VHL PNENs and a cluster containing both sporadic and MEN1-related PNENs. Pathways enriched in epigenetic alterations are intracellular transduction and VEGF-related pathway in VHL cases and RUNX3 transcription regulation and DAG/IP3-signaling pathways for MEN1 and sporadic cases [99].

3.1.2 siNENs

Array-based genome-wide DNA methylation studies on siNENs, performed by Verdugo et al. (n=20) and Karpathakis et al. (n=69), illustrated that these tumors are highly epigenetically dysregulated and that three different subgroups could be distinguished based on DNA methylation profiles. Group A has the most favorable prognosis and is defined by loss of heterozygosity in chromosome 18 and mutations in *CDKN1B* on the genetic level. Group C is characterized by significantly poorer prognosis and the presence of multiple CNVs, while Group B has an intermediate prognosis and is characterized by the absence of CNVs [60, 100]. Pathway analysis of differentially methylated genes between tumor and normal tissue identified multiple cancer-related pathways including MAPK, Wnt, and PI3K–mTOR signaling pathways. A panel of 21 epigenetically dysregulated genes was identified and further analyzed in an additional publication, which showed that in 19 of the genes a trend was seen for progressive hyper/hypomethylation from primary towards metastatic tumor [60, 101]. Barazeghi et al. analyzed in 40 primary siNENs and 47 corresponding metastases the levels of 5-hydroxymethylcytosine, another epigenetic mark, and levels of TET1 and TET2 enzymes. Both enzymes can catalyze the conversion of a 5-methylcytosine to 5-hydroxymethylcytosine and further to 5-formyl- and 5-carboxylcytosine, generating new epigenetic marks or initiating demethylation. They showed epigenetic dysregulation at the level of 5-hydroxymethylcytosine/TET1/TET2 and propose a new class of therapeutics for siNENs [102].

3.2 MicroRNA profiling

miRNA profiles are often altered in cancer cells and can provide information regarding tumor characteristics, such as differentiation state [103]. Therefore, miRNA has been studied for its biomarker potential in cancer, including in GEP-NENs. Interestingly, it has been shown that NENs have a miRNA profile that is different from adenocarcinomas [104]. miRNA profiles of GEP-NENs have been extensively reviewed by Malczewska et al., but we will summarize the most clinically relevant findings [105].

3.2.1 PNENs

A comparison of miRNA profiles of 40 PNENs (28 non-functional PNENs; 12 insulinomas) and 12 paired pancreatic tissue samples showed increased expression of miR-103 and miR-107 and a decreased expression of miR-155 in all cases. Additionally, a set of ten miRNAs (miR-99a, 99b, 100, 125a, 125b-1, 125b-2, 129-2, 130a, 132, and 342) overexpressed in PNENs, was able to differentiate PNEN from pancreatic acinar cell carcinoma (and normal pancreas). miR-204 was only overexpressed in insulinomas. Correlation of the expression profiles with clinical characteristics showed that an overexpression of miR-21, a miRNA involved in regulation of the PI3K/Akt/mTOR pathway component *PTEN*, associated with increased Ki-67 proliferation index and presence of liver metastasis [106]. To determine the optimal control sample, miRNA profiles of 37 PNENs were compared to miRNA profiles of total pancreatic tissue and pancreatic islets. There was no overlap of deregulated miRNAs between PNENs compared to pancreatic tissue and PNENs compared to pancreatic islets [107]. The lack of common deregulated miRNAs indicated that the miRNA profile of pancreatic islets is entirely different from the miRNA profile of pancreatic tissue and stresses the importance of the correct control. Total pancreatic tissue is most likely a less relevant control as PNENs develop from the endocrine cells of the pancreas. When using pancreatic islets as a control, they found a correlation between expression of miR-642 and Ki-67 index and expression of miR-210 and metastatic disease [107]. To identify prognostic miRNA markers, Lee et al. first selected 18 differentially expressed miRNAs, based on the comparison of primary and metastatic tissue of two patients, which were then validated in 37 patients that underwent surgery with curative intent. Only miRNA-196a was significantly associated with grade, stage, DFS and OS, and might be a promising prognostic biomarker for recurrence [108]. Based upon the comparison of human PNENs and neuroendocrine tumors which develop spontaneously in the RipTag2 mouse model, the group of Hanahan could distinguish three PNEN subtypes differing genetically and behaviorally, being (1) islet-like/insulinoma, (2) intermediate and (3) metastasis-like primary (MLP) subtype. The intermediate type was restricted to humans and contained a higher fraction of *MEN1* mutated tumors compared to the other subtypes. The MLP subtype was shown to be the most aggressive subtype with the highest Ki-67 values and a high metastatic potential. Interestingly, it was possible to distinguish the three PNEN subtypes based on their miRNA profiles [109].

