Gastroenteropancreatic High-Grade Neuroendocrine Neoplasms: Histology and Molecular Analysis, Two Sides of the Same Coin

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Abstract
Background: In gastroenteropancreatic (GEP) high-grade neuroendocrine neoplasms (H-NENs), Ki-67 threshold of 55% defines three prognosis subclasses: neuroendocrine tumor (NET) G3, neuroendocrine carcinoma (NEC) <55%, and NEC ≥55%. We investigated whether the molecular profiling of H-NENs differs among these subcategories and evaluated potential therapeutic targets, including PD-L1. ***Methods:*** In GEP-NEN patients, we evaluated: (i) 55% threshold for Ki-67 labeling index for further stratifying NEC and (ii) immunoreactivity and gene mutations by immunohistochemistry and targeted next-generation sequencing (T-NGS). ***Results:*** Fifteen NETs G3 and 39 NECs were identified. Ki-67 labeling index was <55% in 9 NECs and ≥55% in 30 NECs. Gene mutations by NGS (*TP53*, 32.9%; *KRAS*, 5.5%; *BRAF*, 4.1%) were detected in 46.6% NENs, significantly enriched in NEC ≥55% (76.7%) compared to NEC <55% (55.6%) or NET (20.0%). PD-L1 staining in tumor-infiltrating lymphocytes was observed in NEC ≥55% (36.7%; $p = 0.03$). Median OS was 4.3 years in NET G3, 1.8 years in NEC <55%, and 0.7 years in NEC ≥55% ($p < 0.0001$); it was 2.3 years with NGS wild-type, 0.7 years with ≥1 mutation ($p < 0.0001$), 0.8 years in PD-L1-positive patients, and 1.7 years in PD-L1-negative subjects ($p = 0.0004$).

In multivariate analysis, only the proposed subclassification approach yielded statistically significant differences between groups (NEC <55% vs. NET G3, HR 14.1, 95% CI 2.2–89.8, $p = 0.005$; NEC ≥55% vs. NET G3, HR 25.8, 95% CI 3.9–169, $p = 0.0007$). ***Conclusions:*** These findings identify NEC ≥55% as a biologically and prognostically distinct subtype and pave the way for more personalized treatment.

Introduction

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are a heterogeneous group of tumors. Their classification is based on morphology, well-differentiated (WD) or poorly differentiated (PD), and on their proliferative ability, measured by the mitotic index and/or Ki-67 labeling index [1–3]. Based on that, the 2010 World Health Organization (WHO) classification identified three categories: neuroendocrine tumor (NET) grade (G)1 (WD and Ki-67 labeling index ≤2%), NET G2 (WD and Ki-67 labeling index 3–20%), and neuroendocrine carcinoma (NEC) (PD and Ki-67 labeling index >20%) [3].

According to several reports indicating that NEC category is more heterogeneous than expected [4–17] first in 2017 [1] then in 2019 [18], WHO modified the NEN classification, dividing NEC into two subcategories: NETs G3 (WD and Ki-67 labeling index >20%) and NECs (PD and Ki-67 labeling index >20%) [1, 17]. The diagnostic group of NET G3 initially figured only in the pancreatic NET classification; at present, it is considered relevant for the whole gastrointestinal system [18, 19].

The European Neuroendocrine Tumor Society (ENETS) guidelines consider both NET G3 and NEC as high-grade GEP NENs (H-NENs), although highlighting the heterogeneity of this group and importance of discriminating these entities (NET G3 and NEC) [19].

This approach has already been reflected in tumor-reporting protocols according to the College of American Pathologists (CAP protocols) [20].

The evaluation of tumor morphology and/or Ki-67 labeling index, however, is not always reproducible across different pathologists [21]. Thus, immunohistochemical (IHC) and molecular characteristics of the disease were proposed as an aid for proper stratification of GEP-NEN groups [7, 8, 22–24]. The IHC features of PD disease include p53 overexpression, low expression of somatostatin receptors (at least types 2A and 5) and inversely proportional expression of Rb1 or p16 [23]. At the level of genetic alterations, PD NENs showed mutations in *TP53*, *KRAS*, and *BRAF*, while WD disease presented mutations in *MEN1*, *ATRX-DAXX*, *PI3k/AKT/mTOR*, and *TGF-β* pathways, absent in PD NENs [25–27].

The prognosis and subsequent therapeutic approach to GEP-NENs are driven according to WHO classes: (i) NECs show poor prognosis and usually are treated by platinum-based chemotherapy (PBC) regimens derived from those used for the treatment of small cell lung cancer [8, 9, 28]; (ii) NETs G3 are associated with intermediate prognosis with a propensity for early metastases [4, 5], and are treated with systemic non-PBC [4, 6, 9, 11–14, 19]. Having investigated a different group of patients, we reported earlier that in H-NENs the threshold of 55% for a Ki-67 labeling index allows a refinement of prognosis estimation; particularly, NETs G3 were associated with a median overall survival (mOS) of 24.5 months, whereas NECs with a Ki-67 labeling index of <55% (NEC <55%) had a mOS of 12.9 months, and NECs with a Ki-67 labeling index ≥55% (NEC ≥55%) were associated with a mOS of 5.3 months [22]. Of note, this prognostic difference is also shared by mixed neoplasms, mixed adeno-NECs in which Ki-67 labeling index ≥55%, restricted to the NEC component, is associated with the worst prognosis [28].

The prognostic role of Ki-67 labeling index ≥55% has already been validated in several studies [6, 9, 12, 14, 28] and included in ENETS guidelines since 2016 [19], thus recognizing it as a highly reproducible and powerful tool in GEP-NENs.

The aim of this study was to verify if the molecular and IHC profiles of the H-NENs were distinctive in the above-mentioned three categories NET G3, NEC <55%, and NEC ≥55%.

Moreover, considering the lack of tailored NEC treatments and the heterogeneity of the response rate to standard chemotherapy, we aimed to search for new potential therapeutic targets, including the expression of PD-L1, a promising recently described NEC biomarker [4, 5, 29, 30].

Materials and Methods

Case Selection and Study Design

In order to study H-NENs, between 2015 and 2017, the surgical pathology and clinical databases of INT (an Excellence Centre for the therapy of NENs) were retrospectively searched, and patients with one of the following diagnoses were selected: "neuroendocrine neoplasm," "NET," and "NEC."

Exclusion criteria were: (i) cases with MIXED neuroendocrine and non-neuroendocrine components; (ii) cases with inadequate material for next-generation sequencing (NGS) analysis; (iii) not GEP origin.

