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THE ROLE OF MGMT PROMOTER METHYLATION AS A PREDICTIVE FACTOR FOR TEMOZOLOMIDE-BASED TREATMENT IN NEUROENDOCRINE NEOPLASMS: A PROSPECTIVE OBSERVATIONAL STUDY.

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Summary:

1. GENERALITIES OF NEUROENDOCRINE TUMORS

Historical overview

Neuroendocrine neoplasms (NENs) are a heterogeneous group of pathologically related epithelial neoplasms with predominant neuroendocrine differentiation.

Phenotypically, the cells of the GEP-NENs belong to the system of disseminated neuroendocrine cells, also known as "APUD" (amine precursor uptake and decarboxylation) cells. The term "neuroendocrine" derives from the phenotypical relationship to neural cells in the expression of certain proteins, such as synaptophysin, neuron-specific enolase, and chromogranin A [1].

Since they arise from neuroendocrine cells located throughout the body, they can be found in most epithelial organs, but they are most frequently found in the lung, in the gastro-intestinal tract and in the pancreas. These tumors can be highly diverse in terms of origin, mechanism of development, functional status, histologic patterns, and biologic behavior [2].

The entity of "carcinoid tumor" was first proposed by the German pathologist Siegfried Oberndorfer over 100 years ago to describe a type of morphologically distinct intestinal tumor with tight nests of small uniform cells, with an usually favorable course and most often good prognosis. Oberndorfer allocated carcinoid tumors to a category between clearly malignant tumors and clearly benign tumors [3].

Gosset and Masson outlined the recognition of carcinoids as endocrine related tumors in 1914. They noted that the tumors arose from chromaffin cells at the base of the the crypts of Lieberkuhn. These chromaffin cells, or Kulchitsky's cells, exhibited characteristics of amine precursor uptake and decarboxylation [4].

A correlation between the neuroendocrine differentiation and the clinical presentation of these tumors was found after a vasoconstrictor substance (later named “serotonine”) was partially purificated by beef serum by Rapport in 1948 [5] and after Lembeck demonstrated the presence of serotonine in carcinoid tumors in 1953 [6].

In 1954, Thorson provided the first description of flushing, diarrhea, right-sided heart failure and increased urinary levels of 5-hydroxyindoleacetic acid (5-HIAA) in association with carcinoid tumors [7].

In 1963, Williams and Sandler proposed to classify carcinoid tumors into foregut (bronchus, thymus, esophagus, stomach, duodenum, upper jejunum, biliary tract, and pancreas), midgut (lower jejunum, ileum, appendix, cecum, and proximal colon), and hindgut (distal colon and rectum) tumors based upon their embryonic segments [8]. This classification had limited value due to heterogeneous tumors in each category.

In 1971, Soga and Tazawa classified NETs based on histologic architecture into 4 sub-group (type A, B, C, D, and mixed type) [9]. However, the pattern did not reliably predict the primary location of the tumor or prognosis.

In the 1980s Johnson demonstrated how the prognosis was influenced by the tumor primary site [10].

The 1980 WHO classification focused on various silver and other granule staining techniques to classify NENs into enterochromaffin (EC) cell carcinoid, gastrin cell carcinoid, and other carcinoids. It had little consideration of tumor grade or biologic behavior, nor did it predict patient outcome and thus now is obsolete [11].

In 1995 Capella was the first author to propose the idea of using the term neuroendocrine tumor instead of "carcinoid" or "islet cell tumor" [12]. NENs were classified into four groups (I-IV), mainly based upon size and angioinvasion: benign, benign or low-grade malignant, low-grade malignant, and high-grade malignant.

The prognostic value of this revised classification was subsequently validated [13]. Other features were later shown to correlate with malignant behavior, including perineural and capsular invasion, high mitotic index, and tumor necrosis.

In 1997, it was suggested that pancreatic NENs could be separated into prognostically different groups based upon tumor size and mitotic activity [14].

Efforts to refine prognosis culminated in the WHO classification of NENs for the tubular gastrointestinal (GI) tract in 2000 and the pancreas in 2004, which were essentially modified versions of the Capella classification. Based upon a combination of tumor size, vascular and perineural invasion, proliferative activity, local invasion, and lymph node and distant metastases, NENs were separated into well-differentiated neuroendocrine tumor with benign behavior, well-differentiated neuroendocrine tumor with uncertain behavior, well-differentiated neuroendocrine carcinoma, and poorly differentiated neuroendocrine carcinoma [15,16].

WHO classifications are periodically reviewed and modified.

In 2018, WHO released a new classification with a “common classification framework” in order to standardize concepts among NENs of different anatomic sites [17]. However, for digestive NENs, a further classification has been proposed in 2019 [18].

Epidemiology

NENs are relatively rare tumors. In a series of 35,618 NENs, including pancreatic neuroendocrine tumors (NETs) as well as carcinoids at all sites reported to the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute (NCI), the age-adjusted incidence for non-pancreatic primaries was 4.7 per 100,000. The incidence for males was slightly higher than for females (4.97 versus 4.49 per 100,000). The median age at diagnosis for all patients with neuroendocrine tumors was 63 years [1]. Roughly similar incidence rates were found in a database study from a Swedish registry that focused on 5184 carcinoid tumors seen between 1958 and 1998. Incidence rates for men and women were 2.0 and 2.4 per 100,000, respectively. Although clear risk factors have not been identified, a regression analysis of this database suggested that risk was increased in the setting of a family history of carcinoid in a first-degree relative (relative risk 3.6) [19].

The incidence of carcinoid tumors has been rising over time in the United States and elsewhere [1,20,21]. As an example, in the above-mentioned SEER analysis of 35,618 neuroendocrine tumors, there was a significant increase in the age-adjusted incidence for both pancreatic and nonpancreatic primary sites (Figure 1) [1]. For all NENs, the incidence rose from 1 to 5 per 100,000 between 1973 and 2004. The increase is probably partly due to increased detection on radiographic imaging and endoscopy.

Furthermore, given the long survival often experienced by patients with NENs, when considering the prevalence, which estimates the number of people alive affected by a pathology, these tumors become more common than generally believed. For example, when the estimated prevalence of these tumors (35 cases per 100,000) is compared with that of other GI neoplasms, NENs results significantly more common than esophageal cancer, gastric cancer, pancreatic cancer and hepatobiliary cancer in the United States [1].

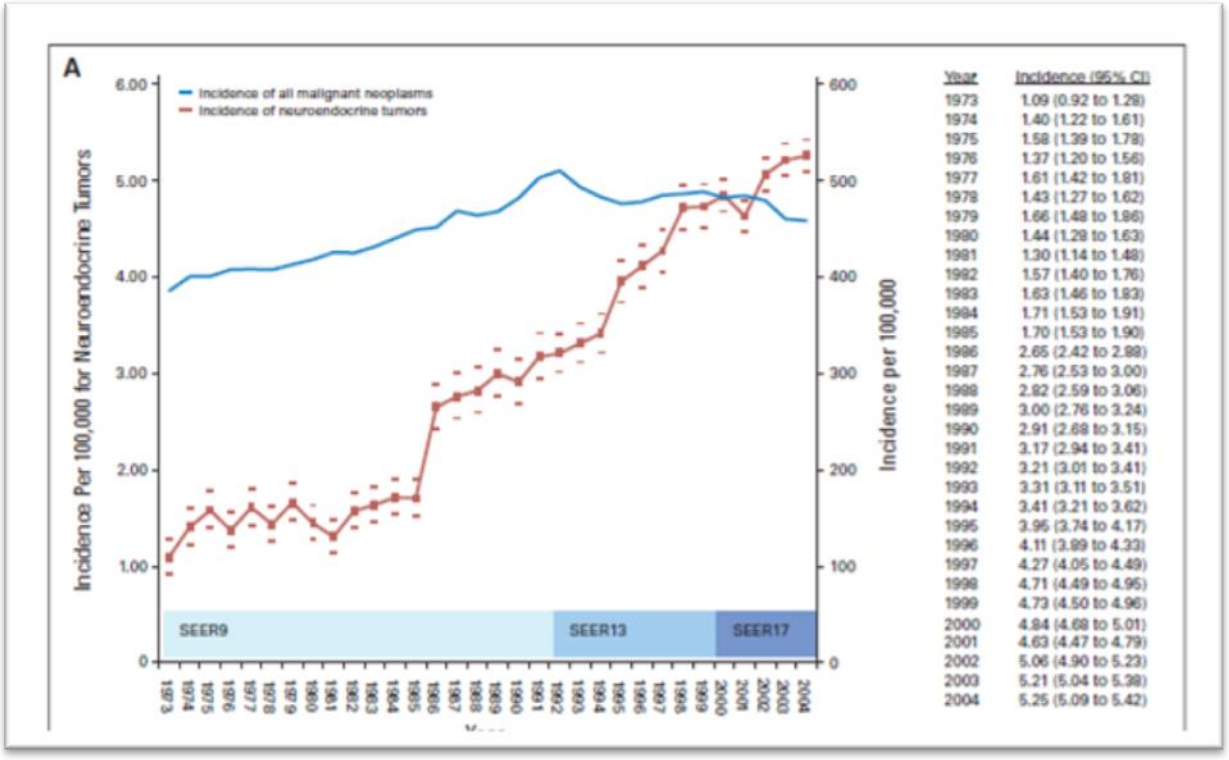


Figure 1. Annual age-adjusted incidence of NENs by year (1973 to 2004) presented as the number of tumors per 100,000 (with 95% CI) age-adjusted for the 2000 US standard population.
Yao JC et al. One Hundred Years after Carcinoids: Epidemiology of and Prognostic Factors for Neuroendocrine Tumors in 35,825 Cases in the United States. JCO 2008;26:3063–72.

The distribution of carcinoids has shifted over time. In a report from the SEER database of 11,427 carcinoid cases treated between 1973 and 1997, the majority were located in the GI tract (55 percent) and bronchopulmonary system (30 percent) [21]. Within the GI tract, most carcinoids arose in the small intestine (45 percent, most commonly in the ileum), followed by the rectum (20 percent), appendix (16 percent), colon (11 percent), and stomach (7 percent). However, since the implementation of screening colonoscopy (approximately in the year 2000), the proportion of patients diagnosed with rectal carcinoids has been greater than the proportion of those diagnosed with small intestinal carcinoids in 12 of 13 SEER registry reporting agencies (Figure 2) [22].

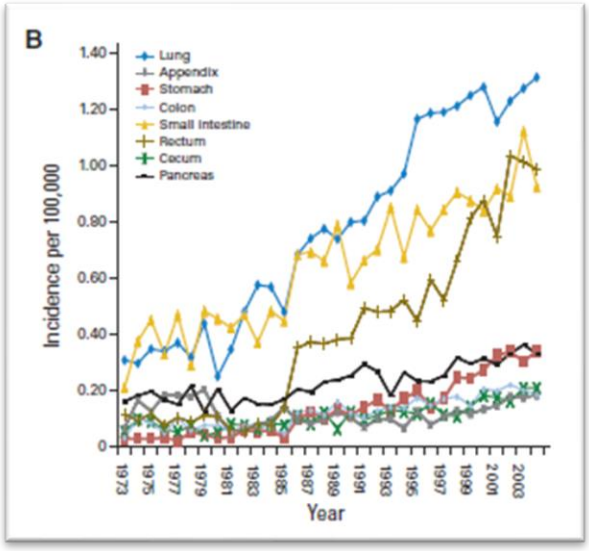


Figure 2. Time-trend analyses of the incidence of NENs by primary tumor site. *Yao JC et al. One Hundred Years after Carcinoids: Epidemiology of and Prognostic Factors for Neuroendocrine Tumors in 35,825 Cases in the United States. JCO 2008:26: 3063–72.*

Distribution varies also depending on sex: females are most commonly affected by NENs of lung, stomach, appendix and cecum, while males most frequently present with thymus, jejunum, pancreas, ileum or rectum NENs.

WHO classifications

The various classification schemes provided by the WHO reflect the status of the knowledge on this topic at the time they were developed.

In the 1980s existed a specific classification for neoplasms of the diffuse endocrine system of the gut and including the carcinoid, separated from a similar one for the pancreas, disconnecting insulinomas (benign and malignant) from tumors of the diffuse endocrine system [23].

The major novelty of the WHO 2000 classification was the concept that tumors were different at different anatomical sites depending on the tumor cell types, the so-called clinical-pathological correlations [15].

A simple three-tier classification scheme was also introduced as common to all anatomical sites, each with comparable dignity. The malignant potential of carcinoids was declared and the word carcinoma (malignant epithelial cell neoplasia) was introduced. Tumors were defined well-differentiated endocrine tumors (WDET) and endocrine carcinomas (WDEC) depending on proven malignancy (presence of metastases and/or deep wall invasion), and these were in contrast to the highly malignant poorly differentiated endocrine carcinomas (PDEC).

In 2010 WHO classification [11] all NENs are definitely accepted as potentially malignant.

A three-tier grading system (G1, G2, and G3) has been introduced, largely based on proliferation fraction of tumor cells according to Ki67 index and mitotic count. “Neuroendocrine tumor” definition is introduced to describe G1 and G2 NENs (previously carcinoids) and “neuroendocrine carcinoma” (NEC) for G3, to mark a separation between low to intermediate-grade versus high-grade NENs. Morphological descriptors are still there, but are substantially disconnected from the grade definition. In specific, the concept of differentiation as utilized in the previous WHO 2000 and 2004 classifications is dropped in WHO 2010. Indeed the classical

carcinoid most of the time lies within the G1 category, but the G2 with a similar morphology is there too, with proven worse prognosis. Further, many carcinoids that originally were approximately baptized as “atypical”, especially in the stomach (type III cases) and in the colon/ rectum, were reclassified as high-grade G3 NENs.

WHO classification changes from 1980 to 2010 are reported in Table 1.

