UNIVERSITY OF INSUBRIA

DEPARTMENT OF MEDICINE AND SURGERY
PHD in EXPERIMENTAL AND TRANSLATIONAL MEDICINE

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AN ITALIAN MULTICENTRE 2-STEP STUDY IN PATIENTS WITH MANECs OF THE GASTRO-ENTERO-PANCREATIC TRACT TREATED WITH CHEMOTHERAPY

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1. Introduction

1.a History and terminology

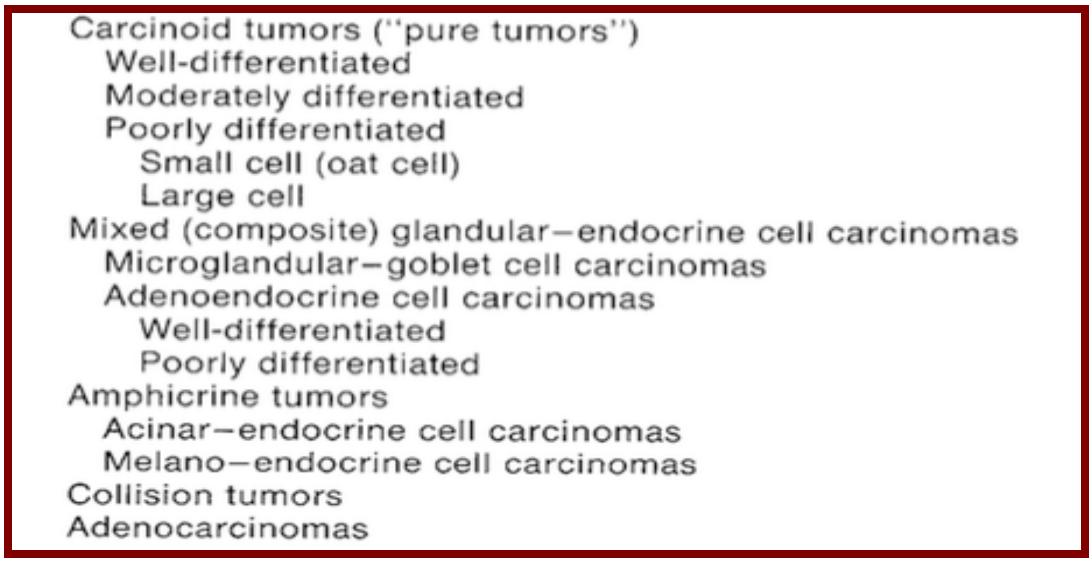
Mixed adeno-neuroendocrine carcinomas (MANECs) represent a group of neoplasms including both an epithelial non-neuroendocrine and a neuroendocrine component in accordance with the 2010 World Health Organization (WHO) classification of tumours of the digestive system (1). The two most important diagnostic criteria are: (i) each component has to comprise at least 30% of the whole tumour taking into account a thresold arbitrarily proposed in 1987 (2, 3), and (ii) both components must be malignant (1).

Although this mixed (non-neuroendocrine/neuroendocrine) feature is frequently observed in routine practice, a true MANEC is rarely found.

The first description of a gastrointestinal tumour involving both the neuroendocrine and exocrine counterpart has been reported by Cordier in 1924(4). Since then, various terms including “composite carcinoid”, “mucin-producing carcinoid”, “argentaffin cell adenocarcinoma”, “goblet cell carcinoid”, “adenocarcinoid”, “small cell undifferentiated carcinoma”, have been used to describe these kind of entities. In 1987 Lewin observed that, between the classic carcinoid and carcinoma, there was also a cluster of distinct tumours, which includes different mixtures of endocrine cells and non-endocrine epithelial cells such as “mucin secreting”, “columnar cells”, “goblet cells”, and “signet ring cells”. Therefore, these lesions have been called “mixed” or “composite” tumours, and have been further divided into three different histologic subtypes: “collision tumours”, “combined tumours”, and “amphicrine tumours” (3)as shown in Figure 1. In accordance with this classification, all tumours containing endocrine and non-endocrine epithelial cells, in whichever amount, could

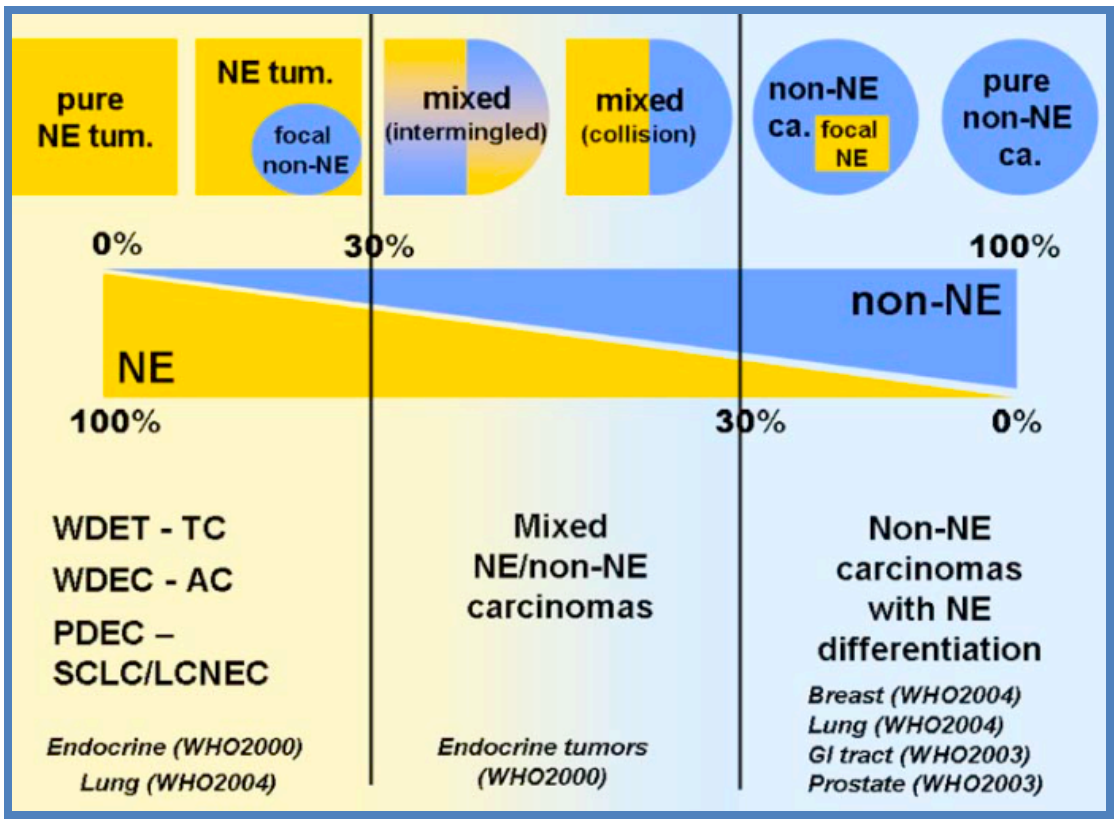
be called “mixed”. However, the authors limited this terminology to those neoplasms in which the endocrine component represented between about a third and half of all cells. They also used the term “amphicrine tumours” for lesions in which endocrine and non-endocrine epithelial differentiation were present within the same cell.

Figure 1. (1987) Nomenclature and classification of pure and mixed endocrine cell tumours(3).



Over the years the use of this varied terminology led to some confusion among clinicians, surgeons, gastroenterologists, and pathologists until the 2000 WHO classification of endocrine tumours(5), where these mixed neoplasms were defined as “mixed exocrine-endocrine tumours”(6). Later on, they were named “mixed adenoneuroendocrine carcinomas” (MANECs) with the 2010 WHO classification of neoplasms of the gastrointestinal (GI) tract(1), that defined the 30% threshold (Fig.2). Therefore neither an adenocarcinoma with scattered immunohistochemistry (IHC) positive neuroendocrine cells, nor a neuroendocrine neoplasm with a focal non-neuroendocrine component can be classified as a MANEC.

Figure 2. Schematic representation of neuroendocrine differentiation in human tumours: *from neuroendocrine neoplasms with a focal exocrine component at one extreme (left) to exocrine carcinomas with interspersed neuroendocrine cells at the other (right). Mixed exocrine-neuroendocrine tumours (middle) are only those neoplasms in which each component represents at least 30% of the lesion* ***(2).***



Legend: ***NE:*** *neuroendocrine;* ***WDET:*** *well differentiated endocrine tumour;* ***TC:*** *typical (lung) carcinoid;* ***WDEC:*** *well-differentiated endocrine carcinoma;* ***AC:*** *atypical (lung) carcinoid;* ***PDEC****: poorly differentiated endocrine carcinoma;* ***LCNEC:*** *large cell neuroendocrine carcinoma;* ***GI:*** *gastrointestinal.*

Moreover, La Rosa et al in 2016(7) reported the most important clinico-pathological and morphological features of the **mixed neuroendocrine-non-neuroendocrine epithelial neoplasms** observed in the pituitary, thyroid, nasal cavity, larynx, lung, digestive system, urinary system, male/female genital organs, and skin, coining the term **MiNEN. This term** was included into the 2017 WHO classification only for pancreatic NEN(8) (9).

It is noteworthy that, the term **MANEC in accordance with the 2010 WHO**

classification (1) still **refers** to the **mixed neuroendocrine-non-neuroendocrine epithelial neoplasms of the digestive system** and not to all the mixed neuroendocrine-non-neuroendocrine epithelial neoplasms. **They are defined as "carcinomas" since both components are histologically malignant but occasionally one or both are low grade**. **Accordingly the rare tumours composed of adenoma and well differentiated neuroendocrine tumour** (NET according to the nomenclature proposed by the 2010 WHO classification) **are not classified into the 2010 WHO classification**.

While in some MANECs the neuroendocrine and exocrine components occur in separate areas of the same lesion **(composite or collision neoplasms)**, in other MANECs they are intimately and diffusely admixed **(combined neoplasms)**. In **amphicrine** tumours exocrine and neuroendocrine features are present in the same neoplastic cell, which shows a divergent immunophenotype.

Goblet cell carcinoids of the appendix are a particular type of mixed exocrine-neuroendocrine neoplasms. In most cases, their neuroendocrine component does not reach 30% and is mostly represented by scattered neuroendocrine cells, therefore they are not included in the present paper. However these neoplasms, based on their particular clinical and biological features, require further investigation to better clarify their histogenesis, molecular profile, and, consequently, a specific classification.

1.b Morphological and prognostic aspects of MANECs

Since the potential various neuroendocrine and non-neuroendocrine histotypes combination, 3 prognostic categories has been previously proposed(6): **high grade**

malignant MANECs (composed of a carcinoma and a NEC component), **intermediate grade malignant** MANECs (including an adenocarcinoma/carcinoma as exocrine component and a well differentiated NET), and **low grade malignant** MANECs (composed of an adenoma and a well differentiated NET).