Table 1. Characteristics of patients with neuroendocrine neoplasms of pancreatic and extrapancreatic origin by prognostic score

Characteristics	Total (n = 54)	NET G3 (n = 15)	NEC <55% (n = 9)	NEC ≥55% (n = 30)	p value*
Age					
<50 years	11 (20.4%)	4 (26.7%)	3 (33.3%)	4 (13.3%)	
50–59 years	15 (27.8%)	3 (20.0%)	1 (11.1%)	11 (36.7%)	
60–69 years	21 (38.9%)	6 (40.0%)	3 (33.3%)	12 (40.0%)	
70+ years	7 (13.0%)	2 (13.3%)	2 (22.2%)	3 (10.0%)	0.55
Sex					
Male	35 (64.8%)	8 (53.3%)	6 (66.7%)	21 (70.0%)	
Female	19 (35.2%)	7 (46.7%)	3 (33.3%)	9 (30.0%)	0.29
Site					
Colon	26 (48.2%)	4 (26.7%)	4 (44.4%)	18 (60.0%)	
Pancreas	17 (31.5%)	11 (73.3%)	3 (33.3%)	3 (10.0%)	
Stomach	11 (20.4%)	0 (0.0%)	2 (22.2%)	9 (30.0%)	<0.0001
Clinical stage					
Local or locally advanced	16 (29.6%)	3 (20.0%)	3 (33.3%)	10 (33.3%)	
Metastatic	38 (70.4%)	12 (80.0%)	6 (66.7%)	20 (66.7%)	0.39
UICC					
I–II	2 (3.7%)	0 (0.0%)	1 (11.1%)	1 (3.3%)	
III	15 (27.8%)	4 (26.7%)	2 (22.2%)	9 (30.0%)	
IV	37 (68.5%)	11 (73.3%)	6 (66.7%)	20 (66.7%)	0.62
Primary tumor surgery					
Yes	38 (70.4%)	12 (80.0%)	6 (66.7%)	20 (66.7%)	
No	16 (29.6%)	3 (20.0%)	3 (33.3%)	10 (33.3%)	0.39
Neoadjuvant treatment					
Yes	12 (22.2%)	3 (20.0%)	2 (22.2%)	7 (23.3%)	
No	42 (77.8%)	12 (80.0%)	7 (77.8%)	23 (76.7%)	0.80
Adjuvant treatment					
Yes	12 (22.2%)	3 (20.0%)	1 (11.1%)	8 (26.7%)	
No	42 (77.8%)	12 (80.0%)	8 (88.9%)	22 (73.3%)	0.53
NGS					
WT	23 (42.6%)	12 (80.0%)	4 (44.4%)	7 (23.3%)	
Mutated	31 (57.4%)	3 (20.0%)	5 (55.6%)	23 (76.7%)	0.0004
Absent	18 (33.3%)	5 (33.3%)	4 (44.4%)	9 (30.0%)	
Present	36 (66.7%)	10 (66.7%)	5 (55.6%)	21 (70.0%)	0.74
Mutations†					
ATM	2 (3.7%)	1 (6.7%)	1 (11.1%)	0 (0.0%)	0.20
VHL	1 (1.9%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	0.14
CTNNB1	2 (3.7%)	0 (0.0%)	1 (11.1%)	1 (3.3%)	0.72
APC	2 (3.7%)	0 (0.0%)	0 (0.0%)	2 (6.7%)	0.24
TP53	23 (42.6%)	0 (0.0%)	1 (11.1%)	22 (73.3%)	<0.0001
STK11	1 (1.9%)	0 (0.0%)	1 (11.1%)	0 (0.0%)	0.75
KRAS	4 (7.4%)	0 (0.0%)	1 (11.1%)	3 (10.0%)	0.26
NRAS	2 (3.7%)	0 (0.0%)	0 (0.0%)	2 (6.7%)	0.24
GNAS	1 (1.9%)	0 (0.0%)	0 (0.0%)	1 (3.3%)	0.41
SMARCB1	1 (1.9%)	0 (0.0%)	0 (0.0%)	1 (3.3%)	0.41
RB1	1 (1.9%)	0 (0.0%)	0 (0.0%)	1 (3.3%)	0.41
FGFR3	1 (1.9%)	0 (0.0%)	1 (11.1%)	0 (0.0%)	0.75
IDH1	2 (3.7%)	1 (6.7%)	0 (0.0%)	1 (3.3%)	0.65
BRAF	3 (5.6%)	0 (0.0%)	0 (0.0%)	3 (10.0%)	0.14
FBXW7	2 (3.7%)	0 (0.0%)	0 (0.0%)	2 (6.7%)	0.24

Table 1 (continued)

Characteristics	Total (n = 54)	NET G3 (n = 15)	NEC <55% (n = 9)	NEC ≥55% (n = 30)	p value*
PD-L1[§]					
Absent	41 (75.9%)	13 (86.7%)	9 (100%)	19 (63.3%)	
Present	13 (24.1%)	2 (13.3%)	0 (0.0%)	11 (36.7%)	0.05
MMR					
Stable	23 (42.6%)	11 (73.3%)	3 (33.3%)	9 (30.0%)	
Unstable	31 (57.4%)	4 (26.7%)	6 (66.7%)	21 (70.0%)	0.02
IHC[††]					
p16 (present)	4 (7.4%)	1 (6.7%)	1 (11.1%)	2 (6.7%)	0.95
Rb1 (absent)	40 (74.1%)	9 (60.0%)	5 (55.6%)	26 (86.7%)	0.04
SSTR2A (0–1)	34 (63.0%)	2 (13.3%)	4 (44.4%)	28 (93.3%)	<0.0001
SSTR5 (0–1)	32 (59.3%)	2 (13.3%)	3 (33.3%)	27 (90.0%)	<0.0001
p53 (overexpressed)	20 (37.0%)	0 (0.0%)	1 (11.1%)	19 (63.3%)	<0.0001

NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; G, tumor grading; *n*, number of patients; UICC, Union for International Cancer Control; NGS, next-generation sequencing; WT, wild type; PD-L1, programmed death ligand; MMR, DNA mismatch repair; IHC, immunohistochemistry. * p value based on the Fisher exact test. [†] No mutations in the HER2 genes found. [§] PD-L1 staining only in microenvironment cells. [††] p53 present or SSTR2A (score 0–1) or SSTR5 (score 0–1).

Selected cases were studied applying tumor grading (G) according to WHO 2010 and WHO 2017 [1, 17], and tumor staging (TNM) according to the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) 8th edition [31].

A number of the patients included in the study had undergone different systemic treatment schemes which are described in detail in supplementary materials and summarized in online supplementary Table 1 (see www.karger.com/doi/10.1159/000503722 for all online suppl. material).

The group of NET G1 and G2, described in online supplementary Table 2, were analyzed separately and used as a reference in order to verify if molecular and IHC profiles of NET G3 go in line with the profiles of other WD tumors.

Targeted Next-Generation Sequencing

Formalin-fixed paraffin-embedded 5-μm cut sections were manually microdissected to isolate the highest possible percentage of neoplastic cells.

Targeted NGS (T-NGS) was performed using the Ion AmpliSeq™ Cancer Hotspot Panel (Thermo Fisher) that amplifies 207 amplicons covering about 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes commonly mutated in human cancers.

This is a commercially available panel widely used for investigation of various tumors; therefore, it is easily reproducible and gives a plenty of data particularly targeting most of the mutations described in H-NENs that were the main focus of this study [15]. In addition, this panel includes a number of genes, not previously reported to be altered in H-NENs but common in other gastrointestinal tumors and interesting in terms of analyzing possible shared molecular pathways. For the details of DNA extraction, quantification, and library preparation, see supplementary material.

Raw sequencing data were processed using Torrent Suite Software™ (version 5.8.0); the variant calling from sequencing data was generated by Variant Caller plugin. The resulting variants were annotated using Ensemble Variant Effect Predictor pipeline, Ion Reporter™ analysis software version 5.6, ClinVar database, COSMIC database, and dbSNP database. Variants with a MAF value greater than 0.01 in 1,000 genomes combined population were considered as SNP and thus excluded. The filtered variants were examined using the Integrative Genomic Viewer IGV tool [32].