WHO 1980	WHO 2000	WHO 2010[a]
I—Carcinoid	1-Well differentiated endocrine tumor (WDET)	1-NET G1 (WDET or WDEC according to staging)
	2-Well-differentiated endocrine carcinoma (WDEC)	2-NET G2 (WDET or WDEC according to staging)
	3-poorly differentiated endocrine carcinoma/small-cell carcinoma (PDEC)	3-NEC G3 (PDEC)
II—mucocarcinoid III—mixed forms carcinoid-adenocarcinoma	4-mixed exocrine–endocrine carcinoma (MEEC)	4-mixed exocrine–endocrine carcinoma, MANEC (MEEC)
IV—pseudotumor lesions	5-tumor-like lesions (TLL)	5-tumor-like lesions (TLL)

G grade, *NET* neuroendocrine tumor, *NEC* neuroendocrine carcinoma

Table 1. Definitions of gastrointestinal neuroendocrine neoplasm in the WHO classifications 1980, 2000 and 2010.
Rindi G, et al. 25 Years of neuroendocrine neoplasms of the gastrointestinal tract. Endocr Pathol 2014;25(1):59-64.

In 2018, WHO released a new classification with the aim of having a more uniform classification for NENs of any anatomical site, to reduce inconsistencies and contradictions among the various systems available. The key feature of the new classification is a distinction between differentiated neuroendocrine tumors (NETs), also designated carcinoid tumors in some systems, and poorly differentiated NECs, as they both share common expression of neuroendocrine markers. This dichotomous morphological subdivision into NETs and NECs is supported by genetic evidence at specific anatomic sites as well as clinical, epidemiologic, histologic, and prognostic differences. In many organ systems, NETs are graded as G1, G2, or G3 based on mitotic count and/or Ki-67 labeling index, and/or the presence of necrosis; NECs are considered high grade by definition [17].

In Table 2, the 2018 WHO NEN classification for pulmonary and pancreatic NENs is summarized.

Site	Category	Family	Type	Grade	Current terminology
Lung	Neuroendocrine neoplasm (NEN)	Neuroendocrine tumor (NET)	Pulmonary neuroendocrine tumor (NET)[a]	G1 G2	Carcinoid Atypical carcinoid[a]
		Neuroendocrine carcinoma (NEC)	Small cell lung carcinoma (Pulmonary NEC, small cell-type)[b]		Small cell lung carcinoma
			Pulmonary NEC, large cell-type		Large cell NE carcinoma
Pancreas	Neuroendocrine neoplasm (NEN)	Neuroendocrine tumor (NET)	Pancreatic neuroendocrine tumor (NET)	G1 G2 G3	PanNET G1 PanNET G2 PanNET G3
		Neuroendocrine carcinoma (NEC)	Pancreatic NEC, small cell-type		Small cell NE carcinoma
			Pancreatic NEC, large cell-type		Large cell NE carcinoma

Table 2. WHO 2018 classification for NEN of lung and pancreas.

However, in 2019 another classification has been proposed for NET of the digestive tract. The basis of the classification includes the integration of both morphological (histological differentiation) and proliferative (grade) features and identifies three main groups: WD-NETs, poorly differentiated NECs, and mixed neuroendocrine/non-neuroendocrine neoplasm (MiNEN). The distinction between NET and NEC relies on morphology and includes specific cellular and architectural criteria. NETs are then classified based on the proliferation index (mitotic count and Ki67-related proliferation index) and are divided in three groups (NET G1, NET G2, and NET G3), while NECs are by definition high grade neoplasms, and the specification G3 has been removed to avoid confusion with NET G3. This approach is particularly useful for the distinction, among the group of G3 neoplasms (Ki67 > 20%), between NET G3 and NEC, two entities showing distinct molecular background, clinical outcome, and therapeutic approach. This classification, based on the combination of both morphology and proliferation, appears as an important evolution of the WHO classification published in 2010, in which the distinction of NETs from NECs was mainly based on the proliferative index [11,18,24].

	Morphological differentiation	Mitotic count/2mm^2	Ki67 index
NET G1	well-differentiated	<2	<3%
NET G2	well-differentiated	2–20	3–20%
NET G3	well-differentiated	>20	>20%
NEC	poorly differentiated	>20	>20%
MiNENs	well or poorly differentiated	variable	variable

Table 3. The 2019 WHO classification for pancreatic NENs.

Tumor grading and differentiation

Tumor grading and differentiation are different but complementary concepts.

Differentiation is a morphological definition and refers to the degree of resemblance of tumor cells with the normal cell counterpart. Well-differentiated (WD) NETs comprise neoplastic cells uniform for size and features organized in organoid, trabecular, ribbon or gyriform architecture. They present abundant content of secretory granules responsible for intense and diffuse staining for general neuroendocrine markers (synaptophysin and chromogranins). Nuclear chromatin is regular with inconspicuous nucleoli, with no atypia. Mitoses are rare or uncommon.

Poorly differentiated (PD) NECs comprising large cell (LC) and small cell (SC) tumors, are neoplasms with pleomorphic and highly atypical nuclei, solid growth pattern and abundant non-ischemic necrosis, arranged to form either ‘‘map” or ‘‘spot” necrosis. Mitoses are plentiful and often atypical (Table 4) [25].

	WD	PD-LC	PD-SC
1 NEN's architectural features			
1a Typical Neuroendocrine architecture with organoid Features	P	A	A
1b Nodular or solid architecture with rosette formation or palisading	A	P	A
2 NEN's cytological features			
2a Low nucleocytoplasmic ratio	P	A	A
2b Hygh nucleocytoplasm ratio	A	P	A
2c Abundant eosinophlic or amphophilic cytoplasm	P	A	A
2d Large amounts of cytoplasm often basophilic	A	P	A
2e Ovoid nuclei and/or salt and pepper chromatin	P	A	A
2f Nuclear Atypia	R	P	P
2g Nuclear pleomorphism	R	P	A
2h Well visible nucleoli	A	P	A
2i Large size tumor cells (by convention larger than three lymphocytes)	A	P	A
3 Tumor necrosis	R	P	P
4 Small cell typical features as definite for LUNG NECs	A	A	P

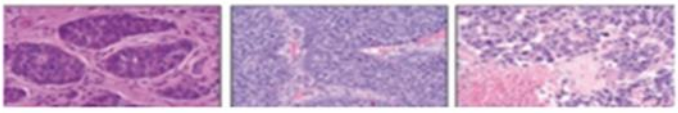
Key: A, Absent; P, present; R, Rare

Table 4. Morphological features distinguishing PD-NECs and WD-NETs, and among PD-NECs large cell NECs against small cell NECs. *Fazio N, et al. Heterogeneity of grade 3 gastroenteropancreatic neuroendocrine carcinomas: New insights and treatment implications. Canc Treat Rev 2016; 50:60-67.*

The grade of a tumor refers to its biologic aggressiveness. For gastroenteropancreatic (GEP) NETs the grading system is based on the rate of proliferation, which is defined by the number of mitoses per 10 high-power microscopic fields or per 2 mm^2 (mitotic rate), or as the percentage of tumor cells that immunolabel positively for the Ki-67 antigen (Ki-67 index) [26].

Mitoses should be counted on hematoxylin and eosin-stained slides in at least 40 HPFs, where possible. The mitoses should be assessed in areas where they are most frequent after a general slide survey. Ki-67 is a non-histonic nuclear protein expressed during the S phase of cell replication. The Ki-67 index should be assessed in 2,000 tumor cells in areas where the highest nuclear labeling is observed [27].

Histological features and Ki-67 index are listed by tumor grade in Table 5.



Histological Classification	Well Differentiated (Low Grade, G1)	Moderately Differentiated (Intermediate Grade, G2)	Poorly Differentiated (High Grade, G3)
Appearance	Monomorphic population of small, round cells	*	Cellular pleomorphism
Prognosis	Prolonged survival	Intermediate	Poor
Mitotic Rate	<2	2–20	>20
Ki-67 Index*	<3%	3–20%	>20%
Necrosis	Absent	*	Present

Table 5. Histopathology of neuroendocrine tumors. *Strosberg J et al. Biology and Treatment of Metastatic Gastrointestinal Neuroendocrine Tumors. Gastrointest Cancer Res. 2008;2:113-125.*

In 1996, La Rosa et al. showed that patients with a NET expressing the MIB-1 epitope of Ki-67 in >2% of cells have poorer prognosis compared with NET patients with MIB-1/Ki-67% < 2% [28]. This finding was confirmed in other studies [29,30]. A 20% threshold was established during the Frascati consensus to define NEC [31]; this figure was validated during the validation clinical studies of the 2010 WHO classification [32,33].

According to the proliferation index, three categories are identified [31]:

-G1 NET: <2 mitosis per 2mm^2 and/or Ki-67 index ≤2%;

-G2 NET: 2–20 mitosis per 2 mm^2 and/or Ki-67 index between 3 and 20%;

-G3 NEC: 21 or more mitosis per 2 mm^2 and Ki-67 index >20%.

In general, G1 and G2 referred to WD NETs displaying diffuse and intense expression of the two general immunohistochemical neuroendocrine markers, chromogranin A and synaptophysin. Punctate necrosis is, per se, indicative of a more aggressive tumor, pointing to a G2 status, which, however, has to be confirmed by the mitotic count. G3 indicates a PD NEC. It has high mitotic counts/Ki-67 index, is often associated with fields of necrosis, and shows significantly reduced chromogranin A expression, while maintaining intense staining for synaptophysin.

Nevertheless, in the last years several reports showed that G3 WHO GEP NECs category is less homogeneous than it can appear [34,35]. Within this group the prognosis can depend on a number of factors including tumor morphology, Ki67 and primary site. Therefore, some authors have proposed a new category of NENs with Ki-67>20% associated to WD morphology, termed GEP NET G3. This subgroup is reported to have prognostic and therapeutic peculiarities. In the vast majority of cases, NET G3 prognosis is more related to tumor morphology rather than Ki-67 value and a therapeutic approach similar to that usually applied in the G2 category could be considered [25].

As mentioned before, recently WHO has released new classifications for NENs.

In 2015 WHO classification, lung NENs are divided in four categories: typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SmCLC) [36]. This classification is based on morphological parameters: mitotic index, the presence of necrosis, and cell size, whereas Ki67 proliferation index is not included in the assessment for classification purposes. A substantial overlapping exists with 2015 WHO classification and the common classification framework proposed in 2018 by Rindi et al [17]. In fact, TC and AC are considered well differentiated NENs (NETs) whereas LCNEC and SmCLC are regarded as poorly differentiated NENs (NECs). Ki67 proliferation index has proven to be a useful parameter in distinguishing a NET from a NEC (LCNEC or SmCLC). Furthermore, Ki67 proliferation index has been shown to be a relevant prognostic factor in lung NETs and its evaluation should be added to the pathological report. In addition, lung carcinoids with high Ki67 proliferation index (between 10% and 20%) have been reported to have peculiar morphological and clinical features, that resemble those of digestive NET G3, and may represent a distinct type of aggressive well differentiated pulmonary NEN [24].

Besides pathology feature-based classifications, for lung NENs a molecular classification has recently been proposed. This classification identifies three different types of lung NENs, showing distinct molecular signatures and clinical behavior [37]. In detail, they listed: 1) primary high grade NENs, which are the most frequent pulmonary NENs (70–75%), are diagnosed on small biopsies of heavy smokers, arise de novo with no recognizable precursor lesions, show classic SmCLC or LCNEC morphology, have low intra- and inter-tumor genetic heterogeneity with consistent inactivation of TP53 and RB1, a high mutation burden, an extremely high Ki67 index, and a very aggressive clinical behavior, with no role for radical surgery; 2) secondary high grade NENs, which represent 20% to 25% of pulmonary NENs, arise in heavy smoker men, have variable morphology (AC, LCNEC, SmCLC), may show

the presence of precursor lesions (neuroendocrine cell hyperplasia/DIPNECH, neuroepithelial bodies, carcinoids, non-small cell lung carcinoma), have high intra- and inter-tumor genetic heterogeneity with involvement of a variety of different pathways (inactivation of TP53, RB1, and NOTCH, KRAS/LKB1/MEN1 mutation, MYC, TERT, SDHA, RICTOR amplification and epithelial-mesenchymal transition), suggesting a multistep pathogenesis, present a heterogeneous Ki67 index, and behave less aggressively than the previous type, being diagnosed mainly on surgical specimen after oncologically radical intervention; 3) indolent low grade NENs, which are the rarest type (5% of lung NENs), are diagnosed in non-smoker women, have well differentiated morphology (TC or AC), are often accompanied by precursor lesions (DIPNECH), may arise in MEN1 or other familial syndromes, show low mutation burden with involvement of chromatin remodeling genes, have an evenly low Ki67 index (10% or less), behave indolently and are successfully treated with surgery. In addition, a growing burden of evidence has been accumulating in support of the hypothesis that at least a subset of high grade NENs (NECs) in this site may arise from the progression of pre-existent NETs (carcinoids) [24].

As for digestive NENs, the most recently updated WHO classification has been published in 2019 [18]. The basis of the classification includes the integration of histological differentiation and proliferative features and identifies three main groups: well differentiated NETs, poorly differentiated NECs, and mixed neuroendocrine/non-neuroendocrine neoplasm (MiNEN). The distinction between NET and NEC relies on morphology and includes specific cellular and architectural criteria. NETs are then graded based on the proliferation index (mitotic count and Ki67-related proliferation index) and are divided in three groups (NET G1, NET G2, and NET G3), while NECs are by definition high grade neoplasms, and the specification G3 has been removed to avoid confusion with NET G3. This approach has proven to be of great help for the prognostic stratification of patients and it is particularly useful for the distinction, among the group of G3 neoplasms

(Ki67 > 20%), between NET G3 and NEC, two entities showing distinct molecular background, clinical outcome, and therapeutic approach.