3.2.2 siNENs

miRNA profiling studies in siNENs have mainly focused on the comparison of primary tumors with metastases, although a few studies have also included adjacent normal tissue or normal enterochromaffin cells as controls. miRNAs that were recurrently identified as differentially expressed during tumor progression are downregulated

miRNAs 129-5p, 133a and 143-3p and upregulated miRNAs 96 and 183 [110-115]. These might be useful for the evaluation of biological behavior of siNENs to detect more aggressive neoplasms. One study identified upregulation of miR-204, which was also identified in insulinomas [113]. *In vitro* analysis of miR-129-5p and let-7 family miRNAs, which are found downregulated in siNENs, has shown that their downregulation induces upregulation of their targets which are known to promote metastasis [114].

3.2.3 Other GEP-NENs

A few miRNA studies have been performed on colorectal NENs. A small study has shown that the miRNA profile of colorectal NENs is different from adenocarcinomas. Comparison with normal tissue has identified differentially expressed miRNAs, including miR-96, 129-5p, 196a and 204, which are also dysregulated in other GEP-NENs [116]. miR-186 showed significant downregulation in tissue, blood and stool of 39 colorectal NEN patients versus controls, while its predicted target, the oncogene PTTG1, showed significant upregulation [117]. In rectal NENs, upregulation of miR-885-5p is significantly associated with invasion state [118].

3.3 Prognostic and predictive epigenetic biomarkers

DNA methylation changes are frequently detected in GEP-NENs and could provide interesting biomarkers. For example, *CDKN2A* methylation has been shown to correlate with poor prognosis in GEP-NENs [119]. *MGMT* hypermethylation is also frequently observed. MGMT is a DNA repair enzyme that removes alkyl groups from an alkylguanine to prevent replication errors due to mismatches. Therefore, promotor hypermethylation of *MGMT* has been proposed as a predictive marker for treatment with temozolomide and other alkylating drugs. Several retrospective studies have found an association between MGMT status and treatment response, making it an interesting predictive marker to be prospectively validated [120-123]. Recently, a prospective trial ("MGMT-NET") has therefore been initiated to study the value of *MGMT* promoter methylation assessment in the prediction of response to alkylating agents [124]. Furthermore, hypermethylation of multiple tumor suppressor genes, such as *RASSF1A, hMLH1* and *MGMT*, which is sometimes termed 'CpG island methylator phenotype (CIMP) positivity', is associated with worse survival [91, 92]. Global hypomethylation, as detected by LINE-1 methylation, was correlated with poor prognosis in a cohort of 56 sporadic PNENs [125]. For siNENs, genome-wide DNA methylation profiles could distinguish subgroups associated with a different prognosis, with the worst prognosis in Group C neoplasms, which also harbor multiple CNVs [60, 100].

In addition, multiple studies have performed miRNA profiling of GEP-NENs, but different approaches, different controls and different sample types have been used. This heterogeneity, together with the small sample sizes, makes comparison difficult and larger validation studies are urgently needed to support use of miRNA as a biomarker. Identifying subtypes with a different prognosis based on miRNA profiles, as illustrated by the group of Hanahan, also provides an interesting application [109].

4. LIQUID BIOPSIES

As previously discussed, genetic and epigenetic alterations have multiple potential clinical applications, both as therapeutic targets and as biomarkers. **Figure 1** summarizes the most frequent genetically and epigenetically altered genes and pathways and their clinical applications. A major disadvantage of using (epi)genetic biomarkers on tissue is the need for enough tumor tissue to perform the genetic analysis and the associated risks of a tissue biopsy. A potential alternative could therefore be the use of liquid biopsies to detect the alterations. A liquid biopsy is a body fluid sample, which also contains information about the tumor, but that is more readily, and non-invasively obtainable than a tumor biopsy. Blood is currently the most studied liquid biopsy, but other sources such as stool or urine might also have potential. Blood contains several components that can be analyzed to learn more about the tumor, including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and circulating tumor RNA (**Figure 2**).

