REVIEW

Changing biological behaviour of NETs during the evolution of the disease: progress on progression

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Abstract

Following improvements in the management and outcome of neuroendocrine neoplasms (NENs) in recent years, we see a subset, particularly of pancreatic NENs, which become more aggressive during the course of the disease. This is reflected by an increase in the Ki-67 labelling index, as a marker of proliferation, which may lead to an occasion of increase in grading, but generally does not appear to be correlated with histologically confirmed dedifferentiation. A systematic review of the literature was performed in *PubMed*, *Cochrane Library*, and *Embase* until May 2020 to identify cases that have behaved in such a manner. We screened 244 articles: only seven studies included cases in their cohort, or in a subset of the cohort studied, with a proven increase in the Ki-67 during follow-up through additional biopsy. In addition to these studies, we have also tried to identify possible pathophysiological mechanisms implicated in advanced NENs, although currently no studies appear to have addressed the mechanisms implicated in the switch to a more aggressive biological phenotype over the course of the disease. Such progression of the disease course may demand a change in the management. Summarising the overall evidence, we suggest that future studies should concentrate on changes in the molecular pathways during disease progression with sequential biopsies in order to shed light on the mechanisms that render a neoplasm more aggressive than its initial phenotype or genotype.

Key Words

- ▶ neuroendocrine tumours
- ▶ molecular biology
- ▶ dedifferentiation
- ▶ Ki-67 index

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Introduction

Neuroendocrine neoplasms (NENs) are tumours which are relatively rare but have shown an ongoing increase in their annual age-adjusted incidence, from 1.09/100,000 persons in 1973 to 6.98/100,000 persons in 2012, according to the most recent database analyses from the National Cancer Institute's *Surveillance, Epidemiology, and End Results* (SEER) Program (Dasari *et al.* 2017).

Over the past few years, the terminology used to describe NENs has evolved in an attempt to reflect their biological behaviour, ranging from the well-differentiated neuroendocrine tumours (NETs) to the poorly differentiated neuroendocrine carcinomas (NECs) (Nagtegaal *et al.* 2020). The Ki-67 protein is a cellular proliferation marker, useful for the determination of the growth capacity of an individual cell population. The grade in various tumour entities has been categorised based on their Ki-67 level of expression. Depending on their Ki-67 index, NENs are classified into well-differentiated NETs of grade G1, G2, or G3, or poorly differentiated G3 NECs (Nagtegaal *et al.* 2020), the boundaries of the grades depending on the specific tumour entity. Thus, both the degree of differentiation and the grade have been utilised as parameters to gauge the aggressiveness of NENs.

Heterogeneity of the neoplastic cellular population of NENs is a well-described histological characteristic of these neoplasms, with Ki-67 values varying both within the primary site and when compared to the synchronous metastatic foci (Miller *et al.* 2014, Singh *et al.* 2014, Richards-Taylor *et al.* 2017, Shi *et al.* 2018), or in relation to other pathological features, such as insulin-like growth factor IEC (IGF-IEC), chromogranin A (CgA) and synaptophysin expression (Alexandraki *et al.* 2017c). Such variation is also demonstrable in terms of the temporal course of the disease. It has been shown that, mainly in pancreatic NENs (pan-NENs), a change in their biological behaviour, as reflected by an increase in the Ki-67 proliferation index or even in their grade, may occur over time. This correlates with worse survival, particularly when occurring early (Hentic *et al.* 2017, Botling *et al.* 2020, Alexandraki *et al.* 2020). An additional example is the emergence of multiple or metachronous hormone secretion in functional NENs, this also correlating with worse survival (de Mestier *et al.* 2015, Crona *et al.* 2016, Zandee *et al.* 2017). Similarly, in some tumours a reduction in the expression of somatostatin receptors (SSTR) during sequential imaging with either ¹¹¹Indium-pentetreotide or

⁶⁸Gallium-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid – PET/CT (⁶⁸Ga-DOTA-compounds PET/CT) – has been associated with an increase in tumoural uptake of ¹⁸F-Fluorodeoxyglucose (FDG) PET/CT, implicating a change from a less to more aggressive behaviour (Paul *et al.* 2016, Chan *et al.* 2017, Carideo *et al.* 2019). One initial possible explanation was that NENs may dedifferentiate during the course of the disease in different areas within the tumour at varying times, and that this process might represent different tumoural sub-clones. However, it is difficult to identify such an accumulation of genetic alterations in NEN patients, although these are clearly present in other cancers (Fendrich *et al.* 2012, Cuny *et al.* 2018). Whether NETs and NECs represent two different forms of the same disease continuum, dependent on dedifferentiation, remains unclear; this is also due to the limitations in the number of tumours examined so far and their precise histopathology. Still, the recent WHO 2019 classification clearly separates NETs from NECs, suggesting that the latter may *ab initio* be quite distinct entities (WHO 2019). Accumulating evidence suggests that changes over time within a given NET do not lead to dedifferentiation *per se* but merely represent an increase in Ki-67 with the possible gain of some additional atypical features (i.e. necrosis or nuclear atypia) but with the maintenance of a well-differentiated morphology (Panzuto *et al.* 2017, Botling *et al.* 2020, Alexandraki *et al.* 2020). In most cases, such changes over time have been reported in pan-NENs (Panzuto *et al.* 2017, Botling *et al.* 2020, Alexandraki *et al.* 2020), with only few seen in those arising from the small bowel (SI), suggesting a dichotomy between pan-NENs' and SI-NENs' progression over time (Singh *et al.* 2014, Grillo *et al.* 2016, Panzuto *et al.* 2017, Shi *et al.* 2018). On the other hand, one hypothesis explaining the peculiar progressive biological behaviour of pan-NENs is that neoplasms can have different cellular origins with well-differentiated G1 and G2, and also G3 NETs, originating from hormonally programmed neuroendocrine precursor cells, as opposed to poorly differentiated G3 NECs deriving from primitive neuroendocrine precursor cells (Kloppel 2017).

In this review, we will attempt to shed light on the phenomenon of the changes in tumour biology and behaviour over time, with particular reference to the questionable concept of 'dedifferentiation', by a critical appraisal of the current literature. The possible pathogenetic mechanisms involved in the process are explored and then linked to the diagnostic tools and therapeutic options available for patients with these neoplasms.

Materials and methods

To identify studies and determine eligibility, we conducted a systematic review of the literature using the *PubMed* database. The search terms strategy included 'Cell Dedifferentiation/etiology' (Mesh) OR 'Cell Dedifferentiation/genetics' (Mesh) OR 'Cell Dedifferentiation/immunology' (Mesh) OR 'Cell Dedifferentiation/physiology' (Mesh) AND (Neuroendocrine Tumors/anatomy and histology' (Mesh) OR 'Neuroendocrine Tumors/epidemiology' (Mesh) OR 'Neuroendocrine Tumors/etiology' (Mesh) OR 'Neuroendocrine Tumors/genetics' (Mesh) OR 'Neuroendocrine Tumors/immunology' (Mesh) OR 'Neuroendocrine Tumors/pathology' (Mesh) OR 'Neuroendocrine Tumors/physiology' (Mesh) OR 'Neuroendocrine Tumors/physiopathology' (Mesh) OR 'Gastro-enteropancreatic neuroendocrine tumor' (Supplementary Concept). The databases were searched through until May 2020. We screened 244 articles: all articles were independently evaluated by four authors (K A, S K, A S, K D) for their relevance according to the research strategy. In addition, studies identified through reference lists were included. In spite of this extensive search, only seven studies included cases which described in their

cohort – or in a subset of the cohort – an increase in the Ki-67 index during follow-up or between primary sites and metastases (Fig. 1).