High grade MANECs

The high grade MANEC is the entity that has been most integrated into the 2010 WHO 2010(1).

This is an aggressive composite or combined MANEC formed by a non-NEC component and a NEC component. **The non-NEC component** is formed by a **carcinomatous** (mainly adenocarcinoma, rarely other variant of ordinary carcinomas linked to the site of origin; acinic in pancreas or squamous in rectum). **The NEC component is represented by poorly differentiated** (small, intermediate or large cell type) **neuroendocrine carcinoma** (NEC). MANECs have been observed in the esophagus(10), stomach(11), ampullary region(12), large bowel(13), and anorectal region(14).

Macroscopically, these neoplasms, independently of the site of origin, appear as polypoid masses or ulcerating stenotic lesions measuring from 0.5 to 14 cm in greatest diameter, with a mean size of about 5 cm. Histologically, the NEC component is morphologically similar to small cell or large cell NEC of the lung and corresponds to a grade 3 neuroendocrine neoplasm, according to the 2010 WHO classification(1) . The small cell component has a diffuse or nesting growth pattern

and is formed by small to intermediate sized cells with scanty cytoplasm and fusiform nuclei with granular chromatin and inconspicuous nucleoli. Diffuse geographic necrosis is common. The mitotic rate is high ranging from 20 to 80 mitotic figures per 10 high-power fields. The large cell component(15), is formed of cells with an abundant cytoplasm showing more vesicular nuclei with prominent nucleoli as well as a more prominent organoid, trabecular and palisading pattern.

As for the immunohistochemical aspects it has been reported that the large or small neuroendocrine cell counterparts are extensively positive for synaptophysin and, although in a lesser way, also for chromogranin A(13, 16, 17). Then, in accordance with the 2010 WHO classification for GEP NEN, the diagnosis of MANEC requires that the NEN counterpart extensively express two neuroendocrine markers (synaptophysin and chromogranin A)(1) and a Ki67 labeling index usually very high (60%–90%)(18). Even though without a clear prognostic relevance, a nuclear accumulation and immunoreactivity for some other proteins such as P53, CDX2, TTF-1, and ASH1 have been observed in NEC(11, 13, 19, 20). Moreover also the CD117 immunoreactivity and vascular invasion have been observed in the NEC component of some colorectal MANECs and these features were associated with a worse prognosis(13).

The non-neuroendocrine component of high grade MANECs can be represented by adenocarcinoma/carcinoma prevalently observed in gastric(20, 21) and colorectal MANECs(13, 15, 22) or, more rarely, by squamous cell carcinoma-like in esophageal and anorectal MANECs (15, 18, 23).

The clinical behavior of high grade MANECs is generally aggressive and the

prognosis depends on biological features(24), and stage(10, 16).

Moreover, given the lack of prognostic and predictive validated factors, some authors suggest that survival differences between MANECs and NECs could depend also on the site of origin(8).

Intermediate grade MANECs

This group include two different tumour categories: **mixed adenocarcinoma-neuroendocrine tumour and amphicrine carcinoma**. In the first the **exocrine** component is represented by an **adenocarcinoma/carcinoma** which can exhibit different degrees of differentiation, while the **neuroendocrine component** is represented by a **well differentiated neuroendocrine** tumour which can show grade 1 (NET G1) or grade 2 (NET G2) differentiation. This entity, since both components are malignant, when referred to the digestive system, is also included in the general group of the so called MANECs. The suffix "NECs" present in the term "MANECs", in this case, should be used carefully although it is in line with the criteria proposed in the 2010 WHO classification of tumours of the gastrointestinal tract(1). In this case the exocrine component is biologically more aggressive than the neuroendocrine one.

The amphicrine carcinoma represents a peculiar tumour in which **exocrine and neuroendocrine features are co-expressed by the same neoplastic cells**, which show a divergent differentiation demonstrable with immunohistochemical or electron microscopic tools(24).

Low grade MANECs or mixed adeno-neuroendocrine tumours ("MANETs")

This group of neoplasms include those consisting of **both a well differentiated neuroendocrine component and an exocrine one which behave in an indolent way**. They represent a particular entity and the term "MANET" has been previously and **provisionally** proposed but **not absorbed nor included in the 2010 WHO classification** (MANETs)(24). From a morphological point of view, these neoplasms appear as polyps ranging from 1.5 to 3 cm in size. While the **exocrine part histologically consists of an adenomatous component with low or high-grade dysplasia**, the **neuroendocrine component is a low-grade neuroendocrine tumour** (NET). In some cases the neuroendocrine cells mixs with the adenomatous glands (combined tumour), while in other cases the neuroendocrine and exocrine components occur in separate areas of the same lesion as separate lesions juxtaposed to one other (collision or composite tumour). In this last case, the polyp appears as a lesion with the neuroendocrine component in the center and the adenomatous component in the periphery of the polyp. Neuroendocrine cells are of small size and form solid nests. They have nuclei with stippled chromatin and they lack significant atypia or mitotic activity. The neuroendocrine component is chromogranin A and/or synaptophysin positive(25, 26). Tumours are generally limited to the mucosa or submucosa and they can be removed by endoscopic polypectomy or trans-anal excision. These morphological characteristics of these rare neoplasms seem to suggest that they are low grade malignant tumours therefore their prognosis seems good because no evidence of tumour recurrence has been found in any of the cases so far reported(25, 26).

1.c Pathogenesis

The pathogenesis of MANECs is still debatable with some controversial histogenic hypotheses still unconfirmed. In accordance with the two most acknowledged theories MANECs may result from either the simultaneous proliferation of multiple cell lineages or the proliferation of stem cells that then differentiate along multiple cell lineages. In fact **the amphicrine entity**, which contains within its cytoplasm both neuroendocrine secretory granules and mucin droplets, **reflects this last hypothesis of a common precursor stem cell capable of divergent differentiation** within an individual neoplastic cell(3, 27).

1.d Recent biological findings and prognosis of MANECs (according to 2010 WHO classification)

In a recent retrospective analysis of 160 surgical samples of the digestive system, the NEC component, in particular the Ki67 index, was the main prognostic driver(28). Therefore the authors found **two main prognostic categories(28) on the basis of Ki67 index (higher or lower than 55%) of the NEC component.** In fact in this regard **Ki67 index of the NEC component showed to be superior to** any other parameter among those **morphological** (prevalence of NEC and non-NEC component, presence of small or large cell in NEC component, histotype), **immunohistochemical** (p53, Rb, Bcl-2, p16, Cdx2, MMR deficiency, TTF1, ALDH, CD117, SSTR2A protein expression, Ki67 index of the non-NEC component) and **molecular** (TP53, KRAS, BRAF mutations) evaluated. Moreover in this analysis, as already observed for pure NEC(29), the survival outcomes were clearly different

between MANEC with Ki67 of the NEC component more or less than 55%. Moreover the overall survival (OS) of these MANECs with > 55% Ki67 in the NEC component seemed to be similar to that of the corresponding pure NECs with Ki67 > 55% [Type C NECs according to Milione et al. 2017(30)]. On the contrary MANECs with < 55% Ki67 showed better survival compared to the pure NECs with 21-55% Ki67 [Type B NECs according to Milione et al 2017(30)].

In the study of Milione et al.(28) the improved survival associated with the expression of Rb, p16, p53, Cdx2, CD117, ALDH, MMR in the NEC component suggests a possible common origin of the two MANEC counterparts(28) from the proliferation of stem cells that go through divergent differentiation during tumour progression. Then some other factors such as tumour stage, subtype (amphicrine and combined), perineural infiltration, high grade budding, gene mutations, and Ki67 of the non-NEC component were significantly associated with survival suggesting a their potential prognostic role(28).

1.e Clinical management of MANECs (according to 2010 WHO classification)

Given the rarity of these entities, the optimal strategy of clinical management of MANECs is largely unknown. In general, when considering treatment, it should be taken into account to prioritise the more aggressive component of MANECs (24, 31).

No specific clinical practice guidelines by Oncological or Neuroendocrine (European or American) Societies have been yet developed worldwide. A chapter on goblet cell carcinoma (which is not within the remit of this dissertation) has been published in

the European Neuroendocrine Tumor Society (ENETS) guidelines some years ago (2012 version)(32). Furthermore, only a limited number of studies on MANEC have been published, mostly retrospective and with a small population.

Some authors maintain that the prognosis of high grade MANECs, **when localized,** is better than that of pure NECs(33) and that they should undergo radical surgery when feasible. This approach has been reported with benefit in terms of survival in patients with pancreatic and colo-rectal MANECs(34, 35). Although some retrospective data seem to suggest a favourable outcomes (10, 36, 37), the role of adjuvant chemotherapy is still undefined and it should be discussed according to patient and tumour features. **When metastatic,** since the NEC component is, almost always, predominant compared to the exocrine part(35), some authors suggest to focus therapy on the neuroendocrine component(24, 31). Otherwise, when metastases contain a prominent adenocarcinoma component, treatment should be similar to that given for "pure" adenocarcinomas(24, 35). In advanced high grade neuroendocrine carcinomas the combination regimen cis-carbo/platinum and etoposide represents the most common chemotherapy proposed(38); it could be offered again if progression occurs more than 3 months from the end of first-line therapy(33). Otherwise the use of combinations of 5-fluorouracil and irinotecan(35) or temozolomide(33) should be considered in some cases. Moreover, as observed in some experiences including patients with NEC from various origins, although extrapolating data from studies on mixed population and not designed to focus on MANECs, the combination of 5-fluorouracil and oxaliplatin(39, 40) should be evaluated. Long-acting somatostatin analogues (SSAs)(41, 42), peptide-radionuclide radiation therapy (PRRT)(43), and biological targeted therapies as everolimus(44,

45), and sunitinib(46) should not be used outside clinical trials in patients with high-grade MANECs as well as "pure" NECs as they have only been studied and approved in low grade NEN populations.

2. Study rationale and study design

2.a Study rationale

The rarity of MANECs, their clinical and biological heterogeneity, the limitation of diagnostic methods, the lack of awareness by researchers, and the lack of uniformity of the available evidence, implicate that a universal shared clinical approach to these neoplasms has not been codified yet. Therefore the real epidemiology and prognosis is not yet precisely known. It is noteworthy that nowadays the median OS of affected patients at any disease stage (or disease stage not specified) varies greatly across the retrospective series ranging between 10 and 78 months(30, 36, 47). So far, in clinical practice, aggressiveness and prognosis of both components drive the treatment choice even if it is not yet known if this type of approach is the most correct.

Therefore, identifying effective therapeutic strategies for MANECs represents an unmet medical need and a major challenge.

