REVIEW

Extrapulmonary poorly differentiated NECs, including molecular and immune aspects

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Abstract

Patients with extrapulmonary poorly differentiated neuroendocrine carcinomas (EP-PD-NECs) have a poor prognosis. Surgery is offered for those with localised disease, but the majority of patients present with advanced disease. Treatment strategies adopted are analogous to that of high grade NECs of the lung, with platinum/etoposide-based regimens advocated in the first-line setting for advanced disease. There is no standard second-line therapy. Research into their molecular and immune pathways may pave the way for novel drug discovery. The molecular drivers of NEC are best identified in small cell lung carcinoma, which present with near universal genomic alterations in *TP53* and *RB1*. The genetics of EP-PD-NEC remain poorly understood; *TP53*, *KRAS*, *PIK3CA/PTEN* and *BRAF* mutations have been identified, with alterations in the BRCA pathway reported additionally in small cell NEC of the cervix and absence of argininosuccinate synthetase 1 expression in NEC of the urinary bladder. The use of cell lines and patient-derived xenografts (PDX) to predict response to treatment in NEC and the emergence of alternative biomarkers, such as circulating tumour cells and cell-free DNA, will also be explored. Despite limited published data on the immune microenvironment of EP-NEC, there are a number of clinical trials investigating the use of immune-targeted agents in this disease category, with conflicting emerging data from studies thus far. This review will summarise the treatment and available molecular and immune data in this under researched diagnosis and may stimulate the direction of future exploratory studies.

Key Words

- extra-pulmonary
- poorly differentiated
- neuroendocrine carcinoma
- treatment
- molecular profile
- immune landscape

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Introduction

High grade neuroendocrine carcinomas (NECs) are usually defined by the combination of a poorly differentiated tumour cell morphology and evidence of high proliferative activity, evaluated either directly through the mitotic index and/or Ki-67 or indirectly through the presence of tumour necrosis (Bosman *et al.* 2010). According to tumour cell morphology, two subtypes of NEC are recognised (Dasari *et al.* 2018). The small cell type is defined by the presence of small- to medium-sized tumour cells, characterised by an elongated, hyperchromatic nucleus, devoid of visible nucleoli. The large cell type, first described in the lung, but later identified in most other body sites, is defined by the presence of medium- to large-sized tumour cells, containing a large, ovoid, vesicular nucleus with well visible nucleoli (George *et al.* 2015, 2018).

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Irrespective of their subtype, patients with NEC usually present with disseminated disease and carry a very poor prognosis (Sorbye *et al.* 2013). The current first-line treatment in the advanced setting is a platinum/etoposide combination (Casas *et al.* 1997, Garcia-Carbonero *et al.* 2016); on progression, there is no consensus for second-line treatment (Garcia-Carbonero *et al.* 2016, McNamara *et al.* 2019).

There has been recent progress in the pathological diagnosis of high grade neuroendocrine neoplasms (NENs) (Lloyd *et al.* 2017, Rindi *et al.* 2018*a*) and in the description of their molecular and immune landscapes (Sahnane *et al.* 2015, Vijayvergia *et al.* 2016, Morgan *et al.* 2019). However, so far, there has been little translation of these new data into clinical management and treatment. Nevertheless, it could be expected that better knowledge of the molecular and immune characteristics of NEC could pave the way for the identification of novel biomarkers of diagnostic, prognostic and predictive relevance, which could potentially enable improvements in patient management and new therapeutic strategy development.

This review will focus on extra-pulmonary NENs, where there is a large clinical and therapeutic unmet need and where very limited progress in patient management has been made in nearly four decades. Secondary NECs developing in treatment-resistant adenocarcinomas such as in the prostate or in the lung will be excluded, as will some well-defined entities closely related to NEC, but of different pathogenesis and thus requiring more extensive systematic review and therefore beyond the scope of this manuscript, such as mixed neuroendocrine non-neuroendocrine neoplasms (see Frizziero *et al.* 2020 for summary of literature) and Merkel cell carcinoma. The current state of the art practice in diagnosis and management will also be briefly mentioned. Recent reports on molecular and immune features will then be discussed and finally how these novel data might translate into new tools for improving patient management and treatment, in the era of precision medicine, molecularly driven therapeutic strategies and immunotherapy.

Extra-pulmonary NEC: current basis for patient diagnosis, management and treatment

Diagnosis and terminology

In a recent series from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program, 14,732 of 162,983 cases of NEC were extrapulmonary (EP-NEC); of these, 5509 were gastrointestinal (37.4%), 4151 were of unknown primary (28.2%) and 5072 were of other sites (34.4%), including the head and neck region and the genito-urinary tract (Dasari *et al.* 2018). There are differences in diagnosis and terminology according to the site of the primary, and this might have consequences for patient management (Klöppel 2017). For example, the morphological diagnosis of high grade pancreatic NENs is challenging, especially when limited pathological material may be available, and so may require additional sections together with immunohistochemical staining for surrogate markers of known genotypes of well differentiated neuroendocrine tumours (NETs) and poorly differentiated NEC (Tang *et al.* 2016). See section on 'Current prognostic and predictive markers' for further details.

High grade, poorly differentiated NECs account for approximately 10% of all gastrointestinal NENs (Janson *et al.* 2010). According to the World Health Organisation (WHO) classification, the diagnosis of digestive NEC is based only on tumour cell morphology; nearly all cases are grade 3 (G3) according to the grading system of digestive NENs (i.e. mitotic index >20 and Ki-67 index >20%) (Bosman *et al.* 2010), but this is not required for the definitive diagnosis. As in the lung, two subtypes are recognised (Dasari *et al.* 2018), but the large cell subtype has been formally recognised in the WHO classification only in 2010; until this date, only the small cell subtype was identified. However, large cell NEC is much more frequent in the GEP sphere than in other body sites (Dasari *et al.* 2018). In the study by Dasari *et al.* (2018), it was reported that there were significant differences in survival of patients with a NEC diagnosis according to morphological subtype ($P<0.001$), with small cell histology being associated with worse median and 5-year survival at most primary sites (Dasari *et al.* 2018). In a multicentre retrospective study investigating the impact of small cell vs non-small cell morphology on outcomes in patients with EP-NEC (poorly differentiated), it was reported that those patients with NEC with non-small cell morphology, compared to small cell, had a lower Ki-67 and were less likely to benefit from first-line platinum/etoposide chemotherapy in the advanced setting (Zaninotto *et al.* 2020).

There are striking differences in the distribution of NEC along the digestive tract; NEC is the main type of NEN encountered in the oesophagus and the anal region (>90%), is frequent in the ampullary region, the colon and the rectum and rare in other digestive locations,

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such as the pancreas, duodenum, the small intestine and the appendix (Brenner *et al*. 2004, Walter *et al*. 2017, Rindi *et al*. 2018*a*). It should also be highlighted that it is now widely accepted that not all high grade digestive NENs are poorly differentiated, and therefore, they should not be labelled and treated as NEC. It has been recognised that some G3 digestive NENs are well-differentiated and that their identification is important, since they carry a better prognosis than true NEC and might justify different therapeutic strategies. This new tumour category, termed neuroendocrine tumour (NET) G3, has been formally recognised, first in the 2017 revision of the WHO classification of pancreatic NENs (Lloyd *et al*. 2017) and then in the 2019 revision of the WHO classification of all digestive NENs (Nagtegaal *et al*. 2020). It is likely that this tumour category also exists in other body sites, but without formal recognition at this time (Rindi *et al*. 2018*b*).