Immunohistochemistry

The investigation of IHC profile included: (i) synaptophysin and chromogranin A (general neuroendocrine markers) in order to confirm the diagnosis of NEN; (ii) Ki-67 labeling index calculation, using the MIB antibody as a percentage of positive cells in 500–2,000 tumor cells counted in areas of strongest nuclear labeling ("hot spots") for defining the grade [1, 2, 18]; (iii) p53, Rb1, p16, somatostatin receptor 2A (SSTR2A), somatostatin receptor 5 (SSTR5), PD-L1, and MMR proteins (MLH1, MSH2, MSH6, PMS2) using the antibodies listed in online supplementary Table 3. p53 staining was evaluated as "negative for mutational pattern" in case of variable low/moderate expression in a minority of cells. Overexpression in a majority of cells and complete loss of IHC expression were evaluated as "positive for mutational pattern." The cases with low percentage of highly intensive nuclear staining were described as a distinct category.

With the exception of SSTR2A, all markers were considered positive regardless of the number of positive cells. SSTR2A was assessed according to Volante et al. [33] (positive: 2+, 3+; negative: 0, 1+ score). Cases with the expression of all MMR proteins were labelled as positive, and cases lacking the expression of at least one of these proteins were evaluated as MMR negative. PD-L1 was evaluated separately in neoplastic cells and intratumoral and peri-

tumoral lymphocytes. A 1% cut-off was used for PD-L1 expression (although all cases evaluated as positive showed expression in >5% lymphocytes). In order to assess the reproducibility of the Ki-67 labeling index, three expert pathologists independently evaluated the samples (online suppl. Table 4).

Evaluation of Proliferative Cut-Offs

The receiver operating characteristic curve with the respective area under the curve was drawn to illustrate the prognostic ability of Ki-67 labeling index to determine 18-month mortality, set as a binary endpoint, and to select the optimal cut-off that maximizes the predictive value of Ki-67 labeling index (online suppl. Fig. 1).

Statistical Analysis

Data were analyzed by descriptive statistics. Differences in frequencies were assessed with the χ^2 or the Fisher's exact test. Interobserver agreement for the evaluation of Ki-67 labeling index was analyzed using kappa statistics and Bland-Altman plots. The primary study endpoint was the correlation of OS with primary tumor site, tumor stage at diagnosis, and NEN differentiation (WD GEP-NENs vs. PD GEP-NENs) according to parameters defined in other solid tumors.

OS was assessed from the date of diagnosis to the date of death or last follow-up. Survival curves were drawn according to the Kaplan-Meier method, and differences between groups were assessed with the log-rank test. The proportions of patients surviving at different time points are presented. Univariate and multivariate Cox proportional hazard regression analysis was used to assess the prognostic significance of various clinical and histopathological characteristics. Data analysis was performed using the SAS software (version 9.4, Cary, NC, USA). All tests were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Clinicopathological Features

A total of 150 candidate cases were identified and, after a pathological revision (detailed in the online suppl. materials), 54 H-NENs were selected consisting of 15 NETs G3 (Fig. 1a, d, g) and 39 NECs (Table 1; Fig. 1b, c, e, f, h, i).

A high level of concordance in Ki-67 assessment between the pathologists (M.M., A.P., and G.P.) involved in its evaluation was reached, with Kappa statistics ranging from 0.87 to 0.96 (online suppl. Fig. 2).

In line with the existing data, also in the present series, the best cut-off for Ki-67 labeling index in predicting NEC patients' survival was 55% (online suppl. Fig. 1).

Applying Ki-67 labeling index at 55%, NECs were subdivided into 9 NECs <55% (Fig. 1h) and 30 NECs ≥55% (Fig. 1i).

Table 1 summarizes the main clinicopathological features of the 54 H-NEN patients enrolled in the study (at the moment of diagnosis). Of those, 35 (64.8%) patients were males and 19 females (35.2%) with a male:female ratio of 1:8. The mean age was 58.5 years. A total of 16 samples (29.6%) were represented by tissue from the primary tumor, while 38 (70.4%) by a specimen of liver metastases from unresectable primary GEP-NENs.

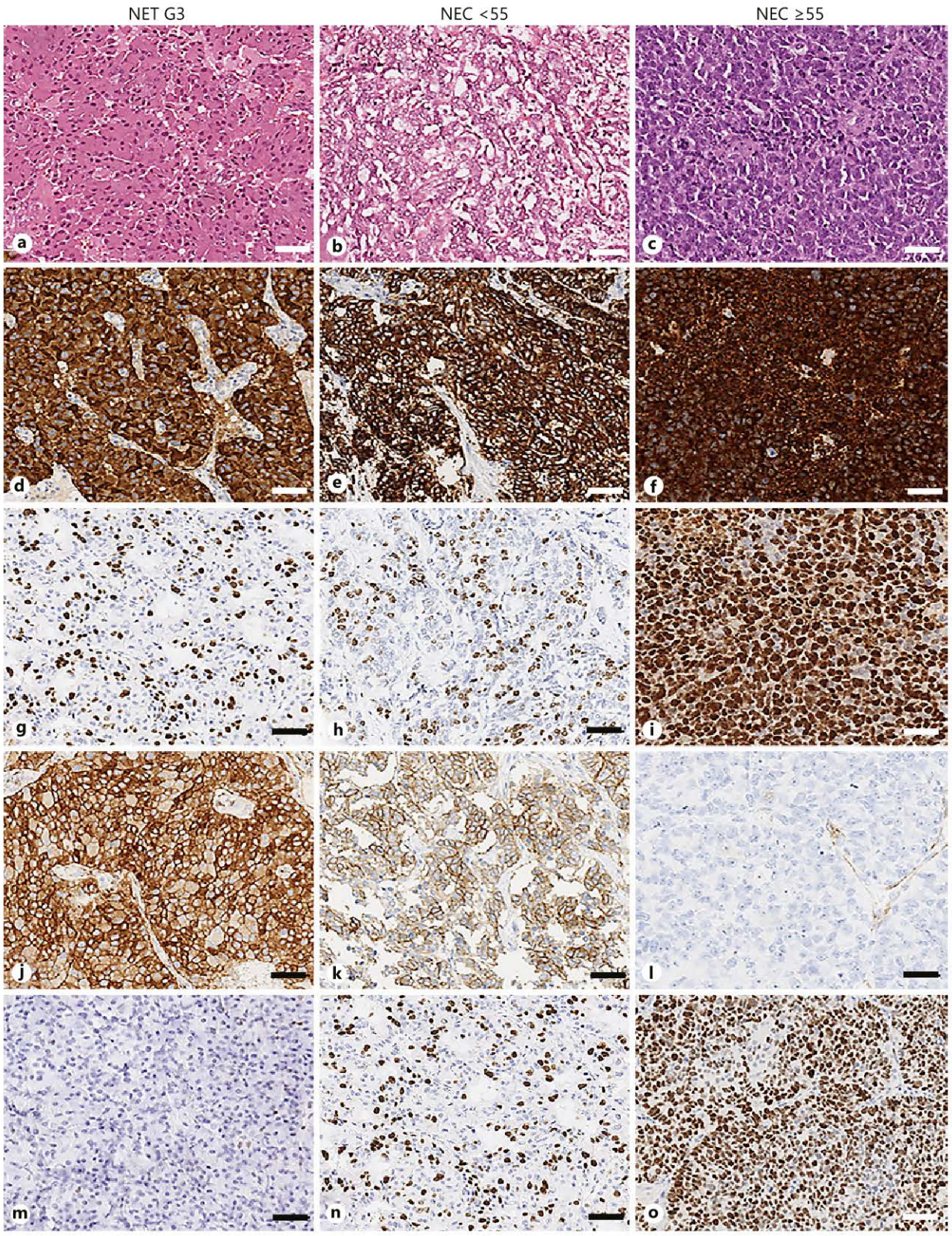
The colon was the most frequent primary site for NECs (22 cases out of 39 NECs): 4 cases of NECs <55% and 18 of NECs ≥55%. A total of 11 cases were located in the stomach (2 NECs <55% and 9 NECs ≥55%) and 6 cases in the pancreas (3 NECs <55% and 3 NECs ≥55%). Interestingly, among the 15 NETs G3, 11 were located in the pancreas, whereas the remaining 4 cases were tumors from the colon (Table 1).