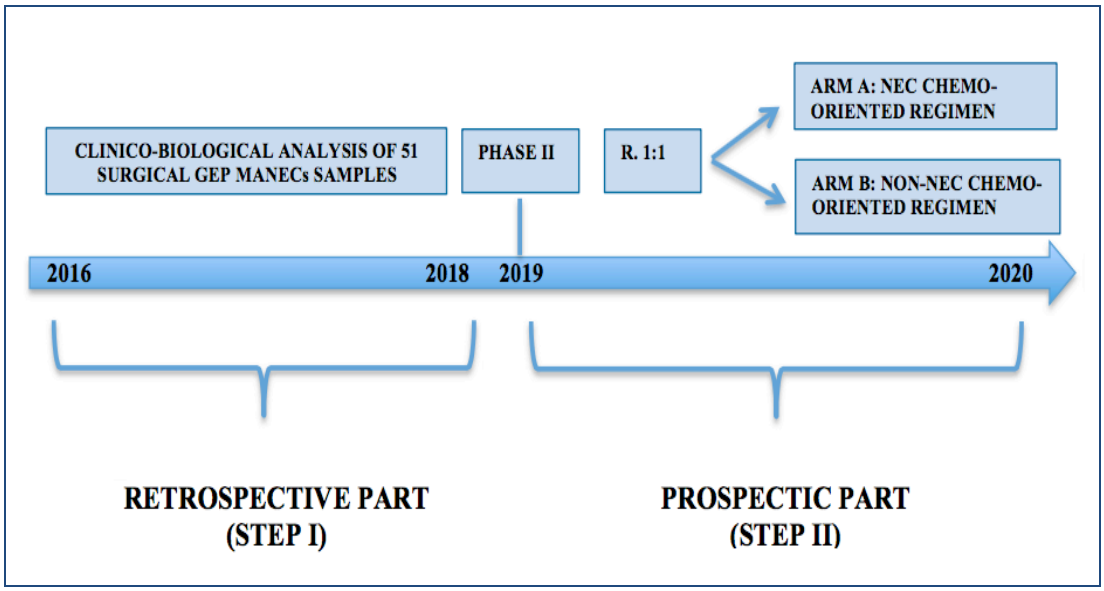
Based on the controversial terminology it should be highlighted that **in this dissertation the author will use the term MANEC to refer to high grade mixed neuroendocrine-non-neuroendocrine carcinomas of the digestive tract as per the 2010 WHO classification(1)**.

2.b Study design

This is an Italian, multicenter, two-step, study, to **retrospectively (step I)** investigate the clinical and biological features of the patients with MANECs of the gastro-entero-pancreatic (GEP) tract treated with chemotherapy, and then to **prospectively (step II)** define the activity and tolerance of two different chemotherapy regimens:

- **Arm A:** NEC-oriented chemotherapy
- **Arm B:** non-NEC-oriented chemotherapy

Figure 3. Study design



The study has been designed in two steps because of the rarity and heterogeneity of MANECs.

Step II is dependent on the results of the retrospective part (step I) which is the real objective of this dissertation.

The step II is planned to be a phase II, 2-arm, multicentre trial aiming to evaluate the

activity and toxicity of 2 regimens of therapies, NEC-oriented and non NEC-oriented as above mentioned, in patients with GEP-MANECs. As a biological point of view morphology and Ki67 of the NEC and non NEC and also somatostatin receptors 2A (SSTR2A) using specific antibodies, mismatch repair protein (MMR) deficiency, will be evaluated. Then, as exploratory analyses, data concerning mutations of RAS, BRAF, TP53 will be detect by PCR pyrosequencing(48) and Next Generation Sequencing (NGS)(49).

3. Material and methods

3.a Search strategy

A comprehensive literature search was designed and conducted by an experienced professional medical librarian (W.R.) with input from the study investigators. We searched the electronic databases Ovid MEDLINE, Embase, Scopus and the Cochrane Library. Various combinations of database-specific controlled vocabulary (subject headings) were used, supplemented by keywords, title and abstract terms for the concepts and synonyms relating to MANEC, mixed adenoneuroendocrine carcinoma, MiNEN and mixed neuroendocrine-non-neuroendocrine neoplasms.

Bibliographies of relevant papers were examined and citing articles were identified using Clarivate Web of Science. Populations searched were limited to Western Countries. Language was restricted to English, and no date restrictions were applied.

The full search strategies employed are reported in the Appendix.

The investigators identified papers eligible for further review by examination of abstracts and titles. If a paper was deemed relevant, the full-text version was

obtained and reviewed, applying the appropriate inclusion and exclusion criteria **(Fig. 4a, 4b, 4c) (Appendix).**

Selection criteria

Clinical and pathological reviews and retrospective papers on mixed neuroendocrine and non-neuroendocrine tumours conducted over the last fifty years were included. Any systemic antitumour therapy and treatment line was admitted. Case reports and case series with less than 10 patients were excluded. Congress abstracts were excluded as well.

Two independent authors (F.S. and M.M.) assessed the eligibility of publications.

3.b Case selection and sample size

Case selection

The clinical case selection of this retrospective study started from the same pathological sample size of a recent previous analysis on 160 surgical samples already published by Milione et al.(28) of which this work is the corresponding clinical retrospective conclusion.

At beginning the surgical pathology and clinical databases of eleven Italian institutions were retrospectively searched between 1995 and 2016, and patients with one of the following diagnoses at histology report sign-out were selected: ‘mixed exocrine-neuroendocrine carcinoma’, ‘adenoneuroendocrine carcinoma (MANEC)’, ‘composite glandular endocrine carcinoma’, ‘carcinoma with neuroendocrine differentiation’, ‘amphicrine/combined, carcinoma or tumour’, ‘mixed adenocarcinoma and neuroendocrine carcinoma’. **Exclusion criteria** were (i) cases

with **only biopsy material available**; (ii) cases with **either NEC or non-NEC component <30%**; (iii) cases in which **the neuroendocrine component was well differentiated** (24).

Sample size

At the beginning a total of 200 candidate cases were identified. Thereafter the case selection established the MANEC diagnosis pathological confirmation. Therefore, the patients' charts and tumour morphology were carefully revised, first by the pathologist of the case-proposing hospital and then by a panel of seven expert pathologists (M.M., A.P., P.S., A.V., L.A., S.L.R. and C.C.) using a multihead microscope. During panel consensus meetings, the original diagnosis was reviewed and further workup was carried out whenever panelists disagreed or quantitative evaluations approached cut-off values. For qualitative parameters a majority decision was adopted, while for quantitative evaluations, the mean of values obtained by the individual panelists was taken as final. MANEC identification, quantitative evaluation of the NEC vs non-NEC components and subtype characterization as collision or combined were based on parallel investigation of at least two consecutive sections from representative blocks, stained with hematoxylin-eosin and synaptophysin, respectively. Ki67 proliferative rate (or other histochemical parameters investigated) was assessed on a third consecutive section. The identification of an amphicrine component was based on finding synaptophysin reactivity within the cytoplasm of cells also showing signet ring or gland-forming patterns after alcian blue counterstaining of the same section or with the help of an adjacent section stained with PAS and alcian blue. At the end, 160 tissue samples

met all the above criteria and were used to start the clinical selection.

The clinical data collection required a rigorous retrospective search among the clinical databases of those eleven Italian institutions. It was performed between 2016 and 2018 and it has been coordinated by a clinical researcher (F. S.), deeply involved in neuroendocrine clinical and research activity. Clinical inclusion criteria were: (i) MANEC diagnosis according with 2010 WHO classification; (ii) primary site from the gastro-entero-pancreatic (GEP) tract; (iii) patients underwent primary site surgery; (iv) availability of follow up information. Goblet cells neoplasms were not included. Other relevant clinical information were: (i) functional status (SSTR2A expression and FDG uptake status) at diagnosis (surgery); (ii) stage at diagnosis (locally advanced or metastatic); (iii) metastases timing (synchronous or metachronous); (iv) timing of chemotherapy received (adjuvant, pre-operative, perioperative, palliative); (v) type of chemotherapy received (regimen); (vi) toxicity; (vii) biochemical expression of specific markers (CEA, CA 19.9, NSE).

The main barriers into collecting clinical data were: (i) second pathological opinions without clear clinical information; (ii) second clinical opinions without clear follow up information; (iii) incomplete clinical traceability due to the lack of recording tools in the analysed period.

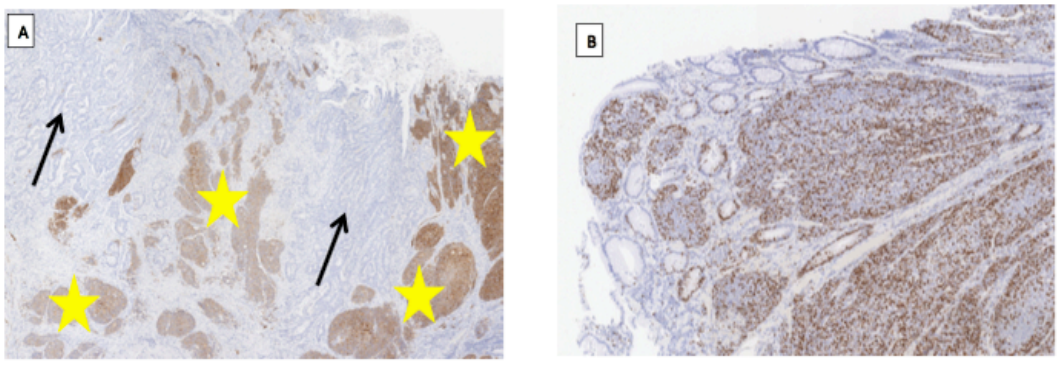
At the end, among the 160 patients with pathological centralised diagnosis of GEP-MANEC above mentioned, 51 patients met all the above clinical criteria and were enrolled in the study: (i) 27 from Istituto Nazionale dei Tumori IRCCS (Milano); (ii) 7 from Istituto Clinico Humanitas IRCCS (Rozzano, Milano); (iii) 11 from Ospedale di Circolo (Varese); (iv) 6 from Istituto Europeo di Oncologia IRCCS (Milano).

An expert pathologist (M.M.), already involved in the previous pathological study, reviewed again the 51 surgical samples to confirm biological features and homogenize the approach.

Since this study is part of a specific research line in which the clinical work represents the consequent conclusion of the previous pathological analysis(28) it has been assumed that the Ethical Committee of Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy) approval (n° INT 21/16) still counted including the present clinical study that was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki.

3.c Histologic and immunohistochemical analyses (Fig. 5A, 5B)

Figure 5A, 5B. MANEC, Immunohistochemical definition.



