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Ki67 proliferative index of the neuroendocrine component drives MANEC prognosis

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ABSTRACT

Mixed adeno-neuroendocrine carcinomas (MANECs) are composed of a poorly differentiated neuroendocrine carcinoma (NEC) and a non-neuroendocrine (non-NEC) neoplastic epithelial component, each representing at least 30% of the tumor. At present, prognostic factors for MANECs remain largely unexplored. We investigated the clinical-pathologic features of a large multicenter series of digestive system MANECs.

Surgical specimens of 200 MANEC candidates were centrally reviewed; diagnosis was confirmed in 160 cases. While morphology, proliferation [mitotic count (MC), Ki67 index] and immunophenotype (p53, SSTR2a, beta-Catenin, Bcl-2, p16, Rb1, ALDH, Mismatch Repair proteins, CD117) were investigated separately in both components, genomic (*TP53*, *KRAS*,*BRAF*) alterations were searched for on the entire tumor. Data were correlated with overall survival (OS).

MANEC sites were: 92 colorectal, 44 gastro-esophageal and 24 pancreato-biliary. Median OS was 13.2 months. After adjustment for primary site, Ki67 index of the NEC component (but not of the non-NEC component) was the most powerful prognostic marker. At multivariable analysis, patients with Ki67 ≥55% had an 8-fold risk of death [Hazard ratio (HR) 7.83; 95% confidence interval (CI) 4.17-14.7; p<0.0001] and a median OS of 12.2 months compared to those with Ki67<55% (median OS 40.5 months). MC (HR 1.51; 95% CI 1.03-2.20, p=0.04) was a weaker prognostic index. Colorectal primary site (HR 1.60; 95% CI 1.11-2.32; p=0.01) was significantly associated with poorer survival. No single immunomarker, in either component, was statistically significant. This retrospective analysis of a large series of digestive system MANECs), showed that the NEC component, particularly its Ki-67 index, was the main prognostic driver.

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INTRODUCTION

The coexistence of neuroendocrine and non-neuroendocrine components in the same neoplasm is a rare but well known phenomenon in digestive system tumors. The 2010 World Health Organization (WHO) classification of tumors of the digestive system proposed the term “mixed adeno-neuroendocrine carcinoma” (MANEC) to define these cancers in which, by definition, each component represents at least 30% of the tumor mass (Bosman F 2010). The pathogenesis of MANECs is still unclear, and different hypotheses have been proposed to explain their biphasic morphology. Molecular investigations have suggested a multistep progression from a common precursor lesion. Indeed, the higher frequency of chromosomal and gene abnormalities found in the neuroendocrine component compared to the non-neuroendocrine component suggests that progression from a non-neuroendocrine towards a neuroendocrine cell phenotype, and not *vice-versa,* is more frequent (Furlan, et al. 2003; Huang, et al. 2002; Jesinghaus, et al. 2017; Kim, et al. 2002; Paniz Mondolfi, et al. 2011; Scardoni, et al. 2014; Volante, et al. 2015; Vortmeyer, et al. 1997; Woischke, et al. 2017).

The clinical behavior of MANECs is generally aggressive, however prognostic markers predictive of MANEC outcome have not been definitively validated to date. Whether prognostic markers of poorly differentiated neuroendocrine carcinomas (NECs), such as proliferation, CD117 expression and microsatellite instability, have the same prognostic relevance in MANECs is, as yet, unknown. Moreover, considering the peculiar morphologic characteristics of MANECs, additional specific parameters may be prognostically important, including the percentage and type of different tumor components, tumor site, morphologic features (i.e. tumor cell budding, vascular and perineural infiltration, intra- and peri-tumoral lymphoid infiltration), type of tumor component in nodal or distant metastases,

immunophenotype and molecular profile. To the best of our knowledge, these diverse characteristics have never been fully investigated in a large series of digestive MANECs.

In this retrospective multicenter study, we collected a large series of MANECs and we extensively investigated clinical, morphologic, immunohistochemical and molecular features in order to characterize the different tumor components and to search for parameters useful in prognostic stratification.

MATERIALS AND METHODS

Case selection and study design

Between 1995 and 2015, the surgical pathology and clinical databases of eleven Italian Institutions were retrospectively searched 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 tumor”, “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 (as discussed by La Rosa, et al. 2012; and Ohike 2017); iv) patients who underwent neoadjuvant chemotherapy.

A total of 200 candidate cases were identified. Patients’ charts and tumor morphology were carefully revised, first by the pathologist of the case-proposing hospital and then by a panel of seven expert pathologists (MM, AP, PS, AV, LA, SLR, and CC) 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 versus non-NEC components, and sub-type 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. In the end, 160 cases met all the above criteria and were enrolled in the study.

