Responses to Reviewers' Comments

Reviewer ID 02445679

The manuscript by Frizziero et al reports data on histological features and clinical management of a series including 69 patients with mixed adeno-neuroendocrine carcinoma (MANEC). Although this work has some limitations (stated by the authors in the discussion) consisting in the retrospective design, the relatively small number of patients, and the long period of observation (> 30 years), it has some merits which make it interesting for physicians dealing with neuroendocrine tumors. Since MANEC is an extremely rare entity, literature focusing on this topic is scanty. This is the reason why also retrospective studies are welcomed. Below are my comments, which should be taken into account before considering this manuscript suitable for publication:

Comment 1: Introduction: it is correctly reported that the recent WHO 2017 changed the definition of MANEC rising from the pancreas into MiNEN. I believe this change should be applied also in the results section. This could make this work more up to date with the current knowledge, and even more easy to use in the clinical practice.

Response 1: This has been amended in the title as well as in the manuscript, tables and figures.

Comment 2: Additional histological revision should be done, in order to add Ki67 value. Although Ki67 role is widely understood in NETs in general, its prognostic accuracy in MANEC (or MiNEN) is unknown, and this could add an additional value to the manuscript

Response 2: Data included in this study were predominantly obtained from neuroendocrine centres of excellence with expertise in the diagnosis and treatment of patients with neuroendocrine tumours. All samples will have undergone pathological review and in some cases, due to availability of tissue (quantity and/or quality), Ki-67 cannot be accurately recorded. It would be interesting from a scientific standpoint to obtain the Ki-67 value and the morphology (small vs large cell) of the neuroendocrine component for these patients, however, neither are proven prognostic or predictive factors in this

disease group (this has now explained in the discussion of the manuscript). These findings reflect normal clinical practice and further review would not be feasible, nor practical, further highlighting the need for prospective studies in this rare poorly studied entity.

Comment 3: Results and table 2. I understand that multivariate analysis was not performed due to the low number of cases included in the subgroups. However, I think it should be attempted, and data (even if not significant) should be given.

Response 3: Multivariable analysis was attempted for recurrence free survival, progression free survival and overall survival in both the localised and advanced setting (see table below). However, in the localised setting, 67.6% of cases were excluded due to lack of complete data and as a result, the number of analysable cases in each subgroup was too small (n=1-11) to allow reliable comparisons (as demonstrated by extremely wide 95% confidence intervals of hazard ratios). Therefore, the multivariable analysis for localised MiNENs has been not reported in the manuscript (this has been explained in the results section), whereas the multivariable analysis for advanced MiNEN has been added to the supplementary material (Table S3).

Cox regression multivariable analysis for recurrence free survival (RFS), progression free survival (PFS) and overall survival (OS) in the localised and advanced disease setting