4.1 Circulating tumor cells

CTCs were first described in 1869 when Ashworth detected cells in the peripheral blood of a metastatic patient that resembled the tumor cells, since then a lot of research has been dedicated to CTCs [126]. The CellSearch® platform was specifically developed to extract and count CTCs by surface marker-based selection, including positivity for epithelial cell adhesion molecules (EpCAM) [127]. In 2011, Khan et al. showed that NENs express EpCAM, enabling the detection of CTCs via the CellSearch® system. Respectively, 43% and 21% of metastatic ileal (N=42) and pancreatic (N=19) NENs had detectable CTCs. Interestingly, the absence of CTCs was associated with stable disease, while the presence of CTCs associated with progressive disease and with PFS and OS in multivariate analysis [128, 129]. Higher CTC levels were detected in patients with a greater disease burden and changes in CTC count were associated with treatment response and OS, suggesting potential as a follow-up marker [130]. In the phase II PAZONET study evaluating pazopanib treatment, patients without baseline CTCs showed a trend towards improved response and longer median PFS [131]. Initial studies have focused only on CTC presence and counts, while more recent studies are also evaluating tumor properties of the CTCs, including the expression

of somatostatin receptors (SSTR) and CXCR4 on the cell surface as potential biomarkers for SSTR-targeted therapies, such as somatostatin anologs (SSAs) and PRRT, and bone metastasis, respectively [132-134]. Presence of CTCs could distinguish between patients with and without bone metastases with an Area Under the Curve (AUC) of 0.79 and 0.65, in PNENs and midgut NENs, respectively. The results of the subset analysis focusing on expression of CXCR4 on CTCs, didn't reach statitistical significance [134]. Another potential application of CTCs is the detection of previously described (epi)genetic alterations, as DNA/RNA can also be extracted from the isolated CTCs and used for molecular analysis, including single-cell DNA or RNA sequencing [135].

4.2 Circulating tumor DNA

ctDNA is DNA that originates from the tumor and is released in the blood as a result of apoptosis, necrosis and active secretion [136]. In 1966, researchers have reported the presence of cell-free DNA (cfDNA) in serum and 10 years later, it was found that cancer patients have higher concentrations of cfDNA than healthy controls [137, 138]. ctDNA can be distinguished from cfDNA originating from healthy cells, as it contains tumor-specific genetic and epigenetic alterations. A lot of research has been performed on ctDNA and cfDNA in cancer patients, highlighting many potential applications, including patient monitoring and detection of relevant mutations such as *EGFR* and *KRAS* mutations to guide therapy in colorectal cancer patients [139, 140]. To do patient monitoring, the absolute amount of ctDNA in the plasma or the ctDNA level, which is the amount of ctDNA in total cfDNA, are used as a measure for tumor burden and are measured over time. For GEP-NENs, ctDNA research is still in its infancy. In a pilot trial on 10 patients, Boons et al. reported the presence of ctDNA in metastatic PNEN patients by performing mutation-specific droplet digital PCR (ddPCR) on cfDNA extracted from the plasma [141]. However, in patients with localized disease, tumor-specific mutations were not detected in the cfDNA. Furthermore, CNVs could be detected in cfDNA of the metastatic cases using shallow WGS, which demonstrates that cfDNA gives a valid representation of the tumor and could be an interesting application as CNVs might have prognostic value. In one patient, for which a follow-up sample was available, an increase in cfDNA concentration, ctDNA level and chromosomal aberrations could be detected in parallel with disease progression. In addition, a *DAXX* mutation could be detected in the tissue and liquid biopsy of a WHO2010 G3 patient, which led to reclassification of the patient as a WHO2017 well-differentiated G3 PNET as confirmed by pathology review [141]. Due to its small size, ctDNA is able to pass the kidney barrier, causing it to be present in urine as well [142]. In a patient suffering from a metastatic rectal NEC, a tumor-specific $BRAF^{V600E}$ mutation could be detected in the urine using ddPCR. After initiation of treatment with BRAF/MEK-inhibitors, to which the patient responded well, the ctDNA level in the urine decreased rapidly, indicating potential for patient monitoring [143].

In general, two ctDNA approaches can be distinguished. One where tissue is first needed for the identification of an alteration to detect in cfDNA and one where tissue is not needed and which provides the most interesting approach. In the previously discussed studies, an alteration was first identified by tumor tissue sequencing, followed by detection of this alteration in the cfDNA. However, also an approach evading the need for tissue was introduced, namely shallow WGS of cfDNA for the detection of CNVs. As NGS is possible on cfDNA, an alternative that would also not need a tissue biopsy, is the use of a gene panel to estimate ctDNA level and to allow detection of relevant mutations directly on plasma [144]. Interesting genes could include *MEN1, DAXX, ATRX, Rb1, TP53* and genes that can propose targeted treatments such as *BRAF* [141, 27, 19, 47, 143]. Two studies that have included a few GEP-NEN cases, were able to detect a variant in the plasma of 1 in 5 and 1 in 2 cases, respectively, based on targeted sequencing [145, 146]. However, they didn't use NEN-adapted gene panels.