Morphologic analysis (Table 1) 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) (La Rosa et al. 2012a); c) morphology of NEC component: small cell or large cell according to WHO 2010 (Bosman F 2010; Rindi G 2010); 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 labelling (“hot spots”)(Rindi G 2010); f) mitotic count (MC) was evaluated in at least 10 HPF (10 HPF=2mm^2)(Rindi G 2010); g) quantitative assessment of NEC, non-NEC or mixed type components in lymph node metastases and/or in distant metastases; h) tumor staging according to the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) 8th edition (Amin2017); i) lymphovascular invasion (evaluated on both hematoxylin-eosin (H&E) and/or

CD31-stained sections); l) perineural invasion; m) intra and/or peritumoral lymphocytic infiltration; n) prevalent tumor 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; p) tumor budding defined according to the International Tumor Budding Consensus Conference 2016 (ITBCC) (Lugli, et al. 2017).

The histochemical and immunohistochemical (IHC) study (Supplementary table 1) 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 (Figure 1B); c) Ki67 staining evaluated in both NEC and non-NEC components (Bosman F 2010; Rindi G 2010); d) IHC assessment and evaluation in both components of several markers including p53, Rb1, p16, Bcl-2, Beta-Catenin, aldehyde dehydrogenase (ALDH), CDX2, thyroid transcription factor-1 (TTF1), mismatch repair (MMR) proteins, CD117 and somatostatin receptor 2A (SSTR2A) using the antibodies listed in Supplementary Table 2.

With the exception of p53 and SSTR2A, all markers were considered positive regardless of the number of positive cells. p53 was considered positive when ≥30% of cells were positive (Ali, et al. 2017); SSTR2A was assessed according to Volante et al. (positive: 2+, 3+; negative: 0, 1+ score) (Volante, et al. 2007). MMR deficiency was established according to the criteria reported by Chiaravalli et al. (Chiaravalli, et al. 2001).

Data concerning mutations (Table 1) of KRAS (codons 12 and 13), BRAF (codon 600) and TP53 (exons 5 to 8) were extracted from investigations performed for therapy decision making, either by PCR pyrosequencig as described by (Sahnane, et al. 2015) or by Next Generation Sequencing analysis (NGS) as detailed by (Meazza, et al. 2016).

This study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethical Committee of Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy (n° INT 21/16).

Evaluation of proliferative cut-offs

We searched for prognostically relevant cut-offs of Ki67 and MC. The results of our exploratory analysis are shown in Supplementary Figure 1.

Statistical analysis

Data were analyzed 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 overall survival (OS) with primary tumor site, tumor 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 intratumoral and peritumoral 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, Bcl-2, Rb1, p16, p53, TTF1, CDX2, CD117, ALDH, Beta-Catenin and SSTR2a.

OS 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% Confidence Interval (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.

RESULTS

Clinicopathologic features

Table 1 and Figure 1 summarize the main clinicopathologic features of the 160 patients enrolled in the study. The series comprised more males than females (72.5% vs 27.5%) and this difference was statistically significant and was maintained across tumor sites (p=0.04). Most patients (74.4%) had Stage IIIB disease. MANECs were more frequently colorectal (n=92, 57.5%), with a prevalence in the right colon (64 cases) *versus* the left colon (10 cases) and rectum (18 cases), followed by gastro-esophageal (n=44, 27.5%) locations, with a prevalence (32 cases) for stomach, versus cardio-esophageal junction (9 cases) and esophageal tumors (3 cases). Finally pancreato-biliary locations were the least common (n=24, 15.0%), the majority of these were pancreatic tumors (14 cases) compared to gallbladder (7 cases) or extrahepatic biliary tract primaries (3 cases). No MANECs were found in the small bowel or appendix.

All neoplasms investigated showed 30% or more reactive cells for synaptophysin, while only 90/160 cases showed significant reactivity for chromogranin-A. Thus, we based our identification of the NEC component on the synaptophysin positive, poorly differentiated

neuroendocrine part of the neoplasm (Figure 1A, 1B). Most MANECs (75.6%) had a NEC component in the 50% to 70% range (Table 1). Considering the NEC component, the large cell type (n=135, 84.4%) was more frequent compared to the small cell type. In the non-NEC component, conventional adenocarcinoma was the dominant histotype (n=105), followed by mucinous (n=17, 11 of which colorectal), signet ring cell (n=10, 8 of which gastric) and squamous cell carcinoma (n=5, 4 of which esophageal or cardio-esophageal). Fifty four (33.8%) tumors showed a *collision* pattern, 82 (51.2%) a purely *combined* pattern, and 24 (15.0%) a mixed *amphicrine* and combined pattern. In *collision* neoplasms, the adenocarcinomatous component was usually overlying a deep, invasive NEC component. In *combined* neoplasms the two components were admixed without distinction between superficial and deep aspects (Figure 1A-1B) however the invasive tumor front (Figure 1C) was predominantly of NEC type with a pure NEC component in 71 (44.4%) cases, mixed NEC/non-NEC component in 79 (49.4%) cases and pure non-NEC carcinomatous component in 10 cases (6.2%) only. Angioinvasion was seen in all MANEC cases. Neoplastic emboli in vessels were prevalently of NEC or mixed NEC/non-NEC type.

