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The Diagnostic Value of EZH2 in Distinguishing NET G3 from NEC: An Immunohistochemical Study of 1618 Neuroendocrine Neoplasms of Various Origins and Grades

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Abstract

The distinction between well-differentiated neuroendocrine tumours of grade 3 (NET G3) and poorly differentiated neuroendocrine carcinomas (NEC) guides treatment but remains challenging. We evaluated Enhancer of Zeste Homolog 2 (EZH2) immunohistochemistry as a supportive marker in 1618 neuroendocrine neoplasms (NEN) from ten centres, including 1083 NET G1/G2 and pulmonary carcinoids, 56 NET G3, 443 NEC and 36 medullary thyroid carcinomas and pheochromocytomas/paragangliomas, using the H-score with three expression groups (low < 100, intermediate 100–200, high > 200). EZH2 expression was low in 99.2% of NET G1/G2 and carcinoids and in all medullary thyroid carcinomas and pheochromocytomas/paragangliomas. NET G3 were EZH2-low in 67.9%, intermediate in 23.2% and high in 8.9% of cases, whereas large and small cell NEC were high in 52.2%, intermediate in 34.0% and low in 13.8% ($p < 0.001$). Low EZH2 expression supported NET G3 with a specificity of 86.2% (positive likelihood ratio 4.9), high expression supported NEC with a specificity of 91.1% (positive likelihood ratio 5.8), and intermediate expression was non-discriminatory. In an exploratory subset, EZH2 and p53/RB1 immunohistochemistry provided complementary information. EZH2 expression was associated with overall survival across the cohort but not within NET G3 or NEC. EZH2 is a simple supportive marker for the distinction of NET G3 and NEC: low expression supports NET G3, high expression supports NEC, and intermediate expression should be resolved by morphology and the established markers.

Introduction

Epithelial neuroendocrine neoplasms (NEN) comprise well-differentiated neuroendocrine tumours (NET) and poorly differentiated neuroendocrine carcinomas (NEC), which differ in their morphomolecular features, clinical behaviour and treatment [1–4]. The WHO classifications of endocrine and digestive system tumours define NET G3 as a distinct category of well-differentiated NEN with a Ki-67 proliferation index above 20% [1, 3, 4]. NET G3 occupy an intermediate prognostic position between NET G1/G2 and NEC, respond less often to platinum-based chemotherapy and are increasingly treated with NET-directed strategies such as somatostatin analogues, peptide receptor radionuclide therapy and temozolomide-based regimens [5–7]. In the lung, carcinoids with elevated mitotic count or Ki-67 index are increasingly recognised as a comparable group, although the current thoracic WHO classification does not yet provide a formal NET G3 category [2, 8–10].

The distinction between NET G3 and NEC therefore informs treatment decisions, but it remains one of the most demanding tasks in neuroendocrine pathology. NET G3 typically show organoid growth, uniform nuclei with finely granular chromatin and a delicate vasculature, but morphology can be ambiguous, particularly in metastases. Immunohistochemical markers reflecting the divergent molecular background — aberrant p53 and loss of RB1 in NEC, retained SSTR2A and, in a subset of pancreatic NET G3, loss of DAXX or ATRX — provide valuable support [11–14]. None of these markers is sufficiently sensitive or specific on its own, and diagnostically challenging cases may remain after integration of

morphology and available markers, with reported interobserver variability in the classification of high-grade NEN [15, 16].

Enhancer of Zeste Homolog 2 (EZH2), the catalytic subunit of Polycomb Repressive Complex 2, mediates trimethylation of histone H3 at lysine 27 and is overexpressed in a wide range of aggressive malignancies [17, 18]. In NEN, high EZH2 expression has been described in high-grade pulmonary neuroendocrine neoplasms, in proliferative intestinal NEN, in small cell lung cancer and in thymic NEC [19–22]. We recently characterised c-MYC expression in a large multicentric NEN cohort as an additional supportive marker, with strong expression almost restricted to NEC [23]. EZH2 is a direct transcriptional target of MYC [24] and therefore a candidate for a related, but more broadly expressed, marker of the NEC phenotype. In a single-centre pilot study of 219 gastroenteropancreatic and pulmonary NEN, including nine NET G3, we found EZH2 to be virtually absent in NET G1/G2, strongly expressed in the majority of NEC and low in NET G3; EZH2 expression was also associated with overall survival in NEN G3 [25]. Based on these findings and the limited number of NET G3 in that study, we analysed EZH2 expression in a multicentric cohort of 1618 pulmonary and extrapulmonary NEN, including 56 NET G3, and derived a practicable three-tier interpretation for routine diagnostics.

Material and Methods

Multicentric NEN Cohort

We established a multicentric cohort of 1618 NEN (1418 primary tumours and 200 metastases) from 1434 patients, based on a previously described multicentric NEN cohort [26] that was extended by additional cases. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were retrieved from the archives of the pathology departments of the University Hospital Marburg, the University Hospital rechts der Isar of the Technical University of Munich, the Munich Municipal Hospital, the University Hospital Mainz, the University Hospital Cologne, the University of Regensburg, the University Hospital Erlangen-Nuremberg, the University Hospital Göttingen, the University Hospital Augsburg and the Bundeswehrkrankenhaus (German Armed Forces Hospital) Ulm. Survival and clinicopathological data were collected from local cancer registries or hospital records; overall survival (OS) was defined as the time from diagnosis to death from any cause. For 184 patients, more than one sample was included (multifocal jejunoileal NET or a metastasis of an included primary tumour); expression analyses were performed on the sample level, all survival analyses were restricted to one sample per patient, and the main diagnostic analyses were additionally repeated with one sample per patient to account for the non-independence of multiple samples.