In addition, DNA methylation changes could also represent interesting alterations to detect in cfDNA. For example hypermethylation of *MGMT*, which was proposed as a predictive marker for temozolomide treatment. In a recently initiated prospective trial to confirm its biomarker potential ("MGMT-NET"), plasma samples will be collected to assess if *MGMT* hypermethylation is also detectable in cfDNA instead of tissue [124]. Furthermore, altered DNA methylation of a specific region has been shown to be a more universal alteration within a specific cancer type, compared to mutations, and could therefore be useful for detection of ctDNA and to estimate ctDNA levels for follow-up [147-149]. Despite several DNA methylation studies in GEP-NENs, such a marker has not been found yet.

4.3 Circulating tumor RNA

Besides DNA and CTCs, RNA can also be extracted from a blood sample to be used as a liquid biopsy biomarker. Reports regarding the use of circulating RNA in plasma or serum of GEP-NENs are still scarce. In 2009, Modlin et al. showed that NEN-related transcripts could be detected by quantitative PCR in the plasma of siNEN patients which could allow diagnosis with acceptable sensitivity and specificity in a small sample set [150]. Since miRNA expression has been shown to be altered in GEP-NEN tissue, several groups are attempting to detect these altered miRNAs in liquid biopsies. In a follow-up study perfomed by Li et al., they showed that the nine miRNAs that were altered on tissue level, could also be detected in serum samples [111]. Furthermore, the serum levels of certain miRNAs were correlated with SSA treatment status or tumor stage [151]. Another study on siNENs was performed by Bowden et al. [152]. They first identified candidate miRNAs by searching for similarly expressed

miRNAs between tissue and plasma. Then, the 31 identified miRNAs were evaluated in an independent cohort of 40 cases and 40 controls, in which 4 of the 31 miRNAs were differentially expressed. In a validation experiment on a larger cohort of 120 cases and 120 controls, 3 of the 4 miRNAs (miR-21-5p, miR-22-3p and miR-150-5p) reached a statistically significant difference. In addition, plasma levels of the 3 miRNAs were associated with presence of metastatis and survival [152]. In the miRNA study in siNENs performed by Heverhagen et al., the most promising miRNA-biomarker, miR-7-5p, was selected from the seven miRNAs with biomarker potential, and tested in serum samples of 32 tumor patients and 25 healthy controls. Sera of siNEN patients had significantly higher levels of miR-7-5p compared to controls [115]. As previously described, Wang et al. found a significant downregulation of miR-186 in colorectal NEN patients, in tissue as well as in blood and stool samples [117].

Another liquid biopsy approach, that was developed by Modlin and his colleagues, is based on the extraction of RNA from whole-blood samples. In 2013, Modlin et al. utilized previously generated tumor and normal tissue microarray datasets, microarray data from peripheral blood of controls and GEP-NEN patients and a literature search to compile a list of candidate biomarkers. Subsequent analysis of the candidate biomarkers using quantitative PCR in a training set of blood samples from 28 cases and 49 controls led to the identification of a 51 marker panel containing genes associated with neoplasticity [153]. An algorithm was developed to calculate a disease-activity score, between 0% (lowest risk) and 100% (highest risk), based on gene expression of the 51 markers. This multianalyte PCR-based test was named the NETest. Follow-up studies to further characterize the NETest showed that the NETest produced robust and reproducible results (interassay and intra-assay variability < 2%) and performed better as diagnostic marker compared to single analyte tests, including chromogranin A (CgA), pancreastatin (PST) and neurokinin A (NKA) [154-156]. Subsequently, the NETest algorithm was extended to include “activity” scoring for prediction of clinical disease status, which could indeed distinguish between stable and progressive disease [157-159]. Furthermore, changes in NETest values over time correlated with treatment responses for SSAs, PRRT and surgery and the baseline levels of a subset of transcripts were able to predict response to PRRT [160-162]. In a diagnostic validation trial on a Dutch cohort of 140 GEP-NEN patients and 113 volunteers, the NETest had a very good sensitivity (93%), but the specificity was relatively low (56%). Sensitivity and specificity for CgA were respectively 56% and 83%. They conclude that the NETest is less suited for screening due to the low specificity, but could be valuable for detection of residual disease after surgery and for follow-up [163]. This trial highlights the importance of independent validation studies to allow integration of the NETest in clinical practice.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The development of high-throuput techniques has clearly accelerated research in GEP-NENs. However, several gaps remain. Due to their low incidence, many studies are being performed on small sample sizes including less than 100 cases. In addition, for some GEP-NENs, such as gastric or appendiceal, there are barely any data available. This stresses the importance of collaborations for the study of rare diseases, whereby both samples and data should be pooled to achieve larger datasets, like available in The Cancer Genome Atlas (TCGA) for various other tumor types. Furthermore, except for some recent studies, most studies have only focused on one aspect of the tumor cell, such as the genetic constitution [19, 97, 30, 99]. However, to fully understand the molecular mechanisms driving development and progression of GEP-NENs, it would be interesting to apply an integrative approach by combining multi-omics data [164]. To understand expression and epigenetic dysregulation, an additional challenge is the use of an appropriate control sample, as expression and epigenetic profiles depend on cell type. The best control would therefore be the cell type of origin. Some studies on PNENs have used paired whole pancreatic tissue, despite the fact that PNENs develop from the endocrine cells of the pancreas, which can give very different results, for example for miRNA profiling [107]. Some groups have chosen to evade this issue by comparing primary tumors with metastatic tissue, as GEP-NENs are often indolent in early stages, to find mechanisms and markers of progression. In addition, functional studies are required to improve our understanding of the implications of these alterations such as *MEN1/DAXX/ATRX* mutations, for which we will need adequate genetic models.

