

Prognostic significance of neuroepithelial transforming gene 1 in adenocarcinoma of the oesophagogastric junction

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Background: Neuroepithelial transforming gene 1 (NET1) mediates tumour invasion and metastasis in a number of cancers, including gastric adenocarcinoma. It is an indicator of poor prognosis in breast cancer and glioma. This study examined NET1 expression and its prognostic significance in patients with adenocarcinoma of the oesophagogastric junction (AOG).

Methods: NET1 expression was measured by immunohistochemistry in a tissue microarray, constructed from biobanked tissue collected over a 10-year interval, and linked to a prospectively maintained clinical database.

Results: Using the Siewert classification for AOG, type I tumours expressed significantly higher levels of NET1, with lowest expression in type III and intermediate levels in type II ($P = 0.001$). In patients with AOG type III, NET1-positive patients were more likely to be female ($P = 0.043$), have advanced stage cancer ($P = 0.035$), had a higher number of transmural cancers ($P = 0.006$) and had a significantly higher median number of positive lymph nodes ($P = 0.029$). In this subgroup, NET1-positive patients had worse median overall (15 *versus* 23 months; $P = 0.025$) and disease-free (11 *versus* 36 per cent; $P = 0.025$) survival compared with NET1-negative patients.

Conclusion: Although existing data show differences in clinical and prognostic indices across AOG subtypes, there are no studies showing differences in tumour biology. These data suggest NET1, a known mediator of an aggressive tumour phenotype in a number of gastrointestinal cancers, is expressed differentially across AOG subtypes and may be of prognostic significance in the clinical management of this condition.

Introduction

The incidence of oesophageal adenocarcinoma has increased markedly in the West over the past 30 years^{1,2}, with most tumours concentrated in the lower oesophagus and oesophagogastric junction. The widely used Siewert classification system³ is based on distance of the centre of the tumour from the anatomical cardia, where the endoscopic cardia is defined as the upper end of the longitudinal folds of the gastric mucosa. Adenocarcinoma of the oesophagogastric junction (AOG) type I involves the distal oesophagus and arises mostly in the specialized intestinal metaplasia of Barrett's oesophagus; AOG type II arises immediately at the junction or anatomical cardia; and AOG type III is a subcardiac gastric carcinoma infiltrating the oesophagogastric junction and distal oesophagus from

below⁴. Previous studies have shown differences in survival among these subgroups⁵, and their clinical management can differ in terms of choice of operation and neoadjuvant treatments^{6,7}. The biology of these tumour subtypes remains unclear, with AOG type I strongly linked to reflux and obesity. It has been suggested that characterization of the molecular pathways across the junctional spectrum might inform clinical decision-making in the future^{5,6}.

Neuroepithelial transforming gene 1 (*NET1*) is located at chromosome 10p15 and encodes a 54-kDa oncoprotein⁸. It is a guanine nucleotide exchange factor involved in cytoskeletal regulation and cancer cell invasion⁹. NET1 expression has been identified in the gastrointestinal tract and other epithelial cancers, where it has been shown to be a marker of aggressive disease with reduced disease-free survival^{10,11}, high tumour grade¹² and lymph

node metastasis¹⁰. It is a mediator of invasion and metastasis in gastric adenocarcinoma^{13,14}. In oesophageal adenocarcinoma, *in vitro* data suggest that NET1 is highly expressed and acts as a promoter of tumour invasion¹⁵. The prognostic significance of NET1 expression in oesophageal adenocarcinoma and across the AOG spectrum is unknown, and was the focus of the present study.