All tumours were diagnosed according to the respective WHO classifications of their organ system, and all cases were morphologically reviewed prior to inclusion [1–3]. NET were defined by well-differentiated neuroendocrine morphology together with expression of synaptophysin and/or chromogranin A and cytokeratins, and were graded according to the Ki-67 index (G1 < 3%, G2 3–20%, G3 > 20%). NET G3 were diagnosed when a tumour with well-differentiated morphology showed a Ki-67 index above 20%,

supported where available by retained RB1 expression, a wild-type p53 staining pattern, SSTR2A expression or, in pancreatic NET, loss of DAXX/ATRX; all NET G3 were independently reviewed by two experienced pathologists in the field of NEN (M.J. and H.B.) blinded to the EZH2 staining results. Pulmonary and thymic carcinoids with a Ki-67 index above 20% (carcinoids with elevated proliferation, conceptually related to digestive NET G3 [10]) were grouped with NET G3 for exploratory analyses, regardless of their classification according to WHO criteria, and are reported separately where relevant. Typical carcinoids (TC) and atypical carcinoids (AC) of the lung or thymus were classified according to the thoracic WHO classification [2]. NEC were classified as small cell (SCNEC) or large cell neuroendocrine carcinomas (LCNEC) according to the respective WHO classifications, as described previously [1–3, 26]. Merkel cell carcinomas (MCC) were included as NEC of cutaneous origin; comparisons between NET G3 and NEC were performed against LCNEC and SCNEC (hereafter LCNEC/SCNEC), with MCC analysed separately. Medullary thyroid carcinomas (MTC) and pheochromocytomas/paragangliomas (PPGL) were included as NEN of C-cell and neural crest origin, respectively. Mixed neuroendocrine-non-neuroendocrine neoplasms were not included. NEN of unknown primary site (CUP) were classified and graded according to the WHO classification of digestive system tumours [1].

Tissue microarrays (TMA) comprising up to four tumour cores per case were assembled using the TMA Grand Master system (Sysmex/3DHistech, Budapest, Hungary). Tumours with insufficient fixation or insufficient tumour material on the TMA were excluded.

Immunohistochemical Analysis of EZH2

TMA sections (2 µm) were stained with an anti-EZH2 antibody (Leica Biosystems, Wetzlar, Germany; EZH2-L-CE, 1:100) after heat-induced epitope retrieval at high pH (EnVision Flex Target Retrieval Solution, Dako/Agilent, Santa Clara, CA, USA), visualised with the EnVision Flex+ polymer system and DAB (Dako/Agilent) and counterstained with haematoxylin, as in the pilot study [25]. Only nuclear staining was considered specific. EZH2 expression was quantified using the H-score [27, 28], calculated as 1 × (% tumour cells with weak nuclear staining) + 2 × (% cells with moderate staining) + 3 × (% cells with strong staining), yielding a range of 0–300 [25]. For each case, the H-score was determined across all evaluable cores. Stainings were evaluated by K.O. and H.B. blinded to the diagnosis; discrepant cases were resolved by consensus.

Cases were assigned to three expression groups: low (H-score 0–99), intermediate (100–200) and high (201–300). The lower threshold of 100 was based on the cut-off of our previous pilot study [25]. The upper threshold of 200 was selected in the present derivation cohort as an operational definition of strong, diffuse EZH2 expression, as an H-score above 200 is only reached when the majority of tumour cells show strong nuclear staining, which is readily recognised at scanning magnification. Diagnostic performance across alternative thresholds was examined in sensitivity analyses (Supplementary Table S1).

p53, RB1 and c-MYC immunohistochemistry was available for subsets of cases from previous studies [23, 25] and routine diagnostics. p53 staining was classified as wild-type or aberrant (overexpression or complete loss) and RB1 as retained or lost; c-MYC was assessed using the immunoreactivity score as described previously [23]. These data were analysed in an exploratory manner.

Statistics

Associations between EZH2 expression groups and clinicopathological parameters were tested using the chi-square test or Fisher's exact test (two-sided) as appropriate, and differences in H-scores between groups with the Mann–Whitney U test. Trends across EZH2 expression groups were assessed with Spearman's rank correlation. The diagnostic performance of low and high EZH2 expression for the distinction between NET G3 and LCNEC/SCNEC, as diagnosed according to WHO criteria, was described by sensitivity, specificity, positive and negative predictive values (Wilson 95% confidence intervals, CI) and positive and negative likelihood ratios (log-method 95% CI). Receiver operating characteristic analysis (area under the curve, AUC) was used to assess the discriminatory performance of the continuous EZH2 H-score. Survival probabilities were estimated with the Kaplan–Meier method and compared using log-rank tests. p-values ≤ 0.05 were considered statistically significant. Analyses were performed with GraphPad Prism version 11.0.2 (GraphPad Software, San Diego, CA, USA) and Python 3.12 (SciPy 1.x, lifelines 0.30).

Results

Clinicopathological Features of the NEN Cohort

The cohort comprised 1618 NEN from 1434 patients (Table 1).

Table 1
Characteristics of the cohort by localisation

Samples, n	Total	GEP	Pulmonary	Skin	Other	CUP
	1618	**1058**	**388**	**53**	**73**	**46**
Patients, n	1434	896	382	46	70	40
Age, median (range), years	63 (6–98)	61 (6–92)	64 (12–94)	79 (57–98)	64 (18–84)	70 (41–89)
Female, n (%)	715 (44.2)	453 (42.8)	187 (48.2)	20 (37.7)	36 (49.3)	19 (41.3)
Male, n (%)	903 (55.8)	605 (57.2)	201 (51.8)	33 (62.3)	37 (50.7)	27 (58.7)
NET G1	724	721	0	0	0	3
NET G2	178	176	0	0	1	1
Typical carcinoid	137	0	137	0	0	0
Atypical carcinoid	44	0	44	0	0	0
NET G3 (incl. carcinoids with elevated proliferation)	56	39	6	0	2	9
LCNEC	181	85	85	1	2	8
SCNEC	210	37	116	0	32	25
Merkel cell carcinoma	52	0	0	52	0	0
MTC / PPGL	36	0	0	0	36	0
Primary tumours / metastases	1418 / 200	998 / 60	318 / 70	32 / 21	62 / 11	8 / 38
Patients with survival data / deaths	1271 / 495	791 / 255	346 / 156	28 / 21	68 / 32	38 / 31

GEP, gastroenteropancreatic; other sites: urogenital tract (n = 31), adrenal gland/paraganglia (n = 24), thyroid (n = 12), head and neck (n = 4), thymus (n = 2); CUP, cancer of unknown primary; LCNEC/SCNEC, large/small cell neuroendocrine carcinoma; MTC, medullary thyroid carcinoma; PPGL, pheochromocytoma/paraganglioma. Survival analyses were performed with one sample per patient and complete follow-up data. Well-differentiated NEN accounted for two thirds of the cohort: 1083 (66.9%) NET G1/G2 including pulmonary carcinoids (724 NET G1, 178 NET G2, 137 TC, 44 AC) and 56 (3.5%) NET G3. NEC comprised 443 cases (27.4%; 181 LCNEC, 210 SCNEC and 52 MCC), and 36 (2.2%) were MTC (n = 12) or PPGL (n = 24) (Fig. 1a). Regarding localisation, 1058 (65.4%) NEN were gastroenteropancreatic, 388 (24.0%) pulmonary, 53 (3.3%) cutaneous and 73 (4.5%) arose at other sites, and 46 (2.8%) were metastases of unknown primary (CUP) (Fig. 1b). The 56 NET G3 (48 patients)

originated from the gastroenteropancreatic tract (n = 39; pancreas n = 25), were CUP in nine cases and included six pulmonary and two thymic carcinoids with elevated proliferation; 25 NET G3 samples were metastases. The median age at diagnosis was 63 years (range 6–98), and 55.8% of patients were male.

