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A comparison of Ki-67 and mitotic count as prognostic markers for metastatic pancreatic and midgut neuroendocrine neoplasms

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Background: The aim of this study was to compare mitotic count (MC) and Ki-67 proliferation index as prognostic markers in pancreatic and midgut neuroendocrine neoplasms (NENs).

Methods: Two hundred eighty-five patients with metastatic NENs were recruited. Concordance between histological grade according to either Ki-67 or MC as defined by the European Neuroendocrine Tumour Society guidelines was assessed and the prognostic significance of Ki-67 or MC were evaluated.

Results: There was a discrepancy of 44 and 38% in grade assignment when using Ki-67 or MC in pancreatic and midgut NENs, respectively. In multivariate analysis, grade using Ki-67, but not MC, was a significant prognostic factor in determining overall survival (hazard ratios: midgut G2 2.34, G3 15.1, pancreas G2 2.08, G3 11.3). The prognostic value of Ki-67 was improved using a modified classification (hazard ratios: midgut G2 3.02, for G3 22.1, pancreas G2 5.97, G3 33.8).

Conclusion: There is a lack of concordance between Ki-67 and MC in assigning tumour grade. Grade according to Ki-67 was a better prognostic marker than MC for metastatic pancreatic and midgut NENs. We suggest that Ki-67 alone should be used for grading pancreatic and midgut NENs and that the current threshold for classifying G1/G2 tumours should be revised from 2 to 5%.

Neuroendocrine neoplasms (NENs) are uncommon, heterogeneous tumours with an increasing incidence and prevalence. Most commonly arising from the gastrointestinal tract, gastroenteropancreatic NENs (GEP-NENs) have a variable prognosis, with survival ranging from 6 months to more than 20 years (Yao *et al*, 2008). As the therapeutic options continue to expand it is increasingly important to define robust prognostic markers to inform clinical decision making (Khan and Caplin, 2011). The Ki-67 proliferation index and mitotic count (MC) have proved to be the most useful prognostic histological markers, and have been incorporated into international grading systems (Ramage *et al*, 2012). However, there is a lack of consensus regarding the best marker and the most appropriate cutoff to define grade. Although some groups have used Ki-67 proliferation index, identifying a cutoff of 2% to define low and intermediate groups in midgut and pancreatic NENs (Furlan *et al*, 2004; Panzuto *et al*, 2005; Rorstad, 2005; Tomassetti *et al*, 2005), other groups have subdivided well-differentiated NENs into low and intermediate-grade based on MC (Hochwald *et al*, 2002; Van Eeden *et al*, 2002).

The European Neuroendocrine Tumour Society (ENETS) has proposed a three-tiered grading system for foregut, midgut and hindgut NENs using either Ki-67 proliferation index or MC, based on findings by Rindi *et al* (2006, 2007). This system has recently been adopted by the WHO (World Health Organisation)

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classification of 2010 as well as the AJCC/UICC (American Joint Committee on Cancer/Union for International Cancer Control) classification. Although the ENETS grading system has been validated (Pape *et al*, 2008b; Jann *et al*, 2011), and adopted into routine practice, definitive data do not exist to determine whether the cutoff values used to distinguish the three grades are optimal and some authors still question the use of Ki-67 proliferation index as an independent prognostic indicator (Durante *et al*, 2009).

The histopathological grading of GEP-NENs according to ENETs proposal suggests equivalence between grade according to Ki-67 index and grade according to MC, but in our experience, the two indices may provide conflicting information about grade. To date, there has been no study systematically investigating the concordance between Ki-67 index and MC when using the ENETS grading system. We therefore investigated the agreement between grade according to Ki-67 index and grade according to MC in midgut and pancreatic NENs. Additionally, we explored the prognostic value of each in terms of overall survival (OS) in order to assess the validity of the three-tiered classification.