Localised stage setting							Advanced stage setting						
			RFS		OS					PFS		OS	
		N pts*	HR (95%-CI)	p	HR (95%-CI)	p			N pts*	HR (95%-CI)	p	HR (95%-CI)	p
Primary tumour	Lower GI (ref.)	11	0.000 (0.000-1.108E+20)	0.466	0.000 (0.000-6.578E+48)	0.757	Primary tumour	Lower GI (ref.)	23	0.180 (0.031-1.051)	0.057	0.104 (0.017-0.654)	0.016
	Upper GI	0						Upper GI	2				
	Pancreato-biliary	1						Pancreato-biliary	4				
Age	<70 y (ref.)	6	0.001 (0.000-570,102.523)	0.483	0.875 (0.000-4.066E+39)	0.998	Age	<70 y (ref.)	16	0.664 (0.262-1.682)	0.388	0.816 (0.292-2.285)	0.699
	≥70 y	6						≥70 y	13				
ECOG PS	0-1	11	0.000 (0.000-1.570E+159)	0.854	0.000 (0.000-1.060E+212)	0.903	ECOG PS	0-1	22	1.343 (0.314-5.736)	0.691	0.154 (0.031-0.769)	0.023
	≥2	1						≥2	7				
Gender	Female (ref.)	7	0.033 (0.000-27,511,701.4)	0.745	0.875 (0.000-2.707E+26)	0.997	Gender	Female (ref.)	11	3.919 (1.260-12.186)	0.018	0.475 (0.145-1.564)	0.221
	Male	5						Male	18				
Predominant component	NE (ref.)	7	0.000 (0.000-164,157,246)	0.433	526.364 (0.000-1.566E+52)	0.914	Predominant component	NE (ref.)	18	6.929 (1.464-32.786)	0.015	1.221 (0.275-5.417)	0.792
	ADC	3						ADC	5				
	Equal	2						Equal	6				
Grading of NE component	G1-G2(ref.)	0	Not applicable				Grading of NE component	G1-G2(ref.)	2	2.844 (0.321-25.179)	0.348	0.055 (0.002-1.441)	0.082
	G3	12						G3	27				
Ki-67 NE component	<55% (ref.)	6	0.000 (0.000-801,900.349)	0.510	0.001 (0.000-4.408E+23)	0.834	Ki-67 NE component	<55% (ref.)	10	0.505 (0.180-1.415)	0.194	1.035 (0.341-3.138)	0.952
	≥55%	6						≥55%	19				
pN+	Positive (ref.)	9	3.561 (0.000-9.501E+15)	0.510	526.364 (0.000-2.163E+60)	0.926	First line active treatment	No (ref.)	5	2.207 (0.594-8.198)	0.237	1.590 (0.383-6.600)	0.523
	Negative	3						Yes	24				
Perioperative treatment	Yes (ref.)	4	29,829.130 (0.000-1.977E+21)	0.602	1.305 (0.000-1.275E+53)	0.997							
	No	8											
%cases not included because of missing data		64.7%					%cases not included because of missing data		47.3%				

Comment 4: Authors should shorten the discussion section by 1/3, particularly in the final section (page 17).

Response 4: The discussion has now been shortened.

Comment 5: Table 1. I wonder why NE proportion was unknown in 1/3 of cases. Please double check, if confirmed add a comment to explain the reason. The same observation refers to % of cell morphology (small vs large), which was unknown in 72.4% of cases. This is quite easy to obtain by checking histological slides.

Response 5: The number of cases for which the predominant histology could not be retrieved from medical records/pathological reports is confirmed to be 23 (~33.3% of the whole cohort). The number of cases for which the morphological subtype of the neuroendocrine component could not be retrieved from medical records/pathological reports is confirmed to be 50 (~72.4% of the whole cohort). A comment has been added to the results section and the discussion of the manuscript regarding this. Although more complete data on the predominant histology in MiNEN, which is a criteria for treatment selection in the palliative setting, would add value to the study, further review of the tumour samples is not feasible nor possible due to tissue availability, further highlighting the need for prospective studies in this rare poorly studied entity.

Comment 6: Table 1, add Ki67 if possible (see point 2).

Response 6: This has now been added to Table 1, Table S1, Table 3 and 4.

Comment 7: Table 2. Add multivariate, if possible (see point 3).

Response 7: Multivariable analysis in the advanced setting has been added to the supplementary material (Table S3).

Comment 8: Figure 2. Please add patients' "number at risk" in the orizontal axis. Try to cut orizontal axis of figures PFS Adv and OS Adv at 36 months.

Response 8: The number of patients at risk has been added to the figures reporting the Kaplan-Meier curves. The length of the horizontal axis of these

figures has been maintained, in order to capture the last progression and death which occurred at 51.6 and 90.3 months from diagnosis, respectively.

Reviewer ID 00050849

This is an interesting manuscript focusing on MANETs by Frizziero MF.

Comment 1: Minor points: At ”Clinical characteristics of patients and pathological data on tumour samples“ last paragraph, “Additional pathological material from synchronous or metachronous metastatic sites was available for 15 patients“ how many patients had synchronous and how many metachronous metastatic sites?

Response 1: This information was available for 9 out of 15 patients with histological material from second biopsies; 3 were synchronous and 6 were metachronous (see Table at Comment 2). This has now been added to the results section of the manuscript.

Comment 2: Major points: Can the authors present biochemical markers (CgA, NSE etc) before and during treatment? Can statistics be applied?

Response 2: Immunohistochemical (IHC) data from samples at diagnosis were collected and are summarised in the table below, and have been added to the results section of the manuscript (Table 2).

