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COMPREHENSIVE GENOMIC PROFILING OF HIGH GRADE NEUROENDOCRINE GASTROINTESTINAL NEOPLASMS

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ABSTRACT

Background. Gastro-entero-pancreatic (GEP) high-grade neuroendocrine neoplasms (H-NENs) are a heterogenous group of aggressive neoplasms which includes neuroendocrine tumours (NETs) G3 and neuroendocrine carcinomas (NECs). Due to the rarity of these neoplasms, a comprehensive molecular characterization is still lacking.

Aim of the study. The aim of this study is to define the genomic profile of H-NENs (NET G3, NEC <55% ki-67 and NEC ≥55% ki-67).

Material and methods. Genomic characterization of 40 cases of GEP-H-NENs (20 cases of NET G3, 8 of NEC <55% ki-67 and 12 of NEC ≥55% ki-67) was subject to DNA and RNA assay targeting 523 genes by Next Generation sequencing, assessing of all variant types including microsatellite instability (MSI) and Tumour Mutational Burden (TMB) (TrueSight Oncology 500, Illumina).

Results. Based on genomic data, our samples were classified as MSI, chromosomally instable (CIN) and genomically stable (GS). MSI was found in 1/20 (5%) NET G3, 0/8 NEC <55% ki-67 and 2/12 (16%) NEC ≥55% ki-67. CIN was found in 6/20 (30%) NET G3, 5/8 (63%) NEC<55% ki-67 and 6/12 (50%) NEC ≥55% ki-67. 13/20 (65%) NET G3, 3/8 (38%) NEC <55% ki-67 and 4/12 (33%) NEC ≥55% ki-67 were GS. A high TMB was found in 0/20 NET G3, 1/8 (13%) NEC <55% ki-67 and 5/12 (42%) NEC ≥55% ki-67. The most commonly found amplifications comprise: *CDK4/6*, *EGFR*, *FGF10*, *RICTOR*, *MYC* family genes, *MET*. Fusions genes were found in 6/40 (15%) cases and included: *HFM1-ETV1*, *SEL1L-EGFR*, *CNTN5-KMT2A*, *KMT2A-EED*, *BCL2-KCTD*, *FLT1-HUWE1*, *SLC37A1-ERG*.

Conclusions. This study sheds light on the biology of H-NENs. Genomic profiling of H-NENs has shown that NET G3, NEC <55% ki-67 and NEC ≥55% ki-67 have are a heterogenous in their molecular profiles, while sharing share some frequently altered genes. Further genomic analysis are required to identify potential druggable alterations and predictive biomarkers.

INTRODUCTION

1.1 Definition

Gastroenteropancreatic neuroendocrine neoplasms (NENs) are an heterogeneous group of tumours. These neoplasms can occur in most of the organs of the body and throughout the digestive system with a wide range of aetiologies, clinical features, histological findings and prognosis (1).

Historically NENs have been classified according to their anatomical location. In 2010 World Health Organization (WHO) published a classification, which distinguished for the first time neuroendocrine tumours (NET) and neuroendocrine carcinomas (NEC) using Ki-67 proliferative index and mitotic rate as the number of mitoses/2 mm^2 to evaluate proliferative activity (2,3). The WHO classification of 2017 and 2019 divides NEC in two subcategories: NETs G3 - well differentiated - and NECs - poorly differentiated - which both have a Ki-67 labeling index >20%. High grade neuroendocrine neoplasms refers to NETs G3 and to all NECs, which are now considered as two distinct entities (4,5).

1.2 Epidemiology

Gastroenteropancreatic NENs incidence has risen in the last decades, particularly in North America, Asia and Europe, however this increase appears to be more profound in North America (6). According to a retrospective analysis of data collected from the Surveillance, Epidemiology and End Results (SEER) program between 1973 and 2012 in the United States, GEP NETs incidence in the group of data collected from 2000-2012 was 3.56 cases per 100,000. In this cohort NETs G3 incidence was around 0.5 cases per 100,000, the maximum incidence rate was among patients aged >70 (in this group it reached 15-16 cases per 100,000) (7). In Germany, data collected from Joint Cancer Registry showed an incidence of all GEP NENs of 2.5 cases per 100,000 in 2006 (8).

Another study analysed data collected from the Surveillance, Epidemiology and End Results (SEER) program between 1975 and 2012 among the population of the U.S.

According to the results, 5509 cases of gastrointestinal NECs were identified. The median age at diagnosis was 68 years. The most affected site was lower gastrointestinal tract, which account for 41%, followed by upper gastrointestinal tract that was the primary site in 23% of cases and the pancreas, which was the primary site in 20% of cases. The incidence increased from 1.5 cases per 1,000,000 in 1973 to 4,6 cases per 1,000,000 in 2012 (9).

The most prevalent primary site of GEP NETs varies among the countries: in North America small intestinal and colorectal NETs are preponderant, in Asia predominate rectal, gastric and pancreatic NETs, while in Europe small intestinal and pancreatic NETs have the highest incidence. NECs real epidemiology is partially unknown due to disease rarity and lack of dedicated analysis (6). Additionally, due to the change of the WHO classifications, it is possible that data collected before 2017 can overrepresent NECs, considering tumours that were NETs G3 as NECs.

Gender differences in the epidemiology of NENs need further investigation. Appendiceal NENs are slightly more frequent in females. Gastric NEC are more frequent in males, while type 1 ECL-cells NETs of the stomach, that are more often high grade (G3) are more frequent between females (5). A recent study that analysed the characteristics of PanNENs from a gender perspective found that females were affected in 54.15% cases and that PanNENs were diagnosed at younger age in females compared to males. In the female group, the presence of type 2 diabetes mellitus (T2DM) was significantly associated with a more advanced disease (10).

1.3 Risk factors

Risk factors for the development of sporadic GEP NENs haven't still been well defined. A large meta-analysis identified some potential risk factors for pancreas, small intestine and rectum NENs.

A first-degree family history of any cancer, obesity, T2DM, heavy alcohol consumption and cigarette smoking were all risk factors for pancreatic NENs, the meta-analysis provided a summary estimated effect (OR) for T2DM of 2.76 and of 2.44 for heavy drinking. For pancreatic NENs the recent onset of T2DM was also important in the evaluation of the risk and OR was 12.80 (11). The same associations have been recently

investigated, highlighting the role of history of cancer and T2DM in the development of pancreatic NENs (12).

This meta-analysis investigated also potential risk factors for small intestinal NENs: an elevated risk was found among individuals with a family history of any cancer and particularly colorectal cancer, also tobacco smoking has a role in the development of small intestinal NENs and for smokers the OR was 1.59 compared with never-smokers. Alcohol consumption, high BMI and T2DM roles seemed to be not significative (11). Recent cholecystectomy was associated with an increased risk – OR 1.78 - in two case-control studies conducted in the USA using SEER data analysis also a history of gallstones or gallstone surgery have a significant association with NENs in small intestine (13,14).

Rectal NENs are more common among tobacco smokers and heavy drinkers, OR were 1.20 and 1.53 respectively. The meta-analysis didn't reported significative associations between NENs in general and T2DM and BMI (11).

Another retrospective case-control study collected data from 148 Italian patients with GEP NENs: for intestinal NENs identified risk factors were obesity and a family history of non-neuroendocrine GEP neoplasms, for pancreatic NENs risk factors were type 2 diabetes mellitus (T2DM) and obesity. Diabetes was also correlated with a more advanced and progressive disease, additionally among diabetic patients a protective role of metformin was suggested (15).

1.4 Pathogenesis

Gastroenteropancreatic NENs arise from cells of diffuse neuroendocrine system of the pancreas and gastrointestinal tract. The term "neuroendocrine cells" refers to endocrine cells producing polypeptides with hormonal significance and/or biogenic amines released in the blood flow with a specific effect on target organs (16). These neoplasms show a large heterogeneity in morphology, clinical signs, behaviour and location that reflects their genetic variability. In the last years there have been great improvement in the comprehension of the genetic pathways involved in NENs, but we are still far from building a common model for their tumorigenesis. While *p53* is a frequent alteration in many cancers, it is rarely mutated in NETs (17). However, it is commonly acknowledged

the central role in the tumorigenesis of p53/Akt imbalance and in the downstream substrates, like PHLDA3 (17). A cooperative tumorigenic effect is supported by many studies: *p53* is altered in a little percentage of PanNETs, but it is
a common finding in association with *RB1* alteration in PanNECs (18,19).

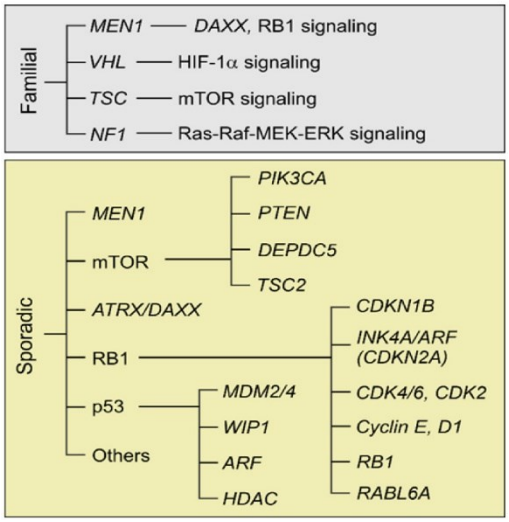

Figure 1. Schematic of frequently altered genes and pathways in familial and sporadic PanNETs (20).