MATERIALS AND METHODS

Patients with metastatic pancreatic and midgut NENs diagnosed between January 1989 and October 2009 were identified from a database at the Neuroendocrine Tumour Unit, Royal Free Hospital. Patients had both a diagnosis of NEN (based on morphology and immunohistochemistry) and had stage IV disease according to the TNM ENETS criteria based on the presence of distant metastatic disease measurable by RECIST 1.1 (Eisenhauer *et al*, 2009). Diagnostic tissue from biopsy or surgical specimens before commencement of treatment were fixed in formalin and embedded in paraffin. Sections were reviewed by a pathologist with expertise in NENs to establish diagnosis, degree of differentiation (well or poorly differentiated) and MC according to ENETS criteria. On light microscopy, mitotic figures (per 10 high power fields (HPF)) were evaluated in at least 40 fields of highest mitotic activity.

Immunohistochemistry. Sections from tumours were submitted for immunohistochemical examination to evaluate Ki-67 proliferation index. Three micron sections of tumour tissue were deparaffinised in xylene, rehydrated in graded alcohols with endogenous peroxidase blocked with 0.5% H_2O_2 in methanol for 10 min. Thereafter, sections were subjected to 3-min heat-mediated antigen retrieval. Immunohistochemical staining was performed with the NovoLink Polymer detection system (Novocastra, Newcastle-upon-Tyne, UK). Sections were incubated with MIB-1 antibody detecting Ki-67 (DAKO, Cambridgeshire, UK) at a dilution of 1 : 200 for 1 h at room temperature, post-primary block for 30 min, followed by Novolink polymer for 30 min. Reaction products were visualised with application of diaminobenzidine substrate chromogen solution. Slides were counterstained in haematoxylin and mounted. The Ki-67 proliferation index was determined by assessing the percentage of positively staining tumour cell nuclei in 2000 neoplastic cells in areas with highest degree of nuclear labelling where possible (Rindi *et al*, 2006, 2007). Positive non-tumour cells (e.g., endothelial cells, intratumoural lymphocytes) were excluded from analysis. Histopathological grading was assigned to each case according to the classification proposed by the European Neuroendocrine Tumours Society (ENETS) (Rindi *et al*, 2006, 2007) as in Table 1. Each case was assigned two grades, one grade according to Ki-67 proliferation index and one grade according to MC. Cases were also classified into grades with according to the parameters described by Scarpa *et al* (2010); G1: Ki-67 $\leqslant$5%, G2: Ki-67 $>$5% and $\leqslant$20%, G3: Ki-67 $>$20%.

Table 1. Grading of NENs as proposed by ENETS

Grade	Ki-67 (%)	Mitoses per 10 HPF
G1	$\leqslant$2	$<$2
G2	3–20	2–20
G3	$>$20	$>$20

Abbreviations: ENET = The European Neuroendocrine Tumour Society; HPF = high power fields; NEN = Neuroendocrine neoplasm.

Inter-observer error. To assess inter-observer error, 44 H&E stained sections (for MC) and 44 sections stained for Ki-67 were independently reviewed by a second expert pathologist blind to initial assessments. Sections were chosen to distribute low- and intermediate-grades evenly, with a small proportion of high-grade sections, reflecting clinical practice. MC and Ki-67 were assessed as above with grade assigned using both indices.

Clinical data. Pre-treatment biochemical data obtained at the time of diagnosis included plasma Chromogranin A (CgA), and for midgut NENs, 24-h urinary 5-hydroxy-indoleacetic acid (5-HIAA). Overall survival was recorded as the time from diagnosis to the patient's death.

Statistical analyses. Statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA) where *P*-values of <0.05 were considered significant. Pancreatic and midgut NENs were analysed separately. Correlation between grades assigned by Ki-67 and MC was assessed using non-parametric correlation. Agreement between grades and inter-observer agreement was assessed with weighted kappas (κ_W). Survival was estimated using Kaplan–Meier methodology, stratified by both grading systems, with differences in survival between groups analysed by log-rank testing. Potential biomarkers were analysed for prognostic significance. Grading assigned by either Ki-67 and MC were analysed as categorical variables. As CgA was not normally distributed, even when log transformed, it was analysed in two groups: $>$and $\leqslant$2 times the upper limit of normal (120 pmol l^{-1}) (Oberg *et al*, 2011). Urinary 5-HIAA was analysed in two groups: $>$and $\leqslant$ the median (Formica *et al*, 2007). Cox-proportional hazards regression analysis was used to obtain univariate and multivariate hazard ratios for OS. Those variables found to be significant on univariate analyses were incorporated into the multivariate models with a significance level of $P<0.05$.