Methods

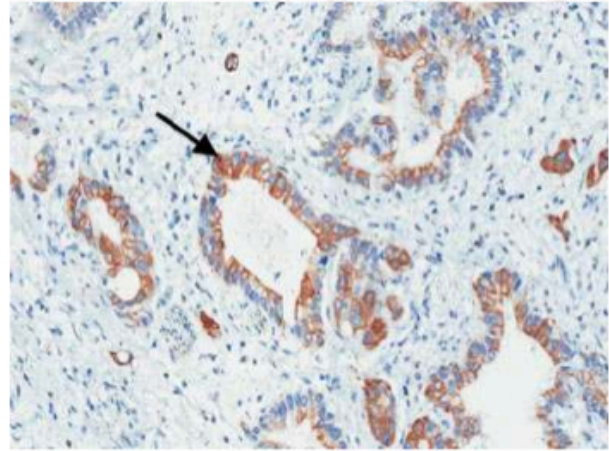
Approval to conduct the study was obtained from the ethics committees of both Mater Hospital (University College Dublin) and St James's Hospital (Trinity College Dublin).

Patient cohort and database

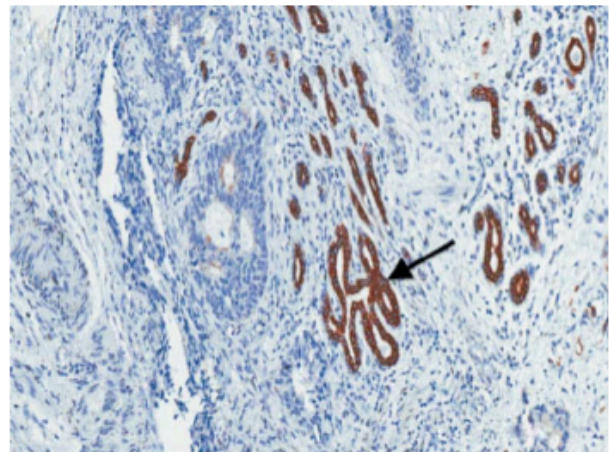
An original cohort of consecutive patients who had surgery for oesophageal and junctional tumours over a 10-year period at a specialist oesophageal cancer surgical centre based at a large tertiary referral university hospital was used. Patient data were maintained prospectively from the date of diagnosis, and included demographic parameters, body mass index, adjuvant or neoadjuvant therapies, duration of follow-up, presence of Barrett's metaplasia (specialized intestinal metaplasia) and/or dysplasia, histology, clinical and pathological staging, and extent of surgical resection. Patients were excluded where: histology was not adenocarcinoma, neoadjuvant therapy was used, cores were not readable after antigen retrieval and staining, there was insufficient clinical information, postoperative death occurred, death was from other causes, or the cause of death was unknown.

Tumour staging and oesophagogastric junction subgroups

Tumours were staged according to the International Union Against Cancer (UICC) sixth edition staging system¹⁶. All



a Grade 1 staining of NET1



b Grade 3 staining of NET1

Fig. 1 Immunohistochemical staining of tissue microarrays for neuroepithelial transforming gene 1 (NET1) protein. Arrows indicate **a** grade 1 (original magnification $\times 40$) and **b** grade 3 (original magnification $\times 10$) positive staining of NET1

Table 1 Clinical characteristics of patients with neuroepithelial transforming gene 1-positive and -negative status

	Overall (n = 89)	NET1-positive (n = 46)	NET1-negative (n = 43)	P†
Age (years)*	67 (27–84)	67 (27–84)	67 (39–82)	0.964‡
Sex ratio (M:F)	76:13	39:7	37:6	1.000
Body mass index (kg/m ²)*	25 (17–48)	24.6 (17–37)	25.1 (18–48)	0.705‡
Length of follow-up (months)*	20 (6–109)	17.5 (7–100)	20 (6–109)	0.202‡
Alive at follow-up	42 (47)	22 (48)	20 (47)	1.000
Disease-free at follow-up	36 (40)	19 (41)	17 (40)	1.000
Barrett's oesophagus	38 of 84 (45)	26 of 44 (59)	12 of 40 (30)	0.009
Barrett's oesophagus with dysplasia	26 of 84 (31)	17 of 44 (39)	9 of 40 (23)	0.156

Values in parentheses are percentages unless indicated otherwise; *values are median (range). NET1, neuroepithelial transforming gene 1. †Fisher's exact test, except ‡Mann–Whitney *U* test.

patients were categorized according to their postoperative pathological status. NET1 expression and relationship to known clinical and prognostic variables, including survival analysis, was examined for all patients. Further analysis also examined NET1 expression and prognostic significance in AOG types I and II compared with that in patients with AOG type III. Patients with tumours more than 5 cm above the anatomical cardia, arising in Barrett's oesophagus, were included with AOG type I for analysis.