MANEC, Immunohistochemical definition: **A (synapthophysin)** Mixed colonic neoplasm stained by synapthophisin (syn); both component adenocarcinoma (arrows) negative for syn and NEC (stars) positive for syn in 100% of neoplastic cells, represent more than 30% of whole neoplasm. NEC component occupy the deeper part of neoplasm. **B:** nuclear staining for Mib-1/Ki67 is extensively diffuse to the majority of nuclei in the NEC component reaching a Ki67 labelling index of 80%

Morphologic analysis considered: (a) assessment of the percentage of NEC and non-

NEC components; (b) morphology of non-NEC component: adenoma, adenocarcinoma, mucinous carcinoma, signet ring carcinoma, squamous cell carcinoma and acinar cell carcinoma (only in pancreatic site)(24); (c) morphology of NEC component: small cell or large cell according to WHO 2010(1); (d) necrosis in the NEC component; (e) Ki67 index was defined using the MIB antibody as a percentage of 500–2000 cells counted in areas of strongest nuclear labeling ('hot spots')(50); (f) mitotic count (MC) was evaluated in at least 10 HPF (10 HPF = 2 mm^2)(50); (g) quantitative assessment of NEC, non-NEC or mixed type components in lymph node metastases and/or in distant metastases; (h) tumour staging according to the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) 8th edition(51); (i) lymphovascular invasion (evaluated on both hematoxylin-eosin (H&E) and/or CD31-stained sections); (l) perineural invasion; (m) intra and/or peritumoural lymphocytic infiltration; (n) prevalent tumour component (NEC, non-NEC or mixed type) on the deep invasive front; (o) type of combination of the NEC and non-NEC component: 'collision' when the two components were clearly demarcated, 'combined' when they were intimately admixed and 'amphicrine/combined', when the same cells displayed both neuroendocrine and non-neuroendocrine phenotype (as a rule this was observed in a combined histological background and (p) tumour budding defined according to the International Tumor Budding Consensus Conference 2016 (ITBCC)(52).

The histochemical and immunohistochemical (IHC) study included: (a) Alcian Blue-Periodic Acid-Schiff (PAS) to better define mucin production in the non-NEC epithelial neoplastic component; (b) synaptophysin and chromogranin-A (general neuroendocrine immunomarkers) in order to confirm the presence and extent of the

NEC component (Fig. 1B); (c) Ki67 staining evaluated in both NEC and non- NEC components (1); (d) IHC assessment and evaluation in both components of several markers including p53, mismatch repair (MMR) proteins, and somatostatin receptor 2A (SSTR2A) using specific antibodies.

p53 was considered positive when ≥30% of cells were positive(53); SSTR2A was assessed according to Volante *et al*. (positive: 2+, 3+; negative: 0, 1+ score) (54). MMR deficiency was established according to the criteria reported by Chiaravalli *et al*.(55).

3.d Biomolecular analysis

Data concerning mutations of KRAS (codons 12 and 13), BRAF (codon 600), and TP53 (exons 5–8) were extracted from investigations performed for therapy decision making, either by PCR pyrosequencing (56) or by next-generation sequencing analysis (NGS) (49).

4.e Statistical analysis

Data were analysed by descriptive statistics. Differences in frequencies were assessed with the chi-square or the Fisher exact test. The primary study endpoint was the correlation of OS with primary tumour site, tumour stage, NEC subtype (large or small cell), non-NEC histotype, MANEC type (collision, combined, amphicrine/combined), percentage of NEC and non-NEC components (evaluated on: whole neoplasm, invasive front, lymph nodes and/or distant metastases), lymphocytic intratumoural and peritumoural infiltrate, angioinvasion, perineural

invasion and necrosis in the NEC component. The following parameters were separately evaluated in NEC and non-NEC components: MC, Ki67, MMR deficiency, p53 and SSTR2A.

Overall survival was assessed from the time of diagnosis to the time of death or last follow-up. Survival curves were drawn according to the Kaplan–Meier method, and difference between groups was assessed with the log rank test. The proportions of patients surviving at different time points are presented with respective 95% CI. Univariable and multivariable Cox proportional hazards regression analysis was used to assess the prognostic significance of various clinical and histopathologic characteristics. Data analysis was performed using the SAS software (version 9.4, Cary, NC, USA). All tests were two sided and P values <0.05 were considered statistically significant.

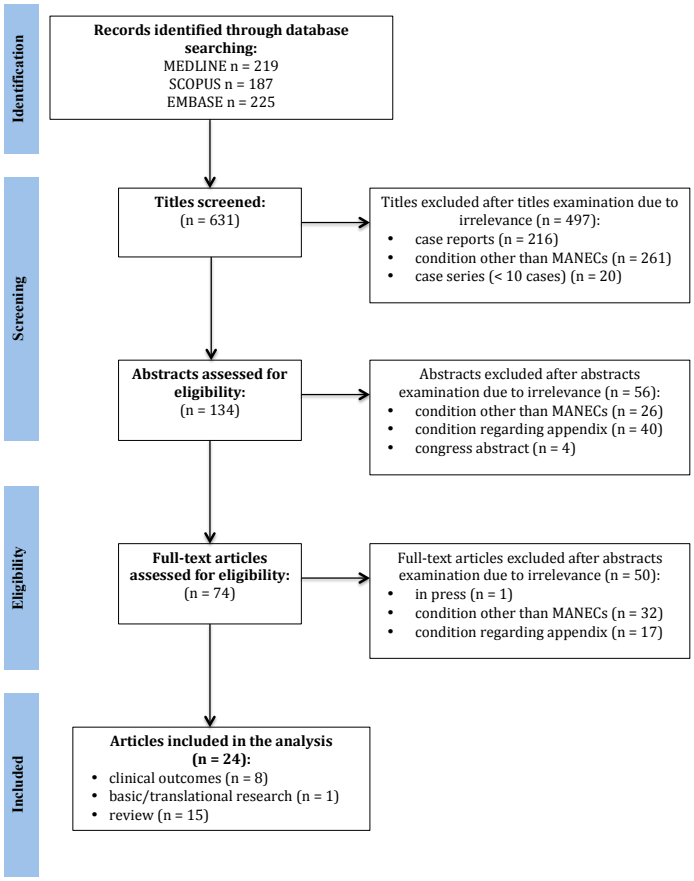
4. Results

4.a Selected studies

The systematic search initially identified 631 articles (Medline: 219 papers; Embase 187 papers; Scopus 225) published between January 1st 1974 and August 1st 2018.

The PRISMA flow diagram (Fig. 4) shows the article selection process. At the end of the research, 24 papers on GEP-MANECs were finally analysed and used as background of this analysis. Publications types included reviews (n = 15)
(2-4, 6, 7, 13, 15, 23, 24, 31, 35-37, 57, 58), retrospective studies (n = 8)(10, 11, 16, 18, 28, 59-61), and basic/translational research (n = 1)(27).

Figure 4. PRISMA flow diagram. Flowchart of study selection process.



4.b Patients features

The main clinicopathologic features of the 51 patients enrolled in the retrospective step of this study are summarized in the Table 1. The series comprised MANECs from 3 main primary sites: **colorectal** (*n* = 32, 63%), with a prevalence in the right colon (23 cases) vs. the left colon (2 cases) and rectum (7 cases), followed by **gastroesophageal** (*n* = 14, 27%) locations, with a prevalence (9 cases) for stomach vs. gastro-esophageal junction (3 cases) and esophageal tumours (1 case). This group did include also 1 duodenal case. **Pancreatobiliary** locations were the least common (*n* = 5, 10%), the majority of these were pancreatic tumours (4 cases) compared to

Ampulla of Vater (1 case). No MANECs were found in the small bowel. The series comprised mainly patients 60-69 years old (*n* = 20, 39%) with colorectal MANECs among all the age ranges evaluated (< 60 years old; 60-69; 70+). There were more males than females (62% vs 37%), and this difference was maintained across tumour sites although not statistically significant (*P* = 0.61). Most neoplasms (74%) were locally advanced (*n* = 38, 74.5%) at diagnosis (surgery) in all the 3 groups of patients. Among the 51 evaluable patients 36 (71%) developed metastases: (i) 12 (24%) synchronous, (ii) 24 (47%) metachronous; liver alone (*n* = 17, 33%) was the most frequent metastatic site. Metabolic imaging by 18-FDG PET/CT was done only in about one third of the whole population (*n* = 14, 27%) (Table 1).

Most MANECs (*n* = 37, 72%) had a NEC component $\geq$ 50% (Table 1) and the large cell type (*n* = 28, 55%) was more frequent compared to the small or intermediate cell type. Thirty-one (61%) tumours showed a 'combined' pattern, 11 (22%) a purely 'amphicrine' pattern, and 9 (18%) a 'collision' pattern (Table 1).

In the non-neuroendocrine component the conventional adenocarcinoma was the dominant histotype (*n* = 29, 57%) (Table 1).

Angioinvasion was seen in all MANEC cases. Neoplastic emboli in vessels were prevalently of NEC or mixed NEC/non-NEC type. Nodal metastases were of pure NEC histotype in 9 (18%) and mixed NEC/non-NEC in 36 (71%) cases. In distant metastases, pure NEC histology was found in 5 (10%) cases, and mixed NEC/non-NEC histology in 2 cases (4%). Pure non-NEC component was not detected in metastases (Table 1).

Focusing only on the NEC component of the 51 MANECs, the proliferation index

evaluated with Ki67 was < 55% in 13 (25.5%) patients, like the so called type B NEC entity (32), and ≥ 55% in 38 (74.5%) as the type C NEC entity(30). Then, while the mitoses [evaluated by mitotic index (MI)], in the neuroendocrine component were mostly in a intermediate range (30-49) (*n* = 21, 41%), in the non-neuroendocrine component they were predominantly (*n* = 23, 45%) in a lower range (0-29).

Biochemical tumour markers more used in the clinical management of non-neuroendocrine carcinomas which are the carcinoembryonic antigen (CEA) and the carbohydrated antigen 19. 9 (CA 19.9), and the marker more often used in clinical practice in the monitoring of high grade neuroendocrine neoplasms which is the neurono-specific antigen (NSE), were evaluated in available patients. Among them, NSE more than 5 ng/mL (normal < 15 ng/mL) was statistically significant (*P* = 0.003).

Biomolecular analysis were performed in 43 (84%) patients in the overall population and specific mutations of KRAS, BRAF, and TP53 were observed in 22 (43%) patients (Table 1).