Nodal metastases (Figure 1D) were of pure NEC histotype in 35 (26.5%) and mixed NEC/non-NEC in 97 (73.5%) cases. In distant metastases pure NEC histology was found in 6 (35.3%) cases, and mixed NEC/non-NEC histology in 11 cases (64.7%). Pure non-NEC component was not detected in metastases.

Survival Analysis

In the overall cohort, median OS was 13.2 months (95% CI 12.4-14.3) (Figure 2A).

Site: OS was significantly shorter in patients with colorectal tumors (median 12.2 months; 95% CI 10.3-13.3) compared to patients with tumors in other digestive system sites: gastro-esophageal or pancreato-biliary (median 17.3 months; 95% CI 13.3-19.9) (p=0.001) (Figure 2B).

Immunohistochemistry markers: The majority of IHC markers showed no statistical association with OS (Supplementary Table 1). Only CD117 (Table 2), evaluated in the NEC component, was associated with OS at univariable analysis (HR=1.59; 95% CI 1.13-2.24; p=0.008), although the association lost statistical significance at multivariable analysis (HR=1.42; 95% CI 0.99-2.04; P=0.06). Loss of MMR proteins (MLH1 and PMS2 in all cases) was found in 8/160 neoplasms with equal involvement of NEC and non-NEC components and no significant influence on OS (Supplementary table 1). Rb, p53, CD117, CDX2 and ALDH were significantly co-expressed in both NEC and non-NEC components (Supplementary Table 3). The distribution of immunohistochemical markers in the NEC and non-NEC components is reported in Supplementary Table 1.

Proliferation: A preliminary evaluation of the NEC component showed 55% to be an optimal prognostic cut-off for Ki67 index and 50 mitoses/10HPF to be optimal for MC (Supplementary Figure 1). Ki67 index turned out to be ≥55% in the large majority (82.5%) of cases, while MC was ≥50 in 30.6% of cases. On the other hand, in the non-NEC component Ki67 was ≥55% in 51.9% and MC was ≥50/10 HPF in 17.5% of cases.

Patients with Ki67 index <55% in the NEC component (median 40.5 months; 95% CI 34.5-53.2) had a significatively longer OS than those with Ki67 ≥55% (median 12.2 months; 95% CI 11.1-13.2) p<0.0001. The latter showed a hazard ratio (HR) of 9.08 (95% CI 5.13-16.1) vs Ki67<55% (p<0.0001) after adjustment for tumor site, which retained high significance at

multivariable analysis (Figure 2c and Table 2). Patients with MC <50 mitoses/10HPF also had significatively longer OS (median 14.1; 95% CI 13.0-16.4) compared to those with ≥50 mitoses/10 HPF (median 11.2 months; 95% CI 9.5-13.2; p=0.002), although with clearly lower HR (Table 2). In the non-NEC component, a Ki67index of ≥55% was associatewd with a tumor site-adjusted HR of 1.81; 95% CI 1.28-2.54 (p=0.0007) vs. <55%, which lost statistical significance at multivariable analysis, while a MC ≥50/10HPF lacked any significant difference compared to MC <50/10HPF.

Stage: Patients with early stage (I-IIIA) tumor (24 cases) had longer OS (median 22.1 months; 95% CI 12.8-36.4) compared to 119 patients with stage IIIB (median 12.7 months; 95% CI 11.2-13.3) or 17 patients with stage IV disease (median 15.7 months; 95% CI 12.2-23.3), with a significant difference (p=0.003) between I-IIIA and IIIB+IV cases (Figure 2D).

When an analysis of OS *adjusted for tumor site* was performed, OS was shown to be significantly associated with tumor stage, MANEC subtype (mixed amphicrine/combined versus collision), perineural infiltration, high grade budding (Figure 1 C) and ~~NGS~~ gene mutations, in addition to Ki67, MC and CD117 in the NEC component, and Ki67 only in the non-NEC component (Table 2).

At *multivariable analysis*, only tumor site, Ki67 and MC in the NEC component and mutations were independently associated with OS (Table 2). Patients with gastro-esophageal tumors (HR=0.64; 95% CI 0.43-0.96; p=0.03) and those with pancreato-biliary tumors (HR=0.58; 95% CI 0.33-1.04; p=0.07) had significantly better survival compared to patients with colorectal MANECs. Patients with Ki67 ≥55% in the NEC component had a 7.83 fold increased risk of death (95% CI 4.17-14.7; p<0.0001) compared to patients with a Ki67 <55%, while patients with MC ≥50 mitoses/10 HPF had a 1.51 fold risk of death (95% CI 1.03-2.20, p=0.04). A

mutation in either *KRAS* or *BRAF* or *TP53* genes (HR for any mutation 2.68; 95% CI 1.50-4.81; p=0.0009) was significantly associated with unfavorable outcome (Table 2). The risk was comparable *KRAS mutations* (HR 2.69; 95% CI 1.24-5.82; p=0.01) and *TP53* mutations (HR 2.90; 95% CI 1.48-5.68; p=0.002) (Footnote Table 2).