In addition to NET and NEC, the WHO classification includes mixed neuroendocrine/ non-neuroendocrine neoplasms (MiNENs).

Immunohistochemical and serum markers

General immunohistochemical markers of neuroendocrine differentiation include chromogranin, synaptophysin, CD56, protein gene product (PGP) 9.5, and neuronspecific enolase (NSE). Chromogranins are a family of glycoproteins associated with dense-core secretory vesicles found ubiquitously in neuronal and endocrine tissues. Chromogranin A (CgA) was first isolated from chromaffin cells of the adrenal medulla. Synaptophysin is a synaptic vesicle membrane protein also found commonly in neuronal tissues and in endocrine tumors. Neuron-specific enolase is a cytoplasmic enzyme detected in tumors of neuroendocrine differentiation, but lacks specificity compared to CgA and synaptophysin [38-39]. Chromogranin positivity generally correlates with the extent of granularity on electron microscopy. WDNETs tend to exhibit diffuse and intense expression of CgA and synaptophysin, whereas PD neuroendocrine carcinomas show significantly reduced CgA expression while maintaining intense staining for synaptophysin [27].

Immunostaining for specific hormones can aid in the diagnosis of NETs. The various hormone-specific markers used in immunophenotyping of pancreatic endocrine tumors include insulin, glucagon, somatostatin, gastrin, VIP, calcitonin, serotonin, ACTH, and neurotensin. This immunoreactivity, however, does not necessarily correlate with serum hormone levels or clinical syndrome. For example, a study of nonfunctional pancreatic endocrine tumors demonstrated that 87% were immunoreactive to at least one peptide hormone, such as insulin or glucagon [39].

To identify the origin of a NEN of unknown primary site, several trascriptional factors may be used, such as TTF-1 (suggestive for a thyroid or lung origin), CDX-2 (intestinal primary), Isl-1 (pancreatic primary), PDX-1 (pancreatic or jejunal origin). The information obtained through these markers though, need to be interpreted in the clinical context. The expression of these transcriptional factors has to be carefully evaluated since it is not rare to observe an aberrant expression of TTF-1, Isl1 and CDX-2 in poorly differentiated tumors of extra-pulmonary or extra-intestinal tumors [40].

Serum and urine tumor markers include hormones and their metabolites (eg, serotonin, 5-HIAA, insulin, glucagon, gastrin) and nonspecific tumor markers such as chromogranin, pancreatic polypeptide (PP), NSE, and substance P. Hormone levels should be assessed in accordance with the patient's clinical syndrome. The specificity of a 24-hour 5-HIAA urine collection approaches 100% in metastatic carcinoid tumors, and sensitivity is high for detection of the carcinoid syndrome. Strict avoidance of serotonin-rich foods during urine collection is necessary to prevent false-positive test results [41].

The most sensitive general serum marker of NETs is CgA. It is released into the circulation in approximately 90% of pancreatic endocrine tumors and 70%–100% in metastatic gastrointestinal carcinoid tumors [40]. False positive tests, however, can occur with renal or hepatic impairment or with atrophic gastritis and proton pump inhibitor use (due to ECL hyperplasia). Serum levels of CgA tend to be highest in metastatic midgut carcinoid tumors and correlate with tumor burden, as well as response to treatment [27,42].

Somatostatin receptors

Around 80% of GEP NETs express somatostatin receptors (SSTRs), located on the cell membrane. There are five different G-protein coupled receptor subtypes (SSTRs 1-5) that are differently expressed in the various types of tumors. Tumors expressing SSTRs often contain one or more receptor subtypes. Several studies have shown that such receptors are preferably expressed in well-differentiated forms and that some advanced tumors lose particular receptor subtypes while keeping others. It has also been reported that SSTR subtypes can form homo/heterodimers at the membrane level, to develop new receptors with different functional features, and that this receptor "association" may be induced by addition of either dopamine or somatostatin [43,44].

In a study examining 81 functioning and non-functioning GEP NETs most tumors expressed SSTRs 1, 2, 3 and 5, while SSTR 4 was detected only in a small minority [45].

Somatostatin receptors have been extensively mapped in different pancreatic tumors by means of autoradiography, reverse-transcription polymerase chain reaction, in situ hybridization and immunohistochemistry; SSTRs 1, 2, 3 and 5 are usually expressed in pancreatic NETS. Pancreatic insulinomas had heterogeneous SSTRs expression while 100% of somatostatinomas expressed SSTR 5 and 100% gastrinomas and glucagonomas expressed SSTR 2.

Somatostatin receptors distribution in different type of NETs is reported in Table 6.

	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
All	68	86	46	93	57
Insulinoma	33	100	33	100	67
Gastrinoma	33	50	17	83	50
Glucagonoma	67	100	67	67	67
VIPoma	100	100	100	100	100
Non-functioning	80	100	40	100	60

Table 6. Somatostatin receptors in GEP NETs (%). *Appetecchia M, et al. Somatostatin analogues in the treatment of gastroenteropancreatic neuroendocrine tumours, current aspects and new perspectives. J Exp Clin Cancer Res 2010;29(1):19.*

Somatostatin is a natural peptide hormone secreted in various parts of the human body, including the digestive tract, able to inhibit the release of numerous endocrine hormones, including insulin, glucagon, and gastrin. The biological effects of somatostatin are mediated through its specific receptors (SSTR 1-5) with a high degree of sequence similarity (39-57%) and which have been cloned in the early 1990s. They all bind natural peptides, somatostatin 14, somatostatin 28 and cortistatin with similar high affinity. Endogenous somatostatin has a short half-life in circulation (1-3 min) and this makes it difficult to use it continuously and has resulted in the development of synthetic analogues [44].

Genetics and hereditary predisposition

Although the majority of GEP tumors are sporadic, several autosomal dominant hereditary syndromes have been identified. The underlying genetic abnormalities yield insight into oncogenic pathways of familial and sporadic tumors. Multiple endocrine neoplasia 1 (MEN1) is an autosomal dominant hereditary syndrome characterized by a predisposition to tumors of the parathyroid glands, anterior pituitary, and pancreatic islet cells [46]. The underlying tumor suppressor gene mutation has been identified in the long arm of chromosome 11 (11q13). Its protein product "menin" has been recently cloned and appears to be a regulator of gene expression. Germline MEN1 genetic testing appears to have a 70%–90% sensitivity in familial MEN1 cases and a somewhat lower sensitivity in sporadic cases [27].

The most frequent manifestation of MEN1 is parathyroid hyperplasia, which typically develops in the second to fourth decade. Pituitary adenomas form in about 15%–20% of patients. Pancreatic endocrine tumors become clinically apparent in about one third of patients, with a higher rate of subclinical disease. Gastrinomas occur most often, followed by insulinomas. Tumors are almost invariably multifocal; consequently, the role of curative surgical therapy is controversial.99,100 An exceptionally indolent growth pattern is characteristic of these tumors; consequently, life expectancy appears to be only modestly diminished in MEN1 patients [47].

Von-Hippel Lindau (VHL) syndrome is caused by an autosomal dominant mutation in the VHL gene located on chromosome 3p25. This gene is involved in the regulation of a hypoxia-inducible gene (HIF-1alpha) expression. Induction of hypoxia-associated cytokines, including erythropoietin, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), is thought to stimulate tumor growth, but the precise mechanism of tumorigenesis is unknown. A variety of tumors can develop in VHL syndrome, including renal cell carcinomas, hemangioblastomas, pheochromocytomas, pancreatic cysts, and pancreatic endocrine

tumors. The latter occur in only 10% of cases and tend to progress in an indolent fashion [48].

Tuberous sclerosis is an autosomal dominant syndrome characterized by lowgrade neoplasms and hamartomas in multiple organs, including skin, brain, and kidney. Pancreatic endocrine tumors occur in 1%–5% of cases. Two variants have been described: TSC1 caused by a mutation on chromosome 9q34106 encoding hamartin and TSC2 on chromosome 16p13 encoding tuberin. A complex of hamartin and tuberin is thought to regulate cell-cycle progression, possibly through upregulation of the mTOR cell-signaling pathway [49].

Hereditary syndromes have not been identified in carcinoid tumors, and a family history is reported in less than 1% of patients. The relative risk of a carcinoid tumor diagnosis in a patient with a first degree affected relative is estimated to be 3.6 thus the absolute risk remains low and does not warrant screening [50].

The genetic aberrations in sporadic GI NETs are poorly understood. Oncogenes and tumor suppressor genes that are mutated in common human malignancies (p53, APC, Rb, K-ras) do not appear to be implicated in NET development. Mutations of the MEN1 gene (chromosome 11q13) occur in about 20% of sporadic, solitary pancreatic endocrine tumors, whereas chromosome 18 deletions are common in midgut carcinoid tumors. Techniques such as comparative genomic hybridization have identified gains and losses in numerous chromosomes. These genetic abnormalities appear to increase in pancreatic endocrine tumor metastases compared to matched primary tumors. Nonfunctional pancreatic endocrine tumors also appear to contain an increased frequency of chromosomal aberrations compared to functional tumors [27,51,52].

Scarpa et al. recently performed a whole-genome sequencing of 102 primary pancreatic sporadic NETs and reported a larger-than-expected proportion of germline mutations, including previously unreported mutations in the DNA repair genes MUTYH, CHEK2 and BRCA2. Together with mutations in MEN1 and VHL, these

mutations are reported in 17% of patients. Somatic mutations, including point mutations and gene fusions, were commonly found in genes involved in four main pathways: chromatin remodelling, DNA damage repair, activation of mTOR signalling (including previously undescribed EWSR1 gene fusions), and telomere maintenance. In addition, a subgroup of tumours associated with hypoxia and HIF signaling was identified [53].

Cell-signaling pathways influence tumor growth and hormonal activity. Neuroendocrine cells can express the insulin-like growth factor (IGF) as well as its receptor (IGFR). Cell line studies indicate that IGF-1 can act in an autocrine and paracrine fashion to inhibit apoptosis and stimulate secretion of chromogranins, possibly by activating the PI3K-AKT pathway [54]. Vascular endothelial growth factor is also expressed by NETs, and elevated levels of circulating VEGF have been associated with tumor progression [55]. Cyclin D1, an important component of cell cycle regulation, has been found to be overexpressed in pancreatic endocrine tumors [56].

Clinical presentation

The clinical behavior of GEP NETs varies based on site of tumor origin and histologic differentiation, which appear to be the most important prognostic factors in the natural history of these tumors.

NETs can be also classified as functioning or nonfunctioning. NETs are considered functioning when a specific clinical syndrome is induced due to the excessive production of hormones by the tumor cells; approximately two-thirds of NETs are not functional and present fairly late, with symptoms of mass effects or distant (usually hepatic) metastases, or both. They are often diagnosed as incidental findings during radiological examinations. The most common symptoms of nonfunctioning tumors are abdominal pain, jaundice, recurrent pancreatitis, weight loss, steatorrhea, GI bleeding, asthenia, hyporexia.

Although functioning tumors cause distinct clinical syndromes, individual symptoms are not recognised as a complex. Delayed diagnosis is typical (5–7 years on average), increasing the probability of metastatic disease.

Functioning NETs are defined based upon the presence of clinical symptoms due to excess hormone secretion by the tumor. Functioning pancreatic NETs are classified according to the predominant hormone they secrete and the resulting clinical syndrome (eg, insulinoma, gastrinoma, glucagonoma, VIPoma, somatostatinoma). Immunohistochemical staining is not a defining criterion for tumor classification. For example, if a tumor stains for gastrin but does not produce symptoms of the Zollinger-Ellison syndrome, it should not be considered a gastrinoma; however, gastrin-secreting NET would be an appropriate term.

Carcinoid tumors:

Approximately 70% of carcinoid tumors arise in the GI tract and about 25% originate in the lungs. Other rare primary sites include larynx, thymus, kidneys, and ovaries.

It was not until the 1950s that the carcinoid syndrome was described, and serotonin was identified as the primary secretory product associated with symptoms such as flushing and diarrhea [7].

Serotonin is derived from the amino acid tryptophan and is inactivated by the liver into 5-hydroxyindoleacetic acid (5-HIAA), its urinary metabolite. Consequently, the carcinoid syndrome occurs primarily in patients with metastatic tumors that secrete serotonin directly into the systemic (rather than portal) circulation. Other vasoactive substances elaborated by carcinoid tumors include biogenic amines (such as histamine, dopamine, and hydroxytryptophan), tachykinins (kallikrein, substance P), and prostaglandins [27].

Carcinoid heart disease typically occurs in patients with high levels of circulating serotonin. Characteristic thickening and fibrosis of right-sided cardiac valves produces tricuspid regurgitation and pulmonary stenosis. The right heart is invariably affected due to its direct exposure to serotonin secreted by liver metastases. Left heart valves are clinically involved in only 10% of cases. The precise underlying mechanism of valvular fibroblast proliferation is uncertain [57].

The most common site of origin is the small intestine, followed by the rectum, appendix, colon, and stomach. As a general rule, midgut (jejunal, ileal, cecal, appendiceal) carcinoid tumors, produce the typical carcinoid syndrome, hindgut (distal colon and rectum) tumors are hormonally inactive, and foregut (bronchial, stomach, duodenal) tumors may be associated with atypical hormonal syndromes.

Gastric carcinoids- Carcinoid tumors of the stomach originate from gastric neuroendocrine cells termed “enterochromaffin-like” (ECL) cells. They can develop sporadically, or arise from the trophic effects of elevated serum gastrin. Three distinct types have been identified [58,59].