Survival data were available for 1271 patients (495 deaths). OS differed significantly between NET G1/TC, NET G2/AC, NET G3 and NEC ($p < 0.0001$, log-rank), with NET G3 occupying an intermediate position between NET G1/G2 and NEC (5-year OS 86%, 77%, 39% and 23%, respectively; Fig. 1c); this was also the case when the analysis was restricted to primary tumours ($p < 0.0001$). Male sex ($p = 0.0001$), age ≥ 65 years ($p < 0.0001$) and gastroenteropancreatic origin of NET G1/G2 ($p = 0.0008$) and of NEC ($p = 0.022$) were associated with shorter OS, as was presentation of NET G3 as CUP (median 9 months; $p = 0.0015$ across localisations) (Fig. 1d–i).

EZH2 Expression Across Neuroendocrine Entities

Across the 1582 NET and NEC, 1167 (73.8%) tumours showed low, 161 (10.2%) intermediate and 254 (16.1%) high EZH2 expression. The distribution of expression groups differed markedly between NET G1/G2, NET G3 and NEC ($p < 0.001$ for each pairwise comparison; Fig. 3a–c). Representative examples of the three expression groups are shown in Fig. 2.

EZH2 expression was low in 1074/1083 (99.2%) NET G1/G2 and pulmonary carcinoids, without site-specific differences among gastroenteropancreatic NET G1/G2 (Fig. 3d). All TC were EZH2-low, whereas six of 44 AC (13.6%; four intermediate, two high) showed elevated expression (AC vs. TC, $p < 0.001$, Fisher's exact test). EZH2 expression was uniformly low in MTC and PPGL: all 12 MTC and all 24 PPGL were EZH2-low (mean H-scores 13 and 9; maximum 70), whereas MCC, the NEC of the skin, showed high expression in 42/52 (80.8%) cases, with a mean H-score of 260 (vs. 198 in LCNEC/SCNEC; $p < 0.001$).

Of 391 LCNEC/SCNEC, 204 (52.2%) showed high, 133 (34.0%) intermediate and 54 (13.8%) low EZH2 expression. The distribution did not differ between SCNEC and LCNEC ($p = 0.57$) or between pulmonary and gastroenteropancreatic NEC ($p = 0.36$), apart from higher H-scores in pulmonary than in gastroenteropancreatic LCNEC (mean 213 vs. 178; $p = 0.009$) (Fig. 3b). In the 226 LCNEC/SCNEC with available c-MYC immunohistochemistry, high EZH2 expression was more frequent in c-MYC-positive than in c-MYC-negative tumours (65% vs. 50%; $p = 0.03$), whereas low EZH2 expression was rare in c-MYC-positive tumours (6 of 92) (Table 2b).

Among the 56 NET G3, 38 (67.9%) showed low, 13 (23.2%) intermediate and 5 (8.9%) high EZH2 expression (mean H-score 90, SD 72); at the patient level (48 patients), the proportions were 64.6%, 27.1% and 8.3%. Intermediate or high expression occurred in NET G3 of the pancreas (11 of 25), the stomach (3 of 5), the thymus (1 of 2) and of unknown primary site (3 of 9), whereas all jejunoileal, colorectal and pulmonary NET G3 were EZH2-low. The five EZH2-high NET G3 were three pancreatic tumours (two metastases, one primary tumour) and two tumours of unknown primary site. Their Ki-67 indices ranged from 26% to 60% (four cases with available data); p53 staining was aberrant in two of three evaluable cases, both pancreatic, and RB1 expression was retained in all three evaluable cases.

Two of the four patients with follow-up died within 9 months. EZH2 expression in NET G3 was not associated with sex, age or pTNM category (all p > 0.09). In metastases, EZH2 expression resembled that of the corresponding primary tumour groups; only metastatic LCNEC/SCNEC showed slightly higher H-scores than primary tumours (mean 216 vs. 191; p = 0.025) (Fig. 3e).

EZH2 Expression in NET G3 Versus NEC: A Three-Tier Interpretation

The distribution of EZH2 expression groups differed significantly between NET G3 and LCNEC/SCNEC (p < 0.001; Fig. 4a–b). Low EZH2 expression discriminated NET G3 from LCNEC/SCNEC with a sensitivity of 67.9% (95% CI 54.8–78.6) and a specificity of 86.2% (82.4–89.3) (positive likelihood ratio 4.9, 95% CI 3.6–6.7; negative likelihood ratio 0.37, 0.25–0.55). High EZH2 expression discriminated LCNEC/SCNEC from NET G3 with a sensitivity of 52.2% (47.2–57.1) and a specificity of 91.1% (80.7–96.1) (positive likelihood ratio 5.8, 2.5–13.6; negative likelihood ratio 0.53, 0.46–0.60). Intermediate expression was present in 23.2% (14.1–35.8) of NET G3 and 34.0% (29.5–38.8) of LCNEC/SCNEC (likelihood ratio 0.68) and was therefore non-discriminatory (Supplementary Table S2). Given the predominance of NEC in our cohort, the positive predictive value of low expression for NET G3 was 41.3% and that of high expression for NEC was 97.6%; for illustration, assuming equal pre-test probabilities of NET G3 and NEC, these likelihood ratios correspond to post-test probabilities of approximately 83% and 85%, respectively. The results were similar when MCC were included in the NEC group, for LCNEC and SCNEC separately, in gastroenteropancreatic NEN (Supplementary Table S2), with one sample per patient and in primary tumours or metastases only (specificities 82.0–93.5%). The area under the receiver operating characteristic curve of the continuous H-score for LCNEC/SCNEC versus NET G3 was 0.83. Across alternative thresholds, a lower threshold of 100 and an upper threshold of 200 each marked the point at which specificity exceeded 85% and 90%, respectively, at a still useful sensitivity (Supplementary Table S1).