As we have described, multiple studies have suggested potential biomarkers, but often no validation studies have been performed. This leads to large amounts of potential biomarkers, without actual advances in the clinic. *DAXX/ATRX* alterations are probably the most extensively studied genetic biomarker for prognosis of mainly PNENs. However, they are not yet used in standard practice. In addition, markers to differentiate between NET and NEC are also very promising to complement histological analysis and deserve further attention. Genome-wide profiling of CNVs, mutations, miRNAs and DNA methylation showed that these profiles are able to distinguish subtypes with different clinical features. This could be interesting to guide for example management of small tumors, to identify the malignant lesions which require immediate action from the benign lesions that would benefit from a wait-and-see approach.

With the development of the NETest and several recent papers assessing biomarkers in body fluids, such as serum, plasma and stool, we can state that the *liquid biopsy* is being introduced in the field of the neuroendocrine tumors. Liquid biopsies have many different potential applications in patient management, that should also be further developed for GEP-NENs. First, they could be used for diagnosis as indicated by the NETest. In addition, they could be used for profiling of certain genetic and epigenetic changes, for example by using NGS panels. This could help classifying neoplasms, for example for NEC vs NET assessment, and pick up signatures, such as CNV profiles, or alterations, such as *DAXX/ATRX* mutations, associated with prognosis. A big advantage of liquid biopsies is that it becomes possible to perform evaluation of the tumor over time, to pick up disease changes and guide treatment. For example, if certain mutations are known to cause resistance, these could be easily picked up in a liquid biopsy, as is being done for *KRAS* mutations before initiating anti-EGFR treatment in colorectal cancer [140]. Furthermore, liquid biopsy markers such as CTC count or ctDNA level can be used to perform follow-up of the tumor load. Therefore, they might be useful to evaluate response to therapy or recurrence. For this purpose, a single alteration could be measured in cfDNA or multiple alterations could be evaluated which can then be combined into one value, like for the NETest.

In addition, liquid biopsies could also be valuable for the detection of targetable molecular alterations to guide treatment decisions, a strategy called precision oncology. To assess the possibility of applying precision oncology on GEP-NENs, Kim. et al. analyzed oncogenic mutations via the Ion AmpliSeq Cancer Hotspot Panel v2 and copy number variations on tissue of 14 GEP-NEN patients. They identified targetable alterations in 50% of the patients, meaning that mutation analysis could provide additional therapeutic options, also in GEP-NENs [165]. Further research on this precision oncology approach in neuroendocrine tumors is being performed within the NCT/DKTK-MASTER precision oncology trial [166, 167].

In summary, genetic and epigenetic alterations have many potential applications for diagnosis, prognosis, therapy response prediction and follow-up, and especially in combination with a liquid biopsy approach they could be extremely useful. The major challenge will now be to rapidly evaluate and validate potential biomarkers, for example within clinical trials for therapeutics, to allow implementation in the clinic.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare no conflict of interest.

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Legends table and figures

Table 1

Studies investigating the prognostic value of mutations in *DAXX*/*ATRX* and loss of DAXX/ATRX in GEP-NENs.