For what concerns PanNENs the most of them are sporadic, but some occur in multi-tumour predisposition syndromes like: multiple endocrine neoplasia type 1 (MEN1), Von-Hippel- Lindau (VHL), neurofibromatosis type 1 (NF-1), tuberous sclerosis complex (TSC), and Cowden syndrome (CS). The origin of sporadic PanNENs is uncertain: some authors believe they arise from stem cells, while others hypothesize a dedifferentiation starting from tumoral islet cells. Frequent genetic alterations in sporadic PanNENs involve *MEN1*, mTOR pathway, *ATRX/DAXX*, *p53, RB1*, the last two exclusively in PanNECs, suggesting different origins of NETs and NECs. It has been also found that virtually all NECs have alterations in RB1/p16 pathways (20). Other genes involved in the genesis of PanNECs are *MYC* oncogene family members: in zebrafish targeted expression of *MYCN* in pancreatic β-cells induces PanNECs. Sonic hedgehog signalling is also altered in NETs and NECs (21). The development of NENs includes also molecules such as cytoskeleton and scaffold proteins, whose mutation modifies dopamine and somatostatin receptors' functionality. A central role is played by genes for histone modification, chromatin remodelling and telomere maintenance like *MEN1*, *ATRX/DAXX*, *SETD2*, *ARID1A*, and *MLL3*, in addition an aberrant methylation in PanNENs has been commonly found (20).

Following steps for invasion and metastasis include expression of VEGF and proinflammatory cytokines that help GEP NENs growth. High IHC expression of TNF-α correlated with a high Ki-67 index and death outcomes. IHC for IL-6, IL-1β, and IL-2 displayed different patterns regardless of Ki-67, most GEP-NENs had high levels of IL-6 and lower levels of IL-1β and IL-2 (22).

1.5 Classification

Classification of NENs has changed over years.

The first WHO classification of NENs was made in 2010, it was based on grading and site. Before this time NENs were classified by tumour diameter and stage as: well-differentiated endocrine tumour benign (WDETB), well-differentiated endocrine tumour with uncertain behaviour (WDETUB), well-differentiated endocrine carcinoma (WDEC), poorly differentiated endocrine carcinoma (PDEC). This classification wasn't widely accepted, because was stage-related and included the category "uncertain behaviour".

WHO 2010 classification divided GEP NENs into three groups: well differentiated neuroendocrine tumours (NETs) grade 1 (G1) with a mitotic count <2/10 high-power field (HPF) and/or ≤2% Ki-67 index or grade 2 (G2) with a mitotic count 2-20/10 HPF and/or 3%-20% Ki-67 index and poorly differentiated neuroendocrine carcinomas (NEC) grade 3 (G3) with a mitotic count >20/10 HPF and/or >20% Ki-67 index (2). It was also still used the term "carcinoid" for NETs G1, that is now outdated.

Terminology	Differentiation	Grade	Mitotic rate[a] (mitoses/2 mm²)	Ki-67 index[a]
NET, G1	Well differentiated	Low	< 2	< 3%
NET, G2		Intermediate	2–20	3–20%
NET, G3		High	> 20	> 20%
NEC, small cell type (SCNEC)	Poorly differentiated	High[b]	> 20	> 20%
NEC, large cell type (LCNEC)			> 20	> 20%
MiNEN	Well or poorly differentiated[c]	Variable[c]	Variable[c]	Variable[c]

Table 1. Classification and grading criteria for neuroendocrine neoplasms (NENs) of the GI tract and hepatopancreatobiliary organs (1).

The current classification was published in 2019 by WHO, it is based on 2017 WHO classification of neoplasms of endocrine organs, specifically it resumes the criteria for the classification of pancreatic NENs. The main change is the introduction of the distinction between well-differentiated neuroendocrine neoplasms grade 3 (G3) and poorly-differentiated neuroendocrine carcinomas (NEC), which are by definition high grade. It

is now clear that the two categories NETs and NECs are two independent neoplasms, with a different origin, even if they share the same neuroendocrine markers chromogranin A and synaptophysin. The main distinction between NET and NEC is morphological: NET have an organoid architecture with nests, cords and ribbons, uniform nuclear features, minimal necrosis, chromatin coarsely stippled, NEC have a less nested architecture, often growing in sheets and abundant necrosis. NECs are also subtyped as small cell NECs (SCNEC), with tightly packed fusiform nuclei with finely granular chromatin and large cell NECs (LCNEC), with rounded atypical nuclei, sometimes with prominent nucleoli. Grade progression is typical of well-differentiated neoplasms (NETs), while poorly-differentiated neoplasms (NECs) arise from a preneoplastic lesions that are usually precursors of non-neuroendocrine carcinomas of the respective organs, like adenomas in the colorectum or squamous dysplasia in the oesophagus. NETs can be defined also by their hormonal functionality, which causes clinical syndromes: glucagonoma, gastrinoma, insulinoma, non-functioning NETs may also produce hormones that can be detected in the serum or in the tumour cells using immunohistochemistry (4,5).

Since NET G3 and NEC differ in clinical behaviour, prognosis and therapies responses, distinguishing between the two entities is a central question for the clinicians. Genomic analysis have provided data supporting the genomic distinction between NEC and NET G3. Mixed neuroendocrine-non-neuroendocrine neoplasms (MiNENs) contain both neuroendocrine and non-neuroendocrine cells that account ≥30% of the neoplasm, the neuroendocrine part is almost always poorly-differentiated. Each NEN's peculiarities are influenced by the primary location and this explains the need for a site-specific classification (5).

Gastric NENs are classified as follows: well-differentiated neuroendocrine tumours (NETs), poorly-differentiated neuroendocrine carcinomas (NECs) and mixed neuroendocrine-non-neuroendocrine neoplasms (MiNENs). NETs are divided into subcategories according to their origin and their secretory activity. Histamine-producing enterochromaffin-like-cell NETs arise from ECL cells. Type 1 ECL-cell NETs are associated with hypergastrinemia due to chronic atrophic gastritis, achlorhydria, hypergastrinemia due to other causes or macrocytic anaemia and they account for about 80% of ECL-cell NETs. Type 2 ECL-cell NETs are associated to hypergastrinemia due to duodenal or pancreatic gastrinoma in multiple endocrine neoplasia type 1 (MEN1) and

hypertrophic hypersecretory gastropathy, they are only the 5-7% of ECL-like NETs. Type 3 ECL-cell NETs arise from normal oxyntic mucosa and they are 10-15% of gastric ECL-cell NETs. High-grade (G3) ECL-cell NETs are usually type 3, exceptional cases are type 2 ECL-cells NETs. Serotonin-producing enterochromaffin-cell NETs are rare and usually non-functioning. Gastrin-producing G-cell NETs and somatostatin-producing G-cell NETs are very rare. Their location depends on their subtype: enterochromaffin-like-cells NETs arise in the corpus/fundus, D-cell and G-cell NETs in the antrum, enterochromaffin cell NETs in the antrum and corpus/fundus. NECs and MiNENs can arise in any part of the stomach, more often in the antrum or cardiac region. NEC - divided into large cell neuroendocrine carcinoma (LCNEC) and small cell neuroendocrine carcinoma (SCNEC) - and MiNENs account for 21% of gastric NENs.

Neuroendocrine neoplasms of the small intestine and ampulla are classified as duodenal, jejunal and ileal neoplasm. These neoplasms can be well-differentiated tumours (NET) grade 1 (G1), grade 2 (G2), grade 3 (G3) or poorly-differentiated neuroendocrine carcinoma (NEC) divided into large cell neuroendocrine carcinoma (LCNEC) and small cell neuroendocrine carcinoma (SCNEC). Other endocrine tumours are: gastrinoma NOS, somatostatinoma NOS, enterochromaffin-cell carcinoid, extra-adrenal paraganglioma NOS. The majority of NETs of the duodenum are located in the first or second part, jejunoileal NETs are mostly in the distal part of ileum, NECs of the small intestine are located in the ampullary region, while jejunoileal NECs are considered exceptional.

Appendiceal neuroendocrine neoplasms are classified as: neuroendocrine tumour grade 1, grade 2 and grade 3, L-cell tumour, glucagon-like peptide-producing tumour, PP/PPY producing tumour, enterochromaffin-cell carcinoid, serotonin-producing carcinoid, large cell neuroendocrine carcinoma (LCNEC) and small cell neuroendocrine carcinoma (SCNEC), there can also be mixed endocrine-non-neuroendocrine neoplasms (MiNENs).

Neuroendocrine neoplasms of the colon and rectum include well-differentiated neuroendocrine neoplasms divided into neuroendocrine tumour grade 1, grade 2 and grade 3, L-cell tumour, glucagon-like peptide-producing tumour, PP/PPY- producing tumour, enterochromaffin-cell carcinoid, serotonin-producing tumour, large cell neuroendocrine carcinoma (LCNEC) and small cell neuroendocrine carcinoma (SCNEC).

In the anal canal neuroendocrine neoplasms are divided into neuroendocrine tumour grade1, grade 2 and grade 3, small cell neuroendocrine carcinoma (SCNEC) which

account for more than one third of all NENs in the anal canal and large cell neuroendocrine carcinoma (LCNEC), that are more frequent than NETs in the anal canal. Classification of pancreatic NENs has been a model for all other organ-specific classifications of NENs. PanNETs are well-differentiated neoplasms of low (G1), intermediate (G2) or high grade (G3) that in cases associated with an hormonal syndrome are subtyped in insulinoma, glucagonoma, somatostatinoma, gastrinoma, VIPoma, serotonin-producing tumour, enterochromaffin-cell carcinoid, ACTH-producing tumour. Non-functioning PanNETs are well differentiated neuroendocrine neoplasms that don't cause an hormonal syndrome, they are divided into neuroendocrine tumours (NET) grade 1, grade 2 and grade 3, oncocytic neuroendocrine tumour non-functioning pancreatic, pleomorphic neuroendocrine tumour non-functioning pancreatic, clear cell neuroendocrine tumour non-functioning pancreatic, cystic neuroendocrine tumour non-functioning pancreatic. PanNECs are divided into large cell neuroendocrine carcinomas (LCNEC) and small cell neuroendocrine carcinomas (SCNEC), also pancreas can have MiNENs.