High grade NENs of the head and neck region are defined by the same diagnostic criteria as that in the lung: poorly differentiated tumour cell morphology, mitotic index >10/2 mm^2 and usually with the presence of necrosis (Uccella *et al*. 2017). As in other body sites, two subtypes are recognised, small cell and large cell. However, the terminology used in the 2016 revision of the WHO classification is different from that employed in the other body sites. All head and neck NENs are termed 'neuroendocrine carcinomas' (uncommon head and neck malignancies), because of their high malignant potential; three categories are recognised: well-, moderately and poorly differentiated. Only the poorly differentiated category is equivalent to what is called NEC in the other body sites (Uccella *et al*. 2017), with treatment varying, consisting of neoadjuvant chemoradiotherapy ± surgical excision ± post-operative chemoradiotherapy (Bouzbouz *et al*. 2020). One meta-analysis of 701 cases of sinonasal NEC reported that the most important predictor of 5-year disease-specific survival (DSS) was tumour type; it was worse for those with sinonasal small cell carcinoma (46.1%) vs those with well- or moderately differentiated tumours (70.2%) (van der Laan *et al*. 2016).

In the other body sites, high grade NENs are more loosely defined than in the lung, the GEP tract and those of head and neck origin. Most of them are labelled as 'small cell' carcinoma, especially in the genito-urinary tract (Gupta *et al*. 2018), even if a closer look confirms the existence of tumours identifiable as large cell NEC in other body sites. Large cell NEC of the prostate is a rare entity, and nearly all patients with primary *de novo* large cell NEC of the prostate present at a late stage and have a poor prognosis, despite systemic chemotherapy (Tu *et al*. 2019).

It is also important to mention that a uniform criteria for demonstrating the neuroendocrine nature of a poorly differentiated carcinoma is not available. In WHO classifications, the only precise criteria are given for large cell carcinoma of the lung, which could be termed 'neuroendocrine' when at least 10% of tumour cells express one of the following neuroendocrine markers: synaptophysin, chromogranin A or complementarity-determining region 56 (CD56) (Travis *et al*. 2015). For small cell and large cell carcinomas of other sites, the expression of only one (in lung and in head and neck), or at least two, including synaptophysin (in GEP NENs), of the above markers in a 'significant' but undefined proportion of tumour cells is required (Lloyd *et al*. 2017). This means that the term 'neuroendocrine carcinoma' is less well defined than it seems and that it may be different from one body site to another.

Clinical and therapeutic issues

Most patients with EP-NEC, irrespective of their primary site, present with metastatic disease and have poor survival (Dasari *et al*. 2018). This is well exemplified by GEP-NEC, in which large clinical series are available. More than two-thirds of patients with GEP-NEC have distant metastases at the time of diagnosis (Sorbye *et al*. 2013, Walter *et al*. 2017). Survival is poor, ranging from 38 months in patients with localised disease to 5 months in the metastatic setting (Sorbye *et al*. 2014). It may be as low as 1 month in those receiving best supportive care alone in the metastatic setting (Sorbye *et al*. 2013). In a population-based retrospective study in the Netherlands, which included 1544 cases (1045 with EP-NEC and 499 with NEC of unknown primary), the overall 5-year relative survival was 38% for patients with local/regional disease (n=447) and 7% for patients with extensive disease (n=582). For patients with NEC of unknown primary (n=499), the 5-year relative survival was 6% (van der Zwan *et al*. 2018).

Surgery, with the potential for cure, is recommended for localised disease, and given the high relapse rate, platinum-based adjuvant chemotherapy is advised (Casas *et al*. 1997, Garcia-Carbonero *et al*. 2016) ± radiotherapy, as appropriate (Garcia-Carbonero *et al*. 2016). To date, systemic therapy does not differ according to location of primary site. In patients with locoregional unresectable disease, locoregional radiotherapy with chemotherapy

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could be considered (Garcia-Carbonero *et al*. 2016). In the advanced setting, systemic therapy is recommended, analogous to that of pulmonary NEC, with platinum-based chemotherapy combined with etoposide advocated in the first-line setting, with a reported response rate (RR) of 67% in one study (Moertel *et al*. 1991) (acknowledging that RR in this study was determined by clinical examination; however, this was the first study to support platinum-based treatment in the first-line advanced setting for patients with EP-PD-NEC), but around 31–58% in more recent publications (Sorbye *et al*. 2013, 2014, Walter et *al*. 2017, Frizziero *et al*. 2019). Radiotherapy may also be considered for symptom control (localised bone metastasis or brain) (Garcia-Carbonero *et al*. 2016).

There is no standard endorsed second-line treatment in the advanced setting for patients with EP-NEC. Re-treatment with a platinum/etoposide combination may be considered in patients that achieved a response to up-front treatment and progressed after a treatment break of at least 3 months, provided that there are no contraindications to re-challenge with platinum-based therapy, such as neurotoxicity or ototoxicity (Garcia-Carbonero *et al*. 2016). In the NORDIC NEC study (Sorbye *et al*. 2013), of 305 patients, 100 (33%) received second-line chemotherapy, with 35 receiving temozolomide-based therapy (at least two other studies have also reported the use of temozolomide in the second-line setting (Welin *et al*. 2011, Olsen *et al*. 2012)) and 20 taxotere-based chemotherapy; 31 patients (10%) received third-line therapy (Sorbye *et al*. 2013). In a French cohort, first-, second- and third-line palliative chemotherapies were given in 176 (69%), 100 (40%) and 51 (20%) patients, respectively; mainly 5-fluorouracil (5-FU)/irinotecan (n=72) and 5-FU/oxaliplatin (n=33) regimens in the post-first-line chemotherapy setting (Walter *et al*. 2017). Some other second-line regimens that have been used in small retrospective series include topotecan (RR: 0%) (Olsen *et al*. 2014), 5-FU/irinotecan (RR: 24–31%) (Hentic *et al*. 2012, Walter *et al*. 2017) and 5-FU/oxaliplatin (RR: 16–29%) (Hadoux *et al*. 2015, Walter *et al*. 2017), with a median reported progression-free survival (PFS) ranging from 2.1–4.5 months and a median overall survival (OS) ranging from 3.2-18 months (where the latter OS was calculated from date of diagnosis of NEC to death (Hentic *et al*. 2012)). In a systematic review and meta-analysis of second-line treatment in 595 patients with advanced EP-NEC, the median RR reported was 18% (range 0–50; 0% for single-agent everolimus, temozolomide, topotecan; 50% with amrubicin), the median PFS was 2.5 months (range 1.2–6.0) and median OS was 7.6 months (range 3.2–22) (McNamara *et al*. 2019). Prospective trials are needed (Walter *et al*. 2018 (NCT02820857), Craig *et al*. 2020 (NCT03837977)).