Type I tumors occur in the setting of chronic atrophic gastritis and account for about 80% of gastric carcinoids. In this condition, serum gastrin rises in response to gastric achlorhydria. Elevated serum gastrin, in turn, causes diffuse ECL hyperplasia and development of multifocal, polypoid carcinoid tumors. These tumors are generally benign, with no reported cases of tumor-related mortality. The diagnosed incidence of type I gastric carcinoid tumors has been rising markedly with increasing use of upper GI endoscopy [60,61].

Type II gastric carcinoids likewise arise in the setting of hypergastrinemia. In these rare tumors, elevated gastrin is produced by pancreatic or duodenal gastrinomas typically in the setting of multiple endocrine neoplasia 1 (MEN1). As is the case in type I gastric carcinoids, tumors tend to be small, multifocal, and clinically indolent. Instances of tumor regression have been described among patients treated with somatostatin analogs [62].

Sporadic gastric carcinoid tumors (type III) occur in about 15% of cases and are not associated with elevated gastrin levels. These tumors have a much higher malignant potential than type I or type II gastric carcinoids, and are typically managed with radical gastrectomy when discovered at an early stage [27].

Ileocaecal carcinoids- The majority of carcinoid tumors originate in the terminal ileum, where the concentration of enterochromaffin cells is highest. Up to 25% of ileal carcinoid tumors are multifocal on pathologic examination. The most frequent sites of distant spread are the liver, bone, and peritoneal cavity. Lymph node

metastases at the root of the mesentery are common, and may be associated with dense desmoplastic fibrosis, rendering them unresectable.

The carcinoid syndrome occurs primarily in patients with serotonin-secreting metastatic small intestinal carcinoid tumors. Common symptoms include flushing (a vasomotor phenomenon described as a sensation of warmth associated with erythema) and diarrhea. Bronchospastic symptoms occur less frequently. The term "carcinoid crisis" describes circulatory collapse caused by an acute release of serotonin and other vasoactive substances into the circulation. Triggers include general anesthesia and epinephrine [63].

Appendiceal carcinoids- Appendiceal carcinoid tumors are found in approximately 1 in 300 appendicectomy specimens, nearly always incidentally. They typically arise from submucosal endocrine cells at the tip of the appendix [64].

Rectal Carcinoids- Carcinoid tumors originating in the rectum are often discovered incidentally during lower endoscopy or as a result of lower GI bleeding. They are not associated with a hormonal syndrome. Malignant potential closely correlates with size. Tumors smaller than 1 cm rarely metastasize and can usually be resected endoscopically or trans-anally, whereas tumors larger than 2 cm metastasize in over 50% of cases [65].

<u>Pancreatic tumors:</u>

Pancreatic endocrine tumors arise from the islet cells of the pancreas. These heterogeneous neoplasms can secrete a variety of peptide and amine hormones, including insulin, gastrin, glucagon, vasoactive intestinal peptide (VIP), ACTH, serotonin, somatostatin, and parathyroid hormone. Approximately 35% to 85% are considered nonfunctional.

Up to 20% are associated with MEN1, an autosomal dominant hereditary syndrome.

Pancreatic endocrine tumors are classified according to the hormone they produce. Insulinomas and gastrinomas are the most common functional subtypes.

The majority of pancreatic endocrine tumors are malignant, with the exception of insulinomas, which are usually benign. Gastrinomas are most commonly associated with MEN1, where they tend to be multifocal [27].

Insulinomas- About 90% of insulinomas are smaller than 2 cm, and less than 10% are considered malignant. Patients typically present with neuroglycopenic symptoms such as dizziness, lethargy, palpitations, and diaphoresis. Diagnosis is established during a monitored fast where serum glucose is measured along with insulin in order to demonstrate hypoglycemia (glucose <45 mg/dL) associated with inappropriate insulin elevation (> 6 μU/mL). C-peptide can also be measured to exclude exogenous insulin administration. Somatostatin receptor scintigraphy using octreotide tagged with radiolabeled 111Indium-pentetreotide (OctreoScan) is relatively insensitive, because up to 40% of insulinomas express insufficient somatostatin receptors. In cases of occult tumor, arterial calcium stimulation with hepatic venous sampling can aid with tumor localization [66].

Gastrinomas- Gastrinomas originate in the duodenum and the pancreas, typically in proximity to the pancreatic head. About 60%–80% are considered malignant and one third of patients present with distant metastases at diagnosis. The MEN1 syndrome is implicated in about 20% of cases and is associated with tumor multicentricity. The gastrinoma syndrome, also known as the Zollinger-Ellison syndrome, is caused by hypersecretion of gastrin stimulating gastric acid release into the stomach. The most common manifestations are dyspepsia, heartburn, and diarrhea. Peptic ulcerations can affect atypical locations such as the jejunum. Diarrhea results from the passage of

excess gastric acid into the small intestine, neutralizing digestive pancreatic enzymes and causing malabsorption.

The diagnosis of gastrinoma can be established when serum gastrin levels exceed ten times the upper limit of normal (ie, > 1,000 pg/mL). It is important to note that acid blocking drugs, such as proton pump inhibitors, can elevate serum gastrin levels and lead to false-positive results. In cases where the diagnosis is equivocal, a secretin stimulation test can help identify gastrinomas: a serum gastrin rise of > 200 pg/mL is considered diagnostic, with a sensitivity and specificity of 83% and 100%, respectively [67].

Prior to the advent of acid blocking medications, the Zollinger-Ellison syndrome was a highly morbid condition necessitating palliative gastrectomy or vagotomy. Today, high-dose proton pump inhibitors effectively control symptoms in the majority of cases [68].

VIPomas- These tumors secrete vasoactive intestinal peptide. The resulting syndome (also known as the Verner-Morrison syndrome) is characterized by profuse watery diarrhea, often exceeding 3 liters a day. Due to the severity of the diarrhea, the syndrome is sometimes described as “pancreatic cholera.” Other complications include flushing, dehydration, hypochlorhydria, and hypokalemia. VIPomas are typically large at presentation (> 3 cm) and usually originate in the tail of the pancreas. The majority are malignant [69].

Glucagonomas- These tumors arise from the alpha cells of the pancreas. The clinical manifestations are protean, and may include hyperglycemia, anorexia, weight loss, venous thromboses, cheilitis, and an unusual rash called necrolytic migratory erythema (NME). NME characteristically manifests as painful, weeping,

erythematous papules or plaques involving the face, perineum, and flexural regions. The underlying mechanism of NME is uncertain [70].

Morphological classification	Main cell type *(hormone)*	P	Stomach		Intestine						Clinical presentation
			CF	An	Small			App	Colon-rectum		
					D	J	I		C	R	
Well differentiated	β *(insulin)*	+									Hypoglycemia
	α *(glucagon)*	+									Glucagonoma
	PP *(pancreatic polypeptide)*	+									-
	D *(somatostatina)*	+	+	+	+	+					Somatostatinoma
	D1 *(VIP)*	+			+						VIPoma or Verner-Morrison syndrome
	EC *(serotonine)*	+	+	+	+	+	+	+	+	+	Carcinoid syndrome
	ECL *(histamin)*		+	+							Atypical Carcinoid syndrome
	G *(gastrin)*	+	+		+	+	+				Zollinger-Ellison syndrome
	L *(GLI/PYY)*		+		+	+	+	+	+		-
Poorly differentiated	s/i/l	+	+	+	+	+	+		+		

Table 7. Association between clinical presentation, site of GEP NET, hormon secretion and histological features. Abbreviations: P: pancreas; CF: corpus-fundum; An: antrum; D: duodenum; J: jejunum, I: ileum; App: appendix; C: colon; R: rectum; EC: enterochromaffin; VIP: vasoactive intestinal peptide; ECL: enterochromaffin-like; GLI: glucagon-like immunoreagents (glicentin, glucagon-37, glucagon-29); PYY: Tyr-amid N-terminal PP-like peptide; s/i/l: small/intermediate/large cells. Adapted from: *Rindi G et al. Pathobiology and classification of digestive endocrine tumors. In: Colombel MMaJ, ed.^, eds. Recent advances in the pathophysiology of inflammatory bowel disease and digestive endocrine tumors. Montrouge-London-Rome: John Libbey Eurotext, 1999; 177–191.*

Diagnosis

The diagnosis of a NET requires a coordinated multidisciplinary approach involving medical oncologists, surgeons, interventional radiologists and pathologists. Results from pathology testing, hormonal testing, and diagnostic and functional imaging need to be integrated to form a comprehensive diagnostic picture.

Imaging

Accurate imaging of NETs is critical to management decisions and should always be tailored to answering relevant clinical questions. These may include suitability for surgery, choice of therapy, response to treatment or evaluation of symptoms.

Computed tomography (CT) constitutes the basic radiological method for primary NET diagnosis, staging, and surveillance after surgery and for therapy monitoring. CT is vastly available and provides fast and detailed contrast enhanced imaging of extended body areas (neck-thorax-abdomen-pelvis). Because of inadequate morphological criteria (short axis measurements) characterization of lymph nodes by CT is difficult and bone metastases are often missed. CT imaging of pancreatic NETs and metastases to the liver and brain is inferior to that of MRI.

Dynamic contrast-enhanced magnetic resonance imaging (MRI) of the liver and pancreas is therefore preferred, for example in the initial staging and for the preoperative imaging work-up. MRI is also preferred for imaging of metastases to brain and bone. MRI is less well suited for examination of extended body areas, because of the comparably longer examination procedure. MRI may miss small lung metastases.

Ultrasonography (US) frequently provides the initial diagnosis of liver metastases and contrast-enhanced US is an excellent method to characterize liver lesions that remain equivocal on CT/MRI.US is the method of choice to guide the biopsy needle

for the histopathological NET diagnosis of abdominal lesions. US cannot visualize lesions in the thorax, brain or bone. CT guided biopsy is therefore used for NET lesions in the thorax and in bone.

Intraoperative US facilitates lesion detection/localization in the pancreas and liver.

Somatostatin receptor imaging by 68Ga-DOTA-somatostatin analog-positron emission tomography (PET)/CT provide high sensitivity for imaging of most types of NET lesions and should always be a part of the tumor staging, preoperative imaging and re-staging.

Somatostatin scintigraphy (SRS) should be performed when PET/CT is not available but is considerably less sensitive.

Bone metastases that are often missed on CT are much better visualized by 68Ga-DOTA-somatostatin analog-PET/CT and lymph node metastases, that are not possible to characterize on CT/MRI, may be diagnosed. Visualization of small peritoneal lesions and primary small-intestinal NETs is facilitated by 68Ga-DOTA-somatostatin analog-PET/CT. 18FDG is better suited for PET/CT of G3 and high G2 NETs, which generally have higher glucose metabolism and less SSTR expression than the low grade NETs. Findings of 18FDG positive NETs at PET/CT indicate worse prognosis [71].

Endoscopy

Upper GI endoscopy is still the gold standard in diagnosing gastro-duodenal NENs. Endoscopic follow up is also recommended following excision, but the correct timing has never been defined. Patients with chronic atrophic gastritis also require careful endoscopic surveillance for apparition of intestinal metaplasia and dysplasia. EUS plays a pivotal role in locoregional evaluation for gastro-duodenal NENs [72].

Direct visualization of small intestine (Si) tumors may be possible with regular colonoscopy if the tumor is prolapsed through the ileocecal valve into the colon, or if intubation of the ileum is performed during the investigation. For investigations of more proximal parts of the ileum or of the jejunum, the newer modalities of enteroscopy including video-capsule endoscopy or double balloon enteroscopy may be effective, although their role in routine staging is still under debate and they are not widely available. Endoscopy is not recommended in the follow up of patients undergoing surgical resection of the tumors [73].

Colonoscopy screening programs are increasingly picking up NENs of the colon and terminal ileum. The incidence rate at screening is 0,17%. Ideally, lesions should be tattooed at the time of removal if thought to be a NET, since further therapy may be needed. EUS is recommended for most rectal NENs except for very small (<5 mm) lesions that have been completely removed where it may not be necessary [74].

Endoscopy is rarely helpful in the diagnosis of appendiceal NETs, unless the tumor is locally advanced and it infiltrates the cecum, which is a very rare situation; thus, colonoscopy for tumor detection is not recommended. In the context of the potentially increased incidence of secondary neoplasms, general recommendation regarding colorectal cancer screening should be followed [75].

The role of EUS in the diagnosis of pancreatic NETs is still under debate. PET/TC with Gallium-labeled somatostatin analogues should be considered as the first line diagnostic imaging method. If not available, SRS/SPECT with EUS and esophagogastroduodenoscopy should be combined, also to allow the execution of biopsies [76].

As for thoracic NENs, if a transthoracic biopsy is not feasible, the diagnosis is carried out with bronchoscopic technique or, less frequently, by mediastinoscopy or endobronchial endoscopic ultrasonography (EBUS).

Treatment

Treatment has to be highly individualised based on the diverse range of tumour burden and symptoms, taking into account the site of the primitive, staging and grading, the presence of SSTRA receptors, the patient's performance status and comorbidities and many other factors.

Surgery is essential in many phases of NET management, and in those with limited disease remains the primary method of cure. Endoscopic tecniques may also be used in some cases for the management of gastroduodenal or colonic NETs.

For patients with advanced disease, cytoreductive surgery is recommended for palliation and increased survival; however, data for these recommendations are not robust and need multicenter prospective assessment. For those with unresectable disease, surgery can obviate bowel obstruction from small-bowel carcinoid fibrosis,and extensive surgery can be done with acceptable morbidity and mortality (range 0–5%). The main limitation of surgery is that more than 80% of patients have liver or lymph-node metastases, or both [77].

Most liver metastases from NETs are hypervascular and embolisation of the hepatic artery by particles or cytotoxic agents effectively generates necrosis. Combination of cytotoxic agents with 10 mL iodised oil can be injected into the branches of the hepatic artery distal to the gastroduodenal artery. Embolisation with gelatine sponge particles or microspheres is used until evidence of a marked decrease in blood flow. Randomised controlled trials that compare the benefits and risks of mechanical embolisation with that by cytotoxic agents are lacking. Contraindications for embolisation are complete portal-vein thrombosis, liver insufficiency, and previous Whipple procedure. Long-lasting complete or partial clinical responses (ie, improved symptoms or performance status) have been noted in about 80% of patients given chemoembolisation. Median time to progression is about 15 months; 5-year survival is about 50%.