Table 1. Main characteristics of patients with MANEC according to tumour site

		All N (%)	Colorectal N	Gastroesophageal N	Pancreatobiliary N	P-value
All		51 (100)	32	14	5	
Center						
	Milan, INT	27 (52.9)	21	4	2	
	Milan, Humanitas	7 (13.7)	3	2	2	
	Varese	11 (21.6)	6	5	.	
	Milan, IEO	6 (11.8)	2	3	1	0.065
Age at diagnosis						
	<60	16 (31.4)	9	5	2	
	60-69	20 (39.2)	12	5	3	
	70+	15 (29.4)	11	4	.	0.66
Year of surgery						
	2000-2004	11 (21.6)	7	3	1	
	2005-2009	7 (13.7)	3	4	.	
	2010-2014	22 (43.1)	15	4	3	
	2015-2016	11 (21.6)	7	3	1	0.68
Gender						

Male	32 (62.7)	21	9	2	
Female	19 (37.3)	11	5	3	0.61
Stage at diagnosis					
Locally advanced	38 (74.5)	22	11	5	
Metastatic	13 (25.5)	10	3	.	0.38
Metastases					
Sinchronous	12 (23.5)	9	3	.	
Metachronous	24 (47.1)	14	5	5	
Non metastatic	15 (29.4)	9	6	.	0.19
Site of metastasis					
Hepatic	17 (33.3)	12	4	1	
Extra-hepatic	12 (23.5)	8	2	2	
Hepatic + extra-hepatic	6 (11.8)	3	2	1	
n/a	1 (2)	.	.	1	0.39
68-Gallium PET/CT /Octreoscan®					
Not done	30 (58.8)	20	7	3	
Positive	4 (7.8)	2	1	1	
Negative	2 (3.9)	1	.	1	0.39
18-FDG PET/CT					
n/a	13 (25.5)	7	6	.	
Not done	24 (47.1)	15	5	4	
Positive	12 (23.5)	8	3	1	
Negative	2 (3.9)	2	.	.	1.00
Pre-operative therapy					
No	40 (78.4)	26	9	5	
Yes	11 (21.6)	6	5	.	0.21
CBDCA+VP16+Radiotherapy	2 (3.9)	*2*	.	.	
CDDP+5-FU	1 (2)	.	*1*	.	
CT + RT	1 (2)	*1*	.	.	
ECF	1 (2)	.	*1*	.	
FOLFOX	2 (3.9)	*1*	*1*	.	
FOLFOX4+AVASTIN	1 (2)	*1*	.	.	
GEMOX	1 (2)	.	*1*	.	
XELOX	2 (3.9)	*1*	*1*	.	
Best response					
CR	1 (2)	.	1	.	
PR	4 (7.8)	3	1	.	
SD	4 (7.8)	2	2	.	
PD	1 (2)	.	1	.	
Too early	1 (2)	1	.	.	0.77
Adjuvant therapy					
No	37 (72.5)	25	8	4	
Yes	14 (27.5)	7	6	1	0.32
Regimen					
CF	1 (2)	.	1	.	
CRT: FOLFOX+RT	1 (2)	1	.	.	
EAP+SSA	1 (2)	.	1	.	
FOLFOX	3 (5.9)	1	1	1	
FOLFOX4	1 (2)	1	.	.	
FU/FA	1 (2)	.	1	.	
GEMOX	1 (2)	.	1	.	
XELOX	4 (7.8)	3	1	.	
XELODA	1 (2)	1	.	.	0.76
Relapse					
No	23 (45.1)	17	6	.	
Yes	28 (54.9)	15	8	5	0.09
First line treatment (metastatic)					
No	28 (54.9)	19	8	1	
Yes	23 (45.1)	13	6	4	0.27
Number of cycles					
1-2	10 (19.6)	6	2	2	
3-5	5 (9.8)	4	1	.	
6+	8 (15.7)	3	3	2	0.69
Best response					
PR	3 (5.9)	1	1	1	
SD	4 (7.8)	2	1	1	
PD	15 (29.4)	9	4	2	
Too Early	1 (2)	1	.	.	0.97

2nd line treatment (metastatic)					
No	16 (31.4)	9	5	2	
Yes	7 (13.7)	4	1	2	0.70
Number of cycles					
1-2	3 (5.9)	2	1	.	
6+	4 (7.8)	2	.	2	0.43
Best response					
SD	3 (5.9)	1	.	2	
PD	4 (7.8)	3	1	.	0.26
3rd line treatment (metastatic)					
No	5 (9.8)	2	1	2	
Yes	2 (3.9)	2	.	.	0.62
Number of cycles					
1-2	2 (3.9)	2	.	.	
Best response					
SD	1 (2)	1	.	.	
PD	1 (2)	1	.	.	
CEA					
<2	10 (19.6)	8	1	1	
2	17 (33.3)	8	7	2	
3	10 (19.6)	6	4	.	
4 +	14 (27.5)	10	2	2	0.38
CA 19.9					
<3	17 (33.3)	8	7	2	
3-9	23 (45.1)	16	5	2	
10+	11 (21.6)	8	2	1	0.56
NSE					
<3	9 (17.6)	4	3	2	
3	16 (31.4)	8	8	.	
4	16 (31.4)	15	.	1	0.003
5 +	10 (19.6)	5	3	2	Trend 0.23
Ki67 NEC					
<55%	13 (25.5)	7	4	2	
≥55%	38 (74.5)	25	10	3	0.70
Ki67 non-NEC					
<55%	20 (39.2)	13	4	3	
≥55%	31 (60.8)	19	10	2	0.51
% NEC component					
<50%	14 (27.5)	10	3	1	
≥50%	37 (72.5)	22	11	4	0.89
% non-NEC component					
<50%	26 (51)	15	8	3	
≥50%	25 (49)	17	6	2	0.76
NGS					
Not performed	8 (15.7)	5	3	.	
Wild type	21 (41.2)	10	6	5	
KRas+ Nras	5 (9.8)	4	1	.	
BRAF	1 (2)	1	.	.	
TP53	13 (25.5)	10	3	.	
Kras	3 (5.9)	2	1	.	0.63
Stage					
IIB	1 (2)	1	.	.	
IIIA	4 (7.8)	3	1	.	
IIIB	35 (68.6)	19	11	5	
IV	11 (21.6)	9	2	.	0.56
% NEC LYMPHNODE METASTASES					
<100% (Mixed)	36 (70.6)	20	12	4	
100% (Pure NEC)	9 (17.7)	7	1	1	
N0	6 (11.8)	5	1	.	0.53
% NEC DISTANT METASTASES					
<100% (Mixed)	2 (3.9)	1	0	1	
100% (Pure NEC)	5 (9.8)	3	4	1	
M0	44 (86.3)	27	13	4	0.43
p53 NEC					
n/a	1 (2)	1	.	.	
0	16 (31.4)	12	2	2	
<50	10 (19.6)	5	4	1	
≥50	24 (47.1)	14	8	2	0.49
P53 non-NEC					
n/a	1 (2)	1	.	.	
0	26 (51)	18	5	3	

<50	19 (37.3)	10	7	2	
≥50	5 (9.8)	3	2	.	0.66
MMR					
Not stable	2 (3.9)	1	1	.	
Stable	49 (96.1)	31	13	5	0.62
NEC MITOSIS					
0-29	15 (29.4)	8	4	3	
30-49	21 (41.2)	15	5	1	
50+	15 (29.4)	9	5	1	0.63
Non-NEC MITOSIS					
0-29	23 (45.1)	13	7	3	
30-49	16 (31.4)	9	6	1	
50+	12 (23.5)	10	1	1	0.42
TYPE OF MANEC					
Small cells	11 (21.6)	7	2	2	
Large cells	28 (54.9)	17	10	1	
Intermediate cells	12 (23.5)	8	2	2	0.36
TYPE OF NON-MANEC					
Adenoma	4 (7.8)	4	.	.	
Adenocarcinoma NAS	29 (56.9)	16	9	4	
Squamous Carcinoma	3 (5.9)	2	1	.	
Acinic Carcinoma	1 (2)	.	.	1	
Mucinous Carcinoma	6 (11.8)	4	2	.	
Signet Ring Carcinoma	2 (3.9)	1	1	.	
Amphicrin Carcinoma	4 (7.8)	4	.	.	
Adenosquamous	2 (3.9)	1	1	.	0.58
BUDDING					
Absent	5 (9.8)	1	4	.	
Mild	12 (23.5)	10	1	1	
Moderate	13 (25.5)	8	4	1	
Severe	21 (41.2)	13	5	3	0.15
SSTR2A					
Negative	27 (52.9)	16	10	1	
Positive 1+	9 (17.6)	7	2	.	
Positive 2+	9 (17.6)	6	1	2	
Positive 3+	6 (11.8)	3	1	2	0.18
Peritumoural infiltration					
Absent	15 (29.4)	9	4	2	
Mild	25 (49)	14	8	3	
Moderate	11 (21.6)	9	2	.	0.72
Intratumoural infiltration					
Absent	20 (39.2)	11	6	3	
Mild	28 (54.9)	20	6	2	
Moderate	3 (5.9)	1	2	.	0.38
HISTOTYPE INVASIVE TUMOUR FRONT					
Non-NEC	3 (5.9)	1	1	1	
NEC	17 (33.3)	12	4	1	
Non-NEC+NEC	31 (60.8)	19	9	3	0.56
Type of MANEC					
Collision	9 (17.6)	7	1	1	
Combined	31 (60.8)	17	11	3	
Amphicrin	11 (21.6)	8	2	1	0.60

Legend: n/a: not available; **CBDCA:** carboplatin**; CDDP:** cisplatin**; CT:** chemotherapy not otherwise specified; **ECF:** epirubicin+cisplatin+5-fluorouracil**; FOLFOX:** 5-fluorouracil+leucovorin+oxaliplatin; **GEMOX:** gemcitabine+oxaliplatin; **CR:** complete response**; PR:** partial response; **SD:** stable disease; **PD:** progression disease; **CF:** cisplatin+5-fluorouracil; **SSA:** somatostatin analogs; **FU/FA:** 5-fluorouracil+leucovorin; **NGS:** next generation sequencing; **MMR:** mismatch repair protein; **SSTR2A:** somatostatin receptor type 2A; **MANEC:** mixed adenoneuroendocrine carcinomas; **NEC**: neuroendocrine carcinoma.

4.c Clinical outcomes

Locally advanced stage

Eleven patients (22%) underwent pre-operative therapy (5 with NEC-oriented regimens and 7 with non-NEC oriented regimen), 14 (27%) adjuvant (only one with a NEC-oriented regimen), and 6 (12%) perioperative chemotherapy (Table 1). In the first group the authors observed 4 (8%) partial responses (PR), 4 (8%) stable disease (SD), one (2%) progression of disease (PD). Eight out of 14 (57%) patients who underwent adjuvant chemotherapy, and 3/6 who received both pre-operative and adjuvant chemotherapy, relapsed.

Metastatic stage

Twenty-three patients (45%) (some metastatic at diagnosis and some relapsed after adjuvant therapy) received first line therapy with a median of 1-2 cycles in 20% patients, 3-5 in 10%, and more than 61 in 6%. Thirteen patients received fluoropyrimidines/oxaliplatin (57%), 1/23 (4%) peptide receptor radionuclide therapy), radiotherapy plus or minus combined with paclitaxel 2/23 (9%), capecitabine/temozolomide 1/23 (4%), cetuximab or bevacizumab as monotherapy 2/23 (9%), fluoropyrimidines/irinotecan 1/23 (4%), gemcitabine 2/23 (9%), and 1/23 (4%) metronomic capecitabine. The overall response rate (ORR = CR+PR+SD) of the global population after first line therapy was 14%. Fifteen out of 23 (30%) patients had PD (Table 1).