Immunohistochemical analysis in samples from patients with a diagnosis of mixed neuroendocrine non-neuroendocrine neoplasm (MiNEN)

VARIABLE	CATEGORY	NUMBER	%
SYNAPTOPHYSIN			
	Positive	60	87.0
	Negative	0	0
	n.a.	9	13.0
CHROMOGRANIN A			
	Positive	37	53.6
	Negative	16	23.2
	n.a.	16	23.2
CD56			
	Positive	26	37.7
	Negative	10	14.5
	n.a.	33	47.8
CK-20			
	Positive	22	31.9
	Negative	11	15.9
	n.a.	36	52.2
CK-7			
	Positive	24	34.7
	Negative	10	14.5
	n.a.	35	50.8
CDX-2			
	Positive	33	47.8
	Negative	4	5.8
	n.a.	32	46.4

n.a. = information not available. Note; the sum of the percentages may not reach 100% due to rounding.

Univariate analysis (Log-rank test for equality of survivors function) for recurrence free survival (RFS), progression free survival (PFS) and overall survival (OS) according to IHC data at diagnosis was run, and results are reported in the table below. This has been added to the results section of the manuscript.

Univariate analysis for RFS, PFS and OS in the localised and advanced setting according to IHC markers

	Localised stage MiNEN										Advanced stage MiNEN									
	Staining	N	RFS				OS				Staining	N	PFS				OS			
			Median (months)	95%-CI		p-value	Median (months)	95%-CI		p-value			Median (months)	95%-CI		p-value	Median (months)	95%-CI		p-value
SYNAPTHOPHYSIN	negative	0	-	-	-	not applicable	-	-	-	not applicable	negative	0	-	-	-	not applicable	-	-	-	not applicable
	positive	28	12.66	8.03	19.21		28.6	18.3	NR		positive	49	6.92	4.66	8.23		9.61	5.64	14.66	
	NA	6	-	-	-		-	-	-		NA	5	-	-	-		-	-	-	
CHROMOGRANIN A	negative	7	8.07	2.66	NR	p = 0.14	28.62	9.28	NR	p = 0.48	negative	15	9.15	5.08	NR	p = 0.039	13.41	5.21	NR	p = 0.26
	positive	17	14.03	9.21	NR		32.56	14.33	NR		positive	30	6.59	4.33	9.44		9.48	5.21	15.21	
	NA	10	-	-	-		-	-	-		NA	9	-	-	-		-	-	-	
CD56	negative	3	18.46	8.07	NR	p = 0.95	34.89	28.62	NR	p = 0.35	negative	9	5.79	4.33	NR	p = 0.5	7.66	5.11	NR	p = 0.4
	positive	12	10.97	5.74	NR		20.39	14.33	NR		positive	20	6.92	5.08	9.80		9.48	5.21	17.93	
	NA	19	-	-	-		-	-	-		NA	25	-	-	-		-	-	-	
CK20	negative	4	10.93	4.16	NR	p = 0.54	18.30	11.21	NR	p = 0.54	negative	10	4.33	3.90	NR	p = 0.46	13.67	5.11	NR	p = 0.4
	positive	10	10.07	6.46	NR		28.62	16.07	NR		positive	19	7.15	4.62	12.85		9.61	5.21	NR	
	NA	20	-	-	-		-	-	-		NA	25	-	-	-		-	-	-	
CK7	negative	3	-	18.46	NR	p = 0.021	-	28.62	NR	p = 0.035	negative	7	6.92	4.66	NR	p = 0.67	13.41	5.21	NR	p = 0.59
	positive	9	9.21	6.36	NR		16.07	14.33	NR		positive	20	4.62	4.33	9.48		9.48	4.56	NR	
	NA	22	-	-	-		-	-	-		NA	27	-	-	-		-	-	-	
CDX-2	negative	1	9.21	-	-	p = 0.33	11.21	-	-	p = 0.0092	negative	4	7.07	2.00	NR	p = 0.74	29.84	2.00	NR	p = 0.76
	positive	17	14.89	10.07	NR		34.43	19.08	NR		positive	29	6.92	4.62	9.48		10.75	9.02	16.30	
	NA	16	-	-	-		-	-	-		NA	21	-	-	-		-	-	-	

NA = not available; 95%-CI = 95%-Confidence Interval; NR = not reached.