Particles or spheres that contain the cytotoxic drug might facilitate an all-in-one procedure. Moreover, radionuclide-labelled microspheres or coupling of embolisation with radioactive somatostatin analogues might improve outcome.

In patients with large tumors (ie, more than >3 cm in diameter), radiofrequency ablation in conjunction with chemoembolisation might be more effective than chemoembolisation alone [78].

Treatment options for advanced or metastatic NENs comprise several agents. However, the best strategy including sequencing is unknown due to the low number of dedicated trials and absence of predictors of response in NENs. Treatment options and sequencing are mostly decided by physician's choice, based on clinical factors, multidisciplinary discussion and patient's preferences.

Peptide-receptor radionuclide therapy has proven to be safe and effective and might become an important treatment strategy for lesions that express adequate densities of somatostatin receptors. GEP NETs overexpressing these receptors, mainly subtype 2, internalize SSTRAs after ligand binding. Therefore, they are targets for cytotoxic drugs coupled to somatostatin. Diagnostic somatostatin receptor imaging (Octreoscan, SRS with indium-111-labelled DTPA0-octreotide ([^{111}In]octreotide) or with 68Gallium PET-CT) can identify tumors that express somatostatin receptors and that are thus suitable for peptide-receptor radionuclide therapy.

Initially, this treatment used high-dose [^{111}In]octreotide. Subsequently, somatostatin peptides with higher receptor affinity were developed and conjugated with the chelator 1,4,7,10 tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), allowing stable labelling with the pure, high-energy β-emitter yttrium-90 or the medium-energy β-emitter lutetium-177 ([^{177}Lu]). Conjugation of octreotide with a chelator can change the affinity profile for particular subtypes of somatostatin receptor. [^{177}Lu]DOTATATE ([^{177}Lu]DOTA-Tyr[3]-octreotate), a selective analogue of

somatostatin receptor 2, has a particularly favourable affinity profile. Its maximum tolerated dose is limited by toxic effects on the kidney and bone marrow [79].

Recently, the NETTER-1 trial showed that treatment with 177Lu-DOTATATE of patients with advanced midgut NETs resulted in markedly longer progression-free survival and a significantly higher response rate than high-dose octreotide LAR among patients with advanced midgut neuroendocrine tumors. The PFS estimated rate at month 20 was 65.2% (95% confidence interval [CI], 50.0 to 76.8) in the 177Lu-Dotatate group and 10.8% (95% CI, 3.5 to 23.0) in the control group. The response rate was 18% in the 177Lu-Dotatate group versus 3% in the control group ($P<0.001$). Preliminary evidence of an overall survival benefit was seen in an interim analysis [80].

The final results of NETTER-1 published in 2021 reported a median OS of 48.0 months in the 177Lu-DOTATATE arm and 36.3 months in the control arm. This difference was not statistically significant, potentially impacted by a high rate (36%) of cross-over of patients in the control arm to radioligand therapy after progression. The NETTER-1 authors concluded that the study demonstrated that 177Lu-DOTATATE yielded a clinically and statistically significant improvement in PFS as well as a clinically meaningful trend towards improvement in median OS of 11.7 months [81].

Systemic therapies established in the management of patients with NETs include somatostatin analogs and alpha-interferon, also referred to as biotherapy. More recently, novel targeted drugs such as the mTOR inhibitor everolimus and the multiple tyrosine kinase inhibitor sunitinib have been introduced in the management of NETs. Other novel targeted agents evaluated in phase 2 trials (bevacizumab, axitinib and pazopanib) remain investigational (pending further validation in phase 3 studies and subsequent licensing) [82].

Somatostatin analogs are indicated to treat symptoms related to peptide hypersecretion in functionally active NETs; this includes distinct clinical syndromes

such as carcinoid syndrome, and syndromes related to duodenal or pancreatic NETs (vipoma, glucagonoma and gastrinoma) and, more rarely, PTH-related peptide-secreting tumors. Malignant somatostatin receptor-2 positive insulinoma may respond to a SSA, however it should be used with caution since hypoglycemia may worsen due to decreased secretion of glucagon [83].

Further, SSA are indicated to inhibit tumor growth in NET, as the PROMID study showed [84]. In this respect octreotide LAR is registered for midgut NET and NET of unknown primary, and lanreotide AG is registered for intestinal and pancreatic NET and NET of unknown primary. In general, somatostatin receptor status should be positive on somatostatin receptor imaging if an SSA is going to be used with antiproliferative intent. Evidence is growing also for the use of non-conventional doses (defined either as increased dose "dose intensity", or shortened interval between administrations "dose density") of SSA as an active and safe option for patients with progressing NETs [85].

Interferon (IFN)-alpha-2b is registered in Europe for the treatment of NETs associated with carcinoid syndrome; it is also used for functionally-active pancreatic NETs (vipoma, glucagonoma, insulinoma) to improve symptoms related to hypersecretion of amines and peptides. In general, it is used as an add-on therapy to SSA in refractory carcinoid syndrome or if SSAs are not the preferred choice (e.g. negative SSTR status) or not tolerated. Uncontrolled and prospective randomized trials have shown activity of IFN similar to that of SSA in GEP NETs. IFN is not registered as an anti-proliferative, but may be considered as an option, particularly in patients with non-pancreatic NET [86]. However, to date, the use of interferon is very rare in clinical practice in Italy.

Everolimus is an inhibitor of the mammalian target of rapamycin (mTOR), an intracellular protein kinase downstream of the phosphatidylinositol 3-kinase/AKT pathway involved in key components of tumorigenesis, including cell growth, proliferation, and angiogenesis. Everolimus is registered for therapy of advanced,

progressive pancreatic NETs and for advanced, progressive G1/G2 non-functional NETs of gastrointestinal or lung origin. Everolimus may improve symptoms from NET-related endocrine hypersecretion; particularly in patients with metastatic insulinomas [87-89].

Sunitinib malate is an oral multi-targeted tyrosine kinase inhibitor of vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), KIT, and RET. It is licensed for patients with progressive, unresectable, locally advanced or metastatic, well-differentiated pancreatic NET based on a placebo-controlled trial [90]; trials are ongoing to evaluate the efficacy of sunitinib in non-pancreatic NET.

Systemic chemotherapy is indicated in progressive or bulky advanced pancreatic NETs and in G3 NENs as per ENETS Guidelines [91,92]. Chemotherapy may be considered in NETs of other sites (lung, thymus, stomach, colon, rectum) under certain conditions (e.g., when Ki-67 is at a high level (upper G2 range), in rapidly progressive disease and/or after failure of other therapies, or if somatostatin receptor imaging is negative).

Streptozocin (STZ)-based chemotherapy has historically been one of the most used treatment options in G1/G2 pancreatic NET, and was preferably recommended in patients with higher tumor burden, with or without associated clinical symptoms, and/or in patients with significant tumor progression within a 6 to 12-months timeframe [93]. In Italy, STZ is not widely available, and other drugs are preferred.

Chemotherapeutic options after failure of STZ-based chemotherapy, or as an alternative if STZ is not available include the following: temozolomide (TMZ) +/- capecitabine, dacarbazine, oxaliplatin combinations with fluoropyrimidines (5-FU or capecitabine) and irinotecan-based therapy. Temozolomide-based chemotherapy is recommended in pancreatic NENs and may be considered in NET G3 and in high risk NET of other primary sites (e.g., pulmonary and ileal NETs) [93].

Overall, temozolomide-based studies have demonstrated objective response rates ranging from 33% to 70%, with the highest response rates reported in studies that combined temozolomide with capecitabine [94-97]. An Eastern Cooperative Oncology Group-sponsored, prospective, randomized, phase 2 trial investigated temozolomide alone versus temozolomide plus capecitabine in 144 patients with progressive G1/G2 pNETs [98]. The combination of temozolomide and capecitabine was associated with a significantly improved PFS (14.4 months in the temozolomide arm vs 22.7 months in the temozolomide/capecitabine arm; HR 0.58) and OS (38 months in the temozolomide arm vs not reached in the temozolomide/capecitabine arm; HR 0.41).

In high grade neuroendocrine carcinomas (NEC G3), chemotherapy is an essential part of the multimodality approach for localized disease and the mainstay of care in advanced or metastatic disease. Platinum-based chemotherapy is generally indicated provided the patient has adequate organ function and performance status. The combination of cisplatin and etoposide, or alternative regimens substituting carboplatin for cisplatin, or irinotecan for etoposide, are recommended as first-line therapy [99,100]. Since response rates of these regimens are lower in patients with Ki-67 in the lower range of G3 (21-55%), other treatment options may be explored in these patients (particularly for G3 NEN of GI origin), although no studies to date have demonstrated improved efficacy of these alternative regimens in this setting.

While 2nd-line and further lines' regimens have not been evaluated rigorously, options include temozolomide-, irinotecan- or oxaliplatin-based schedules as main alternatives.

As for immunotherapy regimens, studies led to approval of immune checkpoint inhibitors for the treatment of small cell lung cancers and of Merkel cell carcinoma. Results in other settings of NEN treatments have been disappointing so far.

Prognosis

Prognosis classification of GEP NENs is based primarily on Ki67 value, and therefore on the distinction between NETs and NENS: while the first usually are characterized by a long survival even in the presence of liver metastasis, the latter have a rapidly deteriorating prognosis due to the aggressiveness of the disease and to the poor response to chemotherapy.

The most relevant negative prognostic factor is staging, in particular the presence of distant metastasis, most frequently to the liver, and secondarily to the lung, peritoneum and bones. The 5-year survival for localized disease is 93% and 20-30% for advanced neoplasms. The great survival difference can be explained by the impact of radical surgery on localized disease.

Primary tumor localization also influences the prognosis: pancreatic NENs have a worse 5-year survival (30-60%) if compared to midgut (54-83%) or rectal NENs (73-81%) [1].

Size of primary, angioinvasion, node metastasis, hepatic tumor burden and positivity to 18FDG PET-CT are risk factors for decreased survival [101-102]. A recent Italian multicenter analysis has demonstrated that prognosis in GI NENs is mostly driven by grading (Ki-67), while tumor burden does not play a significant additional prognostic role. In contrast, in pancreatic NENs, tumor burden seems to be a valid prognosticator. In fact, patients with extensive liver involvement or with bone lesions have a significantly worse survival rate. Thus, these patients should undergo more aggressive therapeutic approaches and intensive follow-up [103].

Interestingly, patients referred to dedicated, high-volume centers for NENs have a better outcome than those in the SEER registry. For example, ileal NENs are reported to have a 54% 5-year overall survival in SEER registry, while it is often superior in specialized centers (56% in UKINETS study, 68% in Spanish registry, 75% in Tampa Single Center, 83% in Berlin and Paris NET centers). Similar data are reported for

pancreatic NETs. This great difference may be related to a more efficient management and a multidisciplinary approach allowing the access to a wider spectrum of treatment (hepatic resections, loco-regional treatment, PRRT, medical therapy and clinical trials) [1].

GEP NECs have a very unfavorable prognosis, even in absence of distant metastasis at the diagnosis. The median survival rate for metastatic disease is 33 months for NET G1-G2, with a 35% 5-year survival, and 5 months in NEC G3 with a 5% 5-year survival [1].

2. CLINICAL STUDY

Abstract

Background. Temozolomide-based treatments have been demonstrated active in advanced NETs of gastro-entero-pancreatic and thoracic origin. Current guidelines suggest the use of TEM in these patients, but treatment selection and sequencing are based solely on clinical parameters. No biomarker is currently available to guide clinical management of NET patients. *MGMT* promoter methylation status has been proven to be a good predictive factor for TEM treatment response in glioblastomas and melanomas; in this setting, its use has been implemented in routine clinical practice. Differently, in NET patients, available evidence on the role of *MGMT* promoter methylation status is scarce; recent studies have reported controversial results and the topic is still under debate. However, studies available to date are all retrospective and including small populations.

Aim. The aim of this study was to prospectively evaluate the role of *MGMT* promoter methylation status in predicting response to TEM-based treatment in patients with advanced gastro-entero-pancreatic and thoracic NETs. Primary endpoint was the correlation of *MGMT* promoter methylation with PFS; secondary endpoint was correlation of this parameter with OS, ORR, DCR; furthermore, we evaluated safety of TEM-based treatment in this cohort. Finally, we conducted an analysis of the costs related to the test.

Material and methods. A single center, prospective observational trial has been conducted at ENETS Center of Excellence Outpatient Clinic of Policlinico Sant'Orsola IRCCS (Bologna, Italy). Patients with advanced, well differentiated NETs of gastro-entero-pancreatic and lung origin candidate to TEM-based treatment (TEM in monotherapy or associated with capecitabine), with tissue available for *MGMT* promoter methylation analysis were enrolled in the trial. *MGMT* promoter methylation status was analyzed by pyrosequencing on tumor tissue from primary tumor or metastases.

Results. Twenty-six patients were enrolled in the study; 4 patients resulted screening failure. The data of 22 patients were finally analyzed. Among these patients, 5 (23%) presented MGMT promoter methylation at pyrosequencing. In the overall population, median PFS was 18 months (95% CI 5-81) while median OS was 23 months (95% CI 6-88). In the *MGMT*-methylated population, median PFS was 34 months [IQR 15-58], compared to 14 [IQR 8-38] in non-methylated patients. Moreover, *MGMT* promoter methylation status was the only independent variable related to PFS. Better outcomes were observed in the *MGMT*-methylated group, also in terms of OS (34 vs 21 months), DCR (100% vs 88%) and ORR (80% vs 24%). TEM-based treatment has been confirmed as a safe treatment, with a low rate of adverse events (G≥3 <10%). The cost of *MGMT* promoter methylation testing by pyrosequencing is very affordable (60 euros per patient) and the test is widely available.