p53 and RB1 immunohistochemistry was available for 26 and 24 NET G3 and for 145 and 88 LCNEC/SCNEC, respectively (Table 2a). Aberrant p53 staining (19% vs. 82%) and loss of RB1 expression (21% vs. 70%) were less frequent in NET G3 than in LCNEC/SCNEC (both p < 0.001). In LCNEC/SCNEC, aberrant p53 staining was observed at similar frequencies across EZH2 groups, whereas loss of RB1 expression increased with EZH2 expression (45%, 63% and 79% in EZH2-low, -intermediate and -high tumours; p = 0.016). In cases with EZH2, p53 and RB1 immunohistochemistry (23 NET G3, 87 LCNEC/SCNEC), EZH2 and p53/RB1 concordantly pointed to the diagnosis in 10 NET G3 and 50 LCNEC/SCNEC (Supplementary Fig. S1a). In EZH2-intermediate tumours, p53 and RB1 staining was normal in all five NET G3 and aberrant in 20 of 24 LCNEC/SCNEC. EZH2 was particularly informative when p53/RB1 immunohistochemistry provided an incomplete signal: among tumours with only one of the two markers altered, six of seven NET G3 were EZH2-low and 15 of 23 LCNEC/SCNEC were EZH2-high (Supplementary Fig. S1b). Combined alteration of p53 and RB1 was restricted to LCNEC/SCNEC.

Table 2
p53, RB1 and c-MYC immunohistochemistry according to EZH2 expression **a** p53 and RB1

NET G3	EZH2 low	EZH2 intermediate	EZH2 high	Total
p53 aberrant	3/18 (17%)	0/5 (0%)	2/3	5/26 (19%)
RB1 loss	5/16 (31%)	0/5 (0%)	0/3	5/24 (21%)
LCNEC/SCNEC				
p53 aberrant	21/26 (81%)	37/46 (80%)	61/73 (84%)	119/145 (82%)
RB1 loss	5/11 (45%)	15/24 (63%)	42/53 (79%)	62/88 (70%)

NET G3 vs. LCNEC/SCNEC: p53 and RB1 $p < 0.001$ (Fisher's exact test). Association with EZH2 group within LCNEC/SCNEC: p53 $p = 0.67$, RB1 $p = 0.016$ (Spearman's rank correlation).

b EZH2 expression according to c-MYC status in LCNEC/SCNEC

c-MYC	n	EZH2 low	EZH2 intermediate	EZH2 high
negative	134	23 (17%)	44 (33%)	67 (50%)
positive	92	6 (7%)	26 (28%)	60 (65%)

High EZH2 expression in c-MYC-positive vs. c-MYC-negative tumours: $p = 0.03$ (Fisher's exact test). p53, RB1 and c-MYC status was available for subsets of cases.

Association of EZH2 Expression With Overall Survival

In the overall cohort, low EZH2 expression was associated with longer OS ($p < 0.0001$, log-rank), in line with its association with grade and entity (Fig. 4d). Within high-grade NEN (NET G3 and NEC combined, the latter including MCC; 381 patients, 286 deaths), EZH2-low tumours showed a numerically longer median OS than tumours with intermediate or high expression (22, 14 and 12 months; $p = 0.13$; Fig. 4e), which partly reflects the composition of the groups, as NET G3 accounted for 39% of the EZH2-low but for only 2% of the EZH2-high high-grade NEN. OS did not differ between expression groups within NET G3 (47 patients; $p = 0.51$) or within LCNEC/SCNEC (307 patients; $p = 0.19$).

Discussion

The distinction between NET G3 and NEC informs prognosis and treatment, and it is rarely settled by a single criterion. In this multicentric analysis of 1618 NEN — to our knowledge the largest series of EZH2 expression in NEN to date, and one that includes a substantial number of NET G3 — EZH2 was low in 99% of NET G1/G2 and pulmonary carcinoids, high in the majority of NEC and predominantly low in NET

G3. The central result is a three-tier framework that may support routine diagnostic assessment: low EZH2 expression argues against NEC, high EZH2 expression argues against NET G3, and intermediate expression is uninformative. EZH2 is not a stand-alone marker — EZH2-high NET G3 and EZH2-low NEC exist in both directions — but an additional supportive marker to be read in the context of morphology and the established markers.

Our single-centre pilot study had suggested an almost dichotomous distribution of EZH2 in NEN G3 [25]. The present cohort, which includes six times as many NET G3, refines this picture: EZH2 emerges as a marker of a proliferative, NEC-like phenotype rather than of the NEC entity itself. The majority of NET G3 retain the low expression of their well-differentiated counterparts, whereas a subset acquires intermediate or high levels, and one in seven LCNEC/SCNEC is EZH2-low. This is consistent with graded EZH2 expression along the spectrum of pulmonary and intestinal NEN [19, 20], with the association of EZH2 with proliferation in thymic NEN [22, 29] and with the high EZH2 levels of small cell lung cancer [21]; within the well-differentiated group, elevated expression was essentially confined to atypical carcinoids, the pulmonary NET with the highest proliferative activity. A subset of NET acquires NEC-like alterations of TP53 or RB1 during high-grade progression [30]; whether EZH2-high NET G3 belong to this group cannot be answered by our data, but the graded expression fits the concept of a biological continuum between NET G3 and NEC. The pattern parallels that of c-MYC in the same cohort [23], a direct transactivator of EZH2 [24]. High EZH2 expression was more frequent in c-MYC-positive NEC; however, half of the c-MYC-negative NEC also showed high EZH2 expression, suggesting additional, MYC-independent routes of EZH2 up-regulation, such as RB1 loss with consecutive E2F activation [31].

The practical value of EZH2 lies in its simplicity. Nuclear staining is readily quantified with the H-score and does not depend on the recognition of subtle cytoplasmic or membranous patterns, which makes EZH2 well suited to metastases, in which the diagnostic performance was at least as good as in primary tumours. Likelihood ratios of 4.9 for low and 5.8 for high expression lie in the range in which a single additional marker is expected to be useful, and they were consistent across all sensitivity analyses. In the proposed algorithm (Fig. 4c), low or high expression adds weight to the diagnosis of NET G3 or NEC, respectively, whereas intermediate expression is disregarded; the outliers in both directions — five EZH2-high NET G3 and 54 EZH2-low LCNEC/SCNEC — are the reason why EZH2 should not be used on its own. As EZH2 is overexpressed in a wide range of non-neuroendocrine carcinomas [17, 18], it is not suited to distinguish NEC from non-neuroendocrine carcinoma. The high expression in MCC and the uniformly low EZH2 expression in MTC and PPGL indicate that, among NEN, EZH2 expression is a feature of highly proliferative epithelial neoplasms.