Targeted Next-Generation Sequencing

Overall, 31 of 54 (57.4%) GEP-NENs showed gene mutations by T-NGS, mainly represented by *TP53* (42.6%), *KRAS* (7.4%), and *BRAF* (5.6%) mutations (Table 1; Fig. 2). In addition, *PIK3CA*, *ATM*, *CTNNB1*, *APC*, *NRAS*, *IDH1*, *FBXW7* sporadic mutations (3.7%), and *VHL*, *STK11*, *GNAS*, *SMARCB1*, *RB1* and *FGFR3* individual mutations (1.9%) were observed (Table 1; Fig. 2). Gene mutations were significantly enriched in NECs ≥55% (23/30, 76.7%; $p = 0.0004$) affecting *TP53* (73.3%), *KRAS* (10.0%), *BRAF* (10.0%), *APC/NRAS/FBXW7* (6.7%), and *CTNNB1/GNAS/SMARCB1/RB1/IDH1* (3.3%) genes (Fig. 2). Five of 9 (55.6%) NECs < 55% showed a single mutation in *ATM*, *CTNNB1*,

Fig. 1. a–c Hematoxylin and eosin (HE), well-differentiated features in **a**, poorly differentiated features in both **b** and **c**; at the HE level, the distinction between the two different prognostic categories NEC <55% and NEC ≥55% is not achievable. **d–f** Synaptophysin intense and diffuse cytoplasmic staining confirms the nature of neuroendocrine neoplasms irrespective of each prognostic category. **g–i** Ki-67/MIB-1 labeling index showing marked differences between <55% (G-H) and ≥55% cases. **j–l** Strong and intense membrane and cytoplasmic staining for somatostatin receptor 2a (SSTR2A) in NET G3 (**j**); weak, prevalently cytoplasmic staining in NEC <55% (**k**); absence of both cytoplasmic and membrane positivity in NEC ≥55% (**l**). **m–o** p53 IHC results showing exactly reversed pattern compared to SSTR2A: negativity for mutational pattern of IHC expression in NET G3 (**m**), rare nuclei with intensive staining in NEC <55% (**n**), virtually all nuclei intensively stained in NEC ≥55% (**o**) so diffusely and intensively that the p53 reported picture resembles the Ki-67 staining for the same category. Scale bars = 50 μm. ×200.

(For figure see next page.)



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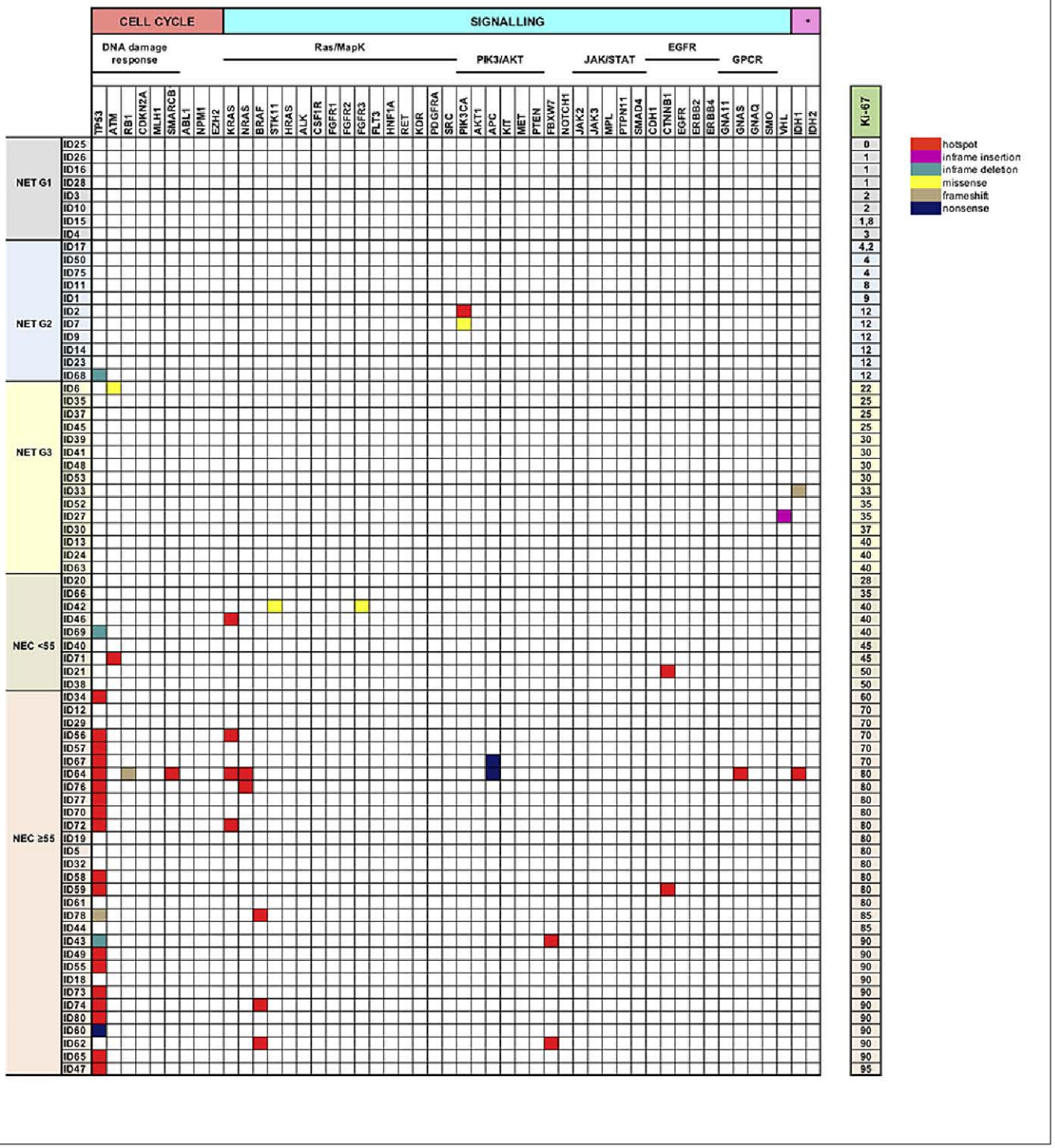


Fig. 2. Genomic alterations in gastro-entero-pancreatic neuroendocrine neoplasms (GEP-NENs). Ki-67 labeling index and somatic mutations are annotated for each patient grouped by different tumor grade. Somatic mutations are classified into hotspot (red), non-hotspot missense (yellow), in-frame insertion (violet), in-frame deletion (light blue), frameshift variant (green), and stop codon (blue). Genes have been grouped into two main functional categories according to their function.