Conclusions. This study has prospectively demonstrated the role of *MGMT* promoter methylation status (tested by pyrosequencing) as a good predictive factor for TEM-based treatment in NET patients. In our cohort, *MGMT* promoter methylation status was the only variable independently related to PFS and identified a subgroup of patients with better response (in terms of PFS, OS, ORR, DCR) to TEM-based treatment. Due to its promising predictive role, the wide availability and low costs of the assay, this biomarker could be implemented in clinical practice to guide treatment selection in this setting. These observations need to be corroborated by a longer follow up and a larger number of patients, to increase the statistical power of the study.

Background and aim

Temozolomide is an oral alkylating agent, showing a good antitumor activity in advanced NETs, when used in monotherapy or in association with capecitabine. Studies have reported ORR ranging from 35 to 70%, with even higher rates in combination therapy studies, in particular with capecitabine, in pancreatic NETS [96,97,104].

On the other hand, TEM treatments efficacy seems to be lower in intestinal NETs, with an ORR of 7% [95].

A recent phase II trial in patients with advanced, progressing well differentiated pancreatic NETs has reported that the association with capecitabine (CAPTEM) led to better outcomes compared to TEM alone (PFS: 14 vs 23 months; OS: 38 months vs not reached. HR 0.41, p=0.012) [98].

Current guidelines (ENETS, AIOM, NANETS, ESMO, NCCN) recommend the use of TEM in monotherapy or in association for the treatment of advanced midgut, thoracic and pancreatic NETs. However, no evidence is available to guide clinicians when choosing treatment sequence and to improve treatment and patient selection.

The cytotoxic activity of TEM is related to DNA alkylation/methylation at the O6 and N7 positions of guanine on the double strand of DNA, resulting in DNA mismatch: altered guanine mispairs with thymine instead of cytosine during DNA replication; futile repair cycles cause DNA replication fork collapse, cell cycle arrest, and ultimately apoptosis [105]. The “suicide” enzyme O6-methylguanine DNA methyltransferase (MGMT) repairs DNA by removing O6-alkylguanine adducts. High levels of MGMT favor the repair of DNA mismatch, counterbalancing TEM effects and causing resistance to treatment. On the contrary, when *MGMT* promoter is silenced through epigenetic modifications, such as methylation of cytosine-phosphate-guanine (CpG) islands located in this region, decreased levels or total absence of MGMT protein can be observed, resulting in a loss of DNA repair activity

and consequently in an enhanced effect of TEM [106]. CpG islands are 200 bp DNA regions rich in CpG dinucleotides (where cytosine is followed by guanine and the two bases are separated by a phosphate group). In most cases, these regions are inside or in close proximity to gene promoters. In these regions cytosine can be methylated by methyltransferase, resulting in gene expression modifications. In MGMT gene promoter, 5 CpG islands are present.

As for the analysis of *MGMT* promoter methylation, different techniques are available. The most commonly used in trials and clinical practice are methylation-specific polymerase chain reaction (a qualitative and semi-quantitative method), pyrosequencing (a quantitative method), and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA, a semi-quantitative method). Taking into consideration prognostic value, cost effectiveness and ease of use, the use of pyrosequencing has been recommended for analyses of *MGMT* promoter methylation [107,108].

In neuro-oncology, MGMT status has been demonstrated to be a good predictor of response to TEM and other alkylating agents for the treatment of glioblastomas, and it is routinely used in clinical practice [109-112]

Differently, in the management of NENs, although several studies have been conducted recently to evaluate the role of MGMT status, evidence is much lower, and the issue is still debated. Indeed, no correlation was observed between MGMT status and TEM response in several studies [113,114].

It must be noted that all the mentioned studies analyzed MGMT expression through immunohistochemistry. However, another study by Raj et al., evaluating MGMT status not only by immunohistochemistry but also with pyrosequencing, did not find a correlation of this marker with TEM response [115].

In three other studies, a predictive value for MGMT status was found only in the sub-group of pancreatic NETs [116-118].

A recent large meta-analysis including 11 studies reported that the proportion of NET patients achieving an ORR when treated with alkylating agent treatment was higher in the MGMT-deficient group than the non-deficient group (OR: 5.00; 95% CI: 3.04–8.22; P <0.001; I 2: 3%). Similar results were noted in the MGMT methylation and MGMT protein expression subgroups. The meta-analysis, in fact, has included studies which evaluated MGMT status with immunohistochemistry, pyrosequencing, or methylation-specific PCR. Therefore, the Authors support the use of MGMT status as a predictive marker [119].

One of the largest retrospective studies on the role of MGMT promoter methylation status was published by our group in 2017 [120]. This multicenter study collected the data of 95 advanced NET patients undergoing TEM-based treatment. *MGMT* methylation was analyzed with two techniques: pyrosequencing and methylation specific-polymerase chain reaction. The results of this study showed that response to treatment, OS and PFS were correlated independently with *MGMT* promoter status. Median PFS was 21 and 8 months for *MGMT* methylated and *MGMT* non-methylated patients, respectively, while median OS was “not reached” in the first group and 23 months in the latter.

A French prospective, multicenter, open label, randomized phase II trial (MGMT-NET; NCT03217097) is currently enrolling NET patients who will be randomized to receive TEM-based or oxaliplatin-based treatments and will be stratified based on *MGMT* methylation. Primary endpoint is ORR at 3 months based on *MGMT* methylation on tumor tissue [121].

Due to the lack of solid evidence, the aim of our study was to prospectively assess the role of *MGMT* promoter methylation status as a predictive biomarker of response to TEM-based treatment in patients with advanced pancreatic gastro-intestinal or thoracic NETs.

The primary endpoint of the study was the correlation of *MGMT* promoter methylation status with progression-free survival (PFS) in advanced NETs of

pancreatic, thoracic or gastro-intestinal origin treated with TEM-based treatment. Secondary endpoints were correlation of *MGMT* promoter methylation status with objective response rate (ORR), disease control rate (DCR), and overall survival (OS). Safety of TEM-based treatments was assessed by monitoring of any adverse events (AEs). Finally, an analysis of the costs correlated with this test was performed.

Material and methods

Study design. A single-center prospective observational study was conducted at the ENETS center of Excellence Outpatient Clinic (head: Prof Davide Campana) at the Oncology Department (Director: Prof Andrea Ardizzoni) of Policlinico Sant'Orsola - IRCCS, Bologna (Italy). All patients with advanced NETs candidate for TEM-based regimens were tested for *MGMT* promoter methylation status before treatment start and were followed-up according to clinical practice. Collected data have been analyzed on October 15th, 2021.

All patients provided written informed consent for treatment and for all the procedures related to the study. This study was approved by local IRB (Comitato Etico Indipendente, Policlinico Sant'Orsola IRCCS, Bologna) and was conducted in accordance with the principles of the Declaration of Helsinki (revision of Edinburgh, 2000).

Study population. All consecutive patients responding to the inclusion criteria referring were included.

Inclusion criteria:

- Age ≥ 18 years
- ECOG-Performance Status: 0-1

- Well differentiated NETs (GEP); typical or atypical carcinoids (thoracic) according to WHO 2019 classification
- Grading 1-2-3 according to WHO 2019 classification
- Primary site: pancreas, gastro-intestinal tract, lung
- Metastatic (stage IV) or locally advanced (stage III) NETs
- Availability of tissue samples for *MGMT* promoter methylation status analysis (formalin-fixed, paraffin-embedded tissue) from biopsy or surgical resection of tumor (primary or metastasis)
- Candidates for TEM-based treatments
- Written informed consent for treatment and study protocol procedures

Exclusion criteria:

- Candidates for radical surgery according to multidisciplinary team evaluation
- Patients unable to provide written informed consent

Data collection. Demographic, clinical, molecular, and pathological data were prospectively collected. A computerized data sheet was created and was updated at each visit.

For each patient the following data were collected: age, gender, date of diagnosis, age at diagnosis, presence of MEN1 syndrome, presence of functioning syndrome, pathological features (tumor primary site, grading, Ki-67 value, WHO 2010 classification and TNM staging according to ENETS), previous treatments (type and time to progression), TEM-based treatment data (regimen, doses, treatment line, start and discontinuation date, reason for discontinuation, cycle number, concomitant medications), adverse events (grading per CTCAE 5.0, correlation with treatment, date of onset and resolution), outcome data (date of progression, death date, best

response and date of best response), molecular data (presence of MGMT methylation). Treatment regimen (TEM in monotherapy or in association with capecitabine) was decided by the investigators.

At baseline evaluation, all patients underwent clinical examination, haematological, liver and kidney function tests, a total body computed tomography scan (CT). Total body CT scans were repeated every 3 months (±1 month) until disease progression according to RECIST 1.1 criteria (unless clinical conditions required shorter intervals). CT scans were performed by a NEN-expert radiologist of the Bologna ENETS Center of Excellence. Patients received treatment until progression or unacceptable toxicity.

MGMT promoter methylation status analysis. The analysis was performed at the Molecular Pathology Laboratory at Policlinico Sant'Orsola IRCCS Bologna.

MGMT promoter methylation status was evaluated using pyrosequencing (PSQ). To be considered fully evaluable, the samples had to contain more than 80% tumor cells. DNA extraction from formalin-fixed, paraffin-embedded tissue (from surgical resection specimen or biopsy of primary tumor or metastases) was performed after deparaffinization using a purification kit (MasterPure DNA, Epicentre, Madison, WI, USA). Genomic DNA was modified by bisulfite conversion (EZ DNA Methylation Gold Kit, Zymo, Irvine, CA, USA).

Pyrosequencing was performed using the PyroMark Q24 CpG MGMT kit (Qiagen, Hilden, Germany) on a PyroMark Q24 System (Qiagen). Data were analyzed and quantified with the PyroMark Q24 Software 2.0.7 (Qiagen). The mean percentage of the five CpG methylated islands detected by the kit was used for analysis. An 8% cut off was used, accordingly to neuro-oncology clinical practice: *MGMT* was considered methylated if methylated alleles were more than not methylated alleles by at least 8%; otherwise *MGMT* was scored as not methylated [122,123].

Study end-points. The primary endpoint of the study was the evaluation of PFS according to *MGMT* promoter methylation status.

Secondary endpoints were the correlations of *MGMT* promoter methylation status and objective response rate (rate of partial response and complete response evaluated per RECIST v.1.1 criteria at CT scans by NEN-expert radiologists), OS, and disease control rate (rate of partial and complete response and stable disease).

Treatment safety has been evaluated through the monitoring of AEs. Another objective of this study was to evaluate the costs of this analysis and its feasibility in clinical practice.

Statistical analysis. Categorical variables were expressed as numbers (percentage), while continuous variables as median and interquartile range [IQR] or mean ± standard deviation (SD), when appropriate. Categorical variables were compared using Pearson's chi square or Fisher exact test, when appropriate. Continuous variables were compared using Mann-Whitney U test or Student t-test. Cox-proportional hazard regression was used to assess odds ratio (OR) and 95% CI of factors related to the primary endpoint, namely PFS, and OS. Kaplan–Meier curves were used to compare PFS and OS and results have been reported as median and 95% confidence intervals. P values < 0.05 were considered statistically significant. MedCalc Statistical Software version 19 (MedCalc Software, Ostend, Belgium; https://www. medcalc.org; 2019) was used.

Results

Study flow-chart. Twenty-six patients meeting inclusion criteria were enrolled in the study. One patient was excluded (screening failure) due to deterioration of clinical conditions before treatment start. Three patients were excluded from the analysis due to inadequate material for *MGMT* promoter methylation status evaluation (insufficient tissue for DNA extraction or technical problems with the assay). Study flow-chart is shown in Figure 3.

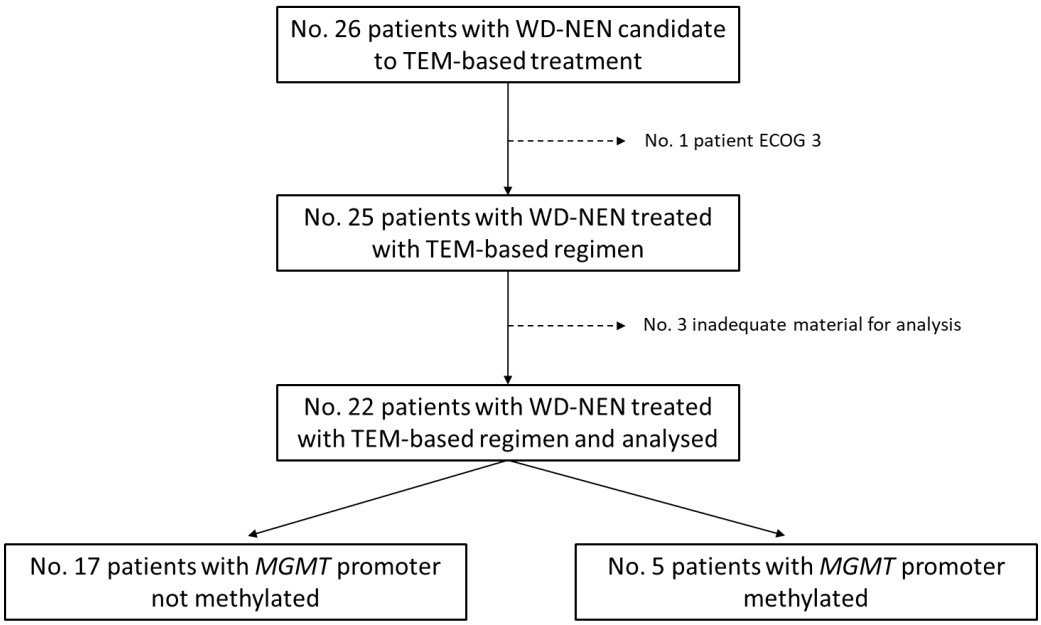


Figure 3. Study flow-chart.

