Prognostic features of gastro-entero-pancreatic neuroendocrine neoplasms in primary and metastatic sites: Grade, mesenteric tumour deposits and emerging novelties

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Abstract

Updates in classification of gastro-entero-pancreatic neuroendocrine neoplasms better reflect the biological characteristics of these tumours. In the present study, we analysed the characteristics of neuroendocrine tumours that could aid in a more precise stratification of risk groups. In addition, we have highlighted the importance of grade (re)assessment based on investigation of secondary tumour lesions. Two hundred and sixty-four cases of neuroendocrine tumours of gastro-entero-pancreatic origin from three centres were included in the study. Tumour morphology, mitotic count and Ki67 labelling index were evaluated in specimens of primary tumours, lymph node metastases and distant metastases. These variables were correlated with overall survival (OS) and relapse-free survival (RFS). Tumour stage, number of affected lymph nodes, presence of tumour deposits and synchronous/metachronous metastases were tested as possible prognostic features. Mitotic count, Ki-67 labelling index, primary tumour site, tumour stage, presence of tumour deposits and two or more affected lymph nodes were significant predictors of OS and RFS. At the same time, mitotic count and Ki-67 labelling index can be addressed as continuous variables determining prognosis. We observed a very high correlation between the

measures of proliferative activity in primary and secondary tumour foci. The presence of isolated tumour deposits was identified as an important determinant of both RFS and OS for pancreatic (hazard ratio [HR] = 7.61, 95% confidence interval [CI] = 3.96-14.6, $P < 0.0001$ for RFS; HR = 3.28, 95% CI = 1.56-6.87, $P = 0.0017$ for OS) and ileal/jejunal neuroendocrine tumours (HR = 1.98, 95% CI = 1.25-3.13, $P = 0.0036$ for RFS and HR 2.59, 95% CI = 1.27-5.26, $P = 0.009$ for OS). The present study identifies the presence of mesenterial tumour deposits as an important prognostic factor for gastro-entero-pancreatic neuroendocrine tumours, provides evidence that proliferative parameters need to be treated as continuous variables and further supports the importance of grade determination in all available tumour foci.

KEYWORDS

GEP-NEN, Ki-67, mesenteric tumour deposit, mitotic count, prognosis, tumour deposit

1 | INTRODUCTION

The classification system for gastro-entero-pancreatic neuroendocrine neoplasms (GEP-NENs), which reliably reflects their biological characteristics and is useful for prognostication, has long been an important topic of discussion with various proposals that have been revised multiple times in the last 20 years. The main variables, which permit distinction between NENs of different clinical behaviour, reflect the proliferative capacity of tumour cells. Mitotic count and proliferation index (measured using Ki-67 labelling) are the main determinants of tumour grade. Clinical data accumulated through many years, together with emerging results concerning molecular alterations, have allowed amendments to the 2017/2019 World Health Organization (WHO) classifications,[1,2] introducing the concept of high grade well differentiated neuroendocrine tumour (NET G3) and clearly distinguishing it from the truly highly aggressive high grade, neuroendocrine carcinoma (NEC G3), even though the proliferation indices of these two entities may be similar.[1-3] Indeed, combining the morphology and proliferation rate for tumour classification has markedly improved prognostication and increased reproducibility, even though there are still cases showing low proliferative activity but recurring shortly after primary surgery.[4] A more complex approach is probably needed for even more accurate prediction of long-term prognosis and some attempts have already been made.[5,6]

The parameters that are included in NEN staging might also need revision. Tumour deposits have only relatively recently been included into the N category of the TNM staging scheme of NETs (AJCC 8th edition).[7] Although some data suggest that tumour deposits in small intestinal NETs are prognostically relevant (indeed even more than lymph node metastases), other studies do not reproduce this finding.[8-10]

WHO classification rules have always been related to primary tumours only, even if NENs may be frequently multifocal and/or with synchronous or metachronous metastatic foci. Tumour proliferation parameter assessment for each of the involved areas may show heterogeneous patterns,[11,12] despite the fact that, morphologically, all of the tumour foci look low-grade and are indistinguishable. Variable Ki-67 labelling index in multiple tumour foci or changes over the course of disease has been found to imply increased risk for tumour progression.[13,14] Ki-67 labelling index and mitotic count in different areas of the same tumour may also correspond to different grade.[15] In all of such discordant cases, the consideration of the highest grade is recommended,[16] although the importance of testing all primary and metastatic tumour loci for proper grading is not well supported by large-scale studies.

A further problem is that Ki-67 labelling index and mitotic count cut-offs are not homogenous throughout multiple studies and often do not match the cut-offs provided in the current WHO grading scheme.[17-20] Although it seems reasonable to conclude that there is no single number that could serve as a universal risk-predictor,[16] clinical decision-making still requires certain cut-offs for standardisation.

Issues with standardisation of GEP-NEN grading, selection of areas for Ki-67 labelling index calculation, counting techniques or choosing cut-offs are well-recognised[21] and further amendments to the current classification may appear as more data accumulate.