Tissue microarrays

Tissue microarrays were constructed using tumour cores from patients undergoing surgery for adenocarcinoma of the oesophagus and oesophagogastric junction between 1999 and 2010. Tumour biopsies from fresh resected tissue were processed in formalin and embedded in paraffin wax by a designated biobank technician¹⁷. Tumour material was identified from tissue blocks and 2-mm cores were assembled in triplicate for each patient on an array for immunohistochemical analysis. Investigators were blinded to all clinical data at the time of staining. Staining was performed using a NET1 antimouse immunoglobulin G primary antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) and biotinylated secondary antibody, following a standard laboratory protocol¹⁸. Diaminobenzidine

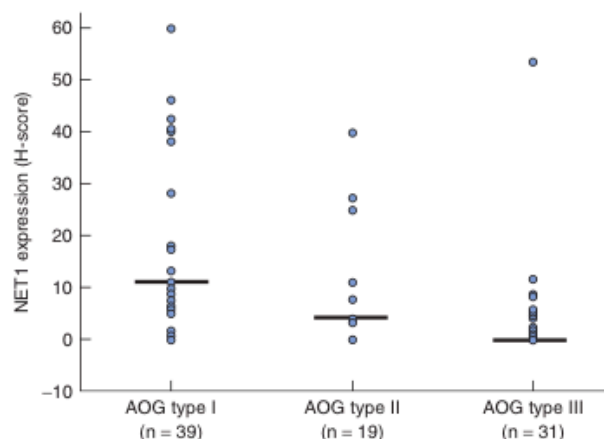


Fig. 2 Comparison of median neuroepithelial transforming gene 1 (NET1) level by H-scores in the three subtypes of adenocarcinoma of the oesophagogastric junction (AOG). Horizontal lines represent median H-scores for each tumour subtype and symbols show the interquartile range of individual values. $P = 0.001$ for all comparisons (ANOVA)

was used to stain NET1 (brown), with haematoxylin and eosin as a counterstain (blue). Tissue microarrays were constructed using two prostate cancer cores (Gleason grade 4) for orientation of the slides, providing a second tissue type and positive control¹⁹.

Table 2 Comparison of pathological details in neuroepithelial transforming gene 1-positive and -negative groups

	Overall (n = 89)	NET1-positive (n = 46)	NET1-negative (n = 43)	P†
Differentiation				
Well	10 (11)	3 (7)	7 (16)	0.187
Moderate	46 (52)	26 (57)	20 (47)	0.399
Poor	30 (34)	16 (35)	14 (33)	1.000
Undifferentiated	3 (3)	1 (2)	2 (5)	0.608
Tumour category				
T1	10 (11)	8 (17)	2 (5)	0.091
T2a	20 (22)	7 (15)	13 (30)	0.210
T2b	2 (2)	0 (0)	2 (5)	0.231
T3	53 (60)	31 (67)	22 (51)	0.135
T4	4 (4)	0 (0)	4 (9)	0.051
Node category				
N0	28 (31)	15 (33)	13 (30)	0.824
N1	52 (58)	27 (59)	25 (58)	1.000
N2	4 (4)	1 (2)	3 (7)	0.349
N3	5 (6)	3 (7)	2 (5)	1.000
No. of positive nodes*	2 (0–32)	3 (0–31)	2 (0–32)	0.859‡
Lymph node ratio*	0 (0–1)	0.15 (0–0.93)	0.15 (0–1)	0.815‡
R0 resection	65 (73)	31 (67)	34 (79)	0.241
Lymphatic invasion	66 (74)	34 (74)	32 (74)	1.000
Vascular invasion	42 of 87 (48)	21 (46)	21 of 41 (51)	0.670
Perineural invasion	36 of 88 (41)	18 (39)	18 of 42 (43)	0.829

Values in parentheses are percentages unless indicated otherwise; *values are median (range). NET1, neuroepithelial transforming gene 1. †Fisher's exact test, except ‡Mann–Whitney *U* test.