In an exploratory subset, EZH2 and p53/RB1 immunohistochemistry provided complementary information. Combined alteration of p53 and RB1 was confined to NEC, whereas EZH2 added information where p53 and RB1 staining was least conclusive: NET G3 with isolated p53 or RB1 alterations were predominantly EZH2-low, and NEC with only one altered marker were predominantly EZH2-high. Conversely, in EZH2-intermediate tumours, in which EZH2 carries no diagnostic weight, p53 and RB1 staining was normal in all NET G3 and aberrant in most NEC, supporting the integration of EZH2

into the established panel rather than its use as a stand-alone marker. The association of RB1 loss with high EZH2 expression in NEC is in line with EZH2 being a downstream target of the pRB–E2F pathway [31].

EZH2 expression was associated with OS in the overall cohort, reflecting its association with grade and entity, but carried no significant prognostic information within NET G3 or NEC, suggesting that its principal utility in NEN is diagnostic rather than prognostic. EZH2 inhibitors are approved for follicular lymphoma and epithelioid sarcoma [32, 33], EZH2 is implicated in the chemoresistance of small cell lung cancer [34], and EZH2 inhibition is being explored in pancreatic NEN [35]; whether EZH2-high NEN are particularly susceptible to such approaches remains to be determined.

The majority of our analyses were performed on TMA and may therefore not fully capture intratumoural heterogeneity of EZH2 expression. The upper threshold and the three-tier framework were derived from the present cohort; their confirmation in independent cohorts is desirable. The diagnosis of NET G3 was based on morphology and available immunohistochemical findings according to WHO criteria; it is demanding, and individual cases might be classified differently by other observers [15, 16]. As in a recent consultation series of NET G3 [36], most NET G3 samples were obtained from metastases and nine presented as CUP; the concordant results at the patient level and in primary tumours and metastases indicate that this did not affect the conclusions. The six pulmonary and two thymic carcinoids with elevated proliferation were too few for separate conclusions.

In conclusion, EZH2 immunohistochemistry is a simple supportive marker for the distinction between NET G3 and NEC. Low expression supports NET G3, high expression supports NEC, and intermediate expression carries no diagnostic weight and should be resolved by morphology and the established markers. Read in this way, EZH2 adds most where p53 and RB1 are least conclusive, and it complements rather than replaces the current diagnostic panel.

Declarations

Ethics Approval

The study was performed in line with the principles of the Declaration of Helsinki and was approved by the ethics committee of the University Medical Center Göttingen (#10/11/20) and by the local ethics committees of the contributing centres: Technical University of Munich (252/16 s and 2022-396-S-DFG-SR, combined vote for all cases from Munich, Regensburg and Augsburg), University Hospital Marburg (AZ 206/10 and AZ 43/21), University Hospital Cologne (13–091), University Hospital Erlangen-Nuremberg (329_16B) and University Hospital Mainz (837.360.16, 10679).

Competing Interests

The authors declare no competing interests directly related to the present study. All other relevant financial and non-financial relationships are listed below for full transparency. H.B. is a member of a Pathology Advisory Board of Boehringer Ingelheim (obrixtamig); this activity is unrelated to the present study. A.K. and M.J. are members of the Editorial Board of Endocrine Pathology; they were not involved in the editorial handling or peer-review process of this manuscript. M.S. provides unpaid scientific advice to Leica Microsystems and ATTO-TEC and has received no honoraria, consulting fees, salary, research funding or other financial compensation from these companies. D.K.B. is a member of the Executive Committee of the European Neuroendocrine Tumour Society (ENETS); the APSIN study led by D.K.B. has received funding from German Cancer Aid. D.C.W. has received honoraria from Agilent, Amgen, AstraZeneca, Janssen, Roche, Bristol Myers Squibb (BMS), MSD, Sanofi and Merck KGaA, and research funding from BMS and Amgen. M.E. (Eckstein) has received consulting fees from Ferring, AstraZeneca, Janssen (Johnson & Johnson), Genomic Health, Diaceutics, Gilead, MSD, Merck and Bicycle Therapeutics; honoraria for lectures, presentations and educational activities from AstraZeneca, Roche, Janssen (Johnson & Johnson), Owkin, Diaceutics, Ferring, Eisai, Merck, Bicycle Therapeutics and Zytomed Systems; payments for expert testimony from AstraZeneca, Janssen (Johnson & Johnson), Astellas, Genomic Health, Diaceutics, Gilead, MSD, Merck and Bicycle Therapeutics; and travel support from AstraZeneca, Roche, MSD, Janssen (Johnson & Johnson), Diaceutics, Astellas, Ferring, Eisai, Merck and Bicycle Therapeutics. He serves on the Clinical Advisory Board of Bicycle Therapeutics and on the boards of the BRIDGE Consortium, the European Society of Uropathology (ESUP) and the International Society of Urological Pathology (ISUP). He has received equipment, materials, services and/or research funding from STRATIFYER, Janssen (Johnson & Johnson), AstraZeneca/MedImmune, Owkin, Gilead, Bicycle Therapeutics, BMS and QUiP GmbH, and reports stock ownership in Bicycle Therapeutics and MSD. K.S. (Steinestel) has received consulting fees and honoraria from AstraZeneca, Merck Sharp & Dohme, Bristol Myers Squibb, Sanofi and Boehringer Ingelheim, and has served on Data Safety Monitoring Boards or Advisory Boards for Merck Sharp & Dohme, Bristol Myers Squibb and AstraZeneca. C.D. has served on advisory boards for MSD Oncology, Daiichi Sankyo, AstraZeneca and BioNTech RNA Pharmaceuticals GmbH; has received speaker honoraria from AstraZeneca and licensing fees from VMscope digital pathology software; and has received institutional research funding from Myriad and the German Breast Group. He is an inventor on patents WO2020109570A1, WO2015114146A1 and WO2010076322A1. A.K. has received consulting and advisory fees from Boehringer Ingelheim GmbH and speaking and lecture fees from Ipsen Pharma GmbH. M.J. has received honoraria from Novartis Pharma. K.O., A.-S.L., A.G., M.K., M.E. (Evert), B.M., A.Q., K.S. (Steiger), S.F., G.K. and P.S. declare no competing interests.

Declarations

of Generative AI and AI-Assisted Technologies in the Writing Process During the preparation of this work, the authors used Claude (Anthropic) exclusively for language editing of existing text written by the authors. The authors reviewed and edited the content and take full responsibility for the content of the publication.