Table 2. Univariate and multivariable analysis (based on selected variables) of overall survival of patients with neuroendocrine neoplasms of pancreatic and extrapancreatic origin

Variable	Categories	Univariate HR (95% CI)	*p* value	Adjusted for site HR (95% CI)	*p* value	Multivariable HR (95% CI)	*p* value
Age	50–59 vs. <50 years	1.92 (0.77–4.76)	0.16				
	60–69 vs. <50 years	1.53 (0.62–3.79)	0.35				
	70+ vs. <50 years	1.11 (0.32–3.81)	0.87				
Sex	female vs. male	0.66 (0.33–1.31)	0.23				
Site	pancreas vs. colon	0.29 (0.12–0.70)	0.01	1.18 (0.42–3.23)	0.76	1.43 (0.42–4.84)	0.56
	stomach vs. colon	1.65 (0.78–3.47)	0.19	1.33 (0.63–2.81)	0.46	1.25 (0.58–2.67)	0.57
New prognostic classes	NET G3 vs. NEC <55%	0.17 (0.04–0.71)	0.02	0.16 (0.03–0.78)	0.02	0.15 (0.03–0.89)	0.04
	NEC ≥55% vs. NEC <55%	3.32 (1.24–8.88)	0.02	3.43 (1.26–9.37)	0.02	1.46 (0.38–5.60)	0.58
Clinical stage	metastatic vs. other	0.77 (0.39–1.52)	0.46				
UICC	IV vs. I–III	0.88 (0.45–1.74)	0.72				
Primary tumor surgery	yes vs. no	0.78 (0.41–1.49)	0.45				
Neoadjuvant therapy	yes vs. no	0.71 (0.31–1.63)	0.42				
Adjuvant therapy	yes vs. no	1.39 (0.67–2.85)	0.37				
PD-L1[§]	present vs. absent	3.78 (1.71–8.35)	0.001			1.95 (0.85–4.46)	0.12
MMR	unstable vs. stable	1.85 (0.97–3.53)	0.06				
p16	present vs. absent	1.57 (0.37–6.67)	0.54				
Rb1	absent vs. present	2.36 (1.08–5.19)	0.03			1.28 (0.47–3.50)	0.63
SSTR2A	0–1 vs. 2–3	4.62 (2.02–10.6)	0.0003				
SSTR5	0–1 vs. 2–3	6.55 (2.66–16.1)	<0.0001				
p53	present vs. absent	4.33 (2.12–8.86)	<0.0001				
p53 or SSTR2A/5[†]	overexpressed or 0–1 vs. other	8.92 (2.71–29.3)	0.0003			1.68 (0.33-8.50)	0.53
NGS[††]	mutated vs. WT	2.52 (1.30–4.89)	0.006				
ATM	mutated vs. WT	–					
VHL	mutated vs. WT	–					
CTNNB1	mutated vs. WT	1.50 (0.36–6.31)	0.58				
APC	mutated vs. WT	2.69 (0.62–11.7)	0.19				
TP53	mutated vs. WT	5.91 (2.78–12.6)	<0.0001				
STK11	mutated vs. WT	–					
KRAS	mutated vs. WT	2.11 (0.73–6.14)	0.17				
NRAS	mutated vs. WT	3.33 (0.76–14.7)	0.11				
GNAS	mutated vs. WT	2.81 (0.37–21.3)	0.32				
SMARCB1	mutated vs. WT	2.81 (0.37–21.3)	0.32				
RB1	mutated vs. WT	2.81 (0.37–21.3)	0.32				
FGFR3	mutated vs. WT	–					
IDH1	mutated vs. WT	0.85 (0.20–3.58)	0.74				
BRAF	mutated vs. WT	4.22 (1.23–14.5)	0.02				
FBXW7	mutated vs. WT	2.75 (0.65–11.7)	0.17				
TP53 or BRAF	mutated vs. WT	6.83 (3.16–14.8)	<0.0001			2.12 (0.85–5.32)	0.11

HR, hazard ratio; NET, neuroendocrine tumor; G, tumor grading; NEC, neuroendocrine carcinoma; UICC, Union for International Cancer Control; PD-L1, programmed death ligand 1; MMR, DNA mismatch repair; NGS, next-generation sequencing. [§] PD-L1 staining only in microenvironment cells. [†] p53 present or SSTR2A (score 0–1) or SSTR5 (score 0–1). [††] No mutations in the PIK3CA or HER2 genes found.

TP53, *STK11*, or *KRAS* (Fig. 2). Among NETs, mutations occurred in 15.8% of G1–2 and 20.0% of G3; interestingly, *PIK3CA* mutations segregated with NETs G1–2 (Fig. 2).

IHC Markers

The distribution of IHC markers is reported in Table 1. All investigated markers showed strong evidence for heterogeneous expression. NETs G3 showed strong

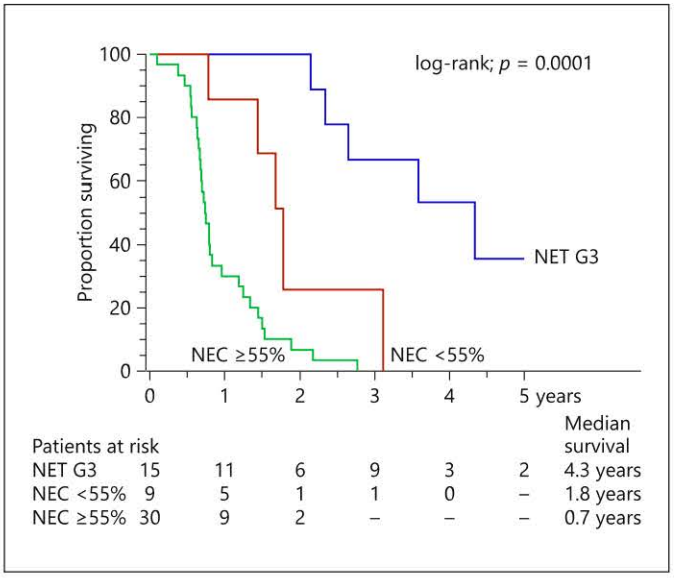


Fig. 3. Overall survival of patients with neuroendocrine neoplasms of pancreatic and extrapancreatic origin by prognostic category. NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; G, tumor grading.

(score 2+/3+) expression of SSTR2A/SSTR5 (Fig. 1j) in more than half of all cases, presence of nuclear Rb1 and negativity for mutational pattern of p53 expression (only one positive case). No significant difference between IHC profile in NET G3 and in NET G1–2 was detected. NEC <55% profile was distinguishable by a lower expression of SSTR2A/SSTR5 (Fig. 1k), presence of some cells with highly intensive p53 staining (Fig. 1n), and markedly reduced Rb1 expression (Table 1). NEC ≥55% is characterized by virtual absence of Rb1 and SSTR2A/SSTR5 staining (Fig. 1l) and the mutational pattern of p53 IHC expression (Table 1; Fig. 1o).