Results

Pathogenesis

In order to investigate the molecular pathogenesis of NENs, especially pan-NENs, in the context of their evolution over time and alterations in biological behaviour during their natural history, three different hypotheses have been put forward: dedifferentiation of mature neuroendocrine cells, successive mutations within progenitors of neuroendocrine cells, and the loss of function of genes in non-neuroendocrine cells (Patel & Galoian 2018), although these hypotheses are not mutually exclusive.

Dedifferentiation is a term used to describe the nuclear reprogramming of cancerous cells following a reverse developmental procedure from a well-differentiated to a less-differentiated status (Yu *et al.* 2014). This process has been well described in several cancers caused by oncogenic

Figure 1

Flowchart for data collection.

alterations in cancer cells but with increasing recognition of the significance of the tumour microenvironment (Friedmann-Morvinski & Verma 2014). Thus, according to this view, the neuroendocrine tumour develops from mature neuroendocrine cells in a progressive ongoing process, with the accumulation of mutations (of oncogenes, tumour suppressor genes and others) that lead to a loss of various tissue-specific functions and hence, dedifferentiation to a less mature phenotype. In parallel, these accumulated mutations result in a more aggressive phenotype (Waldum *et al.* 2018). While the Ki-67 index provides information regarding cell *proliferation*, this is a separate and distinct phenomenon; it does seem to correlate well with the degree of differentiation of various tumours, so this cannot be considered synonymous with dedifferentiation.

Molecular pathogenesis

The molecular pathways leading to dedifferentiation may be responsible for the increase in clinical aggressiveness. During tumour progression, differentiation markers decrease, and stem/progenitor markers become more prominently expressed. Such dedifferentiation will require changes in the panoply of active transcription factors, for example, an increase in activity of the Wnt signalling pathway (Friedmann-Morvinski & Verma 2014). Cadherins are a family of functionally related transmembrane glycoproteins responsible for the calcium-dependent, cell–cell adhesion mechanism of epithelial cells. A lack of E-cadherin (CDH1) expression is related to cellular dedifferentiation which also enhances the metastatic potential of tumours, also accounting for their poor prognosis (Li *et al.* 2002, Fougner *et al.* 2010). Down-regulation of E-cadherin expression is believed to be mostly induced by biochemical modification of the E-cadherin/catenin complex rather than by gene mutations, with the EGF receptor (EGFR) playing a central role. A proposed mechanism is activation of a cascade of tyrosine phosphorylation, caused by the interaction between transforming growth factor- α (TGFA) and EGFR, resulting in down-regulation of the E-cadherin/catenin complex and then nuclear β -catenin accumulation. These effects seem to play a direct role in tumour growth and progression in both well-differentiated NETs as well as poorly differentiated NECs. However, other mechanisms may also trigger dedifferentiation (Barth *et al.* 1997, Semba *et al.* 2000, Clavel *et al.* 2001, Li *et al.* 2002). Decreased or aberrant membranous and nuclear expression of E-cadherin, α -catenin (CTNNA1), and β -catenin (CTNNB1) has been described in NENs of the gastrointestinal tract

(for example, in liver metastases), in this case correlating with the process of dedifferentiation (Rosenau *et al.* 2002). While the presence of E-cadherin characterises most well-differentiated NENs, Yonemori *et al.* have shown that the loss of E-cadherin promotes epithelial–mesenchymal transition (EMT) and correlates with a higher risk for vascular and lymphatic invasion, lymph node and liver metastasis (Yonemori *et al.* 2017). Similarly, loss of the E-cadherin/ β -catenin complex integrity and the increased expression of transcription repressors such as Snail-1 (SNAIL) correlate with the higher malignancy seen in both pulmonary NENs as well as pan-NENs (Fendrich *et al.* 2012, Galvan *et al.* 2013, 2014). In addition, overactivation of the Wnt/ β -catenin signalling pathway represents another mechanism leading to further progression of NETs (Wong *et al.* 2018); such overactivation may relate to mutations or epigenetic silencing of Wnt antagonists (Wnt inhibitors like Axin 2 (AXIN2) and secreted Frizzled-related proteins (SFRPs), Wnt inhibitory factor 1 (*WIF 1*) and DICKKOPFs (*DKKs*)) (Kim *et al.* 2013). While β -catenin shows membranous expression in pan-NENs unrelated to the histological grade of the tumour, some cases of advanced stage disease also showed nuclear β -catenin expression (Weiss *et al.* 2016). Loss of E-cadherin may cause such changes in the localisation of β -catenin, as it normally acts to sequester β -catenin in the membrane and, thus, to inhibit the proliferative action of the WNT pathway.

As noted previously for the EGFR, cell stimulation by various growth factors (GFs) and their concomitant signalling pathways contribute to EMT and facilitate invasion and dissemination. This may also relate to the tumour microenvironment, which can positively feedback onto the tumour cells, so enhancing their invasive and metastatic potential. In a further study, no clear correlation between loss of E-cadherin or Snail and Twist expression (as markers of EMT) could be shown with the occurrence of metastases in pan-NETs (Fendrich *et al.* 2012). While research in pancreatic and pulmonary cancers shows that even though EMT might not be essential for metastasis, it does seem to contribute to chemoresistance. The plasticity of the cancer cell can facilitate the development of therapy resistance and can also be the target of agents which reverse the process and inhibit metastasis (Pastushenko *et al.* 2018).

In gastroenteropancreatic NENs, various changes (genetic and epigenetic) affecting *MEN1* and other complexes (e.g. SWI/SNF and the histone methylase complex) negatively influence chromatin remodelling. Specifically, alternative lengthening of telomeres (ALT) is not only associated with inactivation of the *MEN1* gene

but also with functional loss of *death domain-associated protein (DAXX)/ α -thalassemia/mental retardation X-linked (ATR)*. ALT positivity, detected with large ultrabright nuclear telomere FISH signals, indicative of telomere length heterogeneity, correlates well with mutations of *DAXX/ATR* or loss of nuclear expression of either *DAXX* or *ATR* (Heaphy *et al.* 2011). Tumours with functional loss of *DAXX/ATR* are more aggressive, metastasise more frequently, and have a worse clinical outcome (Marinoni *et al.* 2014, Park *et al.* 2017, Singhi *et al.* 2017, Kyriakopoulos *et al.* 2018). However, in contrast, Jiao and colleagues observed *prolonged* survival in patients with the presence of *DAXX/ATR* mutations (Jiao *et al.* 2011). This contradictory observation was further analysed in more detail: it seems that the impact of these two mutations on survival depends on the stage of the disease, with such mutations associated with worse prognosis in early-stage patients but longer survival when seen in metastatic cases (Boons *et al.* 2019). ALT positivity also demonstrates an association with an increased risk of metastasis in small pan-NENs (Pea *et al.* 2020). Furthermore, *DAXX/ATR* has a feedback interaction with phosphatase and tensin homolog (PTEN), while its inactivation directly influences the PI3K/mTOR pathway (Yao *et al.* 2008). The *PTEN* gene negatively regulates the PI3K-AKT-mTOR pathway, inhibiting neoplastic cell survival and proliferation. It has been shown that the loss of PTEN increases pAKT expression which is associated with more aggressive behaviour in low-grade NETs and a shorter disease-free and overall survival of the patient (Missiaglia *et al.* 2010, Scarpa *et al.* 2017, Jayakumar *et al.* 2018, Uemura *et al.* 2019). The landscape of PI3K/mTOR pathway alterations has recently been enriched with new potential players, including *DEPDC5* and *EWSR1* fusions, and correlated with both the presence of *DAXX/ATR* mutations and poor prognosis (Mafficini & Scarpa 2018).