In addition, classification still proposes grading based on Ki67 labelling index and/or mitotic count; however, a number of studies suggest that both of these parameters are important and should be reported in all cases,[8,22] although this may be questionable. In particular, it is not uncommon to see discordant results of grading based on Ki-67 labelling index and mitotic count and choosing the grade based on the highest index is indicated in such cases. The higher grade is usually shown by Ki-67 labelling index (not mitotic count) and some studies have shown Ki-67 labelling index to be a better predictor of survival.[23]

The present study aimed to better understand the biological behaviour of NETs, both low grade and high grade; compare thr proliferative capacity of the primary tumour, lymph node (LNM) metastases and distant metastases (DM); and assess the value of grading (or regrading) tumour based on Ki-67 labelling index/mitotic count assessment in the primary site, LNM and DM. In addition, we

tested different cut-offs for mitotic count and Ki-67 labelling index in primary and metastatic foci together with other relevant factors (such as tumour location, stage, presence of tumour deposits) for better prediction of long-term effects of NETs.

2 | MATERIALS AND METHODS

2.1 | Case selection and study design

The study was performed in accordance with the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethical Committee of Fondazione IRCCS Istituto Nazionale dei Tumouri (INT), Milan, Italy (no. INT 21/16). All patients provided their written informed consent to allow the use their data and specimen for research purposes.

Patients diagnosed with GEP-NENs between 1 August 1992 and 11 April 2017 at three Italian institutions (Fondazione IRCCS Istituto Nazionale dei Tumouri – INT, Milan; Istituto Europeo di Oncologia – IEO, Milan and Ospedale Policlinico San Martino – Genova) were included in the study. In total, 264 cases were identified.

All neoplasms were surgically resected according to guidelines effective at the time of surgery to ensure surgical margins without disease. In cases of gastric, small and large intestinal NENs, regional lymph node resection was carried out; pancreatic nodules located in body or tail measuring < 2 cm at preoperative imaging staging were resected by enucleation without lymph nodes available for pathology assessment. As supported by the guidelines of the European Neuroendocrine Tumor Society (ENETS) for intestinal NENs, the indication for surgery was determined by risk of bowel obstruction or mesenteric vascular compression. As a result of this approach, some tumours were resected even though they were known to be stage 4 preoperatively.

2.2 | Tumour assessment

For the purpose of analysis, cases were separated into four location groups: gastroduodenal, pancreatic, small intestinal and large intestinal sites. All cases were centrally reviewed and reassessed using the WHO 2019 grading system[2] and Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) 8th staging system,[20] including number of positive lymph nodes. Cases with morphological characteristics of NECs and mixed neuroendocrine–non-neuroendocrine neoplasms were excluded. All gastric NENs were associated with atrophic gastritis in surrounding mucosa (type 1 ECL-cell). No MEN1-related gastric NENs or sporadic tpe 3 gastric NENs were collected.

Data on tumour size were collected, grade and pathological TNM stage have been re-evaluated, and number of tumour-positive lymph nodes was counted. Grading was performed on samples from the primary tumour, regional lymph node metastasis (lymph node with the metastasis of largest size) and distant (liver) metastasis (metastasis of largest size) using mitotic count and Ki67 labelling index. All variables were used independently in downstream analyses. The immunohistochemical staining of Ki-67 was performed using MIB-1 antibody (dilution 1:400; M7240; Dako, Agilent, Santa Clara, CA, USA). Ki-67 labelling index and mitotic count were determined in hot spot areas by manual counting on a slide. This assessment was performed by two expert pathologists independently (MM and FG). For the purpose of this analysis, the means from the calculations were used. Isolated tumour deposits were defined as mesenteric tumour nodules with irregular borders and no evidence of direct extension from the primary tumour or an involved lymph node (Figure 1). Tumour nodules with well-defined round shape or those with considerable number of lymphocytes at the peripheral margin were counted as affected lymph nodes. Deposits were often associated with neurovascular bundles, although it was not a necessary criterion. Distant peritoneal nodules were considered peritoneal metastasis and not tumour deposits.

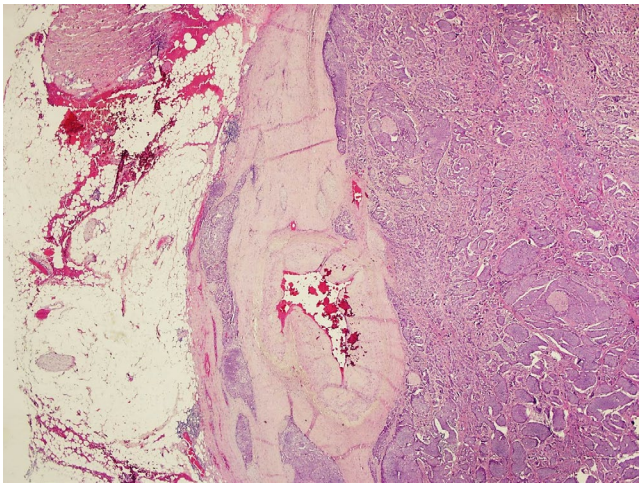


FIGURE 1 Haematoxylin and eosin stained section showing a mesenteric mass, comprising a large, 3-cm, neoplastic deposit of well differentiated neuroendocrine tumour within the mesenteric fat. The lesion shows typical organoid insular architecture and is found adjacent to an artery with intra and peri-lesional fibrosis and peri-neural invasion (magnification ×2). No clear evidence of nodal structures is seen and the mass is focally spiculated

2.3 | Clinical follow-up data

Information on vital status, number and dates of relapses, and dates and causes of death were collected from the medical records.