PD-L1 expression was restricted to intra- and peritumoral lymphocytes of 14 GEP-NENs (19.2%) and was absent in tumor cells. Notably, PD-L1 expression was mainly observed in NECs ≥55% (11/30 = 36.7%; $p = 0.05$) (Table 1; online suppl. Fig. 3).

Survival Analysis

mOS was 4.3 years for NET G3 patients, 1.8 years for the NEC <55% group, and 0.7 years for NEC ≥55% patients ($p = 0.0001$, log-rank test) (Fig. 3). In Kaplan-Meier analysis, the 55% cut-off for the Ki-67 labeling index was statistically correlated with OS ($p < 0.0001$).

Table 2 summarizes the results of univariate and multivariate analysis. In univariate analysis, tumor site (pancreatic vs. colon), NEC <55% or NEC ≥55% versus NET G3, the presence of at least one mutation and mutation of *TP53* and/or *BRAF*, loss of IHC expression of Rb1, SSTR2A, SSTR5, p53, and PD-L1 were predictive of shorter survival ($p < 0.05$) (Fig. 4; online suppl. Fig. 4).

SSTR2A (0–1 vs. 2–3; hazard ratio [HR] 4.62, 95% confidence interval [CI] 2.02–10.6, $p = 0.0003$), SSTR5 (0–1 vs. 2–3; HR 6.55, 95% CI 2.66–16.1, $p < 0.0001$), p53 (present vs. absent overexpression; HR 4.33, 95% CI 2.12–8.86, $p < 0.0001$) and Rb1 (absent vs. present; HR 2.36, 95% CI 1.08–5.19, $p = 0.03$) and p53 or SSTR2/5A (present or 0–1 vs. other; HR 8.92, 95% CI 2.71–29.3, $p = 0.0003$) were associated with OS at univariate analysis (Table 2). No correlation was detected only for p16 in univariate analysis (Table 2).

However, only the presence of NET G3 or NEC ≥55% versus NEC <55% was a prognostic factor after adjustment for tumor site (NET G3 vs. NEC <55%; HR 0.16, 95% CI 0.03–0.78, $p = 0.02$; NEC ≥55% vs. NEC <55%; HR 3.43, 95% CI 1.26–9.37, $p = 0.02$) (Table 2).

In analyses stratified by tumor grade, no single variable was statistically significantly associated with OS (data not shown).

Discussion

This single institution retrospective series of H-NENs showed that the molecular and IHC profiles of NET G3, NEC <55%, and NEC ≥55% were different from each other. Furthermore, NETs G3 showed a distinct molecular profile compared with both NEC groups. This highlights that using only the 55% cut-off for Ki-67 labeling index to stratify all H-NENs will not reliably reflect the biological characteristics of this group.

We have shown molecular heterogeneity among the different H-NEN classes by using a commercial NGS panel targeting the main genes found mutated in H-NENs. Indeed, gene mutations were significantly enriched in NECs ≥55% (67.6%) involving mainly the *TP53* (73.3%), *KRAS* (10.0%), and *BRAF* (10.0%) genes (Table 1). Of note, only NECs ≥55% showed multiple mutations mainly affecting cell cycle-regulating genes (Fig. 2). In contrast, neither of NECs <55% (14.7%) showed more than one mutation (11%) restricted to either of the genes *TP53*, *KRAS*, *ATM*, *CTNNB1*, or *STK11* genes. Moreover, investigated genes were found to be mutated in only 3 cases of the NET G3 group (Fig. 2). The role of TP53 alteration as a potent discriminator of NEN behavior goes along with the existing data [23]. On the other hand, Rb1 mutation,