DISCUSSION

This study demonstrates that the prognosis of MANECs from digestive system is driven mostly by the NEC component with special reference to its Ki67 proliferative index. Ki67 index of the NEC component has proven to be far superior to MC or, indeed, to any other prognostic parameter, either *morphologic* (prevalence of NEC *versus* non-NEC component, NEC small or large cell histology, histologic type), *immunohistochemical* (p53, Rb1, Bcl-2, p16, Cdx2, MMR deficiency, TTF1, ALDH, CD117,SSTR2A protein expression or proliferative index of the non-NEC component) or *molecular* (*TP53* or *KRAS* or *BRAF* mutation). Of such parameters, only primary site, CD117 immunostaining of the NEC component, perineural infiltration, high grade budding and *TP53* or *KRAS* mutations were associated with shorter OS at univariate analysis, while only site and mutations (as well the aforementioned NEC component Ki67 index) retained prognostic value at multivariable analysis (Table 2).

Accordingly, in the diagnostic work up of MANECs, besides identifying the NEC component histologically and immunohistochemically (by synaptophysin and chromogranin-A reactivity), evaluation should also focus on the quantitative assessment of Ki67 index in the neuroendocrine component. While proliferative rate has been widely shown to be an important diagnostic tool for neuroendocrine neoplasms, this study, has added new and interesting findings with regards to the importance of proliferation in MANECs as well. Indeed, as previously reported for pure NECs (Sorbye, et al. 2013) a Ki67 threshold of 55% also distinguishes between two different prognostic classes of MANECs with significant survival differences. These findings

parallel our previously published results on pure gastroenteropancreatic NECs (Milione, et al. 2017), and show that the OS of MANECs with Ki67 ≥55% closely reproduces that of corresponding pure poorly differentiated NECs with Ki67 ≥55% (Type C NECs according to (Milione et al. 2017)(Fig 3b). Conversely, and perhaps more interestingly, with all the limitations of comparing two different studies, MANECs with Ki67 <55% showed better survival compared to pure poorly differentiated NECs with Ki67 between 21-55% (Type B NECs according to (Milione et al. 2017).

Our findings parallel those of a large study on resected colorectal neoplasms, where survival outcomes of NECs were compared with those of patients with high-grade adenocarcinoma (Shafqat, et al. 2015): the results demonstrated that the median survival was significantly shorter (7.1 months) for patients with NECs compared with that of patients affected by high grade adenocarcinoma (36.0 months). Likewise the 5-year OS of patients with gastric NECs (38.7%) was poorer than that of patients with gastric adenocarcinoma (51.8%) (Xie, et al. 2017). The frequent finding of the NEC component only at the invasive front of MANECs or in metastases, suggests that this component is mainly responsible for their aggressiveness.

In our study we showed that the UICC/AJCC staging system developed for site corresponding carcinomas, was prognostically informative also for MANECs. Although most patients were diagnosed at an advanced stage (stage IIIB or IV), with severe prognosis, a small proportion (24 cases, 15%) of potentially curable patients (stage I to IIIA) was identified. As such, our data support the WHO recommendation of considering MANECs as ordinary carcinomas, providing evidence for the application of the relevant dedicated staging in real life pathology reporting.

The significant association of several molecular markers, including Rb, p16, p53 CDX2, CD117, ALDH and MMR that we found in both NEC and non-NEC components ,seems to confirm and to extend previous studies (Furlan et al. 2003; Furlan, et al. 2013; Jesinghaus et al. 2017; Kloppel 2017; Woischke et al. 2017). This finding suggests a possible common origin of the two MANEC components from a pluripotent cancer stem cell which undergoes divergent differentiation, during tumor progression. The mixed NEC/non-NEC pattern of growth we found in 73.5% of nodal and 64.7% of distant metastases may by itself support the proposed origin of the two components from a single, metastatic, bipotent clone, although the origin of some mixed metastases from cytologically mixed emboli cannot be excluded. Our finding of a greater frequency MANECs in the colon and stomach, where ordinary carcinomas are also highly prevalent, coupled with the apparent lack of MANECs originating in the mesenteric small intestine and appendix, where conventional carcinomas rarely arise, suggests a MANEC histogenesis more akin to that of classical (adeno)carcinomas than to that of neuroendocrine tumors.

In conclusion, this study of a large series of digestive system MANECs shows that the outcome of these neoplasms is mainly determined by their poorly differentiated neuroendocrine component and that the Ki67 proliferative index (above or below 55%) is the most important prognostic factor.

DECLARATION OF INTEREST

The authors declare no conflicts of interest. Guido Rindi declares that he has received speaker's fee by Novartis Pharma and Ipsen Pharma

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FIGURE LEGENDS

Figure 1. Two adjacent sections of a combined MANEC illustrate the intimate admixture of the NEC and non-NEC components of the neoplasm. **A**: Hematoxylin/Eosin; **B**: Synaptophysin Immunohistochemistry. Both ×100; **C**: Invasive front of colonic MANEC showing exclusively a NEC component massively reactive for Synaptophysin. Note the associated high grade budding (arrows) ×100; **D**: Synaptophysin immunostained intestinal MANEC in lymph node metastases, massive in the left lymph node and sinusoidal only (arrow) in the right lymph node, both composed exclusively of NEC cells. ×100. **E**: NEC component of a MANEC with 40% of Ki67 proliferative index. ×400; **F**: NEC component of a MANEC with 90% of Ki67 index ×400.