RESULTS

Patient characteristics. A total of 285 cases of NENs, 144 (51%) of pancreatic origin and 141 (49%) of midgut origin were identified. Of these, 131 and 136, respectively, had complete data and were included in the analysis. Patient characteristics and therapy administered are shown in Table 2. Tumour tissue was obtained from biopsy material in 84 (64%) of those with pancreatic NENs and 95 (70%) of those with midgut primary NENs. The remainder had tissue available from surgical resections.

Inter-observer error. Inter-observer error was assessed between grade assigned according to MCs or Ki-67 index. The matrices of this agreement are shown in Table 3. The κ_W for agreement on grade assigned by MC was 0.83 (95% CI: 0.68–0.99) and for grade assigned by Ki-67 was 0.87 (95% CI: 0.74–1.00). The four discrepancies were between G1 and G2.

Comparison of grade assigned by MC and Ki-67 proliferation index. Of 267 cases, 242 were well-differentiated and 25 poorly differentiated NENs. Of the 21 pancreatic NENs that were poorly

Table 2. Background characteristics of patient group

Primary site	Pancreatic ($n=131$)	Midgut ($n=136$)	Total ($n=267$)
Age at diagnosis			
Median years (range)	51.5 (21–81)	56 (22–84)	54 (21–84)
Gender			
Male	65 (50%)	70 (51%)	135 (51%)
Female	66 (50%)	66 (49%)	132 (49%)
Origin of specimen			
Biopsy	84 (64%)	95 (70%)	179 (67%)
Surgery	47 (36%)	41 (30%)	88 (33%)
Grade according to Ki-67			
Low (G1)	34 (26%)	68 (50%)	102 (38%)
Intermediate (G2)	68 (52%)	58 (43%)	126 (47%)
High (G3)	29 (22%)	10 (7%)	39 (15%)
Grade according to MC			
Low (G1)	65 (50%)	84 (62%)	149 (56%)
Intermediate (G2)	55 (42%)	50 (37%)	105 (39%)
High (G3)	11 (8%)	2 (1%)	13 (5%)
Chromogranin A in pmol l^{-1}			
$\leqslant 120$	90 (69%)	68 (50%)	158 (59%)
>120	41 (31%)	68 (50%)	109 (41%)
Urinary 5-HIAA (μmol per 24 h)			
$\leqslant 96$	–	61	–
>96	–	60	–
Missing	–	15	–
Subsequent therapy			
None	1	5	6
Surgical resection	49	70	119
Chemotherapy	78	28	106
Somatostatin analogues	33	84	118
Interferon	5	3	8
Radiofrequency ablation	2	1	3
Embolisation	6	10	16
Radionuclides	14	40	54

Abbreviations: MC = mitotic count; 5-HIAA = 5-hydroxy-indoleacetic acid.

Table 3. Inter-observer Agreement of Grade Assigned by (A) MC and (B) Ki-67 proliferation Index

	Observer 2			
	G1	G2	G3	
A				
Observer 1				
G1	21	4	0	**25**
G2	0	18	0	**18**
G3	0	0	1	**1**
	21	**22**	**1**	**44**
B				
Observer 2				
G1	17	3	0	**20**
G2	1	18	0	**19**
G3	0	0	5	**5**
	18	**21**	**5**	**44**

Abbreviation: MC = mitotic count.