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Author Contribution

K.O., M.J., M.S. and H.B. designed the study. K.O. and H.B. performed the immunohistochemical evaluation. K.O., M.J. and H.B. performed histopathological review and analysed the data. M.S. and M.J. contributed data from previous studies of the cohort and participated in the interpretation of the results. K.O. and H.B. wrote the manuscript with assistance from M.S. and M.J. All other authors provided technical and material support. All authors read and approved the final paper.

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Data Availability

The case-level data supporting the findings of this study are available from the corresponding author upon reasonable request. Tissue is stored at the Institute of Pathology, University Medical Center Göttingen, and the Institute of Pathology, Philipps University Marburg and University Hospital Marburg, and is available from the corresponding authors upon reasonable request.

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Figures

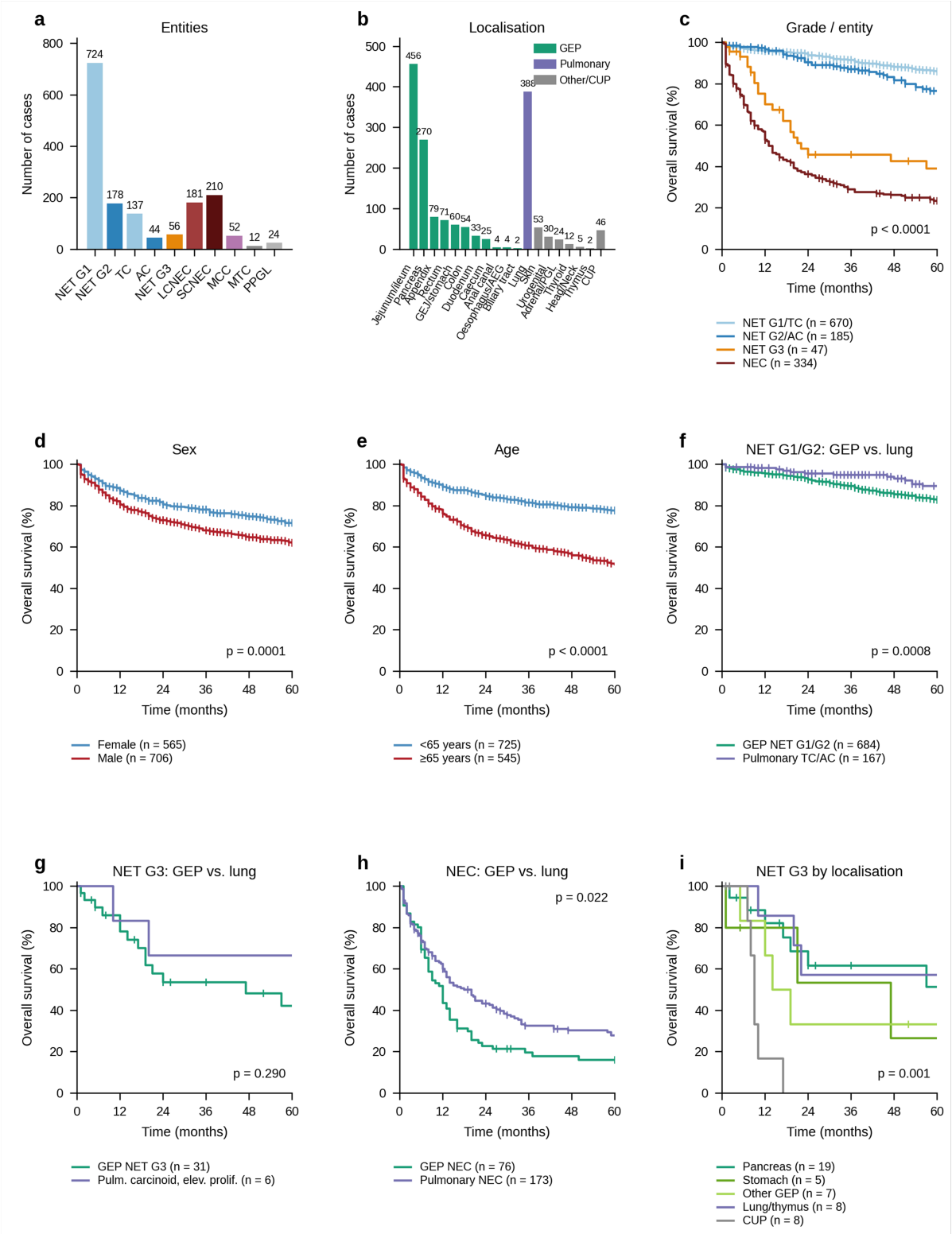


Figure 1

Composition and outcome of the cohort. **a**Frequency of NEN entities in the overall cohort of 1618 NEN. **b**Localisation of the investigated NEN. **c** Overall survival according to grade and entity (NET G1/TC, NET G2/AC, NET G3, NEC). **d–e** Overall survival according to sex (**d**) and age (**e**). **f** Overall survival of gastroenteropancreatic NET G1/G2 vs. pulmonary TC/AC. **g**Overall survival of gastroenteropancreatic NET G3 vs. pulmonary carcinoids with elevated proliferation. **h** Overall survival of gastroenteropancreatic

vs. pulmonary NEC. **i** Overall survival of NET G3 according to localisation (other GEP: jejunoileal, colonic and rectal NET G3). Survival analyses include one sample per patient; Kaplan–Meier estimates, log-rank tests; tick marks indicate censored observations. NEN, neuroendocrine neoplasm; NET, neuroendocrine tumour; NEC, neuroendocrine carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCNEC, small cell neuroendocrine carcinoma; TC, typical carcinoid; AC, atypical carcinoid; MCC, Merkel cell carcinoma; MTC, medullary thyroid carcinoma; PPGL, pheochromocytoma/paraganglioma; GEP, gastroenteropancreatic; CUP, cancer of unknown primary