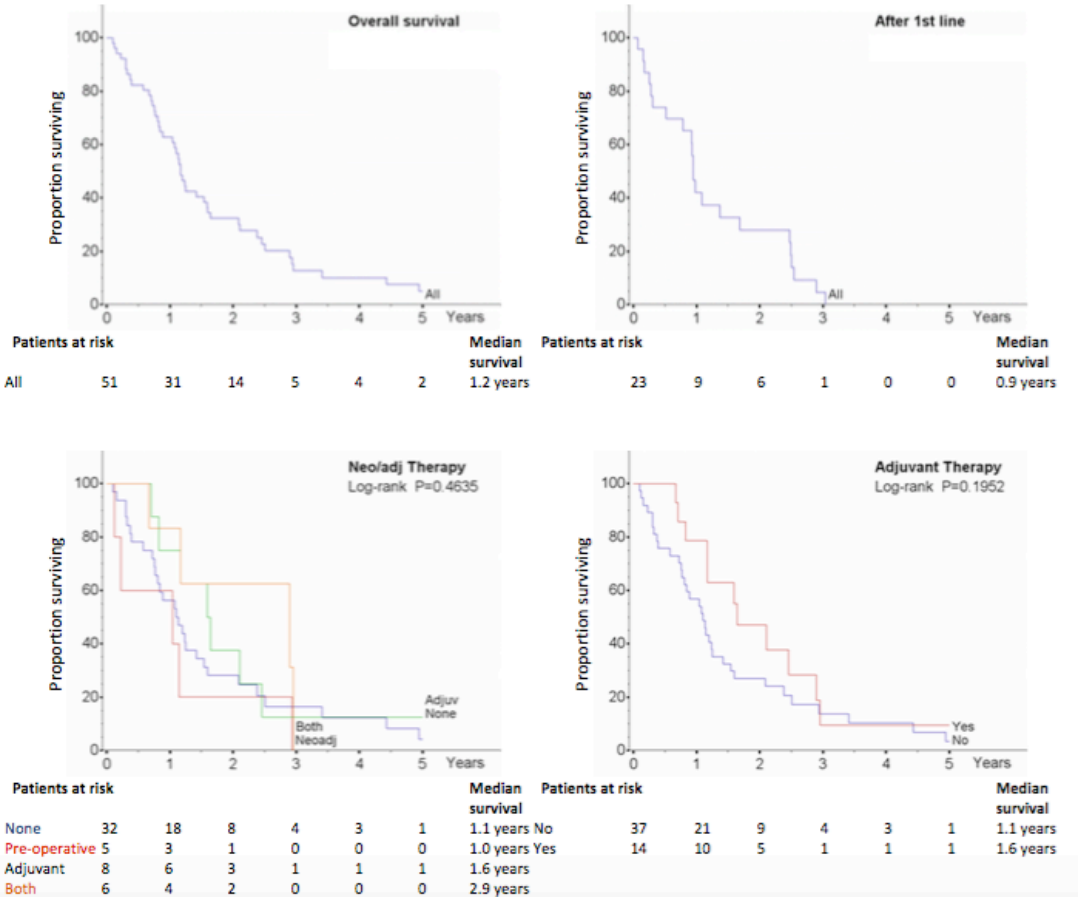
Only 7 patients underwent a second line therapy: 1/7 (14%) cisplatin/5-fluorouracil, 1/7 (14%) fluoropyrimidines/oxaliplatin, 1/7 (14%) PRRT, 1 (14%) irinotecan/cetuximab, 3/7 (43%) not known. There were 6% SD, and 4 (8%) PD.

4.d Survival analysis

Systemic therapy

In the whole cohort, the median OS was 1.2 years (95% CI 0.8-1.6) (Fig. 6A Overall survival), 1.6 years for patients who underwent adjuvant therapy ($P = 0.20$), 1.0 year for those who received neo-adjuvant therapy, 2.9 years for those who received peri-operative therapy ($P = 0.46$), and 0.9 years for patients who received first line therapy (Fig. 6D, C, B).

Figure 6 (A, B, C, D). Overall survival of patients with GEP-MANECs



Legend: Overall survival of the whole 51 GEP-MANECs population (A), of GEP-MANECs during first line therapy (B) or peri-operative therapy (C) or adjuvant therapy (D); Neo/adj: refers to

preoperative/adjuvant therapy.

Proliferation

A preliminary evaluation of the NEC component showed 55% to be an optimal prognostic cut-off for Ki67 index (Fig.7A) and it was ≥55% in the large majority (74.5%) of cases. On the other hand, in the non-NEC component, Ki67 was ≥55% in 61% of cases (Fig.7B).

Patients with Ki67 index <55% in the NEC component (median 2.9 years; 95% CI 2.4-3.4) had a significatively longer OS than those with Ki67 ≥ 55% (median 1.0; 95% CI 0.7-1.2) (*P* = 0.0004). The latter showed a hazard ratio (HR) of 3.61 (95% CI 1.69–7.72) vs Ki67 < 55% (*P* = 0.0009) after adjustment for tumour site, which retained high significance at multivariable analysis (Fig. 7A and Table 2).

Patients with mitotic count (MC) ≥50 mitoses/10 HPF vs <50 mitoses/10 HPF had hazard ratio (HR) of 1.81 (95% CI 0.96–3.44) (*P = 0.07*) after adjustment for tumour site but they revealed high significance at multivariable analysis (Table 2).

In the non-NEC component, patients with Ki67 index <55% (median 1.2 year months; 95% CI 0.8-3.4) had a slightly longer OS than those with Ki67 ≥ 55% (median 1.1; 95% CI 0.8-1.5) although not significantly (*P* = 0.17) (Fig. 7B). Moreover a Ki67 index of ≥55% was associated with a tumour site-adjusted HR of 1.42; 95% CI 0.75–2.71 (*P* = 0.29) vs <55%, while a MC ≥ 50/10 HPF lacked any significant difference compared to MC<50/10 HPF (Table 2).

Figure 7. Overall survival of patients with GEP-MANECs according to Ki67 (NEC and non-NEC)

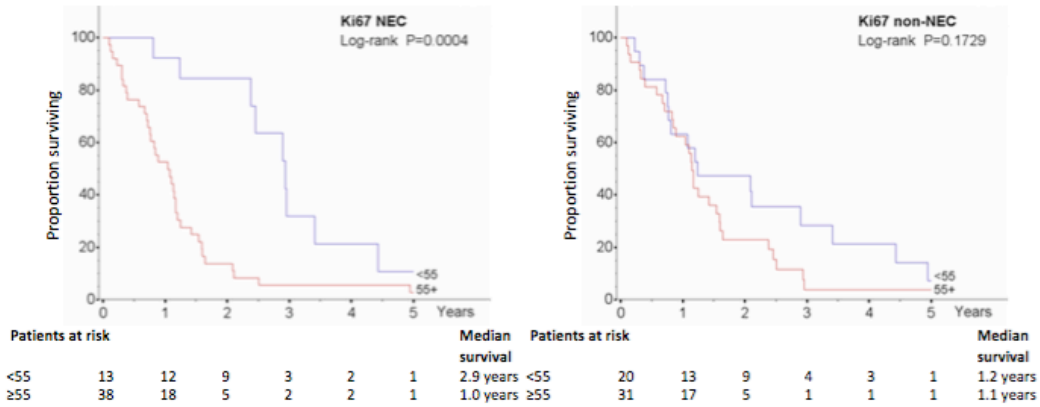
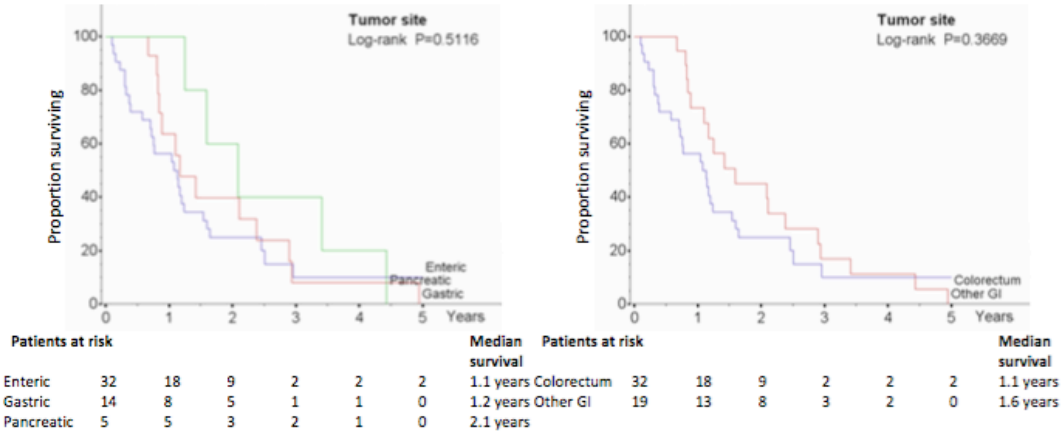


Figure 7 A (left), B (right)

Primary tumour site

Overall survival according to Ki67 in the NEC counterpart was shorter in patients with colorectal tumours (median 1.1 years; 95% CI 0.6-1.5) compared to patients with tumours in other digestive system sites: gastroesophageal (median 1.2 years; 95% CI 0.8-2.4) or pancreatobiliary (median 2.1 years; 95% CI 1.2-4.4) (Fig. 7 C, D and Table 2). The latter had a hazard ratio (HR) of 0.57 (95% CI 0.22–1.50) after adjustment for tumour site, which revealed high significance at multivariable analysis model[3] (which retains only variables showing an association ($P < 0.10$) with OS) with a HR of 0.25 (0.08-0.83) ($P = 0.02$) (Fig. 7C, D and Table 2).