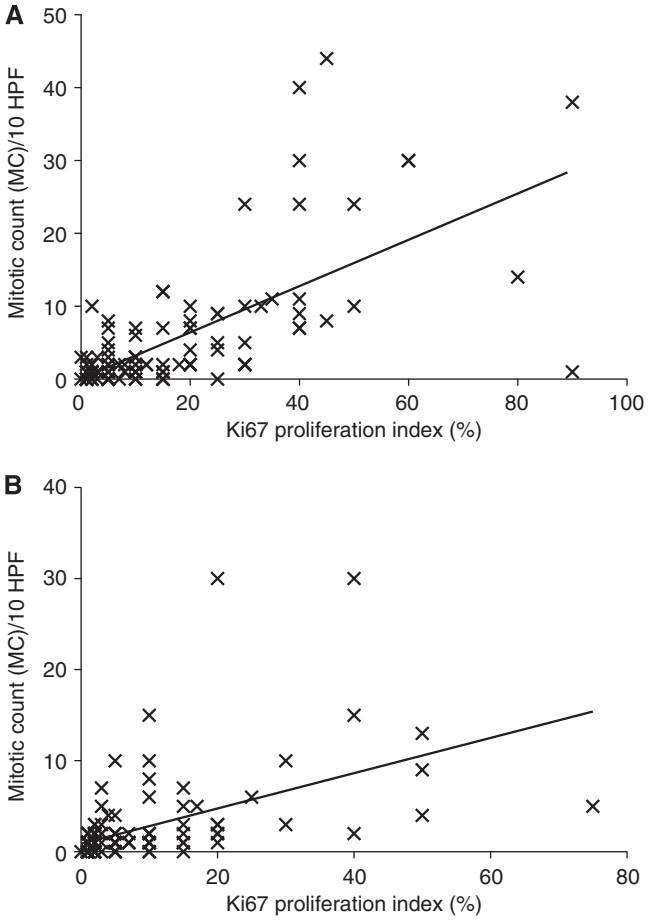

Figure 1. Correlation between Ki-67 proliferation index and MC in (**A**) pancreatic and (**B**) midgut NENs.

differentiated, one was G2 and 20 G3 according to Ki-67; one G1, 11 G2 and 9 G3 according to MC. Four midgut NENs were poorly differentiated, all designated as G3 according to Ki-67 and all G2 according to MC.

There was a moderate correlation between absolute Ki-67 index and MCs ($\rho=0.65$ $P<0.001$ for pancreatic and $\rho=0.59$ $P<0.001$ for midgut NENs) (Figure 1). There was agreement between grade assigned by Ki-67 and grade assigned by MC in 74 of 131 (56%) pancreatic NENs; and in 84 of 136 (62%) of midgut NENs (Table 4). This corresponds to a discordance of 44% and 38%, respectively, with a κ_W of 0.41 (95% CI: 0.30–0.53) and 0.35 (95% CI: 0.22–0.48), respectively. This equates to moderate and fair agreement, when assigning grade with these indices. When surgical and biopsy specimens were compared for agreement between grade using Ki-67 and MC, there was little difference with κ_W 0.42 (0.26–0.59) and κ_W 0.39 (0.29–0.49), respectively.

Survival. Patients were followed up for a median of 46 months (pancreatic) and 42 months (midgut). Survival data are shown in Table 5. Kaplan–Meier survival curves for pancreatic and midgut

NENs are shown in Figure 2. The three-tiered grading systems with either Ki-67 or MCs were able to distinguish significantly different prognostic groups in pancreatic NENs. When analysing midgut NENs, however, only grade according to Ki-67 was able to distinguish the three-tiered prognostically different groups in terms OS. Grading using MC was not able to distinguish G1 from G2 tumours.

Univariate and multivariate analyses are shown in Table 6. Higher grade according to Ki-67 was an independent prognostic indicator of OS in both pancreatic and midgut NENs, whereas grade using MC was not. The only other significant variables were CgA $>120\,\text{pmol}\,\text{l}^{-1}$, which was associated with shorter OS; and age in pancreatic NENs.

Table 4. Agreement between grade assigned by Ki-67 and MCs in (A) pancreatic NENs (agreement in 74/131 cases) and in (B) midgut NENs (agreement in 84/136 cases)

	Grade according to MC/10 HPF			
	G1	**G2**	**G3**	
Grade according to Ki-67				
A Pancreatic				
G1	**29**	5	0	**34**
G2	34	**34**	0	**68**
G3	2	16	**11**	**29**
	65	**55**	**11**	**131**
B MIDGUT				
G1	**55**	13	0	**68**
G2	29	**28**	1	**58**
G3	0	9	**1**	**10**
	84	**50**	**2**	**136**

Abbreviations: HPF = high power fields; MC = mitotic count; NEN = neuroendocrine neoplasm.

Table 5. OS for pancreatic and midgut NENs

	Median	1 Year (%)	3 Year (%)	5 Year (%)	10 Year (%)
Pancreatic	82	89.6	78.6	58.8	35.8
Midgut	84	92.7	73.8	61.3	36.4

Abbreviations: NEN = neuroendocrine neoplasm; OS = overall survival.