Mutations to DNA repair mechanisms appear to be involved not only in carcinogenesis but also in tumour aggressiveness as well. Thus, the mutY DNA glycosylase gene (*MUTYH*) plays a key role in the base excision repair mechanisms by protecting DNA exposed to oxidative stress, and it has been suggested that it is responsible for driving tumorigenesis of both familial and sporadic small intestinal as well as pan-NETs (Dumanski *et al.* 2017, Scarpa *et al.* 2017). Such a mutation would in turn increase the frequency of further disease-enhancing mutations.

In summary, down-regulation of E-cadherin expression, aberrant nuclear E-cadherin and α - and β -catenin expression, increased expression of transcription repressors such as Snail-1, mutations or epigenetic

silencing of Wnt antagonists, functional loss of *DAXX/ATR*, PTEN inactivation and increased pAKT expression can all lead to increased aggressiveness of NENs.

In *pan-NETs*, there is a well-described combination of pathways with *DAXX/ATR* and *MEN1* gene mutations predominantly present in well-differentiated NETs, while *pan-NECs* more frequently demonstrate mutations in tumour protein 53 (*TP53*), *retinoblastoma (RB)1*, cyclin-dependent kinase inhibitor 2A (*CDKN2A*), PTEN, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) catalytic subunit alpha (*PI3KCA*), *KRAS*, and *SMAD4* (Hu *et al.* 2010, Oberg 2013, Singhi & Klimstra 2018, Fang & Shi 2019, Clift *et al.* 2020). Taken together, these pathways involve DNA damage repair, chromatin remodelling, ALT and PI3K/mTOR signalling (International Cancer Genome *et al.* 2010, Di Domenico *et al.* 2017, Scarpa *et al.* 2017). However, to date these two patterns of mutations appear quite distinct, and there is a lack of evidence that tumour progression in pan-NETs involves a shift from one pattern of mutations to another.

The pathological and molecular features that differentiate NETs and NECs, particularly in pancreatic neoplasms, have already been summarised (Singhi & Klimstra 2018, Fang & Shi 2019, Mafficini & Scarpa 2019, Scarpa 2019). The prevalence of alterations in p53 is significantly higher in hepatic metastases than in primary pan-NENs (Gleeson *et al.* 2017). Additionally, several studies have shown that the oncoprotein p53 is almost always normally expressed in well-differentiated NETs but exhibits an abnormal pattern of either overexpression or a complete loss of expression in poorly differentiated NECs (Konukiewicz *et al.* 2017). Physiologically, p53 plays a crucial role in inducing differentiation and preventing dedifferentiation during tumorigenesis. Furthermore, it helps maintain the genomic stability of cells during the generation of induced-pluripotent stem (iPS) cells. Reprogramming factors such as *MYC Proto-Oncogene*, *BHLH Transcription Factor (c-Myc)*, *Kruppel-like factor 4 (Klf4)*, *POU class 5 Homeobox 1 (Oct4)* and *SRY-box transcription factor 2 (Sox2)* may cause DNA damage, and thus, suppression of p53 favours the generation of iPS cells with persistent DNA damage and chromosomal aberrations. Inactivation of the p53 interaction factor *CDKN2A* accelerates the whole process (Yu *et al.* 2014) (Table 1). Thus, while one can pragmatically separate out the various pathways which are altered in pan-NENs, in practice they interweave and cross-regulate such that there is a complex interplay of factors, any one of which may contribute to tumorigenesis alone or in combination. What does appear to be reasonably clear is that, for the

Table 1 Main genetic alterations in pancreatic, small intestine and lung NETs and NECs.

Pathway	Pan-NETs	Pan-NECs	SI-NETs	SI-NECs	Lung-NETs	Lung-NECs
Wnt/ β -catenin	RASSF1A	APC SMAD4	APC EGFR PDGFR RASSF1A	APC CTNNB1 RASSF1A SMAD4		CTNNB1 SMAD4
PI3K/mTOR	PTEN TSC2 VHL	BRAF KRAS PI3KCA		CXCL14 BRAF KRAS SRC	PIK3CA	KRAS PTEN
Chromatin remodelling	ATM MEN1		LOH18	CNV 17q	ARID1A MEN1 SMARCA2 SMARCA4	ATM CREBBP KMT2D SMARCA2 SMARCA4
NOTCH signaling		HES1 NOTCH1	NOTCH1			ASCL1 DLL3 NOTCH1
DNA repair	MUTYH	TP53	MUTYH	TP53		TP53
Cell cycle regulation	CDKN2A	RB1	CDKN1B	RB1		CDKN2A RB1
Altered telomeres	ATRX DAXX					
Miscellaneous				APLP1 RUNX1	EIF1AX	

Pan- and SI-NETs include well-differentiated G1 and G2 tumours and pan- and SI-NECs include poorly differentiated G3 tumours. Lung NETs include typical and atypical carcinoids whereas Lung-NECs include large and small cell neuroendocrine carcinomas.

Information retrieved from: [Fernandez-Cuesta *et al.* \(2014\)](#), [Stalberg *et al.* \(2016\)](#), [Di Domenico *et al.* \(2017\)](#), [Pelosi *et al.* \(2017\)](#), [Scarpa *et al.* \(2017\)](#), [Simbolo *et al.* \(2017, 2018, 2019\)](#), [Derks *et al.* \(2018\)](#), [Fang & Shi \(2019\)](#), [Mafficini & Scarpa \(2019\)](#), [Samsom *et al.* \(2019\)](#), [Scarpa \(2019\)](#), [von Arx *et al.* \(2019\)](#), [Clift *et al.* \(2020\)](#), [Cros *et al.* \(2020\)](#), [Starzynska *et al.* \(2020\)](#).

APC, APC regulator of WNT-signalling pathway; APLP1, amyloid beta precursor-like protein 1; ARID1A, AT-rich interaction domain 1A; ASCL1, achaete-scute family BHLH transcription factor 1; ATM, ataxia-telangiectasia mutated (ATM serine/threonine kinase); ATRX, ATRX chromatin remodeler; ATRX, ATRX chromatin remodeler; BRAF, B-Raf proto-oncogene, serine/threonine kinase; CDKN1B, cyclin-dependent kinase inhibitor 1B; CDKN2A, cyclin-dependent kinase inhibitor 2A; CNV 17q, copy number variation chromosome 17q; CREBBP, CREB binding protein; CTNNB1, catenin beta 1; CXCL14, C-X-C motif chemokine ligand 14; DAXX, death domain-associated protein; DLL3, delta-like canonical Notch receptor; EGFR, epidermal growth factor receptor; EIF1AX, eukaryotic translation initiation factor 1A X-linked; HES1, Hes family BHLH transcription factor 1; KMT2D, lysine methyltransferase 2D; KRAS, KRAS proto-oncogene, GTPase; LOH18, loss of heterozygosity chromosome 18; MEN1, Menin 1; MUTYH, MutY DNA glycosylase; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumour; NOTCH1, Notch receptor 1; PDGFR, platelet-derived growth factor receptor beta; PI3KCA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTEN, phosphatase and tensin homologue; RASSF1A, Ras association domain family member 1; RB1, RB transcriptional corepressor 1; RUNX1, RUNX family transcription factor 1; SI, small intestine; SMAD4, SMAD family member 4; SMARCA2, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 2; SMARCA4, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; TP53, tumour protein P53; TSC2, TSC complex subunit 2; VHL, Von Hippel-Lindau tumour suppressor.

most part, there is an obvious separation of pan-NETs, even when progressive, from pan-NECs.