Figure 7. Overall survival of patients with GEP-MANECs according to Ki67 (NEC and non-NEC)



Patients at risk							Median survival
Enteric	32	18	9	2	2	2	1.1 years
Gastric	14	8	5	1	1	0	1.2 years
Pancreatic	5	5	3	2	1	0	2.1 years

Patients at risk							Median survival
Colorectum	32	18	9	2	2	2	1.1 years
Other GI	19	13	8	3	2	0	1.6 years

Fig. 7 C (left), D (right)

Table 2. Univariate and multivariable analysis for overall survival

	Adjusted for site HR (95% CI)	P-value	Multivariable [1] HR (95% CI)	P-value	Multivariable [2] HR (95% CI)	P-value
Site						
Colorectal	1.00		1.00		1.00	
Gastroesophageal	0.86 (0.44-1.68)	0.67	0.99 (0.39-2.53)	0.99	0.76 (0.37-1.55)	0.45
Pancreatobiliary	0.57 (0.22-1.50)	0.26	0.29 (0.05-1.57)	0.15	0.25 (0.08-0.83)	0.02
Stage						
I/II/IIIA (pN-)	1.00		1.00		1.00	
IIIB (pN+)	2.55 (0.75-8.65)	0.13	43.4 (4.46- 423)	0.001	12.2 (2.55-58.3)	0.002
IV (M+)	5.13 (1.38-19.0)	0.01	58.1 (6.69- 505)	0.0002	16.5 (3.09-88.2)	0.001
MANEC subtype						
Collision	1.00		1.00			
Combined	0.41 (0.18-0.94)	0.03	1.61 (0.48-5.42)	0.44		
Amphicrin/Combined	0.18 (0.06-0.52)	0.001	1.35 (0.28-6.48)	0.71		
NEC type						
Large cells	1.00		1.00			
Small cells	1.70 (0.84-3.46)	0.14	1.40 (0.55-3.61)	0.48		
NEC component						
% NEC component (per 10%)	0.97 (0.79-1.20)	0.79	0.89 (0.65-1.20)	0.43		
Ki67 (≥55% vs. <55%)	3.61 (1.69-7.72)	0.0009	3.73 (1.16-12.0)	0.03	2.91 (1.28-6.60)	0.01
MC (≥50/10HPF vs. <50/10HPF)	1.81 (0.96-3.44)	0.07	4.03 (1.47-11.0)	0.007	4.25 (1.90-9.48)	0.0004
p53 (≥30% vs. <30%)	1.90 (0.94-3.86)	0.08	2.83 (1.16-6.89)	0.02		
SSTR2 (positive vs. negative)	1.85 (1.00-3.42)	0.05	2.83 (0.98-8.16)	0.05	3.45 (1.60-7.45)	0.002
Non-NEC component						
Ki67 (≥55% vs. <55%)	1.42 (0.75-2.71)	0.29	0.66 (0.28-1.59)	0.36		
MC (≥50/10HPF vs. <50/10HPF)	1.93 (0.96-3.91)	0.07	2.82 (0.93-8.56)	0.07		
Budding						
Absent	1.00		1.00			
Mild/moderate	1.27 (0.42-3.88)	0.67	3.51 (0.61-20.2)	0.16		
Severe	2.07 (0.67-6.36)	0.20	2.41 (0.44-13.2)	0.31		
NGS						
Wild type	1.00		1.00			
Mutated	3.33 (1.53-7.28)	0.0025	1.15 (0.32-4.09)	0.83		

Legend: D117: tyrosine-protein kinase Kit; **Ki67:** Ki67 index; **M+:** Liver metastases; **MANEC:** Mixed adeno-neuroendocrine carcinoma; **MC:** Mitotic count; **N+:** Lymph node metastases; **NEC:** neuroendocrine carcinoma; **NGS:** Next Generation Sequencing; **SSTR2**: somatostatin receptor 2A.

Multivariable model 1: includes all factors associated with overall survival after single adjustment for tumour site;
Multivariable model 2: retains only variables showing an association (P<0.10) with overall survival.

Immunohistochemistry markers

The majority of IHC markers showed no statistical association with OS.

Moreover in the NEC-component SSTR2 had a hazard ratio (HR) of 1.85 (95% CI 1–3.42; $P = 0.05$) after adjustment for tumour site, which retained high significance at both multivariable analysis models: model[2] (which includes all factors associated with OS after single adjustment for tumour site) with a HR of 2.83 (0.98-8.16) ($P =$

0.05), and model[3] (which retains only variables showing an association ($P < 0.10$) with OS) with HR 3.45 (1.60-7.45) ($P = 0.002$) (Fig. 2A,B and Table 2).

Loss of MMR proteins (MLH1 and PMS2 in all cases) was found in 2/51 neoplasms therefore no correlation with OS waspossible.

Stage

Patients with early-stage (I–IIIA) tumours (5 cases) had longer OS (median 2.5 years; 95% CI 2.1-3.0) compared to 35 patients with stage IIIB (median 1.2 years; 95% CI 0.8-1.6) or 11 patients with stage IV disease (median 1.0 years; 95% CI 0.3-1.2), with a significant difference ($P = 0.02$) between I–IIIA and IIIB+IV cases (Fig. 4E) in the neuroendocrine component. In the non-neuroendocrine component, patients with early-stage (II–III) tumour (40 cases) had longer OS (median 1.2 years; 95% CI 0.9-2.4) compared to 11 patients with stage IV (median 1.0 years; 95% CI 0.3-1.2), with a significant difference ($P = 0.01$) between II-III and IV cases (Fig. 4F).

Figure 7. Overall survival of patients with GEP-MANECs according to Ki67 (NEC and non-NEC)

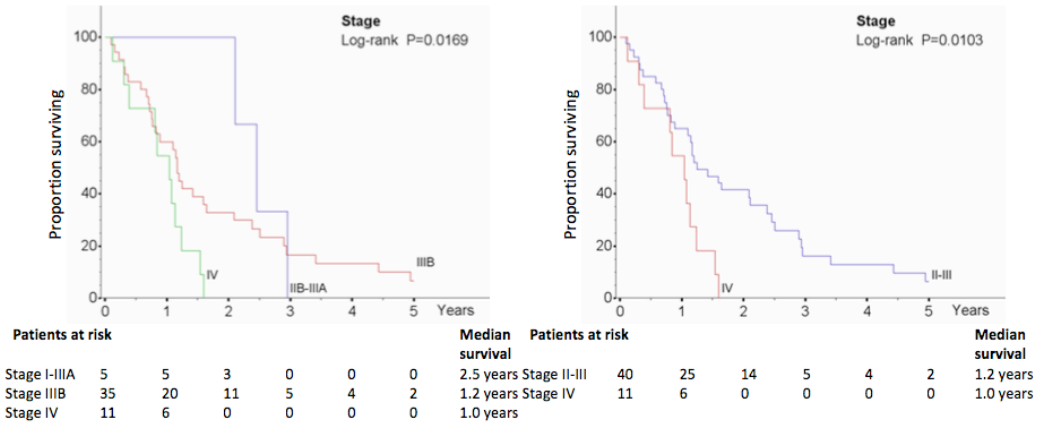


Fig. 7 E (left), F (right)

When an analysis of OS adjusted for tumour site was performed, OS was shown to be significantly associated with tumour stage, MANEC subtype (mixed amphicrine/ combined vs collision), SSTR2 (positive vs negative), NGS gene mutations (mutated vs wild type), in addition to Ki67 in the NEC component (Table 2).

Multivariable analysis

At multivariable analysis, tumour site, stage, Ki67 and MC in the NEC component and SSTR2 were independently associated with OS (Table 2). Patients with pancreatobiliary tumours (HR = 0.15; 95% CI 0.08–0.83; *P* = 0.02) had significantly better survival compared to patients with other gastrointestinal (GI) MANECs. Patients with stage IV in the NEC component had a 16.5 fold increase risk of death (95% CI 3.09–88.2; *P* = 0.001) than those with stage I-IIIA and IIIB.

Patients with Ki67 ≥ 55% in the NEC component had a 3.73 fold increased risk of death (95% CI 1.16–12; *P* = 0.03) compared to patients with a Ki67 < 55%, while patients with MC ≥ 50 mitoses/10 HPF had a 4.03 fold risk of death (95% CI 1.47–11, *P* = 0.007). The tumours with SSTR2 positive had a 2.83 fold risk of death (0.98-8.16) compared to those with SSTR2 negative (Table 2).

5. Discussion

This study confirmed that GEP-MANECs are poor prognosis malignancies, heterogeneously managed. Weaknesses of our work are as follows: (i) proposed chemotherapy regimens were varied, including NEC-oriented and non-NEC-oriented ones, mainly based on the gut-feeling of the treating oncologists; (ii) retrospective design; (iii) lack of clinical information mainly due to the long period of time considered. It was really difficult collecting clinical data, considering that some patients had received chemotherapy outside the referral institute.

Strengths are the following: surgical samples primary tumour series with a clear central pathology review by a team of NEN-dedicated expert pathologists(28); this latter is a critical point, often lacking in published studies, considering the rarity and debated definition of this entity; relatively high number series, considering the poor quality of literature data and rarity of the disease.

Mixed adeno-neuroendocrine carcinoma of the digestive system is a very rare tumour. Thus, most of the published studies concerning this disease entity are case reports. To the best of our knowledge, with exception of studies including also goblet cell carcinomas, the number of patients included in this analysis is in line with those of other clinical retrospective studies on GEP-MANECs from Western Countries over the last forty years, which ranged between 14 and 75 patients(58, 60).

Moreover the anthropometric data of our patients are consistent with other reported cohorts of MANECs(34, 58, 60).

From a strategy-management point of view, despite the small number of patients evaluated, in the locally advanced setting the approach to the patients of this analysis was quite different to that reported in other studies(13, 37, 47, 58, 59, 61), with curative surgery being offered to nearly two-thirds of the whole population and peri-operative treatment delivered only to a handful of resected cases.

Moreover, in the present study, in patients with locally advanced disease, peri-operative or adjuvant chemotherapy regimens were most commonly adherent to the clinical practice guidelines for pure adenocarcinomas from the same sites of origin; this might be explained by the lack of solid evidence advocating the use of adjuvant and/or peri-operative chemotherapy or chemo-radiotherapy for potentially curable pure NENs. Moreover the survival advantage for the patients who received adjuvant therapy (Fig. 1D) was most likely attributable to the better performance status at baseline compared to the untreated arm in which many patients died in the first months after surgery.

So far, the right therapeutic strategy of advanced GEP-MANECs has not yet been codified, therefore a universally shared clinical management is not known. Considering the biological aggressiveness of GEP-MANECs, chemotherapy could play a key role, but the choice of the type of chemotherapy still represents an unmet medical need. Some authors suggest that the therapeutic strategy should be based on the most aggressive neoplastic component(24, 31), but since the lack of validated evidence, at present this theory remains a proposal to be validated with a study specically design on that. Indeed the clinical management of GEP-MANECs in the "real world" setting is quite varied and does not always focused on most aggressive part of the disease.

In our series, considering the whole population, on one hand the NEC component was higher than 50% in quite the 72% while the non-NEC counterpart in the 49%, on the other hand the Ki67 was higher than 55% in the 74.5% of cases in the NEC component, and in the 61% in the non-NEC counterpart. Nevertheless in the present study a fluoropyrimidines/oxaliplatin regimen, clearly most oriented to treat an advanced GI cancer rather than a NEC, has been offered almost to the half of the metastatic group of patients evaluated as first line of therapy with only 6% SD as best response and 10.8 months OS.