2.4 | Statistical analysis

Mitotic count and Ki67 labelling index were analysed both as continuous or ordinal variables, using predefined cut-off values (0%-3%, > 3%-20%, > 20%-55%, > 55%). Concordance between

mitotic count (and Ki-67 labelling index) measured on the primary tumours and measured on nodal or liver metastases were calculated using the Pearson correlation coefficient when using the continuous scale, or the Spearman correlation coefficient when using the ordinal scale. Overall survival (OS) was calculated from the date of surgery to the date of death or last contact with the patient. Relapse-free survival (RFS) was calculated from the date of surgery to the date of relapse or last clinical visit. OS and RFS curves were drawn using the Kaplan-Meier method. Differences in survival between groups were assessed with the log-rank test. Cox proportional hazards regression was used to assess the association between tumours characteristics and survival. Multivariable models were constructed including variables that were significantly associated with outcome at univariate analysis. Analyses were performed using SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ (two-sided) was considered statistically significant.

3 | RESULTS

3.1 | Tumour characteristics

Grade 1 neoplasms comprised the majority of cases (59.1%) except for NENs located in the colon/rectum, for which grade 3 tumours were the most common. For all locations, tumours were most frequently stage III at presentation (stomach/duodenum, 66.7%; pancreas, 47.1%; ileum/jejunum, 61.4%; colon, 81.8%). Some 19.7% of cases were found to have distant metastases at the time of diagnosis, and another 58.7% developed metachronous metastases after a median follow-up of 48 months (interquartile range 23-66 months). NEN deposits were found in 44.3% of cases (Table 1).

3.2 | Grading based on different samples

Calculation of mitotic count and Ki-67 labelling index in samples from primary tumours, regional lymph node or liver metastases showed comparable results (see Supporting information, Table S1). These parameters alone as well as grade groups, defined based on any of the tumour foci, resulted in stratification of the tumours into groups which differed in terms of survival parameters (Figure 2A,B). For some particular subgroups, such as tumours with liver metastases or small bowel tumours with lymph node metastases, the hazard ratio (HR) of high mitotic count or Ki-67 labelling index did not reach statistical significance (see Supporting information, Tables S2 and S3) and this may be explained by the rather small size of these subgroups.

Cumulative analysis shows significant impact of proliferative parameters in any tumour focus on outcome, thereby enabling the use of secondary tumour foci (eg, when a sample of the primary lesion is not available) for grade assessment and risk stratification.

The correlation between mitotic count and Ki-67 labelling index was moderate and uniform between primary and secondary tumour foci (Figure 3; see also Supporting information, Figure S1).

3.3 | Survival analysis

The median follow-up was 100 months (range 2.5-329 months).

At univariate analysis, mitotic count, Ki-67 labelling index, primary tumour site, tumour stage, presence of tumour deposits and two or more affected lymph nodes were significant predictors of OS and RFS.

Mitotic count (continuous and using a cut-off of 2), Ki-67 labelling index (continuous and using a cut-off of 10) and grade groups were determined and were significantly associated with both RFS (see Supporting information, Table S2) and OS (see Supporting information, Table S3).

Tumours of colorectal origin showed significantly poor RFS and OS compared to NENs from ileum/jejunum (HR = 3.85, 95% confidence interval [CI] = 2.31-6.43, $P < 0.0001$ and HR = 5.45, 95% CI = 3.04-9.79, $P < 0.0001$, respectively). Pancreatic origin was found to be a weak negative prognostic factor for OS (HR = 1.78, 95% CI = 1.11-2.84, $P = 0.016$) (Figure 2C). Neither primary gastroduodenal (HR = 0.61, 95% CI = 0.26-1.40, $P = 0.24$), nor pancreatic (HR = 0.96, 95% CI = 0.68-1.36, $P = 0.83$) tumours were significantly different from small intestinal NENs in terms of RFS (Figure 2D).

Tumour stage (stage III compared to stage I-II) was significantly associated with the risk of relapse (HR = 2.02, 95% CI = 1.36-3.01, $P = 0.0005$), but not with OS (HR = 0.86, 95% CI = 0.53-1.39, $P = 0.54$) (see Supporting information, Tables S2 and S3).

Presence of isolated tumour deposits was identified as an important determinant of both RFS (Figure 2F) and OS (Figure 2E) for pancreatic (HR = 7.61, 95% CI = 3.96-14.6, $P < 0.0001$ for RFS and HR = 3.28, 95% CI = 1.56-6.87, $P = 0.0017$ for OS) and for ileal/jejunal NENs (HR = 1.98, 95% CI = 1.25-3.13, $P = 0.0036$ for RFS and HR = 2.59, 95% CI = 1.27-5.26, $P = 0.009$ for OS). Even when compared to the tumours of the same ENETS stage, those with tumour deposits showed significantly lower RFS (see Supporting information, Figure S2).