In *small intestine NENs* (SI-NENs), the mutational landscape remains unclear, with tumours which harbour a loss of heterozygosity at chromosome 18 (LOH18), and *CDKN1B* and adenomatous polyposis coli protein (*APC*) mutations, correlating with a poor prognosis ([Stalberg *et al.* 2016](#), [Di Domenico *et al.* 2017](#), [Simbolo *et al.* 2018](#), [Mafficini & Scarpa 2019](#), [Samsom *et al.* 2019](#), [Scarpa 2019](#), [Starzynska *et al.* 2020](#)). A role for *TP53* in the prognosis of these patients has not been clearly demonstrated to date ([Ali *et al.* 2017](#)). Liver metastases of SI-NENs more frequently display copy number variations (CNVs) and amplification of chromosome 17q, harbouring the

Her2/neu gene, correlating with a more aggressive phenotype ([Karpathakis *et al.* 2017](#)) (Table 1).

On the other hand, well-differentiated *lung-NENs*, such as typical and atypical carcinoids, carry alterations in *MEN1*, *ARID1A*, *SMARCA1*, *SMARCA2*, whereas large and small cell lung carcinomas (NECs) show changes in *RB1*, *TP53*, *PTEN*, *CDKN2A* and *NOTCH1* ([Fernandez-Cuesta *et al.* 2014](#), [Di Domenico *et al.* 2017](#), [Pelosi *et al.* 2017](#), [Simbolo *et al.* 2017, 2019](#), [Derks *et al.* 2018](#), [von Arx *et al.* 2019](#)). Interestingly, in high-grade lung NENs comparative spatial/temporal analyses have confirmed that these tumours emerge from less aggressive clones which, though genetically-heterogeneous, then accumulate 'neuroendocrine carcinoma-like' genetic alterations and

progress along with changes in *TP53* and *RB1* (Cros *et al.* 2020). Whether well-differentiated lung NETs might progress to more aggressive neoplasms, representing a paradigm shift from accepted pathogenesis schemes, has to be clarified (Ie & Boyd 2015, Rekhtman *et al.* 2016, Pelosi *et al.* 2017, Quinn *et al.* 2017). The main genetic alterations observed in pan-NETs compared to pan-NECs, small intestine NETs compared to NECs and lung NETs and NECs are shown in Table 1.

Epigenetic alterations

Epigenetic changes, including DNA methylation and histone modification, also play a crucial role in NET progression (Friedmann-Morvinski & Verma 2014). Hypermethylation in the *RASSF1A* gene may be observed in well-differentiated pancreatic, pulmonary and gastrointestinal NETs but is more pronounced in metastatic tumours and clearly correlates with a worse prognosis (Rahman *et al.* 2010, How-Kit *et al.* 2015). Methylation of two or more tumour suppressor genes correlated with liver metastases in a cohort of pancreatic and small intestine NENs (Liu *et al.* 2005). Further sites of promoter hypermethylation in tumours with a poor prognosis are found in various genes including *DAPK1*, *TIMP3*, *PAX5*, *HIC1* and *CADM1* (Stefanoli *et al.* 2014). Nevertheless, whether these alterations appear *only* with progression so far remains unclear. All these markers of aggressive disease may be putative markers of a neuroendocrine shift during the evolution of the disease.

MicroRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression. More specifically, they interfere with processes such as cell growth, apoptosis, differentiation, and tumour development. They also play a pivotal role in cell–cell communication in tumour biology. Some miRNAs (e.g. miRNA-21 and -155) have been shown to be up-regulated in high-grade tumours but not in low-grade ones, while others can be detected in metastatic low-grade tumours but not in normal tissues (Roldo *et al.* 2006). For our review, most relevant is the demonstration of the differential gene expression of miRNAs during different stages of progression of NENs of intestinal origin (Li *et al.* 2013). Furthermore, some miRNAs (miR-96, -182, -183) have been reported as overexpressed, while others are down-regulated (miR-129-5p and miR-133a), in small bowel NEN metastases compared to their primary lesion. For example, miRNA-133a was found to be down-regulated during progression from primary to metastatic

small intestine tumour, suggesting its potentially important role in the development and progression of NENs (Li *et al.* 2013). Similarly, miR-21 expression, which represses PTEN, is reported to be associated with a high proliferation index in insulinomas and their hepatic metastases (Roldo *et al.* 2006). It is also of interest that the blood levels of some miRNAs may be altered by treatment with somatostatin analogues (SSA) but whether this is a specific treatment effect remains unclear (Malczewska *et al.* 2018, 2019).

Tumour microenvironment

Interleukin-6 (IL-6) induces cell proliferation, down-regulates the expression of neuron-specific enolase (NSE), and it may stimulate tyrosine kinase phosphorylation of STAT3 signalling in non-small cell lung carcinomas that exhibit neuroendocrine properties (Poiana *et al.* 2013). In gastrointestinal NETs, higher IL-6 expression has been associated with disease progression, suggesting a potential modulation of their behaviour under inflammatory conditions (local and systemic) (Pereira *et al.* 2019). Thus, as previously noted, the tumour microenvironment plays a crucial role in determining NET aggressiveness.

One further pathogenetic mechanism is that associated with tumour hypoxia. The role of hypoxia in the genesis of NETs is unclear, but there is limited evidence that it may activate a dedifferentiated phenotype in other malignancies, such as ductal breast carcinoma or neuroblastoma (Axelson *et al.* 2005). Hypoxia induces up-regulation of proangiogenic factors, including the accumulation of hypoxia-inducible factor-1 α (HIF-1 α), leading to a hypoxia-stress expression programme that includes many additional proangiogenic factors (Carmeliet 2005). Of the genes directly regulated by HIF-1, c-Met is involved in the invasive and metastatic behaviour of tumour cells on exposure to hypoxia. Although mTOR down-regulation during hypoxia leads to an increase of proangiogenic HIF-1 levels, an anti-angiogenic effect is observed during malignant transformation using mTOR inhibitors (Hudson *et al.* 2002, Bernardi *et al.* 2006, Thomas *et al.* 2006).