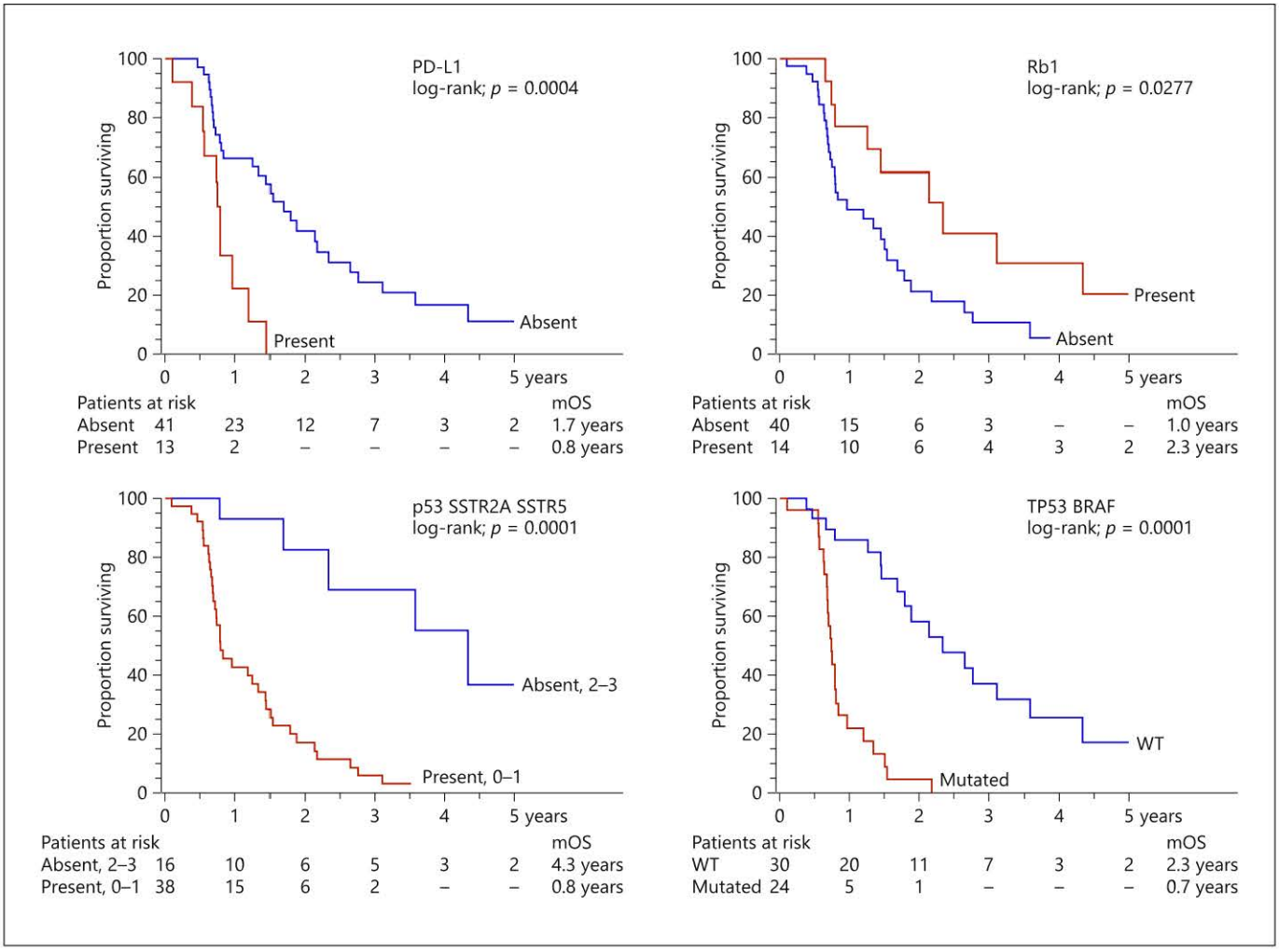
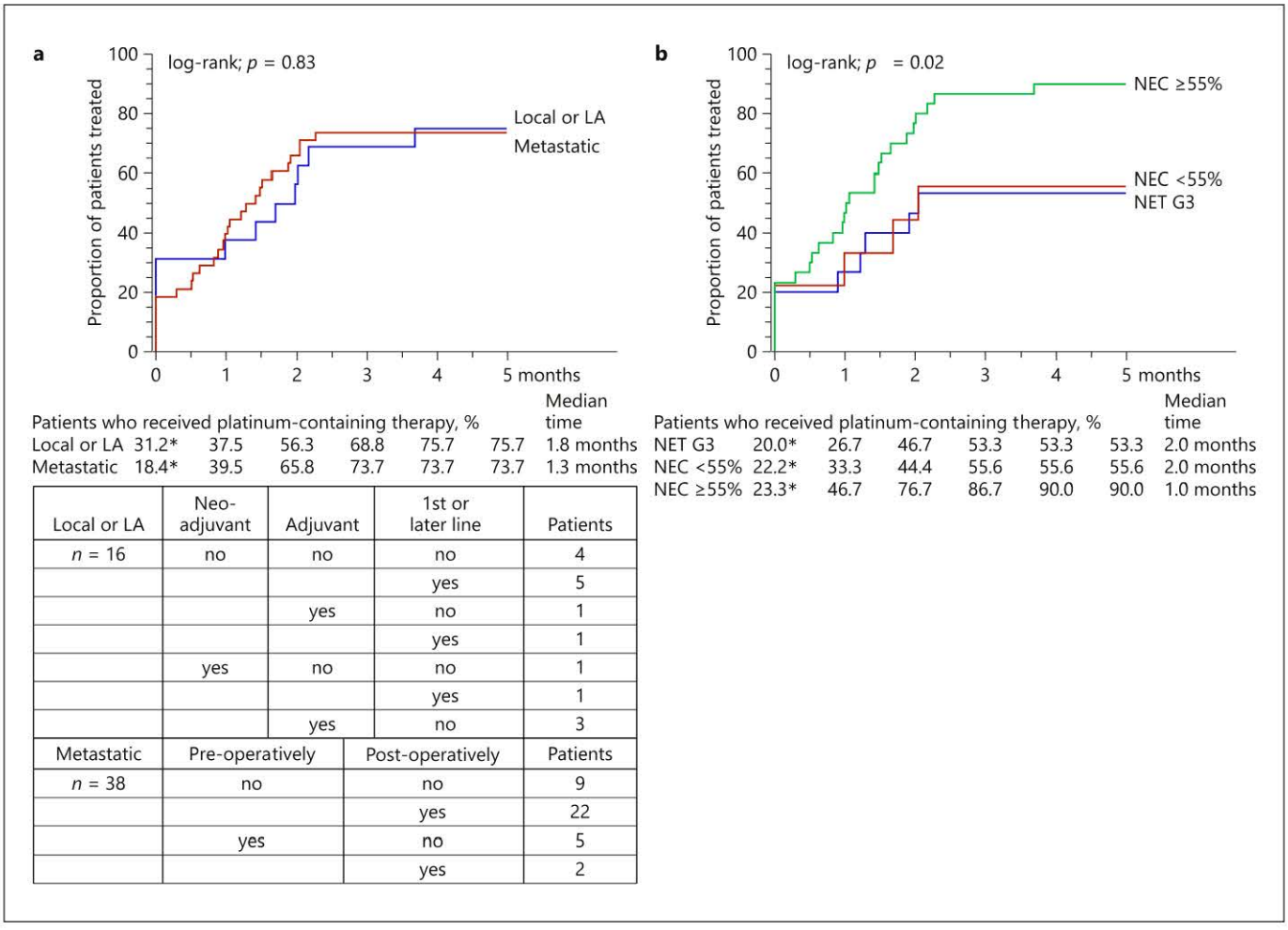


Fig. 4. Overall survival of patients with neuroendocrine neoplasms of pancreatic and extrapancreatic origin according to selected characteristics. mOS, median overall survival.

frequently reported to be a common event in PD-NECs [27, 34] was found in only one case in the current study. This result was discordant with the protein expression loss of Rb1. Most of the studies looking at Rb1 expression in NECs use immunohistochemistry as a tool, and it has also been proposed to use the Rb1 stain for distinguishing the ambiguous cases of H-NENs [12, 35]. The striking difference seen in our data points to the possibility of other molecular events (like methylation) affecting Rb1 protein expression. Epigenetic effects in NECs are documented in a number of studies for a number of genes [36], and it seems like the mechanisms of Rb1 downregulation in NECs still need to be better understood.

The pancreas was confirmed to be the most frequent primary site for NETs G3 (Table 1), which goes in line with the existing data [9, 16, 17, 23] and in part helps understand the fact that this category recognized by the WHO classification is first of all for pancreatic NENs.

As in our previous series [9], the colon was the most common site of NEC ≥55%, by showing at NGS a mutational signature similar to that detected in colorectal adenocarcinoma [28, 34, 37]. Our results strengthen the theory that NEC ≥55% and colorectal adenocarcinoma could share the molecular profile and thus similar therapeutic implications may be considered [22, 38, 39]. Association of NEC with conventional adenocarcinoma precursors has also been documented and further supports the shared molecular background of these 2 tumors [40]. The detection of *RAS* mutations in patients with colorectal carcinoma, in particular *KRAS* and *NRAS*, may



Patients who received platinum-containing therapy, %							Median time
Local or LA	31.2*	37.5	56.3	68.8	75.7	75.7	1.8 months
Metastatic	18.4*	39.5	65.8	73.7	73.7	73.7	1.3 months

Patients who received platinum-containing therapy, %							Median time
NET G3	20.0*	26.7	46.7	53.3	53.3	53.3	2.0 months
NEC <55%	22.2*	33.3	44.4	55.6	55.6	55.6	2.0 months
NEC ≥55%	23.3*	46.7	76.7	86.7	90.0	90.0	1.0 months

Local or LA	Neo-adjuvant	Adjuvant	1st or later line	Patients
n = 16	no	no	no	4
			yes	5
		yes	no	1
			yes	1
	yes	no	no	1
			yes	1
		yes	no	3

Metastatic	Pre-operatively	Post-operatively	Patients
n = 38	no	no	9
		yes	22
	yes	no	5
		yes	2

Fig. 5. a Time to first platinum-containing therapy according to tumor stage. **b** Time to first platinum-containing therapy according to prognostic category. LA, locally advanced. Asterisk indicates patients who received platinum-containing neoadjuvant or preoperative first-line therapy.

have a significant clinical impact. Currently, only *RAS* wild-type (WT) patients are considered for anti-EGFR monoclonal antibodies. We can speculate that EGFR monoclonal antibody-based regimens could be taken into account for pan *RAS* WT NEC patients. Again, moving to a tailored treatment, NEC ≥55% *BRAF* mutated patients may benefit from BRAF-MEK combination therapy as previously described [41, 42]. Frequent RAS mutations have also been described in pancreatic NECs [40], but large-scale studies are lacking due to the rarity of the condition. This may once again question the importance of tumor site and emphasize the molecular-based approach to NENs.