Figure 1

Overview of the (epi)genetically altered genes and pathways and their clinical applications in GEP-NENs. Genes that are altered by mutations are marked with a red border and epigenetically altered genes with a green border. Clinical applications as therapeutic target or biomarker are shown in orange boxes.

Figure 2

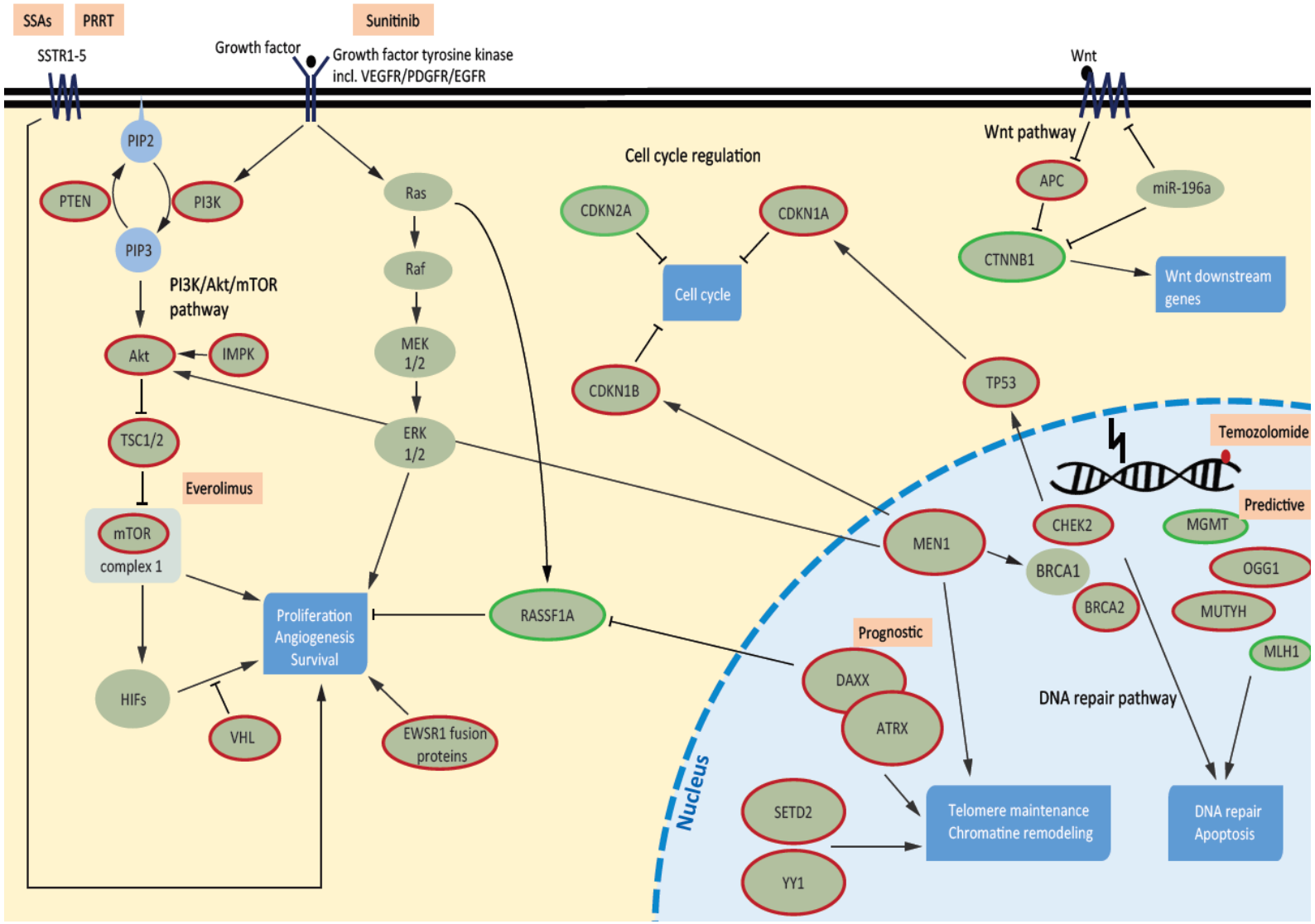
Cellular components with (possible) use as biomarker in liquid biopsies of GEP-NEN patients. The discussed (epi)genetic alterations might be detectable in DNA of circulating tumor cells or in circulating tumor DNA.

Table 1.

Studies investigating the prognostic value of mutations in *DAXX/ATRX* and loss of DAXX/ATRX in GEP-NENs.