1.6 Clinical presentation

Clinical presentation of NENs can be associated to the hormonal function of the neoplasm or to the primary location.

Oesophageal NENs have as common presenting symptoms: dysphagia, pain, weight loss, asthenia, melaena, while some NETs are discovered accidentally. Metastatic NETs may determine a carcinoid syndrome due to biologically active amines and peptides entering the systemic circulation and escaping the first-pass metabolism of the liver that usually inactivates them (5). In the cases of neuroendocrine tumours with liver metastasis, either these bioactive products directly enter into the systemic circulation, or they are not inactivated due to deranged liver function. Carcinoid syndrome is characterized by vasodilatory effects: flushing, wheezing, diarrhoea, malabsorption, pellagra (secondary to niacin malabsorption), cardiac symptoms (usually tricuspid regurgitation), fatigue and rarely cognitive impairment (23). NECs of the oesophagus can also cause paraneoplastic syndromes. On endoscopy NETs appear as polypoid or nodular submucosal masses, while NECs are large, infiltrative and ulcerated (5).

Gastric NENs are usually asymptomatic. ECL-cell NETs type 1 and 2 are associated with hypergastrinemia and atrophic chronic gastritis, while ECL-cell NETs type 3 arise in a normal mucosa, they can be associated with gastritis or other non-specific symptoms due to tumour growth or metastatic dissemination like melaena, pain and weight loss. NENs with liver metastasis can cause an atypical carcinoid syndrome with flushing, facial oedema, lacrimation, headache, bronchoconstriction (5). Serotonin-producing NETs are usually non-functioning or can cause a typical carcinoid syndrome. Gastrin-producing G-cell NETs are usually non-functioning, but can cause a Zollinger-Ellison syndrome characterized by severe peptic ulcer disease, gastroesophageal reflux disease and chronic diarrhoea (24). Gastric NECs and MiNENs can cause non-specific symptoms including dyspepsia and weight loss due to tumour growth or metastases. Ulcerated neoplasms can cause gastric bleeding, anaemia, pain, obstruction in the case of large pyloric lesions.

NENs of the small intestine and ampulla are usually asymptomatic, large neoplasms can cause jaundice and intestinal obstruction. Jejunal and ileal NETs can cause intermittent abdominal pain due to intermittent obstruction or ischaemia. Ampullary NENs can lead to obstructive jaundice and rarely to acute pancreatitis. Occasionally NETs of the small intestine or ampullary region can be functioning causing a syndrome related to their hormonal production. Duodenal NETs are clinically asymptomatic and detected accidentally by endoscopy, while gastrinomas lead to Zollinger-Ellison syndrome, but the symptoms can be treated by proton-pump inhibitor therapy. Somatostatinoma syndrome is characterized by: diabetes mellitus, diarrhoea, steatorrhea, hypochlorhydria or achlorhydria, anaemia, gallstones and it is very rare. Carcinoid syndrome can occur if liver metastases are present, that happens in 10% of patients (5).

For what concerns appendiceal NENs: NETs are usually asymptomatic, they are usually found accidentally after surgery, NECs can lead to symptoms similar to an appendiceal carcinoma which are: acute abdomen and non-specific abdominal pain (25).

Colorectal NETs are usually asymptomatic or they are associated with mass-related symptoms, bleeding, abdominal pain. Some cases have serotonin-producing EC-cells and NENs with liver metastases give rise to a typical carcinoid syndrome (5).

Neuroendocrine neoplasms of the anal canal can present with anal pain, discomfort, anal bleeding and the obstruction of the canal may lead to constipation. LCNECs and NETs

are usually early-staged at the diagnosis, while SCNECs are metastatic. Common sites of metastasis are: liver, lung and bone (5).

PanNENs can be functioning or non-functioning, the functioning ones can give rise to syndromes related to their hormonal production, while the non-functioning ones are discovered accidentally or become clinically visible because of their large size, invasion of adjacent organs or metastasis. Metastasis of PanNENs occur usually to regional lymph nodes and liver and to lung and bone. For the functioning ones the syndromes are various. Insulinomas are the most common functioning PanNENs, hyperinsulinaemic hypoglycaemia leads to autonomic and neuroglycopenic symptoms: palpitations, tremor, sweating, hunger and/or paraesthesia, severe weakness and psychiatric/neurological manifestations. There can also be confusion, agitation, slow reaction pattern, blurred vision, seizures, transient loss of consciousness, hypoglycaemic coma. Gastrinomas are defined by typical Zollinger-Ellison syndrome that includes: duodenal ulcer and/or gastro-oesophageal reflux disease caused by the hypersecretion of acid associated with elevated fasted serum gastrin, these type of neoplasms can also lead to EC-cells hyperplasia and fundic neuroendocrine tumours. VIPoma is characterised by Watery Diarrhea Hypokalemia Achlorhydria (WDHA) syndrome, which entails: large-volume secretory diarrhoea persisting with fasting, achlorhydria, hypokaliemia, acidosis. Other symptoms are: weight loss, flushing, hypercalcemia, glucose intolerance. Glucagonoma gives rise to the typical triad: skin rash, diabetes mellitus and weight loss. Skin lesions are due to a necrolytic migratory erythema located in the groin which migrates to the limbs, buttocks and peritoneum. Some patients present also angular stomatitis, cheilitis or atrophic glossitis. Other symptoms are amino acid deficiency, normochromic normocytic anaemia, widespread venous thrombosis with pulmonary embolism. Metastasis are often present at the diagnosis and affect the liver, lymph nodes, mesentery/peritoneum, bone, lung and spleen. Somatostatinoma syndrome is characterized by: diabetes/glucose intolerance, cholelithiasis, diarrhoea/steatorrhea, usually patients have non-specific symptoms that are absent at the presentation. Some patients have decreased gastric secretion and gastric hypochlorhydria. ACTH-producing NETs are rare and usually low-grade, their main manifestation is Cushing syndrome, sometimes they are preceded by Zollinger-Ellison syndrome or by insulinoma syndrome. Patients with serotonin-producing PanNETs present with carcinoid syndrome only in case

of liver metastasis, usually they have an atypical carcinoid syndrome with: abdominal pain, diarrhoea, weight loss, flushing. PanNECs' presentation is similar to that of pancreatic ductal adenocarcinoma: back pain, jaundice, nonspecific abdominal symptoms. The majority of patients presents with metastases at the diagnosis (5).

1.7 Diagnosis

The diagnosis of NENs can be suspected on the basis of clinical symptoms and general or specific biochemical markers. Morphological and/or functional imaging is crucial for the location of the neoplasm, while histopathological examination is essential for classification, staging and for the final diagnosis.

Clinical symptoms, as previously explained, can be specific or not, they can be related to hormonal syndromes in case of functioning neoplasm, or to tumour's size or metastases.

Biochemical markers are a useful tool for the detection of NENs. General markers are shared by all NENs. One of these is chromogranin A (CgA), that is released by neuroendocrine cells with their secretory products, its circulating levels are elevated in all NENs and correlated with the size of the tumour and with the prognosis of small intestine NENs (26,27). False-positive results affecting the specificity, which is the main weakness of the test, can be due to benign conditions, iatrogenic causes and oncological causes (28). Among the benign conditions there are gastrointestinal disorders, cardiovascular diseases, renal and hepatic dysfunctions and rheumatoid diseases. Some of these are: chronic atrophic gastritis, Helicobacter pylori infection, liver cirrhosis and chronic hepatitis, pancreatitis, inflammatory bowel diseases, irritable bowel syndrome, hypertension, heart failure, acute coronary syndromes, giant cell arteritis, rheumatoid arthritis, systemic lupus erythematosus, pulmonary obstructive disease and hyperthyroidism. Iatrogenic causes of CgA elevation are: PPI and other acid-blocking drugs, which induce G-cells and then EC-cells hyperplasia, responsible for CgA overproduction, histamine 2 receptor antagonists and serotonin reuptake inhibitors. Also other types of tumours can induce CgA elevation, these are: colorectal, gastric, ovarian, breast, prostate, pancreatic, hepatocellular carcinomas. There can also be false negative results, these are mainly associated to the disease extent and function (28). For this reason circulating CgA represents an acceptable marker for advanced functional NENs. Neuron-

specific enolase (NSE) is another general marker that can be evaluated for the diagnosis of NENs. It is a cell-specific isoenzyme of the glycolysis and a gluconeogenesis enzyme. NSE levels aren't dependent on tumour secretory activity and are more useful in low-differentiated NENs. Since NSE can be found also in erythrocytes, false positive results can be due to haemolysis (29). In case of suspected carcinoid syndrome, 5-hydroxyindoleacetic acid (5-HIAA) can be evaluated in 24 h urine collection. 5-HIAA has an acceptable reliability when its values are at least twice the upper cut-off, this because there are many conditions that can lead to its elevation. For example the consumption of pineapples, bananas, eggplant, the common walnut, paracetamol, caffeine, and naproxen can lead to false positive results, while acetylsalicylic acid, adrenocorticotropin, levodopa and phenothiazine derivatives, can cause false negative results (26). Specific tests for Zollinger- Ellison syndrome in gastrinomas are the evaluation of the levels of gastrina and secretin suppression test, which is considered positive for gastrinoma when the levels of gastrina rise after the administration of secretin (26). Insulinoma has a specific diagnostic triad, that is the Whipple triad: hypoglycaemic symptoms, plasma glucose levels <40 mg/dL and symptom relief after glucose administration. The best diagnostic test is prolonged fasting (48-72 hours) with measurement of serum insulin, blood-glucose, C-peptide and proinsulin (5). Specific hormones are used in the suspicious of VIPpoma, somatostatinoma, glucagonoma and other functioning NENs.