By the analysis of the amino acid substitution involving the p53 protein and by the comparison with "The Cancer Genome Atlas" data, we could demonstrate that there is no preferential type of TP53 mutations for NEC. We can assume that a wide range of *TP53* mutations may participate in the pathogenesis of colorectal NECs (online suppl. Table 5).

Further evidence that GEP-NENs are biologically heterogeneous was provided by our IHC investigations. NETs G1–2 are exclusively associated with SSTR2A and SSTR5 expression coupled with absence of Rb1 expression and absence of a mutational pattern of p53 expression; NETs G3 gained only nuclear Rb1 (not p53) positivity in association with SSTR2A and SSTR5 expression. In NECs, p53 and Rb1/SSTR2/SSTR5 expression progressively increased and decreased, respectively, according to the higher Ki-67 labeling index (Table 1;

Fig. 1). These results could be useful in supporting a morphological distinction between NET G3 from NECs (online suppl. Fig. 5) as also reported earlier by Ramage et al. [43].

Our IHC results underlined that one more factor determining different behavior of NEC <55% from NEC ≥55% could be heterogeneous PD-L1 expression in intra- and peritumoral lymphoid cells. Indeed, PD-L1 staining segregates with NEC ≥55% (36.7%, $p = 0.03$) (Table 1). Given the higher rate of mutations and the higher expression of PD-L1 observed in NEC ≥55% compared to NEC <55%, these patients might be considered for immunotherapy, alone or in combination with targeted drugs and chemotherapy for management of these tumors [44]. Ferrata et al. [45] have looked in more detail into PD-L1 expression in NENs of different primaries, stating that although PD-L1 expression is low in most of NETs, immunotherapeutic approaches are promising especially for high-grade NENs. Preliminary data from prospective clinical trials addressing this matter show that monotherapy with immunotherapeutic agents, yet promising, is effective only in a small subset of patients, and further investigation is needed for testing combinations. It may also be meaningful to revise the inclusion criteria of these clinical trials to introduce them earlier in the disease course.

This study also confirmed in an independent series that the combination of morphological categorization and 55% cut-off for the Ki-67 labeling index plays a definitive pivotal role in better stratifying H-NEN patients' prognosis, as already shown in our previous studies [9, 28]. Indeed, the multivariate analysis revealed that patients with NEC ≥55% had poor survival compared with patients with NEC <55% and NET G3 (mOS: 0.7 vs. 1.8 vs. 4.3 years, respectively; $p < 0.0001$) (Fig. 5a). It has to be highlighted that this threshold cannot replace the 20% Ki-67 labeling index threshold for distinguishing WD and PD NENs, but should rather be used as an additional tool for patient stratification and considering early aggressive treatment. Interestingly, although only in univariate analysis, the presence of at least one mutation or *TP53* and/or *BRAF* mutations ($p < 0.05$), as well as PD-L1 expression ($p < 0.05$) were predictive of shorter survival in line with their higher frequency in NEC ≥55%, arguing that the molecular characterization could be of help in properly identifying this group of patients (online suppl. Table 1 and Fig. 5a). Thus, even if in routine practice the H-NEN classification given by the combined use of morphology and Ki-67 labeling index is mandatory, considering its hard reproducibility also across expert NEN pathologists [19], we proposed an integrated IHC (Rb1 in first place) and molecular (TP53 and RAS in first place) approach in order to improve the proper classification of H-NENs applying reproducible and highly sensitive methodologies.

It has to be noted that except for the genes discussed above, none of the other genes from a broad panel used for our study showed relevant association with NEN types or clinical behavior of tumors.

The reported therapeutic approach to our series is only descriptive. No statistical correlations can be drawn. PBC represented the systemic therapy proposed in the vast majority of cases, mainly as first line for metastatic or unresectable locally advanced/oligometastatic GEP-NECs with ≥55%, in line with literature data. In NECs <55%, non-PBC have been reported as potentially more active than platinum/etoposide [14, 46].

A number of limitations could have affected the results of the current study: the investigated population is rather heterogeneous mostly in terms of clinical characteristics and treatments performed. These might have affected the survival data along with the mutation profiles investigated. The main limitation of this study is the relatively small size and the large heterogeneity of the sample analyzed. The analysis of prognostic factors was conducted using data from only 54 patients spread into three major prognostic groups (NET G3, NEC <55%, and NEC ≥55%) and three distinct anatomic sites (colon, pancreas, and stomach), resulting in a low statistical power to detect significant associations after adjustment for these heterogeneous categories. We have shown that NET G3, NEC <55%, and NEC ≥55% have different molecular profiles; however, the strong prognostic value of molecular characteristics, such as Rb1, p53, or SSTR2A/5, in the whole sample decreased drastically and lost statistical significance after adjustment for NET/NEC subclasses. Given the relative rarity of these tumors, collecting homogenous groups of statistically significant sizes seems to be unrealistic. Currently, as there are no strong data supporting recommended clinical approach to these tumors, slightly different treatment options might have impacted the outcome. The study represents a single-center experience, and the data still need to be validated preferably in a larger group with a better possibility to stratify them into more homogenous subgroups.

In conclusion, our study showed that the molecular profile was significantly different in the previously defined three categories of H-NENs, such as NET G3, NEC <55%, and NEC ≥55% G3, and this is mostly in line with existing data. Considering NEC <55% and NEC ≥55%

as distinct categories in molecular analyses of NENs seems to give better understanding of the biology of these tumors.

Overall, categorization based on the Ki-67 labeling index remains the strongest prognostic factor; nevertheless, other findings provide a good insight into the molecular background affecting the prognosis of PD-NENs, and further studies are needed to verify and possibly validate these observations. To this aim, we are going to perform a large-scale molecular analysis of the previously published multicentric series of H-NENs.

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Statement of Ethics

This study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethical Committee of Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy (No. INT 21/16). According to our standard practice, all patients signed informed consent to the use of their data for research purposes.

Disclosure Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author Note

This work is dedicated to Laura Salvaterra, a courageous woman who battled against cancer and invites us to fight every day in her name, even after she was gone.

Author Contributions

Study concept and design: Massimo Milione. Acquisition of data: Adele Busico, Giovanni Centonze, Giovanna Garzone, Elena Tamborini. Analysis and interpretation of data: Massimo Milione, Adele Busico, Natalie Prinzi, Giovanni Centonze, Cinzia Paolino, Antonio Belfiore, Luca Giacomelli. Drafting of manuscript: Luca Giacomelli, Alessandro Mangogna; Ketevani Kankava. Critical revision of the manuscript for important intellectual content: Adele Busico, Natalie Prinzi, Sara Pusceddu, Alessandro Mangogna, Federica Perrone, Alessio Pellegrinelli. Statistical analysis: Patrick Maisonneuve. Obtained funding: Massimo Milione. Administrative, technical, or material support: Alessandro Mangogna, Luca Giacomelli. Study supervision: Nicola Fazio, Giancarlo Pruneri, Massimo Milione.

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