Reference	No. of samples	Technique	Conclusions
Jiao et al, 2011 [27]	68 nonfamilial PNENs	WES (N=10); Targeted sequencing (N=58)	In metastatic patients (N=27): presence of DAXX/ATRX mutations significantly associated with prolonged survival
Chen et al, 2013 [75]	10 gastric, 15 duodenal, 20 rectal, 70 pancreatic NENs	IHC	Loss of ATRX/DAXX is also observed in non-pancreatic NENs. DAXX expression was associated with Ki-67 index.
Marinoni et al, 2014 [62]	243 well-differentiated primary PNENs (Clinical follow-up data obtained for 149 patients)	IHC	Loss of DAXX/ATRX was correlated with a shorter survival and was an independent predictor for patient relapse in multivariate analysis. Subgroup analysis of 20 metastatic patients showed a trend towards longer survival for DAXX/ATRX-negative tumors.
Sato et al, 2014 [66]	16 PNENs	IHC	Loss of DAXX expression was correlated with postoperative hepatic relapse and loss of ATRX/DAXX expression was correlated with DFS.
Yuan et al, 2014 [78]	37 PNENs (Chinese population)	Targeted sequencing	Mutations in *DAXX/ATRX* associated with a shortened survival
Kim et al, 2015 [77]	327 GEP-NENs (colon, stomach, liver, duodenum, pancreas, appendix)	IHC	Overall survival was significantly lower when ATRX/DAXX expression was lost.
Pipinikas et al, 2015 [72]	34 PNENs	IHC	Loss of ATRX/DAXX led to poorer PFS. In a separate analysis of ATRX and DAXX, only a significant association was found between PFS and loss of DAXX.
Geis et al, 2015 [76]	69 siNENs	IHC, quantitative PCR	ATRX expression was lost in 13% and DAXX expression was lost in 0%. Survival was not analyzed and there was no significant association with clinical characteristics.
Cives et al, 2016 [74]	143 metastatic/locally advanced PNENs under CAPTEM treatment	IHC (N=31)	DAXX/ATRX expression was not able to predict response to CAPTEM. DAXX/ATRX loss was associated with improved survival.
Singhi et al, 2016 [69]	321 patients with resected PNEN	IHC	DAXX/ATRX loss was associated with shorter DFS and DSS, but DAXX/ATRX loss was only an independent prognostic factor for DFS.
Park et al, 2017 [67]	76 PNENs	IHC	In curatively resected cases a trend towards longer DFS was seen in cases with positive expression of ATRX/DAXX. In metastatic PNENs, OS was significantly longer in patients with loss of ATRX/DAXX or MEN1 expression.
Kim et al, 2017 [65]	269 PNENs	IHC	DAXX/ATRX loss was not observed in neuroendocrine microadenomas (N=19). DAXX/ATRX loss is a poor prognostic factor for RFS. However, metastatic patients that are negative for ATRX or DAXX have better overall survival.
Chou et al, 2018 [68]	105 PNENs	IHC	ATRX/DAXX loss was associated with poor OS in univariate analysis. ATRX, but not DAXX, loss was also associated with poor OS in multivariate analysis. ATRX/DAXX-negative PNENs with metastasis at presentation showed a trend toward improved OS. Metastatic ATRX-negative tumors demonstrated a trend toward worse OS.
Roy et al, 2018 [73]	347 PNENs	IHC; FISH for CDKN2A	Loss of/deletion in DAXX, ATRX, H3K36me3, ARID1A and/or CDKN2A in primary tumors associated with shorter survival.
Raj et al, 2018 [20]	80 metastatic PNENs	Targeted sequencing	Longer OS was observed in patients with *MEN1* mutations and *DAXX/ATRX* mutations.
Pea et al, 2018 [57]	87 PNENs	IHC	DAXX/ATRX loss is an independent risk factor for liver metastases.