Figure 2. Overall survival of the whole 160 MANECs population (**A**), of MANECs according to tumor site (**B**) or according to Ki67 proliferative index (**C**) or according to AJCC staging system (**D**).

Figure 3. Comparison of overall survival according to Ki67 expression in the current study on MANECs compared to 112 pure poorly differentiated neuroendocrine carcinomas (Type B and C NECs from Milione *et al.* Neuroendocrinology 2017 Used by permission from S. Karger AG, Basel).

Supplementary Figure 1. Overall survival based cut offs for Ki67 proliferative index or mitotic count in the NEC component of the 160 MANECs.

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

	All	Colorectal	Gastro-esophageal	Pancreato-biliary	P-value
Total	**160**	**92**	**44**	**24**	
Sex					
Male	116	61	38	17	
Female	44	31	6	7	**0.04**
Age					
<60	40	25	12	3	
60-69	55	31	14	10	
70+	65	36	18	11	0.66
Stage					
I/II (pN-)	11	3	3	5	
IIIA (pN-)	13	6	1	6	
IIIB (pN+)	119	74	34	11	
IV (M+)	17	9	6	2	**0.002**
MANEC sub-type					
Collision	54	35	12	7	
Combined	82	44	26	12	
Amphicrine/Combined	24	13	6	5	0.63
NEC type					
Large cells	135	78	41	16	
Small cells	25	14	3	8	**0.02**
NEC necrosis					
Absent	40	23	8	9	
Present	120	69	36	15	0.21
% NEC component					
< 50%%	39	19	14	6	
50-60%	68	40	18	10	
>60%	53	33	12	8	0.70
Ki67 (NEC)					
<55%	28	13	7	8	
≥55%	132	79	37	16	0.10
Ki67 (non NEC)					
<55%	77	47	17	13	
≥55%	83	45	27	11	0.34
MC (NEC)					
<50/10HPF	111	62	32	17	
≥50/10HPF	49	30	12	7	0.81
MC (non NEC)					
<50/10HPF	132	73	39	20	
≥50/10HPF	28	19	5	4	0.41
Nerve infiltration					
Absent	20	12	4	4	
Present	106	63	28	15	0.69
Angioinvasion					
Absent	23	14	6	3	
Present	137	78	38	21	1.00
Budding					
Low	47	24	17	6	
Intermediate	47	33	8	6	
High	66	35	19	12	0.21
Peritumoral lymphoid cells					
Absent	70	42	18	10	
Mild	64	32	18	14	
Moderate	23	16	7	0	

Severe	3	2	1	0	0.19
Intratumoral lymphoid cells					
Absent	74	42	22	10	
Mild	69	41	15	13	
Moderate	13	6	6	1	
Severe	4	3	1	0	0.61
N+ (%NEC)					
Median (range)	70 (0-100)	70 (0-100)	70 (20-100)	90 (0-100)	
<100% (Mixed)	97	57	31	9	
100% (pure NEC)	35	23	8	4	0.61
M+ (%NEC)					
Median (range)	85 (20-100)	90 (20-100)	55 (20-100)	88 (85-90)	
<100% (Mixed)	11	5	4	2	
100% (pure NEC)	6	4	2	0	0.80
Molecular analysis					
Wild type	38	14	15	9	
KRAS mutated	12	11	1	0	
BRAF mutated	4	3	0	1	
TP53 mutated	17	9	8	0	**0.004**

* Some immunohistochemical evaluations are missing for some patients;

Ki67: Ki67 index; **M+:** Liver metastasis; **MANEC:** Mixed adenoneuroendocrine carcinomas; **MC:** Mitotic count; **N+:** Lymph node metastasis**; NEC:** neuroendocrine carcinoma;

Budding: single tumor cell or a cell cluster (buds) of up to 4 tumor cells, detected at the invasive tumor front; Low grade Budding:< 4 buds; Intermediate grade Budding: 5-10 buds; High grade budding: >10 buds