Current prognostic and predictive markers

In GEP-NEC, the most common clinical factors reported, to date, that are negatively associated with survival were: poor Eastern Cooperative Oncology Group Performance Score (ECOG PS) (Sorbye *et al*. 2013, Lamarca *et al*. 2017, Walter *et al*. 2017) and stage/presence of liver metastases (Lamarca *et al*. 2017, Walter *et al*. 2017, Dasari *et al*. 2018). Adverse biochemical markers include: lactate dehydrogenase (LDH) (Sorbye *et al*. 2013, Freis *et al*. 2017, Lamarca *et al*. 2017, Walter *et al*. 2017), as in many aggressive neoplasms, but also, somewhat inconsistently, high platelet count (Sorbye *et al*. 2013), high alkaline phosphatase (ALK) (Lamarca *et al*. 2017), aspartate aminotransferase (Freis *et al*. 2017) and neuron-specific enolase (NSE) (Sorbye *et al*. 2013, Lamarca *et al*. 2017, Walter *et al*. 2017). High Ki-67 index has also been proposed as an adverse histological prognostic factor. Sorbye *et al*. reported that patients with G3 GEP-NEN with a Ki-67 >55% had a better RR (42% vs 15%) to systemic treatment, but worse survival (10 vs 14 months) than patients with a Ki-67 <55% (Sorbye *et al*. 2013).

Alese *et al*. reviewed a large series of patients (identified between 2004 and 2013) with NEC (n=1861) from the National Cancer Database, United States, and reported that treatment at an academic centre, age <65 years, and use of chemotherapy, were associated with improved survival (multi-agent was associated with superior survival compared with monotherapy, which was superior to no chemotherapy) (Alese *et al*. 2019). Another study derived a prognostic score (scoring from 0–6 points for 5 variables) for OS in 313 patients (all stages) with GEP-NEC. The five baseline variables included were ECOG PS, presence of liver metastases, ALK, LDH and Ki-67. The score was prognostic for OS on multivariable analysis (P<0.001),and was validated in an external and prospective validation cohort, with two groups identified with incremental risk of death (group A: 0–2 points with good prognosis: median OS 19.4 months, and group B: 3–6 points with poor prognosis: median OS 5.2 months) (Lamarca *et al*. 2017).

The potential clinical importance of immunoreactive p53 protein in tumour tissue was reported from 124 patients with locally advanced or metastatic GEP-NECs treated with platinum-based chemotherapy from the Nordic NEC registry (Ali *et al*. 2017).

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The authors concluded that p53 expression could not be correlated with clinical outcome, but in patients with colorectal NECs, p53 expression was correlated with shorter PFS and OS, and studies are needed to explore its prognostic significance further. In a study of 33 cases of grade 3 NENs of the pancreas, loss of death domain-associated protein (DAXX) or ATP-dependent helicase ATRX (ATRX) protein expression defined well differentiated NETs and abnormal p53, Rb and SMAD4 expression signified poorly differentiated NEC (Tang *et al*. 2016). The disease-specific survival reported was 75 months and 11 months for the well-differentiated neuroendocrine tumour and poorly differentiated NEC group, respectively (Tang *et al*. 2016).

Molecular and immune profile of extrapulmonary NECs: recent results

In recent years, there has been accumulating data on the molecular profile of high grade NENs (Girardi *et al*. 2017). In addition, their immune profiles have been investigated.

Molecular profiling in EP-NEC

Recent data have shown that small cell NEC is associated with a highly distinctive molecular signature, characterised by the bi-allelic inactivation of both *TP53* and *RB1*, as a result of various mechanisms, from mutations to large chromosomal rearrangements. First documented in the lung (George *et al*. 2015, 2018), this molecular signature has subsequently been confirmed in all body sites, including the digestive tract (Jesinghaus *et al*. 2017), the pancreas (Yachida *et al*. 2012, Hijioka *et al*. 2017, Konukiewitz *et al*. 2017, 2018), the head and neck region (Goyal *et al*. 2014, Wasserman *et al*. 2019), the genito-urinary tract (Chang *et al*. 2018, Shen *et al*. 2018) and the uterine cervix (Frumovitz *et al*. 2016, Xing *et al*. 2018). This does not mean that other molecular alterations could not be found in small cell NEC. For instance, the NOTCH pathway is altered in about 25% of lung small cell NEC and in some EP-NECs (Xing *et al*. 2018). Frequent alterations in the phosphatidylinositol 3-kinase/phosphatase and tensin homologue/mammalian target of rapamycin (PI3K/PTEN/mTOR) pathway can be detected in some body sites such as the uterine cervix (Frumovitz *et al*. 2016, Cho *et al*. 2017, Xing *et al*. 2018). Cervical NECs may be associated with human papillomavirus (HPV), especially HPV18, and most will stain positive for p16, with the immunohistochemistry markers synaptophysin and CD56 being the most sensitive (Salvo *et al*. 2019). Targeted next-generation sequencing of ten cases of small cell NEC of the uterine cervix (nine hysterectomy specimens and one biopsy) also identified genetic alterations involving the breast cancer (BRCA) pathway (Xing *et al*. 2018). In the first-line advanced setting in patients with advanced NEC of the cervix, cisplatin combined with etoposide is favoured, and a combination of topotecan, paclitaxel, and bevacizumab has been described as a treatment option in the second-line setting (Salvo *et al*. 2019). The 5-year survival rate has been reported as 36%, with a median overall survival ranging between 22 and 25 months (Salvo *et al*. 2019) (Table 1).

The genomics of large cell NEC is more complex and heterogeneous than that of small cell NEC. In several body sites, it has now been demonstrated that the category, as morphologically defined, can be associated with at least two distinct types of molecular signatures, with an overall comparable proportion of cases: (1) a 'small cell NEC-like' signature, characterised by the double inactivation of *TP53* and *RB1* and (2) a 'carcinoma-like' signature, with the same profile of molecular alterations as in non-neuroendocrine carcinomas of the same location (George *et al*. 2015, 2018, Rekhtman *et al*. 2016). This molecular heterogeneity has been unambiguously documented in the lung (George *et al*. 2015, 2018, Rekhtman *et al*. 2016). In the GEP arena, most of the available studies have included not only 'pure' NECs, but also mixed neuroendocrine-non neuroendocrine neoplasms. Mixed neuroendocrine non-neuroendocrine neoplasms from the gastro-entero-pancreatic tract, by definition, contain a neuroendocrine and an exocrine component, each of them present in at least 30% of the tumour mass and are malignant. It is an aggressive entity with a high-grade neuroendocrine component in the majority of cases and is associated with poor survival outcomes close to those of pure NECs (Frizziero *et al*. 2020). In several studies, it is therefore difficult to determine whether the molecular alterations reported were found in 'pure' NECs, in the high grade neuroendocrine component of a mixed tumour, or even in a whole mixed tumour without distinction between its components. Despite these possible pitfalls, it might be assumed that, in the colon, rectum, pancreas and the stomach, a significant proportion of tumours histologically diagnosed as 'large cell NEC' harbour molecular abnormalities usually found in adenocarcinomas of the same location. Colorectal NECs might harbour mutations in *KRAS* (20–30%), *BRAF* (from 7 to 60% according to the series), adenomatous

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Table 1 Summarised data from some reported literature on immunohistochemistry and molecular profiling of extrapulmonary neuroendocrine carcinoma (NEC) and potentially drugable targets.