The number of tumour-positive lymph nodes affected relapse-free survival: 2-9 positive lymph nodes (HR = 1.66, 95% CI = 1.20-2.31, $P = 0.0024$) and 10 or more positive lymph nodes (HR = 2.63, 95% CI = 1.20-5.78, $P = 0.016$).

Tumour size was significantly associated with RFS when using 2- and 3-cm cut-offs for pancreatic tumours (HR = 2.55 95% CI = 1.00-6.54, $P = 0.05$ and HR = 3.50 95% CI = 1.54-7.95, $P = 0.0027$, respectively) and for all locations analysed together (HR = 1.60 95% CI = 1.05-2.44, $P = 0.027$, HR = 2.17 95% CI = 1.42-3.31, $P = 0.0003$, respectively).

At multivariable analysis, the presence of tumour deposits was the strongest predictor of relapse together with Ki-67 labelling index, tumour stage and site, but not tumour size (Table 2). In terms

TABLE 1 Patient characteristics by centre and tumour site

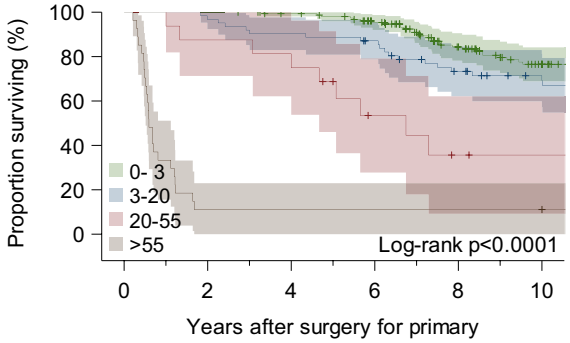
	All	Gastro-duodenum	Pancreas	Small bowel	Large bowel
	N (%)	N (%)	N (%)	N (%)	N (%)
	264 (100.0)	15 (100.0)	87 (100.0)	140 (100.0)	22 (100.0)
Tumour size					
< 2 cm	99 (37.5)	2 (13.3)	23 (26.4)	73 (52.1)	1 (4.5)
2-3 cm	61 (23.1)	1 (6.7)	17 (19.5)	37 (26.4)	6 (27.3)
3-5 cm	59 (22.3)	2 (13.3)	27 (31.0)	20 (14.3)	10 (45.5)
≥ 5 cm	41 (15.5)	9 (60.0)	20 (23.0)	8 (5.7)	4 (18.2)
Stage at diagnosis					
I-II	57 (21.6)	2 (13.3)	39 (44.8)	13 (9.3)	3 (13.6)
III	155 (58.7)	10 (66.7)	41 (47.1)	86 (61.4)	18 (81.8)
IV	52 (19.7)	3 (20.0)	7 (8.0)	41 (29.3)	1 (4.5)
Positive regional lymph nodes					
0	62 (23.5)	1 (6.7)	42 (48.3)	16 (11.4)	3 (13.6)
1-3	97 (36.7)	6 (40.0)	25 (28.7)	60 (42.9)	6 (27.3)
4-9	92 (34.8)	7 (46.7)	19 (21.8)	57 (40.7)	9 (40.9)
10+	13 (4.9)	1 (6.7)	1 (1.1)	7 (5.0)	4 (18.2)
Mitotic count (tumour)					
0-2	203 (76.9)	7 (46.7)	66 (75.9)	127 (90.7)	3 (13.6)
3-30	40 (15.2)	5 (33.3)	16 (18.4)	10 (7.1)	9 (40.9)
>30	21 (8.0)	3 (20.0)	5 (5.7)	3 (2.1)	10 (45.5)
Grade by mitotic count in tumour					
G1	156 (59.1)	7 (46.7)	34 (39.1)	112 (80.0)	3 (13.6)
G2	78 (29.5)	5 (33.3)	45 (51.7)	25 (17.9)	3 (13.6)
G3	30 (11.4)	3 (20.0)	8 (9.2)	3 (2.1)	16 (72.7)
Ki-67 labelling index (tumour)					
< 3	159 (60.2)	3 (20.0)	44 (50.6)	110 (78.6)	2 (9.1)
3-20	62 (23.5)	3 (20.0)	32 (36.8)	25 (17.9)	2 (9.1)
> 20 to < 50	16 (6.1)	2 (13.3)	8 (9.2)	3 (2.1)	5 (22.7)
> 50	27 (10.2)	7 (46.7)	5 (5.7)	2 (1.4)	13 (59.1)
Grade by Ki-67 labelling index in tumour					
G1	145 (54.9)	2 (13.3)	41 (47.1)	101 (72.1)	1 (4.5)
G2	76 (28.8)	4 (26.7)	35 (40.2)	34 (24.3)	3 (13.6)
G3	43 (16.3)	9 (60.0)	11 (12.6)	5 (3.6)	18 (81.8)
Metastasis					
None	57 (21.6)	7 (46.7)	28 (32.2)	20 (14.3)	2 (9.1)
Synchronous	52 (19.7)	3 (20.0)	7 (8.0)	41 (29.3)	1 (4.5)
Metachronous	155 (58.7)	5 (33.3)	52 (59.8)	79 (56.4)	19 (86.4)
Tumour deposit					
Absent	147 (55.7)	10 (66.7)	48 (55.2)	20 (14.3)	9 (40.9)
Present	117 (44.3)	5 (33.3)	39 (44.8)	60 (42.9)	13 (59.1)

of OS, the presence of tumour deposits was again the strongest factor, together with Ki-67 labelling index (Table 2). The significance of proliferative parameters measured in secondary lesions for prediction of RFS and OS was also confirmed at multivariable analysis (see Supporting information, Table S4).