Table 2. Univariate and multivariable analysis for overall survival

	Adjusted for site HR (95% CI) *	P-value	Multivariable [2] HR (95% CI)	P-value	Multivariable [3] HR (95% CI)	P-value
Site						
Colorectal	1.00		1.00		**1.00**	
Gastroesophageal	0.73 (0.50-1.06)	0.10	**0.61 (0.39-0.94)**	**0.02**	**0.64 (0.43-0.96)**	**0.03**
Pancreatobiliary	**0.41 (0.25-0.67)**	**0.0004**	0.54 (0.29-1.03)	0.06	0.58 (0.33-1.04)	0.07
Stage						
I/II/IIIA (pN-)	1.00		1.00		1.00	
IIIB (pN+)	**2.03 (1.20-3.42)**	**0.008**	1.70 (0.90-3.22)	0.10	1.75 (0.95-3.23)	0.07
IV (M+)	1.59 (0.80-3.16)	0.19	1.08 (0.48-2.40)	0.86	1.09 (0.51-2.31)	0.83
MANEC subtype						
Collision	1.00		1.00			
Combined	0.90 (0.63-1.28)	0.56	1.07 (0.72-1.61)	0.73	-	
Amphicrin/Combined	**0.56 (0.33-0.96)**	**0.04**	0.83 (0.45-1.53)	0.55	-	
NEC type						
Large cells	1.00		1.00			
Small cells	1.54 (0.97-2.43)	0.07	1.27(0.77-2.10)	0.35	-	
NEC component						
% NEC component (per 10%)	1.09 (0.96-1.22)	0.18	-		-	
Ki67 (≥55% vs. <55%)	**9.08 (5.13-16.1)**	**<0.0001**	**8.92 (3.96-20.1)**	**<0.0001**	**7.83 (4.17-14.7)**	**<0.0001**
MC (≥50/10HPF vs. <50/10HPF)	**1.81 (1.28-2.58)**	**0.0009**	**1.53 (1.03-2.28)**	**0.04**	**1.51 (1.03-2.20)**	**0.04**
p53 (≥30% vs. <30%)	1.10 (0.78-1.56)	0.58	-		-	
CD117 (positive vs. negative)	**1.59 (1.13-2.24)**	**0.008**	1.45 (0.98-2.15)	0.06	1.42 (0.99-2.04)	0.06
SSTR2a (positive vs. negative)	1.21 (0.87-1.68)	0.25	-			
Non-NEC component						
Ki67 (≥55% vs. <55%)	**1.81 (1.28-2.54)**	**0.0007**	1.29 (0.87-1.92)	0.20	-	
MC (≥50/10HPF vs. <50/10HPF)	1.39 (0.91-2.11)	0.13	-		-	
Nerve infiltration						
Present vs. absent	**3.20 (1.77-5.77)**	**0.0001**	0.88 (0.42-1.82)	0.72	-	
Angioinvasion						
Present vs. absent	0.85 (0.54-1.33)	0.47	-		-	
Budding						
Absent	1.00		1.00			
Mild/moderate	0.82 (0.53-1.27)	0.37	1.29 (0.72-2.28)	0.39	-	
Severe	**1.97 (1.31-2.96)**	**0.001**	1.13 (0.65-1.96)	0.67	-	
Molecular Analysis**						
Wild type	1.00		1.00		1.00	
Mutated	**2.36 (1.41-3.97)**	**0.001**	**2.22 (1.19-4.15)**	**0.01**	**2.68 (1.50-4.81)**	**0.0009**

CD117: 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; **SSTR2a**: somatostatin receptor 2a.

Multivariable model 2 includes all factors associated with overall survival after single adjustment for tumor site; Multivariable model 3 retains only variables showing an association (P<0.10) with overall survival.

** Multivariable risk estimates for single gene mutations from alternative model 3: *KRAS* mutated [n=12; HR=2.69 (1.24-5.82); P=0.01]; *BRAF* mutated [n=4; HR=1.81 (0.51-6.39); P=0.36]; *TP53* mutated [n=17; HR=2.90 (1.48-5.68); P=0.002].