Imaging diagnostic has a key role in the detection of NENs, it can be morphological or functional and includes radiological, endoscopic and nuclear methods. Morphological imaging is represented by abdominal ultrasound (US), contrast-enhanced computed tomography (CECT), and magnetic resonance imaging (MRI) (30). The most widely used imaging is CECT: gastrointestinal NENs may appear as a hypervascular nodular swelling in the intestinal wall or as a localized thickening of the wall, the edges can be smooth and regular or jagged and irregular. Other manifestations detectable at CECT are the invasion of adjacent organs, omental/peritoneal involvement, metastases (lymph nodes and liver) and ascites. US an MRI with contrast are radiation-free examinations and they both have an excellent soft tissues contrast (30). Endoscopic methods like colonoscopy and gastroscopy can be also used according to the location of the tumour. Somatostatin receptor nuclear imaging can help for the diagnosis and staging of NENs and also for the

choice of the best therapeutic approach. Scintigraphy with Indium-111-pentetreotide (Octreoscan) has been recently replaced by PET/TC with 68Ga-labelled-somatostatin analogues (68Ga-DOTA-peptides), which has higher spatial resolution, higher affinity for tumours with moderate SSR expression (31). PET with F-18-tagged glucose, even if isn't a standard for the diagnosis of NENs, can be used in addition to traditional diagnostic imaging to detect low-differentiated lesions. Also FDG uptake correlates with poorer outcomes (31).

Histopathological examination is used for the final diagnosis, additional immunohistochemical markers are used for the diagnosis and the classification of the disease. All NENs show immunohistochemical features of neuroendocrine differentiation like expression of INSM1, synaptophysin, chromogranin, and somatostatin receptors (SSTRs), but also include transcription factors that can identify the site of origin of a metastatic lesion of unknown primary site, as well as hormones, enzymes, and keratins that play a role in functional and structural correlation (32). Particularly G3 NETs exhibit diffuse immunoreactivity for synaptophysin, chromogranin, and INSM1. Functioning G3 NETs also generally demonstrate expression of the hormone responsible for the clinical syndrome. NECs usually exhibit diffuse INSM1 and synaptophysin staining but only focal or dot-like chromogranin A staining and no hormones. Both G3 NETs both NECs show immunoreactivity for cytokeratins, like CAM 5.2 and AE1/AE3, but they don't express high molecular weight cytokeratins. On the basis of their degree of differentiation, NENs can be classified in well-differentiated NETs and poorly-differentiated NECs. Well- differentiated NETs are composed of uniform, round to polygonal cells that grow in nests, acini, ribbons, and trabeculae, some have abundant fibrous stroma, but the majority have fibrovascular stroma with scant fibrous tissue and prominent vessels, often they have monotonous round nuclei with finely stippled chromatin that has a "salt-and-pepper" appearance (30,33). NETs are graded G1,G2 and G3 according to the mitotic rate and/ or Ki-67 labeling index. Mitotic rate is evaluated as number of mitoses/mm^2 that is 10 high-power fields at 40x magnification and an ocular field of 0.5 mm, while Ki-67 is determined counting at least 500 cells in the region of highest labelling. When the two indicators suggest different grades, the highest is assigned (5). Poorly-differentiated NECs have a less nested architecture, often growing in sheets and abundant necrosis, showing a geographic pattern. NECs are by definition

high-grade, they are subtyped as small cell NECs (SCNEC) and large cell NECs (LCNECs). Neoplasms that show neuroendocrine and non-neuroendocrine components are classified as MiNENs if the neuroendocrine component is ≥30%, otherwise the presence of neuroendocrine component <30% may be mentioned, but doesn't affect the categorization (5).

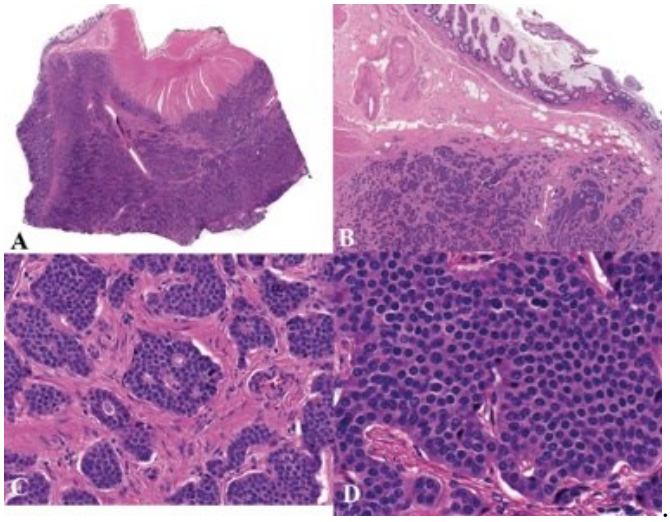

Figure 2. Ileal NET G1. A and B : low and medium power view, the tumour displays architectural patterns of cords, ribbons and nests. C and D: high power view, neoplastic cells with round to oval nuclei containing coarsely clumped, salt and pepper chromatin.. Magnifications: A x2, B x10,C and D x100 (33).

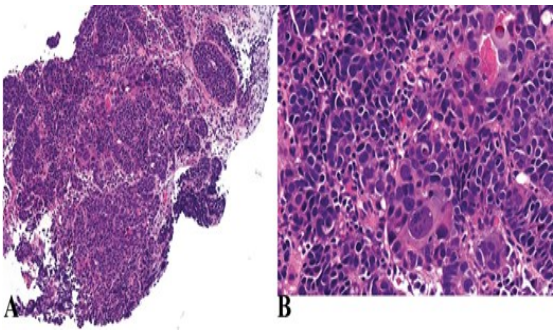

Figure 3. LCNEC composed of sheets of large cells with dispersed chromatin, prominent nucleoli, and moderate to abundant eosinophilic cytoplasm. A:magnification x10. B: x200 (33).

The morphological features of the non-neuroendocrine component vary depending on the site and usually reflect those observed in carcinomas of the related primary location. Adenocarcinoma and squamous cell carcinoma, including their respective organ-specific variants, are the most common subtypes (32).

Sometimes the distinction between NETs G3 and NECs can be challenging; however, they have a different mutational profile. More studies like this are needed to increase our knowledge of their mutational pattern. Loss of *ATRX*, *DAXX*, menin, or *p27* and/or retained SSTR2/5 staining are often used to support the diagnosis of NET G3; in contrast, the identification of global loss of Rb, diffuse positivity or total loss of p53, and/or loss of SSTR2/5 points to the diagnosis of NEC (32).

1.8 Therapy

Treatment recommendations for high grade NENs differ among NETs and NECs. The approach can be surgical or medical.

Only resettable NET G3 can be treated with surgery, an adjuvant therapy with platinum-based chemotherapy can be considered. In case of metastatic G3 NETs surgery can be a valid approach only in patients with resettable liver metastasis, while the presence of NEC is an absolute contraindication for surgery. Palliative surgery is controversial in terms of survival, it is indicated for preventing complications related to bowel obstruction or intestinal ischaemia (34).

Unresectable lesions can be treated with systemic therapies. For what concerns NECs the European Neuroendocrine Tumour Society (ENETS) and European Society for Medical Oncology (ESMO) recommend platinum-based chemotherapy as first-line treatment that has been the golden standard since the late 1980s (35). The therapy combines cisplatin/etoposide or carboplatin/etoposide. Responses to platinum-based therapies are higher in NECs comparted to NETs G3, for this reason identifying other immunohistochemical or genetic markers would be useful also for the choice of a proper therapeutic approach. Second-line treatments that are recommended by ESMO and ENETS include fluorouracil-based chemotherapy in combination with either irinotecan or oxaliplatin as well as temozolomide in monotherapy or in combination with capecitabine (35).

Other therapies that have been evaluated throughout the years are: peptide receptor radionuclide therapy (PRRT) that is a therapeutic option for NETs G1 and G2 and maybe PanNETs G3 with preserved somatostatin receptors expression, immunotherapy, CAR-T therapy, bispecific antibodies and vaccines. Immunotherapy still hasn’t a precise role in

the treatment of NENs, it includes targeting the programmed death protein PD-1 or the cytotoxic T lymphocyte-associated protein 4 CTLA-4.
For what concerns CAR- T therapy, these cells are genetically recombinant T cells consisting of an antigen recognition domain and an intracellular signalling domain that are designed to recognize specific tumour cells and induce their apoptosis (36).

Predicting response to immunotherapy

The potential response of NENs to ICI is still largely unknown. Immunohistochemical (IHC) evaluation of PD-L1 expression and its role in predicting response to ICIs is a topic that needs further investigations, moreover, a high tumour mutational burden (TMB) has been found associated to an increased benefit of ICIs and was represents a possible biomarker to select potential responders to these drugs (36,37). In addition also a high H-MSI phenotype may predict response to immunotherapy. MMR proteins are involved in DNA repair mechanisms, which ensures DNA integrity. Deficiency in MMR proteins (MLH1, MSH2, MSH6, and PMS2), leads to DNA replication errors and mutations and expansion or contraction of microsatellite regions. The resulting phenotype full of neo-antigens, makes cancer cells more recognizable by the host immune system. Additionally, dMMR/H-MSI tumours have prominent lymphocyte infiltrates and are more likely to express PD-L1, which may predict response and clinical benefit to PD-1 axis inhibitors (37).

1.9 Prognosis

The prognosis of high grade NENs is highly variable. It depends on the stage of the tumour, on the primary location, on age of the patient, on the tumour differentiation (36). Patients with localized tumours have a mean 5-year survival of 97%, which is higher than those for regionally and extensively spread tumours, which are 81% and 39%, respectively.
The 5-year survival for patients with tumours originating from the pancreas is 52%; from the stomach, 82%; the small intestine, 84%; the colon, 62%; the appendix, 88%; and the rectum, 96%.