Immunohistochemical data from second biopsy samples (presented in the table below) were available for only a few cases, therefore, were not reported in the manuscript.

Histology and immunohistochemical staining of second biopsy samples

		Histology	Synaptophysin	Chromogranin A	CD56	CK20	CK7	CDX2
1	metachronous	NEC	positive	positive	positive	positive	negative	NA
2	metachronous	NEC	NA	NA	NA	NA	NA	NA
3	metachronous	NEC	positive	NA	NA	NA	NA	NA
4	metachronous	ADC	negative	negative	NA	positive	positive	positive
5	synchronous	MIXED NEC/ADC	positive	positive	positive	positive	positive	positive
6	synchronous	NEC	positive	positive	positive	positive	positive	positive
7	synchronous	NEC	NA	NA	NA	NA	NA	NA
8	metachronous	NEC	positive	positive	NA	negative	negative	negative
9	metachronous	NEC	NA	NA	NA	NA	NA	NA
10	NA	MIXED NEC/ADC	NA	NA	NA	NA	NA	NA
11	NA	NEC	NA	NA	NA	NA	NA	NA
12	NA	NEC	NA	NA	NA	NA	NA	NA
13	NA	MIXED NEC/ADC	NA	NA	NA	NA	NA	NA
14	NA	NEC	NA	NA	NA	NA	NA	NA
15	NA	NEC	NA	NA	NA	NA	NA	NA

	Synaptophysin	Chromogranin A	CD56	CK20	CK7	CDX2
positive	33%	27%	20%	27%	20%	20%
negative	7%	7%	0%	7%	13%	7%
NA	60%	67%	80%	67%	67%	73%

Comment 3: In which studies do the authors base there algorithm to offer surgery in localized disease? A Nordic study included only pancreatic NECs (Annals of Surgical Oncology May 2016, Volume 23, Issue 5, pp 1721–1728).

Response 3:

- **Shen C, Chen H, Chen H, et al. Surgical treatment and prognosis of gastric neuroendocrine neoplasms: a single-center experience. BMC Gastroenterol. 2016; 16: 111.**
- **Brathwaite S, Rock J, Yearsley MM, et al. Mixed Adeno-neuroendocrine Carcinoma: An Aggressive Clinical Entity. Ann Surg Oncol. 2016; 23 (7): 2281-6.**
- **Watanabe J, Suwa Y, Ota M, et al. Clinicopathological and Prognostic Evaluations of Mixed Adenoneuroendocrine Carcinoma of the Colon and Rectum: A Case-Matched Study. Dis Colon Rectum 2016; 59 (12): 1160-1167.**
- **Apostolidis L, Bergmann F, Winkler EC, et al. Prognosis and Treatment Outcomes of Patients with Mixed Adenoneuroendocrine Carcinoma (MANEC)**

- A Single Cancer Centre Experience. Neuroendocrinology 2017; 15 (suppl. 1), abstract [available at https://www.enets.org/prognosis-and-treatment-outcomes-of-patients-with-mixed-adenoneuroendocrine-carcinoma-manec-r-a-single-cancer-center-experience.html]

- **Komatsubara T, Koinuma K, Miyakura Y, et al. Endocrine cell carcinomas of the colon and rectum: a clinicopathological evaluation. Clin J Gastroenterol. 2016; 9 (1): 1-6.**

Comment 4: How the authors address the possible hematogenous metastases of the NEC counterpart before the surgery is done.

Response 4: Although there can be a rationale for considering preoperative systemic treatment against the aggressive NEC component in the localised setting, to reduce the risk of systemic disease spread, there is no evidence supporting such an approach in pure neuroendocrine carcinomas. On the contrary, pre- or peri-operative chemotherapy and/or radiotherapy is recommended by international clinical practice guidelines for curable, pure adenocarcinomas from specific sites of the digestive tract, based on evidence from randomised trials. In the present series, 8 patients with localised stage MiNEN and a primary tumour from the rectum, anus or oesophagus received pre- or peri-operative treatment, in agreement with the standard of care for pure adenocarcinomas from the same site of origin. Possibly, combination regimens with activity against both the components (e.g. 5-fluorouracil/oxaliplatin or 5-fluorouracil/irinotecan) would be preferred, should a preoperative approach be considered.