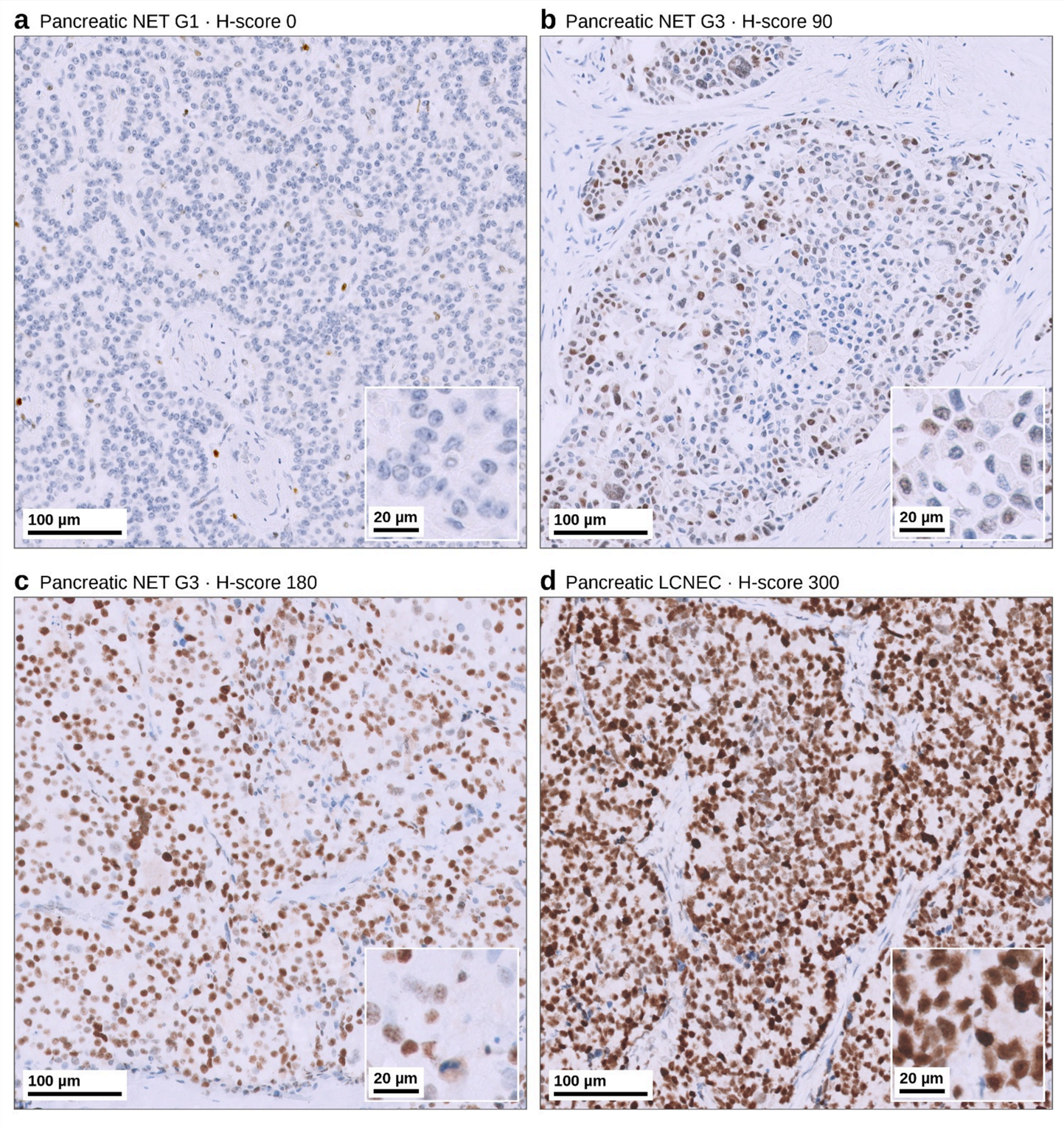


Figure 2

Representative EZH2 immunohistochemistry. **a** Absence of nuclear EZH2 expression in a pancreatic NET G1 (H-score 0). **b** Low expression with scattered positive nuclei in a pancreatic NET G3 (H-score 90). **c** Intermediate expression with weak to moderate staining in the majority of tumour cells in a pancreatic NET G3 (H-score 180). **d** Strong, diffuse nuclear expression in a pancreatic large cell neuroendocrine carcinoma (H-score 300). Original magnification ×20 (scale bars 100 μm); insets ×100 (scale bars 20 μm)