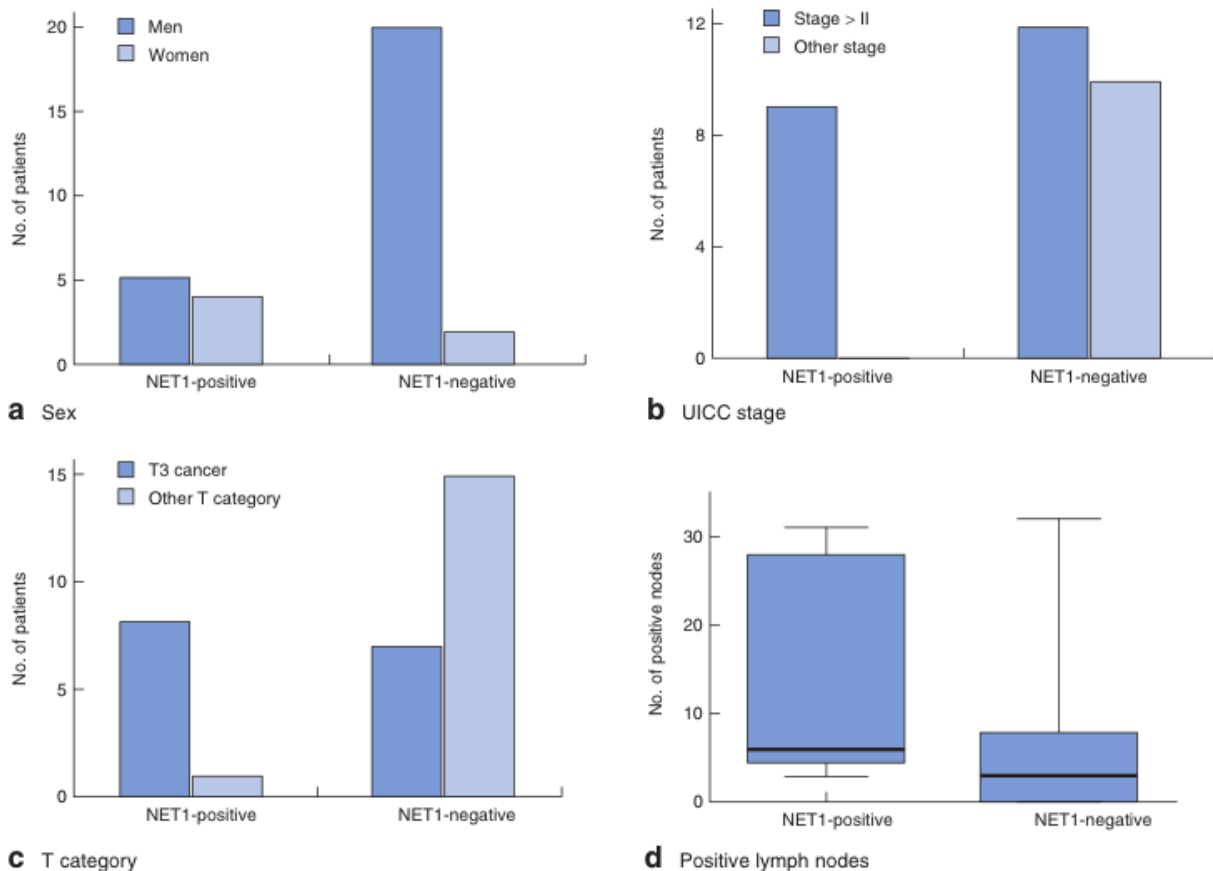


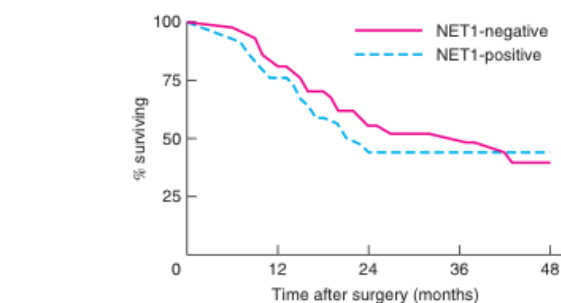
Fig. 3 Differences between patients with neuroepithelial transforming gene 1 (NET1)-positive and -negative type III adenocarcinoma of the oesophagogastric junction with regard to: **a** sex (men *versus* women), **b** International Union Against Cancer (UICC) stage (stage greater than II *versus* other), **c** tumour (T) category (T3 *versus* other) and **d** number of positive lymph nodes (horizontal bars denote median value, boxes the interquartile range, and bars the range). **a** $P = 0.043$, **b** $P = 0.035$, **c** $P = 0.006$ (Fisher's exact test); **d** $P = 0.029$ (Mann–Whitney U test)

Staining was considered positive when membranous with stromal sparing (Fig. 1). Stained slides were photographed electronically using a digital image viewer (Scanscope®; Aperio, Vista, California, USA) for analysis and scoring. NET1 expression was recorded using a qualitative and semiquantitative modified H-score^{20–23}. H-scores were derived from three independent observers. Mean scores were calculated for each patient based on the three representative tissue cores by multiplying intensity score (0–3) by the percentage positivity in quartiles (0–100 per cent). The intensity of the core was graded as the additive expression across heterogeneous sampling. Mean values were then calculated between the three observers. Where the standard deviation of the three scores was greater than half of the total final score, an independent pathologist