Author	Period of enrolment	Number of patients (*n*)	Incidence of alterations (*n* (%))	Primary site (*n*)	Disease stage (*n*)	Method of analysis	Findings	Potentially drugable target ± drugs
Ali *et al.* 2017	1999–2011	124	**p53 immunoreactive**: 48 (39%) **Non-immunoreactive**: 76 (61%)	Colon: 31 Carcinoma unknown primary: 33 Oesophagus: 4 Pancreas: 28 Stomach: 11 Rectum: 17	Local: 3 Regional: 24 Distant: 97	Immunohistochemistry	p53 immunoreactivity correlated with poorer survival in patients with colorectal tumours (hazard ratio 2.1, $P = 0.03$).	-
Bergsland *et al.* 2016	Not reported	274	Not reported	Colon: 92 Pancreas: 123 Other (oesophageal, stomach, small intestine): 59	Not reported	Hybridisation-captured, adaptor ligation-based libraries	*TP53*, *RB1*, *CDKN2A*, *MEN1*, *CDKN2B*, *DAXX*, *APC*, *KRAS* and *CCNE1* mutations detected. Every EP-PD-NEC group had a lower rate of alteration for *TP53* and *RB1* than small cell lung cancer.	-
Gupta *et al.* 2018	1987–2014	74	**ASS1 expression** in 22%	Urinary bladder: 74	I–III: 74	Immunohistochemistry	Ten-year survival from disease-specific death was not statistically significant between ASS1-expressing and ASS1-deficient cases.	High grade NEC of the bladder may be a candidate for arginine deprivation therapy using drugs such as pegylated arginine deiminase (ADI-PEG 20).
Klempner *et al.* 2016	Not reported	108	**BRAF alteration**: 9% (5 colon and 5 rectum; 80% were $BRAF^{V600E}$)	Colorectal: 108	For 9% with BRAF mutation: IV: 10	Next generation sequencing	Two patients with this $BRAF^{V600E}$ alteration had rapid and durable response to combined BRAF-MEK inhibition.	Combined BRAF-MEK inhibition.
Morgan *et al.* 2019	2000–2017	10	**PD-L1 positivity** in 70% **HPV positive** in 7 of 9 (78%) **MMR loss** in 33%	Uterine cervix: 10	Not reported	Immunohistochemistry, HPV in situ hybridisation	PD-L1 expression in >10% of tumour cells seen in a subset of tumours in association with loss of MMR expression.	These patients may be amenable to immune checkpoint inhibitor therapy.

Sahnane *et al.* 2015	Not reported	89 (53 patients with NEC and 36 with MANEC)	**Microsatellite instability** (MSI) in 12% (7 intestinal and 4 gastric) ***BRAF* mutations** in 7% ***KRAS* mutations** in 17%	Colorectal: 37 Duodenum: 4 Gallbladder: 3 Oesophagus: 6 Pancreas: 3 Stomach: 36	I–III: 59 IV: 19	Immunohistochemistry, PCR pyrosequencing	Vascular invasion and MSI (better prognosis) were only independent prognostic factors on multivariable analysis.	*Immune checkpoint blockade may be an option for patients with tumours harbouring MSI. *BRAF inhibitors may be an option for those with tumours harbouring BRAF mutations.
Shamir *et al.* 2019	1994–2017	25	24 (96)	Anus: 2 Colon: 10 Rectum: 13	Local: 5 Distant: 18 Unknown: 2	Next generation sequencing, immunohistochemistry, in situ hybridisation, PCR	Colorectal NECs stratify into three distinct molecular subgroups, differentiated based on Rb protein and HR-HPV status.	HPV-driven NEC may respond to immune checkpoint blockade.
Sinha *et al.* 2018	2000–2015	19 (14 patients with MANEC and 5 with NECs)	Not reported	Colorectal: 19	Local: 3 Distant: 14 Unknown: 2	MSI PCR, fluorescence in situ hybridisation, immunohistochemistry	Frequency of *MYC* amplification was similar to adenocarcinoma, frequency of *PTGER4* amplification was higher than adenocarcinoma.	The discovery of recurrent PTGER4 amplification implies a potential of exploring targeted therapy to the prostaglandin synthesis pathways in a subset of these tumours.
Vijayvergia *et al.* 2016	2013–2015	23	19 (83%) ***TP53*** (57%) ***KRAS*** (30%) ***PIK3CA/PTEN*** (22%) ***BRAF*** (13%)	Colon: 8 Pancreas: 4 Small intestine: 1 Other: 7	I–III: 6 IV: 17	Next generation sequencing	Prevalence of mutations correlated with higher risk of progression within previous year and *TP53* mutation correlated with worse survival.	Potentially actionable mutations: PIK3CA/PTEN and BRAF.

(Continued)

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Table 1 Continued.

Author	Period of enrolment	Number of patients (*n*)	Incidence of alterations (*n* (%))	Primary site (*n*)	Disease stage (*n*)	Method of analysis	Findings	Potentially drugable target ± drugs
Xing *et al.* 2018	Not reported	10	9 (80%)	Uterine cervix: 10	Not reported (9 had hysterectomy; lymph node metastases recorded in 5 patients, biopsy only in 1 patient)	Immunohistochemistry, in situ hybridisation, PCR	Genetic alterations identified in MAPK, PI3K/AKT/mTOR and TP53/BRCA pathways.	Identification of somatic BRCA mutations in patients may lead to clinical trials including poly (ADP-ribose) polymerase (PARP) inhibitors.
Yachida *et al.* 2012	1988–2010	19 (9 small cell, 10 large cell)	7 (37%)	Pancreas: 19	I–III: 19	Immunohistochemistry, PCR	Abnormal immunolabeling patterns of p53 (95%) and Rb (74%) were frequent in small cell and large cell NEC. Overexpression of Bcl-2 protein was observed in 100% of small cell NECs, and 50% large cell NECs.	The finding of Bcl-2 overexpression in poorly differentiated NECs suggests that Bcl-2 antagonists or inhibitors may be a potential treatment option for these patients.

APC: adenomatous polyposis coli; ASS1: argininosuccinate synthetase 1; Bcl-2: B-cell lymphoma 2; BRAF: v-Raf murine sarcoma viral oncogene homolog B; BRCA: breast cancer; CCNE1: cyclin E1; CDKN2A: cyclin-dependent kinase Inhibitor 2A; DAXX: death-associated protein 6; EP-PD-NEC: extrapulmonary poorly differentiated neuroendocrine carcinoma; HPV: human papillomavirus; HR-HPV: high-risk human papillomavirus; KRAS: Kirsten rat sarcoma; MANEC: mixed adenoneuroendocrine carcinoma; MEK: mitogen-activated protein kinase kinase; MEN1: multiple endocrine neoplasia type 1; MMR: mismatch repair protein; PD-L1: programmed death ligand-1; PIK3CA/PTEN: phosphatidylinositol 3'-kinase catalytic subunit alpha/phosphatase and tensin homolog; PTGER4: prostaglandin E receptor 4; Rb protein: retinoblastoma protein; TP53: tumour protein p53.

polyposis coli (*APC*), *PIK3CA* or *PTEN* (up to 25%), as well as *MYC* amplifications (Alitalo *et al*. 1983, Karkouche *et al*. 2012, Takizawa *et al*. 2015, Hammond *et al*. 2016, Klempner *et al*. 2016, Olevian *et al*. 2016, Jesinghaus *et al*. 2017, Idrees *et al*. 2018, Sinha *et al*. 2018). Some cases of pancreatic NECs harbour mutations in *KRAS* or *SMAD4* (Kimura *et al*. 2016, Konukiewitz *et al*. 2018).