Alternative thresholds for grade classification. Tumour grades were reassigned with alternative cutoffs for Ki-67 suggested by Scarpa *et al* (2010) for Ki-67. In this classification grades are defined as follows: G1: Ki-67 $\leqslant$5%, G2: Ki-67 $>$5% and $\leqslant$20%, G3: Ki-67 $>$20%. Univariate analyses with survival curves for this alternative grading classification are shown in Figure 3, which demonstrates that that this grading system was able to distinguish three prognostically different groups. Multivariate analyses confirmed that grade according to ki-67 was an independent prognostic factor (Table 7). The hazard ratios using the alternative threshold were higher than those using the ENETS thresholds suggesting that the alternative thresholds may be more discriminatory than those of ENETS. Chromogranin A $>120\,\text{pmol}\,\text{l}^{-1}$ was also prognostic.

DISCUSSION

We found a correlation between absolute Ki-67 index and MC, which is to be expected as both are markers of cell division and measure proliferation. However, we demonstrate when using these indices to assign grade, there was 44 and 38% discordance in pancreatic and midgut NENs; moderate and poor agreement defined by κ_W. Using ENETS guidelines, either MC or Ki-67 can be used to assign grade but the lack of concordance may result in different classification of the same tumour depending on which parameter is used. This could result in different patient management as G3 tumours are usually treated with chemotherapy first line.

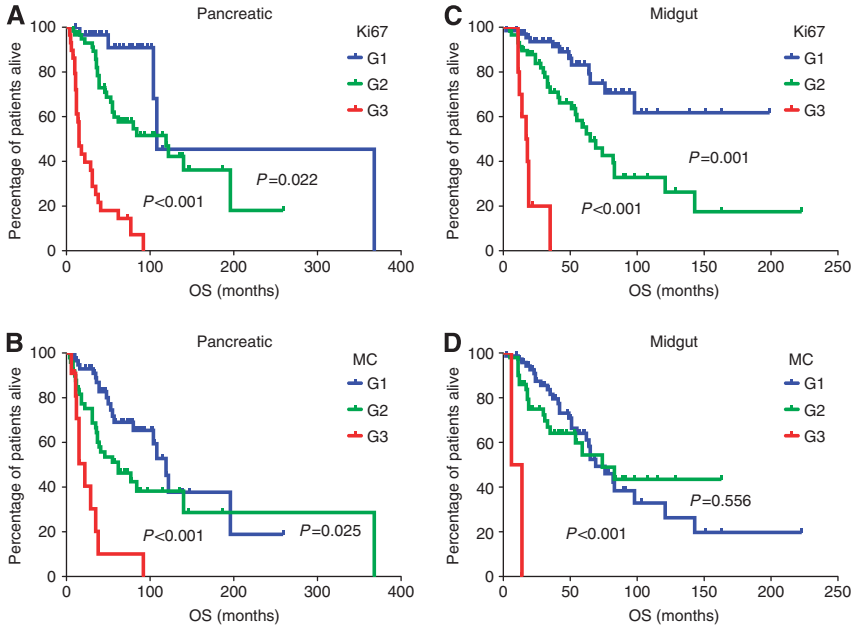

Figure 2. Survival curves for pancreatic and midgut NENs using grade according to Ki-67 (**A** and **C**, respectively) or grade according to MC (**B** and **D**, respectively); blue (G1), green (G2) and red (G3).

Table 6. Univariate and multivariate analyses of independent prognostic factors for pancreatic and midgut NENs using the ENETS grading system