reviewed these cores and assigned a final score. Patients with a final H-score of less than 5 were judged to be NET1-negative, as described elsewhere¹⁰.

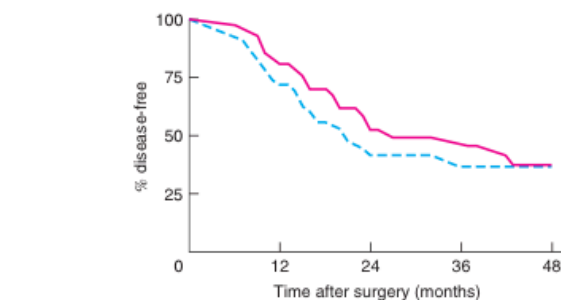
Statistical analysis

Univariable statistical analysis and Kaplan–Meier survival analyses were carried out using Mann–Whitney U , Fisher's exact, Kruskal Wallis/ANOVA and log rank tests, as appropriate. Multivariable survival analysis was performed using a Cox proportional hazards model and forward stepwise regression, adjusting for variables found to be significant predictors of survival ($P < 0.050$) on univariable analysis. The statistical package SPSS® version 20 (IBM, Armonk, New York, USA) was used for statistical analysis. $P < 0.050$ was considered statistically significant for all analyses.



No. at risk	0	12	24	36	48
NET1-positive	46	35	23	23	23
NET1-negative	43	35	26	25	22

a Overall survival



No. at risk	0	12	24	36	48
NET1-positive	46	33	21	20	20
NET1-negative	43	35	25	25	21

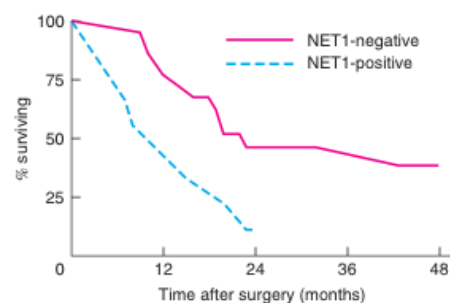
b Disease-free survival

Fig. 4 Kaplan–Meier analysis of **a** overall and **b** disease-free survival for all 89 patients, according to neuroepithelial transforming gene 1 (NET1) status. **a** $P = 0.546$, **b** $P = 0.358$ (log rank test)