Intratumoral heterogeneity and cell clones

There are various molecular pathways by which sub-clones, of a relatively quiescent NET, may acquire some of the changes noted above, and thus, gain an ‘evolutionary advantage’ and become the dominant cancer in a heterogeneous tumour. Such heterogeneity can be demonstrated in many NETs by the demonstration

of non-homogeneous radionuclide tracer uptake (^{68}Ga -DOTATATE or ^{18}F -FDG), due to variable cellular differentiation within the same tumour mass (Kayani *et al.* 2008). One example supporting this theory is given by pan-NET (insulinoma) cells lacking insulin-like growth factor II (IGF-II), which grow slowly *in vitro* but display a proliferation advantage in mixed cultures where they can access the IGF-II provided by other subclones, that is, IGF-II-producing cells (Archetti *et al.* 2015). Thus, intratumoural cell heterogeneity is advantageous to the tumour and such heterogeneity may persist. On the other hand, if a therapy targets a GF receptor, then subsequent loss of that GF may render the tumour refractory to further similar therapy (Archetti *et al.* 2015). Changes in the expression of IGF-I, IGF-II, IGF-IR, or their combinations, have been documented in various malignancies, and the role of the IGF system in metastasis, disease progression and poor prognosis has already been demonstrated in several human cancers (Samani *et al.* 2007). Accordingly, IGF-1Ec expression is increased in secondary compared to primary foci in NENs, predominantly in pan-NENs and small bowel NENs, when compared to less aggressive NENs originating from the appendix (Alexandraki *et al.* 2017c).

Circulating tumour cells (CTC) originate from a primary tumour and can become seeds for distant metastases. The expression of epithelial cell adhesion molecule (EpCAM) on their surface allows for their detection within the circulation. Strong homogeneous, membranous EpCAM expression was observed in ileal and pan-NETs, and CTC detection was associated with progressive NENs and could be used as prognostic marker. However, the differential expression of synaptophysin and CD-56 in CTCs from NENs suggest that CTCs are heterogeneous. This heterogeneity may have diagnostic implications as mutations may arise from the primary tumour *per se* or could occur *de novo* with the circulation, the latter possibly as an escape mechanism to a specific therapy (Khan *et al.* 2011). The CTC count does not correlate with either tumour Ki-67 or CgA expression, and thus, its role in NET progression remains unclear.

As an alternative to the gradual appearance of cancer cell clones with differing mutational profiles, with the less differentiated showing more aggressive characteristics and, hence, a selective advantage, the cancer stem cell (CSC) hypothesis has been proposed to explain tumour heterogeneity. It is noteworthy that mutations in the stem cells may generate the so-called CSCs which are pluripotent and able to self-renew. This subpopulation of stem-like cells plays a role in tumour growth,

giving rise to highly heterogeneous tumour lesions (Visvader & Lindeman 2008). Furthermore, the possibility of “tumour cell plasticity”, where non-CSC can dedifferentiate and acquire CSC-like properties under certain conditions, has also been reported. Unfortunately, there are currently no data supporting this hypothesis with reference to NETs. It is unclear whether some CTCs are indeed CSCs. In animal models, ‘aggressive’ CTCs have been shown to colonise their tumour of origin with self-seeding. This may explain relationships between tumour size, vascularity, prognosis and local recurrence seeded by disseminated cells following complete excision (Kim *et al.* 2009). CSCs have also been identified in several NETs, including those arising in the pancreas, midgut, and bronchial NETs, the latter having the highest levels, and representing a potential therapeutic target for this entity (Gaur *et al.* 2011, Oberg *et al.* 2013).

NET-specific therapeutic agents

There is limited evidence that tumour progression may also be associated with NET-specific therapeutic agents. Although SSAs are associated with high symptomatic response rates in NETs, patients may develop resistance to treatment over time (De Martino *et al.* 2010). It is now suspected that truncated sst5 receptor variants with a distinct tissue distribution and cellular localisation display selective responses to somatostatin and its analogues (Duran-Prado *et al.* 2009). More specifically, two different sst5 receptor isoforms were recently identified, one with four (sstTMD4) and one with five transmembrane domains (sstTMD5). These isoforms are not expressed in the normal pituitary gland but are expressed in various other normal tissues and are also present in both functioning and non-functioning pituitary adenomas. They differentially respond to stimuli such as somatostatin and cortistatin, have distinct subcellular localisations, while the presence of the sst5TMD4 isoform correlates with increased aggressive features and a worse prognosis in somatotrophinomas. This provides a potentially useful tool to predict the outcome of clinical responses to SSA-therapy and the development of new therapeutic targets (Luque *et al.* 2015).

Similar considerations may apply to NENs. Basu *et al.* reported two cases of grade 2 NETs with a relatively high Ki-67 that showed a significant increase in SSTR expression following treatment with everolimus. The authors suggested that this agent might be associated with tumour redifferentiation (Basu & Ostwal 2016). Furthermore, it may be noted that the improved progression-free survival (PFS) seen under combined long-acting octreotide and everolimus

treatment of intermediate grade NETs may relate to a change in tumour genotype. However, the RADIANT trials showed that the combination of everolimus with octreotide LAR improved PFS but not overall survival in patients with advanced NENs, suggesting that redifferentiation as such effect may not actually occur (Pavel *et al.* 2011, 2017, Yao *et al.* 2011). Moreover, resistance to rapalogs/everolimus treatment has alternatively been attributed to various mechanisms such as PI3K/Akt/mTOR signalling mutations, activation of the PI3K/Akt/mTOR feedback loop, growth signalling mutations (*FGFR4*), activation of the Raf/MEK/ERK pathway, dysregulation of the RB pathway, activation of PIM kinases, increased oxidative stress, stimulation of anti-apoptotic signals and/or up-regulation of proangiogenic factors. On the other hand, sunitinib resistance seems to rely on alternative activation of proangiogenic signalling, with recruitment of bone-marrow-derived cells, an increase of pericyte coverage and lysosomal sequestration of the sunitinib (Beyens *et al.* 2019). Thus, such evidence implies that the development of tumour resistance to therapeutic agents may involve either a substitution of intracellular signalling pathways within a treated cell or the clonal escape of cells activating such alternative pathways rather than specific dedifferentiation. This can be shown to occur in the pan-NEN BON1 cell line in culture with everolimus (Aristizabal Prada *et al.* 2018).

In summary, there is strong evidence that the genetic interface of tumours is dynamic and may alter over time due to the accumulation of mutations (Burrell *et al.* 2013). This condition may lead to tumour heterogeneity due to the growth of several clonal patterns within the same tumour. Recently, Vandamme *et al.* reported on several genetic alterations being identifiable only within a small fraction of the respective tumour sample. The presence of such so-called 'low-abundance' mutations is in line with pan-NET heterogeneity and might explain tumour progression, and possibly dedifferentiation, during the course of the disease as these clones obtain a survival advantage (Vandamme *et al.* 2019). However, clear evidence for such dedifferentiation at a molecular or pathological level is lacking.

Diagnostic tools for progression

Circulating markers

NENs can secrete a wide range of amines and polypeptide hormones into the circulation. Classic biomarkers include CgA, NSE and pancreatic polypeptide as well as hormones

that elicit clinical syndromes, such as serotonin, insulin, glucagon and gastrin. Given the complexity and heterogeneity of NENs as well as the interactions within the tumour microenvironment, no such biomarker has proven to be uniformly effective to estimate proliferation, metabolic activity or metastatic potential (Modlin *et al.* 2016). Of the common biomarkers investigated to date, CgA correlates better with the tumour burden and the biological activity of the NEN, especially in small bowel and pan-NENs, and can serve as an independent prognostic factor (Oberge 2011, Fuksiewicz *et al.* 2018). Nevertheless, there is no correlation between CgA levels and tumour mass in patients treated with SSAs, and CgA is not always sufficient for the detection of early recurrence with treatment (Oberge 2011). Although CgA does not have a very good sensitivity and specificity, it is more frequently elevated in G1 and G2 tumours compared to G3 NETs and can better predict prognosis in the subgroup of low-grade NETs (Modlin *et al.* 2010, Rossi *et al.* 2015). Patients with poorly differentiated NECs may present with neoplastic syndromes secondary to ectopic hormone production (e.g. ACTH) but rarely with the carcinoid syndrome or with functional hormone hypersecretion (Tang *et al.* 2016). Interestingly, as previously noted, the secondary appearance of hormonal secretion in an initially non-functional pan-NET (metachronous hormonal syndrome) correlates with the presence of metastases, radiological progression and worse survival, signs suggestive of tumour progression (de Mestier *et al.* 2015, Crona *et al.* 2016). Unlike pan-NETs, small bowel NETs are only rarely present with metachronous hormone secretion (Crona *et al.* 2016).