In line with the theory to treat the most aggressive component of GEP-MANECs, on one hand, considering the NEC component, it should be highlighted that the clinical behavior of NECs is similar to that of small-cell lung cancer (SCLC). Since SCLC is known to be responsive to cisplatin and etoposide, this combination has been the most widely used combination in extra-pulmonary NECs although, the literature evidence on this regimen are based on old studies conducted in mixed population and not designed to evaluate the efficacy of these regimens in NEC(62, 63). On the other hand, when considering the exocrine component, the typical first-line chemotherapy backbone comprises a fluoropyrimidine (intravenous 5-FU or oral capecitabine) used in various combinations and schedules depending on the site of primary tumours with irinotecan or oxaliplatin(64, 65) +/- anti-VEGF or anti-EGFR(64) in the case of colon cancer or gemcitabine in case of pancreatic cancer(66).

In the current study the prognosis of GEP-MANECs is driven mostly by the NEC component with special reference to its Ki67 proliferative index like in the recent evidence from the Italian pathologists(28). These similar results could suggest that (i)

the real prognosis of GEP-MANECs could depend only on the biology of both components regardless the type of therapy or that (ii) the therapy administered really influenced the prognosis also in the previous pathological report(28).

Indeed, as previously reported for pure NECs, patients with Ki-67 <55% were less responsive to platinum-based chemotherapy, but had a longer survival(29) and moreover that a Ki67 threshold of 55% identified two main different prognostic classes of MANECs with significant survival differences(30). Therefore, although comparing different studies, on one side the findings of this analysis seem to show that the OS of MANECs with Ki67 ≥ 55% is closely similar to that of the corresponding pure poorly differentiated GEP-NECs with Ki67 ≥ 55% (type C NECs according to Milione *et al*. 2017)(30), and, on the other side, that the GEP-MANECs with Ki67 < 55% of our series showed better survival (35 months) compared to that of 24.5 months of the pure NECs with 21-55% Ki67 (type B NECs according to Milione *et al*. 2017)(30). Moreover for KRAS mutations in our series, the significant correlation with OS ($P = 0.0025$) after adjusted for side at univariate analysis seems to suggest a join point(64) with the pure adenocarcinoma counterpart, at least as for colorectal primary site. Moreover, the significantly shorter OS observed in the patients with primary colorectal tumours seem to reflect previous data (59) and reinforce the hypothesis that presence of the NEC component at the invasive front of MANECs or in metastases, could explain their aggressiveness and drive their prognosis(28). And then, although in our study BRAF mutations have been detected only in one patient among those twenty-two evaluated, considering our findings in terms of OS in this group of patients, and that colorectal NECs frequently exhibit BRAF mutations and are associated with a poor prognosis (67), mutations of BRAF

and moreover of RAS and TP53, should be prospectively investigated in a more homogeneous population of GEP-MANECs.

In our study, stage was one of the factors independently associated with OS (Table 2). Indeed our patients with stage IV showed a higher increase risk of death than thoose with stage I-IIIA and IIIB as happened for pure site corresponding carcinomas. Therefore, our data support the WHO recommendation of considering MANECs as ordinary carcinomas, providing evidence for the application of the relevant dedicated UICC/AJCC staging system(5, 8) in the pathological reports.

In the real life practice of our study, the functional characterization of the disease in some cases (6 cases) led to the 111In-pentetreotide or OctreoScan® (SRS, somatostatin receptor scintigraphy) or 68-gallium PET/CT for the evaluation of somatostatin receptors, in other cases (14 cases, 12 with positive uptake) to the 18-FDG PET/CT, based on the expertise of the clinicians. While the receptors evaluation lost sense in these type of aggressive tumours, the role of 18-FDG PET/CT could be taken into account. The prognostic role of 18-FDG PET/CT has been evaluated in a phase II trial including 98 patients with NEN enrolled after surgery and scheduled for various therapies(68). It showed that among 18-FDG PET/CT, Ki67, chromogranin A (CgA), and the presence of hepatic metastases, the only parameter that correlated with the prognosis was the positivity to 18-FDG PET/CT. In our analysis, although the small number of cases evaluated, the positive uptake of almost all cases evaluated could suggest at least to include this parameter as part of a specific clinical score in a prospective way in such aggressive tumours.

As for biochemical markers, although the positive trend of the neuron-specific enolase (NSE) for patients with more than 5 ng/ml which is not useful unless it is

contextualized at exact time points, its real meaning should be prospectically evaluated with rigorous blood sampling at specific times within the clinical strategy (i.e. histological diagnosis, beginning of therapy, every morphological/functional evaluation, disease progression).

Finally, the significant association of some molecular markers, including p53, and SSTR2 (Table 2), that we found in both NEC and non-NEC components, seems to suggest a possible common origin of the two MANEC components from a pluripotent cancer stem cell, which undergoes divergent differentiation, during tumour progression as observed in previous studies(2, 7, 24).

Conclusion

In conclusion, this study of a clinical series of GEP-MANECs, confirms that GEP-MANECs are an aggressive disease in which the prognosis is mainly driven by the NEC component, in particular to its Ki67 proliferative index. Moreover since the confirmed biological heterogeneity of GEP-MANECs, their rareness, the many diagnostic difficulties, and the lack of a universally shared clinical approach, this study cannot suggest a therapeutic strategy for GEP-MANECs nor that chemotherapy really impacts on the prognosis but it could be useful as "hypothesis generating study". Therefore the STEP II of this work could be the right tool for collecting clinical-pathological information on GEP-MANECs in a prospective way and treat these patients with a more homogenous clinical approach.

Moreover this work highlights again that (i) an expert pathologist in this field should review all the diagnosis dubious for MANEC to confirm diagnosis and evaluate all the most important prognostic factors, that (ii) the GEP-MANECs should be

managed in accordance with a reference centre for NENs to clearly characterise the patient and the disease and to homogenise the clinical therapeutic approach, and finally that (iii) since the NEC component is confirmed as the most important driver influencing the prognosis of GEP-MANECs, the second step of this reasearch is warranted. Therefore a prospective trial in a more homogeneous and well selected populations of GEP-MANECs, including a centralised revision of pathological samples, should be performed to evaluate the clinical activity and toxicity of two different regimens of chemotherapy NEC-oriented and non-NEC oriented with a prospectic exploratory analysis of the main morphological, immunohistochemical, and biomolecular prognostic factors.

PUBLISHED ARTICLES ON THIS TOPIC

- **Spada F,** Antonuzzo L, Marconcini R, Radice D, Antonuzzo A, Ricci S, Di Costanzo F, Fontana A, Gelsomino F, Luppi G, Nobili E, Galdy S, Cella CA, Sonzogni A, Pisa E, Barberis M, Fazio N. Oxaliplatin-Based Chemotherapy in Advanced Neuroendocrine Tumors: Clinical Outcomes and Preliminary Correlation with Biological Factors. Neuroendocrinology. 2016;103(6):806-14.

- Fazio N, **Spada F,** Giovannini M. Chemotherapy in gastroenteropancreatic (GEP) neuroendocrine carcinomas (NEC): a critical view. Cancer Treat Rev. 2013 May;39(3):270-4.

- Milione M, Maisonneuve P, **Spada F,** Pellegrinelli A, Spaggiari P, Albarello L, Pisa E, Barberis M, Vanoli A, Buzzoni R, Pusceddu S, Concas L, Sessa F, Solcia E, Capella C, Fazio N, La Rosa S. The Clinicopathologic Heterogeneity of Grade 3 Gastroenteropancreatic Neuroendocrine Neoplasms: Morphological Differentiation and Proliferation Identify Different Prognostic Categories. Neuroendocrinology. 2017;104(1):85-93.

- **Spada F,** Maisonneuve M, Fumagalli C et al. Temozolomide alone or in combination with capecitabine in patients with advanced neuroendocrine neoplasms: an Italian multicenter experience. *To be submitted (Neuroendocrinology)*

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Appendix

Search strategy and identification of eligible papers.

For this systematic reviewed we searched:

1.a Medline search strategy

Database: Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) <1946 to August 17, 2018>

Search Strategy:

---

1 manec.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (127)

2 (Mixed adj2 adenoneuroendocrine).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (159)

3 (Mixed adj2 "adeno-neuroendocrine").mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (20)

4 1 or 2 or 3 (197)

5 (amphicrine adj1 (carcinoma$1 or tumor$1 or tumour$1 or cancer$1 or neoplas$)).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (43)

6 4 or 5 (234)

7 minen$1.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (25)

8 "Mixed neuroendocrine-nonneuroendocrine ".mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (7)

9 "Mixed neuroendocrine-non-neuroendocrine ".mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms] (4)

10 7 or 8 or 9 (28)

11 6 or 10 (257)

12 limit 11 to (english or french or german or italian or spanish) (219)

1b. Scopus search strategy

1 (((manec) OR (mixed PRE/2 adenoneuroendocrine) OR (mixed PRE/2 “adenoneuroendocrine”)) OR (TITLE-ABS-KEY (minen)) OR (TITLE-ABS-KEY (“mixed neuroendocrine-nonneuroendocrine”)) OR (TITLE-ABS-KEY (“mixed neuroendocrine-non-neuroendocrine”))) AND NOT (INDEX (medline)) AND (LIMIT-TO (LANGUAGE, “English”) OR LIMIT-TO (LANGUAGE, “German”) OR LIMIT-TO (LANGUAGE, “ French”) OR LIMIT-TO (LANGUAGE, “Spanish”))

2 (((manec) OR (mixed PRE/2 adenoneuroendocrine) OR (mixed PRE/2 “adenoneuroendocrine”)) OR (TITLE-ABS-KEY (minen)) OR (TITLE-ABS-KEY (“mixed neuroendocrine-nonneuroendocrine”)) OR (TITLE-ABS-KEY (“mixed neuroendocrine-non-neuroendocrine”))) AND NOT (INDEX (medline))

3 INDEX (medline)

4 (((manec) OR (mixed PRE/2 adenoneuroendocrine) OR (mixed PRE/2 “adenoneuroendocrine”)) OR (TITLE-ABS-KEY (minen)) OR (TITLE-ABS-KEY (“mixed neuroendocrine-nonneuroendocrine”)) OR (TITLE-ABS-KEY (“mixed neuroendocrine-non-neuroendocrine”)))

5 TITLE-ABS-KEY (“mixed neuroendocrine-non-neuroendocrine”) (187)

1c. Embase serach strategy

1 manec (242)

2 mixed NEXT/2 neuroendocrine (171)

3 'mixed adeno-neuroendocrine' (38)

4 'minen':ti,ab,kw (18)

5 'mixed neuroendocrine-nonneuroendocrine' (8)

6 'mixed neuroendocrine-non-neuroendocrine' (6)

7 1 OR #2 OR #3 OR #4 OR #5 OR #6 (373)

8 #7 AND [embase]/lim NOT ([embase]/lim AND [medline]/lim) (225)