Results

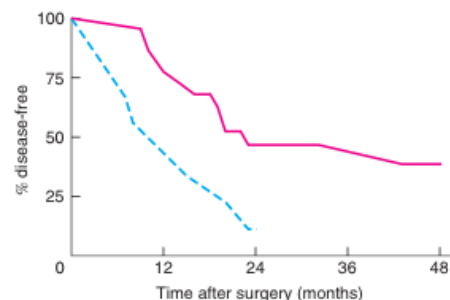
From an original cohort of 210 patients, 89 patients remained after exclusions and formed the basis of this analysis. Exclusions were: cores not readable after antigen retrieval and staining (5), insufficient clinical information (3), in-hospital death (4), death from other causes (23), cause of death unknown (14), squamous cell carcinoma (41) and use of neoadjuvant treatment (31). Most patients (57, 64 per cent) underwent transthoracic oesophagectomy, seven (8 per cent) had a transhiatal approach and 24 (27 per cent) underwent gastrectomy; operative data for one patient were not available.

Baseline clinical and demographic information is presented in *Table 1*. Median duration of follow-up was 20 (range 6–109) months. NET1 staining was highly variable



No. at risk	0	12	24	36	48
NET1-positive	9	5	1	1	1
NET1-negative	22	17	11	11	10

a Overall survival



No. at risk	0	12	24	36	48
NET1-positive	9	5	1	1	1
NET1-negative	22	17	11	11	10

b Disease-free survival

Fig. 5 Kaplan–Meier analysis of **a** overall and **b** disease-free survival for 31 patients with type III adenocarcinoma of the oesophagogastric junction, according to neuroepithelial transforming gene 1 (NET1) status. **a** $P = 0.025$, **b** $P = 0.025$ (log rank test)

across patients. *Fig. 1* shows representative images of different NET1 staining intensities. Some 46 patients (52 per cent) were NET1-positive. There were no differences in NET1 status in terms of age, sex, body mass index or duration of follow-up, or in the proportion alive and disease-free at follow-up.

Data regarding Barrett's metaplasia were recorded for 84 (94 per cent) of the 89 patients. Barrett's oesophagus was detected more frequently in the tumour cores of NET1-positive than in NET1-negative patients (59 *versus* 30 per cent respectively; $P = 0.009$) (*Table 1*). More dysplastic Barrett's was also detected in the NET1-positive group, although this was not statistically different (39 *versus* 23 per cent respectively; $P = 0.156$). Of seven patients with high-grade dysplasia, five (71 per cent) were NET1-positive,

compared with 12 (63 per cent) of 19 patients with low-grade dysplasia ($P = 1.000$).

Barrett's oesophagus was seen more frequently in AOG type I tumours (31 of 39, 79 per cent) than in AOG types II and III (7 of 45, 16 per cent) ($P < 0.001$) (Fig. S1, supporting information). In 39 patients with AOG type I tumours, which included five patients with tumours more than 5 cm above the cardia, Barrett's oesophagus was present in 24 (86 per cent) of 28 patients in the NET1-positive group, compared with seven (64 per cent) of 11 in the NET1-negative group ($P = 0.187$) (Fig. S2, supporting information).

Expression of NET1 according to subtype of oesophagogastric junction adenocarcinoma

Of 89 patients with AOG, 39 (44 per cent) had AOG type I, 19 (21 per cent) AOG type II and 31 (35 per cent) AOG type III tumours. There was significant variation in NET1 staining (by H-score) (Fig. 2). Median H-scores were 11.1 (i.q.r. 1.7–46.3), 4.2 (0–40.0) and 0 (0–5.8) for AOG I, II and III respectively ($P = 0.001$).

Pathological findings and NET1 expression

Pathological correlations by NET1 status for the overall group are presented in Table 2. NET1-positive and -negative groups were similar in terms of level of differentiation, tumour (T) category, node (N) category, number of positive lymph nodes, lymph node ratio (ratio of positive lymph nodes to excised lymph nodes), R0 resection rate, and lymphatic, perineural and vascular invasion.

There was higher overall NET1 expression in AOG type I and II patients combined, compared with AOG type III (median H-score 9.6 *versus* 0 respectively; $P = 0.001$).