As previously described, methods for detecting circulating transcripts and tumour cells have been developed to improve diagnostic efficacy (Hofland *et al.* 2018). The detection of CTCs correlates to some degree with the tumour burden but only weakly or not at all with CgA or the tumour Ki-67 labelling index. The low number of NEN patients with detectable CTCs, together with the heterogeneity of these tumours, suggests that this biomarker currently requires further investigation. Specific miRNA overexpression or down-regulation has been reported with tumour progression in various NENs, as noted above, but a lack of standardisation of this method renders it unavailable for current clinical use. The presence of tumour-specific genetic alterations in cell-free (cf) DNA has recently been demonstrated in cases of metastatic pan-NETs, together with a clear correlation of the cf-DNA concentration with disease progression, suggestive of additional genetic alterations as drivers

for progressive disease (Boons *et al.* 2018). Ultrahigh-throughput sequencing technology – whole-genome sequencing of germ-line DNA – might be used in the future to identify rare, highly penetrant, high-risk alleles that can be used to screen individuals at high risk. Evaluation of early- and late-stage samples could be used to identify biomarkers associated with progressive disease (Modlin *et al.* 2014). Furthermore, a NET liquid biopsy strategy with blood mRNA measurement (NETest) has been shown to have clinical utility in the accurate identification of NETs and their post-operative monitoring for the early identification of residual or metastatic disease (Malczewska *et al.* 2019, Laskaratos *et al.* 2020, Oberg *et al.* 2020). Thus, future and developing techniques will hopefully provide a more personalised molecular disease signature indicative of the disease status, progress and prognosis, and indicate appropriate tumour-based therapeutic options.

Pathology

According to the most recent 2019 WHO classification, the classification of a gastroenteropancreatic (GI)-NEN is based on both its differentiation and proliferative activity (Nagtegaal *et al.* 2020). Thus, well-differentiated G1 NETs present a mitotic count $<2/2 \text{ mm}^2$ and/or a Ki-67 index <3 and G2 NETs have a mitotic count $2\text{--}20/2 \text{ mm}^2$ and/or a Ki-67 index between 3–20%. The latest group of NENs includes very heterogeneous neoplasms. Within the same histological grade, morphological features characterising these tumours are not uniform in all sites (Zatelli *et al.* 2018). Thus, the WHO classification of 2019 distinguishes between well-differentiated G3 NETs with a mitotic count $>20/2 \text{ mm}^2$ and/or a Ki-67 $>20\%$ and poorly differentiated NECs also with a mitotic count $>20/2 \text{ mm}^2$ and/or a Ki-67 $>20\%$ (Nagtegaal *et al.* 2020). On the other hand, the classification of lung NENs is based on mitoses and necrosis according to 2015 WHO classification. Typical carcinoid is defined by a mitotic count <2 mitoses/ 2 mm^2 and lacking necrosis; atypical carcinoid is defined by a mitotic count $2\text{--}10$ mitoses/ 2 mm^2 , and/or foci of necrosis. Large and small cell NECs are both characterised by extensive necrosis and a high mitotic count (>10 mitoses/ 2 mm^2) (Travis *et al.* 2015).

For GI-NENs, new ‘sub-categories’ suggested – but not yet adopted by the WHO – include: G3 NETs, with well-differentiated morphology and a Ki-67 of 21–55%, G3 NECs with poorly differentiated morphology and a Ki-67 of 21–55%, and finally NECs (tentatively referred to as G4) that are poorly differentiated and show a Ki-67 $>55\%$ (Fazio & Milione 2016, Pellat & Coriat 2020).

However, even apparently well-differentiated NETs may include high-grade components in both primary and metastatic sites, occupying more than 20% of the tumour, with large heterogeneity in mitotic rate and Ki-67 index. While the presence of a high-grade component in well-differentiated pan-NETs was associated with an unfavourable clinical outcome, its prognosis is not as dismal as that of a truly poorly differentiated NEC (Tang *et al.* 2016). It is conceivable that NEN subclones of NETs might acquire further genetic changes and phenotypic features of NECs but overall retaining the characteristics of well-differentiated NENs. Even when the high-grade regions may resemble large-cell NECs, the association with a low-grade component may maintain these neoplasms in the well-differentiated NET group. Similarly, tumours at the site of metastasis have both the low/intermediate grade and the high-grade components. Morphological characteristics distinguishing G3 NETs from NECs are organoid growth pattern, capillary network in direct contact to tumour cells, and an absence of desmoplastic stroma; these characteristics also correlate with improved overall survival, suggestive of a less aggressive tumour behaviour (Elvebakken *et al.* 2020). Support for the dichotomy of the pan-NENs also comes from the observation that some G3 NENs with a Ki-67 index above 20% show the typical DAXX/ATRX or *MEN1* mutations but do not harbour *TP53* or *RB1* mutations and respond much better to therapies appropriate for well-differentiated NETs than to systemic platinum-based chemotherapy (Kloppel 2017). The large heterogeneity of pan-NENs can additionally be seen on investigation of the subcategories of small cell vs large-cell G3 NECs, where one study demonstrated that small cell NECs display significantly lower SSTR-1 and SSTR-2A expression when compared to large-cell NECs (Mizutani *et al.* 2012). However, other studies could neither identify differential expression of immunohistochemical markers such as SSTR-2A, CgA and p53 nor differential genetic profiles between these two groups (Yachida *et al.* 2012, Nielsen *et al.* 2020). Histological examples of the large heterogeneity in these tumours can be found in Figs 2, 3 and 4.

An increase in the Ki-67 index in metachronous metastases during the evolution of the disease has been assessed in terms of possible dedifferentiation. In several studies, particularly on pan-NETs, follow-up biopsy of the metastatic lesions along with clinical evidence of disease progression demonstrated an increase in the Ki-67 index, sometimes leading to progression to a higher-grade NET (Singh *et al.* 2014, Grillo *et al.* 2016, Panzuto *et al.* 2017, Botling *et al.* 2020, Alexandraki *et al.* 2020).

Figure 2

Haematoxylin and Eosin staining of a pancreatic neuroendocrine neoplasm: before (left) and after (right) the increment in Ki-67 index.

More recently, morphological features usually associated with a NEC such as necrosis or nuclear atypia were observed during progression of a pan-NEN but lacking a clear dedifferentiated pattern (Botling *et al.* 2020) and displaying a change in tumour pathology over the progression of disease (Singh *et al.* 2014). Pan-NENs developed more aggressive growth characteristics compared to non-pan-NENs with not only an increase in Grade from G1 to G2 but also from G1 to G3 (Panzuto *et al.* 2017, Botling *et al.* 2020). High-grade progression with an increase in Ki-67 ranging from 17 to 55% has been reported. Similarly, we have recently presented a series of 15 patients with Pan-NENs with over-expression of p53 documented in three out of seven patients assessed but

without clear evidence of histological dedifferentiation (Alexandraki *et al.* 2020). The main features of these seven studies are summarised in Table 2.