Figure 1

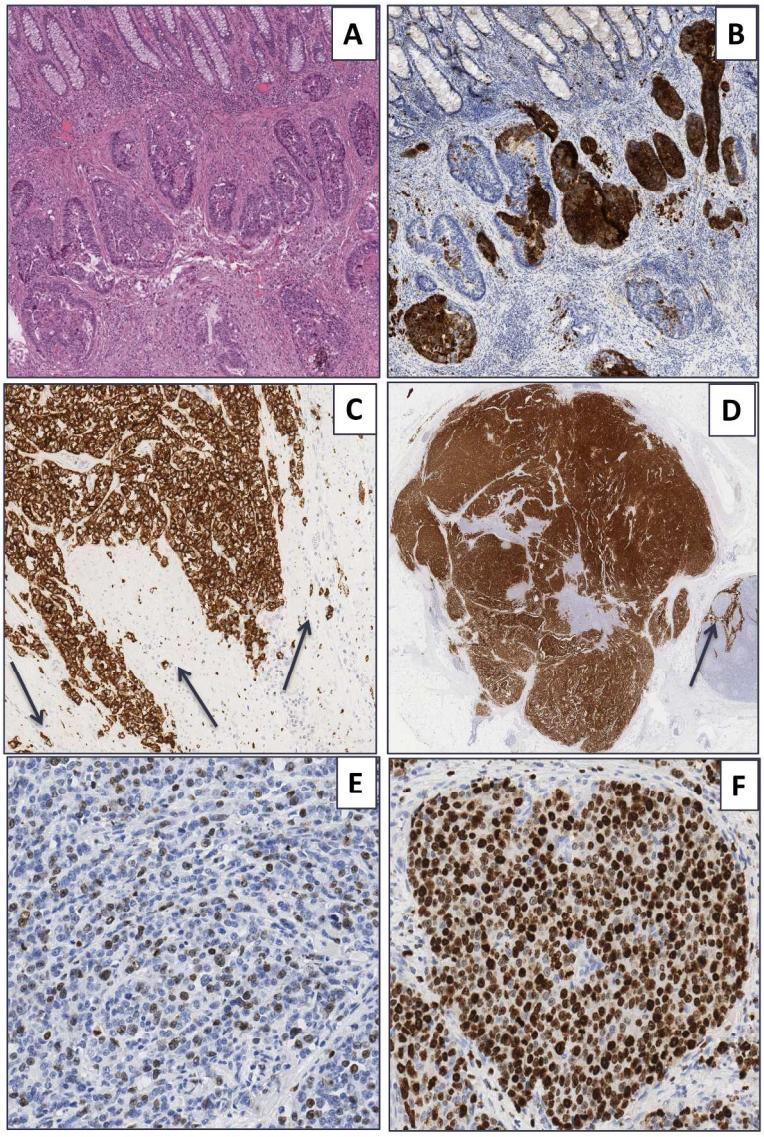

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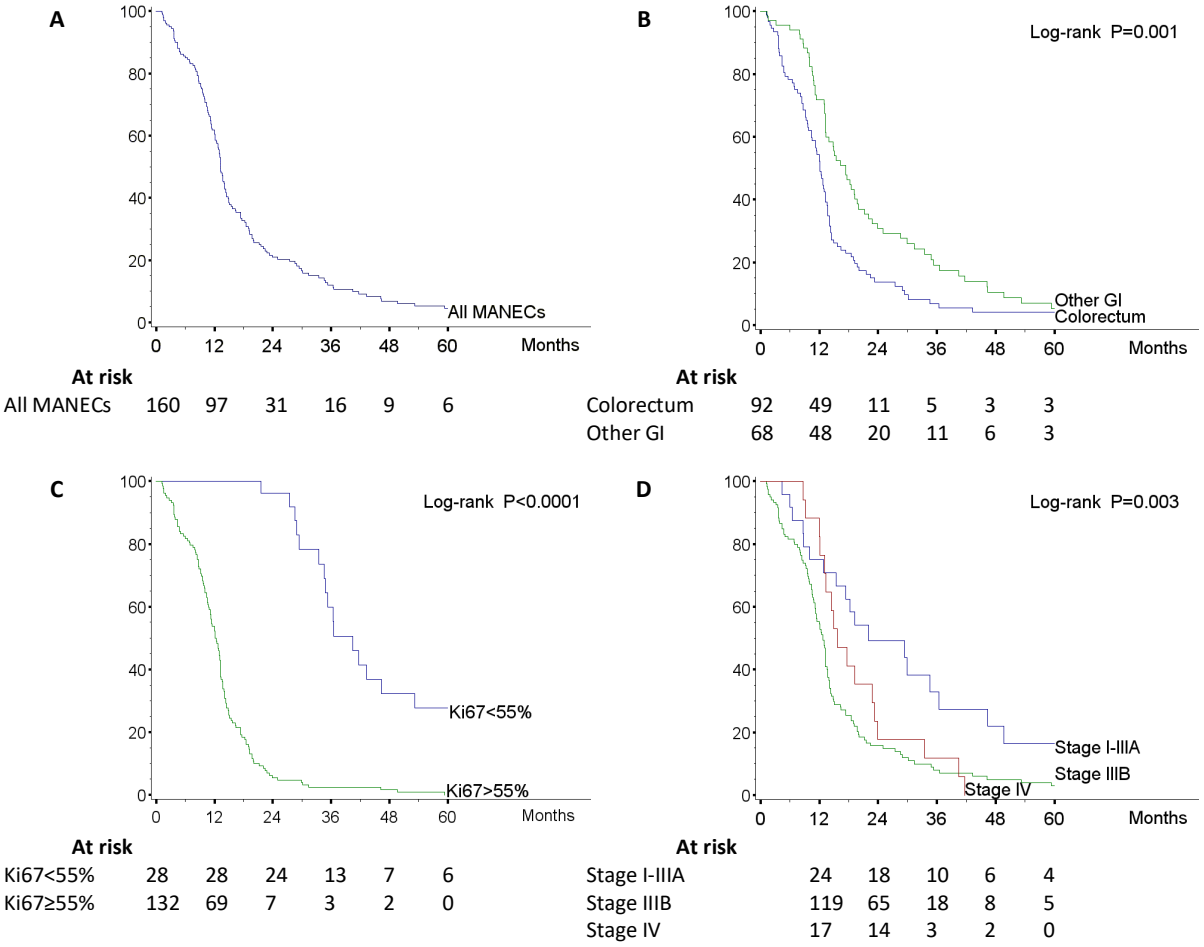
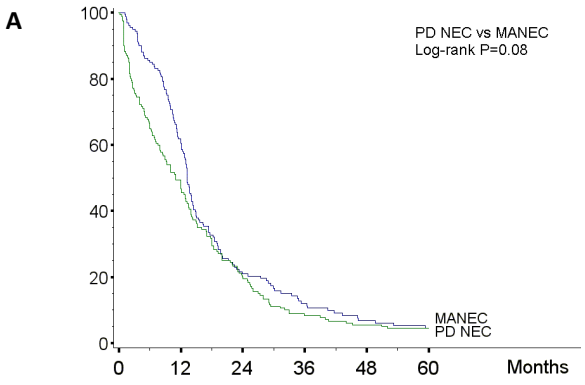
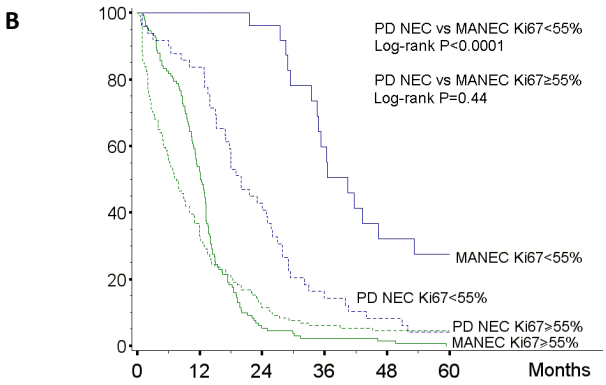
Figure 2. Overall survival in MANEC, by tumor site, Ki67 labelling index and tumor stage