Study population baseline characteristics. Patients' baseline characteristics are described in Table 8. In detail, among the final study population (no. 22), 13 patients were females (59%) and 9 (41%) males; median age at enrollment was 64 (IQR 56-74) years. Eighteen (82%) patients had an ECOG-PS 0; 4 (18%) patients an ECOG-PS of 1. One patient was affected by MEN-1 syndrome and one patient had a functioning NET. Primary site of NET was pancreas in 14 patients (64%), lung in 5 (23%), gastro-intestinal tract in 2 (9%); one patient (4%) had a double primary (pancreas and small bowel). Among the 5 patients with lung NETs, 4 had an atypical carcinoid and 1 a typical carcinoid. Using WHO 2019 classification, grading was 1 in 3 (14%) patients, 2 in 12 (54%), 3 in 7 (32%). Median Ki-67 value was 15% (IQR 8-25). Five patients (23%) presented *MGMT* promoter methylation. All methylated patients had a pancreatic NET.

Table 8. Baseline characteristics of the study population.

Characteristic	Patients
Gender (female), *no. (%)*	13 (59%)
Age (years), *median [IQR]*	64 (56-74)
ECOG PS 0, *no. (%)*	18 (82%)
MEN-1 syndrome, *no. (%)*	1 (5%)
Functioning NET, *no. (%)*	1 (5%)
Primary tumor site	
Pancreas, *no. (%)*	14 (64%)
Lung, *no. (%)*	5 (23%)
Atypical carcinoid, *no.*	4
Typical carcinoid, *no.*	1
Gastro-intestinal, *no. (%)*	2 (9%)
Double site, *no. (%)*	1 (5%)
WHO 2019 grading	
G1, *no. (%)*	3 (14%)
G2, *no. (%)*	12 (54%)
G3, *no. (%)*	7 (32%)
Ki-67 (%), *median [IQR]*	15 [8-25]
MGMT promoter methylation, *no. (%)*	5 (23%)

Abbreviations: no. – number; IQR - interquartile range, ECOG PS – eastern cooperative oncology group performance status; NET – neuroendocrine tumor; WHO – world health organization; MEN – multiple endocrine neoplasia; MGMT – O6-methyl-guanine-DNA methyl-transferase.

TEM-based treatment. Treatment characteristics are reported in Table 9. Treatment regimens used were TEM 180-200 mg/mq (from day 10 to 14, monthly), with or without capecitabine 1500 mg/mq (from day 1 to 14 in two daily doses, monthly).

Eleven (50%) patients received the association of capecitabine and temozolomide (CAPTEM); 11 (50%) patients received TEM in monotherapy. Of the 11 patients undergoing CAPTEM treatment, 2 discontinued capecitabine while continued TEM treatment: one patient for toxicity, while one as maintenance treatment after 42 cycles of CAPTEM.

Patients received TEM-based treatment as first line in 8 (36%) cases, as second line in 5 (23%), and as third or further line in 9 (41%). Median time to progression observed in the previous treatment line was 7.5 months (95% CI 4-15).

Median duration of TEM-based treatment was 13 months (95% CI 9-34). Median number of cycles was 12 (IQR 9-22). TEM-based treatment characteristics are reported in Table 9.

Median follow-up time was 23 (IQR 13-44) months. At the time of data analysis (October 15th, 2021), 11 patients (50%) are still receiving TEM-based treatment, 2 (9%) are on “chemo-break” (discontinuation of treatment during long term response), 3 (14%) discontinued treatment for AEs, 2 (9%) discontinued for PD, while the remaining 4 (18%) died after PD.

Median PFS was 18 months (95% CI 5-81). Median OS was 23 months (95% CI 6-88). Best response was CR in one case (5%), PR in 7 (32%), SD in 12 (55%). Objective response rate, defined as the rate of patients achieving CR or PR as best response, was 36%. Disease control rate, defined as the rate of patients achieving CR, PR or SD as best response was 91%.

Table 9. Treatment characteristics.

	Total (no. 22)
Treatment regimen	
TEM, *no. (%)*	11 (50%)
CAPTEM, *no. (%)*	11 (50%)
Duration (months), *median (95% CI)*	13 (9 – 34)
Cycle number, *median [IQR]*	12 [9-22]
Ongoing treatment, *no. (%)*	11 (50%)
TEM-based treatment line	
First, *no. (%)*	8 (36%)
Second, *no. (%)*	5 (23%)
Third or further, *no. (%)*	9 (41%)
Previous line TTP (months), *median (95% CI)*	7.5 (4 – 15)

Abbreviations: TEM – temozolomide; CAPTEM – capecitabine and temozolomide; no. – number; 95% CI – 95% confidence interval.

Primary outcome analysis: correlation of MGMT promoter methylation status and PFS. Factors related to PFS (tested with Cox proportional-hazards regression analysis) were reported in Table 10. In detail, age, gender, primary tumor site, TEM-based treatment regimen and line, WHO 2019 grading and Ki-67 value were not correlated with PFS. MGMT promoter methylation status was the only variable related to PFS (OR 0.00 [0.00-0.62]; p=0.02).

Table 10. Variables related to progression free survival.

	Univariate analysis (Odd ratio [95% CI)	P
Age (years)	0.97 [0.92 – 1.02]	0.22
Gender (male)	2.13 [0.47 – 9.61]	0.32
Primary tumor site		
Panreatic NET	0.63 [0.11 – 3.60]	0.61
Lung NET	0.77 [0.09 – 6.70]	0.81
Gastro-intestinaI NET	5.98 [0.53 – 67.6]	0.15
TEM-based treatment		
TEM	3.01 [0.54 – 16.7]	0.19
1st line	0.60 [0.12 – 3.14]	0.54
2nd line	1.66 [0.32 – 8.60]	0.53
3rd or further line	3.57 [0.68 – 18.62]	0.13
WHO 2019 Tumor grading		
G1	2.40 [0.40 – 14.3]	0.36
G2	0.42 [0.07 – 2.49]	0.37
G3	0.32 [0.04 – 2.69]	0.29
Ki-67 (%)	0.96 [0.89 – 1.03]	0.25
MGMT promoter status		
MGMT methylation	0.00 [0.00 – 0.62]	0.02

Abbreviations: OR – odd ratio; no. – number; IQR - interquartile range, ECOG PS – eastern cooperative oncology group performance status; NET – neuroendocrine tumor; WHO – world health organization; MEN – multiple endocrine neoplasia; MGMT – O6-methyl-guanine-DNA methyl-transferase; TEM – temozolomide.

Median PFS was 14 (IQR 8-38) months in patients with non-methylated *MGMT* promoter status and 34 (IQR 15-58) months in methylated patients. Kaplan-Meier curve for PFS is shown in Figure 4.

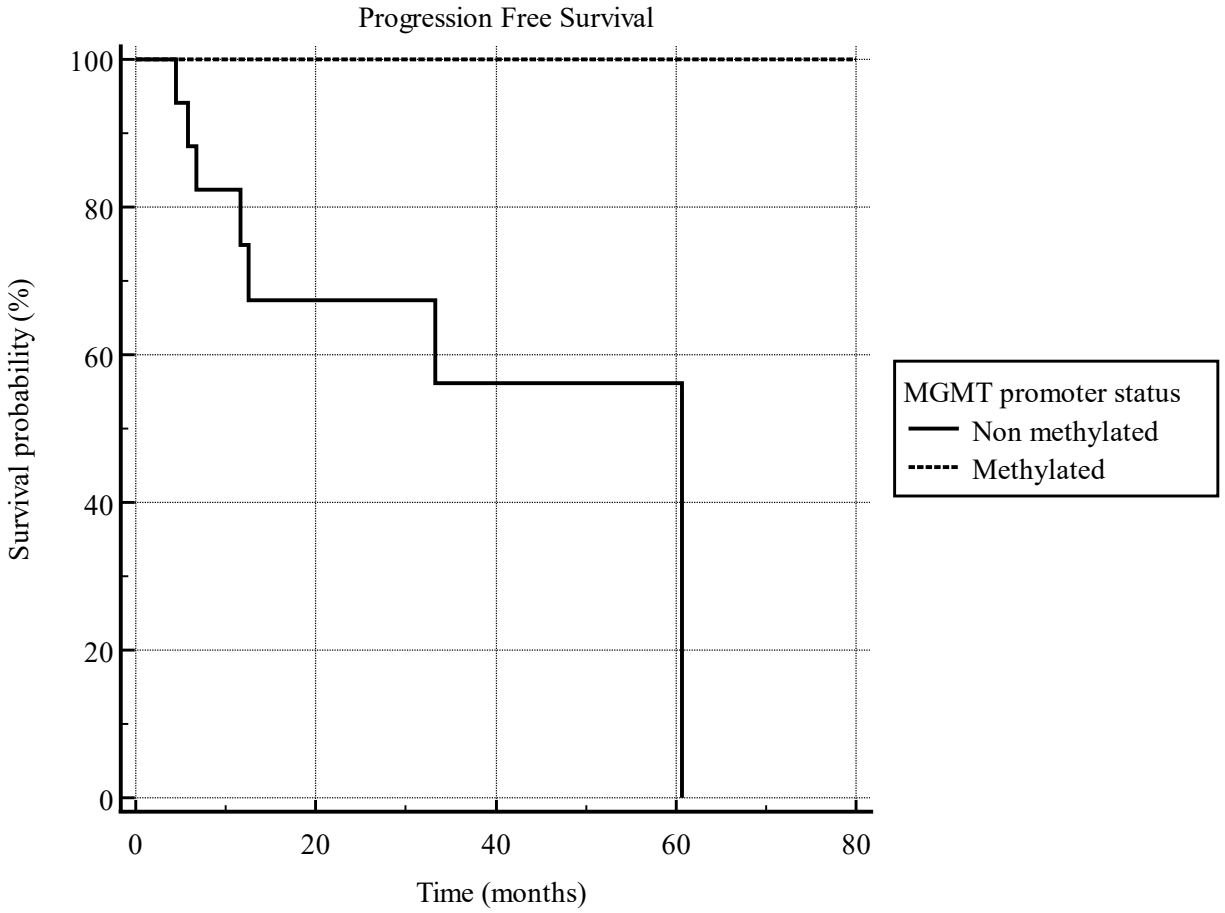


Figure 4. Kaplan-Meier curves for progression free survival according to *MGMT* promoter methylation status.

Progression free survival in patients with pancreatic NET. Since all patients with methylated *MGMT* promoter had pancreatic NET, we conducted a subgroup analysis limited to patients with pancreatic NETs. *MGMT* promoter status was confirmed to be the only variable correlated to PFS in patients with pancreatic NET (OR 0.00 [0.00-0.50]; P=0.03). Kaplan-Meier curve for PFS in patients with pancreatic NET is shown in Figure 5.

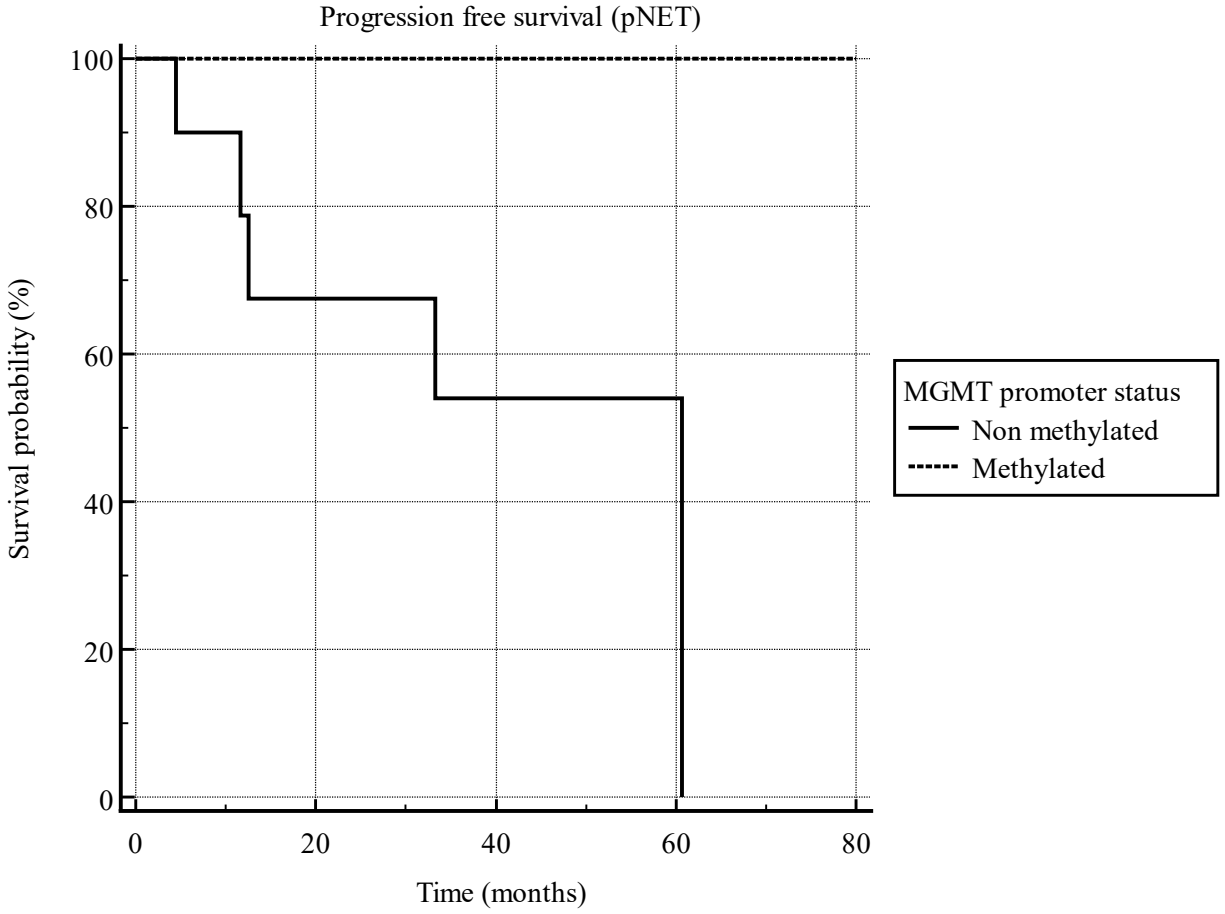


Figure 5. Kaplan-Meier curves for progression free survival in pancreatic NETs according to *MGMT* promoter methylation status.