3.4 | Cut-offs for proliferation assessment

ROC analysis showed that both mitotic count and Ki-67 labelling index are highly sensitive and specific for prediction of 5-year OS and also optimal for prediction of 5-year RFS (Figure 4). We identified

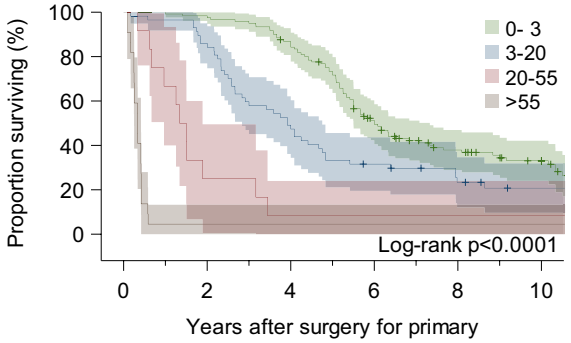
(A)



Patients at risk

0-3	159	159	153	137	97	70
3-20	62	60	56	52	40	32
20-55	16	14	13	6	3	2
>55	27	3	3	3	3	3

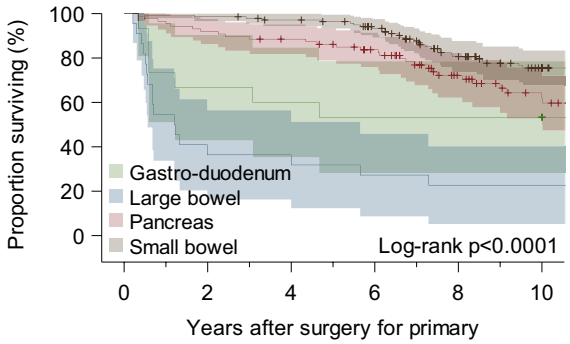
(B)



Patients at risk

0-3	121	119	104	55	35	24
3-20	57	49	28	17	11	7
20-55	12	3	1	1	1	1
>55	22	1	1	1	1	1

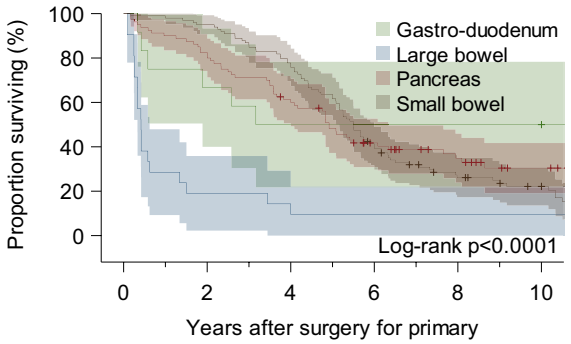
(C)



Patients at risk

Gastro-duo.	15	10	9	8	8	8
Large bowel	22	8	8	6	5	5
Pancreas	87	80	75	65	41	27
Small bowel	140	138	133	119	89	67

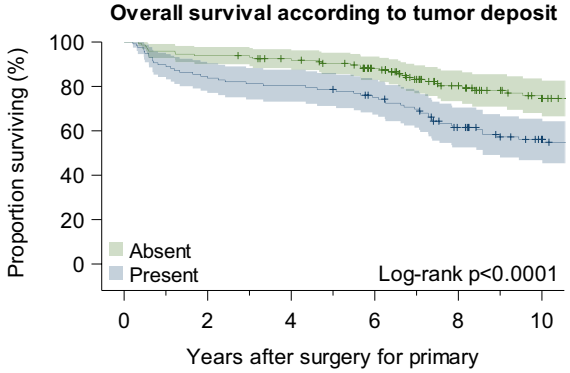
(D)



Patients at risk

Gastro-duo.	12	8	6	6	6	6
Large bowel	21	4	3	2	2	2
Pancreas	80	66	48	28	18	10
Small bowel	99	94	77	38	22	15

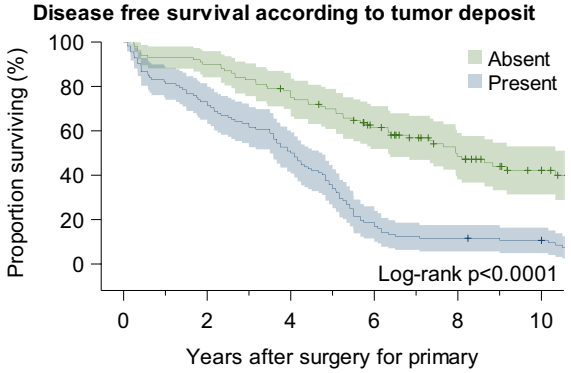
(E)



Patients at risk

Absent	147	138	131	113	80	60
Present	117	98	94	85	63	47

(F)