Worse survival is also associated with higher age; patients younger than 30 years have a 5-year survival of 92%, those aged between 30 and 60 years have a 5-year survival of 87%, and those aged older than 60 years have a 5-year survival 72%.

Patients with NET G3 and NEC have also a lower 5-year survival compared to patients with NET G1 or G2, that of patients with NETs G1 is 91%, 78% for patients with NETs G2, 21% for patients with NETs G3 and 21% for patients with NECs (36). Another study analysed the median survival time of NET G3 patients and NEC patients, the first group had a significantly better survive of 99 months, than that of the NEC group that had a median survive of 17 months (HR =8.3; $p < 0.001$) (38).

A study compared female overall survival with male patients among all substrata of patients (according to stage, histology, and differentiation), the result was that females with all types of NENs have a better prognosis compared to male counterpart (38).

Some researchers analysed PanNET G3 and PanNEC patients undergoing surgical treatment, the median overall survival (OS) of patients with PanNET G3 and PanNEC was 41.8 and 11.3 months.

AIM OF THE STUDY

The aim of this study is to define the genomic profile of H-NENs GEP, particularly among the three categories NET G3, NEC with a Ki-67 labeling index of <55% and NEC with a Ki-67 labeling index ≥55%, in order to shed the light on the biology of these tumours and to determine new potential diagnostic molecular markers or therapeutic targets.
We investigated: i) MSI status between NET G3, NEC<55 ki-67 and NEC ≥55 ki-67 samples; ii) TMB between NET G3, NEC <55 ki-67 and NEC ≥55 samples; iii) prevalence of amplifications and fusion genes.

METHODS AND MATERIALS

3.1 Study cohort

A series of 49 formalin-fixed and paraffin-embedded (FFPE) HG-NETs samples were collected from the pathology databases of Istituto Nazionale dei Tumori (INT), Milan, Italy. Clinical and pathological data included patient sex, primary tumour location, tumour grade and site of sampling (primary tumour or metastatic site).
Exclusion criteria were: i) cases with inadequate material for NGS analysis (criteria for assessing sample quality will be described below) ii) cases not classified as H-NENs; iii) cases with MIXED neuroendocrine and non-neuroendocrine components; iv) not GEP origin. Of these 49 samples, 1 was excluded due to scarce sample quality. Among the 48 cases included, 40, which are included in this work, have been analysed until today, while the remaining 8 are still undergoing analysis.

3.2 Targeted Next-Generation Sequencing

Microdissection, DNA and RNA extraction and quantification

For each patients, five formalin-fixed paraffin-embedded (FFPE) 10-µm cut sections were used to extract the DNA from neoplastic cells, using the QIAmp FFPE tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was quantified using the Qubit® 3.0 fluorometer and the Qubit® dsDNA Broad Range Assay kit (both Thermo Fisher Scientific, Waltham, MA, USA).
The same process was repeated for each patient to extract the total RNA using the RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Extracted RNA was quantified using the Qubit® 3.0 fluorometer and the Qubit® RNA HS (High Sensitivity) Assay kit (both Thermo Fisher Scientific).

Assessing sample quality

In order to assess DNA and RNA samples quality before using the TruSight Oncology 500 assay (Illumina, San Diego, CA, USA) DNA samples were analysed using the

Infinium FFPE QC Kit (Illumina), while RNA samples were analysed using the Agilent Tape Station (Agilent Technologies, Santa Clara, CA, USA). Accepted DNA samples had a delta Cq value ≤5, while used RNA samples had a DV_{200} value of ≥20%.

Library preparation

The TruSight Oncology 500 protocol was followed to convert DNA and RNA extracted from formalin-fixed paraffin embedded (FFPE) tissue samples into libraries enriched for cancer-related genes that can be sequenced on Illumina sequencing systems. This panel allows the detection of low-frequency somatic variants across 523 target genes, recurrently associated to several cancers. Genes included are: *BRAF*, *FGFR1*, *FGFR2*, *FGFR3*, *ERBB2*, *TP53*, *PTEN* and many more. Particularly, DNA biomarkers include single nucleotide variants (SNVs), insertions, deletions, copy number variants (CNVs), multinucleotide variants (MNVs), it also detects immunotherapy biomarkers for tumour mutational burden (TMB) and microsatellite instability (MSI) in DNA. Fusions and splice variants are detected in RNA. Particulary in this thesis we focus on TMB, MSI and CNVs. Briefly, 200 ng of total RNA was reverse transcribed to single strand cDNA using random primers, while DNA was fragmented using the Covaris (Woburn, MA, USA) sonication instrument. Libraries were quantified using the KAPA library quantification kit (Roche, Basel, CH) and pooled to equimolar concentration. The Next-generation sequencing (NGS) was performed on a NextSeq-550 Platform (Illumina) and results were analysed using the PierianDx software.

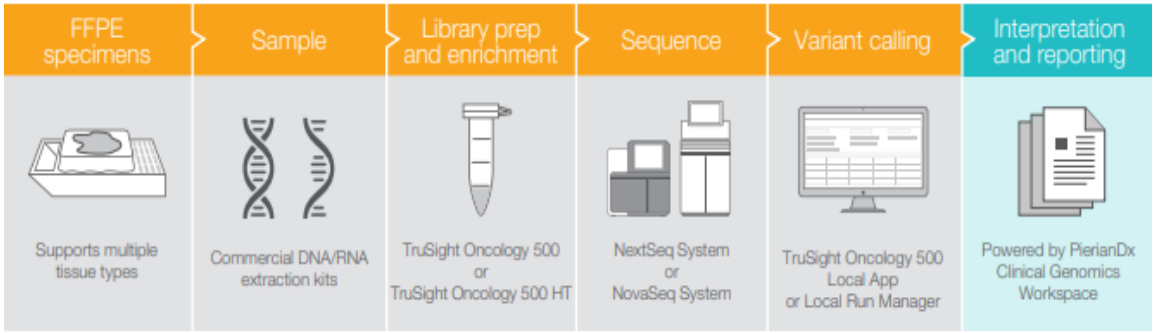

Figure 4. TruSight Oncology 500 workflow.

ABL1	BRD4	CUX1	FAM175A	GATA6	IGF1	MAP3K13	NOTCH4	POLE	RPTOR	TAF1
ABL2	BRIP1	CXCR4	FAM46C	GEN1	IGF1R	MAP3K14	NPM1	PPARG	RUNX1	TBX3
ACVR1	BTG1	CYLD	FANCA	GID4	IGF2	MAP3K4	NRAS	PPM1D	RUNX1T1	TCEB1
ACVR1B	BTK	DAXX	FANCC	GLI1	IKBKE	MAPK1	NRG1	PPP2R1A	RYBP	TCF3
AKT1	C11orf30	DCUN1D1	FANCD2	GNA11	IKZF1	MAPK3	NSD1	PPP2R2A	SDHA	TCF7L2
AKT2	CALR	DDR2	FANCE	GNA13	IL10	MAX	NTRK1	PPP6C	SDHAF2	TERC
AKT3	CARD11	DDX41	FANCF	GNAQ	IL7R	MCL1	NTRK2	PRDM1	SDHB	TERT
ALK	CASP8	DHX15	FANCG	GNAS	INHA	MDC1	NTRK3	PREX2	SDHC	TET1
ALOX12B	CBFB	DICER1	FANCI	GPR124	INHBA	MDM2	NUP93	PRKAR1A	SDHD	TET2
ANKRD11	CBL	DIS3	FANCL	GPS2	INPP4A	MDM4	NUTM1	PRKCI	SETBP1	TFE3
ANKRD26	CCND1	DNAJB1	FAS	GREM1	INPP4B	MED12	PAK1	PRKDC	SETD2	TFRC
APC	CCND2	DNMT1	FAT1	GRIN2A	INSR	MEF2B	PAK3	PRSS8	SF3B1	TGFBR1
AR	CCND3	DNMT3A	FBXW7	GRM3	IRF2	MEN1	PAK7	PTCH1	SH2B3	TGFBR2
ARAF	CCNE1	DNMT3B	FGF1	GSK3B	IRF4	MET	PALB2	PTEN	SH2D1A	TMEM127
ARFRP1	CD274	DOT1L	FGF10	H3F3A	IRS1	MGA	PARK2	PTPN11	SHQ1	TMPRSS2
ARID1A	CD276	E2F3	FGF14	H3F3B	IRS2	MITF	PARP1	PTPRD	SLIT2	TNFAIP3
ARID1B	CD74	EED	FGF19	H3F3C	JAK1	MLH1	PAX3	PTPRS	SLX4	TNFRSF14
ARID2	CD79A	EGFL7	FGF2	HGF	JAK2	MLL	PAX5	PTPRT	SMAD2	TOP1
ARID5B	CD79B	EGFR	FGF23	HIST1H1C	JAK3	MLLT3	PAX7	QKI	SMAD3	TOP2A
ASXL1	CDC73	EIF1AX	FGF3	HIST1H2BD	JUN	MPL	PAX8	RAB35	SMAD4	TP53
ASXL2	CDH1	EIF4A2	FGF4	HIST1H3A	KAT6A	MRE11A	PBRM1	RAC1	SMARCA4	TP63
ATM	CDK12	EIF4E	FGF5	HIST1H3B	KDM5A	MSH2	PDCD1	RAD21	SMARCB1	TRAF2
ATR	CDK4	EML4	FGF6	HIST1H3C	KDM5C	MSH3	PDCD1LG2	RAD50	SMARCD1	TRAF7
ATRX	CDK6	EP300	FGF7	HIST1H3D	KDM6A	MSH6	PDGFRA	RAD51	SMC1A	TSC1
AURKA	CDK8	EPCAM	FGF8	HIST1H3E	KDR	MST1	PDGFRB	RAD51B	SMC3	TSC2
AURKB	CDKN1A	EPHA3	FGF9	HIST1H3F	KEAP1	MST1R	PDK1	RAD51C	SMO	TSHR
AXIN1	CDKN1B	EPHA5	FGFR1	HIST1H3G	KEL	MTOR	PDPK1	RAD51D	SNCAIP	U2AF1
AXIN2	CDKN2A	EPHA7	FGFR2	HIST1H3H	KIF5B	MUTYH	PGR	RAD52	SOCS1	VEGFA
AXL	CDKN2B	EPHB1	FGFR3	HIST1H3I	KIT	MYB	PHF6	RAD54L	SOX10	VHL
B2M	CDKN2C	ERBB2	FGFR4	HIST1H3J	KLF4	MYC	PHOX2B	RAF1	SOX17	VTCN1
BAP1	CEBPA	ERBB3	FH	HIST2H3A	KLHL6	MYCL1	PIK3C2B	RANBP2	SOX2	WISP3
BARD1	CENPA	ERBB4	FLCN	HIST2H3C	KMT2B	MYCN	PIK3C2G	RARA	SOX9	WT1
BBC3	CHD2	ERCC1	FLI1	HIST2H3D	KMT2C	MYD88	PIK3C3	RASA1	SPEN	XIAP
BCL10	CHD4	ERCC2	FLT1	HIST3H3	KMT2D	MYOD1	PIK3CA	RB1	SPOP	XPO1
BCL2	CHEK1	ERCC3	FLT3	HLA-A	KRAS	NAB2	PIK3CB	RBM10	SPTA1	XRCC2
BCL2L1	CHEK2	ERCC4	FLT4	HLA-B	LAMP1	NBN	PIK3CD	RECQL4	SRC	YAP1
BCL2L11	CIC	ERCC5	FOXA1	HLA-C	LATS1	NCOA3	PIK3CG	REL	SRSF2	YES1
BCL2L2	CREBBP	ERG	FOXL2	HNF1A	LATS2	NCOR1	PIK3R1	RET	STAG1	ZBTB2
BCL6	CRKL	ERRFI1	FOXO1	HNRNPK	LMO1	NEGR1	PIK3R2	RFWD2	STAG2	ZBTB7A
BCOR	CRLF2	ESR1	FOXP1	HOXB13	LRP1B	NF1	PIK3R3	RHEB	STAT3	ZFHX3
BCORL1	CSF1R	ETS1	FRS2	HRAS	LYN	NF2	PIM1	RHOA	STAT4	ZNF217
BCR	CSF3R	ETV1	FUBP1	HSD3B1	LZTR1	NFE2L2	PLCG2	RICTOR	STAT5A	ZNF703
BIRC3	CSNK1A1	ETV4	FYN	HSP90AA1	MAGI2	NFKBIA	PLK2	RIT1	STAT5B	ZRSR2
BLM	CTCF	ETV5	GABRA6	ICOSLG	MALT1	NKX2-1	PMAIP1	RNF43	STK11	
BMPR1A	CTLA4	ETV6	GATA1	ID3	MAP2K1	NKX3-1	PMS1	ROS1	STK40	
BRAF	CTNNA1	EWSR1	GATA2	IDH1	MAP2K2	NOTCH1	PMS2	RPS6KA4	SUFU	
BRCA1	CTNNB1	EZH2	GATA3	IDH2	MAP2K4	NOTCH2	PNRC1	RPS6KB1	SUZ12	
BRCA2	CUL3	FAM123B	GATA4	IFNGR1	MAP3K1	NOTCH3	POLD1	RPS6KB2	SYK	