Overall survival. Median OS was 34 (IQR 15-58) months in patients with methylation of *MGMT* promoter and 21 (IQR 13-44) in the non-methylated group. Age, gender, TEM-based treatment regimen and line, WHO 2019 grading and Ki-67 value *MGMT* promoter methylation status were not correlated with OS on Cox proportional-hazards regression analysis; the only variable significantly correlated with OS was gastro-intestinal primary tumor site (p=0.02). Kaplan-Meier curve for OS is shown in Figure 6.

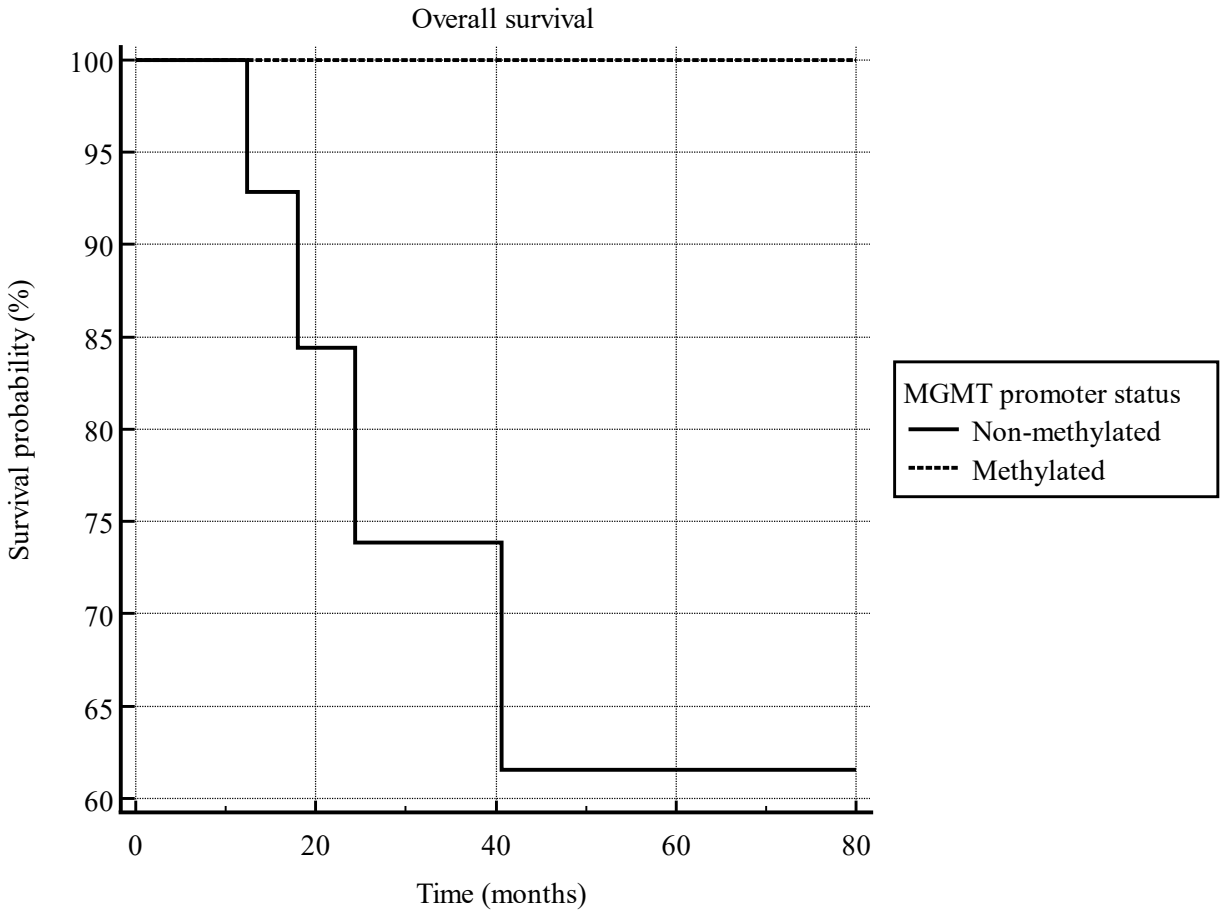


Figure 5. Kaplan-Meier curves for overall survival in NETs according to *MGMT* promoter methylation status.

Overall response rate (ORR) and disease control rate (DCR). In the overall population, ORR was 36%, whereas DCR was 91%.

Among the 5 patients with *MGMT* promoter methylation, best response was CR for one patient, PR for 3, SD for 1. ORR in patients with methylated MGMT promoter was 80%, while DCR was 100%.

Among the 17 non-methylated patients, best response was PR in 4 cases, SD in 11. No patient experienced complete response in this group. Thus, ORR in non-methylated patients was 24%, while DCR was 88%.

Safety. Treatment-related AEs are reported in Table 11. Three patients (14%) discontinued treatment due to adverse events. Among the 3 patients discontinuing TEM treatment for AEs, only one had treatment-related toxicities (G3 nausea and diarrhea), while the other 2 patients stopped treatment for unrelated events.

One patient discontinued capecitabine due to G3 thrombocytopenia, and continued TEM treatment with no further toxicity.

The most commonly reported AEs were fatigue, hematological (anemia, non-febrile neutropenia, thrombocytopenia), and gastro-intestinal toxicity. Overall, G1-2 AEs were reported in 14 patients (64%), while G3 AEs in 2 cases (9%). No G4 AEs were reported.

Table 11. Treatment-related adverse events.

	Total (no. 22)
Fatigue	
G1-G2, *no. (%)*	4 (18%)
G≥3, *no. (%)*	0
Thrombocytopenia	
G1-G2, *no. (%)*	3 (14%)
G≥3, *no. (%)*	1 (5%)
Neutropenia	
G1-G2, *no. (%)*	1 (5%)
G≥3, *no. (%)*	0
Anemia	
G1-G2, *no. (%)*	2 (10%)
G≥3, *no. (%)*	0
Gastro-intestinal events	
G1-G2, *no. (%)*	4 (18%)
G≥3, *no. (%)*	1 (5%)
Total	
G1-G2, *no. (%)*	14 (64%)
G≥3, *no. (%)*	2 (9%)

Abbreviations: no. – number; G – grade as per CTCAE 5.0.

Cost analysis and feasibility. Costs related to MGMT promoter methylation analysis have been evaluated and reported in Table 12. For each patient, three assays had to be performed: one on tumor sample, one on non-neoplastic sample, one on a positive control (a *MGMT* methylated sample). Processing each assay costs around 20 euros, thus the cost for the final analysis of each patient is 60 euros.

Table 12. Costs related to *MGMT* promoter methylation analysis.

Item	Cost (euro)
DNA extraction	5
DNA quantification	3.2
Bisulfite treatment	3.4
PyroMark Q24 MGMT kit	4.4
Pyro PCR kit	1
Consumables and reagents	1.5
Total amount for each sample	18.5
Total amount for each patient	55.5

Discussion and conclusions

The results of our study confirm that *MGMT* promoter methylation status may be considered a good predictive factor of response in NET patients treated with TEM-based treatment. To our knowledge, the present study is the first prospective trial reporting results on the role of *MGMT* promoter methylation status in NET patients receiving TEM/CAPTEM.

In our cohort, *MGMT* promoter methylation resulted to be the only predictive factor for PFS in NET patients treated with TEM-based treatment. In fact, patients with *MGMT* promoter methylation had a median PFS of 34 months, compared to a PFS of 14 months in non-methylated patients. Interestingly, in our cohort, type of regimen (TEM vs CAPTEM), line of treatment, grading and Ki-67 value, were not predictors for PFS. However, these observations could be influenced by the small population.

In our overall population, PFS and OS resulted comparable to outcomes reported in other studies evaluating TEM-based treatment activity [97], while outcomes for *MGMT* methylated patients seems longer than what reported in previous retrospective studies [120]. Moreover, an extended follow-up time is needed to have mature data, since half of our population is still receiving study treatment.

The ORR (36%) and DCR (91%) of our overall population are comparable to data reported by previous studies [94, 97, 120]. Indeed, a very good activity of TEM-based treatment has been observed in terms of ORR and DCR in the *MGMT* methylated patients of our cohort. In the subgroup of methylated patients, in fact, ORR was 80% compared to 24% of non-methylated patients; DCR was 100% in the methylated group compared to 88% in the other group.

These encouraging results, with very good responses to treatment, could support the choice of TEM-based regimens in patients that need a shrinkage of a bulky disease, in case of symptomatic disease, or in patients where surgery does not appear indicated

as upfront approach due to local extension of disease, as a conversion or as a "bridge-to-surgery" strategy.

Another interesting observation is that no patient in the methylated group, after a median follow up time of 23 months, has experienced PD yet. Furthermore, it has to be noted that, in the *MGMT* methylated group, 2 patients have stopped treatment (one for an unrelated AE, the other for "chemo-break" after complete response) and have a stable disease despite treatment discontinuation. Further evidence is needed on the optimal treatment duration of TEM in patients experiencing long term SD or response, and in the management of maintenance treatment or in the application "chemo-break" strategies.

Surprisingly, the study failed to demonstrate a correlation among *MGMT* promoter methylation and OS. We speculate that this endpoint has not been reached due to the long life-expectancy of NET patients even when diagnosed at advanced stages.

In our cohort, we observed a rate of *MGMT* promoter methylation of 23%. Notably, all the methylated patients had a pancreatic NET; in the group of pancreatic NETs, the prevalence of *MGMT* promoter methylation was 35%. Literature reports that, overall, *MGMT* promoter methylation is present in 25–50% of NENs; however, this rate differs depending on primary site. In fact, *MGMT* promoter methylation has been described as a more frequent event in pancreatic NETs (about 50%), whereas in lung and gastro-intestinal NETs the incidence is much lower (0-15%) [117, 118, 124].

This could be one of the reasons for different sensitivity to TEM-based treatment of NENs of different primary sites.

Since among our study population no patient with thoracic or gastro-intestinal NETs had methylated *MGMT* promoter, we acknowledge that the results of our study could be applied only to pancreatic NETs. We are planning to enroll a larger population based on the low methylation prevalence observed in these groups.

In this study we also conducted a cost analysis. The cost of *MGMT* promoter methylation status by pyrosequencing is very affordable (60 euros for each patient); also, this technique is widely available in diagnostic laboratories, is easily reproducible and, very importantly, obtaining the results is very fast. Therefore, this analysis could be offered to most NET patients at diagnosis of metastatic disease or just before starting treatment, thus giving the clinicians further useful information to guide treatment selection and tailoring of therapy sequencing based on a promising biomarker. In fact, in the scenario of NET management, to date sequencing of treatment is based solely on clinical factors and the physicians cannot have the support of any further parameter to decide treatment strategy. The use of *MGMT* promoter methylation status could instead be very useful to help clinical management. Further studies are needed to validate these observations, in particular in the issue of treatment sequencing and other biomarker in NET patients.

One of the main limits of this study is the relatively low number of patients enrolled. Since trial IRB approval, a substantial part of the study has been conducted during the COVID-19 pandemia, which probably has negatively affected the accrual of patients. It has to be kept in mind that NETs are relatively rare tumors and, despite the fact that the coordinating center of the study is listed among the ENETS Center of Excellence, many patients may have not been referred from other centers in this period due to the emergency state and to travel limitations. The low number of patients has affected the statistical power of the study. A time extension of enrollment, and potentially the implementation of a multicenter part of the trial, is currently under evaluation, in order to include more patients and increase the power of the study.

Another limit of this study is the imbalance in the primary sites. In fact, most patients (64%) have pancreatic NETs and all observed *MGMT* methylated patients belong to this group. This issue limits the evaluation of *MGMT* promoter methylation status in patients affected by other primary sites. In order to exclude possible bias due to the imbalance of tumor with different prognosis, we conducted a sub-group analysis

limited to pancreatic NETs patients that confirmed the good predictive value in this homogeneous setting.

Finally, the relatively short follow-up (23 months) could be responsible of some of the unreached goals of the study; in fact, none of the *MGMT* methylated patients presented progressive disease yet. Indeed, these preliminary observations will be corroborated by a longer follow-up.

Further studies should focus on the development of other biomarkers to guide clinical management of NET patients. Another topic to be developed should be the role of liquid biopsy in this population. In fact, this approach could help unravel the molecular and genomic landscape of NETs, allowing the characterization of tumor profile and its changes during the natural history of disease and to tailor treatment based on molecular features.

In conclusion, this study has prospectively demonstrated the role of *MGMT* promoter methylation status evaluation as predictive factor for TEM-based treatment response in patients with advanced NETs. According to these results, *MGMT* promoter methylation status could help identifying a subgroup of patients with better response (in terms of PFS, OS, ORR, DCR) to TEM-based treatment. Due to its good predictive role, the wide availability and low costs of the assay, this biomarker could be implemented in clinical practice to guide treatment selection in this setting, especially in pancreatic NET patients.

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