Abbreviations. CAPTEM, Capecitabine/Temozolomide treatment; DFS, disease-free survival; DSS, disease-specific survival; FISH, fluorescence in situ hybridization; GEP-NENs, gastroenteropancreatic neuroendocrine neoplasms; IHC, immunohistochemistry; PFS, progression-free survival; PNENs, pancreatic neuroendocrine neoplasms; RFS, relapse-free survival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PRRT
Sunitinib
SSTR1-5
Growth factor
Growth factor tyrosine kinase
incl. VEGFR/PDGFR/EGFR
Wnt
Wnt pathway
APC
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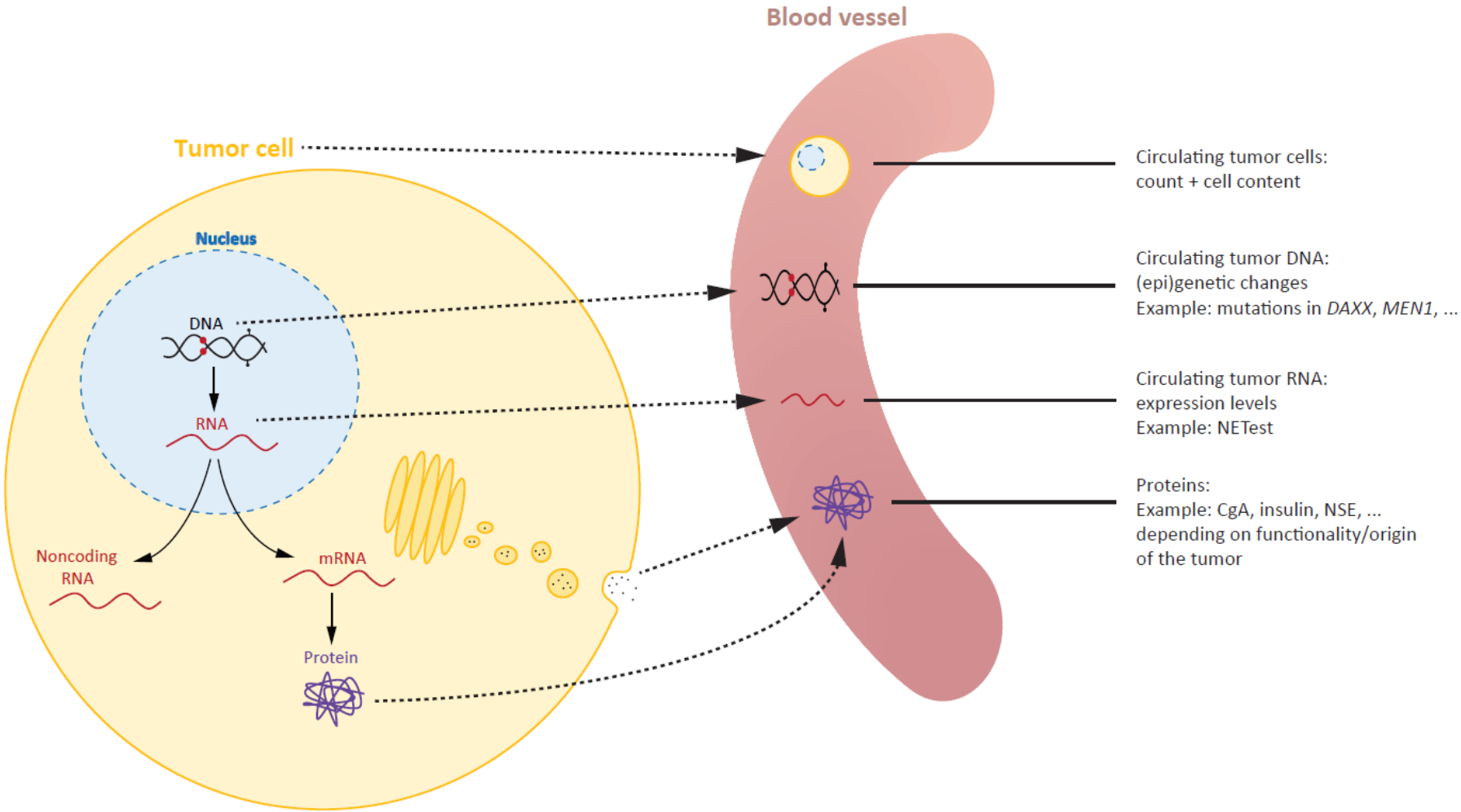
CTNNB1
Wnt downstream genes
PIP2
PTEN
PI3K
PIP3
PI3K/Akt/mTOR pathway
Ras
Raf
MEK 1/2
ERK 1/2
Cell cycle regulation
CDKN2A
CDKN1A
Cell cycle
CDKN1B
TP53
Akt
IMPK
TSC1/2
Everolimus
mTOR complex 1
Proliferation Angiogenesis Survival
RASSF1A
HIFs
VHL
EWSR1 fusion proteins
Temozolomide
Predictive
MEN1
CHEK2
MGMT
BRCA1
BRCA2
OGG1
MUTYH
MLH1
Prognostic
DAXX
ATRX
DNA repair pathway
Nucleus
SETD2
YY1
Telomere maintenance
Chromatine remodeling
DNA repair
Apoptosis

Figure 1.



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1139 Figure 2.