	Pancreatic NENs				Midgut NENs			
	Univariate analyses		Multivariate analyses		Univariate analyses		Multivariate analyses	
Risk factor	**OS HR (95% CI)**	***P*-value**	**OS HR (95% CI)**	***P*-value**	**OS HR (95% CI)**	***P*-value**	**OS HR (95% CI)**	***P*-value**
Differentiation								
Well	1.00		1.00		1.00		1.00	
Poorly	6.94 (3.93–12.2)	<0.001	1.24 (0.48–3.15)	0.658	10.6 (3.02–37.5)	<0.001	1.90 (0.36–10.0)	0.451
CgA (pmol l^{-1})								
CgA⩽120	1.00		1.00		1.00		1.00	
CgA>120	5.47 (3.0–10.0)	<0.001	2.32 (1.23–4.36)	0.009	2.12 (1.20–3.75)	0.01	1.65 (0.88–3.08)	0.117
Urinary 5-HIAA (μmol per 24 h)								
5-HIAA ⩽96					1.00			
5-HIAA>96	–	–	–	–	1.38 (0.67–2.82)	0.379	–	–
Grade (Ki-67)								
G1	1.00		1.00		1.00		1.00	
G2	3.17 (1.11–9.09)	0.032	2.08 (0.69–6.28)	0.192	2.94 (1.50–5.74)	0.002	2.34 (1.1–4.80)	0.021
G3	17.5 (6.03–50.8)	<0.001	11.3 (2.88–44.4)	0.001	21.9 (8.33–57.5)	<0.001	15.1 (3.94–58.1)	<0.001
Grade (MC)								
G1	1.00		1.00		1.00		1.00	
G2	1.92 (1.08–3.41)	0.026	0.912 (0.46–1.81)	0.793	1.19 (0.67–2.09)	0.557	0.848 (0.43–1.68)	0.635
G3	6.99 (3.19–15.3)	<0.001	1.32 (0.43–4.04)	0.627	22.5 (4.79–105)	<0.001	2.69 (0.39–18.3)	0.313
Age (for every 10 years)	1.22 (1.00–1.49)	0.046	1.29 (1.03–1.62)	0.028	1.21 (0.97–1.52)	0.097	1.14 (0.89–1.45)	0.122
Histology sample								
Surgical	1.00		1.00				1.00	
Biopsy	2.35 (1.26–4.37)	0.007	1.84 (0.92–3.7)	0.082	2.35 (1.14–4.83)	0.020	1.87 (0.85–4.17)	0.122

Abbreviations: CI = confidence interval; CgA = chromogranin A; ENET = The European Neuroendocrine Tumour Society; HR = hazard ratio; NEN = neuroendocrine neoplasm; OS = overall survival.

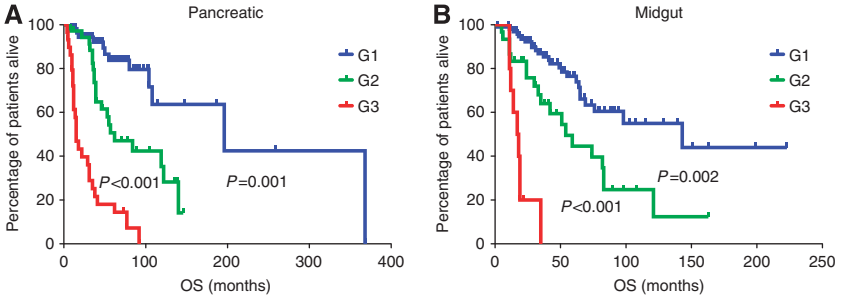

Figure 3. Survival curves demonstrating OS in (**A**) pancreatic NENs and (**B**) midgut NENs with grade (G1, G2 and G3) according to Ki-67 classifications according to Scarpa *et al* (2010).

Our findings conflict with findings by Strosberg *et al* (2009), who demonstrated complete agreement between grade by Ki-67 and MC. However, they used a two-tiered rather than three-tiered grading system, which is a simplification of the ENETS grading classification. A recent study compared methods of assessing proliferation: 'hotspot' Ki-67 assessment in one field; field average over 10 consecutive fields using digital imaging analysis; and MC (Goodell *et al*, 2012). Although they highlighted discordance in grade assignment in a small sample of pancreatic NENs, comparing those methods did not reflect current clinical practice, and there was no prognostic evaluation or survival data in the study.

We have assessed OS rather than disease specific survival and this is a potential limitation. However, others have shown, in this patient group, that non-cancer related mortality is 2.6% (Scarpa *et al*, 2010) and our survival data for metastatic pancreatic NENs appears to be comparable to previous series with 5- and 10-year survival rates of 59% and 36%, respectively (median follow-up 54 months). A Swedish series of 324 pancreatic NENs had 5- and 10-year survival of 64 and 44% (median follow-up 54 months), but only 180/324 in that series had metastatic disease (Ekeblad *et al*, 2008). A more recent series has reported survival rates 35% and 17%, respectively, in a metastatic subgroup (Scarpa *et al*, 2010).