A potentially interesting alteration is the acquisition of microsatellite instability (MSI) by GEP NEC. The frequency is variable according to the series, from 0 to more than 10% (Arnold *et al*. 2008). One of the most detailed studies is that of Sahnane *et al*., who investigated the incidence of MSI in 89 cases of GEP NECs and mixed adenoneuroendocrine carcinomas (MANECs) (53 NECs and 36 MANECs) (6 oesophageal, 77 gastrointestinal, 3 pancreatic and 3 gallbladder) (Sahnane *et al*. 2015); MSI was observed in 11 NEC/MANECs (12.4%) (7 intestinal and 4 gastric). A *BRAF* mutation was identified in 6 of 88 cases (7%) and *KRAS* mutations were identified in 15 cases (17%); *BRAF* mutations were associated with MSI ($P<0.0008$), while *KRAS* status did not correlate with any clinicopathological or molecular features (Sahnane *et al*. 2015). It was concluded that MSI identifies a subset of gastric and intestinal NECs/MANECs with distinct biology and a better prognosis; vascular invasion ($P=0.0003$) and MSI ($P=0.0084$) were identified as the only independent prognostic factors on multivariable analysis (Sahnane *et al*. 2015). A high incidence of MSI phenotype, higher than 30%, has also been recently reported in small cell NEC of the uterine cervix (Morgan *et al*. 2019).

The recent progress in molecular profiling of small and large cell NEC has also underlined the striking molecular differences existing between well- and poorly differentiated NENs. This is particularly obvious in the GEP arena where well-differentiated NETs usually harbour no mutation in *TP53* or *RB1*, while poorly differentiated NECs show none of the mutations found with a high incidence in their well-differentiated counterparts (such as mutations in multiple endocrine neoplasia type 1 (*MEN1*), *ATRX* and *DAXX* in the pancreas) (Yachida *et al*. 2012).

In 2016, Bergsland *et al*. examined the genomic alterations (using 192 cancer-related genes) of 867 NECs, including 593 small cell lung cancer samples and 274 EP-NECs (123 pancreas, 92 colon and 59 others from the oesophagus, stomach and small intestine (stage not stated) (Bergsland *et al*. 2016). Only *TP53* crossed a 15% threshold in every group; *MEN1* and *DAXX* were genes with alterations in >15%, specific to the pancreas, while *APC* and *KRAS* were altered in >15%, specific to the colon, whereas *Cyclin E1* (*CCNE1*) was altered in >15% of the 'other' gastrointestinal primary NECs. A lower rate of alterations in *TP53* and *RB1* were reported in all EP-NECs when compared to small cell lung cancer. The authors concluded that optimal therapy for EP-NECs may be site specific and different from small cell lung cancer (Bergsland *et al*. 2016).

The immune context in EP-NECs

The immune context, including the use of selected immune checkpoint targeted agents, has recently been explored, mainly in pulmonary NECs, but also in some extra-pulmonary tumours. In a study where 94 cases of pulmonary ($n=61$) and extra pulmonary ($n=33$) small cell NECs were analysed by immunohistochemistry for programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) protein expression, PD-L1 was expressed in tumour-infiltrating macrophages and was correlated with tumour-infiltrating lymphocytes (Schultheis *et al*. 2015). The available data on the immune environment or the use of immunotherapy in patients with EP-NECs is limited. Morgan *et al*. reported that PD-L1 expression (predominantly focal) was present in 70% of ten cases of SCNEC of the uterine cervix (eight of whom received platinum/etoposide chemotherapy, one received radiotherapy alone and one best supportive care) (Morgan *et al*. 2019).

Indirect evidence for a hyperimmune state in some cases of NEC has been provided by molecular analysis. A high tumour mutational load (or burden) has been detected in lung NECs (Peifer *et al*. 2012, Rudin *et al*. 2012), but also in several types of EP-NECs (Sharabi *et al*. 2017, Chang *et al*. 2018, Salem *et al*. 2018).

New perspectives for diagnosis, management and treatment

New diagnostic and predictive markers

The translation of NEC molecular profiling into clinical practice may be made easier by the fact that most of the main and most constant genetic abnormalities associated with EP-NEC, that is, mutations in *TP53* and *RB1* genes, can be screened for using immunohistochemistry. Mutations in the *TP53* gene are usually, while not consistently, associated with the accumulation of the abnormal protein within the nucleus of altered cells, making it detectable by conventional anti-p53 antibodies (Konukiewitz *et al*. 2017).

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In contrast, in the normal state, the p53 protein is expressed at very low levels, well below the sensitivity threshold of conventional immunohistochemistry. A strong and uniform nuclear labelling of tumour cells by anti-p53 antibodies is therefore suggestive of the presence of *TP53* mutations. Mutations in *RB1* genes are usually associated with a loss of expression of the corresponding protein in altered cells, which can be easily detected by conventional immunohistochemistry (Konukiewitz *et al*. 2017).

The demonstration of p53 accumulation and Rb loss in tumour cells has therefore been proposed in the recent WHO classifications as a useful tool to strengthen a diagnosis of NEC, as opposed to NET, including NET G3, which are constantly p53 negative and Rb positive (Lloyd *et al*. 2017). This has also been reported in a study analysing the histological and molecular properties of gastroenteropancreatic high grade NENs (15 grade 3 NETs and 39 NECs) (Busico *et al*. 2019). The sensitivity and specificity of this approach remains to be validated in large series, but the published results are encouraging (Basturk *et al*. 2014, Konukiewitz *et al*. 2017).

Rb loss might also prove to be a predictive marker of response to platinum salts in NECs. Some studies, in both pulmonary (Derks *et al*. 2018) and EP-NECs (Terashima *et al*. 2012), suggest that Rb loss in NEC may be associated with a better response to standard treatment. These results need to be validated in larger, prospective, multicentre studies.

New therapeutic perspectives

The three main perspectives are: (1) actionable molecular abnormalities, (2) identification of cellular targets and (3) immunotherapy.

BRAF mutations, so frequently found in NECs, could be actionable. A dramatic response has been reported to combination BRAF-mitogen-activated protein kinase kinase (MEK) inhibition in two cases of metastatic rectal NEC, refractory to standard therapy (Klempner *et al*. 2016). Urinary $BRAF^{V600E}$ circulating tumour DNA monitoring correlated with disease response, and it was concluded that $BRAF^{V600E}$ may be an oncogenic driver responsive to BRAF-MEK combination therapy in this disease site (Klempner *et al*. 2016). Capdevila *et al*. have recently reported that NEC from the colon and colorectal carcinomas are similar in their mutational repertoire, with NEC from the colon being particularly enriched in $BRAF^{V600E}$ mutations (Capdevila *et al*. 2020). They concluded that $BRAF^{V600E}$ mutant colon NEC may benefit from BRAF inhibition in monotherapy, or with the addition of anti-EGF receptor (EGFR) antibodies, instead of MEK inhibitors for efficient blockade of acquired resistance (Capdevila *et al*. 2020).