Patients at risk

Absent	100	90	77	55	35	22
Present	112	82	57	19	13	11

FIGURE 2 Overall survival and relapse-free survival according to Ki-67 evaluated in the primary tumour (T), tumour site and the presence of tumour deposits. A - Overall survival according to primary tumour size (T), B - Disease free survival according to primary tumour size (T), C - Overall survival according to tumour site, D - Disease free survival according to tumour site, E - Overall survival according to presence of tumour deposits, F - Disease free survival according to presence of tumour deposits

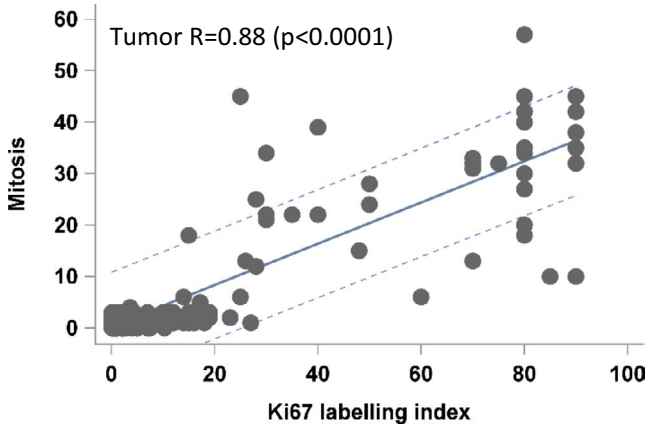


FIGURE 3 Correlation between Ki67 labelling index and mitotic count in the primary tumour

best cut-off values for Ki-67 labelling index and mitotic count to predict 5-year tumour progression as 2.8% and 1, respectively. The best cut-off values for 5-year OS prediction were 11.2% for Ki-67 labelling index and 4 for mitotic count.

Our results support the effectiveness of the current grading system, which uses Ki-67 labelling index cut-offs of 3% and 20% for a reliable prediction of tumour outcome (see Supporting information, Figure S3). In addition, a cut-off of 55% was confirmed as an additional poor prognostic factor for Grade 3 NENs of any origin and measured in any tumour focus (primary or metastatic) (see Supporting information, Figure S4).

3.5 | Survival analysis in "re-graded" tumours

All tumours were graded based on calculation of mitoses/Ki-67 labelling index in primary and metastatic lesions. There were cases for which secondary lesions showed higher or lower grade: 49% of G1 tumours showed G2 lesions in at least one secondary focus. Some 3.7% and 15% of G2 tumours showed at least one G3 and G1 secondary lesion, respectively. Interestingly, this was found in G1 and G2 tumours only and survival parameters for these patients were determined by highest grade identified. None of the G3 tumours were associated with lower-grade secondary lesions.

3.6 | Survival analysis by centre

The results were stratified by centres. Survival data, separated by tumour site, were different, although this was mainly attributable to the low number of cases in some subgroups, making the data from different centres incomparable (see Supporting information, Tables S2 and S3). On the other hand, in the case of small bowel NENs, which were found in relatively higher numbers in all three centres, there was a significant difference in OS among the centres (see Supporting information, Figure S6 and Table S3). This difference could be explained by the fact that accrued patients were treated within a time frame of more than 20 years, meaning that a standardised management approach cannot be guaranteed.

4 | DISCUSSION

According to the data, a number of parameters were found to significantly influence prognosis of GEP-NENs.

The most underappreciated factor, which definitely needs to be highlighted and taken into account in clinical decision-making, is the presence of tumour deposits. Even irrespective of tumour grade, the presence of tumour deposits should be considered an alarming feature in NENs because it appears to affect outcome more than the presence of positive lymph nodes. Data on tumour deposit assessment have been somewhat controversial. On the one hand, a report exists to support that deposits influence prognosis independent from tumour grade in a large series of the midgut (jejunal/ileal) tumours.[10] On the other hand, another study shows non-statistically significant association of tumour deposits with peritoneal disease but not OS or PFS.[8] The current staging system for small intestinal NENs requires that mesenteric tumour deposits have a maximum dimension of > 2 cm to allow upstaging to pN2.[7] At the same time in some studies size of the deposit has not been shown to determine increased risk of progression, whereas the number of deposits appears to be more predictive.[8] On the whole, our data supports the importance of tumour deposit assessment independent of size and number for tumours of all grades and all gastrointestinal locations, especially in pancreatic and small intestinal tumours. Pathologists should be aware of this and make every effort to incorporate data on tumour deposits in their pathology reports, even though distinction between a mesenteric deposit and a nodal metastasis may be challenging.

Our results confirm Ki-67 labelling index and mitotic count to be the best predictors of long-term effects of GEP-NENs. At the same time, in the great majority of cases, these parameters, measured in secondary tumour foci (in lymph nodes or other organs), are highly concordant with the findings in primary tumours. This proves to be an important aspect when dealing with unresectable tumours where grading based on the more easily accessible metastatic site may be suggested as an alternative approach.