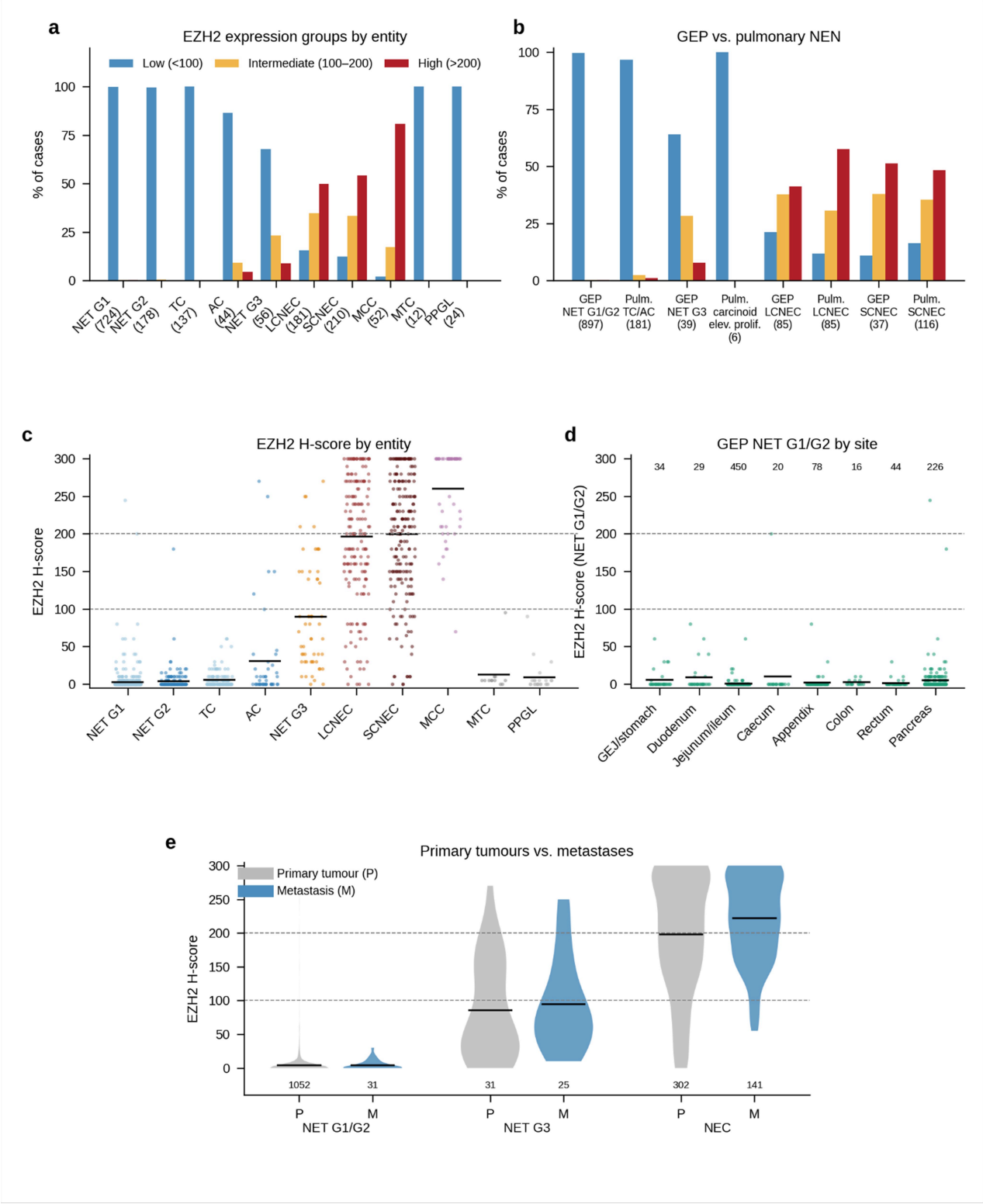


Figure 3

EZH2 expression across neuroendocrine entities. **a** Frequency of EZH2 expression groups (low <100, intermediate 100–200, high >200) according to entity. **b** Frequency of EZH2 expression groups in gastroenteropancreatic and pulmonary NEN by entity. **c**EZH2 H-scores of individual cases by entity; horizontal lines indicate means, dashed lines the group thresholds. **d** EZH2 H-scores of gastroenteropancreatic NET G1/G2 by site. **e** EZH2 H-scores in primary tumours (P) and metastases (M)

within NET G1/G2, NET G3 and NEC. Numbers indicate cases; Pulm., pulmonary; other abbreviations as in Fig. 1

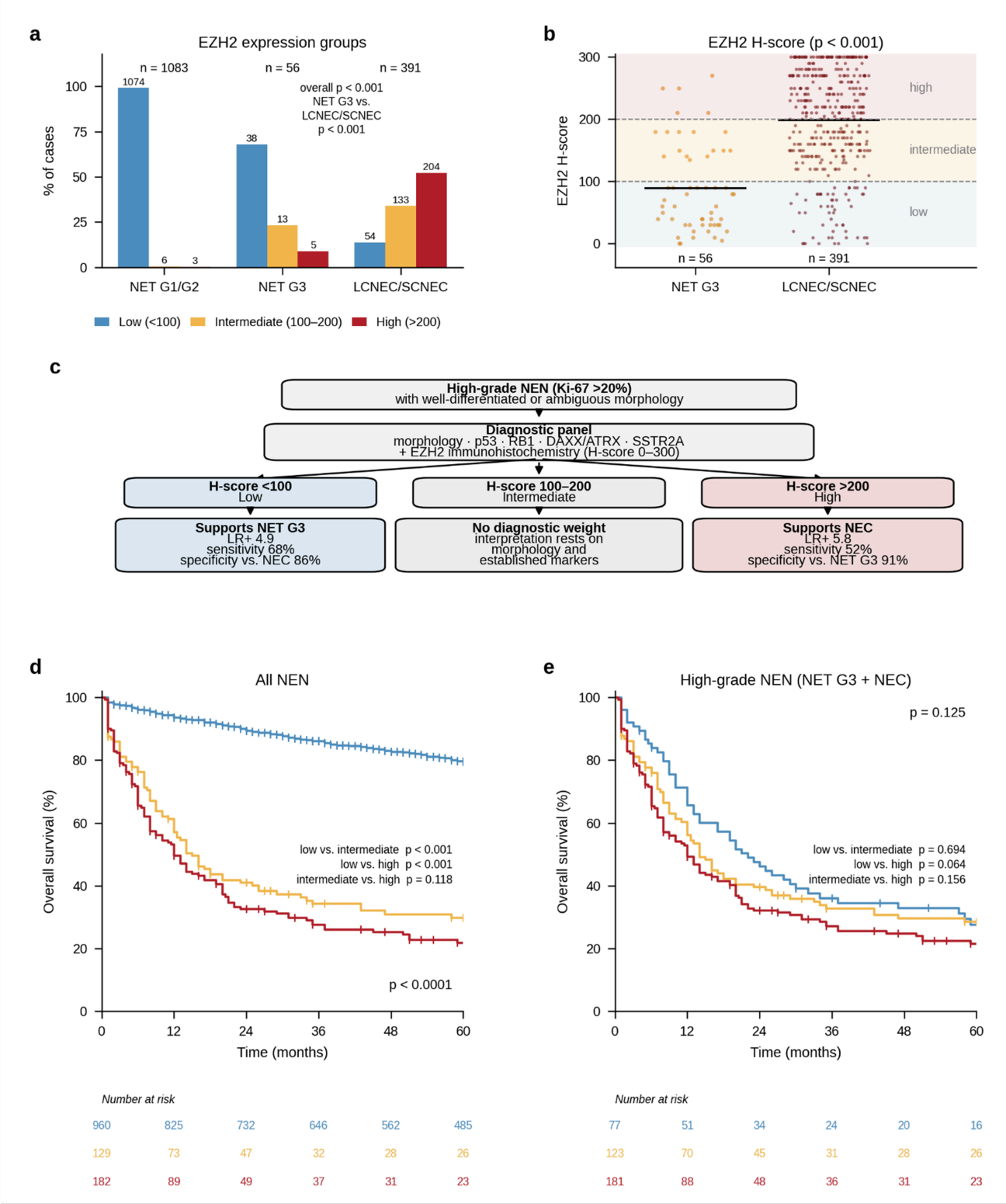


Figure 4

EZH2 in the distinction between NET G3 and NEC and its relation to survival. **a** Frequency of EZH2 expression groups in NET G1/G2, NET G3 and LCNEC/SCNEC (chi-square test, overall and NET G3 vs.

LCNEC/SCNEC); numbers above the bars indicate cases. **b** EZH2 H-scores of individual NET G3 and LCNEC/SCNEC with the three expression groups shaded; horizontal lines indicate means (Mann–Whitney U test). **c**Proposed three-tier interpretation of EZH2 within the diagnostic panel for high-grade NEN: low expression (H-score <100) supports NET G3, high expression (>200) supports NEC, intermediate expression (100–200) carries no diagnostic weight; sensitivity, specificity and positive likelihood ratio (LR+) refer to the comparison of NET G3 with LCNEC/SCNEC. **d–e** Overall survival according to EZH2 expression group in the overall cohort (**d**) and in high-grade NEN (NET G3 and NEC including MCC; **e**); one sample per patient, Kaplan–Meier estimates with numbers at risk, overall and pairwise log-rank tests. Abbreviations as in Fig. 1

Supplementary Files

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