Interestingly, a recent study has reported the efficacy and safety of the monoclonal antibody against vascular endothelial growth factor receptor 2 (VEGFR2), ramucirumab, combined with chemotherapy in patients with pre-treated metastatic gastric NEC ($n=13$). The authors concluded that the ramucirumab/chemotherapy combination demonstrated promising activity, without severe or unexpected safety issues and may be due to higher VEGF receptor 2 (VEGFR2) expression in gastric NEC (Mishima *et al*. 2018). This may be an avenue for further therapeutic research.

Additional potential cellular targets in EP-NECs have been identified, such as in breast NEC (which is rare), farletuzumab and mirvetuximab soravtansine (FOLR1), sacituzumab govitecan (TROP-2) and HDAC inhibitors (H3K36Me3) (Vranic *et al*. 2019). Another study reported that nuclear and cytoplasmic thymidylate synthase, nuclear and cytoplasmic neuron-specific enolase and nuclear p27 were significantly overexpressed in neuroendocrine breast carcinoma ($P<0.01$); cytoplasmic somatostatin receptor-2A (SSTR-2A) expression was associated with better distant disease-free survival ($P=0.013$), cytoplasmic menin expression with worse relapse-free survival ($P=0.022$) and nuclear p27 with longer breast cancer-specific survival ($P=0.022$), compared with invasive ductal carcinomas (Roininen *et al*. 2019). Others targets of potential relevance are now detailed. Gupta *et al*. have reported on argininosuccinate synthetase (ASS1) expression, assessed by immunohistochemistry, in 74 patients who had radical cystectomy for NEC of the urinary bladder (63 small cell, 5 large cell and 6 mixed morphology) (Gupta *et al*. 2018). It was reported that 58 patients (78%) had absent ASS1 expression, including all patients with large cell and mixed morphology. The 10-year survival from disease-specific death was not statistically significant between ASS1-expressing and deficient cases ($P=0.75$). The authors hypothesised that arginine deprivation therapy may offer therapeutic benefit in these patients (Gupta *et al*. 2018), but validation studies would be needed.

The possible indications and relevance of immunotherapy in NECs, especially in cases with high mutational burden and/or MSI phenotype, are currently under investigation, prompted by the success encountered in Merkel cell carcinoma (cutaneous), an entity closely related to NEC.

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Table 2 Selected clinical trials including immunotherapy for patients with extrapulmonary neuroendocrine carcinoma (NEC).

Immunotherapy approach	Trial description	Key eligibility criteria	Planned recruitment (*n*)	Primary endpoint	Clinical Trials.gov reference	Status ± preliminary results
(Checkpoint inhibitor monotherapy) Avelumab	Single centre, pilot trial	Unresectable or metastatic, grade 3, poorly differentiated NEC, having received 0, 1 or 2 prior lines of systemic therapy.	10	Overall response rate (RECIST 1.1)	NCT03278405	Completed
Avelumab	Single centre, phase II	Locally advanced, unresectable or metastatic gastroenteropancreatic NEC, Grade 3; must have had radiographic or clinical disease progression during or after first-line therapy containing any platinum/etoposide.	30	Best response	NCT03147404	Completed
Humanised anti-PD-1 antibody JS001 (Zhang *et al*. 2018)	Single-centre, open-label, phase Ib	Locally advanced or metastatic non-functional neuroendocrine tumours, including well- and poorly differentiated NEC, Ki-67 ≥10% who have failed previous systemic therapy.	40	Overall response rate (RECIST 1.1)	NCT03167853	Completed (23 patients recruited: 15 with a diagnosis of NEC; overall response rate in NEC was 25%, with median PFS in entire cohort of 2.8 months)
Pembrolizumab (Vijayvergia *et al*. 2018)	Single-centre, open-label, phase II	Metastatic high grade neuroendocrine tumour (Ki-67 >20%), excluding high grade neuroendocrine tumours of large or small cell type of lung/thymus origin and Merkel cell carcinoma; must have received at least one platinum-containing regimen.	21	Objective response rate (RECIST 1.1)	NCT02939651	Active (not recruiting) (21 patients enrolled; similar toxicities to that previously described: grade 3 toxicities observed in 28%, with 19% possibly related to pembrolizumab (elevated liver enzymes, fatigue, hypercalcaemia, hyperkalaemia))
Pembrolizumab	Single-centre, open-label, phase II	Metastatic or unresectable poorly differentiated NEC of non-pulmonary origin (Ki-67 >20%); must have failed at least one line of therapy (platinum or temozolomide-based).	40	Overall response rate (using immune-related RECIST)	NCT03190213	Terminated (principal investigator discontinued)
Anti-PD1 PDR001	Multi-centre, open-label, phase II	Advanced or metastatic, well-differentiated, non-functional neuroendocrine tumours of pancreatic, gastrointestinal, or thoracic origin or poorly differentiated gastroenteropancreatic NEC; must have disease progression while on/or after prior treatment.	110	Overall response rate (RECIST 1.1)	NCT02955069	Active, not recruiting

(Continued)

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Table 2 Continued.

Immunotherapy approach	Trial description	Key eligibility criteria	Planned recruitment (*n*)	Primary endpoint	Clinical Trials.gov reference	Status ± preliminary results
Rovalpituzumab tesirine	Multi-centre, open-label	Advanced solid tumours (delta-like protein 3-expressing) including high grade gastroenteropancreatic neuroendocrine carcinoma, with disease progression after at least one prior systemic therapy.	378	Maximum tolerated dose and adverse events	NCT02709889	Terminated (strategic considerations)
(Dual checkpoint inhibition) Durvalumab and tremelimumab	Multi-centre, open-label, single-arm, phase II (DUNE trial)	Different cohorts of patients, including those with grade 3 gastroenteropancreatic neuroendocrine neoplasms, or unknown primary site (excluding lung primaries), after progression on previous therapy.	126	Clinical benefit rate (by RECIST 1.1, defined as percentage of patients achieving complete or partial response or stable disease at month 9 after commencement of treatment)	NCT03095274	Recruiting
Nivolumab monotherapy or nivolumab plus ipilimumab	Multi-centre, open-label, randomised	Advanced, refractory pulmonary or gastroenteropancreatic poorly differentiated NEC with tumour progression after one or two lines of treatment.	180	Objective response rate (RECIST 1.1)	NCT03591731	Recruiting
Nivolumab and ipilimumab (DART) (Patel *et al.* 2020)	Multi-centre, open-label	Rare tumours including endocrine carcinoma of pancreas and digestive tract and lung; must have progressed following at least one line of standard systemic therapy.	707	Overall response rate (RECIST 1.1)	NCT02834013	Recruiting (preliminary results from patients with non-pancreatic neuroendocrine tumours published: 32 patients received therapy; 18 had high-grade disease; patients with high grade NEC had an overall response rate of 44%, with 0% overall response rate in patients with low/intermediate grade disease)
(Checkpoint inhibition plus chemotherapy) Pembrolizumab (Part A) (Mulvey *et al.* 2019), pembrolizumab plus physician's choice: paclitaxel or irinotecan (Part B)	Multi-centre, using adaptive Simon's two-stage design	Locally advanced or metastatic high grade NEC excluding pulmonary neuroendocrine carcinoma and Merkel cell carcinoma; must have progressed during or after completion of first-line systemic therapy.	42	Overall response rate (RECIST 1.1)	NCT03136055	Recruiting (preliminary results (Part A): 14 patients enrolled; overall response rate 7%, median PFS was 58 days: conclusion was that pembrolizumab was not effective in this pretreated, biomarker-unselected population)