Table 7. Multivariate analyses of prognostic factors in pancreatic and midgut NENs with grade (G1, G2 and G3) according to Ki-67 using thresholds according to Scarpa ***et al*** (2010)

	Pancreatic NENs		Midgut NENs	
Risk factor	**OS HR (95% CI)**	***P*-value**	**OS HR (95% CI)**	***P*-value**
CgA (pmol l^{-1})				
CgA ⩽ 120 CgA > 120	1.00 2.09 (1.08–4.06)	0.029	1.00 2.03 (1.12–3.68)	0.020
Grade (Ki-67)				
1 2 3	1.00 5.97 (1.97–18.14) 33.8 (9.50–120)	0.002 <0.001	1.00 3.02 (1.58–5.75) 22.1 (7.12–68.4)	0.001 <0.001
Grade (MC)				
1 2 3	1.00 0.72 (0.32–1.58) 0.91 (0.29–2.88)	0.409 0.872	1.00 0.61 (0.30–1.21) 1.57 (0.27–9.21)	0.158 0.620
Age (for every 10 years)	1.022 (0.99–1.05)	0.103	1.12 (0.89–1.43)	0.001

Abbreviations: CI = confidence interval; HR = hazard ratio; NEN = neuroendocrine neoplasm; OS = overall survival.

For our series of metastatic midgut NENS (1989–2009), we report a 5-year OS of 61.3%, which compares with results of the Surveillance, Epidemiology and End Results (SEER) registry data (1992–1999) in which the overall 5-year survival of midgut NENs was 61%, and 50% for those with metastatic disease(Modlin *et al*, 2003). Although a higher 5-year survival rate was quoted with more recent SEER (2004–2007) data for midgut NENs (68%), a breakdown by stage was not given (Lawrence *et al*, 2011). A higher 5-year survival rate (83%) was reported in a recent European series but a number of slow growing hindgut NENs were included (Jann *et al*, 2011).

AS there was a lack of concordance between grade assigned by Ki-67 and MC, we investigated which index was more clinically valuable by analysing the prognostic value of each. In pancreatic NENs, although grade according to MC was prognostic on univariate analyses, it was not an independent prognostic factor on multivariate analyses and was not able to distinguish G1 from G2 in terms of OS. Only grade according to Ki-67, and not MC, was prognostic in multivariate analyses in both pancreatic and midgut NENs. Baseline CgA $>120\,\text{pmol}\,\text{l}^{-1}$ was the only other risk factor, apart from age, found to be associated with worse OS on multivariate analysis, and only in metastatic pancreatic NENs. This suggests that grade according Ki-67 is a better prognostic variable than grade according to MC. One of the reasons for this finding may be that MC is affected by pre-analytical or analytical factors such as delay in tissue fixation (Donhuijsen *et al*, 1990; Bergers *et al*, 1997), problems in identification of a mitotic figure (Baak *et al*, 1989), selection of measurement area (Paulus *et al*, 1984; Verhoeven *et al*, 1990), or assessment of mitotic cells in relation to tumour tissue in the sample (Woosley, 1991). The discrepancy in grading could also arise from the fact that the mitotic phase represents the smallest portion of the cell cycle and Ki-67, which detects cells from mid-G1 through S and G2 phases, will detect proliferating cells that do not show mitotic figures.

The ENETS guidelines stipulate that Ki-67 is assessed in areas of highest proliferative activity (hot spots), whereas mitoses are expressed by 10 separate HPF over an average of 40 HPF and many fields may not have any proliferative activity, contributing to the discrepancy. Also, in NENs, Ki-67 assessment has been standardised to 2000 cells with highest activity, whereas the consensus in breast cancer is 500–1000 cells and assessment of 'hot spots' being less consistent (Dowsett *et al*, 2011).

According to ENETS grading criteria, when the amount of tumour tissue is limited such as in a core biopsy, it is not possible to perform an accurate MC as it does not contain the recommended 40 microscopic fields of tumour. In these cases, Ki-67 may provide a more accurate proliferative index, although the MC can be readily performed on the routine H&E slides and the Ki-67 index requires the performance of an immunostain.