Imaging

Multiphasic contrast-enhanced abdominal and pelvic CT or MRI are the most common imaging modalities recommended for all patients with suspected NENs (Alexandraki *et al.* 2017b). For the evaluation of disease progression, multi-slice contrast-enhanced CT is the established technique for extra-hepatic lesions, while MRI is considered superior in the detection of hepatic lesions (Merino-Casabiel *et al.* 2018). Endoscopy and

Figure 3

A pancreatic neuroendocrine neoplasm. Left panels: p53 immunohistochemical staining of the primary tumour (upper panel) and a metachronous liver metastasis (lower panel) with a markedly increased p53 expression in the metastasis in comparison to the initial pattern, with an increment in Ki-67 index from 5 to 70%. Middle and right panels: the tumour (primary: upper panel – metastasis: lower panel) shows a retained pattern of membranous expression of β -catenin (middle) and E-cadherin (right).

Figure 4

Left panel: A well-differentiated pancreatic neuroendocrine neoplasm with middle-sized uniform cells, round nuclei, salt-and-pepper chromatin, granulated cytoplasm, and a trabecular pattern, without crowding of the cells. Right panel: change to an atypical morphology, with nuclear polymorphism, with both round and spindle-shaped nuclei, dense chromatin and scant cytoplasm, with a solid growth pattern.

endosonography combined with diagnostic biopsy can often be used for diagnosis (Alexandraki *et al.* 2017a, Daskalakis *et al.* 2019).

Until recently, the standard conventional modality for functional NET-imaging was SSTR imaging (SRI) scintigraphy with ^{111}In -labelled pentetreotide (DTPA-octreotide, Octreoscan). ^{18}F -fluorodihydroxyphenylalanine (^{18}F -DOPA)-PET/CT or ^{11}C -5-hydroxytryptophan (^5HTP)-PET/CT have also been used, especially if SRI is negative (Ober *et al.* 2012), whilst ^{111}In -DOTA-exendin-4 (GLP-1R scintigraphy) is most helpful in cases with a high suspicion for the presence of an insulinoma (Antwi *et al.* 2019a), including patients with

MEN1 (Antwi *et al.* 2019b). However, ideally, SRI should nowadays be performed by PET (SRI-PET) when ^{68}Ga -DOTA-TOC/-NOC/-TATE is available. SRI is indicated for the initial diagnosis of a small primary NET, for staging of the disease in a patient with a histologically proven NET, and pre-therapeutically in situations where peptide receptor radionuclide therapy (PRRT) is being considered. SRI-PET shows high sensitivity for most low-grade NETs but not in intermediate or high-grade pulmonary NETs (Tirosh & Kebebew 2018). The usefulness of SRI for the follow-up of NET patients needs to be further validated (Antwi *et al.* 2019a). Interestingly, several studies have documented that a high SUVmax value correlates with

Table 2 Characteristics of studies investigating progression and de-differentiation in NENs.

Study	*No. of patients with ki-67% increase	Origin of NENs	Ki-67% at diagnosis in the whole cohort	Change in ki-67% in the whole cohort	Grade upgrading
Alexandraki <i>et al.</i> 2020	15/264	All pancreatic	5% (range: 1–25%)	+45% (range: +6 to +90%)	2 from G1 to G2; 2 from G1 to G3; 1 from low-to-high G2; 9 from G2 to G3; 1 from low-to-high G3
Botling <i>et al.</i> 2020	34/46	All pancreatic	7% (range 1–38%), G1 $n = 8$, G2 $n = 36$, G3 $n = 2$	+14% (range –11 to +80%)	7 from G1 to G2; 2 from G1 to G3; 25 from G2 to G3
Shi <i>et al.</i> 2018	12/30	GEP	N/A	N/A	**4 from G1 to G2; 1 from G2 to G2; 1 from G2 to G3; 6 from G3 to G3
Panzuto <i>et al.</i> 2017	28/43	24 pancreatic 19 small intestine	3% (range 1–20% in 43)	+5% (range +1 to +70%) in 43	8 from G1 to G2 (6 pan-NEN, 2 SI-NEN); 4 from G2 to G3 (all pan-NEN)
Grillo <i>et al.</i> 2016	10/60 (metachronous metastases)	GEP	N/A	N/A	9 from G1 to G2; 1 from G2 to G3
Shingh <i>et al.</i> 2014	12/43 [†]	GEP, bronchial	N/A	N/A	5 from G1 to G2; 2 from G2 to G3; 5 from G1 to G3
Poiana <i>et al.</i> 2013	1	Ovarian	25–30%	+40%	N/A

**Sixteen cases had change, (12 up-regulation, 4 down-regulation) but without distinction between the 10 cases with simultaneous detection and the 6 cases with the change during the course of the disease change in Ki-67; *patients with an increase in Ki-67/entire cohort; [†]patients with an increase in ki-67 leading to a change in grade.

G, grade; GEP, gastroenteropancreatic; NEN, neuroendocrine neoplasms.

a better clinical outcome, independent of the primary tumour site, suggesting this parameter as a good prognostic factor (Carideo *et al.* 2019).

As most NETs are well-differentiated tumours, they generally do not have a high glucose turnover rate. Thus, the sensitivity of ^{18}F -FDG PET/CT is low, in particular in well-differentiated NETs (G1 and G2). As such, ^{18}F -FDG-PET is not routinely used for diagnostic purposes. However, poorly differentiated NETs (G3) display high glucose metabolism, usually associated with a poor prognosis. Most well-differentiated NETs, given their low proliferative activity, are usually negative on ^{18}F -FDG-PET scans (Merino-Casabiel *et al.* 2018). Thus, SRI-PET is useful for the evaluation or progression of metastases in G1 NET patients, whereas in G2 NETs both ^{18}F -FDG and SRI-PET can be utilised. Additionally, a 'flip/flop' phenomenon with negative ^{18}F -FDG PET but positive ^{68}Ga -DOTA-TOC PET in some lesions and positive ^{18}F -FDG-PET but negative ^{68}Ga -DOTA-TOC PET in other lesions, implying a synchronous heterogeneity in tumour aggressiveness, has also been observed (Nilica *et al.* 2016). Additionally, it has been suggested that ^{18}F -FDG PET should be limited to SRI-negative NET patients, although G1–2 patients may also initially have ^{18}F -FDG-negative tumours and may develop ^{18}F -FDG-positive lesions during follow-up. This latter group of lesions may represent the ones previously considered as 'dedifferentiated' or at least switching to a higher-grade NET. Gradually, NETs may present progression with some elements of dedifferentiation, losing their ability to express SSTR and increasing their metabolism, such that a positive correlation between the Ki-67 labelling index and ^{18}F -FDG-SUV_{max} has been documented in many studies (Carideo *et al.* 2019). As hypoxic cancer-associated fibroblasts that promote tumour progression carry out anaerobic glycolysis, high fibroblast density increases ^{18}F -FDG SUV_{max}, leading to intratumoral heterogeneity on functional imaging (Peppicelli *et al.* 2020). Adopting a dual-tracer approach, assessing SSTR expression and glycolytic metabolism, may, thus, support more individualised therapy selection in NET patients. The combination of ^{18}F -FDG and SRI-PET only slightly improves the accuracy of detection in pan-NETs in comparison to SRI-PET alone but significantly assisted in the characterisation of pulmonary NETs (Kayani *et al.* 2009, Partelli *et al.* 2014, Lococo *et al.* 2015). The combination of both modalities has a significant impact on the individual therapeutic approach, in particular by identifying high-risk lesions, which can be selected for biopsy/re-biopsy to identify changes in

tumour biology (Tirosh & Kebebew 2018). The use of combined ^{18}F -FDG and SRI-PET may also reveal areas of different tumour grading within the same lesion. Thus, dual imaging may provide relevant information which cannot be assessed by a single biopsy of a given metastatic lesion, as these may show heterogeneous metabolic activity and SSTR expression (Carideo *et al.* 2019). Dual imaging should generally be considered in G2 NETs, when there is heterogeneous SSTR expression of different tumour lesions, in patients with disease progression after prolonged stable disease, and in cases of discrepancy between conventional imaging and biochemical assessment.