Table 2. DNA content included in the TruSight Oncology 500.

ABL1	BCL2	CSF1R	ESR1	EWSR1	FLI1	KIF5B	MSH2	NRG1	PAX7	RAF1
AKT3	BRAF	EGFR	ETS1	FGFR1	FLT1	KIT	MYC	NTRK1	PDGFRA	RET
ALK	BRCA1	EML4	ETV1	FGFR2	FLT3	MET	NOTCH1	NTRK2	PDGFRB	ROS1
AR	BRCA2	ERBB2	ETV4	FGFR3	JAK2	MLL	NOTCH2	NTRK3	PIK3CA	RPS6KB1
AXL	CDK4	ERG	ETV5	FGFR4	KDR	MLLT3	NOTCH3	PAX3	PPARG	TMPRSS2

Table 3. RNA content included in the TruSight Oncology 500.

RESULTS

4.1 Clinico-pathologic features

A total of 48 samples were included in this study, 40 of which have been already analysed. Among the 40 cases of HG-NENs included in this work, 20/40 (50%) were NET G3, 8/40 (20%) NEC with a ki-67 index <55% and 12/40 (30%) NEC with a ki-67 index ≥55%. There were 10/40 (25%) females and 30/40 (75%) males, 13/40 (32.5%) were aged less than 65 years, while the remaining 27/40 (68%) were over 65 years.

All the samples were of NENs of GEP origin, particularly the primary site was pancreas in 21/40 (52.5%) cases, in 6/40 (15%) cases was stomach, in 4/40 (10%) colon, in 3/40 (7.5%) sigma, in 2/40 (5%) oesophagus and ileum, 1/40 (2.5%) case originate from gallbladder, 1/40 (2.5%) originate from papilla of Vater. Sampling site coincided with the primary site in 32 cases, while in 8 cases sampling site wasn't the primary site. In detail the sample was obtained from liver metastasis in 5 cases, in 2 cases from nodal metastasis and 1 sample was from soft tissues metastasis.

Characteristics	***Total (N=40)***
SEX	
Male	30 (75%)
Female	10 (25%)
AGE	
<65 years	13 (32.5%)
≥65 years	27 (68%)
GRADE	
NET G3	20 (50%)
NEC < 55%	8 (20%)
NEC ≥55%	12 (30%)
PRIMARY SITE	
Pancreas	21 (52.5%)
Colon	4 (10%)
Stomach	6 (15%)
Sigma	3 (7.5%)
Oesophagus	2 (5%)
Ileum	2 (5%)
Gallbladder	1 (2.5%)

Papilla of Vater	1 (2.5%)

Table 4. Characteristics of patients analysed.

4.2 Genomic analysis of H-NENs

HISTOTYPE	**TMB**	**MSI**	**CIN**	**STABLE**	**TOTAL** (N=40)
NET G3	0	1 (5%)	6 (30%)	13 (65%)	20 (50%)
NEC <55%	1 (13%)	0	5 (63%)	3 (38%)	8 (20%)
NEC ≥55%	5 (42%)	2 (16%)	6 (50%)	4 (33%)	12 (30%)

HISTOTYPE / **ALTERATION**	NET G3 (N=20)	NEC <55% ki-67 (N=8)	NEC ≥55% ki-67 (N=12)	TOTAL (N=40)
Amplifications				
CDK4/6	2 (10%)	2 (25%)	2 (17%)	6 (15%)
EGFR	2 (10%)	1 (12.5%)	2 (17%)	5 (13%)
FGFR10	1 (5%)	0	4 (34%)	5 (13%)
RICTOR	1 (5%)	1 (12.5%)	4 (34%)	6 (15%)
MYC family genes	2 (10%)	3 (38%)	6 (50%)	11 (28%)
MET	1 (!%)	3 (38%)	1 (8.5%)	5 (13%)
Fusion genes	*HFM1-ETV1, SEL1L-EGFR*	*CNTN5-KMT2A, KMT2A-EED, KMT2A-EED*	*BCL2-KCTD, FLT1-HUWE1, SLC37A1-ERG*	

Table 5. Results of genomic analysis of H-NENs.

Among the 40 selected patients 20/40 (50%) had NET G3, 8/40 (20%) NEC <55% ki-67, and 12/40 (30%) NEC ≥55% ki-67.

Our cases were classified as follows: MSI, chromosomally instable and genomically stable.

We considered to display microsatellite instability (MSI) the samples that showed mutations in 40% or more of the investigate microsatellites, while we defined as chromosomal instability (CIN) the samples with ≥3 genetic alterations (either amplifications and/or fusions genes), the remaining samples in which we didn't find MSI

or CIN were considered as genomically stable. We also investigated tumour mutational burden (TMB) that represent the measurement of tumour mutation frequency and we considered classified sample with more than 10 mut/Mb as with high TMB.
We found MSI in only 3/40 (7.5%), CIN in 20/40 (50%) and a high TMB was found in 6/40 (15%) cases. The cases that were found to have a stable genome were 19/40 (47.5%). Correlation between MSI and high TMB was found only in 2/40 (5%) cases, all of which were NEC ≥55 % ki-67.

NET G3

Out of 20 NET G3 samples, MSI was found in 1/20 (5%), CIN in 6/20 (30%) and 13/20 (65%) were considered stable. None of them had a high TMB.
The following amplifications had a prevalence of 2/20 (10%) cases: *AKT, BRAF, BRCA1, CCND3, CCNE1, CDK4, EGFR, ERCC2, FGF2, FGF, JAK2, PDGFRA, MYC* family genes, *RPS6KB1*.
Fusion genes *HFM1-ETV1* and *SEL1L-EGFR* were found in 2 different samples.

NEC <55% ki-67

Out of 8 NEC <55% ki-67, MSI was found in none of them, CIN in 5/8 (63%) and 3/8 (38%) were considered stable, 1/8 (13%) had a significative TMB.
The following amplifications had a prevalence of 2/8 (25%) cases: *CDK4, FGFR1*, while *MET* and *MYC* had a prevalence of 3/8 (38%) cases.
Fusion genes *CNTN5-KMT2A*, *KMT2A-EED*, *KMT2A-EED* were found only in 1/20 (5%) samples.