Moreover, as the grade of secondary tumour focus correlates with prognosis, it might be considered reasonable to re-evaluate the management approach for the tumours, which demonstrate

TABLE 2 Multivariable analysis

	Overall survival		Relapse-free survival	
	HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**
Center				
INT	1.00		1.00	
IEO	0.24 (0.10-0.55)	0.0007	0.34 (0.20-0.58)	< 0.0001
Genova	2.46 (1.34-4.53)	0.0039	0.96 (0.59-1.59)	0.89
Tumour size				
< 2 cm	1.00		1.00	
2-3 cm	1.11 (0.58-2.11)	0.76	1.01 (0.63-1.59)	0.98
3-5 cm	0.89 (0.46-1.74)	0.74	1.52 (0.96-2.40)	0.07
≥ 5 cm	1.24 (0.61-2.54)	0.56	1.42 (0.80-2.52)	0.23
Site of primary tumour				
Small bowel	1.00		1.00	
Gastroduodenal	0.74 (0.22-2.44)	0.62	0.06 (0.02-0.24)	< 0.0001
Pancreas	1.51 (0.84-2.71)	0.16	1.11 (0.73-1.68)	0.62
Large bowel	1.17 (0.55-2.48)	0.69	1.21 (0.60-2.41)	0.60
Ki67 labelling index (tumour)				
Continuous (5-point increase)	1.27 (1.20-1.34)	<.0001	1.36 (1.27-1.45)	<.0001
Stage (ENETS)				
I-II	1.00		1.00	
IIIA	0.64 (0.34-1.23)	.18	2.49 (1.51-4.11)	.0004
IIIB	0.59 (0.32-1.09)	.09	1.65 (1.03-2.66)	.038
IV	0.58 (0.24-1.37)	.21	-	-
Tumour deposits				
No	1.00		1.00	
Yes	1.96 (1.21-3.18)	.006	2.99 (2.08-4.29)	<.0001

Note: CI, confidence interval; ENETS, European Neuroendocrine Tumor Society; HR, hazard ratio; IEO, European Institute of Oncology; INT, Istituto Nazionale dei Tumouri.

metachronous metastases with increased grade. Assessment of grade in all synchronous tumour foci[24] and regrading of NENs based on newly developed secondary foci were both previously reported to be important for the proper prediction of prognosis.[25] Shi et al[13, 14] showed that any Grade 3 tumour (primary or secondary) significantly influences the outcome. Numbere et al[11] proposed the immunohistochemical re-evaluation of secondary foci, and this is clinically important when secondary foci show markedly increased proliferation/mitotic count. Therefore, sampling as many sites as possible could help correctly stratify patients into treatment and prognostic groups, although this is obviously clinically unrealistic. Considering that the lesion with the highest grade drives the prognosis, our approach would be to recommend a biopsy of the metachronous metastatic focus following a multidisciplinary decision, which takes into account clinical aspects, imaging (eg. fluorodeoxyglucose-positron emission tomography positivity) and the available treatments of the patients.

As shown, Ki-67 labelling index is an extremely accurate prognostic factor for OS and RFS of GEP-NENs when analysed on a continuous scale. Understandably, using cut-offs is more convenient for the purpose of categorisation. Several studies propose a 5% cut-off