Although there were no differences between NET1-positive and -negative patients regarding clinical, demographic and histopathological parameters for AOG types I and II (data not shown), this was not the case for the 31 patients with AOG type III tumours (Fig. 3). In the NET1-positive subgroup, there were more women (4 (44 per cent) of 9 *versus* 2 (9 per cent) of 22 in the NET1-negative subgroup; $P = 0.043$), more advanced UICC stage cancers (9 (100 per cent) of 9 greater than stage 2 *versus* 12 (55 per cent) of 22 respectively; $P = 0.035$), more T3 cancers (8 (89 per cent) of 9 *versus* 7 (32 per cent) of 22; $P = 0.006$) and a higher median number of positive lymph nodes (6.0 *versus* 3.0; $P = 0.029$).

Survival analysis

Overall and disease-free survival analyses for all 89 patients according to NET1 status are shown in Fig. 4,

and those for the 31 patients with AOG type III tumours in Fig. 5. Median overall survival was numerically greater in the NET1-negative than in the NET1-positive group, but this difference was not significant (37 *versus* 23 months respectively; $P = 0.546$). NET1-positive patients with AOG type III tumours had worse median overall survival (15 *versus* 23 months for NET1-negative patients; $P = 0.025$). In the NET1-negative group, 8 (36 per cent) of the 22 patients were disease-free at last follow-up, compared with one (11 per cent) of the nine patients in the NET1-positive group ($P = 0.025$).

Univariable analysis identified sex, pathological T category, number of positive lymph nodes and UICC stage as predictors of survival (data not shown). On multivariable analysis, NET1 independently predicted both disease-free and overall survival (odds ratio for overall survival 2.66, 95 per cent confidence interval 1.06 to 6.64; $P = 0.037$).

Discussion

Although some authors suggest that subclassification of AOG is not necessary, and that management protocols should not differ between tumour subtypes²⁴, this study has demonstrated clear differences in biology between AOG subtypes. The most recent edition of the American Joint Committee on Cancer TNM staging manual provides a uniform classification for AOG and clarifies previous discrepancies in relation to oesophageal and gastric staging systems^{25–27}. However, pathological and prognostic variations between these tumour subtypes^{5,6} suggest underlying biological differences that warrant further investigation.

In colorectal cancer, the identification of several biomarkers has changed management protocols^{28–31}. Identification and validation of similar biomarkers in AOG may help to identify cellular pathways that may explain the phenotypic differences observed in subtypes of these cancers. Previous gene expression studies in oesophageal and gastric cancer have identified potential biomarkers for targeted therapy in this field, in relation to both identification of responders to therapy^{32,33} and overall prognosis³⁴.

The strong predominance for NET1 positivity seen in the AOG type I tumours compared with AOG type III suggests that these represent distinct biological entities. The status of AOG type II in the literature has been controversial. Some authors contend that these are reflux-related tumours and should be classified with AOG type I, with the absence of specialized intestinal metaplasia being related to tumour overgrowth³⁵. Conflicting data suggest a weak relationship between reflux and AOG type II³⁶. The present study showed an intermediate level of NET1 expression in AOG type II tumours, and it remains

unclear whether these tumours represent a third, discrete, biological entity or simply a collection of type I and type III tumours, not requiring further subclassification.

Overall survival in the present cohort, considering the relative proportion of patients with N0 disease (31 per cent) and exclusion of those undergoing neoadjuvant therapy, is consistent with the literature³⁷.

NET1 status did not predict survival in the overall cohort, but there was a clear survival advantage in patients with AOG type III who were NET1-negative. NET1 positivity in these patients was associated with greater tumour size, larger number of positive lymph nodes and higher UICC stage, all of which are established markers of poor prognosis³⁸.

There are certain limitations to this study. The study design did not allow for an *a priori* sample size calculation. Patients receiving neoadjuvant treatment were excluded, potentially excluding a greater proportion of locally advanced and more aggressive tumour types.

The present study represents a novel observation in relation to NET1 expression for AOG and suggests that there are biological differences between tumour subtypes. Further characterization of the role of NET1 and its related proteins in AOG models may provide valuable insights into the biology of these tumours and how that biology translates to clinical practice.

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