NEC ≥55% ki-67

Out of 12 samples of NEC ≥55% ki-67, MSI was found in 2/12 (16%), CIN in 6/12 (50%) and 4/12 (33%) were considered stable, 5/12 (42%) had a high TMB.
The following amplifications had a prevalence of 2/12 (17*%): CCNE1, CDK4, EGFR, ERBB3, FGF23, FGF6, KRAS, LAMP1, MDM2, RAF1*, *FGF10* had a prevalence of 4/12 (33%) samples, *MYC* family members in 6/12 (50%) samples, *RICTOR* in 4/12 (33%) samples.

The following fusion genes: *BCL2-KCTD*, *FLT1-HUWE1*, *SLC37A1-ERG* were found in 3 different samples.

DISCUSSION

5.1 Microsatellite instability

In our pool of cases 1/20 among NET G3, 2/12 among NEC ≥55% ki-67 and none of the NEC <55% ki-67 show MSI, representing the 7.5% of the total, similar to the percentage observed in colorectal cancers (39). This result underlines the existence of a small group of H-NENs with MSI, that has been recently defined as a clinical-pathological entity, with peculiarities in clinical behaviour and therapy response (40). This subset of H-NENs with MSI was associated with a more favourable prognosis than H-NENs defined as stable, with a median survival of 60 vs 5.5 months respectively (P=0.0048) and this suggests a possible role of MSI in predicting patients' survival. (40).

The presence of MSI can be also used to predict response to therapies. It is well-known that 5-fluorouracil therapy isn't effective in cancers with MSI because of the deficiency in the induction of apoptosis by proteins of mismatch repair (dMMR), the main mechanism of response to 5-fluoruracil (41). The same ineffectiveness has been observed in human colon cancer cells line with dMMR treated with cisplatin and carboplatin therapies, while there was no difference in sensitivity to oxaliplatin, tetraplatin and transplatin (42). On the other hand in 2017 FDA approved two checkpoint inhibitors: pembrolizumab and nivolumab for the treatment of dMMR–MSI-H colorectal cancers. Indeed, pembrolizumab is now FDA-approved for the treatment of all dMMR–MSI-H metastatic solid tumours (43,44). It has also been demonstrated that colorectal cancers with MSI have a better response to Irinotecan, a topoisomerase-1 inhibitor, suggesting that a similar therapeutic approach could be effective also on this subset of GEP H-NENs with MSI (45).

These findings highlight the need for more pharmacological trials on this specific group of H-NENs and suggest the potential role of the IHC analysis in the identification of this group of patients that are more likely to have a cancer resistant to traditional first-line treatments with cisplatin/etoposide and carboplatin/etoposide and to second line treatments with 5-fluoruracil recommended by ENETS (European Neuroendocrine Tumour Society) and ESMO (European Society for Medical Oncology).

5.2 Chromosomal instability

In our analysis 6/20 NET G3, 5/8 NEC <55% ki-67, 6/12 NEC ≥55% ki-67 had chromosomal instability, resulting in 50% of our pool of cases.
Chromosomal instability is here defined as the presence of ≥3 amplifications and/or fusion genes.
The percentages of tumours with chromosomal instability found in gastric cancers is 50%, while in sporadic colorectal cancers it is 65-70% (46,47).

Amplifications

The most frequent amplifications in our pool included: CDK4/6, *EGFR*, *FGFR10*, *MET*, *MYC* family genes and *RICTOR*.

CDK4/6

In detail, *CDK4* and *CDK6* amplifications have been found in 6/40 (15%) cases and 3/40 (7.5%) cases respectively.
These genes catalyse the phosphorylation of key proteins and transcription factors implicated in cell cycle transition, specifically CiclinD-CDK4 and CiclinD-CDK6 regulate in quiescent cells the G0–G1 transition and the early G1 phase in proliferating cells by phosphorylating the tumour suppressor retinoblastoma protein pRb and activating E2F (48). The amplification of these genes results in increased proliferation.
The presence of this amplification suggests a possible usage of CDK4/6 inhibitors therapies, like Palbociclib, in H-NENs. The PALBONET trial investigated the possible usage of Palbociclib as a single agent in molecularly unselected and heavily pre-treated patients with advanced G1/2 pNETs, showing the lack of activity for this patients (49). An ongoing, phase II trial of a more potent CDK4/6 inhibitor, Abemaciclib, is currently being conducted in patients with advanced and refractory well-differentiated GEP NETs (NCT03891784) (50). However, since mutation of *Rb* results in a primary resistance to CDK4/6 inhibitors, as suggested by Milione et al., more studies should be performed to evaluate the effectiveness of these therapies, particularly in wild-type *Rb1* H-NENs (51). Another study highlights that all the trials with CDK4/6 inhibitors has been conducted in monotherapies and suggests the possibility of his usage in combination therapies (20).

CDK4/6 inhibitors could still have a role in combination therapies in wt*Rb1* H-NENs and for this reason further studies are needed.

MYC family members

Other amplifications included *MYC* family members, which have been found in 11/40 (28%) cases.

Myc is a transcription factor that activates and represses transcription of discrete gene sets, leading to changes in cellular state that can impact on global RNA production and turnover (52).

It has been shown that *MYC* amplification in SCLC is linked to response to therapies with Aurora kinase inhibitors, which combined with chemotherapy strongly suppresses tumour progression and increases survival (53). A selective aurora B inhibitor, ZM447439, potently suppresses proliferation and induces apoptosis of pNET cells lines and exhibits potentiated activity when combined with cisplatin or streptozotocin. (54). Additional studies are needed to investigate the efficacy of Aurora kinase inhibitors in H-NENs with *MYC* amplifications.

It has also been observed that inhibition of *MYC* through shRNA or pharmacologic (10058F4, CPI-203) approaches enhances the sensitivity of pNETs to mTOR inhibitors and reverses pNET resistance to mTOR inhibition by suppressing Akt activation (55,56).

It has also been found that Myc drives tumour angiogenesis by upregulating VEGF and other angiogenic proteins, so Myc inhibition disrupts the pNET vasculature and causes tumour regression in Myc-driven pNET mouse models and in RIP-Tag2 mice (20,57–59).

For all this reasons Myc surely represents a therapeutic target of emerging interest.

RICTOR

One amplification that has also been found in 6/40 (15%) cases is *RICTOR,* particularly 4 cases with the amplification were NET G3.

RICTOR is a gene that encodes for a protein called Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), that has a key role in mTORC2 formation and Akt activation, implicated in the pathway downstream receptor tyrosine kinases

(RTK) family (which includes among others: *FGFR, EGFR, VEGFR, PDGFR*).

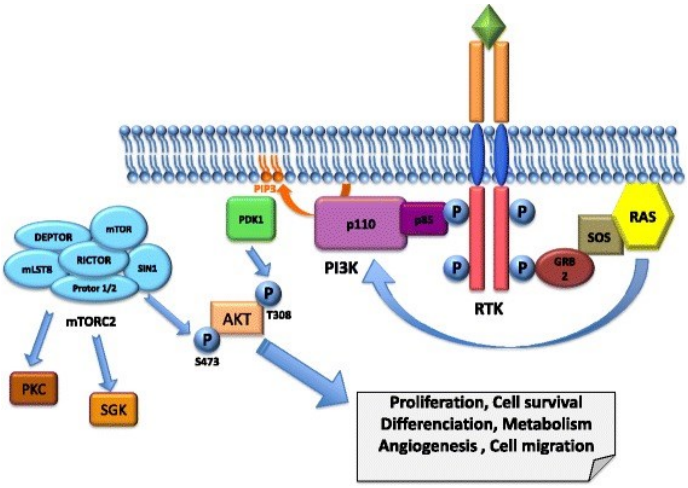

Figure 5. Schematic representation of an RTK and the downstream PI3K/AKT pathway. mTORC2 is defined by its scaffold protein RICTOR and promotes the stability and activation of AKT, SGK and PKC. AKT activates downstream signals involved in cell proliferation, differentiation, survival and migration (60).

The amplification of *RICTOR* could have important effects in tumour development either because it cooperates with altered RTKs to transform cells or as a critical regulator of a major pathway downstream of RTKs. This amplification has been found in many cancer analysing the Cancer Genome Atlas database for *RICTOR* amplification, particularly in neuroendocrine prostate cancer (18%) and lung squamous cell carcinoma (16%), followed by sarcoma (12%) and oesophagus and stomach cancer (10%), interestingly was the finding of a tendency for co-occurrence of *RICTOR* and *RTK* alterations in these tumours (60).

One study that analysed the amplification of *RICTOR* in SCLC, found that this gene was altered in 14% of patients, similar to the percentage found in our study, and co-amplified with *FGF10* (fibroblast growth factor 10) and *IL7R* all of which localize on chromosome 5p13 (61). In our analysis 5/6 cases with *RICTOR* amplification have also *FGF10* co-amplification. In all the cited studies tumours with *RICTOR* amplification had a poor prognosis, in addition *RICTOR* was seen as a potential target of mTORC1/2 inhibition therapies.

Another recent study on pNETs has investigated the resistance to Everolimus, that inhibits the activity of mTORC1 by preventing this complex to interact with its intracellular receptor FKPB12. Resistance to Everolimus and other mTOR inhibitors has been mainly attributed to mTORC2 in a feedback reaction to mTORC1 inhibition. They

showed that the response to Everolimus synergize with p21 activated kinase (PAK4) and nicotinamide adenine dinucleotide biosynthesis enzyme nicotinamide phosphoribosyl transferase (NAMPT) dual inhibitor KPT-9274 in vitro against pNETs tumour models. (62). The usage of KPT-9274 alone or in combination with Everolimus represent an interesting possible therapeutic approach for H-NENs showing *RICTOR* amplification. To strengthen the importance role of mTORC2 in the escape phenomenon to Everolimus another study on small intestinal neuroendocrine tumours (SINETs) found that the resistance to this therapy is due to increased mTORC2 activity, which resulted in either activation or incomplete inhibition of AKT phosphorylation, with consequent resistance to mTOR inhibitor treatment (63).