It is recognised that the proliferative rate with Ki-67 is not always uniform throughout a given NEN. Whether the Ki-67 index obtained from a core biopsy, which may contain the recommended 2000 cells, represents the whole tumour has been investigated by Yang *et al* (2011). Despite the intratumoural heterogeneity in Ki-67 labelling found in nearly half of metastatic well-differentiated NENs to the liver, Yang demonstrated that Ki-67 grading based on virtual biopsies had significant prognostic value similar to that using whole slides. Thus, Yang's data support Ki-67 staining of core biopsies as an adequately reliable method of proliferation assessment for prognosis.

The distinction between G1 and G2 NENs is based on a very subtle difference in the proliferative rate, which may not be optimal grading threshold and hence accountable for the discrepancy in grading and prognostic value. The 2% threshold for Ki-67 was derived from previous data (Furlan *et al*, 2004; Panzuto *et al*, 2005; Rorstad, 2005; Tomassetti *et al*, 2005). However, the thresholds may not apply to all populations of NENs studied, as there is heterogeneity in terms of primary tumour, stage of disease and subsequent treatments among studies. We have separated midgut and pancreatic NENs in the analysis and have also focussed on cases with metastatic disease as this population constitutes the majority of clinical practice.

The identification and definition of optimal cut-points to distinguish the three grades remains the subject of debate. The three-tiered ENETS grading system has been validated in retrospective series in foregut (Pape *et al*, 2008b), midgut and hindgut NENs (Jann *et al*, 2011) with most using Ki-67. More recently, Scarpa *et al* (2010) found the Ki-67 cutoff of 2% was unable to distinguish G1 and G2 prognostically in a multivariate analysis of 237 pancreatic NENs . However a cut of 5%, as originally suggested Pelosi *et al* (1996), was found to be more discriminatory. Pape *et al* (2008a) also reported that cutoffs of 5 and 10% were prognostic in a heterogeneous series of 239 NENs.

Here, we also investigated thresholds of 5 and 20% for Ki-67 as used by Scarpa *et al* (2010) for both midgut and pancreatic NENs. In both midgut and pancreatic NENs, on univariate and multivariate analyses, Ki-67 was again confirmed as an independent prognostic indicator. Raising the cutoff between G1 and G2 to 5% resulted in better differentiation of G1 from G2 NENs in terms of OS with hazard ratios higher than with ENETS thresholds, suggesting that these alternative thresholds may be more optimal when prognosticating.

The guidelines for the management of NENs continue to evolve with some discrepancy between the ENETS and AJCC/UICC staging systems. In Europe, the TNM staging system suggested by ENETS has been adopted, whereas the recent 2010 WHO guidelines suggest the AJCC/UICC system should be used; however, it also mentions the ENETS TNM system and proposes a grading system resembling that of ENETS, based on Ki-67 or MC (Bosman *et al*, 2010). In 2009, the AJCC/UICC introduced TNM staging of gastrointestinal and pancreatic NENs, which differ in several aspects from the ENETS guidelines but do concede that Ki-67 is a useful prognostic marker (Sobin *et al*, 2009). In 2010, the

NANETS provided guidelines for clinical management and refer to diagnosis by either ENETS and AJCC/UICC systems, but states that it should be indicated which is used (Klimstra *et al*, 2010; Kulke *et al*, 2010; Strosberg *et al*, 2010; Vinik *et al*, 2010a,b).

Here, we have validated the grading system proposed by ENETS guidelines in patients with NENs with metastatic disease, a more homogenous group than the large published pathology data sets. As the majority of patients present with metastatic NENs at the time of diagnosis, this is a clinically relevant population. Our data suggest that despite the ENETS grading guidelines, one should not assume agreement between Ki-67 and MC, which can impact on therapeutic decisions. We also conclude that grade according to Ki-67 is better in predicting prognosis than MC. Furthermore, the alternate grading strata suggested by Scarpa *et al* (2010) were found to be prognostic in both metastatic pancreatic and midgut NENs and more optimal than ENETS guidelines. In conclusion, as both MC and Ki-67 measure proliferation and MC provides no additional information, future clinical guidelines should define grade in NENs solely with Ki-67.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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