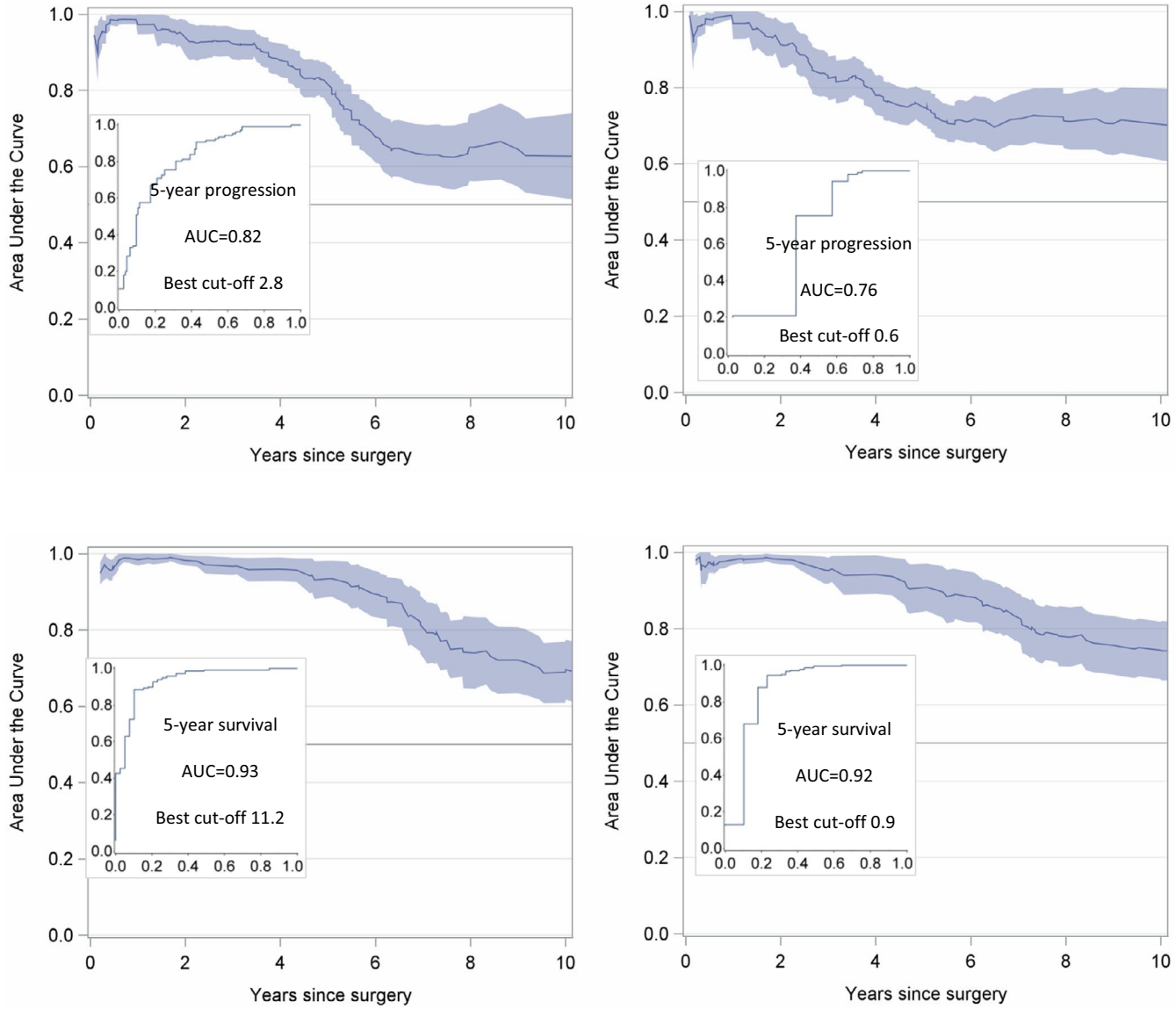


FIGURE 4 Time-dependent area under the curve (AUC) for Ki-67 (left) and mitotic count (right) and respective ROC curve for the prediction of 5-year tumour progression (top) and 5-year overall survival (bottom)

for Ki-67 labelling index with respect to recurrence risk prediction.[17] Furthermore, emerging data show that a cut-off of 55% separates significantly distinct groups of NENs.[18] The idea of Ki-67 labelling index being not only a determinant of tumour grade, but also a tool for treatment selection and response prediction is perfectly highlighted by ESMO and ENETs guidelines,[19,20] which support the importance of the 5%, 10% and 15% cut-offs for selecting chemotherapy, somatostatin analogues or liver transplantation as treatment options and when planning follow-up. Based on our analyses, the cut-offs used in the current grading system, as well as cut-offs set at 10% for Ki-67 labelling index and 2 for mitotic count, reflect the tumour prognosis, even though, in terms of clinical decision-making, Ki-67 labelling index should desirably be addressed as a continuous variable.

As expected, GEP-NENs from various locations showed significantly different long-term effects. The primary site has been found to affect prognosis even for metastatic NENs.[26] Tumours originating from the large bowel should be managed with early treatment and more caution compared to the tumours from other primaries. At the same time, these tumours are much rarer. In most cases, they are grade 3 and have spread to secondary sites at the moment of presentation. This limits the availability of tissue for a thorough analysis of the prognostic factors in these tumours.

A number of limitations of the present study have to be noted. First of all, the cases have been collected over a long period of time in three institutions, and so the treatment schemes for the patients were not homogenous. The standards of diagnostic and treatment procedures for these lesions have been changed gradually. New diagnostic tools allow the stratification of patients with stage 4 disease, which was not a practice until accurate imaging technologies were made available. For this particular study, in most cases, a thorough analysis of the medical care procedures received by each patient was not possible. The composition of the investigated group is

another factor worthy of note. The analysis was performed by subdividing the study sample into multiple categories and, as a result, some of the subgroups turned out to have a low number of cases, making analysis of those particular groups impossible. This mainly applies to tumours of gastroduodenal and colorectal origin, which also show secondary foci of different grade. A number of associations could not show statistical significance as a result of the low number of cases in a subcategory. For example, we consider that, if a higher number of gastroduodenal tumours is analysed, tumour deposits could have been a significant risk modifier in this particular subgroup as well.

In conclusion, the present study further strengthens the importance of evaluation of grade in GEP-NENs at any site and underlines the importance of mesenteric tumour deposits as a novel and significant prognostic factor.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Ketevani Kankava: Investigation; Writing – original draft. **Patrick Maisonneuve:** Formal analysis; Software; Validation; Writing – original draft. **Alessandro Mangogna:** Investigation. **Giovanni Centonze:** Formal analysis; Investigation. **Laura Cattaneo:** Writing – review and editing. **Natalie Prinzi:** Data curation; Investigation. **Sara Pusceddu:** Data curation; Investigation. **Nicola Fazio:** Data curation; Writing – review and editing. **Eleonora Pisa:** Formal analysis. **Stefano Di Domenico:** Data curation. **Emilio Bertani:** Data curation. **Vincenzo Mazzaferro:** Data curation. **Manuela Albertelli:** Data curation; Validation. **Federica Grillo:** Conceptualisation; Investigation; Writing – review and editing. **Massimo Milione:** Investigation; Project administration; Supervision; Writing – review and editing.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets obtained during the present study are not publicly available because of privacy/ethical restrictions, but are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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