Pembrolizumab in combination with chemotherapy (carboplatin/cisplatin with etoposide or docetaxel and carboplatin)	Single centre, phase Ib	Locally advanced or metastatic small cell/neuroendocrine cancers of urothelium or prostate. Different cohorts, with no prior systemic chemotherapy allowed for primary small cell prostate cancer.	30	Different outcome measures including durable response rate, overall response rate (RECIST1.1), duration of response, PFS, OS, radiographic PFS, incidence of adverse events	NCT03582475	Recruiting
(Checkpoint inhibitor plus tyrosine kinase inhibitor) Pembrolizumab (+ lenvatinib)	Open-label, phase II	Metastatic poorly differentiated and/or high grade neuroendocrine tumour/carcinoma originating outside of the lung (including unknown primary), and have received at least one prior line of systemic treatment.	30	Objective radiographic response rate (RECIST)	NCT03290079	Suspended (investigator working on amendment)

www.ClinicalTrials.gov search was last updated on 30th March 2020.
NEC: neuroendocrine carcinoma; OS: overall survival; PFS: progression-free survival; RECIST: Response Evaluation Criteria In Solid Tumours.

Microsatellite instability and high mutational load are more pronounced in high grade NENs (Sahnane *et al*. 2015), and to date, clinical experience of immune checkpoint blockade in NENs mainly exists for Merkel cell carcinoma (D'Angelo *et al*. 2018), and results of on-going immunotherapy trials in NEC with translational end-points will be informative (Table 2). There has been a case report of a patient with mismatch repair deficient platinum-resistant colorectal NEC, with a history of Lynch syndrome, who received pembrolizumab and subsequently underwent resection, with lack of radiologically evident metastatic disease 24 months after immunotherapy discontinuation (Whitman *et al*. 2019).

Despite the paucity of data published on the immune microenvironment of EP-NEC, there are a number of clinical trials investigating the use of immune-targeted agents in this disease category (Table 2). Emerging data from studies, thus far, have been conflicting. In preliminary analysis of a phase II study (NCT 02955069), the activity and safety of spartalizumab (PDR001), a high-affinity, humanised, anti-PD-1 IgG4 antibody that blocks PD-L1 and PD-L2 binding to PD-1, was assessed in 21 patients with GEP-NEC who had progressed on one line of chemotherapy; confirmed RR was 5%, and the authors concluded that T-cell immunoglobulin and mucin-domain containing-3 (TIM3) expression in immune cells may be associated with the lack of response in patients with GEP NEC (Yao *et al*. 2018). In contrast, a non-randomised phase Ib trial (NCT 03167853) examined the efficacy and safety of PD-1 blockade with Toripalimab (JS001) in 28 patients with NEC who had failed standard treatment; the RR reported was 17.9% by Response Evaluation Criteria in Solid Tumours (RECIST) (Zhang *et al*. 2018), and it was concluded that patients with PD-L1 positive NENs might preferentially respond to JS001 treatment, but larger studies were recommended.

A phase II trial of the anti-PD-L1 antibody avelumab in 27 patients with advanced, metastatic high-grade NENs G3 (16 with G3 NEC and 11 with moderately differentiated G3 NETs) who had progressed after first-line chemotherapy (AVENEC) reported (interim analysis) that the disease control rate (stable disease or partial remission according to immune-related RECIST) after 8 weeks was 32%, and in responders, the mean duration of disease control was 20 weeks, with four patients having stable disease or partial response ≥6 months (with excellent tolerability) (Fottner *et al*. 2019).

Two studies have presented preliminary results of pembrolizumab monotherapy in previously treated patients with extrapulmonary poorly differentiated

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NEC (NCT 03136055 and NCT02939651); treatment was well tolerated, but initial results concluded that pembrolizumab in monotherapy was not effective in these biomarker-unselected populations of patients with EP-NEC (poorly differentiated), arising in different organs (Vijayvergia *et al*. 2018, Mulvey *et al*. 2019).

The results from the neuroendocrine cohort of the Southwest Oncology Group (SWOG) S1609 study, dual anti-cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and anti-PD-1 blockade in rare tumours (DART) (NCT02834013), have recently been reported (Patel *et al*. 2020). This was a multi-centre phase II clinical trial of ipilimumab (1 mg/kg every 6 weeks) plus nivolumab (240 mg intravenously every 2 weeks) across multiple cohorts of rare tumours. In the neuroendocrine cohort, 32 eligible patients received therapy; 58% ($n=19$) had high grade disease, with the most common sites being gastrointestinal (non-pancreatic) (45%; $n=15$) and lung (18%; $n=16$). The patients had received a median of two lines of prior therapy. The overall RR was 25% and patients with NEC had a RR of 44%. All patients who had a complete or partial response had high grade disease. The 6-month PFS reported was 31% and the median OS was 11 months. It was hypothesised that NEC may have a higher tumour mutational burden, indicating a better response to immunotherapy, but this needs to be verified (MSI status was not readily available for the patients with non-pancreatic NETs enrolled in this study).

Recently, Li and colleagues have reported that combining immunotherapy with modified formulations of traditional cancer therapies, such as radiotherapy and chemotherapy, may stimulate an inflammatory response, eliciting a more effective response to immunotherapy, thereby turning immunologically 'cold' tumours (contain few infiltrating T cells and so are not recognised and do not provoke a strong response by the immune system) into 'hot' ones (Li *et al*. 2018). In EP-NEC, to date, the studies reported have not adopted this rationale, and this may potentially explain the predominantly less than favourable results.

Other options for combination with immunotherapy may include peptide receptor radionuclide therapy (PRRT) (Kong & Hicks 2019). However, the use of PRRT in patients with grade 3 gastroenteropancreatic NETs is less well defined (Ezziddin *et al*. 2011). Sorbye *et al*. have advised that PRRT could be considered for patients with increased uptake on somatostatin receptor imaging, in patients with grade 3 gastroenteropancreatic NETs as well as in cases of NEC with a Ki-67 of 21–55% (Sorbye *et al*. 2020). Information on the combination of tyrosine kinase inhibitors with immunotherapy in patients with NEC is lacking and a phase 2 study of pembrolizumab with the multiple kinase inhibitor lenvatinib in previously treated patients with extra-pulmonary (including unknown primary) metastatic poorly differentiated and/or high grade NETs/carcinoma has been suspended (investigator working on amendment) (NCT03290079). There are no trials utilising chimeric antigen receptor (CAR) T-cell therapy recorded on clinicaltrials.gov enrolling patients specifically with a NEC diagnosis to date, and further research on NEC immune mechanisms is required prior to embarking on this treatment pathway.