Metabolic grading according to SRI or ^{18}F -FDG PET of ambiguous lesions allows prognostic stratification in metastatic GI-NENs, and the newly introduced NETPET scoring system combining results of ^{18}F -FDG and SRI-PET can accurately describe the patient's disease burden (Ezziddin *et al.* 2014, Haug *et al.* 2014, Chan *et al.* 2017). Metabolic changes typically precede anatomic changes, and combination of these functional modalities enables early detection of poorly differentiated sites to redirect appropriate management (Hofman & Hicks 2012, Merola *et al.* 2017). Similarly, ^{18}F -FDG PET plays an important role in the assessment of the post-treatment response by identifying a significant metabolic response even in the absence of a corresponding morphological imaging response (Calabro *et al.* 2020).

Management

In the era of personalised treatment, the appropriate management of each NET patient depends on the stage of the disease at diagnosis along with symptoms, the grade of the neoplasm, and other factors such as patient comorbidities. The development of classification systems along with advances in anatomical and functional imaging should define a better risk stratification for each individual patient. In patients with tumour recurrence or metastatic foci recurrence and/or a rapid or unexpected progression in morphological or functional imaging studies, the re-evaluation of Ki-67 can play a role in producing a more accurate stratification. Thus, repeated biopsy in the recurrent mass or in the foci with the more aggressive features, or in new or rapidly growing tumour foci, can in these cases be useful. While clear dedifferentiation from a well-differentiated to a poorly differentiated phenotype is very rarely identified, an increase in the previous value of Ki-67 may dictate a change in treatment strategy (Knigge *et al.* 2017). The development of a new stratification score

such as the NETPET scoring scheme may simplify the indications for a follow-up biopsy (Chan *et al.* 2017).

Surgery remains the mainstay of treatment for cure, but the substantial evolution of medical therapies allowed prolonged survival in cases with locally advanced or metastatic disease not amenable to surgical resection. Long-acting SSAs are usually the first-line treatment for functional and well-differentiated low-grade NENs, resulting in symptomatic control and mainly stabilisation of tumour growth; slow progression may necessitate an increase in dose or frequency. On progression, interferon- α , targeted-therapy, either molecular or radionuclide (PRRT), or chemotherapy may follow and in a higher-grade NEN, immunotherapy may be used depending on the progression of the disease, imaging or histological features. Therapeutic agents are used in sequential order or in combination depending on tumour origin and differentiation, disease burden, clinical symptoms, and the patient's performance status (Tsoli *et al.* 2020). As tumours move along a path of progression, the appropriate therapy may change, and it is, therefore, important that the varying biochemical, pathological, and imaging modalities are considered. We have recently published a case with pan-NEN which progressed from G2 to G3, by increasing Ki-67 from 18 to 60%, and has responded to nivolumab, an anti-programmed cell death 1 agent for more than 34 months (Koumariou *et al.* 2021). This case illustrates the clinical utility of being aware of the biological progression of the tumour in order to optimise appropriate treatment.

Nevertheless, over time, there may be a change in the Ki-67 index (mean time in one study, 123 months or median time in another more recent study 36.8 months) with concomitant rapid disease progression leading to an adverse outcome, independent of treatment, in parallel with the histological evidence of change in grade (Grillo *et al.* 2016, Alexandraki *et al.* 2020). In the largest published study with pan-NENs, a negative outcome in terms of overall survival was documented in patients showing progression, with 83% patients in a cohort of 46 patients rapidly dying from their disease (Botling *et al.* 2020). Patients with progression to a high-grade (grade 3) NET had a shorter overall survival (median 50.2 months) compared to those without progression (median 115.1 months), resulting in a hazard ratio (HR) of 3.89 (95% CI 1.91–7.94, $P < 0.001$) (Botling *et al.* 2020). Progression from a low-grade to a high-grade NET based on the increase in Ki-67 index was selected as a useful prognostic marker. In a multivariate analysis including age, high-grade progression and change of disease

behaviour, all three parameters remained independently correlated with a poor prognosis; HR 3.78 (95% CI 1.72–8.69) for high-grade progression, HR 2.47 (95% CI 1.042–5.87) for change of disease behaviour, and HR 1.057 (95% CI 1.015–1.1) for age (Botling *et al.* 2020). Patients with progression to a high-grade pan-NET had a median survival of 12.2 months, while those without high-grade progression had a median survival of 51.6 months (HR 4.34, 95% CI 2.02–9.35, $P < 0.001$). Survival was also different among patients with any grade increase compared to those without an increase in grade, with a HR 2.95 (95% CI 1.37–6.34, $P = 0.006$). Suspicion of a change in disease behaviour defined by clinical, biochemical, and/or radiological criteria, as stated by the authors, was also associated with a negative prognosis, HR 4.17 (95% CI 1.9–9.13, $P < 0.001$). Moreover, in our recent study with a similar design, a shorter overall survival was seen in patients who had a change in Ki-67 labelling index within the first 36 months of diagnosis, compared to those who had a change of Ki-67 at a later time point (Alexandraki *et al.* 2020). Documentation of such progression provides a strong prognostic factor and plays an important role in determining further therapeutic management (Singh *et al.* 2014, Grillo *et al.* 2016, Panzuto *et al.* 2017, Botling *et al.* 2020, Alexandraki *et al.* 2020) (Table 2).

Conclusions

A number of pathogenetic mechanisms has been identified to be involved in both the genesis and the progression of NENs but prognostically it is difficult to predict such changes. Many NENs, especially pan-NENs, progress over time, probably due to an accumulation of additional mutations leading in selective advantage of specific clones in a heterogeneous tumour but without leading to a true dedifferentiation process. This may be identified by changes in pathological characteristics of the biopsy, by biochemical markers, or by a switch from SSTR radionuclide activity to ^{18}F -FDG PET positivity, an important trait; these alterations will determine a change in therapeutic options. Indeed, there is also evidence that molecular therapy may lead to the development of resistance by the adaptation of signalling pathways (Vandamme *et al.* 2016, Aristizabal Prada *et al.* 2018). However, this progression will rarely lead to a true NEC, whose molecular pathogenesis appears to be determined by quite different factors to classic NETs, at least for gastroenteropancreatic NETs. Nevertheless, awareness of the time-dependent progression and processes of

dedifferentiation are vital in selecting the most appropriate therapy at any particular time.

Declaration of interest

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

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