	Overall Survival (95% Confidence Intervals)					
	12-month	**24-month**	**36-month**	**48-month**	**60-month**	**Median OS**
All MANECs	61.1 (53.1-68.2)	20.8 (14.8-27.5)	12.3 (7.7-18.2)	6.9 (3.5-12.0)	4.6 (1.9- 9.1)	13.2 (12.4-14.3)
Colorectum	53.3 (42.6-62.8)	13.3 (7.3-21.2)	7.0 (2.8-13.7)	4.2 (1.2-10.3)	4.2 (1.2-10.3)	12.2 (10.3-13.3)
Other GI	71.9 (59.5-81.0)	31.0 (20.4-42.2)	19.6 (11.0-30.1)	10.7 (4.5-20.0)	5.3 (1.4-13.2)	17.3 (13.3-19.9)
Ki67<55%	100	96.2 (76.1-99.5)	62.0 (39.6-78.1)	33.4 (15.2-52.8)	28.6 (11.9-48.0)	40.5 (34.5-53.2)
Ki67≥55%	52.9 (44.0-61.0)	5.4 (2.4-10.2)	2.3 (0.6- 6.0)	1.5 (0.3- 4.9)	-	12.2 (11.1-13.2)
Stage I-IIIA	75.0 (52.6-87.9)	48.5 (27.4-66.7)	33.2 (14.9-52.8)	22.1 (7.4-41.8)	16.6 (4.3-35.8)	22.1 (12.8-36.4)
Stage IIIB	55.3 (45.9-63.7)	15.9 (9.9-23.0)	8.4 (4.2-14.4)	5.2 (2.1-10.7)	3.1 (0.9- 8.0)	12.7 (11.2-13.3)
Stage IV	82.4 (54.7-93.9)	17.6 (4.3-38.3)	11.8 (2.0-31.2)	-	-	15.7 (12.2-23.3)

Figure 3. Overall survival according to Ki67 expression in MANEC and poorly differentiated NEC





At risk

NEC	180	88	36	15	10	8
MANEC	160	97	31	16	9	6

At risk

PD NEC Ki67<55%	49	41	21	7	4	2
PD NEC Ki67≥55%	131	47	15	8	6	6
MANEC Ki67<55%	28	28	24	13	7	6
MANEC Ki67≥55%	132	69	7	3	2	0

Overall Survival (95% Confidence Intervals)

	12-month	24-month	36-month	48-month	60-month	Median OS
PD NEC	48.9 (41.4-55.9)	20.0 (14.5-26.1)	8.3 (4.9-12.9)	5.6 (2.8- 9.6)	4.4 (2.1- 8.2)	11.0 (8.4-13.0)
MANEC	61.1 (53.1-68.2)	20.8 (14.8-27.5)	12.3 (7.7-18.2)	6.9 (3.5-12.0)	4.6 (1.9- 9.1)	13.2 (12.4-14.3)
PD NEC Ki67<55%	83.7 (70.0-91.5)	42.9 (28.9-56.1)	14.3 (6.3-25.5)	8.2 (2.6-17.9)	4.1 (0.8-12.3)	20.0 (16.9-25.0)
PD NEC Ki67≥55%	35.9 (27.8-44.0)	11.5 (6.7-17.6)	6.1 (2.9-11.1)	4.6 (1.9- 9.1)	4.6 (1.9- 9.1)	7.6 (5.5-10.0)
MANEC Ki67<55%	100	96.2 (76.1-99.5)	62.0 (39.6-78.1)	33.4 (15.2-52.8)	28.6 (11.9-48.0)	40.5 (34.5-53.2)
MANEC Ki67≥55%	52.9 (44.0-61.0)	5.4 (2.4-10.2)	2.3 (0.6- 6.0)	1.5 (0.3- 4.9)	-	12.2 (11.1-13.2)