Future directions

The use of cell lines and patient-derived xenografts (PDX) to predict response to treatment in NEC

Insights into the biology of NEC are crucial for identification of potential therapeutic molecular targets, and cell lines derived from tumour tissue may be helpful. Cell lines have been derived from liver (NEC-DUE1) or lymph node metastases (NEC-DUE2) from patients with large cell gastro-oesophageal junction and large intestine NECs, respectively (Krieg *et al*. 2014). Both cell lines retained malignant potential *in vitro* and *in vivo*. Resistance to chemotherapy such as cisplatin, etoposide and oxaliplatin were exhibited by NEC-DUE1 and -DUE2, but the NEC-DUE1 cell line was sensitive to 5-FU (Krieg *et al*. 2014).

Another study examined the antitumour effects of three chemotherapy agents, cisplatin, etoposide and irinotecan, and their combinations in three small cell GEP-NEC cell lines: a pancreatic NEC (A99), an oesophageal NEC (TYUC-1) and a duodenal NEC (TCC-NECT-2) (Ohmoto *et al*. 2018). The oesophageal cell line was the most susceptible to all agents, whereas the pancreatic NEC cell line was refractory. These preclinical models indicated that cisplatin was a key agent in treatment of NEC and that the cisplatin/irinotecan combination may be a reasonable option, although efficacy was moderate (Ohmoto *et al*. 2018). Whether these tools can enable establishment of novel targeted therapies is unknown, given the differences in cell line sensitivity to chemotherapeutic agents reported.

Shinji *et al*. recently established a novel cell line (SS-2) derived from resection of an ascending colon tumour (Shinji *et al*. 2019). The SS-2 cell line maintained characteristic features of the resected tumour, which were also retained when implanted into the s.c. tissue of

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nude mice, and the authors concluded that these cells may add to the current knowledge of the biological behaviour of midgut NEC and thus may provide a novel therapeutic examination platform (Shinji *et al*. 2019).

Gastric NEC is rich in blood vessels and has highly malignant biological behaviour (Lin *et al*. 2019). In Lin *et al*., immunohistochemistry was used to assess expression of CDK5RAP3 in tumour tissue and adjacent non-tumour tissue from gastric NEC. Cell lines with stable overexpression or knockdown of CDK5RAP3 were developed using lentiviral transfection; protein levels of CDK5RAP3 were reduced in tissue from gastric NEC and low expression correlated with more advanced stage, increased tumour micro-vessel density and poor prognosis. The authors reported that CDK5RAP3 inhibited angiogenesis and thus could be a potential therapeutic target in gastric NEC (Lin *et al*. 2019).

Cell lines may exhibit significant genetic divergence compared to the primary tumour in patients with cancer. The establishment of a PDX model of NEC potentially will recapitulate the heterogeneity of the patient's primary tumour and possess more biological stability of gene expression and mutational status (Tentler *et al*. 2012). A human gastric NEC-derived xenograft model called GA0087 has been previously established, which demonstrates high gene expression of VEGF-A and B and high potential for lung metastasis, and it was concluded by the authors that this may provide a robust platform for cancer research, as well as novel anti-cancer drug development (Jiang *et al*. 2015), but this awaits confirmation.

The emergence of alternative biomarkers

Development of new circulating biomarkers that can inform patient management and facilitate novel drug development are needed. In a single centre prospective study, Khan *et al*. concluded that circulating tumour cells (CTCs), enumerated using CellSearch (Veridex, Raritan, NJ, USA) were promising prognostic markers for patients with NENs (Khan *et al*. 2013). In total, 176 patients were recruited in this study (primary sites: midgut, pancreatic, bronchial and unknown), including 29 patients with G3-NENs (differentiation not reported, nor primary site of those with G3-NENs). Of those, 28% had ≥50 CTCs and 66% had ≥1 CTC. The presence of ≥1 CTC was associated with worse PFS and OS, and on multivariable analysis, CTCs remained significant when the other prognostic markers, grade, tumour burden and chromogranin A, were included (Khan *et al*. 2013). In a follow-up study by the same authors, it was reported that changes in CTCs were associated with response to treatment and OS in metastatic NENs, suggesting that CTCs may be useful as surrogate markers to direct clinical decision making (Khan *et al*. 2016). One hundred and thirty-eight patients with metastatic NENs who were commencing therapy were prospectively recruited in this study; 26 patients had G3 NENs. The best prognostic group were those with 0 CTCs before and after therapy, followed by those with ≥50% reduction in CTCs (HR 3.31), and those with <50% reduction, or increase in CTCs, had the worst outcome (HR 5.07) (Khan *et al*. 2016).

The role of miRNAs (small endogenous non-coding RNAs of 19–25 nucleotides in length that control eucaryotic gene expression post-transcriptionally through inhibition of degradation or translation of specific messenger RNAs (He & Hannon 2004)) as prognostic markers in NENs is not well defined, and prospective studies are required to elucidate their prognostic ability (Zatelli *et al*. 2017).

Genomic subtyping using cell-free DNA (cfDNA) analysis has been successfully reported in 63 patients with pulmonary large-cell NEC and may have potential in prognostication and therapeutic decision making for these patients (Zhuo *et al*. 2020). To date, results using this technology have not been reported in EP-NEC. Tumour-specific genetic alterations in cfDNA of patients with metastatic pancreatic NETs have been reported (Boons *et al*. 2018) and results in EP-NEC are eagerly awaited.

Conclusion

There are many unanswered questions in relation to the most effective prognostic markers and treatment strategies for patients with EP-NEC, both localised and advanced. Eastern Cooperative Oncology Group PS, presence of liver metastases, haematological markers (platelets), biochemical markers such as ALK and LDH, histological markers such Ki-67 and Rb, CTCs and use of chemotherapy may have prognostic value (Khan *et al*. 2013, 2016, Sorbye *et al*. 2013, Lamarca *et al*. 2017, Alese *et al*. 2019).

Preliminary data have reported that the median survival in patients with advanced disease receiving platinum-based chemotherapy up-front is better in patients with small cell lung cancer than in EP-NEC (Terashima *et al*. 2012). A lower rate of alterations in *TP53* and *RB1* were reported in EP-NECs, when compared to small cell lung cancer, and so optimal therapy for EP-NECs may be site specific and different from small cell lung

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cancer (Bergsland *et al.* 2016). A better understanding of the underlying biology of EP-NEC is critical. Better models of this diagnosis are vital, and human NEC-derived xenograft models may provide a unique platform for cancer research, as well as for pre-clinical evaluation of therapeutic efficacy of novel anti-cancer agents. Additionally, there are limited pre-clinical data published on the immune microenvironment of EP-NEC, and further research in this area is necessary.

Whether differential treatment strategies are required for small cell and large cell NECs is uncertain, as is the necessity for individualised primary tumour site therapy, with potential molecular stratification. Collaborative prospective trials with translational correlates are warranted and may inform future biomarker-driven studies.

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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Author contribution statement

M G McNamara developed the review concept and drafted the manuscript. All authors were involved in design, critical revision and approval of the manuscript.

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