FGF10

Amplifications of *FGF10* were found in 5/40 (13%) cases, these amplifications were always associated with *RICTOR* co-amplification, since they both localize on chromosome 5p13 (61).

FGF10 is a growth factor that interacts with the receptor FGFR2, a family member of receptor tyrosine kinases (RTK), which activates several intracellular signalling cascades, resulting in cell proliferation, differentiation, and invasion (64). Overexpression of *FGF10* and *FGFR2* has been found also in pancreatic, gastric and breast cancers (65–67) resulting in poor prognosis. *FGF10* amplification have been proposed as a potential target of therapies for tumours, but also in this case more studies are needed (64).

EGFR

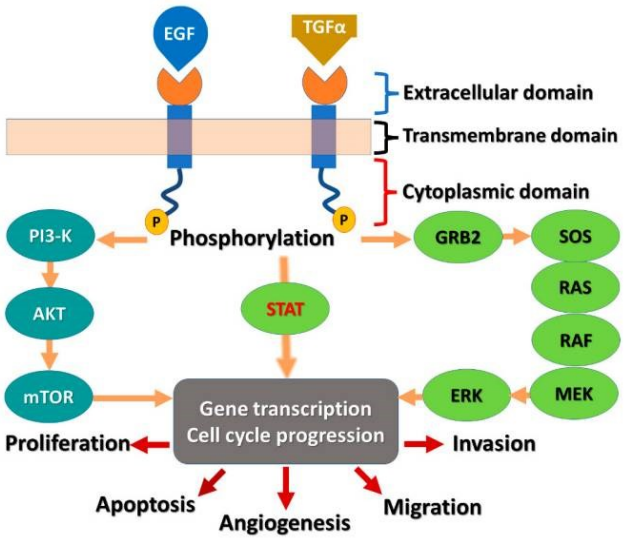

Figure 2. Diagram of the EGFR receptor and the signalling cascade (68).

Amplifications of *EGFR* have been found in 5/40 cases (13%).

EGFR is a RTK member of the ErbB family which includes EGFR (ErbB-1) and three other members, HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4) (68).

EGFR in pathological settings, mostly in lung and breast cancer and in glioblastoma, is a driver of tumorigenesis (69). EGFR is increasingly recognized as a biomarker of resistance in tumours, as its amplification or secondary mutations have been found to arise under drug pressure.

In GEP NENs overexpression of *EGFR* has been already observed and the possible usage of TKI (tyrosine kinase inhibitors), like Erlotinib and Gefitinib has been analysed. A phase II study of Gefitinib that included 57 patients with GEP-NENs demonstrated that only one of 40 evaluable patients achieved a radiological response; however, 32% had an increased time to progression (21). Since the development of these tumours includes the over-lapping signalling from many pathways to achieve efficacy these tumours should be targeted at many levels.

MET

MET amplifications have been found in 5/40 (13%) cases. *MET* encodes for hepatocyte growth factor (HGF) receptor, a RTK family member and its activation promotes tumour survival, proliferation, invasion, and metastasis in many cancers (20).

It has been shown that patients with pNETs with high expression of *MET* had decreased overall survival compared with patients with tumours with low expression of *MET* (71). MET could represent a potential therapeutic target and a randomized phase III clinical trial in advanced or metastatic NETs with ki-67<20% of any origin is ongoing with Cabozantinib, a potent non-selective RTK inhibitor against VEGFRs 1, 2, 3, and *Met*, along with other RTKs (NCT03375320) (72).
However, more studies in H-NENs are required to investigate the potential effect of MET inhibitors.

Fusion genes

Eight fusion genes have been identified in our analysis.

NET G3

In NET G3 we found fusion *HFM1-ETV1* mapping on chromosomes 1 and 7 respectively and fusion *SEL1L-EGFR*, mapping on chromosomes 14 and 7.
Hfm1 is an ATP-dependent DNA helicase and is expressed mainly in germ-line cells, participates in Golgi-associated spindle assembly and division in mouse oocyte meiosis, it has a role in premature ovarian failure (73).
ETS variant 1 (ETV1) is a member of a transcription factor family that comprises 28 proteins in humans and is characterized by the ETS DNA-binding domain, which interacts with GGAA-containing target sequences. Knockout of ETV1 in mice led to defective connections between sensory and motor neurons, which caused motor discoordination and eventually death approximately one month after birth. ETV1 is required for rapid conduction in the heart, and overexpression of ETV1 induced atrial arrhythmias in mice and has been observed in respective human patients. ETV1 has also been implicated as a promoter of oncogenesis. The ETV1 gene was found to be translocated in 5-10% of all human prostate carcinomas, which results in overexpression of full-length ETV1, N-terminal deletions, or fusion proteins that retain most of the ETV1 amino acids, including the ETS domain. Overexpression of ETV1 in transgenic mouse models led to the development of prostatic intraepithelial neoplasia, a precursor of prostate adenocarcinoma. Overexpression of ETV1 was also found in colorectal tumours and its downregulation compromised the growth of colon cancer cells (74).

All these studies suggest a possible role of this fusion in the development and progression of this specific NEN.
The other fusion found was *SEL1L-EGFR. EGFR* encodes for an RTK and its alteration can lead to resistance to TKI.

NEC<55% ki-67

Fusion genes *CNTN5-KMT2A*, *KMT2A-EED*, *KMT2A-EED* were found only in 1 sample, all in chromosome 11.
KMT2A encodes a histone H3 lysine 4 specific methyltransferase, which plays important roles in many mouse tissues and at different tumour stages. Rearrangements of the human *KMT2A* gene by chromosomal translocation are associated with acute myeloid and lymphoid leukaemia. The functions of *KMT2A* in solid tumours have not been well clarified.
What is already known is that the *KMT2A* gene is located on chromosome segment 11q23 which frequently undergoes LOH in PanNETs, so the fusions we found are coherent with was has been observed in other studies.
It has been also reported that inactivation of Kmt2a in Men1-deficient mice accelerated pancreatic islet tumorigenesis and shortened the average life span. Increases in cell proliferation were observed in mouse pancreatic islet tumours upon inactivation of both *KMT2A* and *MEN1* (75).
This finding suggests a possible role of *KMT2A* fusions in the progression of tumorigenesis.

NEC ≥55% ki-67

Fusion genes *BCL2-KCTD*, *FLT1-HUWE1*, *SLC37A1-ERG* were found in 3 different samples.
BCL2-KCTD fusion was found in chromosome 18.
BCL2 is a gene that encodes for a protein, Bcl-2 (B-cell lymphoma 2) of the family of Bcl-2 proteins, that has an antiapoptotic function (76). Translocation involving *BCL2* was initially observed in indolent B cell non-Hodgkin lymphoma called follicular lymphoma (77). Copy number variation on chromosome 18q have already been observed in sporadic neuroendocrine tumours of the small intestine and were mainly LOH (78). Additionally

in colorectal cancer patients with low Bcl-2/MMR demonstrate a significantly shorter disease free progression, whereas patients with high expression of the two markers obtain the greatest benefit from 5-FU-based chemotherapy (79).

Gene *KCTD* is involved in neurologic disorders (80).

FLT1-HUWE1 fusion was between chromosomes 13 and X.

HUWE1 encodes for a enzyme of E3 ligases family, that can catalyse the transfer of ubiquitin (Ub) from an E2 enzyme to the substrate, that has been found to be altered in many cancers (81).

FLT1 encodes for VEGF1 that is implied in normal and pathological angiogenesis in many tumours (82).

SLC37A1-ERG was found in chromosome 2.

SLC37A1 encodes for a ionic exchanger of endoplasmic reticulum that seems to be required for lipid biosynthesis in cancer cell lines (83).

ERG is an oncogene that encodes a member of the erythroblast transformation-specific family of transcription factors involved in cell proliferation, differentiation, angiogenesis, inflammation and apoptosis. Prostate cancer-specific TMPRSS2-ERG gene fusion in chromosome 21 is detectable by FISH in approximately 50% of PD-NECs of the prostate and adenocarcinomas (84). We can affirm that the presence of this fusion isn't new in the landscape of tumour genetic analysis.

5.3 Tumour mutational burden

Significative TMB was found in 6/40 (15%) cases. Correlation between MSI and high TMB was found only in 2/40 (5%) cases, all of which were NEC ≥55 % ki-67, in the remaining 3 cases the high TMB was probably caused by other genetic mutations than MSI.

High TMB is a predictor of response to immunotherapies, but it has not fully entered the clinical practice yet (85,86). Our result of 15% of high TMB is comparable to the percentage of 29.3% found in a retrospective analysis of TMB in NET by Shao et al.(87). Furthermore, high TMB has been associated with poor prognosis across many solid tumours. Accordingly, in our case series the percentage of cases with an high TMB is the highest in NEC ≥55% ki-67, according to other authors, correlates with a poor prognosis.

CONCLUSION

This is the first study to provide a comprehensive genomic profile of HG-NENs by using targeted NGS.

We proposed a molecular classification of this group of neoplasms based on the genomic data, identifying three categories with a different distribution across NET G3, NEC with ki67 <55% and NEC with Ki67 ≥55%, with an enrichment of MSI and CIN in the latter.

We also investigated the prevalence of several amplifications and fusion genes and found *CDK4/6*, *EGFR*, *FGFR10*, *MET*, *MYC* family genes and *RICTOR* particularly of great interest for their therapeutic implications.

In conclusion, this study shed lights on the biology of HG-NENs and demonstrates that HG-these tumours have a composite molecular landscape, with some of the molecular alterations identified representing potential targets for precision oncology.

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